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Impact of Enoxaparin +α Lipoic acid combination on oxidative stress, follicle development and apoptotic damage in ovarian ischemia reperfusion model

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Abstract. – OBJECTIVE: With this study we aimed at investigating the impact of enoxaparin (E) and α-lipoic acid (α-LA) on ischemia/reperfusion (I/R) damage in an experimental ovarian torsion (T) detorsion (D) model.

MATERIALS AND METHODS: The study comprised 80 female albino rats aged 12 weeks. They were divided into 8 groups as follows, with 10 rats in each group: Group 1 (Sham), Group 2 (Ischemia, 3 hours), Group 3 (I/R, 6 hours reperfusion), Group 4 (I/R, 3 days reperfusion), Group 5 (I/R, E, 6 hours), Group 6 (I/R, E, 3 days), Group 7 (I/R, E+α-LA, 6 hours), and Group 8 (I/R, E+α-LA, 3 days). Immediately after detorsion, enoxaparin (0.5 mg/kg/day, sc) or α-LA (100 mg/kg/day, ip) were administered. Ovarian levels of malondialdehyde (MDA), glutathione peroxidase (GPx), and catalase were measured. In addition to total Antioxidant Status (TAS) and Total Oxidative Stress (TOS), the Oxidative Stress Index (OSI) was calculated. Serum levels of AMH, FSH, LH, E2, and progesterone were measured. Ovarian samples were histopathologically examined and they underwent Tag staining for apoptosis.

RESULTS: Compared with the sham group, there was a significant increase in TOS, OSI, and MDA levels in I/R group ($p<0.001$). Compared to Group 2, a significant increase was determined in TOS and OSI in Group 3 ($p<0.05$). In Group 4, a significant increase was found compared to Group 2 ($p<0.01$). The lowest level of I/R damage and the highest level of antioxidants were found in Group 2, 7, and 8. The highest values of TOS, OSI, and MDA were found in Group 3, Group 4, Group 5, and Group 6 ($p<0.001$). When the sham group and Group 4 were compared, it was found that AMH and E2 levels decreased ($p<0.001$), while FSH levels increased ($p<0.001$). In Group 6, AMH and E2 values were significantly decreased compared to the sham group ($p<0.001$). In the histopathological examination, the findings were observed to be parallel to those of the biochemical markers.

CONCLUSIONS: Combined use of α-Lipoic acid and enoxaparin prevents oxidative stress and ovarian damage due to ischemia-reperfusion injury better than the use of drugs alone.

Key Words:
α-Lipoic, Enoxaparin, Ovarian torsion, Ischemia/eperfusion, Oxidative stress, Apoptosis.

Introduction

Ovarian torsion is defined as the rotation of the ovary, fallopian tubes and the vascular structures around their own axis. It constitutes 2-3% of gynecological emergencies, and usually occurs in childhood and during reproductive period[1]. Following torsion, the ovaries cannot be fed adequately and subsequently ischemia and damage to the ovarian tissue develops. Ischemia is defined as the inability to meet the need for oxygen and other metabolites due to insufficient blood supply of the tissues[2]. With the decrease or cessation of blood flow, oxidative phosphorylation decreases, and thus the synthesis of adenosine triphosphate (ATP) and phosphocreatine decrease. The

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anaerobic shift of intracellular metabolism leads to acidosis, increased calcium concentration, and activation of intracellular proteases[3]. Reperfusion occurring with detorsion leads to formation of reactive oxygen derivatives (ROS) due to the increased blood flow to the hypoxic tissue[4].

By causing damage to lipid structures in cell membranes, intracellular proteins, and DNA[5] ROS leads to cell damage. The cell damage starts with ischemia and intensifies during the reperfusion period, resulting in cell death[6]. Ischemia and subsequent reperfusion may cause many serious clinical conditions including damage in the follicle development. Many studies have been carried out to minimize the ischemic and subsequent reperfusion injury with the use of antioxidants[6,7], anticoagulants[8], and anti-inflammatory agents[9,10]. Enoxaparin is a low molecular weight heparin which is used for prophylaxis against potential thromboembolic events. It minimizes I/R damage and helps accelerate tissue regeneration. It has been shown that enoxaparin administration plays an important role in the recovery of ischemia/reperfusion injury[8]. α-LA is an antioxidiant agent that plays an important role in the mitochondrial dehydrogenase reaction[11]. It acts as an antioxidant in scavenging free radicals and has been shown to decrease the effect of I/R damages[12]. When reviewing the literature there is no study investigating the combined use of enoxaparin and α-LA in I/R injury. The study was, therefore, planned to examine the effects of enoxaparin and α-LA combination on I/R damage in an experimental rat model of the ovarian torsion/detorsion. Impcat of these two drugs on oxidative stress, antioxidant enzymes, ovarian reserve marker and apoptosis were evaluated in collected ovarian samples.

Materials and Methods

This study was approved by Mustafa Kemal University Animal Experiments Local Ethics Committee (Approval number: 2020/05-05). All animal experiments were performed according to the National Health and Medical Research Council guidelines for Experimental Animal Care. The study was reported in accordance with the ARRIVE (Animal Research: Reporting of *In vivo* Experiments) guidelines[13]. All the rats were kept in standard laboratory conditions with free access to food and water (12:12 h light: dark cycle, 55% ± 10% relative humidity, 22 ± 2°C ambient temperature). A total of 80 Wistar Albino adult female rats, aged 10-12 weeks, and each weighing 300-350 g, were separated into 8 equal groups (n=10) as follows: group 1 (Sham), group 2 (Ischemia: I, oophorectomy after 3 hours), group 3 (Ischemia/reperfusion: I/R, oophorectomy after 6 hours), group 4 (I/R, oophorectomy after 3 days), group 5 (I/R, enoxaparin: E, oophorectomy after 6 hours), group 6 (I/ R, enoxaparin oophorectomy after 3 days), group 7 (I/R, enoxaparin +α-LA), oophorectomy after 6 hours), group 8 (I/R, enoxaparin + α-LA, oophorectomy after 3 days).

Medications

Enoxaparin sodium (Atabay Kim.,Turkey), 98% α-lipoic acid (Sigma-Aldrich-Merck, Germany), Ketamine hydrochloride (Ketasol, Richter Pharma, Austria), Xylazine hydrochloride (Rompun, Bayer Health Care, Germany), Unacefin 1 gr. flac. intramuscular (Yavuz Pharmaceutical Warehouse Medical Products Industry and Trade Inc. Istanbul-Turkey).

Application Dose of Drugs

Enoxaparin sodium 0.5 mg/kg/day was subcutaneously administered, while α-lipoic acid 100 mg/kg/dayx1/3 was administered intraperitoneally. Ketamine hydrochloride 80 mg/kg and Xylazine hydrochloride 12 mg/kg were administered intraperitoneally, and anesthesia was provided for 45-60 minutes. For antibiotic prophylaxis, Unacefin 12.5 mg/kg/day was administered intramuscularly.

Surgical Procedures

The animals were administered ketamine hydrochloride and xylazine intramuscularly as anesthetic agents. The abdomen of the rats was shaved and disinfected with povidone-iodine solution. Following a midline incision to the lower abdomen, uterine, horns and ovaries were explored. In Group 1, after observing the ovaries and uterus, the parietal peritoneum, abdominal muscle, fascia, and subcutaneous and skin were closed in 2 layers without any further procedure. Bilateral oophorectomy was performed 3 hours after this procedure. 15 ml of blood samples were taken from the heart for routine analysis of AMH, FSH, LH, E_2, Progesterone, TAS, and TOS. Bilateral ischemia was performed in rats from the Groups 2-8. The bilateral ovaries and tubes of the rats were turned 360° clockwise 3 times and then were sutured to the anterior wall of the abdomen with 5/0 prolene, which was maintained for 3 hours[8,14,15]. Group 2 underwent oophorectomy 3 hours after this procedure, and 15 ml of blood sample were taken from the heart. In Group 3, detorsion was performed 3 hours after the ovarian torsion, then bilateral oophorectomy was performed

6 hours after and 15 ml of blood samples were taken from the heart. In Groups 4-8, bilateral oophorectomy was performed after I/R and blood samples were taken. One of the ovaries was placed in liquid nitrogen postoperatively and frozen at -80°C until oxidative stress tests were applied. The other ovary was sent to the pathology in 10% formaldehyde for histopathological examination. After taking intracardiac blood, all the rats were sacrificed.

Collection, Storage and Study of Biochemical Samples

Blood samples were taken into plain or hemogram tubes with EDTA. Samples were centrifuged at 1,500 g for 15 minutes and their serum was portioned and stored at -80ºC until analysis. The total antioxidant capacity (TAC) and total oxidative stress (TOS) parameters in serum samples were measured spectrophotometrically using the Erel method[16] in an autoanalyzer (Siemens Advia 1800, Tarrytown, NY, USA). OSI (arbitrary unit) was calculated as TOS (μmol H_2O_2 Eq/l)/TAS (μmolTroloxEq/l) x 100. Antimullerian hormone (AMH), Follicle Stimulating Hormone (FSH)), Luteinizing hormone (LH), estradiol (E_2), and progesterone levels in the serum samples were studied using the chemoluminescent method (Siemens Advia Centaur XP, Tarrytown, NY, USA).

Tissue samples were thawed to room temperature on the day of assay, then the weights were recorded. Each tissue sample was homogenized in a knife homogenizer using phosphate buffer (PBS) (pH=7.4). The protein level in the obtained homogenates was measured spectrophotometrically with the Bradford method and calculated according to the standard radiograph prepared with bovine serumalbümin (BSA). Tissue malondialdehyde levels were measured using the double heating method and the Drapper and Hadley method[17]. The homogenates were then centrifuged at 15,000 xg and the supernatants were separated. The protein levels were measured again using the Bradford method. The catalase activities were measured according to the Aebi method[18] in which the H_2O_2 decomposition was spectrophotometrically monitored, and the Glutathioneperoxidase (GPX) activity was measured spectrophotometrically according to the Paglia method[19] (Schimadzu UV, 1800 Japan). All results were given in proportion to the protein values measured in the tissue.

Histopathological Examination

Ovarian I/R damage and the effects of treatment were examined with Hematoxylin Eosin (H&E) staining sections (Figure 1). The morphological character of the ovarian cortex was histopathologically examined. In order to assess ovarian reserve, the number of primordial and primary follicles were recorded in the largest section of the ovary. The following criteria were used to assess tissue damage: congestion, interstitial edema, hemorrhage, inflammatory cell infiltration, degeneration of follicular cells (loss of cohesion in granulosa cells: parenchymal separation in normal ovarian cortex and follicles, damage in granulosa cells). Scoring was applied according to Osman[20] as 0+3; 0) no pathological finding; 1) ≤ 33% mild; 2) 33-66% moderate; 3) 66% ≤ severe. All sections were examined with a light microscope (Olympus BX53F Binocular, Tokyo, Japan) and photographed. Five different microscopic fields were examined in each sample. The number and histological examination of follicles were performed according to the previous studies[8,21].

TUNEL Staining for Apoptosis

The TUNEL Apoptosis-Tag staining method was used to examine follicular cell degeneration. Staining was performed according to the manufacturer's instructions using the ApopTag Peroxidase *in situ* Apoptosis Detection Kit (Merck Millipore, Saint Louis, MO, USA). Stained sections were examined under the microscope according to the classification as follows[7]: 0) < 5% of follicular cells positive, 1) 5-25% of follicular cells positive, 2) 26-50% of follicular cells positive, 3) 51% ≤ of follicular cells positive.

Statistical Analysis

All statistical analyses were performed using SPSS version 21.0 software (Statistical Package for the Social Sciences, IBM Corp., Armonk, NY, USA). After testing the normality of the data with the Shapiro-Wilk test, parametric and non-parametric tests were performed. Of the parametric tests, the Student's *t*-test, Paired *t*-test and ANOVA, Kruskall-Wallis, Wilcoxon test, Mann-Whitney U-test, Friedman and Chi-square tests were used. Mean and standard deviation values were used to express descriptive statistics. A value of $p<0.05$ was considered statistically significant. All analyses were calculated with a 95% confidence interval. $*p<0.05$ was considered significant with 95% of accuracy and 5% of tolerance (0.05). $**p<0.01$ was considered significant with 99% of accuracy and 1% of tolerance (0.01). $***p<0.001$ was considered significant with 99.9% of accuracy and 0.1% of tolerance (0.001).

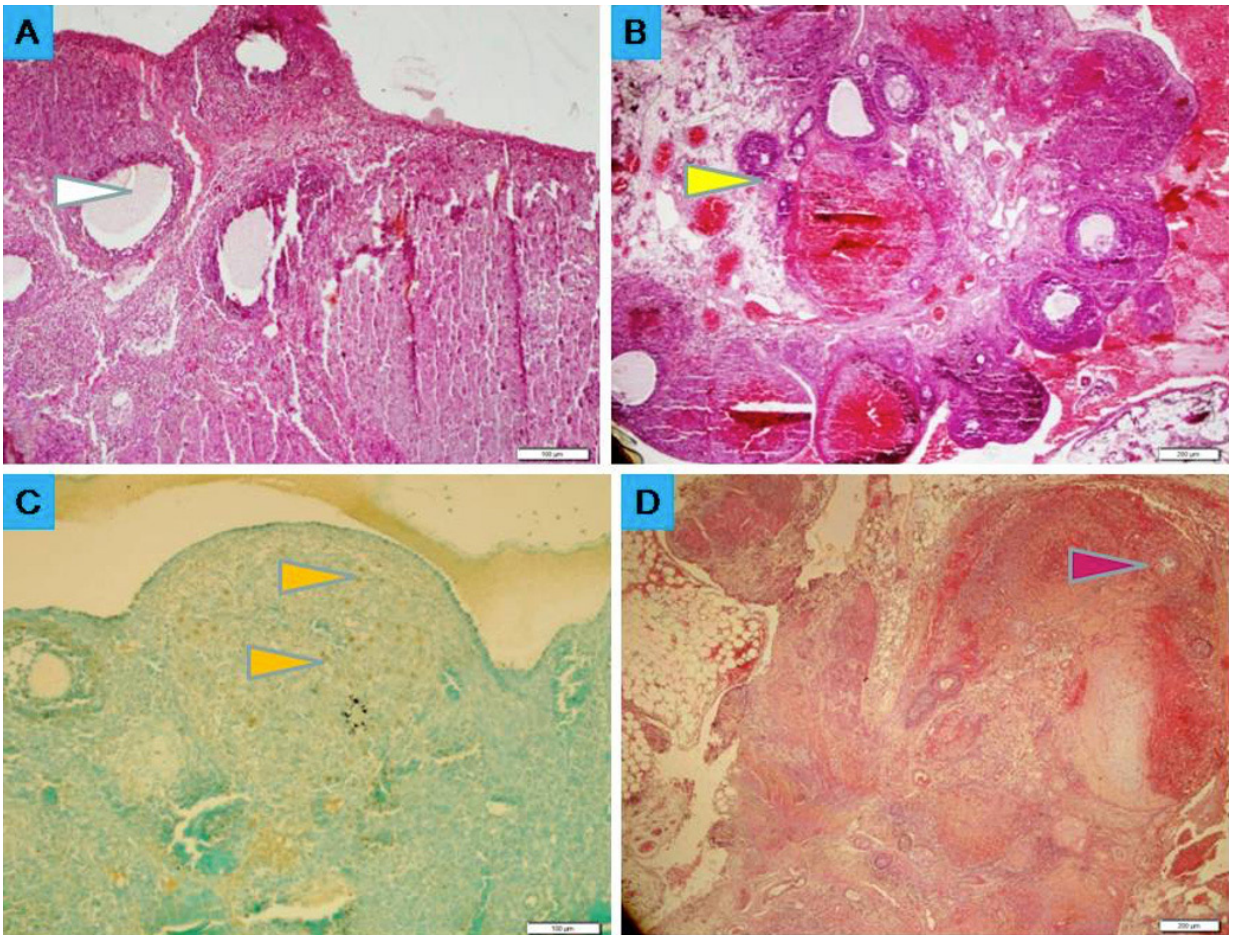

Figure 1. **A**, Normal histological findings and healthy follicle in ovarian sections of rats in the Sham group (white arrowhead, H&Ex10). **B**, Diffused hemorrhage and follicular damage in the I/R group (yellow arrowhead, H&EX10). **C**, Diffused apoptotic cells on TUNEL staining in the I/R group (pink arrowheads, TUNELX10). **D**, Normalization of I/R-related damage in parenchyma and follicle with a combination of α-Lipoic acid enoxaparin (red arrowhead, H&EX10).

Results

Biochemistry Results

The results of oxidative stress markers (TOS, OSI, MDA), antioxidant markers (TAS, GPx, CAT), and ovarian reserve markers (AMH, FSH, LH, E_2, progesterone) are shown in Tables I, II, and III. When the sham group was compared with the other groups, while TOS, OSI, and MDA were significantly increased in Group 3, Group 4, Group 5, and Group 6 ($p<0.001$), GPx, and CAT were significantly decreased ($p<0.001$). There was a significant decrease in serum OSI ($p<0.05$) and tissue MDA ($p<0.01$) levels in Group 7 compared to Group 3. Significant increases in serum TAS levels ($p<0.05$), tissue GPx ($p<0.001$) and CAT ($p<0.05$) activity were found. There was a significant decrease in serum TOS ($p<0.01$), OSI and tissue MDA levels ($p<0.05$) in Group 7 compared to Group 5, and a significant increase in tissue GPx activity ($p<0.01$). When Group 8 was compared with Group 4, a significant decrease in serum TOS ($p<0.001$), OSI ($p<0.05$) and tissue MDA levels ($p<0.001$) was registered. Also, a significant decrease in serum TAS ($p<0.05$) levels and a significant increase in tissue GPx activity ($p<0.001$) were detected. When Group 8 was compared with Group 6, a significant decrease was found in serum TOS ($p<0.05$) and tissue MDA levels ($p<0.001$), and a significant increase was registered in GPx activity ($p<0.001$) (Tables I-III).

Ovarian Resve Results

There was a significant decrease in AMH, FSH, and E_2 in Group 4 compared to the Sham group ($p<0.001$). A significant increase ($p<0.01$) in AMH and E_2 levels in Group 6 were detected when

Table I. Mean and standard deviation values of the data of biochemical markers, according to groups.

Variables	Experimental Groups								*p*
	Sham	Ischemia	I/R- Bilateral Oopherectomy after 6 hours	I/R- Bilateral Oopherectomy after 3 days	I/R+ Enoxaparin Oopherectomy after 6 Hours	I/R+ Enoxaparin Bilateral Oopherectomy after 3 days	I/R+ Enoxaparin +α-Lipoic Acid; BO after 6 hours	I/R+ Enoxaparin +α-Lipoic Acid; after 3 days	
TAS	1.42 ± 0.25	1.24 ± 0.15	1.15 ± 0.13	1.31 ± 0.4	1.36 ± 0.25	1.1 ± 0.2	1.43 ± 0.25	1.08 ± 0.2	0.003
TOS	22.6 ± 5.65	30.92 ± 9.19	40.5 ± 8.99	43.78 ± 10.58	46.66 ± 8.16	36.13 ± 11.7	33.94 ± 8.55	28.06 ± 7.97	<0.001
OSI	1.65 ± 0.55	2.51 ± 0.71	3.55 ± 0.84	3.75 ± 1.74	3.51 ± 0.81	3.32 ± 1.11	2.41 ± 0.65	2.72 ± 0.99	<0.001
AMH	4.05 ± 1.19	3.94 ± 1.88	3.86 ± 1.06	1.91 ± 1.04	3.78 ± 1.19	2.08 ± 1.13	3.96 ± 2.02	3.18 ± 1.27	0.001
E2	30.93 ± 6.61	28.63 ± 7.79	28.74 ± 6.63	18.96 ± 7.64	27.06 ± 5.2	20.95 ± 6.97	25.28 ± 9.53	25.21 ± 8.64	0.009
FSH	2.36 ± 0.92	2.67 ± 0.93	2.59 ± 0.52	3.99 ± 1.59	2.69 ± 1	2.66 ± 0.68	2.92 ± 0.9	2.81 ± 0.96	0.022
LH	12.13 ± 4.69	11.46 ± 4.41	9.66 ± 4.56	15.58 ± 4.58	11.02 ± 5.82	11.54 ± 3.59	11.64 ± 5.79	12.21 ± 3.83	0.276
Progesterone	6.2 ± 2.02	5.78 ± 1.47	5.5 ± 1.49	5.16 ± 1.29	6.25 ± 1.92	6.74 ± 2.77	5.76 ± 1.73	6.05 ± 1.5	0.660
BRADFORD	80.94 ± 7.09	89.81 ± 9.06	88.47 ± 7.77	91.27 ± 13.17	99.35 ± 12.71	92.93 ± 10.37	95.45 ± 10.02	97.18 ± 12.84	0.009
MDA	0.77 ± 0.24	2.39 ± 0.76	4.74 ± 0.85	4.68 ± 1.28	4.42 ± 1.15	4.29 ± 2.5	2.9 ± 1.9	2.15 ± 0.82	<0.001
GPX	482.64 ± 63.37	148.9 ± 14.74	174.69 ± 25.85	104.11 ± 8.29	292.52 ± 27.32	197.64 ± 47.59	336.43 ± 39.33	296.1 ± 33.8	<0.001
CAT	82.57 ± 36.22	25.06 ± 4.16	29.46 ± 7.04	35.61 ± 18.26	46.28 ± 24.52	50.78 ± 29.44	49.52 ± 22.07	54.75 ± 18.66	<0.001

TAS: Total Antioxidant Level; TOS: Total Oxidative Stress Level; OSI: Oxidative Stress Index; AMH: Anti Mullerian Hormone; E2: Estradiol; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; MDA: Malondialdehyde; GP_x: Glutathione Peroxidase; CAT: Catalase.

Table II. Comparison of the biochemical markers of the Sham group with the other groups.

Groups	Groups 1-2 *p*	Groups 1-3 *p*	Groups 1-4 *p*	Groups 1-5 *p*	Groups 1-6 *p*	Groups 1-7 *p*	Groups 1-8 *p*
TAS	0.085	0.015	0.285	0.573	0.003	0.934	0.002
TOS	0.043	<0.001	<0.001	<0.001	<0.001	0.006	0.180
OSI	0.056	<0.001	<0.001	<0.001	<0.001	0.088	0.017
BRADFORD	0.066	0.117	0.033	<0.001	0.014	0.003	0.001
MDA	0.010	<0.001	<0.001	<0.001	<0.001	<0.001	0.026
GPX	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
CAT	<0.001	<0.001	<0.001	<0.001	0.002	0.002	0.007
AMH	0.857	0.761	<0.001	0.660	0.002	0.887	0.169
FSH	0.474	0.604	<0.001	0.448	0.487	0.200	0.307
LH	0.753	0.247	0.106	0.600	0.783	0.819	0.967
E2	0.494	0.514	0.001	0.251	0.004	0.096	0.092
Progesterone	0.608	0.393	0.209	0.956	0.511	0.588	0.857

TAS: Total Antioxidant Level; TOS: Total Oxidative Stress Level; OSI: Oxidative Stress Index; AMH: Anti Mullerian Hormone; E_2: Estradiol; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; MDA: Malondialdehyde; GP_x: Glutathione Peroxidase; CAT: Catalase.

compared to the Sham group, while a significant decrease in AMH and E_2 and significant increase in FSH were observed in Group 4 compared to Group 2. A significant decrease was found in FSH in Group 4 compared to Group 6. A significant increase in AMH and a significant decrease in FSH were found in Group 8 compared to Group 4 (Tables II-III).

Histopathology Results

The comparison of histopathological parameters is shown in Tables IV-VI. When the Sham group was compared with the other groups, the lowest values of Total Damage Score (TDS), and Mean Apoptosis Index (MAI) were found in Group 7 and Group 8. TDS and OAI were found to be significantly decreased in Group 7 compared to Group 3 ($p<0.001$). When compared to Group 5 and Group 7, while TDS and OAI were similar, a significant decrease in interstitial Apoptosis index (IAI) was recorded ($p<0.05$). TDS and OAI were found to be significantly decreased in Group 8 compared to Group 4 ($p<0.001$, $p<0.05$). A significant decrease in TDS was found in Group 8 compared to Group 6 ($p<0.05$). The biochemistry results were consistent with the histopathological findings obtained from H&E, or TUNEL stained samples.

Table III. Pairwise comparisons between groups.

Variables	Groups 2-1	Groups 3-2	Groups 4-2	Groups 5-3	Groups 7-3	Groups 6-4	Groups 8-4	Groups 7-5	Groups 8-6
TAS	0.085	0.455	0.506	0.057	0.012	0.059	0.037	0.518	0.839
TOS	0.043	0.020	0.002	0.131	0.108	0.062	<0.001	0.002	0.049
OSI	0.056	0.022	0.006	0.934	0.012	0.329	0.023	0.016	0.183
BRADFORD	0.066	0.048	0.512	0.025	0.146	0.727	0.217	0.648	0.374
MDA	0.010	<0.001	<0.001	0.601	0.004	0.519	<0.001	0.015	0.001
GPx	<0.001	0.119	0.008	<0.001	<0.001	<0.001	<0.001	0.009	<0.001
CAT	<0.001	0.662	0.296	0.098	0.049	0.134	0.060	0.748	0.693
AMH	0.857	0.902	0.002	0.892	0.872	0.787	0.045	0.766	0.081
FSH	0.474	0.844	0.004	0.810	0.443	0.003	0.009	0.597	0.742
LH	0.753	0.398	0.055	0.524	0.352	0.060	0.115	0.767	0.751
E2	0.494	0.975	0.005	0.617	0.597	0.553	0.066	0.597	0.207
Progesterone	0.608	0.731	0.453	0.364	0.753	0.058	0.280	0.551	0.403

TAS: Total Antioxidant Level; TOS: Total Oxidative Stress Level; OSI: Oxidative Stress Index; AMH: Anti Mullerian Hormone; E_2: Estradiol; FSH: Follicle Stimulating Hormone; LH: Luteinizing Hormone; MDA: Malondialdehyde; GP_x: Glutathione Peroxidase; CAT: Catalase.

Table IV. Data of histopathological markers, according to groups.

	Experimental Groups								
Variables	Sham Group 1	Ischemia Group 2	I/R- Bilateral Oopherectomy after 6 hours Group 3	I/R- Bilateral Oopherectomy after 3 days Group 4	I/R+ Enoxaparin BO after 6 Hours Group 5	I/R+ Enoxaparin BO after 3 days Group 6	I/R+ Enoxaparin + Lipoik Asit; BO after 6 hours Group 7	I/R+ Enoxaparin + Lipoik Asit; BO after 3 days Group 8	*p*
EDEMA	0.3 ± 0.48	1.5 ± 0.53	1.9 ± 0.74	1.1 ± 0.32	1.2 ± 0.42	0.7 ± 0.48	0.9 ± 0.32	0.5 ± 0.53	<0.001
VAS. CON.	0.2 ± 0.42	2.2 ± 0.42	2 ± 0.47	1.2 ± 0.42	1.6 ± 0.52	0.8 ± 0.42	1.1 ± 0.32	0.5 ± 0.53	<0.001
HEMORRHAGE	0.2 ± 0.42	2.1 ± 0.32	2.1 ± 0.57	1.4 ± 0.52	2.3 ± 0.48	1.4 ± 0.7	2.5 ± 0.53	1 ± 0.47	<0.001
PNL INFILTRA	0.3 ± 0.48	2 ± 0.67	2.1 ± 0.57	1.6 ± 0.52	1.5 ± 0.53	0.9 ± 0.57	1.1 ± 0.32	0.8 ± 0.42	<0.001
FOL. DE.	0.3 ± 0.48	1.6 ± 0.52	2.7 ± 2.26	1.2 ± 0.79	1.3 ± 0.67	0.7 ± 0.48	0.8 ± 0.42	0.5 ± 0.53	<0.001
TDS	1.3 ± 1.64	9.4 ± 0.97	10.1 ± 1.6	6.5 ± 1.43	8 ± 1.94	4.7 ± 1.34	6.6 ± 0.7	3.3 ± 1.25	<0.001
FOL. NUMBER.	10.3 ± 3.27	8.2 ± 2.49	6.5 ± 4.06	6.2 ± 2.78	8.5 ± 3.66	7.2 ± 5.2	7.5 ± 1.51	8.2 ± 3.19	0.099
COR. LUT. C.	4.7 ± 1.42	4.3 ± 1.25	5.2 ± 2.62	4.2 ± 1.87	2.9 ± 1.2	2.8 ± 1.03	3.9 ± 1.79	3.1 ± 1.37	0.008
TOT. FOL. NUM	15 ± 2.75	12.5 ± 3.06	11.7 ± 5.48	10.4 ± 2.99	11.4 ± 3.03	9.7 ± 5.66	11.4 ± 2.99	11.3 ± 2.98	0.045
IN. AP. INDEX	0.3 ± 0.48	2.1 ± 0.32	2.5 ± 0.53	1.4 ± 0.52	1.7 ± 0.67	1.1 ± 0.57	1.1 ± 0.32	0.8 ± 0.63	<0.001
FOL. H. AP. I.	0.2 ± 0.42	1.8 ± 0.42	2.2 ± 0.42	1.2 ± 0.79	1.3 ± 0.95	0.7 ± 0.67	0.7 ± 0.48	0.5 ± 0.71	<0.001
MEAN. AP. INDEX	0.25 ± 0.42	1.85 ± 0.41	2.35 ± 0.41	1.15 ± 0.47	1.5 ± 0.78	0.9 ± 0.57	0.9 ± 0.32	0.65 ± 0.63	<0.001

VAS. CON.: Vascular congestion; PNL INFILTRA: PNL infiltration; FOL. DE: Follicular degeneration; TDS: Total Damage Score; FOL. NUMBER: Number of follicles; COR. LUT. C.: Corpus Luteum Count; TOT. FOL. NUM: Total number of follicles; IN. AP. INDEX: Interstitium Apoptotic Index; FOL. H. AP. I.: Apoptosis index in follicle cells; MEAN. AP. INDEX: Mean apoptosis index; BO: Bilateral Oopherectomy.

Table V. Comparisons of the histopathological parameters according to groups.

HP	Groups 2-1	Groups 3-2	Groups 4-2	Groups 5-3	Groups 7-3	Groups 6-4	Groups 8-4	Groups 7-5	Groups 8-6
EDEMA	<0.001	0.208	0.057	0.022	0.002	0.045	0.01	0.088	0.374
VC	<0.001	0.329	<0.001	0.09	0.001	0.051	0.007	0.022	0.17
HEMORRHAGE	<0.001	0.957	0.004	0.423	0.125	0.865	0.09	0.374	0.118
PNL INF	<0.001	0.721	0.165	0.03	0.001	0.014	0.003	0.057	0.689
FD	<0.001	0.06	0.24	0.013	<0.001	0.11	0.042	0.061	0.374
TDS FN(x)	<0.001	0.197	<0.001	0.034	<0.001	0.011	0.001	0.072	0.02
CLC TFS	0.72	0.583	0.561	0.02	0.278	0.045	0.181	0.177	0.527
IAI	<0.001	0.057	0.004	0.013	<0.001	0.24	0.038	0.021	0.264
FCAI	<0.001	0.051	0.056	0.016	<0.001	0.144	0.053	0.112	0.45
MEAN AI	<0.001	0.018	0.005	0.009	<0.001	0.268	0.046	0.058	0.288

HP: Histopathological parameter; VC: Vascular congestion; PNL INF: PNL infiltration; FD: Follicular degeneration; TDS: Total Damage Score; FN(x): Number of follicles (primordial, preantral, minor antral, major antral); CLC: Corpus Luteum Count; TFS: Total number of follicles; IAI: Interstitium Apoptotic Index; FCAI: Apoptotic index in follicle cells (primordial, preantral, small antral, large antral); MEAN AI: Mean Apoptosis Index.

Table VI. Histopathological comparisons of the sham group with other groups.

GROUPS HP	2;1 p	3;1 p	4;1 p	5;1 p	6;1 p	7;1 p	8;1 p
EDEMA	<0.001	<0.001	<0.001	<0.001	0.081	0.008	0.374
VC	<0.001	<0.001	<0.001	<0.001	0.009	<0.001	0.17
HEMORRHAGE	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002
PNL	<0.001	<0.001	<0.001	<0.001	0.024	<0.001	0.028
FD	<0.001	<0.001	0.012	0.003	0.081	0.028	0.374
TDS FS	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.011
CLC TFC	0.72	0.927	0.258	0.007	0.003	0.272	0.011
IAI	<0.001	<0.001	<0.001	<0.001	0.006	<0.001	0.067
FC API	<0.001	<0.001	0.005	0.005	0.066	0.028	0.3
MAI	<0.001	<0.001	<0.001	<0.001	<0.011	0.003	0.102

HP: Histopathological parameter; VC: Vascular congestion; PNL: Polymorpho Nuclear Leukocytes; FD: Follicular degeneration; TDS: Total Damage Score; FS: Number of follicles (primordial, preantral, minor antral, major antral); CLC: Corpus Luteum Count; TFC: Total Follicles Counts; IAI: Interstitium Apoptotic Index; FC API: Apoptotic index in follicle cells (primordial, preantral, small antral, large antral follicles); MAI: Mean Apoptotic Index.

Discussion

Ovarian torsion is an important clinical emergency which results in ischemia of ovarian tissues. Detorsion of affected tissues is the only treatment. Reperfusion occurring following detrosion leads to damage of ovarian tissues and is defined as I/R damage. Actually, anticoagulant treatment is administered after detorsion to minimize the I/R damage. The effects of enoxaparin in reducing I/R damage have been shown in previous publications[7,8]. α-LA is a powerful antioxidant and its curative effect on I/R damage has been demonstrated in ovarian torsion models[11,12]. However, the effect of their combined use on ovarian I/R damage has not been studied yet. The results of the current study demonstrated that the combined use of enoxaparin and α-LA has a more protective effect against tissue damage caused by I/R than the use of enoxaparin alone.

Both serum TOS levels and tissue MDA levels were significantly higher in Group 2 than in Group 1. Significant decreases were found in the levels of GPx and CAT, which are antioxidant markers, and significant increase in TDS and MAI was found during histopathological examination. Serum TOS, OSI values and tissue MDA levels were significantly higher in Group 3 and Group 4, compared to Group 2. In view of the findings, it can be said that the severity of the tissue damage increased after reperfusion. The I/R damage was seen to be greater than the damage caused by ischemia alone. In addition, the increase in serum TOS and OSI levels showed that the oxidative stress caused by this damage at the tissue level was also reflected in the systemic circulation. Parallel findings were found in the histopathological examination. In Group 2, significant damage occurred in the stroma and granulosa cells of the ovaries. It has been stated in the literature that the damage caused by ischemia is exacerbated by reperfusion[1-10,12,14,15]. The results of a previous study on ovarian torsion were similar[4]. Comparisons were made of a Sham group, ischemia group and reperfusion group in the I/R model and it was reported that the highest ovarian damage score was obtained in the detorsion group. While inhibin B and E_2 levels were not much affected after torsion, AMH levels decreased after I/R[14]. A significant decrease was determined in serum OSI levels and tissue MDA levels in Group 7 compared to Group 3, and there was laso a significant decrease in the serum TAS levels of the antioxidant markers ($p<0.05$). A significant increase was found in tissue GPx and CAT activity, while a significant reduction was detected in TDS and MAI. These results show that the concomitant use of enoxaparin and α-LA reduces I/R injuries. In the oxidative stress tests of Group 7 compared to Group 5, a significant decrease was determined in TOS, OSI, and MDA levels. Histopathological examination showed improvements in TDS and MAI in ovarian tissues, but this did not reach statistically significance.

In the oxidative stress tests of Group 8 compared to Group 6, a significant decrease was found in TOS and MDA levels, while a significant increase in GPx. Histopathologically, a significant decrease was found in TDS in ovarian tissues. These results show the protective effect of the co-administration of enoxaparin and α-lipoic acid against tissue damage caused by I/R. In line with our results, Sahin Ersoy et al[7] stated that the use of enoxaparin in ovarian torsion was effective in reducing I/R damage. They also reported that AMH values were higher in the enoxaparin group compared to the drug-free group.

A significant increase in GPx was found in Group 6 compared to Group 4. Biochemical and histopathological findings were similar to these findings. Combined use of enoxaparin and α-LA led to significant decrease in TOS, MDA, and GPx levels compared to the use of enoxaparin alone. Ozler et al[14] stated that detorsion alone is not sufficient to reduce I/R damage. Kaya et al[8] also showed that use of enoxaparin in T/D model protects the AMH values. Tuncer et al[12] reported that α-lipoic acid and coenzyme Q had a protective effect in ovarian I/R damage. Likewise, Sak et al[9] reported the protective effect of curcumin against I/R injury. In a review by Firuzi et al[11] the application of antioxidant therapy was shown to have a beneficial effect on ischemic injury. There are also reports that the administration of N-actylcysteine[7] magnesium[22], Genistein[23], Melatonin[6], Atorvastatin[24], and Platelet Rich Plasma (PRP)[25] have a protective effect against the ovarian I/R damage.

Conclusions

The current study demonstrated that an increase in oxidative stress and a decrease in antioxidants occurred following I/R. Parallel with these markers, evidence of ovarian damage was observed in H&E and Apoptosis Tag staining. When enoxaparin and α-LA are administered together, I/R damage was remarkable reduced compared drugs used alone. In the groups applied with enoxaparin, the ovarian functions were preserved. Surgical detorsion alone is insufficient to reduce I/R damage, and enoxaparin and α-LA combination can be used together to reduce this damage.

Conflict of Interest

The authors declare that they have no conflict of interest.

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