EXPERIMENTAL STUDY

Comprehensive evaluation of the antioxidant, anti-inflammatory, and anti-apoptotic effects of hydroxytyrosol in skeletal muscle ischemia–reperfusion injury

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ABSTRACT

BACKGROUND: Skeletal muscle ischemia–reperfusion (I/R) injury is a complex pathological process characterized by excessive reactive oxygen species production, activation of inflammatory cascades, and apoptosis, ultimately resulting in substantial structural and functional tissue damage. Hydroxytyrosol (HT), a naturally occurring phenolic compound predominantly in olive-derived products, exhibits potent antioxidant and anti-inflammatory properties. This study aimed to comprehensively evaluate the potential protective effects of HT against lower-extremity skeletal muscle I/R injury in an experimental rat model.

METHODS: Twenty-four adult Wistar albino rats were randomly assigned to four experimental groups: Sham, HT-Control, I/R, and HT+I/R. Hind-limb ischemia was induced for two hours, followed by two hours of reperfusion. In the treatment group, HT (10 mg/kg, intraperitoneally) was administered 30 minutes before ischemia induction. Serum oxidative stress parameters, including total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI), were measured. Skeletal muscle tissues were evaluated histopathologically, and interleukin-1β (IL-1β) levels, caspase-3 expression, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive apoptotic cells were assessed.

RESULTS: Compared with the Sham group, the untreated I/R group exhibited significantly higher TOS and OSI levels, increased histopathological injury scores, marked inflammatory cell infiltration, and enhanced apoptotic activity ($p<0.05$). HT administration significantly attenuated oxidative stress, reduced inflammatory responses, decreased tissue injury severity, and lowered apoptotic indices compared with the I/R group ($p<0.05$).

CONCLUSION: Hydroxytyrosol exerted significant protective effects against skeletal muscle I/R injury by attenuating oxidative stress, suppressing inflammatory mediators, and reducing apoptosis. These findings highlight the therapeutic potential of HT as a promising adjunctive strategy in the prevention and management of ischemia–reperfusion-induced tissue damage.

Keywords: Apoptosis; hydroxytyrosol; ischemia–reperfusion; oxidative stress; skeletal muscle.

Cite this article as: Özdem T, Kaymak Ş, Kartal H, Demirdaş E, Erol G, Ertaş E, et al. Comprehensive evaluation of the antioxidant, anti-inflammatory, and anti-apoptotic effects of hydroxytyrosol in skeletal muscle ischemia–reperfusion injury. Ulus Travma Acil Cerrahi Derg 2026;32:885-894.

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Ulus Travma Acil Cerrahi Derg 2026;32(8):885-864 DOI: 10.14744/tjtes.2025.49540
Submitted: 17.02.2026 Revised: 26.02.2026 Accepted: 14.05.2026 Published: 10.08.2026
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INTRODUCTION

Skeletal muscle ischemia–reperfusion (I/R) injury is a major pathological condition encountered in a variety of clinical settings, including vascular surgery, trauma, organ transplantation, and tourniquet application. It may result in substantial tissue damage and functional impairment.[1] During ischemia, interruption of blood flow causes cellular hypoxia. Paradoxically, restoration of blood flow during reperfusion exacerbates tissue injury and contributes to impaired microvascular perfusion.[2]

Reperfusion is associated with excessive production of reactive oxygen species (ROS), leading to disruption of cellular redox homeostasis and activation of inflammatory pathways. Together with intracellular calcium overload and mitochondrial dysfunction, these processes play a central role in the pathogenesis of tissue injury, ultimately leading to cell death through apoptosis and necrosis.[3] Therefore, agents capable of attenuating oxidative stress and inflammation while inhibiting apoptotic pathways have attracted considerable interest as potential therapeutic strategies for skeletal muscle I/R injury.[4]

Hydroxytyrosol (HT) is a major bioactive phenolic compound derived from olive oil and olive leaves and is recognized for its potent free radical–scavenging activity and its ability to modulate inflammatory and apoptotic pathways.[3,5] In skeletal muscle, HT has been reported to ameliorate mitochondrial dysfunction and protect against muscle degeneration.[6] However, studies evaluating the effects of HT on oxidative balance, inflammation, and apoptosis in skeletal muscle I/R injury remain limited.

The present experimental study investigated the protective effects of hydroxytyrosol against ischemia–reperfusion–induced injury in rat hindlimb skeletal muscle. Systemic redox status was assessed by measuring serum total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI). Histopathological alterations were evaluated using hematoxylin–eosin staining, inflammatory responses were assessed through interleukin-1β (IL-1β) expression, and apoptotic activity in skeletal muscle tissue was examined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and caspase-3 activity.

MATERIALS AND METHODS

Ethical Approval and Experimental Animals

Ethical approval for this study was obtained from the Local Ethics Committee of Kobay DHL Inc., Ankara, Türkiye (Approval No. 693; December 8, 2023). All experimental procedures were conducted in accordance with institutional guidelines and current regulations governing the care and use of laboratory animals.

The experimental study was performed at the Experimental Animal Research Facility and completed within a one-week period in December 2023.

Adult male Wistar albino rats weighing 250–300 g were included in the study. Animals were housed under standardized environmental conditions at a temperature of 22±2°C, relative humidity of 50±5%, and a 12-hour light/dark cycle. Standard laboratory chow and water were provided ad libitum. Food was withheld for 12 hour before the experimental procedures.

Surgical Procedure and Ischemia–Reperfusion Model

All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals.[7]

Prior to surgical intervention, general anesthesia was induced using ketamine (50–100 mg/kg) and xylazine (10 mg/kg), administered either intramuscularly or intraperitoneally. Body temperature was maintained throughout the procedure using a thermostatically controlled heating pad and an external warming source.

After shaving and sterile preparation of the surgical field, the animals were placed in the supine position. A right inguinal incision was made to expose the femoral vessels, and the femoral artery and vein were carefully dissected free from surrounding connective tissue. To minimize thrombus formation, heparin (100 U/kg) was administered intravenously through the femoral vein in all animals.

Ischemia was induced by applying an atraumatic microvascular clamp to the femoral artery. Successful ischemia was confirmed by limb pallor, cooling, and loss of distal pulse, and absence of pulsation in the distal artery. In the hydroxytyrosol-treated groups, HT (10 mg/kg, intraperitoneally) was administered 30 minutes before ischemia induction.[8]

Following 2 hours of ischemia, the vascular clamp was removed to initiate reperfusion. Restoration of blood flow was confirmed by normalization of limb color and temperature. The incision was closed using 4-0 silk sutures, and the animals were observed for an additional two hours during the reperfusion period.

Experimental Groups and Drug Administration

Animals were randomly assigned to four groups (n=6 per group):

Sham Group: Animals underwent anesthesia and vascular isolation to control for the effects of surgical manipulation; however, no ischemia was induced and no treatment was administered.

HT-Control Group: To assess the potential effects of HT in the absence of ischemia, animals underwent anesthesia and vascular isolation, followed by intraperitoneal administration of HT. No ischemia was induced.

Ischemia–Reperfusion (I/R) Group: Animals were subjected to 120 minutes of hindlimb ischemia followed by 120 minutes of reperfusion without pharmacological treatment.

I/R + Hydroxytyrosol (HT) Group: HT (10 mg/kg, intraperitoneally) was administered 30 minutes before ischemia induction as a prophylactic treatment, followed by the ischemia–reperfusion protocol.

At the end of the reperfusion period, animals were euthanized under deep anesthesia. Blood samples were collected by cardiac puncture for biochemical analyses, including TAS, TOS, and OSI. Plasma was separated by centrifugation and stored at −80°C until analysis.

Gastrocnemius muscle specimens were harvested from the affected hindlimb for histopathological (hematoxylin–eosin staining), immunohistochemical (TUNEL, caspase-3), and biochemical (IL-1β, tissue homogenization) analyses. Tissue samples designated for histopathological and immunohistochemical evaluation were fixed in 10% formalin for subsequent processing and analysis.

Histological Evaluation by Hematoxylin–Eosin Staining

At the end of the experimental period, gastrocnemius muscle specimens were collected and immersed in 10% neutral-buffered formalin for 48 hours to ensure adequate preservation of tissue morphology.

Subsequently, the samples underwent routine histological processing, including stepwise dehydration in ascending concentrations of alcohol followed by xylene clearing. The processed tissues were then embedded in paraffin, and serial sections (5 μm thick) were cut using a microtome (Leica RM 2125RT).

After deparaffinization and rehydration, the tissue sections were stained using a hematoxylin–eosin (H&E) staining kit (ab245880). Microscopic evaluation was performed using a light microscope equipped with a digital camera system (Olympus CX43).

Structural alterations in the muscle tissue were assessed using a semi-quantitative scoring system based on the presence of edema, inflammatory cell infiltration, and necrosis. Each parameter was scored as follows: 0=none, 1=mild, 2=moderate, and 3=severe (Fig. 1).

Caspase-3 Immunohistochemical Analysis

Caspase-3 expression was evaluated immunohistochemically to assess apoptosis in skeletal muscle tissue.

Paraffin-embedded tissue sections (4–5 μm) previously fixed in formalin were cleared in xylene and subsequently rehydrated through graded alcohol concentrations. Heat-induced antigen retrieval was performed in citrate buffer (pH 6.0) using a microwave oven for 15 minutes, after which the slides were allowed to cool to room temperature.

To block endogenous peroxidase activity, sections were treated with 3% hydrogen peroxide (H_2O_2) for 10 minutes. After blocking nonspecific background staining, the slides were incubated overnight at +4°C with an anti-caspase-3 primary antibody (Anti-Caspase-3 [EPR18297], ab184787).

Following rinsing with phosphate-buffered saline (PBS), sections were incubated with a biotinylated secondary antibody and subsequently treated with a streptavidin–peroxidase complex. The antigen–antibody reaction was visualized using 3,3′-diaminobenzidine (DAB) chromogen (UltraView Universal DAB Detection Kit, 5269806001). Finally, the slides were counterstained with Mayer's hematoxylin and coverslipped.

Cells exhibiting brown cytoplasmic and/or nuclear immunoreactivity were considered positive. The apoptotic index was graded semi-quantitatively according to staining intensity and distribution as follows: 0, none; 1, mild; 2, moderate; and 3, severe/diffuse (Fig. 2).[4,9]

IL-1β Immunohistochemical Analysis

IL-1β expression was evaluated as a tissue-level marker of the inflammatory response associated with ischemia–reperfusion injury.

Paraffin sections underwent the same deparaffinization, rehydration, and antigen retrieval procedures described for caspase-3 analysis. After blocking endogenous peroxidase activity, sections were incubated overnight at +4°C with an anti-IL-1β primary antibody (Anti-IL-1 beta [RM1009], ab283818).

Following incubation with the secondary antibody and strep-

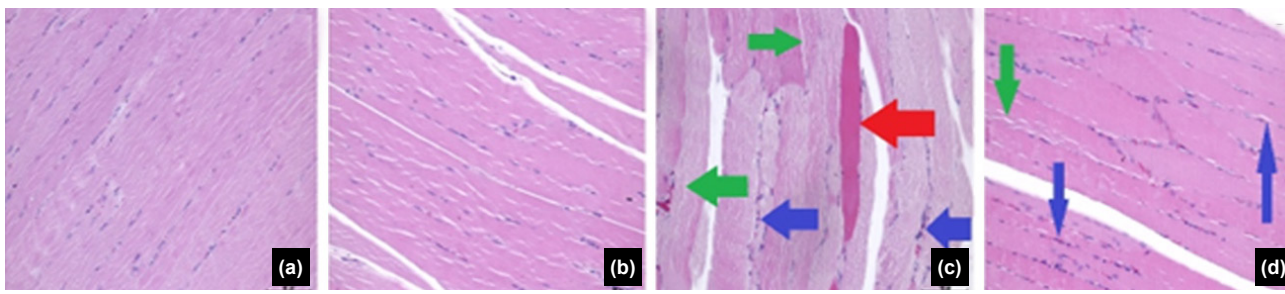


Figure 1. Hematoxylin–eosin histological evaluation of skeletal muscle. Light microscopic images of skeletal muscle sections stained with hematoxylin–eosin (H&E) obtained from the experimental groups. Panels a, b, c, and d correspond to the Sham, HT-Control, I/R, and HT+I/R groups, respectively. Red arrows indicate areas of necrosis, green arrows indicate edema, and blue arrows indicate inflammatory cell infiltration (magnification ×200). HT: Hydroxytyrosol.

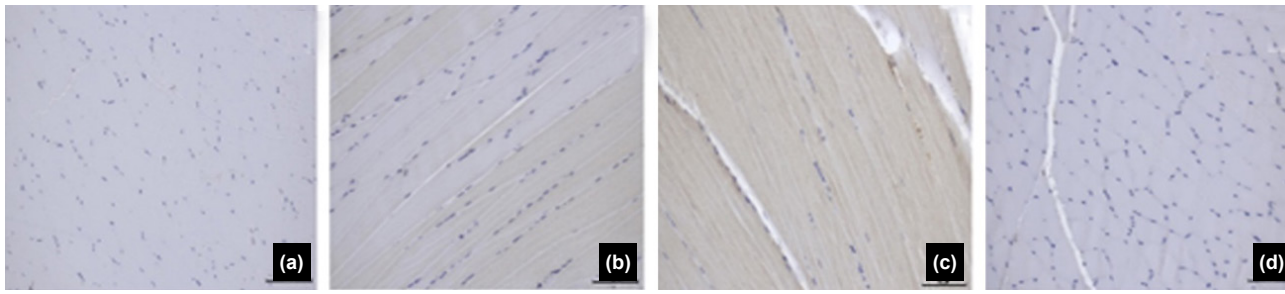


Figure 2. Caspase-3 immunohistochemical evaluation of skeletal muscle. Light microscopic images of skeletal muscle sections immunostained with a primary anti-caspase-3 antibody. Panels a, b, c, and d correspond to the Sham, HT-Control, I/R, and HT+I/R groups, respectively (magnification ×200). HT: Hydroxytyrosol.

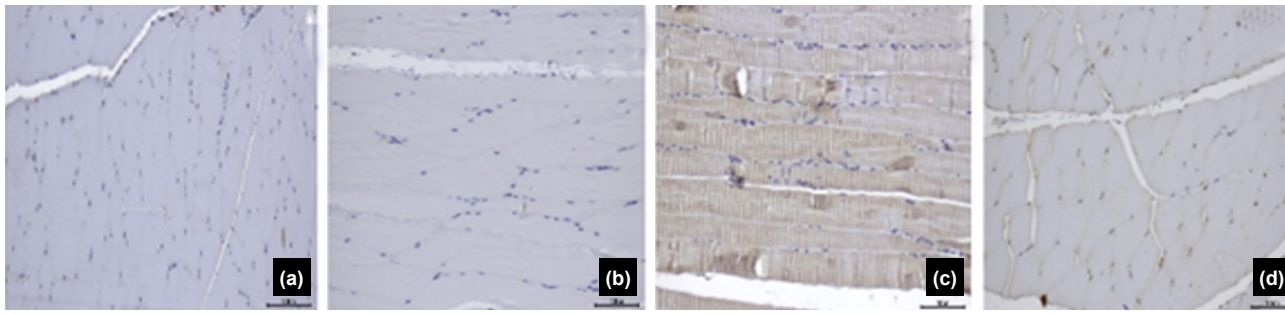


Figure 3. Interleukin-1β immunohistochemical evaluation of skeletal muscle. Light microscopic images of skeletal muscle sections immunostained with a primary anti-interleukin-1β (anti–IL-1β) antibody. Panels a, b, c, and d correspond to the Sham, HT-Control, I/R, and HT+I/R groups, respectively (magnification ×200). HT: Hydroxytyrosol.

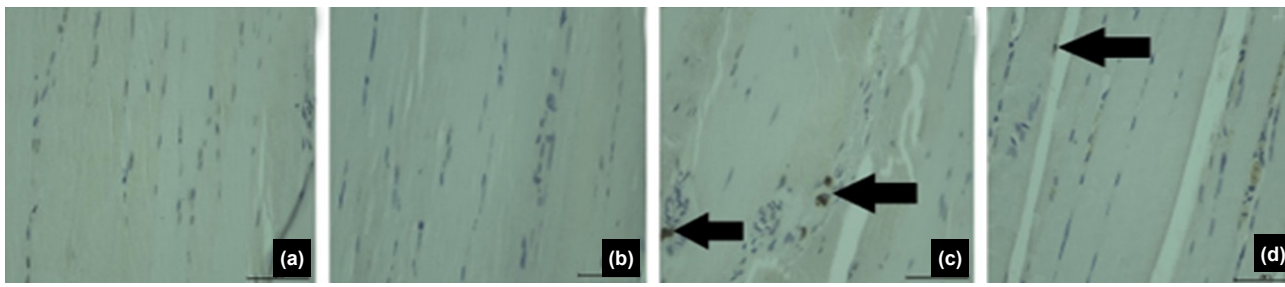


Figure 4. TUNEL immunohistochemical evaluation of skeletal muscle. Light microscopic images of skeletal muscle sections stained using the TUNEL method. Panels A, B, C, and D correspond to the Sham, HT-Control, I/R, and HT+I/R groups, respectively. Black arrows indicate TUNEL-positive apoptotic cells (magnification ×400). HT: Hydroxytyrosol.

tavidin–horseradish peroxidase (HRP), IL-1β immunoreactivity was visualized using DAB chromogen. The slides were counterstained with hematoxylin and evaluated in a blinded manner.

Immunopositive staining observed in inflammatory cells and interstitial regions surrounding muscle fibers was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. Scores were compared among the study groups (Fig. 3).[10]

TUNEL Immunohistochemical Analysis

Apoptotic cell death in skeletal muscle tissue was detected in situ using the TUNEL method.

DNA fragmentation was detected using a commercially available assay kit (ApopTag® Plus Peroxidase In Situ Apoptosis Kit, S7101). Tissue sections were deparaffinized and rehydrated, followed by incubation with proteinase K to enhance membrane permeability and facilitate reagent penetration.

Subsequently, sections were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) enzyme and labeling solution at 37°C for 60 minutes in a humidified chamber. After washing, a peroxidase-conjugated reagent was applied. Signal development was achieved using DAB chromogen (UltraView Universal DAB Detection Kit, 5269806001).

Cells exhibiting brown nuclear staining were considered TUNEL-positive. For quantitative analysis, five randomly selected microscopic fields from each specimen were evaluated. The apoptotic index (AI) was calculated as the percentage of positively stained cells relative to the total number of cells counted (Fig. 4).[4,9]

Total Antioxidant Status, Total Oxidant Status, and Oxidative Stress Index Analysis

At the end of the experimental protocol, blood samples were collected into plain tubes without a clot activator. Following clot formation, samples were centrifuged at 3,000 g for 10 minutes at +4°C. The separated serum was aliquoted and stored at −80°C until biochemical analysis.

Serum TAS and TOS were measured spectrophotometrically using commercially available automated colorimetric assay kits (TAS: MBS2567994; TOS: MBS2567995).

TAS was determined based on the ability of serum antioxidants to reduce the blue-green 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation to its colorless form, with absorbance measured at 660 nm. Results were expressed as mmol Trolox equivalent/L.

For TOS measurement, oxidant molecules in the serum converted the ferrous ion complex to ferric ions under acidic conditions. The resulting color formation was measured at 530 nm and expressed as μmol H_2O_2 equivalent/L.

The OSI, representing the overall oxidant–antioxidant balance, was calculated after conversion of TAS values from mmol to μmol (×1000) to ensure unit compatibility. OSI values were expressed as arbitrary units (AU), calculated using the TOS/TAS ratio.[1]

Statistical Analysis

Sample size was determined using the Mead method, which indicated that six rats per group were sufficient for the Sham, HT-Control, I/R, and HT+I/R groups, yielding a total sample size of 24 animals.

Continuous variables are presented as mean ± standard deviation, whereas categorical variables are expressed as frequencies and percentages.

Differences in TAS, TOS, OSI, and TUNEL values among groups were analyzed using one-way analysis of variance (ANOVA). When overall statistical significance was detected, intergroup comparisons were performed using Tukey's post hoc multiple-comparison test.

Associations between experimental groups and histopathological parameters, including edema, necrosis, inflammatory cell infiltration (ICI), caspase, and IL-1β expression, were evaluated using the chi-square exact test.

A p value <0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics version 25.

RESULTS

Biochemical Evaluation

Biochemical findings according to the treatment group are summarized in Table 1.

Mean TOS values were 0.12±0.01 in the Sham group, 0.16±0.02 in the HT-Control group, 0.28±0.03 in the I/R group, and 0.14±0.03 in the HT+I/R group. Overall comparison revealed a significant difference among the groups (p<0.001).

Post hoc analysis demonstrated that TOS levels were significantly higher in the I/R group than in the Sham, HT-Control, and HT+I/R groups (all p<0.05). Furthermore, TOS levels were significantly lower in the Sham group than in the HT-Control and I/R groups (p<0.05). HT treatment significantly reduced TOS levels in the HT+I/R group compared with the untreated I/R group (p<0.05).

Total Antioxidant Status

Mean TAS values were 0.24±0.05 in the Sham group, 0.30±0.03 in the HT-Control group, 0.25±0.03 in the I/R group, and 0.26±0.05 in the HT+I/R group. No statistically significant differences were observed among the groups (p=0.11).

Oxidative Stress Index

Mean OSI values were 0.05±0.01 in the Sham group, 0.06±0.01

Table 1. Comparison of biochemical parameters among experimental groups

Treatment group	TOS (Mean±SD)	TAS (Mean±SD)	OSI (Mean±SD)
Sham (a)	0.12±0.0^{b,c}	0.24±0.05	0.05±0.01^{c}
HT-Control (b)	0.16±0.02^{a,c}	0.30±0.03	0.06±0.01^{c}
I/R (c)	0.28±0.03^{a,b,d}	0.25±0.03	0.11±0.01^{a,b,d}
HT+I/R (d)	0.14±0.03^{c}	0.26±0.05	0.06±0.01^{c}
p	<0.001	0.11	<0.001

SD: Standard deviation; TAS: Total Antioxidant Status. Comparisons among groups were performed using one-way analysis of variance (ANOVA). Different superscript letters (a, b, c, d) indicate statistically significant differences between groups (p<0.05).

in the HT-Control group, 0.11±0.01 in the I/R group, and 0.06±0.01 in the HT+I/R group. Overall comparison demonstrated a significant difference among the groups (p<0.001).

Post hoc analysis showed that OSI levels were significantly higher in the I/R group than in the Sham, HT-Control, and HT+I/R groups (p<0.05). No significant differences were observed among the Sham, HT-Control, and HT+I/R groups (p>0.05).

Histopathological Examination Using Hematoxylin–Eosin Staining

Histopathological findings are presented in Table 2.

Edema

No edema was observed in the Sham and HT-Control groups, with all specimens classified as grade 0. In the I/R group, 83.3% of specimens exhibited grade 2 edema and 16.7% exhibited grade 3 edema. In the HT+I/R group, 83.3% of specimens showed grade 1 edema and 16.7% showed grade 2 edema. Edema severity differed significantly among the groups (p<0.001).

Necrosis

No necrotic changes were observed in the Sham or HT-Control groups, and all specimens were classified as grade 0. In the I/R group, necrosis was graded as 1 in 50% of specimens, 2 in 33.3%, and 3 in 16.7%. The HT+I/R group exhibited the same grading distribution. Overall, necrosis scores differed significantly among the groups (p=0.002).

Inflammatory Cell Infiltration

All animals in the Sham and HT-Control groups were classified as grade 0. In the I/R group, severe infiltration (grade 3) was observed in all animals (100%). In the HT+I/R group, inflammatory cell infiltration was graded as 1 in 33.3% of animals and 2 in 66.7%. A significant association was observed between treatment group and ICI severity (p<0.001).

Immunohistochemical Evaluation

Immunohistochemical findings are summarized in Table 3.

Mean TUNEL-positive cell counts were 1±0 in both the Sham and HT-Control groups, 4.33±2.1 in the I/R group, and 1.33±0.75 in the HT+I/R group. Intergroup comparison demonstrated a significant difference (p<0.001). Post hoc analysis revealed significantly higher TUNEL values in the I/R group than in all other groups (p<0.05).

Caspase-3 Expression

All animals in the Sham and HT-Control groups were classified as grade 0. In the I/R group, all animals (100%) exhibited grade 2 caspase-3 immunoreactivity. In the HT+I/R group, 83.3% of animals were classified as grade 0 and 16.7% as grade 1. Caspase-3 expression differed significantly among the groups (p<0.001).

IL-1β Expression

No IL-1β immunoreactivity was observed in the Sham and HT-Control groups, with all specimens classified as grade 0. In the I/R group, 66.7% of animals exhibited grade 3 staining and 33.3% exhibited grade 2 staining. In the HT+I/R group, 66.7% of animals exhibited grade 1 staining and 33.3% exhibited grade 2 staining. IL-1β expression differed significantly among the groups (p<0.001).

DISCUSSION

The beneficial health effects of olive oil and adherence to the

Table 2. Comparison of histopathological scores among experimental groups

Parameter	Grade	Sham n (%)	HT-Control n (%)	I/R n (%)	HT+I/R n (%)	p
Edema	0	6 (100)	6 (100)	0 (0)	0 (0)	<0.001
	1	0 (0)	0 (0)	0 (0)	5 (83.3)	
	2	0 (0)	0 (0)	5 (83.3)	1 (16.7)	
	3	0 (0)	0 (0)	1 (16.7)	0 (0)	
Necrosis	0	6 (100)	6 (100)	0 (0)	0 (0)	0.002
	1	0 (0)	0 (0)	3 (50)	3 (50)	
	2	0 (0)	0 (0)	2 (33.3)	2 (33.3)	
	3	0 (0)	0 (0)	1 (16.7)	1 (16.7)	
Inflammatory cell infiltration (ICI)	0	6 (100)	6 (100)	0 (0)	0 (0)	<0.001
	1	0 (0)	0 (0)	0 (0)	2 (33.3)	
	2	0 (0)	0 (0)	0 (0)	4 (66.7)	
	3	0 (0)	0 (0)	6 (100)	0 (0)	

Comparisons among groups were performed using the chi-square exact test. A p value <0.05was considered statistically significant.HT: Hydroxytyrosol.

Table 3. Comparison of immunohistochemical findings among experimental groups

Parameter	Sham[a]	HT-Control[b]	I/R[c]	HT+I/R[d]	p
TUNEL-positive cells (Mean±SD)	1±0[c]	1±0[c]	4.33±2.1[a,b,d]	1.33±0.75[c]	<0.001*
Grade 0	6 (100)	6 (100)	0 (0)	5 (83.3)	<0.001†
Grade 1	0 (0)	0 (0)	0 (0)	1 (16.7)	
Grade 2	0 (0)	0 (0)	6 (100)	0 (0)	
Grade 0	6 (100)	6 (100)	0 (0)	0 (0)	<0.001†
Grade 1	0 (0)	0 (0)	0 (0)	4 (66.7)	
Grade 2	0 (0)	0 (0)	2 (33.3)	2 (33.3)	
Grade 3	0 (0)	0 (0)	4 (66.7)	0 (0)	

SD: Standard deviation, HT: Hydroxytyrosol. *Data were analyzed using one-way analysis of variance (ANOVA). †Variables were analyzed using the Chi-square exact test. Different superscript letters (a, b, c, d) indicate statistically significant differences between groups (p<0.05).

Mediterranean diet have been demonstrated in numerous epidemiological studies. Hydroxytyrosol, one of the principal bioactive compounds found in olive products, is characterized by high bioavailability and a favorable safety profile in humans.[11] The European Food Safety Authority (EFSA) has approved a health claim indicating that olive oil polyphenols contribute to the protection of blood lipids from oxidative damage.[5]

In the present study, the protective effects of hydroxytyrosol on oxidative stress, edema, inflammation, necrosis, and apoptosis were evaluated in a rat hindlimb I/R model using histopathological, biochemical, and immunohistochemical analyses. As expected, I/R injury significantly increased OSI, TOS, pro-inflammatory cytokine IL-1β expression, and apoptotic markers, including caspase-3 and TUNEL positivity. Pre-ischemic administration of HT significantly attenuated these pathological alterations, preserved tissue architecture histologically, and reduced the overall oxidative burden.

Ischemia–Reperfusion Pathophysiology and Oxidative Stress

The pathophysiology of I/R injury is characterized by depletion of energy stores during ischemia and paradoxical exacerbation of tissue damage following reperfusion due to excessive generation of reactive oxygen species. During ischemia, intracellular adenosine triphosphate (ATP) levels decline, anaerobic glycolysis increases, and intracellular calcium accumulates. Upon reperfusion, reoxygenation promotes electron leakage from the mitochondrial respiratory chain and activates enzymatic systems such as xanthine oxidase, resulting in a surge of cytotoxic ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radicals.[7,4]

In the present study, significantly elevated TOS and OSI levels in the I/R group compared with the Sham group confirm the oxidative stress induced by reperfusion. Oxidative stress promotes membrane damage through peroxidation of polyunsaturated fatty acids within phospholipid bilayers, thereby compromising cellular integrity. Previous studies have consistently reported increased levels of lipid peroxidation by-products, such as malondialdehyde (MDA), in experimental I/R models.[12]

The significantly lower TOS and OSI levels observed in the HT+I/R group compared with the untreated I/R group, approaching those of the Sham group, demonstrate the potent antioxidant effects of HT.

Although TAS values did not differ significantly among the groups (p=0.11), this finding is consistent with previous observations. During the early phase of I/R injury, antioxidant reserves may be rapidly consumed as part of the cellular defense response to oxidative stress. The reduction in TOS without a corresponding increase in TAS suggests that HT may not primarily enhance systemic antioxidant capacity. Rather, HT appears to suppress excessive ROS production at its source or directly scavenge free radicals, thereby reducing the overall oxidant burden. Previous studies have demonstrated that HT decreases mitochondrial ROS generation and preserves mitochondrial membrane potential, thereby limiting oxidative damage.[8,13] This mechanism may explain the reduced OSI values observed in the present study. Because OSI reflects the balance between oxidants and antioxidants, the reduction in OSI is likely attributable to the ability of HT to decrease the oxidant component of this ratio.

Modulation of the Inflammatory Response and IL-1β Expression

Oxidative imbalance associated with skeletal muscle I/R injury triggers a non-infectious inflammatory response. Excessive ROS generation activates transcriptional regulators that promote the expression of pro-inflammatory cytokines.[14,15] In the present study, marked IL-1β immunoreactivity in the I/R group indicated activation of this inflammatory pathway. As a key mediator of the early inflammatory response, IL-1β promotes leukocyte recruitment and amplifies tissue injury. Consistent with these findings, the substantial inflammatory cell infiltration and edema observed histologically in the I/R

group likely represent local tissue manifestations of cytokine-mediated inflammation.

Previous studies have suggested that the anti-inflammatory effects of HT are mediated, at least in part, through inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. Activated by oxidative stress and cytokines, NF-κB induces the transcription of pro-inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), IL-1β, IL-6, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2).[16,17] In the present study, the significant reductions in IL-1β immunostaining scores and histopathological indicators of inflammation, including ICI and edema, in the HT-treated group support the anti-inflammatory effects of HT.

Prevention of Apoptosis and Cell Death

Cell death following I/R injury occurs primarily through necrosis and apoptosis. Whereas necrosis is typically associated with severe ischemic damage, apoptosis is a regulated process that is predominantly activated during reperfusion and contributes to the loss of potentially viable tissue.[3,18]

In the present study, the I/R group exhibited marked increases in both TUNEL positivity, indicative of DNA fragmentation, and caspase-3 expression, a key executor of apoptosis. These findings demonstrate that reperfusion injury induces a substantial apoptotic response in skeletal muscle tissue.

During I/R injury, opening of the mitochondrial permeability transition pore (mPTP) facilitates the release of cytochrome c into the cytoplasm, leading to the sequential activation of caspase-9 and caspase-3. Previous studies have demonstrated that HT modulates the mitochondrial apoptotic pathway.[19] In the present study, HT treatment reduced both TUNEL-positive cell counts and caspase-3 immunostaining intensity to levels comparable to those observed in the Sham group. These findings suggest that HT exerts not only antioxidant but also potent anti-apoptotic effects.

Skeletal Muscle Integrity and Mitochondrial Function

Studies investigating the effects of HT on exercise physiology have demonstrated its role in regulating muscle metabolism and enhancing resistance to fatigue. Skeletal muscle is a highly energy-dependent tissue whose function is closely linked to mitochondrial capacity. I/R injury leads to structural disorganization of muscle fibers, sarcomere damage, and ultimately necrosis.[20]

The histopathological improvement and reduction in necrosis observed in the present study may be attributable to the preservation of mitochondrial integrity and attenuation of the cellular energy deficit induced by injury. These findings suggest that HT acts not only as a local antioxidant but also as a bioactive molecule capable of modulating cellular metabolism and energy balance.

Systemic Implications

The findings of the present study are consistent with previously reported protective effects of HT in I/R injury affecting other organ systems. In myocardial I/R models, HT has been shown to reduce infarct size.[8,21] In hepatic I/R injury, HT exerts hepatoprotective effects by enhancing antioxidant enzyme activity and suppressing inflammation.[22] Similarly, in renal I/R injury, HT has been reported to attenuate acute kidney injury and preserve renal function.[23]

The consistency between the findings of the present study and those reported in other organ systems suggests that the protective effects of HT are not tissue-specific but rather result from modulation of fundamental cellular pathological mechanisms, including oxidative stress, inflammation, and mitochondrial dysfunction.

CONCLUSION

Hydroxytyrosol demonstrated significant protective effects against lower-extremity ischemia–reperfusion injury in rats. Biochemical analyses revealed that HT treatment significantly reduced oxidative stress, as evidenced by lower TOS and OSI values. Histopathological and immunohistochemical evaluations further demonstrated reductions in tissue necrosis, edema, inflammatory cell infiltration, IL-1β expression, and apoptotic markers, including caspase-3 and TUNEL positivity, in the HT+I/R group compared with the untreated I/R group.

These findings indicate that HT protects skeletal muscle tissue from reperfusion-induced injury through redox-modulating, anti-inflammatory, and anti-apoptotic mechanisms. HT may therefore represent a promising adjunctive therapeutic strategy in clinical settings associated with I/R injury, including vascular surgery, trauma, and organ transplantation. Nevertheless, further studies investigating different dosing regimens, administration timing (during ischemia or at the onset of reperfusion), and long-term outcomes are needed before these findings can be translated into clinical practice.

Limitations

This study has several limitations. First, only a single dose of HT (10 mg/kg) and a single administration time point were evaluated; therefore, dose–response relationships and the efficacy of post-reperfusion administration could not be assessed. Second, although the effects of HT on oxidative stress and inflammation were demonstrated, the underlying molecular signaling pathways were not investigated in detail. Finally, because this study was conducted in an experimental animal model, additional well-designed clinical studies are required to determine the translational relevance of these findings in humans.

Ethics Committee Approval: Ethical approval for this study was obtained from the Local Ethics Committee of Kobay DHL Inc., Ankara, Türkiye (Date: 08.12.2023, Decision No: 693).

Peer-review: Externally peer-reviewed.

Authorship Contributions: Concept: T.Ö., S.K., H.T.Ö.; Design: T.Ö., S.K., H.K.; Supervision: H.K., T.B.; Resource: T.Ö.; Materials: M.E.Ö., H.T.Ö., E.D., G.Ü., H.K.; Data collection and/or processing: E.E., B.K., F.M.G., R.S.; Analysis and/or interpretation: E.E., B.K., F.M.G.; Literature review: E.D., H.K., T.B.; Writing: T.Ö., H.K., G.Ü., R.S.; Critical review: G.Ü., E.D., H.T.Ö., M.E.Ö.

Conflict of Interest: None declared.

Financial Disclosure: The author declared that this study has received no financial support.

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DENEYSEL ÇALIŞMA - *ÖZ*

İskelet kası iskemi–reperfüzyon hasarında hidroksitirozolün antioksidan, anti-enflamatuvar ve anti-apoptotik etkilerinin kapsamlı değerlendirilmesi

AMAÇ: İskelet kası iskemi-reperfüzyon (I/R) hasarı; reaktif oksijen türlerinin aşırı üretimi, enflamatuvar yanıtın aktivasyonu ve apoptoz ile karakterize, sonuçta belirgin yapısal ve fonksiyonel doku hasarına yol açan karmaşık bir patolojik süreçtir. Zeytin ve zeytin ürünlerinde doğal olarak bulunan fenolik bir bileşik olan hidroksitirozol (HT), güçlü antioksidan ve antienflamatuvar özelliklere sahiptir. Bu çalışmada, alt ekstremite iskelet kası I/R hasarı oluşturulan deneysel sıçan modelinde HT'nin olası koruyucu etkilerinin kapsamlı olarak değerlendirilmesi amaçlandı.

GEREÇ VE YÖNTEM: Yirmi dört erişkin Wistar albino sıçan rastgele dört gruba ayrıldı: Sham, HT-kontrol, I/R ve HT+I/R. Arka ekstremitede 2 saat iskemi ve ardından 2 saat reperfüzyon uygulandı. Tedavi grubunda, iskemi indüksiyonundan 30 dakika önce HT (10 mg/kg, intraperitoneal) uygulandı. Serum total antioksidan kapasite (TAS), total oksidan durum (TOS) ve oksidatif stres indeksi (OSI) ölçüldü. İskelet kası dokuları histopatolojik olarak incelendi; ayrıca interlökin-1β (IL-1β), kaspaz-3 ekspresyonu ve TUNEL-pozitif apoptotik hücreler değerlendirildi.

BULGULAR: Tedavi uygulanmayan I/R grubunda, Sham grubuna kıyasla TOS ve OSI düzeyleri, histopatolojik hasar skorları, enflamatuvar hücre infiltrasyonu ve apoptotik aktivite anlamlı derecede artmıştı ($p<0.05$). HT uygulaması, I/R grubuna göre oksidatif stres belirteçlerini, enflamatuvar yanıtı, doku hasarının şiddetini ve apoptotik indeksleri anlamlı olarak azalttı ($p<0.05$).

SONUÇ: Hidroksitirozol, oksidatif stresi azaltarak, enflamasyonu baskılayarak ve apoptozu inhibe ederek iskelet kası I/R hasarına karşı belirgin koruyucu etki göstermiştir. Bu bulgular, HT'nin iskemi-reperfüzyona bağlı doku hasarının önlenmesi ve tedavisinde umut verici bir yardımcı tedavi stratejisi olabileceğini düşündürmektedir.

Anahtar sözcükler: Apoptozis; hidroksitirozol; iskemi-reperfüzyon; iskelet kası; oksidatif stres.

Ulus Travma Acil Cerrahi Derg 2026;32(8):885-894 DOI: 10.14744/tjtes.2025.49540