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Effects of alpha-lipoic acid on skeletal muscle ischemia-reperfusion injury in mice

Farelerde iskelet kasında iskemi reperfüzyon hasarında alfa lipoik asidin etkileri

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Abstract
Aim: The main problem in the treatment of cardiovascular diseases, which is the most common cause of death in the world today, is reperfusion injury. In our study, we investigated the effects of alpha-lipoic acid on lower limb ischemia-reperfusion injury.
Methods: In this study, 30 male Swiss breed albino mice weighing 45-65 g were used. Mice were randomly divided into 5 groups (Control group (C), Dimethyl Sulfoxide (DMSO) group, ischemia-reperfusion (IR) group, alpha-lipoic acid control group (ALA), alpha-lipoic acid - IR group (ALA+IR)), 6 mice in each group. All groups were administered 100 IU/kg intravenous heparin bolus 30 minutes before the procedure. Lower limb ischemia (2 hours) and reperfusion (2 hours) protocols were applied to the IR and ALA+IR groups by clamping the main femoral artery. Alpha-lipoic acid was administered to ALA and ALA+IR groups 1 hour before the experimental procedure (100 mg/kg intraperitoneal). After the experiment protocol, the lower limb gastrocnemius muscles were collected from the sacrificed mice and total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), Myeloperoxidase (MPO) and Malondialdehyde (MDA) levels were determined in the tissue. In addition, edema, necrosis, and inflammatory cell infiltration levels in the skeletal muscle were determined by histologic grading, and apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method.
Results: TAS measurements of ALA group subjects were higher than the control group (P=0.01). The TOS level of the ALA+IR group was lower than the IR group (P=0.01), but higher than the C, ALA, and DMSO groups. The OSI index of the IR group was significantly higher than the ALA+IR group (P=0.01). It was observed that the IR group was at higher levels than the ALA+IR group (P=0.01). MDA measurements of IR group subjects were higher than that of the C, ALA, DMSO, ALA+IR groups (P=0.01). Edema of the IR and ALA+IR group subjects were higher than the C, ALA, DMSO groups (P=0.01). Necrosis measurements of IR and ALA+IR group subjects were higher than C, ALA, DMSO groups (P=0.01). Inflammatory cell infiltration measurements of IR and ALA+IR group subjects were higher than C, ALA, DMSO groups (P=0.01). Apoptosis level was significantly lower in the ALA+IR group than in the IR group. (P=0.01).
Conclusion: The results showed us that alpha-lipoic acid has a protective effect against oxidative damage, inflammation, and apoptosis in the tissue in lower limb ischemia-reperfusion.
Keywords: Alpha-lipoic acid, Ischemia-reperfusion injury, Skeletal muscle

Öz
Amaç: Günümüzde dünya üzerinde en sık ölüm nedeni olan kardiovasküler hastalıkların tedavisnde temel sorun reperfüzyon hasarıdır. Çalışmamızda alfa lipoik asid'in alt ekstremite iskemi reperfüzyon hasarında etkilerini araştırdık.
Yöntemler: Çalışmada ağırlıkları 45-65 gr arasında değişen 30 adet, erkek Swiss cinsi albino fare kullanıldı. Fareler her grupta 6 tane olmak üzere, rastgele 5 gruba ayrıldı (Kontrol grubu (C), Dimethyl Sulphoxyde (DMSO) grubu, iskemi- reperfüzyon (İR) grubu, alfa-lipoik asid kontrol grubu (ALA), alfa-lipoik asid - İR grubu (ALA+İR)). Tüm gruplara işlemden 30 dakika önce 100 IU/kg heparin intravenöz bolus olarak uygulandı. İ-R ve ALA+İR gruplarına ana femoral arter klemplenerek alt ekstremite iskemisi (2 saat) ve reperfüzyon (2 saat) protokolü uygulandı. ALA ve ALA+İR gruplarına deney prosedüründen 1 saat önce alfa lipoic asid uygulaması (100 mg/kg intraperitoneal) yapıldı. Deney protokolü sonrası farelerin alt ekstremite gastroknemus kası sakrifiye edildi ve dokudan totalantioksidan status (TAS), total oksidan status (TOS), oksidatif stress indexi (OSI), Myeloperoxidase (MPO) ve Malondialdehyde (MDA) düzeyleri bakıldı. Ayrıca iskelet kasından histolojik gradeleme ile ödem, nekroz ve inflamatuar hücre infiltrasyon düzeyleri ile Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) yöntemi ile apoptozis tayini yapıldı.
Bulgular: ALA grubu deneklerinin TAS ölçümlerinin kontrol grubunda göre daha yüksek düzeylerde olduğu tespit edilmiştir (P=0,01). ALA+İR grubunun TOS seviyesi IR grubuna göre daha düşük çıkmış (P=0,01) ancak C, ALA ve DMSO gruplarına göre yüksek olarak tespit edilmiştir. IR grubunun OSI indeksi, ALA + İR grubunda anlamlı derecede yüksek çıkmıştır (P=0,01). İR grubun ALA+İR grubundan daha yüksek düzeylerde olduğu görülmüştür (P=0,01). İR grubu deneklerinin MDA ölçümlerinin C, ALA, DMSO, ALA+İR gruplarına göre daha yüksek düzeylerde olduğu tespit edilmiştir (P=0,01). İR ve ALA+İR grubu deneklerinin ödem ölçümlerinin C, ALA, DMSO gruplarına göre daha yüksek düzeylerde olduğu tespit edilmiştir (P=0,01). İR ve ALA+İR grubu deneklerinin nekroz ölçümlerinin C, ALA, DMSO gruplarına göre daha yüksek düzeylerde olduğu tespit edilmiştir (P=0,01). İR ve ALA+İR grubu deneklerinin inflammatory cell infiltration ölçümlerinin C, ALA, DMSO gruplarına göre daha yüksek düzeylerde olduğu tespit edilmiştir (P=0,01). Apoptozis düzeyi ALA+İR grubunda İR grubuna göre anlamlı olarak düşük çıkmıştır (P=0,01).
Sonuç: Sonuçlar bize alfa lipoik asid'in alt ekstremite iskemi reperfüzyonunda dokuda oluşan oksidan hasara ve inflamsyona ve apoptozise karşı koruyucu etkinliğinin olduğunu gösterdi.
Anahtar kelimeler: Alfa lipoik asit, İskemi reperfüzyon hasarı, İskelet kası

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Ethics Committee Approval: Our study was carried out at Gazi University Experimental Animals Research Center (GÜDAM) with the approval received from Gazi University Animal Experiments Local Ethics Committee, dated 21/01/2020, numbered E.2321 with the code number G.Ü.E.T.-20.004.
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Introduction

Ischemia-reperfusion injury is common in many clinical conditions such as acute coronary syndromes, peripheral vascular diseases, and organ transplant procedures [1]. Ischemia-reperfusion (IR) injury is the damage caused by the return of blood flow to the tissue, especially after the lack of oxygen. The lack of oxygen and nutrients during ischemia induces many events related to oxidative damage and inflammation. The main mechanism of cardiovascular diseases, the most common cause of death worldwide, is ischemia-reperfusion injury [2]. For these reasons, ischemia reperfusion injury has been an area of interest for researchers [3,4].

Alpha-lipoic acid (α-LA) is a short-chain fatty acid that occurs naturally and contains 2 sulfur molecules [5]. Although alpha-lipoic acid is sufficiently found in the human diet, it is synthesized de novo by lipoic acid synthase in mitochondria. It dissolves in both lipid and aqueous media is easily absorbed and transported to the cells and reduced to DHLA [6]. It is the dithiolane ring that provides the chemical reactivity of α-LA and its reduced form, DHLA. This structure makes α-LA unique among other known biomolecules containing thiol [7]. Due to its low redox potential and unique reduction capacity, α-LA, which is involved in the capture of reactive oxygen derivatives and the reduction of oxidized forms of other antioxidants, is called the 'antioxidant of antioxidants' [8].

In our study, we investigated the effects of alpha-lipoic acid on changes caused by skeletal muscle ischemia-reperfusion injury.

Materials and methods

The study was conducted at Gazi University Laboratory Animal Breeding and Experimental Researches Center (GÜDAM) with the permission obtained from Gazi University Animal Experiments Local Ethics Committee with the code number G.Ü.ET-20.004. Alpha-lipoic acid used in the study was provided from Sigma-Aldrich company without any institutional or company support. The study protocol was drawn up with the permission obtained from Gazi University Faculty of Medicine Animal Experiments Ethics Committee with the code number G.Ü.ET-20.004. All procedures were carried out in accordance with the Standards for the Care and Use of Laboratory Animals.

Technical procedure

General anesthesia was achieved with Ketamine (90 mg/kg) + Xylazine (10 mg/kg) before surgery. All groups were administered 100 IU/kg intravenous heparin bolus 30 minutes before the procedure. Subsequently, the abdominal area was shaved and excess hair was removed. The surgical area was cleaned with an antiseptic solution. Before starting the surgical procedure, it was tested whether anesthesia was sufficient. The surgical procedure was performed under a heat lamp while the mice were in the supine position. A longitudinal skin incision was made on the mice in the inguinal region. Common-superficial and profundal femoral artery were explored. An atraumatic microvascular clamp was placed on the common femoral artery. Following the 120-minute ischemic period, the microvascular clamp on the common femoral artery was removed and reperfusion was achieved for 120 minutes. The same time (240 minutes) was awaited by applying an inguinal incision on the mice forming the control group, but IR was not generated in these groups.

In the study, 30 male Swiss breed albino mice weighing 45-65 g were used. Mice were randomly divided into 5 groups, 6 mice in each group. (Control group (C), Dimethyl Sulfoxide (DMSO) group, ischemia-reperfusion (IR) group, alpha-lipoic acid control (ALA) group, alpha-lipoic acid - IR (ALA+IR) group)

Control group (Gr-I, n=6): After heparin administration, only an inguinal incision was made and closed without ischemia in this group of mice. The mice were sacrificed under anesthesia four hours after the procedure.

DMSO group (Gr-II, n=6): After heparin administration, Dimethyl Sulfoxide (DMSO), in which alpha-lipoic acid is dissolved, was intraperitoneally administered to mice in this group without ischemia, only an inguinal incision was made and closed, and four hours after the procedure the mice were sacrificed under anesthesia.

Ischemia-Reperfusion group (Gr-III, n=6): After heparin administration to the mice in this group, an inguinal incision was made without injection of alpha-lipoic acid. Atraumatic microvascular clamp was placed on the main femoral artery, and mice were sacrificed under anesthesia after 120 minutes of ischemia and 120 minutes of reperfusion.

Alpha-lipoic acid group (Gr-IV, n=6): After heparin administration to the mice in this group, a dose of 100 mg/kg alpha-lipoic acid was administered intraperitoneally without ischemia, only an inguinal incision was made and closed, and four hours after the procedure the mice were sacrificed under anesthesia.

Alpha-lipoic acid Ischemia-Reperfusion group (Gr-V, n=6): In this group, alpha-lipoic acid was administered intraperitoneally at a dose of 100 mg/kg 1 hour before ischemia. An inguinal incision was made 30 minutes after heparin administration. Atraumatic microvascular clamp was placed on the main femoral artery, and mice were sacrificed under anesthesia after 120 minutes of ischemia and 120 minutes of reperfusion.

Tissue homogenization

The hind limb tissue was collected in a sterile Eppendorf tube and was stored at −80°C until total antioxidant/oxidant status and oxidative stress index analysis. Tissues were quickly weighed on a precision scale without allowing them to dissolve. These frozen tissue samples were ground in a porcelain mortar by adding liquid nitrogen. The pulverized tissue was transferred to the homogenization tube adding 140 mM KCl solution per gram of tissue, with a dilution of 1/10. To prevent temperature rise during homogenization, the tube was kept in a glass beaker filled with ice for 2 minutes at 50 rpm rotation speed before and after homogenization with homogenizer. Homogenates were transferred to Eppendorf tubes and the tubes were covered with paraffin and then centrifuged at 3,000 rpm for 10 minutes. After centrifugation, the supernatant was collected in another Eppendorf tube and made suitable for the measurement of total oxidative status (TOS) and total antioxidant status (TAS) [9].

TAS measurement

Samples were studied on the fully automated Mindray BS300 device with the Relassay® kit. 300 µL of reagent 1 (measuring buffer) and 18 mcL of the sample were taken and mixed in the cuvette. The first reading was made at 660 nm after 30 seconds. Next, 45 µL of reagent 2 (colored 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was added to the mixture, and absorbance was measured at 660 nm after 5 minutes of incubation. For standard measurement, the Trolox Eq solution at a concentration of 1 mmol/L was equally used instead of the sample. The first and second measurements were performed three times, and their average were calculated. The absorbance change (ΔAbs) was calculated by subtracting the first absorbance value (A1) from the second absorbance value (A2). TAS levels were calculated using the formula given in the kit and expressed as mmol Trolox Eq/L. TAS=$\Delta AbsH_{2nd}O$ - Abs sample / $\Delta AbsH_{2nd}O$ - Abs standard

TOS measurement

Samples were studied on the fully automated Mindray BS300 device with the Relassay® kit. 300 µl of reagent 1 (measuring buffer) and 45 µl of the sample were taken and mixed in the cuvette. After 30 seconds, the first reading was obtained at 530 nm. Subsequently, 15 µl of reagent 2 (Pro-chromogenic solution) was mixed and left for 5 minutes in the incubator and the second reading was taken at 530nm. A standard solution containing 10 µmol/L hydrogen peroxide (H_20_2) equivalent/liter supplied in the kit was used for standard measurement. The first and second measurements were performed three times, and their averages were calculated. The absorbance change (ΔAbs) was calculated by subtracting the first absorbance value (A1) from the second absorbance value (A2). TOS levels were calculated using the formula provided in the kit and expressed as mmol H202 Eq/L. TOS= Δ Abs sample /Δ Abs standard X Standard Concentration 10 µmol/L

Oxidative Stress Index (OSI)

The ratio of TOS to TAS was accepted as the oxidative stress index (OSI). For calculation, the resulting TAS unit was converted to µmol/L and the OSI value was calculated according to the formula below. OSI (arbitrary unit) =TOS (µmol H2O2 equivalent/L)/ TAC (µmol Trolox equivalent/L) [10-12].

Myeloperoxidase (MPO) measurement

MPO possesses various catalytical activities. It exhibits the main catalytical activity by the production of hypochlorous acid (HClO) from hydrogen peroxide (H2O2) and chloride anion, Cl- (or halide). MPO also exhibits peroxidase activity that catalyzes the oxidation of several substrates by H_2O_2. These reaction categories have been widely used to assess the activities of MPO [13].

The Relassay Myeloperoxidase Chlorination Activity Assay Kit and the Relassay Myeloperoxidase Peroxidation Activity Assay Kit are quantitative and colorimetric assay kits for measuring the myeloperoxidase activity within a sample. In the Relassay Myeloperoxidase Chlorination Activity Assay Kit, MPO catalyzes the formation of hypochlorous acid, which reacts with taurine to form taurine chloramine. Taurine chloramine reacts with the chromophore TNB, resulting in the formation of the colorless product DTNB. One unit of MPO activity is defined as the amount of enzyme that hydrolyzes the substrate and generates taurine chloramine to consume 1.0 µmole of TNB per minute. In the Relassay Myeloperoxidase Peroxidation Activity Assay Kit, MPO catalyzes o-dianisidine to colored o-dianisidyl radical using H2O2. The increasing absorbance is monitored at 412 nm and the activity is measured kinetically. This kit can be used manually and is easily adapted to automated analyzers.

Malondialdehyde (MDA) measurement

The MDA level is an end-product of lipid peroxidation at the end of the reperfusion process and an indication of increased free radical production [14]. MDA has been an important biochemical parameter measured in many experimental animal studies to evaluate the oxidative stress level [15].

The tissue MDA level was determined by a method based on the reaction with thiobarbituric acid (TBA) at 90-100°C. In the TBA test reaction, MDA or MDA-like substances and TBA react with the production of a pink pigment with maximum absorption at 532 nm. The reaction was performed at pH 2–3 at 90°C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid for the precipitation of protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed as nmol/g wet tissue.

Histopathological analysis

Tissue samples taken from the patient were rapidly thrown into 10% neutral buffered formalin. Routine histopathological processing steps were performed after 48 hours of fixation. At the end of processing, tissue samples were embedded in paraffin blocks. 5-micron sections from the blocks were stained with Hematoxylin-Eosin. Stained sections were graded according to necrosis, edema, and inflammatory cell infiltration criteria as follows (0: No damage, 1: Slight damage, 2: Moderate damage, 3: Severe damage) [16,17]. The evaluation of the stained sections prepared from the tissues was performed with the microscope with Olympus CX43 camera attachment.

Determination of Apoptosis by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) Method: 4-micron sections from paraffin blocks were painted using commercial TUNEL kit (ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit, LOT: 2789455 Merc) after deparaffinization and hydration procedures. In order to determine the apoptotic index (AI), 5 randomly selected regions of each section were chosen under x400 magnification. Cells stained brown or black were considered TUNEL-positive apoptotic cells. The AI of hepatocytes was determined as the percentage of TUNEL positive cells with respect to the total number of cells counted using the

Formula: Apoptotic index (AI)= (Number of positive cells / Total number of cells counted) x100

Statistical analysis

In the analysis of the data, descriptive statistics were presented with mean, and standard deviation values. In the study, the Kruskal-Wallis test was used to analyze the difference between the measurement values of 6 different groups. Mann-Whitney U test was used for each pair to identify the groups which differ. In the study, p values less than 0.05 were

considered statistically significant. All analyses were made using SPSS 22.0 software package.

Results

TAS measurements differed among the study groups (*P*=0.01): That of the ALA group subjects were higher than that of the C group (*P*=0.01).

TOS measurements differed among the study groups (*P*=0.01). TOS measurements of the IR group were higher than the C, ALA, DMSO, ALA+IR groups (*P*=0.01). Besides, the TOS level of the ALA+IR group was lower than the IR group (*P*=0.01), and higher than C, ALA, and DMSO groups.

OSI measurements differed among the study groups (*P*=0.01). OSI measurements of the IR group were higher than the C, ALA, DMSO, ALA+IR groups (*P*=0.01). The OSI index of the IR group was significantly higher than the ALA+IR group (*P*=0.01).

MPO measurements differed according to the study groups (*P*=0.01). MPO measurements of the IR group were higher than the C, ALA, DMSO, ALA+IR groups (*P*=0.01). Besides, that in the IR group was higher than that in the ALA+IR group (*P*=0.01).

MDA measurements differed according to the study groups (*P*=0.01). MDA measurements of the IR group subjects were higher than the C, ALA, DMSO, ALA+IR groups (*P*=0.01).

Edema measurements differed according to the study groups (*P*=0.01). Edema levels of the IR and ALA+IR group subjects were higher than those of the C, ALA, DMSO groups (*P*=0.01). There was no significant difference between IR and ALA+IR groups (*P*=0.06) (Table 1)

Necrosis differed with the study groups (*P*=0.01). Necrosis in the IR and ALA+IR group subjects were higher than those in the C, ALA, DMSO groups (*P*=0.01). There was no significant difference between IR and ALA+IR groups (*P*=0.06).

Inflammatory cell infiltration measurements differed with the study groups (*P*=0.01). Inflammatory cell infiltration measurements of IR and ALA+IR group subjects were higher than those in the C, ALA, DMSO groups (*P*=0.01).

TUNEL measurements were different among the study groups (*P*=0.01). TUNEL measurements of the IR and ALA+IR group subjects were higher than those in the C, ALA, DMSO groups (*P*=0.01). Apoptosis was significantly lower in the ALA+IR group than in the IR group (*P*=0.01) (Table 2, Figure 1, 2).

Table 1: TAS (mmol/L), TOS, OSI, MPO, MDA, and groups

Group	Control (1) X (SD)	Alfa lipoic acid (2) X (SD)	DMSO (3) X (SD)	Mouse ischemia reperfusion (4) X (SD)	Alfa lipoic acid + IR (5) X (SD)	*P*-value
TAS mmol/L	0.27 (0.02)	0.44 (0.03)	0.31 (0.04)	0.30 (0.03)	0.35 (0.03)	0.01
	Difference: 1-2 (*P*=0.01)*, 1-3 (*P*=0.46), 1-4 (*P*=0.49), 1-5 (*P*=0.08), 2-3 (*P*=0.12), 2-4 (*P*=0.11), 2-5 (*P*=0.21), 3-4 (*P*=0.86), 3-5 (*P*=0.36), 4-5 (*P*=0.51)					
TOS µmol/L	3.29 (0.41)	3.31 (0.11)	3.54 (0.9)	7.17 (0.8)	4.22 (0.31)	0.01
	Difference:1-2 (*P*=0.56), 1-3 (*P*=0.32), 1-4 (*P*=0.01)*, 1-5 (*P*=0.10), 2-3 (*P*=0.27), 2-4 (*P*=0.01)*, 2-5 (*P*=0.12), 3-4 (*P*=0.01)*, 3-5 (*P*=0.14), 4-5 (*P*=0.01)*					
OSI	1.21 (0.12)	0.76 (0.06)	1.25 (0.25)	2,43 (0.27)	1.21 (0.1)	0.01
	Difference: 1-2 (*P*=0.12), 1-3 (*P*=0.67), 1-4 (*P*=0.01)*, 1-5 (*P*=0.92), 2-3 (*P*=0.09), 2-4 (*P*=0.01)*, 2-5 (*P*=0.13), 3-4 (*P*=0.01)*, 3-5 (*P*=0.81), 4-5 (*P*=0.01)*					
MPO U/L	54.42 (4.19)	55.55 (4.39)	53.71 (1.34)	80.34 (2.82)	61.92 (4.15)	0.01
	Difference: 1-2 (*P*=0.74), 1-3 (*P*=0.63), 1-4 (*P*=0.01)*, 1-5 (*P*=0.01)*, 2-3 (*P*=0.58), 2-4 (*P*=0.01)*, 2-4 (*P*=0.01)*, 3-4 (*P*=0.01)*, 2-4 (*P*=0.01)*, 4-5 (*P*=0.01)*					
MDA nmol/L	0.43 (0.04)	0.38 (0.19)	0.45 (0.03)	0.69 (0.04)	0.55 (0.03)	0.01
	Difference: 1-2 (*P*=0.16), 1-3 (*P*=0.32), 1-4 (*P*=0.01)*, 1-5 (*P*=0.10), 2-3 (*P*=0.37), 2-4 (*P*=0.01)*, 2-5 (*P*=0.06), 3-4 (*P*=0.01)*, 3-5 (*P*=0.12), 4-5 (*P*=0.01)*					

** Kruskal-Wallis test, * shows significant difference, ** Mann-Whitney U test as difference test, SD: Standard deviation

Table 2: TUNEL, inflammatory cell infiltration, edema, necrosis measurements by groups

Group	Control (1) X (SD)	Alfa lipoic acid (2) X (SD)	DMSO (3) X (SD)	Mouse ischemia reperfusion(4) X (SD)	Alfa Lipoic acid + IR(5) X (SD)	*P*-value
Edema	0 (0)	0 (0)	0 (0)	2.5 (0.55)	2.17 (0.98)	0.01
	Difference: 1-2 (*P*=0.99), 1-3 (*P*=0.99), 1-4 (*P*=0.01)*, 1-5 (*P*=0.01)*, 2-3 (*P*=0.99), 2-4 (*P*=0.01), 2-5(*P*=0.01)*, 3-4 (*P*=0.01)*, 3-5 (*P*=0.01)*, 4-5 (*P*=0.18)					
Necrosis	0.17 (0.41)	0.50 (0.55)	0.17 (0.41)	2.67 (0.52)	1.83 (1.17)	0.01
	Difference: 1-2 (*P*=0.13), 1-3 (*P*=0.99), 1-4 (*P*=0.01)*, 2-3 (*P*=0.13), 2-4 (*P*=0.01)*,2-5 (*P*=0.01)*, 3-4 (*P*=0.01)*, 3-5 (*P*=0.01)*, 4-5 (*P*=0.06)					
Inflammatory cell infiltration	0 (0)	0.33 (0.52)	0.17 (0.41)	2.67 (0.52)	1.83 (0.75)	0.01
	Difference: 1-2 (*P*=0.01)*, 1-3 (*P*=0.01)*, 1-4 (*P*=0.01)*, 1-5 (*P*=0.01)*, 2-3 (*P*=0.18), 2-4 (*P*=0.01), 2-5 (*P*=0.01)*, 3-4 (*P*=0.01)*, 3-5 (*P*=0.01)*, 4-5 (*P*=0.07)					
TUNEL	1 (1.17)	7 (1.41)	6.58 (3.14)	3.5 (2.17)	30.17 (6.79)	0.01
	Difference: 1-2 (*P*=0.01)*, 1-3 (*P*=0.01)*, 1-4 (*P*=0.01)*, 1-5 (*P*=0.01)*, 2-3 (*P*=0.41), 2-4 (*P*=0.08), 2-5 (*P*=0.01)*, 3-4 (*P*=0.09), 3-5 (*P*=0.01)*, 4-5 (*P*=0.01)*					

** Kruskal-Wallis test, * shows significant difference, ** Mann-Whitney U test as difference test, SD: Standard Deviation

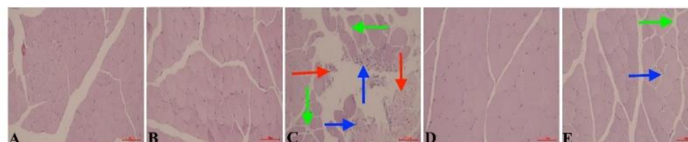

Figure 1: H&E stained photographs of histological sections of experimental groups. A, B, C, D, and E belong to the Control, DMSO, IR, ALA, and ALA+IR groups, respectively. The photographs show areas of necrosis (red arrow), edema (green arrow), and inflammatory cell infiltration (blue arrow) (Magnification x200).

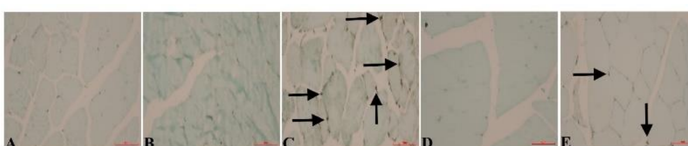

Figure 2: Photographs of sections stained with the TUNEL method for the evaluation of apoptosis in experimental groups. A, B, C, D, and E belong to the Control, DMSO, IR, ALA, and ALA+IR groups, respectively. In the photos, arrows show apoptotic cells (Magnification x400).

Discussion

Alpha-lipoic acid, a naturally occurring thiol compound, has long been known as an essential cofactor for mitochondrial bio-energetic enzymes. Alpha-lipoic acid and its reduced form of dihydrolipoic acid (DHLA) have been observed to strongly cleanse reactive oxygen radicals in vitro experiments [18]. However, more in vivo studies are needed to determine whether ALA and DHLA act directly as antioxidants [19]. Therefore, we investigated the effects of alpha-lipoic acid on lower limb ischemia-reperfusion injury, which is very common among cardiovascular diseases, on the animal model. We found that alpha-lipoic acid therapy has protective effects in lower limb ischemia-reperfusion injury, especially against oxidative damage and inflammation and apoptosis in skeletal muscle.

In total antioxidant status (TAS) measurements, the antioxidant levels of the ALA group were significantly higher than of the control group. However, although the TAS level of the ALA group was numerically higher than the TAS level of the other groups, no statistically significant difference was observed. Though not statistically significant, the level of TAS in the ALA+IR group being higher than the IR and ALA groups may be an indicator of the antioxidant activity of alpha-lipoic acid.

In total oxidant status (TOS) measurements, the TOS level of the IR group was significantly higher than the other groups. Also, the TOS level of the ALA+IR group was significantly lower than that of the IR group. These results show us that alpha-lipoic acid reduces the oxidative status in the tissue.

The oxidative stress index (OSI), which is the ratio of total oxidative status and total anti-oxidative status in the tissue, is a better indicator for the level of tissue damage associated with reperfusion compared to TAS and TOS as it reflects the balance between the oxidants and antioxidants in the tissue. The fact that the OSI level was significantly lower in the ALA+IR group

compared to the IR group indicates that alpha-lipoic acid shifts the balance between oxidants and antioxidants formed in the tissue in reperfusion injury in favor of antioxidants. According to these results, it can be claimed that alpha-lipoic acid protects the tissue against oxidative damage in reperfusion injury.

In a study, it was observed that MDA and MPO levels increased in all periods of reperfusion in ischemia-reperfusion. In addition, it has been determined that the longer the reperfusion period, the higher the damage to the skeletal muscle [20]. Therefore, measuring the MDA and MPO, both products of lipid peroxidation that occur during ischemia-reperfusion injury, can give an idea about the degree of reperfusion injury. As a matter of fact, in this study, MDA and MPO measurements were made to evaluate reperfusion injury [21].

In our study, the fact that the MDA level was significantly higher in the IR group compared to the ALA+IR group shows that alpha-lipoic acid has protective effects against lipid peroxidation in reperfusion injury. The fact that the MDA level of the ALA+IR group is significantly higher than DMSO and ALA groups shows that alpha-lipoic acid decreases the MDA level in reperfusion injury but cannot decrease it to a level close to the control groups.

The values of MPO secreted from leukocyte and mononuclear cells, which is an inflammatory indicator, in the treatment group were significantly lower than that detected in the IR group. The low level of MPO in the treatment group shows that alpha-lipoic acid also prevents leukocyte and mononuclear cell migration into ischemic-reperfused muscle tissue and thus may create an anti-inflammatory effect. These effects that reduce inflammatory cell migration show that alpha-lipoic acid can also be effective in reducing damage in muscle cells in reperfusion injury.

In the case of ischemia-reperfusion, reactive oxygen molecules are produced, and the oxygenation and nutrient intake of the cell decreases, and eventually necrosis develops in the cells [22]. In one study, it was found that alpha-lipoic acid reduced cardiac dysfunction by reducing necrosis, inflammation, and apoptosis after myocardial ischemia-reperfusion injury [23]. In another study on people with type II diabetes mellitus, the effects of oral alpha-lipoic acid therapy on oxidative stress and inflammation were investigated. Patients receiving alpha-lipoic acid therapy were similar to those receiving placebo treatment. It is stated that this result may be dose-dependent [24]. However, the route of drug administration is important in the anti-inflammatory activity of alpha-lipoic acid. The effectiveness of oral administration of treatment is questioned [25,26].

In our study, histologic grading was performed on skeletal muscle and edema, necrosis, inflammatory cell infiltration parameters were examined. In histologic grading, it was observed that the ALA+IR group had numerically lower edema, necrosis, and inflammatory cell infiltration values than the IR group. However, no statistically significant difference was observed. Evaluating our pathology results together with tissue MPO in the evaluation of inflammatory cell infiltration, it can be claimed that alpha-lipoic acid reduces inflammation in reperfusion injury.

In the assessment of apoptosis with the TUNEL method, the significant difference between the number of apoptotic cells in the ALA+IR group and those in the IR group indicates that the alpha-lipoic acid has antiapoptotic activity in reperfusion injury.

Similar to our study on skeletal muscle, in another study, it was found that alpha-lipoic acid treatment reduced the production of reactive oxygen radicals, MDA production, and apoptosis in cardiac myocytes [27].

Limitations

The fact that edema, necrosis, and inflammation were numerically high but not statistically significant in histologic grading showed us that more animals should be used in the study groups.

Conclusion

It can be claimed that alpha-lipoic acid has a protective effect against oxidant damage, inflammation, and apoptosis in the tissue in lower limb ischemia-reperfusion injury. An important aspect of our study is that it was conducted in vivo. However, larger doses and clinical studies of the protective effect of alpha-lipoic acid against reperfusion injury are needed.

References

1. Carter SE, Faulkner A, Rakobowchuk M. The role of prostaglandin and antioxidant availability in recovery from forearm ischemia-reperfusion injury in humans. J Hypertens. 2014 Feb;32(2):339-51. doi: 10.1097/HJH.0000000000000033. PMID: 24296519; PMCID: PMC3914903.
2. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. J Pathol. 2000 Feb;190(3):255-66. doi: 10.1002/(SICI)1096-9896(200002)190:3<255::AID-PATH526>3.0.CO;2-6. PMID: 10685060.
3. Geldi O, Kubat E, Ünal CS, Canbaz S. Acetaminophen Mitigates Myocardial Injury Induced by Lower Extremity Ischemia-Reperfusion in Rat Model. Braz J Cardiovasc Surg. 2018 May-Jun;33(3):258-64. doi: 10.21470/1678-9741-2017-0218. PMID: 30043918; PMCID: PMC6089134.
4. Eken H, Kurnaz E. Biochemical and histopathological evaluation of taxifolin: An experimental study in a rat model of liver ischemia reperfusion injury. J Surg Med. 2019;3(7):494-7.
5. Biewenga GP, Haenen GR, Bast A. The pharmacology of the antioxidant lipoic acid. Gen Pharmacol. 1997 Sep;29(3):315-31. doi: 10.1016/s0306-3623(96)00474-0. PMID: 9378235.
6. Cakatay U. Pro-oxidant actions of alpha-lipoic acid and dihydrolipoic acid. Med Hypotheses. 2006;66(1):110-7. doi: 10.1016/j.mehy.2005.07.020. Epub 2005 Sep 13. PMID: 16165311.
7. St Clair D, Zhao Y, Chaiswing L, Oberley T. Modulation of skin tumorigenesis by SOD. Biomed Pharmacother. 2005 May;59(4):209-14. doi: 10.1016/j.biopha.2005.03.004. Epub 2005 Mar 17. PMID: 15862717.
8. Smith AR, Shenvi SV, Widlansky M, Suh JH, Hagen TM. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. Curr Med Chem. 2004 May;11(9):1135-46. doi: 10.2174/0929867043365387. PMID: 15134511.
9. Kılıç Y, Özer A, Tatar T, Zor MH, Kirişçi M, Kartal H, et al. Effect of picroside II on hind limb ischemia reperfusion injury in rats. Drug Des Devel Ther. 2017 Jun 26;11:1917-25. doi: 10.2147/DDDT.S132401. PMID: 28721011; PMCID: PMC5500556.
10. Yumru M, Savas HA, Kalenderoglu A, Bulut M, Celik H, Erel O. Oxidative imbalance in bipolar disorder subtypes: a comparative study. Prog Neuropsychopharmacol Biol Psychiatry. 2009 Aug 31;33(6):1070-4. doi: 10.1016/j.pnpbp.2009.06.005. Epub 2009 Jun 12. PMID: 19527764.
11. Kosecik M, Erel O, Sevinc E, Selek S. Increased oxidative stress in children exposed to passive smoking. Int J Cardiol. 2005 Apr 8;100(1):61-4. doi: 10.1016/j.ijcard.2004.05.069. PMID: 15820286.
12. Harma M, Harma M, Erel O. Increased oxidative stress in patients with hydatidiform mole. Swiss Med Wkly. 2003 Nov 1;133(41-42):563-6. PMID: 14691728.
13. Tullis MJ, Brown S, Gewertz BL. Hepatic influence on pulmonary neutrophil sequestration following intestinal ischemia-reperfusion. J Surg Res. 1996 Dec;66(2):143-6. doi: 10.1006/jsre.1996.0386. PMID: 9024826.
14. Witt EH, Reznick AZ, Viguie CA, Starke-Reed P, Packer L. Exercise, oxidative damage and effects of antioxidant manipulation. J Nutr. 1992 Mar;122(3 Suppl):766-73. doi: 10.1093/jn/122.suppl_3.766. PMID: 1514950.
15. Tural K, Ozden O, Bilgi Z, Kubat E, Ermutlu CS, Merhan O, et al. The protective effect of betanin and copper on heart and lung in end organ ischemia reperfusion injury. Bratisl Lek Listy. 2020;121(3):211-7. doi: 10.4149/BLL_2020_032. PMID: 32115979.
16. Elmali N, Esenkaya I, Karadağ N, Taş F, Elmali N. Effects of resveratrol on skeletal muscle in ischemia-reperfusion injury. Ulus Travma Acil Cerrahi Derg. 2007 Oct;13(4):274-80. PMID: 17978908.
17. Erkanli K, Kayalar N, Erkanli G, Ercan F, Sener G, Kirali K. Melatonin protects against ischemia/reperfusion injury in skeletal muscle. J Pineal Res. 2005 Oct;39(3):238-42. doi: 10.1111/j.1600-079X.2005.00240.x. PMID: 16150103.
18. Smith AR, Shenvi SV, Widlansky M, Suh JH, Hagen TM. Lipoic acid as a potential therapy for chronic diseases associated with oxidative stress. Curr Med Chem. 2004 May;11(9):1135-46. doi: 10.2174/0929867043365387. PMID: 15134511.
19. Packer L, Witt EH, Tritschler HJ. alpha-Lipoic acid as a biological antioxidant. Free Radic Biol Med. 1995 Aug;19(2):227-50. doi: 10.1016/0891-5849(95)00017-r. PMID: 7649494.
20. Lu S, Wang X, Wen L, Han Z, Tao T, Guan D. [Effect of superoxide dismutase on adhesion molecules expression in skeletal muscle ischemia/reperfusion injury in rats]. Zhonghua Yi Xue Za Zhi. 2002 Jun 25;82(12):840-3. Chinese. PMID: 12126535.
21. Alaçam B, Ek RO, Yıldız Y, Serter M, Boylu NT, Temoçin S. Abdominalaorta iskemi-reperfüzyonuna bağlı gelişen akciğer hasarında karnozinin etkisi ADÜ Tıp Fakültesi Dergisi. 2010;11(3):41-7.
22. Govind J. Lumbar radicular pain. Aust Fam Physician. 2004 Jun;33(6):409-12. PMID: 15253601.
23. Deng C, Sun Z, Tong G, Yi W, Ma L, Zhao B, et al. α-Lipoic acid reduces infarct size and preserves cardiac function in rat myocardial ischemia/reperfusion injury through activation of PI3K/Akt/Nrf2 pathway. PLoS One. 2013;8(3):e58371. doi: 10.1371/journal.pone.0058371. Epub 2013 Mar 7. PMID: 23505496; PMCID: PMC3591314.
24. Mendoza-Núñez VM, García-Martínez BI, Rosado-Pérez J, Santiago-Osorio E, Pedraza-Chaverri J, Hernández-Abad VJ. The Effect of 600 mg Alpha-lipoic Acid Supplementation on Oxidative Stress,

Inflammation, and RAGE in Older Adults with Type 2 Diabetes Mellitus. Oxid Med Cell Longev. 2019 Jun 12;2019:3276958. doi: 10.1155/2019/3276958. PMID: 31285784; PMCID: PMC6594273.
25. Petersen Shay K, Moreau RF, Smith EJ, Hagen TM. Is alpha-lipoic acid a scavenger of reactive oxygen species in vivo? Evidence for its initiation of stress signaling pathways that promote endogenous antioxidant capacity. IUBMB Life. 2008 Jun;60(6):362-7. doi: 10.1002/iub.40. PMID: 18409172.
26. Yan W, Li N, Hu X, Huang Y, Zhang W, Wang Q, et al. Effect of oral ALA supplementation on oxidative stress and insulin sensitivity among overweight/obese adults: a double-blinded, randomized, controlled, cross-over intervention trial. Int J Cardiol. 2013 Jul 31;167(2):602-3. doi: 10.1016/j.ijcard.2012.09.232. Epub 2012 Oct 25. PMID: 23102606
27. He L, Liu B, Dai Z, Zhang HF, Zhang YS, Luo XJ, et al. Alpha lipoic acid protects heart against myocardial ischemia-reperfusion injury through a mechanism involving aldehyde dehydrogenase 2 activation. Eur J Pharmacol. 2012 Mar 5;678(1-3):32-8. doi: 10.1016/j.ejphar.2011.12.042. Epub 2012 Jan 12. PMID: 22266491.

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