
















# Investigation of the protective effects of hydroxytyrosol on erythrocyte deformability during hind limb ischemia-reperfusion injury in rats

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## ABSTRACT

**BACKGROUND:** Ischemia-reperfusion (IR) injury significantly reduces erythrocyte deformability, leading to increased oxidative stress, inflammation, and impaired microvascular perfusion. This study aimed to examine the protective effects of hydroxytyrosol (HT), a powerful antioxidant derived from olives, on erythrocyte deformability and related oxidative stress markers in a rat model of hind limb ischemia-reperfusion injury.

**METHODS:** Twenty-four rats were randomly divided into four groups: Sham, HT-Sham, ischemia-reperfusion (IR), and IR treated with HT (IR-HT). HT was administered intraperitoneally at a dose of 10 mg/kg prior to ischemia induction. Following reperfusion, biochemical parameters, including malondialdehyde (MDA), superoxide dismutase (SOD), endothelial nitric oxide synthase (eNOS), erythrocyte deformability, and morphology, were evaluated.

**RESULTS:** HT administration significantly improved erythrocyte deformability, decreased MDA levels, increased SOD activity, and moderated eNOS expression in the IR-HT group compared to the untreated IR group ( $p < 0.001$ ). Morphological analysis demonstrated substantial preservation of erythrocyte integrity, with fewer pathological changes such as echinocytes and dacryocytes.

**CONCLUSION:** This study provides preliminary insights into the protective mechanisms of HT, highlighting its therapeutic potential in reducing erythrocyte dysfunction and oxidative damage during ischemia-reperfusion events. Further research exploring comprehensive signaling pathways and long-term clinical outcomes is advisable.

**Keywords:** Hydroxytyrosol; ischemia-reperfusion injury; erythrocyte deformability; oxidative stress; rats.

## INTRODUCTION

Ischemia-reperfusion (IR) injury initially results from ischemia-induced hypoxia and worsens significantly upon res-

toration of blood flow. During ischemia, reduced oxygen delivery causes anaerobic glycolysis, adenosine triphosphate (ATP) depletion, lactic acidosis, and intracellular  $Ca^{2+}$  accu-

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mulation due to impaired ATP-dependent ion pumps.<sup>[1-4]</sup> Elevated intracellular  $\text{Ca}^{2+}$  triggers proteolytic enzymes, causes mitochondrial dysfunction, and leads to the opening of mitochondrial permeability transition pore (mPTP), promoting apoptosis and necrosis.<sup>[2,4,5]</sup> Reperfusion introduces oxygen, which increases reactive oxygen species (ROS) production through mitochondrial electron transport chains, xanthine oxidase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, leading to significant oxidative stress.<sup>[4]</sup> This stress causes endothelial nitric oxide synthase (eNOS) to become uncoupled, decreasing nitric oxide (NO) levels and impairing vasodilation.<sup>[4,5]</sup> Endothelial dysfunction promotes leukocyte-endothelial interactions, initiating inflammation through neutrophils, macrophages, lymphocytes, and cytokines (tumor necrosis factor- $\alpha$  [TNF- $\alpha$ ], interleukin-1 [IL-1], IL-6, IL-8, and platelet-activating factor [PAF]), thereby worsening tissue damage. Complement activation enhances leukocyte chemotaxis, causes vascular leakage, and leads to edema. Adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], vascular cell adhesion molecule-1 [VCAM-1], and selectins) facilitate leukocyte migration into tissues.<sup>[1,2]</sup> Malondialdehyde (MDA), a marker of lipid peroxidation, accumulates during reperfusion, indicating membrane oxidative damage.<sup>[1-3]</sup> Reduced superoxide dismutase (SOD) activity impairs ROS detoxification, leading to increased oxidative damage.<sup>[2,3]</sup> Additionally, erythrocyte deformability, which is crucial for microcirculatory perfusion, declines significantly due to oxidative damage to the membrane and cytoskeleton, as well as disrupted calcium balance, leading to microvascular obstruction.<sup>[1-3]</sup> Elevated inflammatory cytokines and oxidative stress further damage erythrocytes, exacerbating tissue hypoxia and injury.<sup>[2,4,6]</sup>

Erythrocyte deformability, which is essential for effective microcirculation and tissue oxygenation, allows red blood cells (RBCs) to change shape dynamically when passing through narrow capillaries.<sup>[6,7]</sup> This property depends on membrane elasticity, cytoskeletal integrity, intracellular viscosity, and cellular geometry, which are largely influenced by proteins such as spectrin, ankyrin, band 3, and glycophorin.<sup>[8]</sup> During reperfusion, excessive ROS-hydroxyl radicals, hydrogen peroxide, and superoxide anions damage RBC membranes through lipid peroxidation, altering membrane fluidity and increasing rigidity, as reflected by elevated MDA levels.<sup>[1,2,9]</sup> Oxidative stress also damages cytoskeletal proteins (spectrin and actin), leading to cross-linking and fragmentation, which further decreases RBC flexibility.<sup>[8,10]</sup> NO, primarily synthesized by eNOS, maintains vascular tone and erythrocyte deformability. Endothelial dysfunction during IR injury impairs NO synthesis, leading to increased vascular resistance and decreased erythrocyte deformability.<sup>[11]</sup> Enhancing NO production shows protective effects on RBC deformability in IR models.<sup>[9,12]</sup> Ischemia-induced ATP depletion disrupts ionic gradients essential for maintaining cytoskeletal function, leading to cell swelling, increased intracellular viscosity, and decreased deformability.<sup>[7]</sup> Reperfusion initially worsens these metabolic

disturbances before they eventually return to normal. Additionally, intracellular calcium accumulation during ischemia activates enzymes such as calpain, leading to cytoskeletal degradation and membrane stiffening, significantly impairing erythrocyte deformability.<sup>[13]</sup> Several pharmacological agents, including alprostadil, dexmedetomidine, thymoquinone, and esmolol, reduce oxidative stress and lipid peroxidation, thereby improving erythrocyte deformability during IR and highlighting the therapeutic potential of targeting oxidative pathways to preserve erythrocyte deformability and attenuate IR injury.<sup>[9,14-17]</sup>

Hydroxytyrosol (HT) is a potent phenolic compound predominantly found in olives, olive leaves, and extra virgin olive oil, chemically known as 3,4-dihydroxyphenylethanol. It is characterized by robust antioxidant and anti-inflammatory properties, primarily through its capacity to neutralize ROS, thereby mitigating oxidative stress.<sup>[18]</sup> Studies have consistently demonstrated that HT significantly reduces oxidative markers such as MDA, a marker of lipid peroxidation, and enhances endogenous antioxidant enzyme activities, notably SOD, thereby safeguarding cellular integrity under oxidative conditions.<sup>[19-22]</sup> Moreover, HT has been shown to improve vascular function by upregulating eNOS expression and increasing NO bioavailability, which is critical for maintaining vascular homeostasis and promoting vasodilation.<sup>[22-26]</sup> Its anti-inflammatory effects are mediated by inhibition of nuclear factor-kappa B (NF- $\kappa$ B) signaling pathways, leading to reduced production of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$ , interleukin-1 beta, and interleukin-6, as well as adhesion molecules such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1.<sup>[18,20,25]</sup> HT's protective mechanism against IR injury also involves inhibition of mPTP opening, a critical event leading to apoptosis and cellular damage during reperfusion.<sup>[22,27]</sup> Additionally, HT's protective effects on erythrocyte deformability highlight its crucial role in reducing blood viscosity and maintaining microcirculatory flow under oxidative stress conditions, thus improving overall microvascular function during IR events.<sup>[19,21,28,29]</sup> Collectively, these multi-targeted protective mechanisms position HT as a promising therapeutic agent for conditions involving oxidative stress and inflammation, particularly IR injuries.

The primary aim of this study is to evaluate the potential protective effects of HT on erythrocyte deformability and related oxidative stress parameters in a rat model of hind limb IR injury. Specifically, the study seeks to determine whether HT administration can reduce erythrocyte dysfunction, maintain cellular integrity, and improve microcirculatory flow during IR conditions. Using comprehensive biochemical, hematological, and histological methods, this investigation aims to uncover the underlying protective mechanisms of HT, providing important insights into its therapeutic potential and guiding future dietary strategies for managing and preventing IR injury.

## MATERIALS AND METHODS

### Ethical Approval

The experimental protocol of this study received approval from the Local Ethics Committee of the Kobay Experimental Animals Laboratory in Ankara, Türkiye, dated November 24, 2023 (Approval No: 692). All procedures involving animal handling, surgery, and sampling were meticulously performed in accordance with the ethical guidelines and standards established by the approving ethics committee.

The HT used in this study was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA; CAS No. 10597-60-1). All reagents and materials were supplied by the researchers, and no external funding sources supported this study.

### Experimental Design and Animals

The study utilized a total of 24 rats, systematically allocated into four experimental groups (n=6 per group): Sham, HT-Sham, IR, and ischemia-reperfusion treated with hydroxytyrosol (IR-HT). Sample sizes for each group were determined using Mead's method to ensure sufficient statistical power to detect meaningful differences in primary outcomes, including MDA, SOD, eNOS, erythrocyte morphology assessed via May-Grünwald-Giemsa (MGG) staining, and erythrocyte deformability.

Animals were housed individually under carefully regulated environmental conditions, with room temperature consistently maintained at 20–21°C and relative humidity kept between 45% and 55%. A standardized 12-hour light/dark cycle was used to align with circadian rhythms. Throughout the study, all rats had free access to standardized laboratory rodent chow and purified drinking water to ensure optimal nutrition and hydration.

### Induction of Hind Limb Ischemia-Reperfusion Injury

The hind limb IR injury model was used to evaluate the protective effects of HT on erythrocyte deformability and oxidative stress parameters. Before the procedure, all rats fasted overnight but had free access to water. Anesthesia was induced by intraperitoneal injection of ketamine (80 mg/kg) combined with xylazine (10 mg/kg). The level of anesthesia was confirmed by the absence of withdrawal reflexes upon paw pinch. Rats were then placed on a heated surgical table in a supine position, with body temperature maintained at approximately 37°C throughout the procedure.

Hydroxytyrosol was administered intraperitoneally at a dose of 10 mg/kg, 30 minutes prior to ischemia induction, in the HT-Sham and IR-HT groups to assess its potential protective effects against erythrocyte dysfunction and oxidative stress during IR injury.

The right hind limb was carefully prepared by shaving and then disinfected with povidone-iodine solution. A small longitudinal incision was made in the right inguinal area to access the femoral artery. For rats in the IR and IR-HT groups,

the femoral artery was gently isolated and occluded with a non-traumatic microvascular clamp. Complete ischemia was visually confirmed by pallor and decreased temperature of the distal limb. The ischemic period was strictly maintained for two hours. In the Sham and HT-Sham groups, the femoral artery was similarly exposed but left unclamped, serving as surgical controls.

Following the ischemic period, reperfusion was initiated by gently removing the microvascular clamp. Successful reperfusion was confirmed by the restoration of normal limb color and temperature. Afterwards, the surgical incision was closed with 4-0 silk sutures, and the animals were carefully monitored during recovery. The reperfusion phase lasted an additional two hours for all relevant groups.

### Measurement of Biochemical and Hematological Parameters

At the end of the reperfusion period, blood samples were collected via cardiac puncture, and gastrocnemius muscle tissue from the affected limb was harvested for subsequent biochemical, hematological, and histological analyses.

**Erythrocyte Deformability:** Erythrocyte deformability was evaluated to determine the flexibility of RBCs under different treatment conditions. To reduce the risk of hemolysis, blood samples were carefully collected, and measurements were performed promptly. The samples were initially centrifuged at 1,000 × g for 10 minutes. The serum and buffy coat layers above the erythrocyte pellet were then gently removed and discarded. The erythrocyte pellet was washed with isotonic phosphate-buffered saline (PBS), followed by centrifugation at 1,000 × g for an additional 10 minutes. This washing process was repeated three times to ensure the isolation of pure erythrocytes. The purified erythrocytes were resuspended in PBS to achieve a final hematocrit of 5%, creating a standardized erythrocyte suspension used for deformability measurements, which were consistently performed at 22°C.

The evaluation of erythrocyte deformability was performed using a constant-flow filtration system. For each sample, a 10 mL suspension of erythrocytes in PBS was prepared. A steady flow rate of 1.5 mL/min was maintained using an infusion pump. The filtration setup included a nucleoporin polycarbonate filter with a pore size of 5 µm and a diameter of 28 mm. Pressure changes caused by erythrocytes passing through the filter were continuously monitored with a pressure transducer, and the data were recorded digitally using an MP30 data acquisition system (BIOPAC Systems Inc., Goleta, CA).

Before each measurement, the system's pressure calibration was performed to ensure accuracy. Initially, the PBS buffer alone was passed through the filtration apparatus to establish a baseline measurement. The erythrocyte suspension was then introduced, and pressure values were recorded. Relative resistance ( $R_{rel}$ ) was calculated as the ratio of the pressure generated by the erythrocyte suspension to that of the PBS buffer alone. The deformability index was interpreted in-

versely; higher  $R_{rel}$  values indicated decreased erythrocyte deformability.

**Measurement of Malondialdehyde Levels:** MDA levels were measured as a marker of lipid peroxidation and oxidative stress using a commercially available ELISA (enzyme-linked immunosorbent assay) kit (SunRed Biological Technology Co. Ltd., Cat. No. 201-11-0157, Shanghai, China; Ref: DZE201110157, Lot: 202311). The assay was performed strictly according to the manufacturer's protocol. Briefly, all reagents were equilibrated to room temperature prior to use. Serial dilutions of the standard stock solution were prepared at concentrations of 40, 20, 10, 5, 2.5, and 1.25 ng/mL. Then, 50  $\mu$ L of each standard solution was pipetted into the respective wells. Since the standard solutions already contained a biotin-labeled antibody, no additional antibody was added to these wells. For experimental samples, 40  $\mu$ L of each sample was dispensed into separate wells, followed by the addition of 10  $\mu$ L of anti-MDA antibody.

Subsequently, 50  $\mu$ L of streptavidin-horseradish peroxidase conjugate was added to all standard and sample wells. The plate was incubated at 37°C for 60 minutes. After incubation, the wells underwent five washing cycles with 350  $\mu$ L of washing solution per well using an automated ELISA plate washer. Next, 50  $\mu$ L of Chromogen A and 50  $\mu$ L of Chromogen B solutions were added sequentially to each well, followed by incubation in the dark at 37°C for an additional 10 minutes. The reaction was stopped by adding 50  $\mu$ L of stop solution to each well, resulting in a color change from blue to yellow. Absorbance was measured at 450 nm using a microplate reader, with readings taken within 15 minutes of stopping the reaction.

**Measurement of Superoxide Dismutase Activity:** SOD activity was measured to evaluate antioxidant enzyme status using a commercial ELISA kit (SunRed Biological Technology Co. Ltd., Cat. No. 201-11-0169, Shanghai, China; Ref: DZE201110169, Lot: 202311). The assay was performed strictly according to the manufacturer's instructions. Briefly, all reagents were brought to room temperature before starting. SOD standards were serially diluted from a stock solution to concentrations of 64, 32, 16, 8, 4, and 2 ng/mL. Then, 50  $\mu$ L of each standard dilution was added to the respective wells of the microplate. Since the standard solutions already contained a biotin-labeled antibody, no additional antibody was added to these wells. For the experimental samples, 40  $\mu$ L of each sample was added to individual wells, followed by the addition of 10  $\mu$ L of anti-SOD antibody solution.

Thereafter, 50  $\mu$ L of streptavidin-horseradish peroxidase conjugate was added to all wells containing standards and samples, followed by incubation at 37°C for 60 minutes. After incubation, each well was washed five times with 350  $\mu$ L of wash solution using an automated ELISA plate washer to remove unbound components. Subsequently, 50  $\mu$ L of Chromogen A and 50  $\mu$ L of Chromogen B solutions were added

sequentially to all wells. The plate was then incubated in the dark at 37°C for an additional 10 minutes. The enzymatic reaction was stopped by adding 50  $\mu$ L of stop solution to each well, resulting in a color change from blue to yellow. Absorbance was immediately measured at 450 nm using a microplate reader, with readings taken within 15 minutes of stopping the reaction.

### Histological Analysis

To examine erythrocyte morphology, blood smear samples were stained using the MGG method. Photomicrographs were taken at 1000 $\times$  magnification to allow detailed visualization of cell structure and changes.

### Immunohistochemical Analysis

Blood smear samples fixed in ethanol underwent antigen retrieval, followed by washing with Tris-buffered saline containing Tween 20. Subsequently, the samples were treated with hydrogen peroxide to inhibit endogenous peroxidase activity and minimize nonspecific staining. Slides were incubated overnight at 4°C with a primary antibody specific for eNOS, diluted 1:100. The following day, the slides were incubated with a biotinylated secondary antibody and then treated with a streptavidin-peroxidase complex. Diaminobenzidine was used as the chromogenic substrate for visualization, and Mayer's hematoxylin served as the counterstain. Immunoreactivity for eNOS was quantitatively assessed using the H-score method.

### Statistical Analysis

Data were expressed as mean $\pm$ standard deviation. Statistical comparisons of continuous variables among treatment groups were conducted using one-way analysis of variance (ANOVA). When ANOVA indicated statistically significant differences, pairwise comparisons between individual groups were performed using Tukey's post hoc test. A p-value of <0.05 was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics software version 25 (IBM Corp., Armonk, NY, USA).

## RESULTS

The results of biochemical, hematological, and histopathological analyses across the experimental groups are summarized comprehensively in Table 1. Statistically significant differences were observed among the groups for all measured parameters ( $p < 0.001$ ).

### Erythrocyte Deformability

Erythrocyte deformability was significantly reduced in the IR group ( $3.12 \pm 0.35$ ) compared to the Sham group ( $1.74 \pm 0.12$ ,  $p < 0.001$ ), indicating decreased red blood cell flexibility under ischemic conditions. However, HT administration notably preserved erythrocyte deformability in the IR-HT group ( $2.02 \pm 0.24$ ) compared to the untreated IR group ( $p < 0.001$ ), demonstrating a significant protective effect of HT against IR-induced erythrocyte rigidity.

**Table 1.** Mean  $\pm$  standard deviation values of deformability, May-Grünwald-Giemsa (MGG) score, endothelial nitric oxide synthase (eNOS) expression, malondialdehyde (MDA) levels, and superoxide dismutase (SOD) activity according to treatment groups

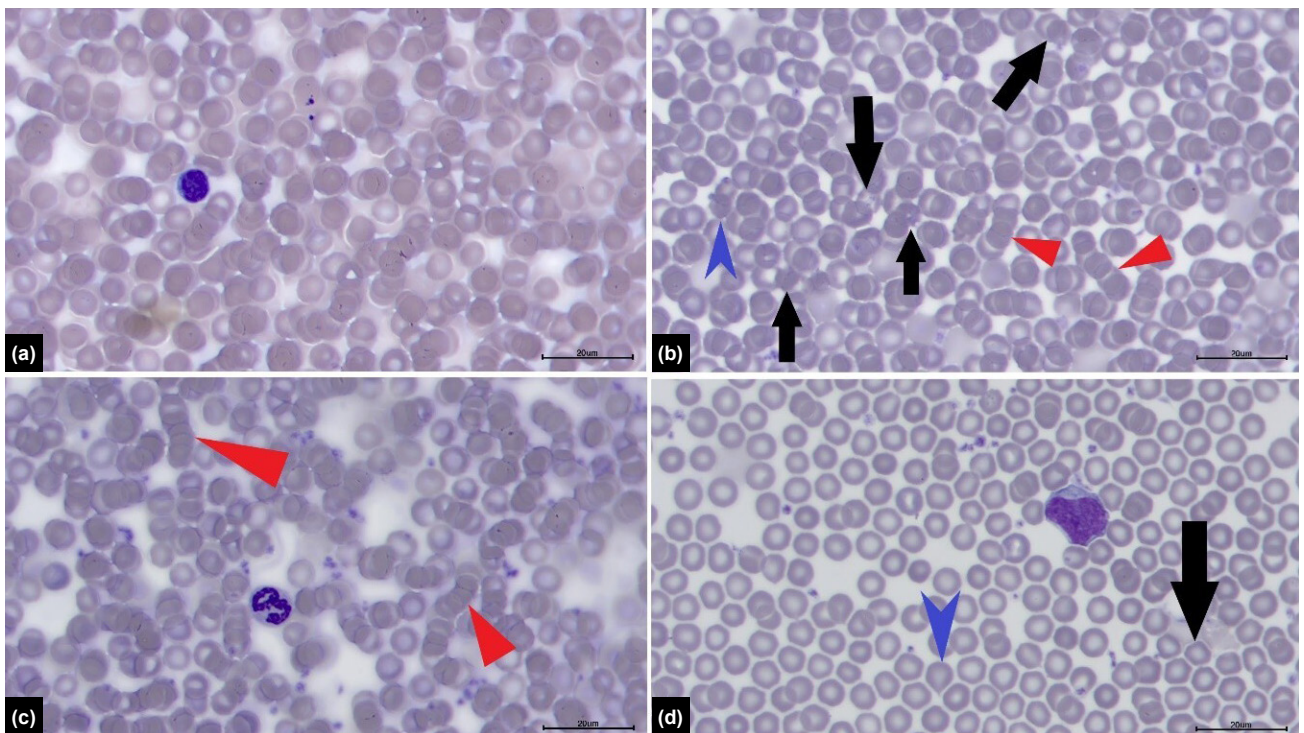
Groups	DEFORMABILITY	MGG	E-NOS	MDA	SOD
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Sham	1.74 $\pm$ 0.12	2.11 $\pm$ 0.47	70.18 $\pm$ 14.15	12.91 $\pm$ 1.41	4.68 $\pm$ 0.96
HT-Sham	1.91 $\pm$ 0.06	2.93 $\pm$ 0.33	152.53 $\pm$ 16.24	15.09 $\pm$ 1.59	9.18 $\pm$ 0.88
IR	3.12 $\pm$ 0.35	4.42 $\pm$ 0.52	222.05 $\pm$ 23.34	21.97 $\pm$ 5.72	9.69 $\pm$ 1.36
IR-HT	2.02 $\pm$ 0.24	3.09 $\pm$ 0.41	161.67 $\pm$ 18.36	16.02 $\pm$ 1.76	12.45 $\pm$ 1.91
p <sup>†</sup>	<0.001	<0.001	<0.001	<0.001	<0.001
p <sup>††</sup>					
1 vs. 2	0.59	0.02	0.001	0.63	0.001
1 vs. 3	0.001	0.001	0.001	0.001	0.001
1 vs. 4	0.18	0.005	0.001	0.34	0.001
2 vs. 3	0.001	0.001	0.001	0.006	0.91
2 vs. 4	0.83	0.92	0.91	0.96	0.002
3 vs. 4	0.001	0.001	0.002	0.02	0.01

<sup>†</sup>One-Way ANOVA; <sup>††</sup>Tukey post hoc test ( $p < 0.05$ : statistical significance). SD: Standard deviation.

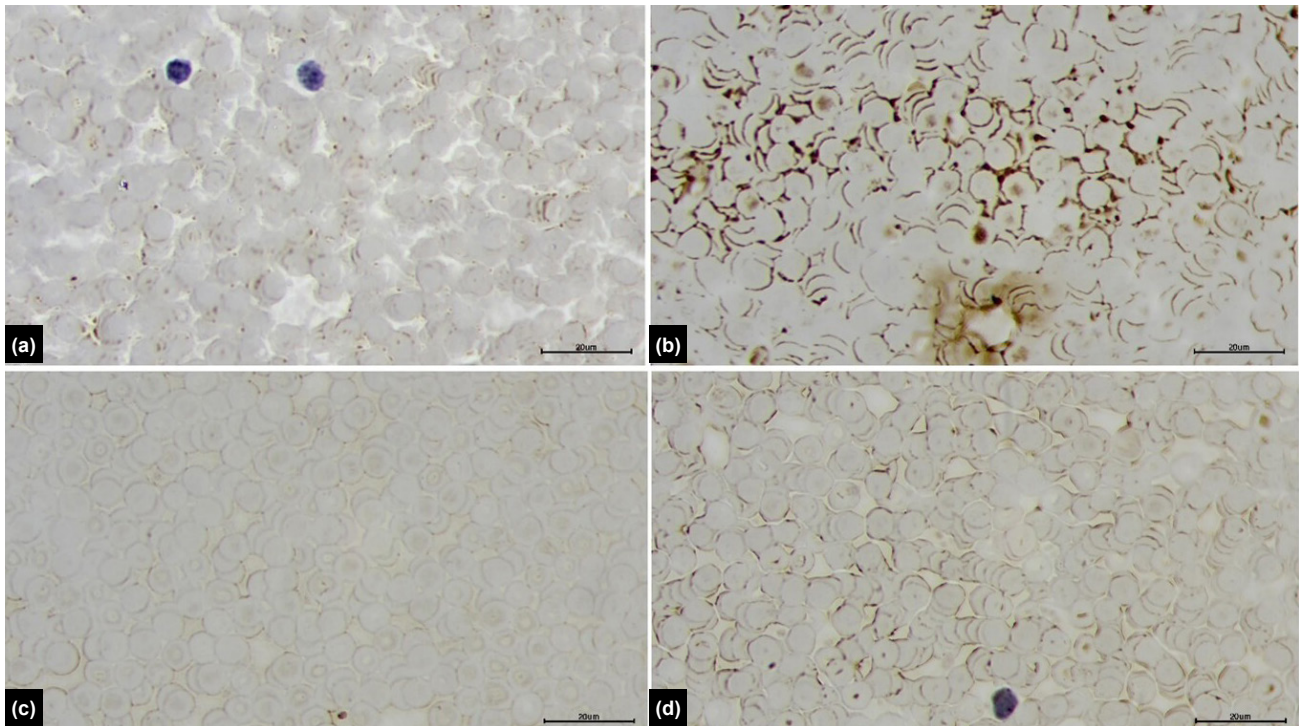
### Malondialdehyde (MDA) Levels

Malondialdehyde, a key biomarker of lipid peroxidation and oxidative damage, was significantly higher in the IR group (21.97 $\pm$ 5.72) compared to the Sham group (12.91 $\pm$ 1.41,

$p < 0.001$ ). HT treatment significantly lowered MDA levels in the IR-HT group (16.02 $\pm$ 1.76) compared to untreated IR animals ( $p = 0.02$ ), demonstrating effective attenuation of oxidative stress.



**Figure 1.** May-Grünwald-Giemsa (MGG)-stained photographs of the experimental groups Control, IR, HT-Sham, and HT-IR are shown in **a, b, c, and d**, respectively. Black arrows indicate echinocytes, blue arrowheads indicate dacrocytes, and red arrowheads indicate rouleaux formation (magnification  $\times 1000$ ).



**Figure 2.** Immunohistochemically-stained photographs of the experimental groups Control, IR, HT Control, and HT-IR are shown in **a, b, c, and d**, respectively (magnification  $\times 1000$ ).

### Superoxide Dismutase (SOD) Activity

Superoxide dismutase activity, a key antioxidative enzyme, was significantly higher in the IR-HT group ( $12.45 \pm 1.91$ ) compared to the IR group ( $9.69 \pm 1.36$ ,  $p=0.01$ ). This increase underscores the protective antioxidative effect of HT during IR injury.

### Histological and Immunohistochemical Observations

Morphological changes in erythrocytes, assessed by MGG staining, showed significantly increased pathological features, including more echinocytes, dacrocytes, and rouleaux formations, in the IR group ( $4.42 \pm 0.52$ ) compared to the Sham ( $2.11 \pm 0.47$ ) and HT-Sham ( $2.93 \pm 0.33$ ) groups (both  $p < 0.001$ ). HT treatment notably reduced these morphological changes in the IR-HT group ( $3.09 \pm 0.41$ ) compared to the IR group ( $p < 0.001$ ), highlighting its protective effects on erythrocyte structure (Fig. 1).

Immunohistochemical staining showed markedly increased eNOS immunoreactivity in the IR group, indicating vascular endothelial activation under ischemic stress. In contrast, the IR-HT group exhibited moderate immunoreactivity, which aligns with biochemical data indicating improved endothelial function due to hydroxytyrosol treatment (Fig. 2). The IR group had significantly higher eNOS levels ( $222.05 \pm 23.34$ ), suggesting increased endothelial activity in response to ischemic stress compared to all other groups ( $p < 0.001$ ). HT treatment significantly lowered eNOS levels in the IR-HT group ( $161.67 \pm 18.36$ ) compared to the IR group ( $p < 0.001$ ).

## DISCUSSION

Ischemia-reperfusion injury is a complex pathological process characterized by cellular and vascular damage that occurs not only during ischemia but also paradoxically increases upon reperfusion. The initial ischemic event results in hypoxia-related metabolic disturbances, including a shift from aerobic to anaerobic glycolysis, leading to lactic acidosis, ATP depletion, and dysfunction of ATP-dependent ion pumps.<sup>[1-3]</sup> This energy failure causes accumulation of intracellular calcium ( $\text{Ca}^{2+}$ ), which activates proteolytic enzymes and contributes to mitochondrial dysfunction and opening of the mPTP, ultimately leading to cell death through necrosis or apoptosis.<sup>[4,5]</sup> Paradoxically, reperfusion amplifies injury due to the sudden reintroduction of oxygen, significantly increasing ROS production via mitochondrial electron transport, xanthine oxidase, and NADPH oxidase pathways.<sup>[2,3]</sup> This increase in ROS results in eNOS uncoupling, reducing NO bioavailability and impairing vascular relaxation and homeostasis.<sup>[3,5]</sup> At the cellular level, oxidative stress during reperfusion causes extensive lipid peroxidation, which can be measured by increased MDA levels, a reliable marker of membrane oxidative damage.<sup>[3-5]</sup> Concurrently, the activity of SOD, a crucial antioxidant enzyme, decreases markedly, impairing the body's ability to detoxify ROS and exacerbating oxidative injury.<sup>[4]</sup> These redox imbalances significantly affect RBC physiology, particularly erythrocyte deformability, a key determinant of microcirculatory flow and oxygen delivery.<sup>[6]</sup>

Erythrocyte deformability describes the ability of RBCs to

change shape as they pass through capillaries smaller than their normal diameter. This feature is mainly governed by membrane elasticity, cytoskeletal structure, internal viscosity, and cell shape, and is largely maintained by cytoskeletal proteins such as spectrin, ankyrin, and band 3.<sup>[6-8]</sup> IR injury-induced oxidative stress damages both lipid membranes and cytoskeletal proteins, leading to cross-linking, fragmentation, and stiffening of RBCs, thereby impairing their ability to navigate microvascular beds.<sup>[1,2,10]</sup> Elevated MDA levels are associated with reduced deformability and increased membrane rigidity.<sup>[9]</sup> Simultaneously, ischemic ATP depletion disrupts ionic gradients, resulting in increased cytoplasmic viscosity and alterations in cell shape.<sup>[7]</sup> Furthermore, intracellular  $Ca^{2+}$  overload activates calpain and other calcium-dependent enzymes, accelerating cytoskeletal breakdown and increasing cell stiffness.<sup>[13]</sup> NO, produced by eNOS, plays a key role in maintaining erythrocyte deformability by promoting vasodilation, inhibiting platelet aggregation, and preserving cytoskeletal integrity.<sup>[11]</sup> However, eNOS uncoupling during IR injury markedly reduces NO bioavailability, worsening vascular tone regulation and decreasing RBC flexibility. Restoring NO synthesis or availability has been shown to improve deformability and microcirculatory flow.<sup>[9,12]</sup>

This study demonstrates that HT significantly improves erythrocyte deformability, reduces oxidative stress, and increases eNOS expression in a rat model of hind limb IR injury. These findings support a growing body of research indicating HT's protective role against oxidative damage through multiple mechanisms, including antioxidant, anti-apoptotic, anti-inflammatory, and gene-regulatory pathways.

Hydroxytyrosol's ability to significantly reduce MDA levels and enhance SOD activity observed in our study highlights its potent antioxidant capacity. These findings align with those of Manna et al.,<sup>[19]</sup> who reported that HT protected erythrocytes against hydrogen peroxide-induced oxidative stress, thereby preserving membrane integrity. The antioxidant effects of HT stem not only from direct radical scavenging but also from activation of mitochondrial SOD, which is crucial for reducing intracellular superoxide buildup during reperfusion, as previously shown by Pei et al.<sup>[22]</sup> in myocardial IR models. Additionally, HT's role in maintaining mitochondrial integrity and activating anti-apoptotic signaling pathways is well documented; Pei et al.<sup>[22]</sup> demonstrated that HT significantly inhibited apoptosis in cardiomyocytes after IR injury by lowering cleaved caspase-3 expression and promoting phosphorylation of Akt and glycogen synthase kinase-3 beta (GSK3 $\beta$ ), thereby stabilizing mitochondrial membranes. Although mature erythrocytes lack mitochondria, these systemic protective mechanisms may extend beneficial effects to erythroid precursors and endothelial cells, ultimately supporting overall hemorheological stability. Importantly, HT also plays a key role in preventing eryptosis, the programmed cell death pathway specific to erythrocytes. Tortora et al.<sup>[28]</sup> showed that HT counteracts lysophosphatidic acid-induced

eryptosis by restoring intracellular ATP and glutathione levels and maintaining calcium balance. These mechanistic insights closely match our morphological findings, in which HT administration reduced echinocyte and dacrocyte formation, indicating membrane stabilization and preservation of erythrocyte deformability under IR conditions.

Consistent with our results showing reduced impairment in erythrocyte deformability, decreased MDA levels, and increased SOD activity after HT treatment, Pan et al.<sup>[21]</sup> also reported that HT administration significantly lowered oxidative stress markers (ROS and MDA) and increased the activity of antioxidative enzymes such as SOD and catalase through increased expression of SOD1, SOD2, and catalase in a mouse model of liver IR injury. Although our study does not involve direct hepatoprotective outcomes, the underlying antioxidative mechanisms identified by Pan et al.<sup>[21]</sup> align closely with our observed improvements in erythrocyte oxidative status and deformability, suggesting a similar protective pathway mediated through upregulation of endogenous antioxidant defense. Additionally, the anti-inflammatory effects of HT demonstrated by Pan et al., through decreased levels of pro-inflammatory cytokines (TNF- $\alpha$ , IL-6, and macrophage inflammatory protein-2 [MIP-2]), may further explain our findings of improved endothelial function and reduced inflammatory responses following HT administration. Collectively, these mechanisms offer a plausible molecular basis for the beneficial effects of HT on protecting erythrocytes and the endothelium in our IR model.

At the cellular signaling level, HT activates key redox-sensitive pathways, including phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), nuclear factor erythroid 2-related factor 2/heme oxygenase-1 (Nrf2/HO-1), and sirtuin 1 (SIRT1), which are essential for maintaining vascular homeostasis.<sup>[22,30,31]</sup> Zrelli et al.<sup>[31]</sup> provided further mechanistic evidence supporting HT's protective effects by demonstrating its potent antioxidant properties in vascular endothelial cells. Their study revealed that HT significantly increased endothelial cell proliferation, promoted cytoprotection against  $H_2O_2$ -induced oxidative damage, and enhanced wound repair through activation of the PI3K/Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways. Importantly, HT treatment led to activation and nuclear translocation of nuclear factor-E2-related factor-2 (Nrf2), a crucial transcription factor that regulates antioxidant enzyme expression. Once activated, Nrf2 induced expression of heme oxygenase-1, thereby strengthening the cellular antioxidant defense system. These findings align well with our results, in which HT administration considerably reduced MDA levels and increased SOD activity, along with improvements in erythrocyte deformability and morphology, indicating an antioxidative mechanism. The enhanced HO-1 induction through Nrf2 activation, as highlighted by Zrelli et al.,<sup>[31]</sup> may represent a central pathway through which HT provides protection against oxidative stress and inflammation observed in our IR model. Specifically, HT-induced Nrf2 translocation promotes upregulation

of HO-1, a cytoprotective enzyme responsible for degrading pro-oxidant heme, thereby reducing oxidative stress and inflammation. These effects are consistent with the notable improvements in erythrocyte deformability and morphological preservation observed in our findings, suggesting improved membrane integrity.

In another study, Zrelli et al.<sup>[30]</sup> provided valuable mechanistic insights into the cytoprotective effects of HT, highlighting its role in vascular endothelial cells. They showed that HT increased HO-1 expression in a dose- and time-dependent manner through activation and nuclear accumulation of Nrf2. This effect required new synthesis and stabilization of Nrf2, mediated through the PI3K/Akt and ERK1/2 pathways. Additionally, their findings suggested that HO-1 induction was crucial for HT-mediated endothelial wound repair, largely dependent on HO-1-derived carbon monoxide production. Although our study does not directly examine endothelial wound repair, the mechanisms described by Zrelli et al.<sup>[30]</sup> are consistent with the improved endothelial function and reduced oxidative stress observed in our IR model. Therefore, activation of the Nrf2/HO-1 pathway, stabilization of Nrf2, and the ensuing antioxidative and anti-inflammatory actions outlined by Zrelli et al.<sup>[30]</sup> are likely key mechanisms underlying the protective effects of HT observed in our research.

Our findings show a significant decrease in eNOS expression in the IR-HT group compared to the untreated IR group. Elevated eNOS expression usually indicates compensatory endothelial activation in response to ischemic stress; however, persistently high eNOS levels in the IR group may also reflect pathological endothelial activation driven by severe oxidative stress and inflammation. The decrease in eNOS expression following HT administration suggests normalization of endothelial function and reduced vascular stress. This observation aligns with evidence from a review by Serreli et al.,<sup>[23]</sup> who reported that HT enhances eNOS activity by promoting phosphorylation at Ser1177 through PI3K/Akt and AMP-activated protein kinase (AMPK) signaling pathways, thereby increasing NO bioavailability.<sup>[22]</sup> Therefore, the balanced regulation of eNOS expression by HT observed in our study likely contributes to improved NO-mediated vascular responses, including increased erythrocyte flexibility via downstream cyclic guanosine monophosphate-dependent (GMP-dependent) mechanisms. Overall, these positive effects of HT may reduce vascular resistance and enhance microcirculatory outcomes during IR injury.

A significant body of research has demonstrated the vascular protective effects of HT, highlighting its ability to counteract endothelial dysfunction induced by inflammatory stimuli.<sup>[25]</sup> Specifically, these studies showed that HT significantly reduces TNF- $\alpha$ -induced endothelial cell damage by decreasing apoptosis and promoting cell survival. These protective effects are mediated through suppression of caspase-3 activation and inhibition of the NF- $\kappa$ B signaling pathway. Additionally, HT treatment increases eNOS phosphorylation and NO production, leading to inactivation of NF- $\kappa$ B and enhanced

endothelial cell viability.

Moreover, Wang et al.<sup>[26]</sup> reported that HT directly activates SIRT1, which subsequently enhances antioxidant pathways such as SIRT1-FOXO1-SOD1, reduces ROS production, and preserves endothelial health. HT-mediated activation of SIRT1—a NAD<sup>+</sup>-dependent deacetylase—may also explain the moderated eNOS expression observed in our study, as SIRT1 activation increases eNOS activity through deacetylation and simultaneously inhibiting NF- $\kappa$ B-mediated inflammatory signaling.<sup>[32,33]</sup> This interpretation is consistent with the balanced decrease in eNOS expression observed in the IR-HT group, indicating normalized endothelial function.

Clinically relevant, these cellular mechanisms likely also underlie the reduced erythrocyte rigidity and vascular resistance observed in our findings, highlighting HT's ability to inhibit platelet aggregation and thrombosis. López-Villodres et al. and Muñoz-Marin et al.<sup>[35]</sup> reported that HT plays a role in suppressing platelet activation and thromboxane B2 production, mechanisms that are particularly beneficial under IR conditions, where platelet-endothelial interactions exacerbate microvascular occlusion.<sup>[34]</sup> Moreover, HT's nutrigenomic and epigenetic modulation further supports the sustained vascular protection observed in our experimental findings. Carluccio et al.<sup>[36]</sup> demonstrated HT's ability to modify endothelial transcriptomic profiles and regulate microRNA expression, thereby positively influencing inflammatory suppression and vascular repair. These molecular changes align with the preserved erythrocyte morphology and reduced oxidative stress observed in the IR-HT group. Finally, HT's potential to prevent endothelial-to-mesenchymal transition (EndMT), as shown by Terzuoli et al.,<sup>[37]</sup> may further explain the improved endothelial function and reduced vascular stress indicated by our eNOS expression data, highlighting its regenerative therapeutic potential in IR injury.

Our findings regarding HT's protective effects align well with related research on structurally similar compounds such as p-tyrosol, which has demonstrated significant antioxidative and hemorheological benefits in IR models. Osipenko et al.<sup>[38]</sup> showed that intravenous administration of p-tyrosol markedly reduced blood and plasma viscosity, enhanced erythrocyte deformability, and subsequently improved oxygen delivery to cerebral tissues following global cerebral IR injury in rats. They attributed these protective effects to substantial reductions in lipid peroxidation products, including diene and triene conjugates, as well as fluorescent lipid oxidation products, which effectively lowered the lipid oxidation index in brain tissues. Correspondingly, our results demonstrate that HT exhibits a similar capacity to mitigate lipid peroxidation, as indicated by significantly reduced MDA levels and improved erythrocyte deformability, highlighting comparable antioxidative mechanisms. This parallel suggests that both tyrosol derivatives may exert their beneficial effects through similar pathways by reducing oxidative stress, stabilizing erythrocyte membranes, and enhancing microvascular perfusion. Conse-

quently, the neuroprotective effects of p-tyrosol described by Osipenko et al.<sup>[38]</sup> provide additional mechanistic insights that support and reinforce the vascular protective mechanisms identified in our study with HT, indicating broad potential for these phenolic compounds in IR injury management.

A limitation of this study is that it assessed a limited range of biochemical, hematological, and morphological parameters without directly exploring detailed molecular signaling pathways. Specifically, although antioxidant status, erythrocyte deformability, eNOS expression, and oxidative stress markers were measured, crucial signaling pathways such as Nrf2/HO-1, PI3K/Akt, SIRT1, and NF- $\kappa$ B were not examined. Additionally, the study employed only a single dosage and route of HT administration, which limits conclusions regarding optimal dosing strategies or dose-dependent effects. Another limitation was the relatively short reperfusion period, which may not fully reflect long-term progression and delayed effects associated with IR injury. Furthermore, the study utilized a rat hind limb IR model, which, despite its experimental relevance, may not fully represent the complexity of clinical IR injury in humans. Therefore, caution is needed when applying these preliminary results to clinical settings. Future research incorporating broader molecular assessments, multiple dosages, longer observation periods, and translational clinical models is necessary to fully understand HT's therapeutic potential and practical applicability.

## CONCLUSION

In conclusion, this study demonstrates that HT effectively reduces erythrocyte dysfunction in a rat model of hind limb IR injury by preserving erythrocyte deformability, lowering lipid peroxidation, enhancing antioxidant enzyme activity, and regulating eNOS expression. Given the limited research specifically addressing lower limb IR injury, these findings provide initial insights into HT's protective mechanisms and emphasize its therapeutic potential. Further research is necessary to verify these results and investigate the underlying molecular pathways in greater detail, ultimately supporting the potential clinical application of HT in the management of IR injury.

**Ethics Committee Approval:** The experimental protocol of this study received approval from the Local Ethics Committee of the Kobay Experimental Animals Laboratory in Ankara, Türkiye (Date: 24.11.2023, Decision No: 692).

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## ORIJİNAL ÇALIŞMA - ÖZ

## Şıçanlarda alt ekstremite iskemi-reperfüzyon hasarı sırasında hidrokstitirozolün eritrosit deformabilitesi üzerindeki koruyucu etkilerinin araştırılması

**AMAÇ:** İskemi-reperfüzyon (İR) hasarı, eritrosit deformabilitesini anlamlı derecede azaltarak oksidatif stresin ve inflamasyonun artmasına, buna bağlı olarak da mikrovasküler perfüzyonun bozulmasına yol açan karmaşık bir patofizyolojik süreçtir. Bu çalışmada, zeytinden elde edilen güçlü bir fenolik antioksidan olan hidrokstitirozolün (HT), şıçan alt ekstremite iskemi-reperfüzyon modeli üzerinde eritrosit deformabilitesi ve ilişkili oksidatif stres belirteçleri üzerindeki koruyucu etkilerinin incelenmesi amaçlanmıştır.

**GEREÇ VE YÖNTEM:** Toplam 24 şıçan rastgele dört gruba ayrılmıştır: Sham, HT-Sham, İR ve hidrokstitirozol ile tedavi edilen İR grubu (İR-HT). Hidrokstitirozol, iskemi indüksiyonundan önce 10 mg/kg dozunda intraperitoneal olarak uygulanmıştır. Reperfüzyon döneminin ardından malondialdehit (MDA), süperoksit dismutaz (SOD), endotelial nitrik oksit sentaz (e-NOS) düzeyleri ile eritrosit deformabilitesi ve morfolojisi gibi biyokimyasal parametreler değerlendirilmiştir.

**BULGULAR:** Hidrokstitirozol uygulaması, tedavi edilmeyen İR grubuna kıyasla İR-HT grubunda eritrosit deformabilitesini anlamlı düzeyde artırmış, MDA düzeylerini belirgin şekilde düşürmüştür, SOD aktivitesini yükseltmiş ve e-NOS ekspresyonunu dengeleyici yönde düzenlemiştir ( $p < 0.001$ ). Morfolojik analiz sonuçları, eritrosit bütünlüğünün büyük ölçüde korunduğunu, ekinosit ve dakrosit gibi patolojik şekil değişikliklerinin belirgin şekilde azaldığını göstermiştir.

**SONUÇ:** Bu çalışma, hidrokstitirozolün iskemi-reperfüzyon sürecinde eritrosit disfonksiyonunu azaltma ve oksidatif hasarı sınırlama konusundaki koruyucu mekanizmalarına ilişkin öncü bulgular sunmaktadır. Bulgular, HT'nin terapötik bir ajan olarak potansiyelini vurgulamakta olup, daha geniş moleküler yollar ve uzun dönemli klinik sonuçları değerlendiren ileri düzey çalışmalara ihtiyaç olduğunu göstermektedir.

**Anahtar sözcükler:** Hidrokstitirozol, İskemi-Reperfüzyon Hasarı, Eritrosit Deformabilitesi, Oksidatif Stres, Şıçanlar

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