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Original Article

In-vivo antioxidant and therapeutic effects of ellagic acid on ischemia-reperfusion injury in skeletal muscle

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Abstract

Aim: Skeletal muscle ischemia-reperfusion (IR) injury is a critical clinical issue characterized by oxidative stress, inflammation, and tissue damage, potentially leading to systemic organ dysfunction. Ellagic acid (EA), a naturally occurring polyphenolic compound, is widely recognized for its strong antioxidative, anti-inflammatory, and antiapoptotic effects demonstrated in various preclinical studies. This study sought to assess the therapeutic effects of EA in a rat model of lower extremity IR injury, focusing on histopathological and biochemical parameters.

Material and Methods: 24 male Albino Wistar rats were randomly divided into four groups: Sham, EA, IR, and IR+EA. IR injury was induced by occluding the infrarenal abdominal aorta for 45 minutes, followed by 120 minutes of reperfusion. EA (40 mg/kg) was administered intraperitoneally prior to reperfusion. Left gastrocnemius muscle samples were collected for histopathological and biochemical analyses, including TOS, TAS, OSI, levels and PON-1 enzyme activity.

Results: The IR group showed marked muscle injury, with a significantly higher total injury score (10.00±0.63) compared to the Sham (2.00±0.58) and EA groups (2.00±0.52) ($p<0.001$, both). The IR-EA group demonstrated notable improvement, with a reduced total injury score (6.17±0.54), which was also significantly lower than the IR group ($p<0.001$). Biochemically, TAS levels and PON-1 activity significantly decreased while TOS and OSI levels increased in the IR group compared to the sham and EA groups. In addition, EA treatment significantly increased TAS levels and PON-1 activity while reducing TOS and OSI levels in the IR-EA group compared to the IR group ($p=0.039$, $p=0.045$, $p=0.045$, $p=0.007$, respectively).

Conclusion: EA effectively mitigated skeletal muscle damage induced by IR injury through its antioxidative, anti-inflammatory, and antiapoptotic mechanisms. The results suggest that EA exhibits potential effects as a therapeutic agent in managing IR-related injuries.

Keywords: Ischemia-reperfusion, ellagic acid, oxidative damage, reperfusion injury

CITATION

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INTRODUCTION

Ischemia-reperfusion (IR) injury is a multifaceted pathological process initiated by a transient interruption in blood flow followed by reperfusion, resulting in profound metabolic disturbances, accelerated adenosine triphosphate (ATP) depletion, and elevated oxidative stress. During reperfusion, tissue damage is frequently increased as a result of the excessive creation of reactive oxygen species (ROS), the activation of pro-inflammatory pathways, and the induction of apoptosis. All of these factors contribute considerably to the development of multiple organ dysfunction syndrome [1-4].

Lower extremity IR injury, commonly associated with conditions such as arterial embolism, peripheral artery disease, surgical interventions, and trauma, is marked by pronounced oxidative stress, lipid peroxidation, and DNA damage. The infiltration of neutrophils intensifies the inflammatory response, elevating the risk of irreversible muscle necrosis, compromised tissue regeneration, and systemic complications in the kidney and lungs [5-10].

Natural antioxidants with inherent anti-inflammatory effects are increasingly explored for their therapeutic applications in IR injury management [11-14]. Ellagic acid (EA), a naturally derived polyphenolic compound found in high concentrations in red berries and nuts, is shown to have promising protective properties against IR injury [15,16]. It is believed that its ability to neutralize free radicals, reduce the peroxidation of lipids, and regulate inflammatory and apoptotic signaling pathways is responsible for its positive benefits. EA contributes to the preservation of mitochondrial integrity and stabilization of cell membranes [6,17,18]. Furthermore, it regulates caspase activity, thereby attenuating apoptosis and enhancing cell survival [19].

Although numerous preclinical studies have demonstrated the antioxidant and anti-inflammatory effects of EA in various models of IR injury, the specific mechanisms underlying its protective effects in skeletal muscle IR injury have not yet been fully elucidated. This study aims to investigate the antioxidative, anti-inflammatory, and antiapoptotic properties of EA in a rat model of skeletal muscle IR injury through biochemical analyses and histopathological evaluation, assessing its potential as a therapeutic intervention.

MATERIAL AND METHODS

Animals

This research utilized 12-week-old male Albino Wistar rats, weighing between 250 and 350 grams, which were obtained from the experimental research facility of Gazi University in Ankara, Türkiye. All experimental protocols were conducted in strict compliance with ethical standards approved by the Gazi University Experimental Animal Ethics Committee (G.Ü.ET-23058). Animals were maintained under controlled laboratory conditions, with ambient temperatures (20–21°C), humidity (45–55%), and a controlled 12-hour light/dark cycle. They were granted ad libitum access to standard laboratory chow and water. Measures were implemented during the study to reduce animal distress, in strict accordance with institutional and international guidelines for the humane care and use of laboratory animals.

Drug

EA administration was performed according to established experimental protocols [20]. The EA used in this study ($C_{14}H_6O_8$, molecular weight: 302.19 g/mol) was obtained from Sigma-Aldrich (E2250, Merck KGaA, Germany) with a purity greater than 95%, as verified by high-performance liquid chromatography (HPLC) analysis. According to the findings of earlier research, EA was given intraperitoneally at a dose of forty milligrams per kilogramme in order to make the most of its therapeutic potential in reducing inflammatory responses, oxidative stress, and intraperitoneal damage [20].

Experimental Protocol

The research included 24 male Albino Wistar rats, randomly assigned to four experimental groups, each comprising six rats (n=6): Sham, Ellagic Acid (EA), Ischemia-Reperfusion (IR), and Ischemia-Reperfusion with EA (IR-EA). A probability sampling method was used in this study. The animals were randomly assigned to each experimental group using simple randomization to ensure unbiased group allocation. General anesthesia was achieved through intramuscular injection of ketamine (50 mg/kg; Ketax®, Vem İlaç) and xylazine (10 mg/kg; Alfazyne®, Ege Vet). To maintain adequate anesthesia depth, supplementary doses of ketamine (20 mg/kg) and xylazine (5 mg/kg) were given upon the observation of any indicators of surgical stress [10]. After a 30-minute stabilization period, experimental procedures commenced with the rats placed in a supine position under a warming lamp to maintain body temperature.

In the Sham Group, a midline laparotomy was conducted, followed by an intraperitoneal injection of 0.3 cc of 0.9% saline 45 minutes post-surgery. No further treatment was administered, and the left gastrocnemius muscle was collected after a 2-hour observation and sacrifice. The EA Group underwent the same surgical procedure but received 40 mg/kg of EA intraperitoneally at 45 minutes post-surgery, with muscle samples collected post-sacrifice. In the IR Group, ischemia was induced through occlusion of the infrarenal abdominal aorta for 45 minutes, succeeded by a 120-minute reperfusion period. Following the protocol, the rats were sacrificed, and muscle samples were collected. In the IR-EA Group, 40 mg/kg of EA was administered intraperitoneally prior to aortic clamping, followed by equivalent durations of ischemia and reperfusion, with muscle collection conducted post-sacrifice. The 45-minute ischemia and 120-minute reperfusion model was chosen based on established protocols known to trigger oxidative stress and inflammation [21].

Sacrifice was conducted under deep anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg) [10]. Death was confirmed by the complete cessation of cardiac and respiratory functions, followed by a 2-minute observation to verify the absence of vital signs. Subsequently, the left gastrocnemius muscle from each group was harvested for histopathological and biochemical analyses.

Although no formal power analysis was performed, the sample size (n=6 per group) was determined based on our previous experimental studies and other similar studies in the literature with comparable endpoints, and was considered adequate to detect statistically significant histopathological and biochemical changes [14,22,23].

Histopathological Evaluation

Histopathological analyses were performed at the NOROM facility, Gazi University, Ankara, Türkiye. Muscle tissues were fixed in 10% neutral buffered formalin for 48 hours, followed by conventional processing and paraffin embedding. Serial 4 µm sections were cut at 20-µm intervals using a microtome (HistoCore MULTICUT, Leica, Germany). Hematoxylin and eosin (H&E) staining was applied, and the slides were analyzed using a light microscope (Leica DM 4000 B, Leica, Germany) using LAS V4.12 software at 200× and 400× magnifications.

Two blinded observers independently conducted histopathological scoring to avoid bias. Evaluations included hemorrhage severity, neutrophil infiltration, muscle fiber disorganization, degeneration, and edema. Each parameter was scored on a semiquantitative scale from 0 to 3, contributing to a total injury score ranging from 0 to 12 [24,25].

Biochemical Evaluation

The left gastrocnemius muscle samples were instantly flash-frozen in liquid nitrogen and meticulously preserved at −80°C to ensure optimal biochemical stability for subsequent analyses. A comprehensive evaluation of oxidative stress biomarkers was conducted, including Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Oxidative Stress Index (OSI), and paraoxonase-1 (PON-1) activity. The methodologies and calculation formulas for assessing TAS, TOS, OSI, and PON-1 were precisely adapted from our previous research, ensuring consistency and scientific rigor [26-28].

Statistical Analysis

All analyses were conducted using SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA), under an institutional academic license. The primary outcome measures of this study were the histopathological injury scores and oxidative stress biomarkers (TAS, TOS, OSI, levels and PON-1 enzyme activities), which were used to assess the protective effects of ellagic acid in skeletal muscle IR injury. The assumptions of normality and homogeneity of variances were assessed using the Shapiro-Wilk test and Levene's test, respectively. Data distribution was also evaluated through histograms and probability plots. Quantitative data were summarized using descriptive statistics and presented as mean±standard error (SE). There were no qualitative variables in the dataset. All statistical analyses were performed using one-way analysis of variance (ANOVA) to test for differences among the groups. Tukey's post-hoc test was applied for multiple pairwise comparisons. Statistical significance was set at $p<0.05$.

RESULTS

Histopathological Results

Histopathological evaluation of H&E-stained sections showed significant differences in tissue injury parameters among the experimental groups, and p-values were given in Table 1. Hemorrhage differed significantly across groups ($p=0.002$). Hemorrhage severity varied notably, with the IR group displaying significantly higher hemorrhage than both the Sham and EA groups ($p<0.001$, both), (Table 1, Figures 1-4).

Neutrophil infiltration differed significantly across groups ($p<0.001$). The IR group exhibited a marked increase in neutrophil infiltration compared to the Sham and EA groups ($p<0.001$, both). Similarly, the IR+EA group showed higher infiltration than the Control and EA groups ($p<0.001$, $p=0.007$, respectively) (Table 1, Figures 1-4).

Significant disparities in muscle fiber disorganization and degeneration were observed ($p<0.001$). The IR group had considerably greater muscle fiber disruption and degeneration compared to the Sham and EA groups ($p<0.001$, $p=0.002$, respectively). The IR+EA group also demonstrated significantly more muscle fiber damage than the Sham and EA groups ($p<0.001$, both). Notably, muscle fiber disorganization and degeneration were significantly reduced in the IR+EA group compared to the IR group ($p=0.006$) (Table 1, Figures 1-4).

The levels of interstitial edema differed significantly between the groups ($p <0.001$). The IR group demonstrated significantly higher edema levels compared to the Sham and EA groups ($p<0.001$, $p=0.006$, respectively). The IR+EA group also exhibited increased edema relative to the Sham and EA groups ($p<0.001$, $p=0.001$, respectively). Nonetheless, edema levels were significantly lower in the IR+EA group than in the IR group ($p<0.001$) (Table 1, Figures 1-4).

Total muscle injury scores also varied significantly among the groups ($p<0.001$). The IR group showed markedly higher injury scores compared to the Sham and EA groups ($p<0.001$, both). Similarly, the IR+EA group had significantly higher scores compared to the Sham and EA groups ($p<0.001$, both). Importantly, the IR+EA group exhibited a significantly lower injury score than the IR group ($p<0.001$) (Table 1, Figures 1-4).

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Table 1. Histopathological findings in H&E stained muscle tissue specimens (Mean±SE)

	Control group (n=6)	EA group (n=6)	IR group (n=6)	IR+EA group (n=6)	P-value
Hemorrhage	0.50±0.22	0.50±0.22	1.83±0.33*,**	1.17±0.17	0.002
Neutrophil infiltration	0.17±0.17	0.50±0.22	2.33±0.23*,**	1.67±0.33*,**	<0.001
Disorganization and degeneration of muscle fibers	0.67±0.21	0.50±0.22	2.83±0.17*,**	1.83±0.31*,**,***	<0.001
Interstitial edema	0.67±0.21	0.50±0.22	3.00±0.00*,**	1.50±0.22*,**,***	<0.001
Total injury	2.00±0.58	2.00±0.52	10.00±0.63*,**	6.17±0.54*,**,***	<0.001

Data are presented as Mean±Standard Error of the Mean (SEM); ANOVA test was used for statistical analysis; significance level was set at p<0.05; *p<0.05: significant difference compared to the control group (C), **p<0.05: Significant difference compared to EA group; ***p<0.05: significant difference compared to IR group

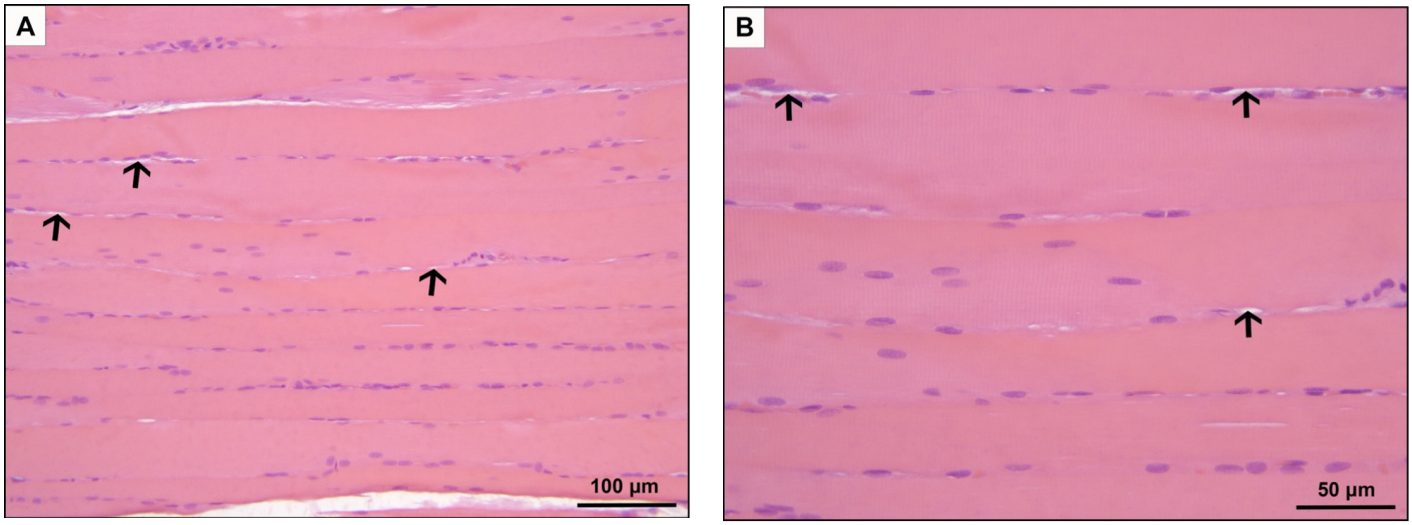

Figure 1. Micrographs representing the H&E stained muscle sections from sham group; normal muscle fibers with cross-striation and peripherially located nuclei are seen in longitudinal section plane at 200× (**A**) and 400× (**B**) magnifications; slight interstitial edema was seen (arrows)

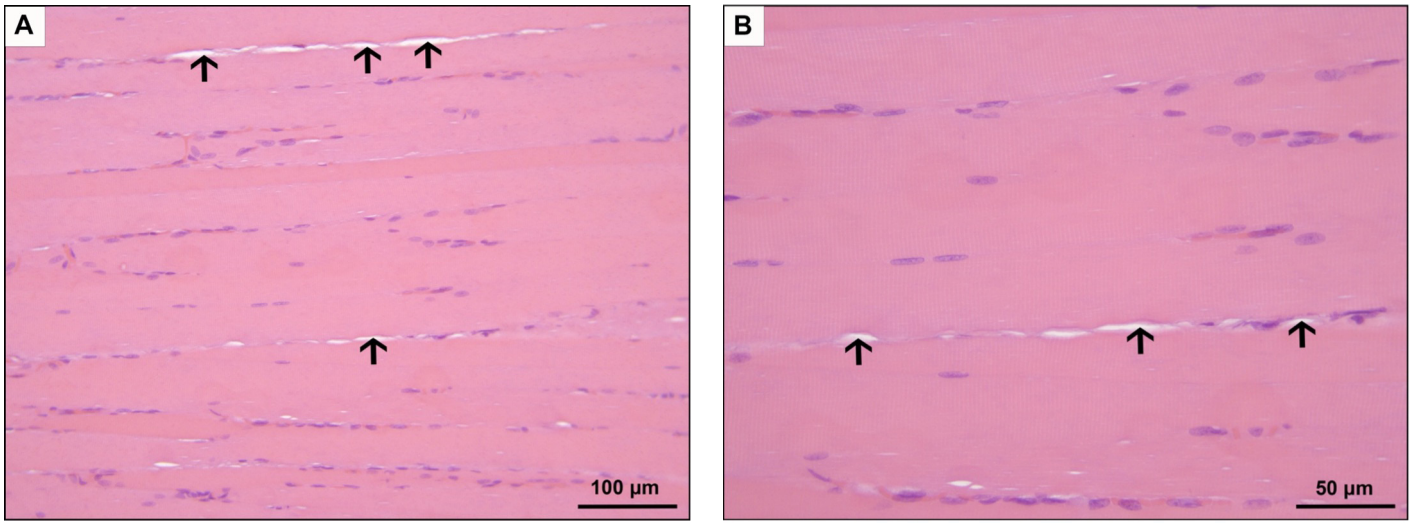

Figure 2. Micrographs representing the H&E stained muscle sections from EA group; longitudinal sections of normal muscle fibers at 200× (**A**) and 400× (**B**) magnifications display cross-striation and peripherially located nuclei. Interstitial edema was noted (arrows)

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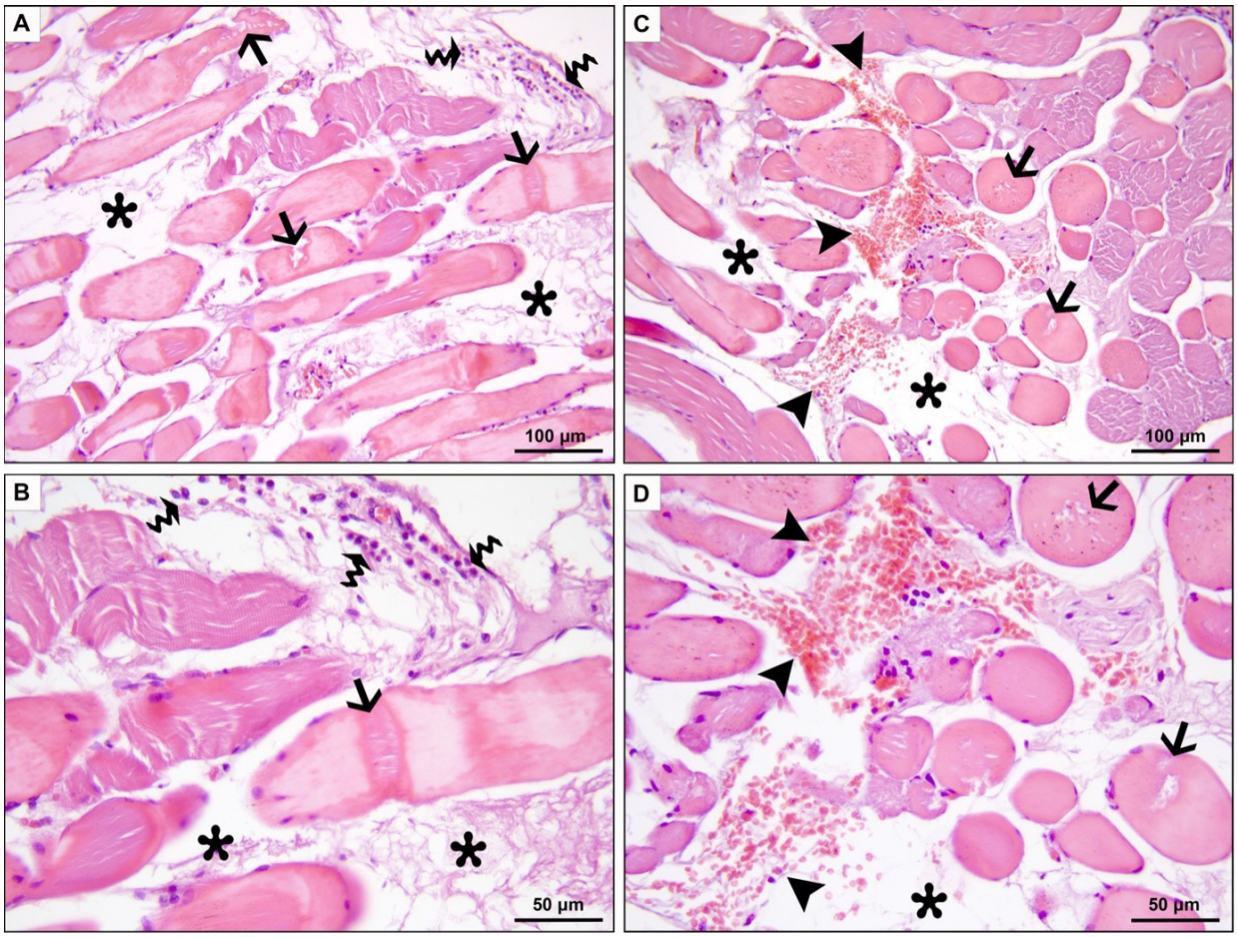

Figure 3. Micrographs representing the H&E stained muscle sections from IR group. Cytoplasmic disintegration (arrows) and swelling of varying degrees were obsorved in myocytes; severe interstitial edema (asterisk) expanding the intecellular space greatly was accompanied by hemorrhage (arrow heads) and neutrophil infiltration (wavy arrows) in some regions; Figures **A** and **C** were captured at 200× magnification; figures **B** and **D** were captured at 400× magnification

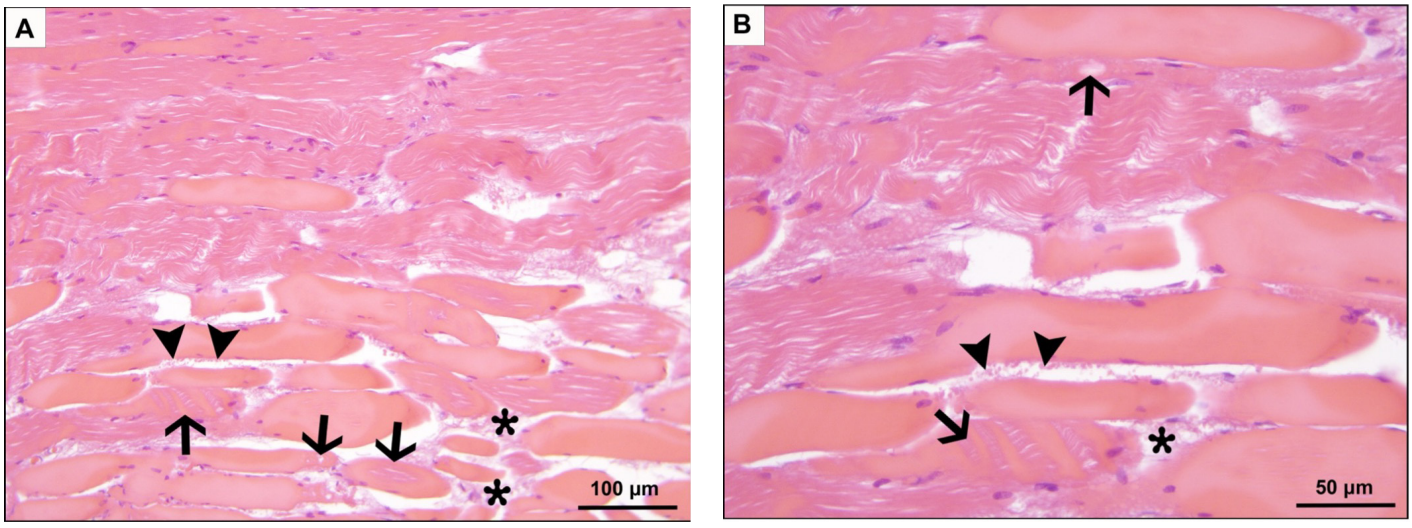

Figure 4. Micrographs representing the H&E stained muscle sections from IR+EA group; myocyte swelling and cytoplasmic disintegration (arrows) were milder compared to IR group; compared to IR group, milder interstitial edema (asterisk) and erythrocyte extravasation (arrow heads) were present; Figure **A** was captured at 200× magnification and figure **B** was captured at 400× magnification

Biochemical Results

Statistically significant differences were observed in oxidative stress parameters among the experimental groups, as detailed in Table 2. TAS levels showed a significant variation (p=0.003), with the IR group presenting significantly lower TAS levels compared to both the Sham and EA groups (p=0.016, p<0.001, respectively). Notably, the IR+EA group demonstrated a marked increase in TAS levels relative to the IR group (p=0.039), reflecting the antioxidant capacity of EA. Similarly, TOS levels varied significantly among the groups (p=0.042). The IR group exhibited significantly elevated TOS levels compared to the Sham and EA groups (p=0.013, p=0.014, respectively), whereas EA treatment in the IR+EA group significantly reduced TOS levels compared to the IR group (p=0.048), highlighting EA's efficacy in mitigating oxidative stress.

Table 2. Oxidative status parameters in muscle tissue (Mean±SE)

	Control group (n=6)	EA group (n=6)	IR group (n=6)	IR+EA group (n=6)	P-value
TAS (mmol/L)	0.24±0.05	0.35±0.05	0.08±0.03*,**	0.22±0.04***	0.003
TOS (µmol/L)	7.30±0.43	7.34±0.70	11.71±2.13*,**	8.33±0.30***	0.042
OSI	3.50±0.58	2.45±0.51	34.85±14.43*,**	4.39±0.57***	0.012
PON-1 (U/L)	39.26±6.75	43.88±13.06	6.66±1.40*,**	28.70±2.07***	0.010

Data are presented as Mean±Standard Error of the Mean (SEM); ANOVA test was used for statistical analysis; significance level was set at p<0.05; *p<0.05: significant difference compared to the control group (C); **p<0.05: significant difference compared to EA group, ***p<0.05: significant difference compared to IR group

In addition, OSI levels differed significantly between groups (p=0.012). The IR group had significantly higher OSI values than the Sham and EA groups (p=0.006, p=0.005, respectively). Conversely, OSI levels were significantly reduced in the IR+EA group compared to the IR group (p=0.007), reinforcing the antioxidative effects of EA. Furthermore, PON-1 enzyme activity demonstrated significant differences among the groups (p=0.010). The IR group showed significantly reduced PON-1 activity compared to the Sham and EA groups (p=0.006, p=0.002, respectively). Notably, EA treatment in the IR+EA group significantly increased PON-1 activity compared to the IR group (p=0.045), indicating that EA not only reduces oxidative stress but also enhances antioxidant enzyme function.

DISCUSSION

Our findings clearly demonstrate that ellagic acid exerts significant protective effects against skeletal muscle IR injury by diminishing oxidative stress, reducing inflammatory responses, and ameliorating histopathological damage. These results provide compelling evidence for the therapeutic potential of EA in the context of IR-related muscle injury.

Previous studies have also indicated the beneficial role of EA in IR injury models [29-31]. For instance, Ekinci Akdemir et al. demonstrated that EA significantly reduces oxidative damage and improves antioxidant capacity in skeletal muscle IR injury, which aligns well with our observations [31]. Although several studies have confirmed the antioxidative and anti-inflammatory properties of EA, its therapeutic efficacy specifically in lower extremity skeletal muscle IR injury has not been sufficiently clarified. Our study contributes to bridging this knowledge gap by providing both biochemical and histopathological evidence of EA's protective mechanisms.

Our study findings highlight the substantial protective effects of EA against IR-induced skeletal muscle damage. EA administration notably diminished oxidative stress, reduced inflammatory responses, and minimized histopathological deterioration, further supporting its therapeutic potential in managing IR-related injuries [29].

In line with existing literature, our results demonstrated that IR exposure significantly elevated TOS and OSI, while decreasing TAS and PON-1 activity. EA treatment effectively reversed these deleterious effects by enhancing TAS and PON-1 levels while concurrently reducing TOS and OSI, which aligns with previous studies highlighting EA's antioxidative efficacy [29,30]. Our findings corroborate previous studies demonstrating the systemic antioxidative and cytoprotective effects of EA across various IR injury models.

Moreover, EA's capacity to enhance PON-1 activity is well-documented, contributing to its antioxidative and anti-inflammatory effects. Kara et al. investigated the impact of EA on exercise-induced endothelial damage and reported that EA significantly increased PON-1 activity, thereby reducing oxidative stress and preventing the accumulation of oxidized low-density lipoprotein (LDL) [32]. Uzar et al. further demonstrated that EA upregulates PON-1 in diabetic rats, offering neuroprotection by

attenuating oxidative damage [33]. In a related study, Goudarzi et al. showed that EA boosts PON-1 levels and suppresses lipid peroxidation in an acrylamide-induced neurotoxicity model [34]. These studies collectively reinforce EA's role in modulating antioxidant defense through PON-1 activity.

Beyond its antioxidative properties, EA demonstrates substantial anti-inflammatory effects. Our histopathological evaluations revealed that EA administration significantly decreased neutrophil infiltration, interstitial edema, and muscle fiber disorganization following IR injury, effectively attenuating the inflammatory cascade. While some studies suggest a potential discrepancy between histological improvements and biochemical recovery, necessitating further investigation, others present opposing evidence that supports a consistent therapeutic effect of EA [35]. Rizk et al. also observed that EA enhances muscle fiber organization and maintains intracellular structural integrity [36]. Furthermore, Hseu et al. demonstrated that EA effectively reduces interstitial edema and promotes cellular regeneration in skeletal muscle tissue [37]. Similarly, Ding et al. showed that EA reduces post-ischemic hemorrhage, tissue injury, and necrosis in IR models [7]. While our study specifically addresses the protective effects of EA in skeletal muscle I/R injury, previous studies have reported that EA exhibits similar antioxidative and cytoprotective effects in various organ systems, including the kidney, liver, and lungs [35,38]. These findings collectively support the broader systemic potential of EA, complementing our results at the skeletal muscle level.

EA's therapeutic benefits are not confined to skeletal muscle. Dianat et al. highlighted EA's cardioprotective effects, demonstrating its ability to ameliorate cardiac arrhythmias following cerebral IR injury, thereby reducing cardiovascular complications [39]. Gurmen et al. also reported that EA lowers oxidative stress markers in renal IR injury, suggesting its broad-spectrum protective potential across multiple organ systems [40].

Despite the promising outcomes, our study indicates that EA does not confer complete protection against IR-induced damage. Although EA administration significantly reduced hemorrhage and neutrophil infiltration, not all improvements reached statistical significance. This observation implies that EA monotherapy may not be sufficient to achieve optimal therapeutic outcomes, highlighting the need for further research on dosage optimization, potential combination therapies, and long-term safety and efficacy.

This study has certain limitations. The small sample size (24 male Albino Wistar rats) may limit the generalizability of the findings, necessitating larger-scale studies for validation. The use of a single-dose EA (40 mg/kg) prevented the assessment of dose-response relationships and the optimal therapeutic window, highlighting the need for studies with varying doses and repeated administrations. The study focused on acute IR injury with a 120-minute reperfusion period; thus, the long-term effects of EA on chronic IR injury and functional muscle recovery remain uncertain. Although the antioxidative, anti-inflammatory, and antiapoptotic effects of EA were demonstrated, specific molecular mechanisms were not investigated, warranting further studies incorporating Western blotting or gene expression analyses. The clinical translation of these findings requires careful evaluation, and human studies are necessary to confirm the safety, efficacy, and therapeutic potential of EA. Furthermore, since this study did not involve the co-administration of other pharmacological agents, the potential interactions of ellagic acid with commonly used drugs such as statins, antihypertensives, and antidiabetic agents remain unknown. Future studies should also assess possible drug-drug interactions and organ-specific toxicity profiles to better define the safety and therapeutic applicability of EA in clinical settings.

CONCLUSION

In conclusion, this study demonstrates the significant antioxidative, anti-inflammatory, and antiapoptotic effects of EA in mitigating IR-induced skeletal muscle injury. EA administration effectively reduced oxidative stress markers, enhanced antioxidant capacity, and ameliorated histopathological damage, including hemorrhage, neutrophil infiltration, muscle fiber disorganization, and edema. These findings support the potential of EA as a therapeutic agent for IR-related injuries. However, while the results are encouraging, further research is warranted to explore optimal dosing, long-term effects, molecular mechanisms, and translational applicability in human studies. By addressing these aspects, future studies could solidify EA's role in the clinical management of ischemic conditions.

Ethics Committee Approval: All experimental protocols were conducted in strict compliance with ethical standards approved by the Gazi University Experimental Animal Ethics Committee (G.Ü.ET-23058).

Patient Consent for Publication: Patient consent form is not required.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: All authors contributed equally to the article.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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