EXPERIMENTAL STUDY

# Effects of epigallocatechin gallate on ischemia-reperfusion injury: an experimental study in rats

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## ABSTRACT

**BACKGROUND:** Skeletal muscle ischemia-reperfusion injury (IRI) is a significant concern in various clinical settings. Oxidative stress and neutrophil infiltration play central roles in its pathophysiology. However, clinicians have very few therapeutic options for the prevention and treatment of IRI. The present study aimed to investigate the effects of epigallocatechin gallate (EGCG) on (i) skeletal muscle injury, (ii) oxidative stress, and (iii) markers related to neutrophil infiltration.

**METHODS:** This was an experimental study conducted on rats. The IRI model involved the application of an elastic rubber band for 4 + 2 hours (IRI group). In the sham control (SC) group, all procedures were identical except for the rubber band application. Saline (10 mL/kg, intraperitoneally) and EGCG (25 or 50 mg/kg, intraperitoneally) were administered 30 minutes before reperfusion (IRI-SF, IRI-25, and IRI-50 groups, respectively). Creatine phosphokinase (CPK) was the primary endpoint. Other parameters included lactate dehydrogenase (LDH), total oxidant status (TOS), total antioxidant status (TAS), myeloperoxidase (MPO), E-selectin, P-selectin, L-selectin, intercellular adhesion molecule-1 (ICAM-1), and various cytokines (interleukin-1 beta [IL-1β], IL-6, and tumor necrosis factor-alpha [TNF-α]), which were measured in serum or gastrocnemius muscle samples.

**RESULTS:** CPK, LDH, and TOS levels were higher in the IRI group compared to the SC group (p=0.001, p=0.0001, and p=0.005, respectively). Although not statistically significant, decreases in these parameters were observed in the IRI-50 group compared to the IRI group (p=0.628, p=0.167, and p=0.444, respectively). Regarding TAS, a noticeable decrease was observed in the IRI group compared to the SC group (p=0.054), which was significantly increased by treatment with 50 mg/kg EGCG (p=0.011). For the remaining parameters (except IL-6), there were no statistically significant increases in the IRI group compared to SC, nor decreases in the EGCG-treated groups compared to the IRI group.

**CONCLUSION:** We propose that EGCG possesses antioxidant activity. However, any beneficial effect related to its interaction with neutrophil infiltration markers remains only suggestive.

**Keywords:** Cytokines; epigallocatechin gallate; ischemia-reperfusion injury; neutrophil infiltration; oxidative stress; skeletal muscle.

## INTRODUCTION

Ischemia-reperfusion injury (IRI), characterized by paradoxical cellular damage following the restoration of blood flow to previously ischemic tissues, is a major concern in various clinical settings.[1] These include thrombolytic therapy, organ transplantation, limb trauma, and aortic cross-clamping during abdominal aortic aneurysm repair.[1] The acute inflammatory response observed in IRI is driven by multiple factors, including the formation of reactive oxygen species (ROS), lipid peroxidation, eicosanoid generation, neutrophil infiltration, complement cascade activation, and cytokine release.[1] One of the

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most significant ROS is the superoxide anion ($O_2$-), which is primarily generated during the reperfusion phase and plays a role in the activation of the endothelium. Along the activated endothelium, leukocytes begin to roll due to the expression of P- and E-selectins on the endothelium and L-selectin on neutrophils.[1] Thereafter, neutrophil adhesion to the endothelium is intensified by the interplay between leukocyte integrins (such as platelet endothelial cell adhesion molecule-1 (PECAM-1/CD-31) and CD-11/CD-18) and adhesion molecules like intercellular adhesion molecule-1 (ICAM-1/CD54), followed by transmigration through the endothelium under the influence of chemotactic agents such as interleukin-8 (IL-8).[1]

Our previous study utilizing a rat model provided evidence for the role of oxidative stress in the pathogenesis of skeletal muscle IRI, as demonstrated by elevated levels of total oxidative status (TOS).[2] Another rat model of skeletal muscle IRI showed a positive association between myeloperoxidase (MPO) activity, a marker of neutrophil infiltration, and the severity of IRI.[3] A recent *in vivo* study using a rat model also demonstrated a significant increase in E-selectin expression in reperfused limbs compared to controls.[4] Plasma P-selectin levels of rats, measured by enzyme-linked immunosorbent assay (ELISA), were significantly elevated in the skeletal muscle IRI group compared to the sham control group.[5] Furthermore, a previous study reported that IRI significantly increased the expression of E-selectin, L-selectin, and P-selectin in the cremaster muscles of Sprague Dawley rats.[6] Although PECAM-1 expression has not been studied in skeletal muscle, administration of platelet endothelial cell adhesion molecule-1/immunoglobulin G (PECAM-1/IgG) was shown to reduce peroxynitrite-mediated extremity reperfusion injury in rabbits, suggesting a role for PECAM-1 in neutrophil transendothelial migration.[7] Consistent with the observed upregulation of selectins, plasma levels of ICAM-1, a key adhesion molecule involved in neutrophil transmigration, were also significantly elevated after reperfusion in a rat model of IRI.[5] Regarding chemotactic agents that stimulate neutrophil transmigration, plasma levels of IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) were increased following the ischemia-reperfusion challenge in rat skeletal muscle.[8]

Epigallocatechin gallate (EGCG), a major component of green tea extract derived from the Camellia sinensis plant, demonstrates potent antioxidant activity.[9] This property has attracted interest for its potential therapeutic role in conditions such as IRI, where oxidative stress plays a key role. In this context, an *in vitro* study showed that EGCG exhibits strong radical scavenging activity against $O_2$-, with an effect over 100-fold more potent than that of ascorbic acid.[10] Similarly, EGCG was found to act as an $O_2$- scavenger under *in vivo* conditions in a rat skeletal muscle IRI model.[11] In the same study, EGCG administration significantly reduced levels of malondialdehyde (MDA), a marker of free radical-induced lipid peroxidation.[11] Additionally, histological evaluation revealed reduced tissue damage and neutrophil accumulation in the skeletal muscle following EGCG treatment.[11] EGCG administration was also shown to prevent the upregulation of ICAM-1 and P-selectin in a rat model of intestinal IRI.[12] While this finding was observed in the intestine, it suggests a potential mechanism by which EGCG might exert protective effects in skeletal muscle IRI through modulation of neutrophil adhesion molecules. Furthermore, EGCG administration in hypercholesterolemic rats attenuated the expression of TNF-α induced nuclear factor of activated T cells (NF-AT) in liver tissue homogenates.[13] This resulted in a more pronounced downregulation of its downstream targets (ICAM-1 and E-selectin) compared to the nuclear factor kappa B (NF-κB)-mediated downstream targets, vascular cell adhesion molecule-1 (VCAM-1) and P-selectin.[13]Indeed, pretreatment with EGCG effectively prevented the induction of ICAM-1, E-selectin, and monocyte chemotactic protein-1 (MCP-1) expressions caused by oxidized low-density lipoprotein (oxLDL) in human umbilical vein endothelial cells (HUVECs).[14] In line with its ability to prevent neutrophil infiltration, EGCG also diminished the production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in a dose-dependent manner in a rat skeletal muscle IRI model.[15]

In conclusion, elucidating the mechanisms underlying EGCG's previously demonstrated protective effects in striated muscle IRI is of critical importance. Accordingly, the present study aimed to evaluate the beneficial actions of EGCG using specific markers related to striated muscle damage and oxidative stress. Subsequently, the potential effects of EGCG on neutrophil infiltration were investigated. Finally, the study sought to explore the involvement of E-, P-, and L-selectins, ICAM-1, and cytokines (IL-1, IL-6, and TNF-α) in EGCG's preventive effects on neutrophil infiltration.

## MATERIALS AND METHODS

### Experimental Animals

The present study was approved by the Local Animal Experimentation Ethics Committee (Protocol number: 2020/04-01) and conducted in accordance with the EU Directive 2010/63/EU for animal experiments. The study is reported in compliance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments). Forty male Wistar rats (200-300 g), selected through haphazard sampling, were housed in the Experimental Research Unit under controlled conditions: temperature (24±2°C), humidity (60%-70%), and a 12-hour light-dark cycle. Animals had ad libitum access to standard laboratory rodent chow and water. Groups of four rats were randomly assigned to separate cages and allowed to acclimate for one week prior to the start of the experiment.

### Study Protocol

Building upon our previous work,[2] the following protocol was implemented. After induction of anesthesia with urethane (1 g/kg, intraperitoneally (i.p.); Sigma-Aldrich), each rat was placed in the supine position on a wooden board and

secured with foot restraints. A tourniquet was applied to the right hindlimb, proximal to the knee joint, using an elastic band. This procedure induced ischemia in the right lower limb for four hours, after which the band was removed to allow reperfusion for two hours. Ischemia and reperfusion were confirmed by visual inspection of color changes in the paws. Anesthesia was maintained during the surgical procedure and throughout the experiment with supplemental urethane as needed to ensure adequate depth. After establishing the IRI method, rectal temperature was monitored hourly using a digital thermometer and maintained at 37±0.5°C with a 60 W domestic lamp to prevent anesthesia-induced hypothermia until the end of the experiment. The sham-control group underwent all procedures described above, except for the application of the elastic band. At the end of the experiment, venous blood (>5 mL) was collected from the right ventricle into plain tubes and centrifuged at 2500 rpm for 10 minutes to obtain serum samples. These samples were stored at -80°C until biochemical analysis. The right gastrocnemius muscle was excised, transferred into tubes containing saline (SF), placed on dry ice, and then stored at -80°C until the day of biochemical evaluation. The personnel performing the biochemical analyses were blinded to the study groups. After cardiac puncture, rats that remained alive but moribund were euthanized by cervical dislocation under urethane anesthesia.

### Experimental Groups

The experimental groups were as follows (n=8 per group):

1) Sham control (SC),

2) IRI (4 + 2 h) (IRI),

3) IRI + SF (10 mL/kg, i.p.) (IRI-SF),

4) IRI and EGCG (25 mg/kg, i.p.) (IRI-25), and

5) IRI and EGCG (50 mg/kg, i.p) (IRI-50).

EGCG and its vehicle (SF) were administered 30 minutes before the onset of reperfusion. EGCG doses of 25 and 50 mg/kg, based on our previous study, were dissolved in a 10 mL/kg SF solution. The number of animals per group was determined through a power analysis based on a previous study. G*Power 3.1.9.2 software (Franz Faul, Univerzität Kiel, Germany) was used with $\alpha$=0.05, power=0.80, and an effect size of 0.88. The effect size was calculated using parameters from a previous study,[2] with the primary endpoint being creatine phosphokinase (CPK) levels. This analysis indicated a minimum of five animals per group.

### Measurement of Serum Parameters

Serum levels of lactate dehydrogenase (LDH: U/L) and creatine phosphokinase (CPK: U/L) were measured spectrophotometrically using a calibrated and validated analyzer (ADVIA 1800, Germany).

### Measurement of Total Oxidant and Antioxidant Status

Tissues were weighed, blotted on filter paper, and homogenized in three volumes of ice-cold 1.15% potassium chloride (KCl). The supernatant was obtained from centrifugation at 14,000 rpm. TOS (mmol/g protein) and total antioxidant status (TAS) (mmol/g protein) were measured in gastrocnemius muscle homogenates using a commercially available kit (REL Assay Diagnostics, Mega Tip, Gaziantep, Türkiye) with an enzyme-linked immunosorbent assay device (Thermo Fisher Scientific). Protein levels of the homogenized samples were determined using the Lowry method.[16]

### Measurements Related to Neutrophil Infiltration

Levels of MPO, E-selectin, P-selectin, L-selectin, ICAM-1, IL-1$\beta$, IL-6, and TNF-$\alpha$ were measured in gastrocnemius muscle homogenates using ELISA.

### Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 21, Chicago, IL) and are presented as mean ± standard error of the mean (SEM) and median. Initially, data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Subsequently, one-way analysis of variance (ANOVA) was used to analyze the data, with the significance of individual comparisons assessed using Tukey's test. In cases where normality and/or homogeneity of variance were violated, the Kruskal-Wallis and Dunn-Sidak nonparametric tests were used instead. A p value of <0.05 was considered statistically significant.

## RESULTS

### Effect of EGCG on Parameters Related to Tissue Injury

A statistically significant difference in CPK levels was observed among the groups (Kruskal-Wallis test: $X^2$=23.843, p=0.0001) (Table 1). CPK levels were higher in the IRI group compared to the SC group (Dunn-Sidak test: p=0.001). Similarly, the IRI-SF and IRI-25 groups showed elevated CPK levels compared to the SC group (Dunn-Sidak test: p=0.0001 and p=0.009, respectively). In contrast, although the IRI-50 group (50 mg/kg EGCG) showed a notable reduction in CPK levels compared to the IRI group, this difference was not statistically significant (Dunn-Sidak test: p=0.628). Additionally, CPK levels in the IRI-50 group were not significantly different from those in the SC group (Dunn-Sidak test: p=0.308).

The statistical analysis revealed a significant difference among the groups in terms of LDH levels (one-way ANOVA: F=8.93, p=0.0001) (Table 1). Further analysis showed that LDH levels in the IRI group were higher than those in the SC group (Tukey's test: p=0.0001). Similarly, LDH levels were significantly elevated in both the IRI-SF and IRI-25 groups compared to the SC group (Tukey's test: p=0.0001 and p=0.001, respectively). Although LDH levels in the IRI-50 group were lower than in the IRI group, the difference was not statistically significant (Tukey's test: p=0.167). However, LDH values in the IRI-50 group were also not significantly different from those in the SC group (Tukey's test: p=0.083).

**Table 1.** Creatine phosphokinase and lactate dehydrogenase levels in experimental groups

| Group | Creatine Phosphokinase (U/L) | Lactate Dehydrogenase (U/L) |
|---|---|---|
| SC | 1994.50±278.85 | 725.62±62.70 |
| (n=8) | (1971.50) | (767.00) |
| IRI | 11161.00±1156.52* | 2037.37±297.48* |
| (n=8) | (11625.50) | (2067.00) |
| IRI-SF | 11413.37±1001.91* | 1997.25±169.56* |
| (n=8) | (11484.50) | (2059.00) |
| IRI-25 | 10061.50±632.62* | 1933.12±200.96* |
| (n=8) | (10245.50) | (1734.00) |
| IRI-50 | 7416.25±1098.13 | 1426.50±116.93 |
| (n=8) | (7589.50) | (1406.00) |

SC: Sham-control; IRI: Ischemia-reperfusion injury; IRI-SF: IRI+Saline; IRI-25: IRI + 25 mg/kg epigallocatechin gallate; IRI-50: IRI + 50 mg/kg epigallocatechin gallate. Creatine phosphokinase data (mean ± standard error, median) were analyzed using the Kruskal-Wallis and Dunn-Sidak tests. Lactate dehydrogenase data (mean ± standard error, median) were analyzed using one-way analysis of variance and Tukey's test. p<0.05: *compared with SC

**Table 2.** Total oxidant status and total antioxidant status in experimental groups

| Group | TOS (μmol $H_2O_2$ Eq/g protein) | TAS (μmol Trolox Eq/g protein) |
|---|---|---|
| SC | 1.39±0.30 | 99.21±19.34 |
| (n=8) | (1.40) | (79.57) |
| IRI | 6.08±1.14* | 44.48±6.07 |
| (n=8) | (5.98) | (43.56) |
| IRI-SF | 6.09±1.30* | 47.68±2.77 |
| (n=8) | (5.87) | (48.60) |
| IRI-25 | 3.95±0.73 | 64.53±2.09 |
| (n=8) | (3.69) | (67.1641) |
| IRI-50 | 2.77±0.47 | 84.08±9.65**, *** |
| (n=8) | (2.77) | (78.76) |

SC: Sham-control; IRI: Ischemia-reperfusion injury; IRI-SF: IRI+Saline; IRI-25: IRI + 25 mg/kg epigallocatechin gallate; IRI-50: IRI + 50 mg/kg epigallocatechin gallate. Total oxidant status (TOS) and total antioxidant status (TAS) data (mean ± standard error, median) were analyzed using the Kruskal-Wallis and Dunn-Sidak tests. p<0.05: *compared with SC; **compared with IRI; ***compared with IRI-SF.

### Effect of EGCG on Parameters Related to Oxidative Stress

The study groups were statistically analyzed in terms of TOS levels, and a significant difference was found among the groups (Kruskal-Wallis test: $X^2$=16.238, p=0.003) (Table 2). TOS levels were significantly elevated in both the IRI and IRI-SF groups compared to the SC group (Dunn-Sidak test: p=0.005 and p=0.012, respectively). Although the IRI-25 and IRI-50 groups exhibited a reduction in TOS levels compared to the IRI group, these differences were not statistically significant (Dunn-Sidak test: p=1.000 and p=0.444, respectively). Moreover, the TOS levels in the IRI-25 and IRI-50 groups were not significantly different from those in the SC group (Dunn-Sidak test: p=0.176 and p=1.000, respectively).

Similarly, the analysis of TAS levels revealed a significant difference among the groups (Kruskal-Wallis test: $X^2$=20.009, p=0.0001) (Table 2). In this context, an apparent decrease in TAS levels was observed in the IRI group, although it did not reach statistical significance (Dunn-Sidak test: p=0.054) (Table 2). A similar trend was noted in the IRI-SF and IRI-25 groups (Dunn-Sidak test: p=0.051 and p=1.000, respectively). However, the IRI-50 group showed a statistically significant increase in TAS levels compared to both the IRI and IRI-SF groups (Dunn-Sidak test: p=0.011 and p=0.010, respectively).

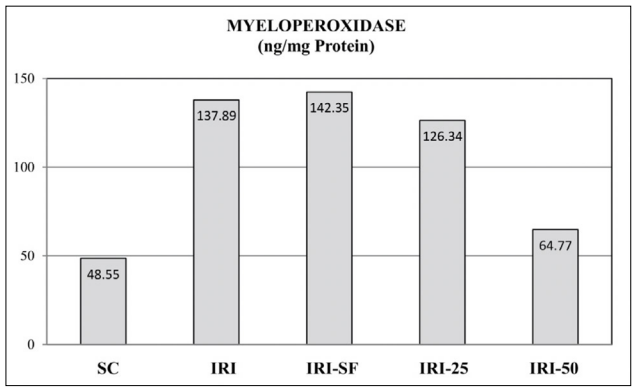


**Figure 1.** Myeloperoxidase levels across study groups. Myeloperoxidase levels (median) were evaluated in the SC (sham-control), IRI (ischemia-reperfusion injury), IRI-SF (IRI+saline), IRI-25 (IRI + 25 mg/kg epigallocatechin gallate), and IRI-50 (IRI + 50 mg/kg epigallocatechin gallate) groups. Data were analyzed using the Kruskal-Wallis and Dunn-Sidak tests. No significant differences were observed among the groups.

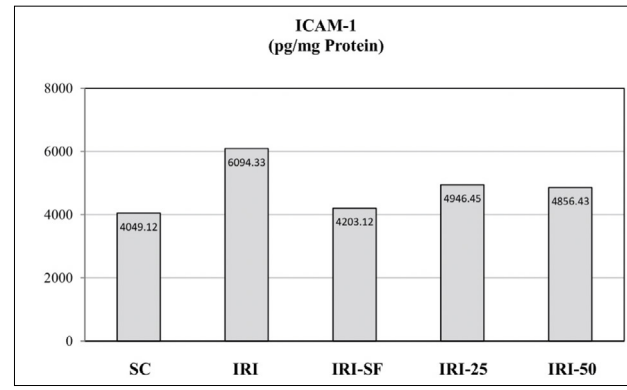


**Figure 2.** Intercellular adhesion molecule-1 (ICAM-1) levels across study groups. ICAM-1 levels (median) were measured in the SC (sham-control), IRI (ischemia-reperfusion injury), IRI-SF (IRI+saline), IRI-25 (IRI + 25 mg/kg epigallocatechin gallate), and IRI-50 (IRI + 50 mg/kg epigallocatechin gallate) groups. Statistical analysis was performed using the Kruskal-Wallis and Dunn-Sidak tests. No significant differences were detected among the groups.

### Effect of EGCG on Parameters Related to Neutrophil Infiltration

Myeloperoxidase levels did not differ significantly among the groups (Kruskal-Wallis test: $X^2$=5.416, p=0.247) (Fig. 1). Nonetheless, there was a trend toward increased MPO levels in the IRI group compared to the SC group, and a tendency toward decreased levels in the IRI-50 group relative to the IRI group.

No statistically significant differences were found between the groups in terms of ICAM-1 levels (Kruskal-Wallis test: $X^2$=2.711, p=0.607) (Fig. 2). However, the median ICAM-1 value in the IRI group (6094.33) was somewhat higher than that in the SC group (4049.12). The values in the remaining groups were similar to that of the SC group (IRI-SF: 4203.12; IRI-25: 4946.45; IRI-50: 4856.43). Although EGCG-treated animals showed lower ICAM-1 levels compared to untreated IRI animals, a similar reduction was also observed in the saline-treated group, suggesting that EGCG's beneficial effect is unlikely to be mediated through interference with ICAM-1.

As for E-selectin, P-selectin, and L-selectin, no statistically significant differences were detected among the groups (one-way ANOVA: F=1.759, p=0.159; F=0.931, p=0.457; F=1.958, p=0.123, respectively) (Fig. 3). Nevertheless, a clear trend of increased levels was observed in the IRI group compared to the SC group, and slight decreases were noted in response to 50 mg/kg EGCG.

No statistically significant differences were observed among the groups for IL-1β (Kruskal-Wallis test: $X^2$=7.509, p=0.111) and TNF-α levels (Kruskal-Wallis test: $X^2$=8.607, p=0.072). However, a significant difference was found for IL-6 levels (Kruskal-Wallis test: $X^2$=13.557, p=0.009) (Fig. 4). IL-6 levels were higher in the IRI group compared to the SC group (Dunn-Sidak test: p=0.012). Although not statistically significant, moderate decreases were observed in the groups treated with 25 and 50 mg/kg EGCG (Dunn-Sidak test: p=1.000 and p=0.166, respectively).

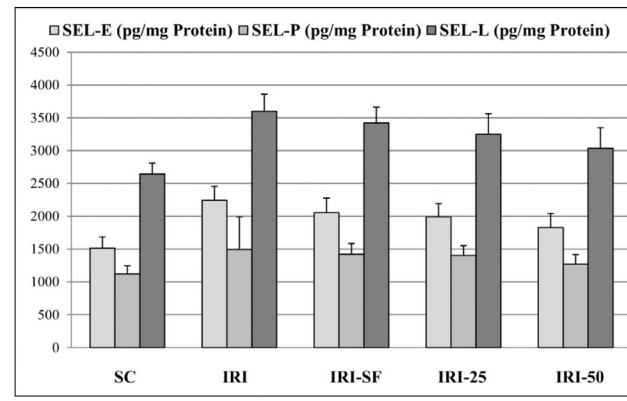


**Figure 3.** Selectin levels across study groups. E-selectin (SEL-E), P-selectin (SEL-P), and L-selectin (SEL-L) levels (mean ± standard error) were assessed in the SC (sham-control), IRI (ischemia-reperfusion injury), IRI-SF (IRI+saline), IRI-25 (IRI + 25 mg/kg epigallocatechin gallate), and IRI-50 (IRI + 50 mg/kg epigallocatechin gallate) groups. One-way analysis of variance and Tukey's test were used for statistical analysis. No significant differences were found among the groups.

## DISCUSSION

In the present study, IRI in rats resulted in marked tissue damage and oxidative stress, along with a slight decrease in the antioxidant capacity of skeletal muscle. Additionally, the outcomes in the IRI group indicated a statistically insignificant but noticeable increase in neutrophil infiltration within the tissue. Interleukin-6, a chemotactic signal associated with neutrophil infiltration, was significantly elevated in response to the injury. Despite mild increases, no statistically significant

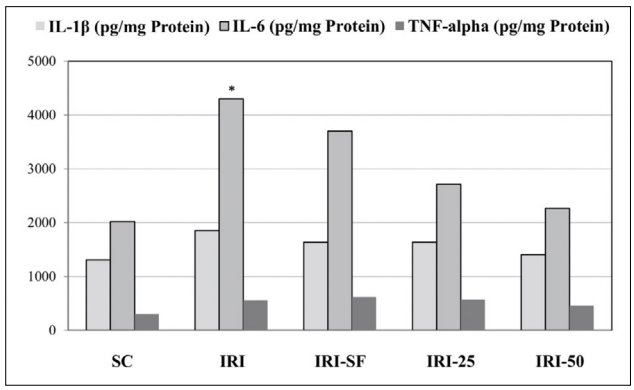


**Figure 4.** Interleukin levels across study groups. Interleukin levels (median) were analyzed in the SC (sham-control), IRI (ischemia-reperfusion injury), IRI-SF (IRI+saline), IRI-25 (IRI+25 mg/kg epigallocatechin gallate), and IRI-50 (IRI + 50 mg/kg epigallocatechin gallate) groups using the Kruskal-Wallis and Dunn-Sidak tests. *Compared with SC, $p<0.05$.

changes were observed in other parameters associated with neutrophil infiltration, including IL-1β, TNF-α, ICAM-1, E-selectin, P-selectin, and L-selectin. In summary, high doses of EGCG demonstrated beneficial effects on tissue injury, oxidative stress, and antioxidant capacity. However, only weak evidence was found to support its role in modulating markers associated with neutrophil infiltration.

The elevated serum levels of CPK and LDH in the IRI group compared to the SC group confirmed skeletal muscle injury in this model. CPK, a cytosolic enzyme found predominantly in skeletal muscle, is widely recognized as a biomarker of muscle damage.[17] Its elevation likely reflects IRI-induced myocyte necrosis and the disruption of muscle membrane integrity, resulting in the leakage of intracellular enzymes into the bloodstream. Although LDH is also present in the liver, erythrocytes, and most other tissues of the body in addition to skeletal muscle,[18] this widespread distribution can limit its specificity as a marker for skeletal muscle injury. However, it remains useful in evaluating muscle damage, particularly when LDH and CPK levels show close concordance, as observed in our study. Ideally, histological examination of muscle tissue would have provided more direct evidence of IRI-induced morphological changes. However, this technique was not employed due to limitations in our laboratory resources. Future studies incorporating histological analysis alongside current biochemical markers (e.g., CPK, LDH) would enhance the strength of the findings and offer a more comprehensive assessment of tissue injury. Similar to our previous study,[2] the highest dose of EGCG in the current experiment slightly attenuated the elevation of CPK and LDH levels in the IRI group, although the changes were not statistically significant. This suggests a mild protective effect of EGCG against skeletal muscle injury. Supporting our results, a previous study using histological evaluation demonstrated the protective effect of EGCG against reperfusion injury in rat skeletal muscle tissue.[11] The observed muscle damage in our study, as well as the protective mechanism of EGCG, may be explained by etiological factors such as oxidative stress and leukocyte infiltration.

The increased TOS and decreased TAS values in the IRI group of the present study are particularly significant in highlighting the central role of oxidative stress in skeletal muscle injury. In the context of IRI, oxidative stress can impair mitochondrial function, induce calcium overload, and trigger inflammation, apoptosis, and necrosis.[15] The preventive effect of EGCG against IRI-induced oxidative stress observed in this study further supports the critical involvement of oxidative stress, especially considering that EGCG has been shown to possess antioxidant properties in previous studies using various methodologies.[9,10] EGCG may exert its antioxidant effects either by inhibiting the production of ROS or by reducing the levels of ROS that have already been generated.

Regarding the former mechanism, dysfunction of the mitochondrial respiratory chain, activation of xanthine oxidase (XO), and neutrophil activation have been proposed as potential sources of ROS production in skeletal muscle during IRI.[19] Under physiological conditions, electron leakage from the mitochondrial electron transport chain contributes minimally to ROS generation through interaction with molecular oxygen.[20] However, during ischemia, failure of electron transfer leads to the accumulation of electrons within the mitochondrial respiratory chain. Reperfusion then introduces a sudden surge of oxygen, which readily reacts with the accumulated electrons, generating excessive ROS.[20] The resulting ROS burst triggers the opening of the mitochondrial permeability transition pore (mPTP), leading to mitochondrial swelling, adenosine triphosphate (ATP) depletion, and cytochrome c release.[20] This cascade of events ultimately activates the cellular apoptosis program.[20] In this context, in a severe IRI rat model induced by cardiac arrest/cardiopulmonary resuscitation, EGCG was shown to attenuate IRI and restore mitochondrial function, thereby reducing ROS levels, mPTP opening, and the release of cleaved-caspase 3.[21] Similarly, in a previous study on skeletal muscle IRI in rats, EGCG suppressed apoptosis, as indicated by the measurement of apoptotic markers such as Bax, cleaved-caspase 3, and cleaved-caspase 9.[15] With respect to another source of ROS, XO, which is converted from oxidized nicotinamide adenine dinucleotide-dependent dehydrogenase under ischemic conditions, generates large quantities of superoxide anions in the presence of molecular oxygen during reperfusion.[19] Although not specifically evaluated in skeletal muscle IRI, EGCG's known inhibitory effect on XO in the context of uric acid metabolism suggests a potential additional mechanism underlying its protective effects.[22] Regarding neutrophils, these cells contribute to ROS production through the action of nicotinamide adenine-dinucleotide phosphate oxidase (NOx) in tissues they infiltrate.[19] In this context, our results showed that administration of 50 mg/kg EGCG slightly reduced MPO levels in muscle tissue. Although histological confirmation

was lacking (a major limitation of the study), this finding moderately suggests a preventive effect of EGCG on neutrophil infiltration and neutrophil-derived ROS production. However, our data do not allow us to distinguish whether the observed reduction in MPO levels following EGCG treatment is due to decreased neutrophil infiltration or reduced MPO activity within infiltrating neutrophils. Future studies incorporating histological techniques, such as myeloperoxidase staining or flow cytometric analysis of neutrophil-specific markers, could provide a more definitive assessment of neutrophil infiltration and help clarify whether the observed decrease in MPO levels with EGCG treatment reflects reduced infiltration or diminished enzymatic activity within neutrophils. Additionally, the possibility that EGCG directly inhibits enzymatic activity in neutrophils, even when neutrophil counts are within the normal range, requires further investigation.

ROS generated from sources mentioned above can be neutralized by enzymatic systems, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and heme oxygenase (HO), as well as by endogenous small molecule scavengers such as glutathione and urate.[23] In this context, EGCG administered prior to reperfusion has been shown to exert protective effects through the activation of serum SOD in rat models of skeletal and testicular IRI.[15,24] Similarly, a study on IRI in isolated rat hearts demonstrated that lipid peroxidation was inhibited, and the expression of Mn-SOD and catalase was increased in the presence of EGCG.[25] Furthermore, decreased SOD and GSH-Px activities resulting from oxygen-glucose deprivation/reperfusion in primary cultured cerebral cortical neurons were reversed by EGCG pretreatment.[26] Additionally, EGCG was shown to induce the expression of HO-1, a downstream signaling molecule and rate-limiting antioxidant enzyme involved in heme metabolism in a rat model of IRI. While HO-1 is expressed at low levels under normal physiological conditions, its expression is significantly upregulated under stress.[15]

Regarding scavenging activity, $O_2$-, hydrogen peroxide ($H_2O_2$), hydroxyl radical (OH), and peroxynitrite (ONOO-) are considered the primary radicals generated via the aforementioned mechanisms.[27,28] Such an in vivo effect of EGCG on $O_2$- was specifically demonstrated in a rat skeletal muscle IRI model.[11] In line with this, the scavenging activities of green tea extract and EGCG were shown to eliminate $H_2O_2$ radicals in the brain and retina.[29,30] Likewise, EGCG was capable of scavenging $O_2$- and OH radicals in guinea pigs with ischemic myocardial dysfunction.[31] Additionally, green tea extract and EGCG were shown to reduce nitrotyrosine formation, a marker of ONOO- generation, in the intestine and kidney.[12,32]

In the present study, MPO levels, a marker of neutrophil infiltration, showed an increasing trend in the IRI group, which was modestly attenuated by EGCG treatment. Although tissue levels of E-selectin, P-selectin, L-selectin, and ICAM-1, which were involved in neutrophil adhesion to the endothelium, did not differ significantly between groups, a slight increase was observed in the IRI group compared to the SC group. Nevertheless, other studies have demonstrated that E-selectin, L-selectin, P-selectin, and ICAM-1 actively contribute to striated muscle IRI, as their levels were significantly higher in IRI groups compared to the control group.[4-6] The relatively paradoxical results of these two studies, compared with our findings, may stem from differences in the experimental models of IRI employed. For example, we used a 4 + 2 h IRI model (relatively short-term), while two of the other studies used 2 + 24 h and 6 + 24 h models (relatively long-term).[4,5] The expression of such molecules may require a longer time to reach detectable levels, which could explain why our shorter experimental protocol resulted in only a slight, statistically non-significant increase. In contrast, extended protocols may provide a more conducive environment for these inflammatory markers to participate more fully in the infiltration process. Supporting this, previous studies have shown that EGCG administration inhibited the upregulation of ICAM-1, P-selectin, and E-selectin in models of intestinal IRI in rats, hypercholesterolemic rat liver, and human umbilical vein cell cultures.[12,13] Although a potential effect of EGCG on E-selectin, P-selectin, L-selectin, and ICAM-1 could not be confirmed due to the lack of significant differences between groups in the present study, this is the first report suggesting a subtle interaction between EGCG and these markers in the context of skeletal muscle IRI.

In addition to neutrophil rolling and adhesion, the release of chemotactic signals is also a critical step in the neutrophil infiltration process. In this study, the level of IL-6, a cytokine associated with chemotactic signaling, increased in the IRI group compared to the SC group; this increase was markedly prevented by 50 mg/kg EGCG. Meanwhile, IL-1$\beta$ and TNF-$\alpha$ levels showed slight but statistically non-significant changes in the same context. While other neutrophil infiltration markers remained unchanged, the significant elevation of IL-6 suggests potential neutrophil involvement in IRI-induced damage. This dissociation between IL-6 and other markers may reflect early neutrophil recruitment triggered by IL-6, occurring before significant changes in other markers. These results are, however, only partially consistent with our previous study, in which these parameters were measured in serum samples and no statistically significant differences were found between the study groups.[2] Nonetheless, all three cytokines have been convincingly implicated in the etiology of striated muscle IRI. Moreover, these cytokines (TNF-$\alpha$, IL-1$\beta$, and IL-6) have been shown to be suppressed by EGCG in a rat skeletal muscle IRI model.[15]

In recent years, in addition to the studies mentioned above, an accumulating body of evidence has demonstrated the beneficial effects of EGCG and its mechanisms of action in various IRI models in animal experiments, including the brain,[33,34] heart,[35] intestine,[36] spinal cord[37] germ cells,[38] and retina.[39] EGCG has been shown to exert its protective effects through

multiple mechanisms, such as restoring Na+/K+-ATPase (NKA) activity and increasing membrane glutamate transporter-1 (GLT-1) expression via NKA–GLT-1 interaction;[33] downregulating pro-inflammatory IL-1β and upregulating anti-inflammatory IL-10;[34] inhibiting ferroptosis, apoptosis, and autophagy through modulation of 14–3-3η;[35] activating nuclear receptor-related protein 1;[36] modulating caspase-3, TNF-α, and inducible nitric oxide synthase (iNOS);[37] suppressing apoptosis via the Survivor Activating Factor Enhancement (SAFE)/Nrf2 signaling pathway;[38] and activating the nuclear factor erythroid 2-related factor 2/heme oxygenase-1 (Nrf2/HO-1) pathway.[39] Taken together, our previous and current findings, along with these studies, support the conclusion that EGCG is a promising drug candidate with a broad range of protective actions across various tissues affected by IRI.

## CONCLUSION

Epigallocatechin gallate has the potential to exert a protective effect against skeletal muscle injury during IRI, an effect that appears to be primarily mediated through its antioxidant capacity. While a slight modulatory effect of EGCG on neutrophil infiltration was observed, its influence on specific inflammatory markers, including E-selectin, P-selectin, L-selectin, ICAM-1, IL-1β, IL-6, and TNF-α, could not be conclusively established. Given the limitations of the current study, future experiments with higher concentrations of EGCG may help determine whether statistically significant effects on IRI can be observed in this experimental model. Additionally, extending the reperfusion period to 12, 24, or 48 hours may better simulate delayed inflammatory responses, allowing for a more detailed analysis of neutrophil infiltration-related markers and the detection of statistically significant differences among study groups.

**Ethics Committee Approval:** This study was approved by the Kahramanmaraş Sütçü İmam University Animal Experimentation Ethics Committee (Date: 19.04.2020 Decision No: 2020/04-0).

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DENEYSEL ÇALIŞMA - *ÖZ*

## Epigallokateşin gallat'ın iskemi-reperfüzyon hasarı üzerindeki etkileri: Sıçanlarda deneysel bir çalışma

AMAÇ: İskelet kası iskemi-reperfüzyon hasarı (İRH), çeşitli klinik durumlarda önemli bir endişe kaynağıdır ve oksidatif stres ve nötrofil infiltrasyonu bu olguda merkezi bir rol oynar. Ne yazık ki, klinisyenlerin hem İRH'nin önlenmesi hem de tedavisi için çok az tıbbi ürün seçeneği vardır. Mevcut çalışmanın amaçları, epigallokateşin gallat'ın (EGCG) (i) iskelet kası hasarı, (ii) oksidatif stres ve (iii) nötrofil infiltrasyonuyla ilişkili belirteçler üzerindeki etkilerini incelemektir.

GEREÇ VE YÖNTEM: Bu, sıçanlar üzerinde yapılan deneysel bir çalışmadır. Elastik bir lastik bant kullanan İRH modeli 4 + 2 saatti (İRH grubu). Sahte kontrol (SC) grubunda, bant bağlaması hariç tüm adımlar korundu. Serum fizyolojik (10 mL/kg/i.p.) ve EGCG (25 ve 50 mg/kg/i.p.), reperfüzyondan 30 dakika önce uygulandı (İRH-SF, İRH-25 ve İRH-50 grupları). Kreatin fosfokinaz (CPK) (primer uç-nokta), laktat dehidrogenaz (LDH), total oksidan durum (TOS), total antioksidan durum (TAS), miyeloperoksidaz (MPO), E-selektin (Endotelyal selektin), P-selektin (Plazma selektini), L-selektin (Lenfosit selektini), hücreler arası adhezyon molekülü-1 (ICAM-1) ve çeşitli sitokinler (IL-1β, IL-6, TNF-α) serum veya gastroknemius kas örneklerinde ölçüldü.

BULGULAR: CPK, LDH ve TOS düzeyleri İRH grubunda SC grubuna göre daha yüksekti (p=0.001; p=0.0001; p=0.005); İRH-50 grubunda İRH grubuna göre istatistiksel olarak anlamlı olmayan düşüşler gözlendi (p=0.628; p=0.167; p=0.444). TAS açısından, İRH'de SC grubuna göre görünür bir düşüş görüldü (p=0.054) ve bu, 50 mg/kg EGCG ile önemli ölçüde arttı (p=0.011). Geri kalan parametreler açısından (İL-6 hariç), İRH'de SC'ye göre istatistiksel olarak önemsiz artışlar ve EGCG ile tedavi edilen gruplarda İRH gruplarına göre düşüşler tespit edildi.

SONUÇ: EGCG'nin antioksidan aktiviteye sahip olduğu söylenebilir. Ancak nötrofil infiltrasyonuyla ilişkili belirteçlerle etkileşiminden kaynaklanan yararlı bir etki yalnızca öneri niteliğinde olacaktır.

Anahtar sözcükler: İskelet kası; epigallokateşin gallat; nötrofil infiltrasyonu; oksidatif stres; reperfüzyon hasarı; sitokinler.

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