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RESEARCH ARTICLE

# A comparative study on the antioxidant effects of hesperidin and ellagic acid against skeletal muscle ischemia/reperfusion injury

Fazile Nur Ekinci Akdemir[1], İlhami Gülçin[2,3], Berna Karagöz[4], Recep Soslu[5], and Saleh H. Alwasel[3]

[1]*Department of Nutrition and Dietetics, Health School, Ağrı İbrahim Çeçen University, Ağri, Turkey,* [2]*Department of Chemistry, Atatürk University, Faculty of Sciences, Erzurum, Turkey,* [3]*Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia,* [4]*Department of Pharmacology, Faculty of Pharmacy, Ağrı İbrahim Çeçen University, Ağri, Turkey, and* [5]*Physical Education and Sports, Bartın University, Bartin, Turkey*

## Abstract

The antioxidant effects of ellagic acid (EA) and hesperidin (HES) against skeletal muscle ischemia/reperfusion injury (I/R) were performed. Hindlimb ischemia has been induced by tourniquet occlusion for 2 h on left hindlimb. At the end of ischemia, the tourniquate has been removed and initiated reperfusion for 2 h. EA (100 mg/kg) has been applied orally before ischemia/reperfusion in the EA + I/R group. HES (100 mg/kg) has been given orally in the HES + I/R group. The left gastrocnemius muscle has been harvested and stored immediately at −80 °C until assessed for the levels of MDA and antioxidant enzymes activities. MDA level has statistically increased in I/R group ($p < 0.05$) compared to other groups. The muscle tissue antioxidant enzymes activities were lower than the other groups in the I/R group ($p < 0.05$). EA and HES treatments significantly reversed the damage level in I/R, also activity of tissue SOD increased in the EA + I/R and HES + I/R groups.

## Keywords

Antioxidant activity, ellagic acid, hesperidin, ischemia/reperfusion injury, skeletal muscle

## History

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## Introduction

Reperfusion injury occurs when the tissues reintake molecular oxygen after ischemia. It is quite an important clinical condition, which includes a large spectrum of vascular events such as organ transplantation, thrombolytic therapy, limb trauma and cardiovascular surgery. Ischemia/Reperfusion (I/R) affects many different organs such as the brain, heart, lung and skeletal muscle in varying degrees[1]. Skeletal muscle I/R can start numerous detrimental events in tissue such as release and production of oxygen-derived radicals by the cells when exposed to ischemia, degradation of phospholipids by peroxidation, damage in endothelial and parenchymal cells, cytosolic calcium overload and edema due to increased permeability in the microcirculation[2]. Reactive oxygen species (ROS) are known as reactive oxygen derivatives occurred from free radicals such as superoxide anion radicals ($O_2^{\bullet-}$) and hydroxyl radicals (OH•)[3–6]. ROS assault to membrane lipids and this event results in lipid peroxidation[7–10]. Also, they can affect the cellular proteins, lipids, nucleic acids and other potential susceptible substances. This process results eventually with excess production of free radicals and organ dysfunction, also ROS can lead to many diseases[11–14]. They are mainly responsible for I/R injury. Different treatment strategies that are applied to reduce I/R injury include various antioxidant vitamins, bioflavonoids and drugs[15].

Ellagic acid (EA) is a polyphenolic compounds that exist in plants and fruits such as strawberries, walnuts, nuts and blueberries[16]. It was reported that EA shows different pharmacological effects, including anti-inflammatory, antioxidant and chemoprevention inhibition of tumorigenesis[17–20]. Hesperidin (HES), a natural flavonoid, exists in fruits and vegetables[21]. HES also have wide pharmacological effects such as anti-inflammatory, antioxidant, anticancer and antiallergic effects[22–24].

The main purpose of this study is to investigate the potential antioxidant effects of EA and HES against skeletal muscle injury induced I/R in rats. MDA level, as a specific biomarker of lipid peroxidation, and antioxidant enzyme activities such as SOD, CAT and GSH-Px, were measured to show the potential protective capacities of EA and HES.

## Materials and methods

### Chemicals

Ellagic acid, hesperidin and sodium carboxymethylcellulose (CMC) were obtained from Sigma Chemicals, St. Louis, MO and stored in dark at 2–4 °C until use.

### Animals

The experimental permission for this study was confirmed by the Experimental Animal Local Ethic Committee of the Veterinary Faculty of Atatürk University (2016–2013). In this experimental study, 30 adult Sprague-Dawley rats between 200 and 230 g weight were used. We kept the rats in standard polypropylene cages (four rats/cage) in a temperature-controlled room (22 ± 2 °C), humidity (55 ± 5%), alternating 12 h light–dark cycles and gave water *ad libitum*. Also, they were acclimatized before the experiment for 1 week. Animals were fasted 8 h before the study.

Address for correspondence: Prof. Dr İlhami Gülçin, Department of Chemistry, Atatürk University, Faculty of Sciences, Erzurum, Turkey. Tel: +90 442 2314375. Fax: +90 442 2314109. E-mail: igulcin@atauni.edu.tr

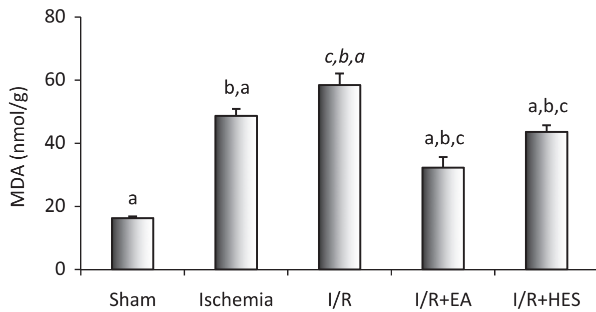


Figure 1. The effects of ischemia, I/R and the treatment with EA and hesperidin (HES) on the MDA levels ((a) $p<0.01$; (b) $p<0.05$ and (c) $p<0.05$). The comparison between the sham group with other groups are denoted by ''a''. The comparison between the ischemia group with IR, EA and HES groups is denoted by ''b''. The comparison between the IR group with EA, and HES groups is denoted by ''c''.

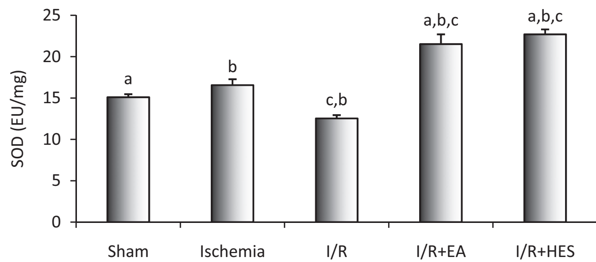


Figure 2. The effects of ischemia, I/R and the treatment with EA and hesperidin (HES) on the SOD activity ((a) $p<0.01$; (b) $p<0.05$ and (c) $p<0.05$). The comparison between the sham group with EA, and HES groups is denoted by ''a''. The comparison between the ischemia group with IR, EA, and HES groups is denoted by ''b''. The comparison between the IR group with EA, and HES groups is denoted by ''c''.

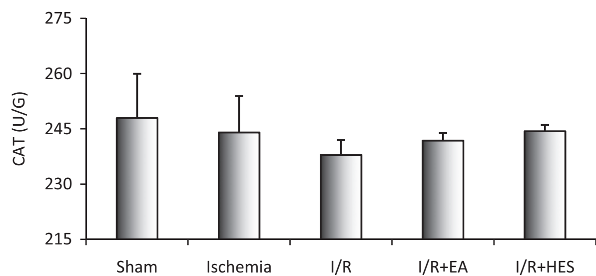


Figure 3. The effects of ischemia, I/R and the treatment with EA and hesperidin (HES) on the CAT activity.

## Experimental design

The rats were weighed and anesthetized with Sodium Pentothal 40–50 mg/kg dose intraperitoneally and the sevoflurane (1,1,1,3,3,3-hexafluoro-2-(fluoromethoxy)propane). They were randomly allocated into five groups of six rats. Group 1 (Sham group, $n=6$), which was subjected to all operative processes, except ischemia reperfusion. Five hundred microliters of CMC (0.5%) were given orally per 100 g weight to this group. Group 2 (ischemia group, $n=6$); 0.5% CMC was administered prior to ischemia period for 1 week and was exposed to ischemia for 2 h. Group 3 (I/R group, $n=6$); rats were exposed to ischemia for 2 h and reperfusion for 2 h. Also, 0.5% CMC was performed. Group 4 (EA + I/R group, $n=6$); I/R was applied. A solution of EA 100 mg/kg as used in the previous study[25] was suspended into 0.5% CMC solution and administered before I/R period for 1 week. Group 5 (I/R + HES group, $n=6$); a solution of HES 100 mg/kg as used in the previous study[26] was suspended into 0.5% CMC solution and administered before I/R period for 1 week. Later the rats were subjected to I/R.

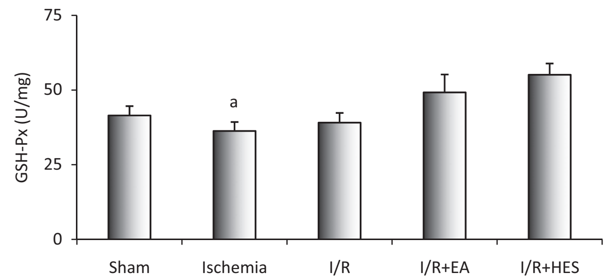


Figure 4. The effects of ischemia, I/R and the treatment with EA, the treatment with hesperidin (HES) on the GSH-Px activity ((a) $p<0.05$). The comparison between the ischemia group with other groups is denoted by ''a''.

## Surgery and specimens collection

Surgery was performed at the Research Laboratory of Animals Experimental, Atatürk University. Operative procedure was similar in all groups but the tourniquate was not applied to the sham group. The animals were placed on the carton in dorsal recumbency position and their limbs immobilized with sticky band. Body temperature was maintained with a heating lamb. They were anesthetized with thiopental sodium prior to ischemia (40–50 mg/kg, intraperitoneally) and additional doses were given when it is necessary throughout the ischemic period. The decline of cyanosis and temperature in the extremity was accepted as ischemia. Later then, tourniquate was removed and reperfusion started. The extremities such as normalization of the temperature, edema and change in color to pink were evaluated as reperfusion. Muscle tissue was harvested for biochemical evaluations after completing the experiment. The gastrocnemius muscles were cleaned in cold isotonic saline. Euthanasia was performed at the end of experiment using an overdose of thiopental sodium (300 mg/kg, ip) to all rats.

## Biochemical assays

MDA level, SOD, CAT and GSH-Px activities were detected in the samples obtained from the skeletal muscle. Each tissue was frozen at $-80\,^{\circ}C$ until the date of analysis. Then, the tissues were homogenized. GSH-Px activity measurement was performed according to the method of Matkovics et al.[27] as described previously[28]. The results were expressed as Units per milligrams of protein tissue. CAT activity in muscle tissue was measured using spectrophotometric methods[29,30]. Results were expressed as units per grams of protein. The total SOD activity determination was made kinetically by using the method described by Sun et al.[31]. The method is based on the inhibition of nitroblue tetrazolium (NBT) reduction[32–34] via the xanthine–xanthine oxidase enzyme system, which is an $O_2^{\bullet-}$ generator. One unit of SOD was defined as the SOD concentration causing 50% inhibition in the NBT reduction rate. SOD activity was expressed as endotoxin units per milligrams of protein. MDA levels were determined spectrophotometrically using thiobarbituric acid (TBA) reaction as described by Placer et al.[35]. The origin of this method is related to the measurement of the red-pink color produced by the interaction of barbituric acid with MDA. A portion of the MDA is formed during the peroxidation, but majority of the MDA occurs as a result of combustion of lipid peroxides in heating step after the acidified environment. Results were measured using a standard calibration curve and were calculated as nanomoles per grams of protein.

## Statistical analysis

Data were analyzed with IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY). Groups were compared with analysis of variance (ANOVA), Bonferroni's multiple comparison tests were performed. Results of the study were given as Mean ± SEM. $p < 0.05$ was accepted as statistically significant.

## Results and discussion

The effects of skeletal muscles I/R were quite devastating. Ischemia induces the damage but this damage increases paradoxically in reperfusion, which causes most significant damage in the muscle and other tissues. The consequence of the I/R injury depends on many factors and is important to understand the pathophysiology of I/R injury[36,37]. The effectiveness of many antioxidant agents has been investigated for palliative I/R injury[38,39]. Scientific data have shown that skeletal muscle I/R injury is an unavoidable event on several surgical procedures. The reblood supply to ischemic tissues results with the intensive formation of ROS, which causes damage to the biological molecules such as nucleic acids, membrane lipids and enzymes[40]. ROS can cause tissue damage and it leads to various diseases[5,41–44]. ROS, which involves free radicals such as OH• and $O_2^{•-}$ radicals, and nonfree radical species such as $H_2O_2$ and singlet oxygen ($^1O_2$) were known as different forms of activated oxygen[45–48]. On the other hand, cellular injury can initiate with various defensive mechanisms by ROS. Firstly, defensive mechanisms contain antioxidant enzymes including CAT, SOD and GSH-Px. Consequently, these enzymes produce the less reactive species. Normally, the pro-oxidant and antioxidant activities are balanced in all physiological processes[49–51]. If this balance is disrupted toward pro-oxidant activity, it causes hazardous damage in functional tissues and organs. Free radicals assault on polyunsaturated fatty acids in cell membrane[52,53]. Antioxidants prevent and stop the free radical activity against lipid peroxidation. Free radicals can alter the structure and feature of biological molecules such as proteins, lipids, carbohydrates and DNA[54–56]. Antioxidants can scavenge oxygen-derived radicals by delaying the process of polyunsaturated fatty acids peroxidation[57,58]. Consequently, the human body can be protected by antioxidants against the damage, depending on the free radicals and ROS[59–61].

The animals well tolerated the experimental procedure and have not died during the experiment. Results are presented as mean ± SEM in all groups. The MDA levels of the muscle tissue were as follows: sham group, 16.26 ± 0.54; ischemia group, 48.70 ± 2.17; I/R group, 58.43 ± 3.70; EA group 32.29 ± 3.30 and hesperidin group 43.61 ± 2.08. The tissue MDA levels in the I/R group were markedly higher than the sham, ischemia, ellagic acid and hesperidin groups. The difference between all the groups was also statistically significant ($p < 0.01$) (Figure 1).

The SOD levels of the muscle tissue were decreased as follows: sham group (15.11 ± 0.36); ischemia group (16.57 ± 0.71); I/R group (12.54 ± 0.40); EA group 21.53 ± 1.18; hesperidin group (22.71 ± 0.60). Muscle tissues SOD activity decreased in I/R group significantly compared to sham group. SOD levels were significantly increased in EA and hesperidin groups and there were significant differences in EA and hesperidin groups ($p < 0.01$) (Figure 2).

The CAT levels of the muscle tissue were as follows: sham group (247.94 ± 12.05); ischemia group (244.03 ± 9.88); I/R group (237.97 ± 3.97); EA group (241.84 ± 2.06) and HES group (244.36 ± 1.72). CAT levels were enhanced in EA and HES groups but there were no significant differences in sham, ischemia and I/R compared to EA and HES groups (Figure 3).

Finally, the GSH-Px levels of the muscle tissue were as follows: sham group (41.52 ± 3.12); ischemia group (36.32 ± 3.00); I/R group (39.11 ± 3.25); EA group (49.25 ± 5.99) and HES group (55.16 ± 3.75). By comparing all the groups, it can be seen that there were significant differences between I/R and HES groups ($p < 0.05$) (Figure 4).

EA shows effective scavenging action on free radicals *in vitro*, as well as the protective effects against lipid peroxidation[62]. HES is a potential antioxidant agent, with strong superoxide radical scavenging activity against free radicals[63]. A lot of scientists have evaluated the antioxidant activity and $O_2^{•-}$, OH• and $H_2O_2$ scavenging activities of HES[64,65]. Previous studies have shown that oxidative damage was attenuated by EA and HES after I/R in animals[64,66,67]. Several studies have reported various antioxidants that can reduce the tissue MDA level[1]. The intestinal ischemia reperfusion study has reported a significant decrease of MDA level in EA group[67]. Also, the decrease of MDA levels in HES group was in accordance with the results of the lung ischemia reperfusion study by Bayomy et al.[66] The results obtained from the present study suggest that I/R injury increased MDA levels in muscle tissue. In contrast, EA and HES led to decrease in the MDA levels in muscle tissue. Depending on these results, in the I/R group, we can clearly say that greater muscle damage occurred only due to ischemia. Also, EA and HES have antioxidative effects that attenuated the muscle damage reducing lipid peroxidation levels. Normally, living organisms are protected from the dangerous effects of $O_2^{•-}$ by SOD, which converts $O_2^{•-}$ into $H_2O_2$[68–70]. Whereas, during reperfusion of ischemic tissues, these natural defences may not be overcome and $H_2O_2$ is converted into OH•, this transformation has the capacity to damage a wide range of biomolecules species containing amino acids, membrane proteins and nucleic acids[71–73]. CAT is an oxidoreductase that catalyzes the conversion of $H_2O_2$ to $H_2O$ and $O_2$. Also, it can protect the cells from damage induced by I/R[74]. It has reported that HES treatment increased SOD, CAT and GSH-Px activities and reduced the tissue damage caused by oxidative stress-induced ischemia and I/R[75]. It was suggested that HES has positive effects on SOD, CAT and GSH-Px activities and has protective effects against oxidative stress in rat brain[76]. In another study, it has been demonstrated that EA enhanced increased tissue SOD, CAT and GSH-Px activities[77]. According to our results, it has been detected that SOD, CAT and GSH-Px activities increased in EA and HES groups compared to ischemia and I/R groups. The results show the most important point that the EA and HES with antioxidant properties also have cell protective effects against lipid peroxidation.

## Conclusion

We have detected the antioxidant effects of EA and HES against the skeletal muscle injury-induced I/R. Further studies should be performed to use these drugs, which have clinically protective effects against the I/R injury in skeletal muscle.

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## Declaration of interest

The authors report there is no conflict of interest.

## ORCID

İlhami Gülçin http://orcid.org/0000-0001-5993-1668


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