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AMELIORATIVE EFFECT OF ASTAXANTHIN ON ISCHEMIA-REPERFUSION INJURY OF SKELETAL MUSCLES

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ABSTRACT

This experimental study aimed to investigate the ameliorative effect of astaxanthin (AST) on the prevention of skeletal muscle injury resulting from lower extremity ischemia/reperfusion (I/R). Twenty-eight (250-300g) male Wistar albino rats were divided into 4 groups as Control, I/R, I/R+AST and AST. In the control group, only anesthesia was induced for 2 h without I/R. In the I/R group, 2 h of reperfusion was facilitated following ischemia under anesthesia. For the I/R+AST group, 7 days prior to ischemia, 125 mg/kg AST was given through a gavage, and 2 h of ischemia and 2 h of reperfusion were facilitated under anesthesia. At the end of the study, blood and gastrocnemius muscle tissue samples were taken for biochemical, histopathological and immunohistochemical examinations. Compared to the control group, there were increased Malondialdehyde (MDA) levels and decreased Superoxide dismutase (SOD) and Catalase (CAT) enzyme activities in the I/R group ($p<0.001$). Degeneration, necrosis, inflammation, loss of striation, interfibrillar and interfascicular edema were seen in the histopathological examination of the skeletal muscles in the I/R group. These histopathological findings were minimal in the I/R+AST group. In the immunohistochemical examination of muscle tissue with the GPx1 primary antibody, a mild degree of GPx1 reactivity was observed in the I/R group, and a moderate degree of GPx1 reactivity was seen in the I/R+AST group. As a result, the strong ameliorative effect of AST on ischemia-reperfusion injury and its complications on skeletal muscles was demonstrated by biochemical, histopathological and immunohistochemical examinations.

Keywords: Ischemia-reperfusion, Muscle, Astaxanthin, MDA, SOD, CAT, Histopathology

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INTRODUCTION

Ischemia is defined as the inability of the tissue to supply oxygen and other metabolites and remove residual products (Majino and Jorris, 1995). Reperfusion is the recovery of blood circulation in this ischemia tissue (Zimmerman and Granger, 1992). Ischemia/reperfusion (I/R) injury is a complex series of events leading to cell and tissue damage. Many factors such as the size of the occluded artery, the duration of occlusion and the characteristics of the tissue influence the severity of damage to the tissue as a result of ischemia (Vardanian *et al*., 2008). The damages in ischemic tissues in many organs (lung, kidney, liver, heart, brain and intestines) following reperfusion have been evaluated in previous studies (Weight *et al.,* 1996; Jaeschke *et al.,* 1988; Collard and Gelman, 2001). The main cause of ischemia-reperfusion injury is oxidative stress caused by the oxidative burst starting from the reintroduction of blood supply and oxidative damage caused by polymorphonuclear leukocytes during the development of inflammatory response (Ritenour *et al.,* 2010). Reperfusion in the extremity following acute limb ischemia may cause severe local tissue damage and systemic complications. In these cases, the mortality rate is 25-50%, and the amputation rate is 15-40%. This is due to toxic metabolites that occur during reperfusion rather than ischemia itself (Saba *et al.,* 2000). The main purpose of reperfusion is the repair the tissue or organ to restore and regain its functions (Collard and Gelman, 2001). The number of studies on finding methods to prevent I/R injury is increasing. Failure to resolve reperfusion injury following ischemia has a negative effect on treatment. Immunosuppressives and corticosteroids are extensively used to prevent the damage caused by an I/R injury, and antioxidants are also important substances (Ulusoy *et al.,* 2012). Astaxanthin is found in various organisms such as salmon, sea bream, trout, shrimp, lobster, roe and algae

(Akkoyun *et al.,* 2019). AST has the effect of eliminating most free radicals. It also protects cell membranes against oxidative stress by preventing lipid peroxidation. This shows that AST is a powerful biological antioxidant (Lim *et al.,*1992).

In this study, the ameliorative effect of astaxanthin on an experimental rat skeletal muscle I/R injury model was investigated by using biochemical, histopathological and immunohistochemical methods.

MATERIALS AND METHODS

Experimental Animals: This experimental study was conducted with 28 male Wistar albino rats weighing between 250 and 300 g, following approval by the Bingol University Animal Experiments Local Ethics Committee. The rats were kept in cages in a controlled room with a constant temperature of 20-22ºC and a twelve (12h)-hour light-dark cycle (dark at 07: 00-19: 00; 19: 00-07: 00 dark). Water and standard food were provided *ad libitum*. This way, the rats were acclimatized to their cages for a week, and then, the trials were started. All experimental procedures were performed under anesthesia with 50 mg/kg Ketamine hydrochloride and a 10 mg/kg dose of Xylazine hydrochloride. In the experimental animals, ischemia was created by the constriction of the femoral artery in the right hind leg. The rats were divided into four groups (n=7 in each group).

Control Group: The rats in this group underwent only anesthesia (2 hours) without ischemia-reperfusion.

Ischemia/Reperfusion (I/R): Two hours after ischemia under anesthesia, reperfusion was facilitated for two hours (Bilgiç *et al.,* 2018).

Ischemia/Reperfusion (I/R) + AST (125mg / kg): Astaxanthin (AST) at a dose of 125 mg/kg was given via oral gavage 7 days before inducing ischemia. Two hours of ischemia and two hours of reperfusion were facilitated under anesthesia.

Astaxanthin (AST) 125 mg/kg: The rats in this group received astaxanthin (AST) at a dose of 125 mg/kg by oral gavage for 7 days, but only anesthesia (2h) was induced without ischemia (Gross *et al.,* 2006).

Biochemical assays: At the end of the study, the rats were anesthetized by an intraperitoneal (i.p.) injection of 50 mg/kg ketamine + 10 mg/kg xylazine. Blood samples were obtained by cardiac puncture for biochemical analyses. Lipid peroxidation was measured at 532 nm by means of Malondialdehyde (MDA) measurement with thiobarbituric acid (TBA). The results are expressed as nmol/mg protein (Ohkawa *et al.,*1979). Superoxide dismutase (SOD) enzyme activity was determined as the amount of the enzyme that inhibited nitro blue tetrazolium reduction by 50%. The results are expressed as U/mg protein (Fhole and Otting, 1984). Catalase (CAT) enzyme activity was determined as a decrease in the optical density per minute, and the enzyme activity is expressed in U/mg protein (Aebi, 1984). The protein concentrations of the homogenates from the muscle tissues were determined according to the method reported by Bradford (1976).

Histopathological and Immunohistochemical Examination: Necropsy was performed on all rats, and tissue samples of the gastrocnemius muscle were taken and fixed in 10% buffered formalin solution for 48 h for subsequent histopathological and immunohistochemical examinations. Within the scope of routine tissue follow-up, the tissue samples were blocked in paraffin after they were dehydrated by passing them through a graded alcohol series (70, 80, 90, 100%), and they were made transparent by passing them through a graded series of xylol. Serial 4-5-micron-thick sections were taken from these paraffin blocks by a microtome (Leica RM 2135), and the sections were stained using the Hematoxylin-Eosin (H.E.) staining technique. Histopathologically, loss of striation, separation of muscle fibers, inflammatory cells, interfibrillar and interfascicular edema, degeneration and necrosis were evaluated semi-quantitatively. For the immunohistochemical examination, 4-5-µm-thick sections were placed on polylysine-coated slides. The sections were kept in a 60°C drying oven for 1 h and then made transparent in two separate xylol concentrations, rehydrated with a decreasing graded alcohol series, and kept in distilled water for 5 min. The sections were kept in a citrate buffer solution for 20 min at room temperature, and then, they were kept in a 3% hydrogen peroxide solution for 5 min to prevent endogenous peroxidase. The Anti-GPx1 (abcam-ab22604) primary antibody was added to the sections that were washed 3 times with PBS for 5 min and left overnight at + 4°C. In order to determine whether the GPx1 immunoreactivities were specific or not, no additional antibody was added to the sections in the negative controls. The next day, the sections were washed 3 times with PBS and then incubated with biotinylated secondary antibody for 30 min. After the addition of the secondary antibody, the antibody- avidin-biotin-peroxidase complex sections were visualized by dropping the chromogen material, 3,3'-Diaminobenzidine (DAB). Background staining was performed with Mayer's hematoxylin. After the sections were dehydrated in a graded alcohol series, they were made transparent with xylol, and they were closed with Entellan. Immunohistochemical staining intensity was evaluated independently by a pathologist. GPx1 staining grade was scored as the absence of staining (-), weak staining (+1), medium staining (+2) and strong staining (+3). For the histopathological and immunohistochemical analyses, the stained specimens were examined using a Nikon 80i-DS-R12 microscope.

Statistical analysis: The values are given as mean ± standard error of mean. Analysis of variance (ANOVA) was conducted, and group comparisons were performed with post-hoc Tukey' test. The SPSS 17 statistical program was used for this purpose. The level of statistical significance was set as $p<0.05$.

RESULTS

The MDA levels in the I/R and I/R+AST groups were significantly higher than those in the control group ($p<0.001$). It was determined that there was a decrease in the I/R+AST and AST group in comparison to the I/R group (Figure I). The SOD enzyme activity was lower in the I/R group compared to the control group ($p<0.001$), and a higher value was determined in the AST group compared to the I/R+AST group (Figure II). CAT enzyme activity was lower in the I/R group in comparison to the control group ($p<0.001$), and it was higher in the I/R+AST group than the I/R group (Figure III).

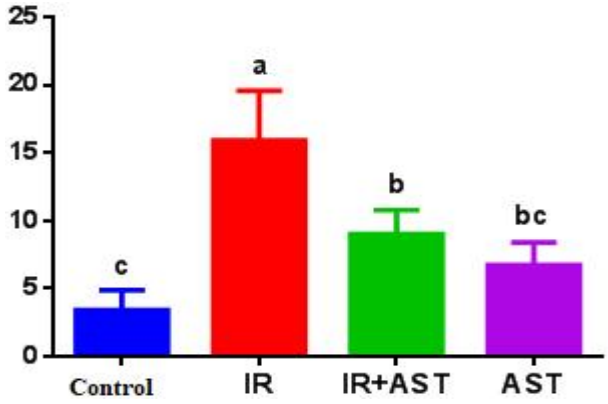

Figure I. MDA levels of groups (a,b,c: p<0.001)

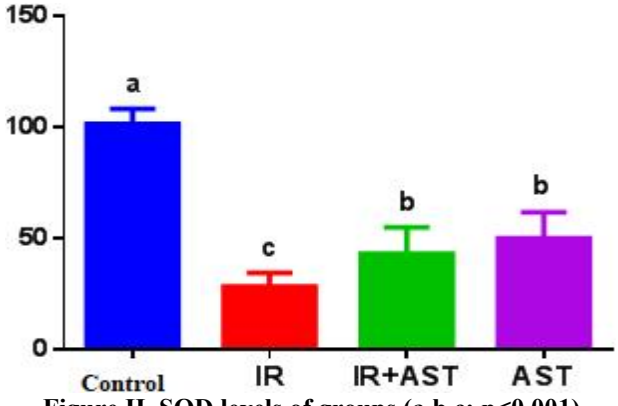

Figure II. SOD levels of groups (a,b,c: p<0.001)

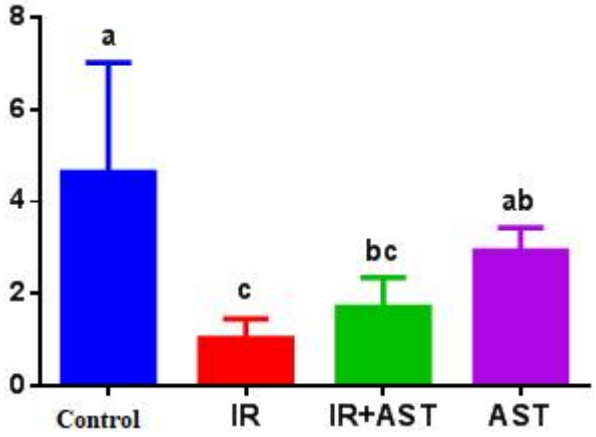

Figure III. CAT levels of groups (a,b,c: p<0.001)

Table 1 and Figure IV show the distribution of the results of the microscopic examinations of the protective efficacy of astaxanthin on I/R injury in the gastrocnemius muscle of the rats. No histopathological findings were found in the microscopic examination of the muscle tissue of the rats in the control group and the astaxanthin group. The muscle fibers, which were intact, had transversal striation. Myocytes with flat nuclei were just below the sarcolemma and poriferous. The vascular structure of the perimysium was normal, there was no degeneration in the peripheral nerves, and the fat and connective tissues were normal around these nerves. The pathological examination did not show inflammatory cell infiltration (Figures IVA and E). The I/R group showed loss of striation and fascicules characterized by degenerated muscle fibers. In some parts, the cytoplasm of myocytes was stained with the eosinophilic dye, and the nuclei were pycnotic. Intensive inflammatory cell infiltration was observed in the interfibrillar and interfascicular area with edema. Degeneration was also observed in the peripheral nerves (Figures IVB and C). In the group given astaxanthin with ischemia-reperfusion, there was a slight degree of edema between the muscle areas and myofibrils. In general, the histological structure of the muscle fibers was preserved in both transverse and longitudinal sections, and a similar situation was observed to that in the control group. The transverse lines were prominent in the muscle fibers, and the nuclei in the myocytes had a peripheral appearance as in the control group. The peripheral nerves had a normal appearance, and no damage in the connective tissue and adipose tissue surrounding the muscle tissue was observed. In this group, the differences in the findings were generally negligible in comparison to the I/R group (Figure IVD).

Table 1. The muscle tissue microscopic results concerning the effects of astaxanthin on ischemia-reperfusion in rats.

Parameters		Control	I/R	I/R + AST	AST
Loss of striations		-/7^{b}	7/7^{a}	2/7^{a}	-/7^{b}
	Mild	-	1	1	-
	Moderate	-	2	1	-
	Severe	-	4	-	-
Separation in muscle fibers		-/7^{b}	7/7^{a}	2/7^{a}	-/7^{b}
	Mild	-	-	1	-
	Moderate	-	2	1	-
	Severe	-	5	-	-
Inflammatory cells infiltration		-/7^{b}-	7/7^{a}	3/7^{ab}	1/7^{b}
	Mild	-	-	2	1
	Moderate	-	1	1	-
	Severe	-	6	-	-
Interfibrillar and interfascicular edema		-/7^{b}	7/7^{a}	3/7^{b}	-/7^{b}
	Mild	-	-	1	-
	Moderate	-	3	2	-
	Severe	-	4	-	-
Degeneration and necrosis		-/7	7/7^{a}	2/7^{a}	-/7
	Mild	-	1	1	-
	Moderate	-	3	1	-
	Severe	-	3	-	-

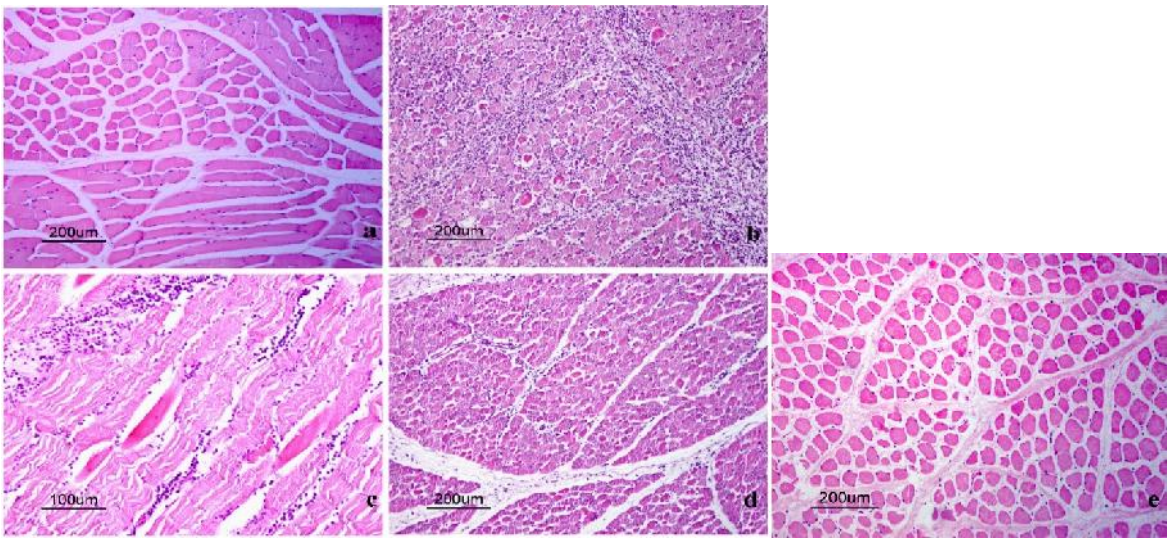

Figure IV (A-E): (A) Normal view of the transverse and longitudinal sections of the skeletal muscle of the control group (H.E. × 10). (B) Myofibrillar bundles with intense inflammatory cell infiltrations and degenerative and necrotic changes in the cross-section of the I/R group's skeletal muscle (H.E. × 10). (C) Striation loss in the longitudinal section of the I/R group skeletal muscle and inflammatory cell infiltrations in the interstitial area (H.E. × 20). (D) Slight focal inflammatory cell infiltrations in the interstitial area in the cross-section of the I/R + AST group (H.E. × 10). (E) Normal view of the cross-section of the AST group (H.E. × 10)

Immunohistochemical findings: The distributions of the GPx1 reactivity of the ameliorative effect of astaxanthin on ischemia and reperfusion injury in the gastrocnemius muscle in the rats are presented in Table 2, and the mean GPx1 scores according to the groups are presented in Figure V.

The microscopic views of the degree of staining regarding GPx1 reactivity in the muscle tissue by ischemia-reperfusion injury to astaxanthin according to the groups are presented in Figure VI.

Table 2. The distribution of the protective efficacy of astaxanthin on ischemia-reperfusion in the rats according to the groups.

Parameters	Control	I/R	I/R + AST	AST
Immunohistochemical GPx1 reactivity	+3	+1	+2	+3

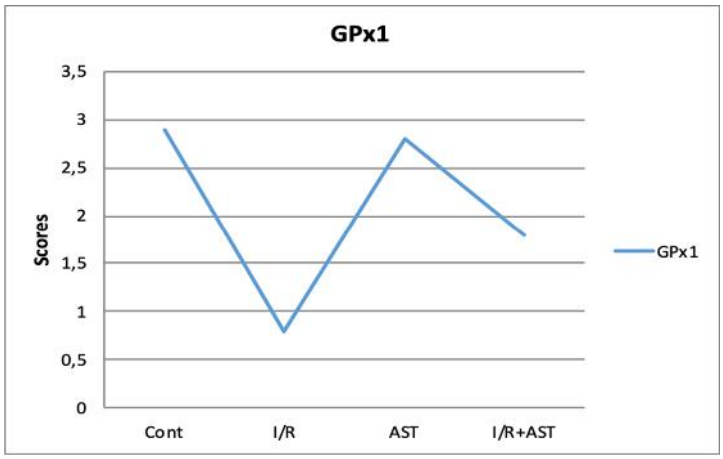

Figure V: Mean GPx1 scores in the groups

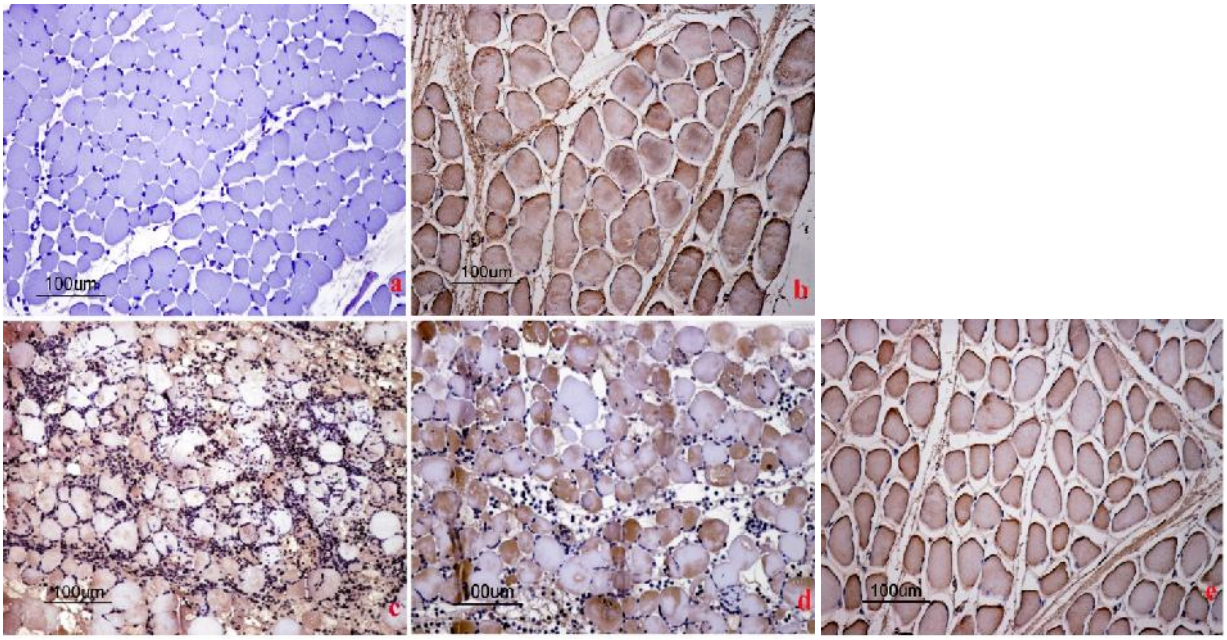

Figure VI (A-E): GPx1 immunoreactivity grades in muscle tissue sections. (A) Negative control group, no staining (-). (B) Control group, strong staining. (C) I / R group, slight staining. (D) I/R + AST group, Moderate staining. (E) AST group, strong staining. (IHC-avidin-biotin-peroxidase).

DISCUSSION

I/R damage in skeletal muscles is a clinical phenomenon that has an increasing effect on the morbidity rates of patients (Arslan *et al.,* 2013). This leads to a decrease in the energy level of the cell due to ischemia and the accumulation of toxic metabolites in the tissue, leading to the initiation of a series of biochemical reactions ranging from cell dysfunction to cell death (Engin *et al.,* 2003). Reflow to the tissue, i.e., is called reperfusion, is necessary for the recovery of energy requirements and removal of toxic metabolites from the environment. However, serious metabolic disorders may occur due to toxic metabolites and various inflammatory mediators formed during ischemia, and reperfusion may lead to further tissue damage (Blaisdell, 2002; Eltzschig

and Collard, 2005). In some surgeries, the application of a tourniquet is a method that aims to provide as much blood-free environment as possible in the operating area, and thus, to facilitate the operation of the surgeon. However, this method has the drawback of causing I/R injury. For this reason, it will be a useful approach to apply drugs that will protect the patient by reducing the potential damage to the tissue during the tourniquet application (Bilgiç *et al.,* 2018). AST has many antioxidant properties, since each ion ring has a unique and remarkable molecular structure of hydroxyl and keto fragments (Liu and Osawa, 2007). Recent studies on animal models have shown that AST has a protective impact by reducing oxidative damage in I/R-related liver (Curek *et al.,* 2010), brain (Lu *et al.,* 2010; Shen *et al.,* 2009) or cardiovascular (Lauver *et al.,* 2005) injuries. However, the protective effect of AST against skeletal muscle I/R injuries is still unknown. Therefore, in this study, it was planned to investigate the possible beneficial effects of substances with antioxidant capacities in the rat I/R injury model.

Reactive oxygen species (ROS) have an important place in the formation of I/R injury (Seekamp and Ward, 1993; Carden and Granger, 2000; Sucu *et al.,* 2002). These are superoxide anions, hydroxyl radicals, hydrochloric acid, hydrogen peroxide and peroxynitrite derived from nitric oxide. The initial event in oxidative damage caused by oxygen-derived free radicals is the production of xanthine oxidase-based superoxide anions (Toyokuni, 1999). ROS can also react with proteins, nucleic acids and lipids and lead to the lipid peroxidation of biological membranes and cause the formation of lipid peroxides such as MDA (Tuna *et al.,* 2001). As a conclusion, lipid peroxidation results in cellular damage, causing structural and functional alterations in the cells (Henderson *et al.,* 2010). Free radical scavengers are various enzymes that react with reactive oxygen species and convert them into harmless substances (Toyokuni, 1999; Collard and Gelman, 2001). SOD, CAT and GPx are known as endogenous antioxidants and the first-line defense mechanism against oxidative damage (Halliwell and Gutteridge, 1990). These antioxidant enzymes have a significant role in the antioxidative defense system. Antioxidant enzyme status was evaluated in a previous study after I/R in skeletal muscles (Takhtfooladi *et al.,* 2013). In this study, it was determined that the level of MDA, the most important product of lipid peroxidation, increased significantly in the I/R group ($p<0.001$) and decreased significantly in the I/R+AST group. It was determined that the enzyme activity levels of SOD and CAT, which are important elements of the antioxidant defense system, decreased significantly in the I/R group compared to the control group ($p<0.001$), and there was a significant increase in the I/R+AST group (Figs. II, III). Qiu *et al.* (2015) also found an increased level of MDA in the I/R group and a decreased level in the I/R group given astaxanthin in their study on the protective effects of astaxanthin against I/R-induced kidney injury in mice. In their study on the protective effect of astaxanthin on learning and memory deficits and oxidative stress in a mouse model of repeated brain I/R, Xue *et al.* (2017) stated that there was a decrease in the I/R group related to the SOD enzyme activity level and an increase in the I/R+AST group. Regarding this increase in the activities of antioxidant enzymes after reperfusion, the over-produced ROS in the I/R event stimulate the antioxidant defense system, and the antioxidant astaxanthin strengthens the antioxidant defense system, possibly by causing an increase in the expression of these enzymes, known as the antioxidant enzymes SOD, CAT and GPx1. Thus, a compensatory mechanism created by the body to detoxify the high quantity of free radicals formed in the skeletal muscle after reperfusion and more antioxidant enzyme production with astaxanthin occurs. Various vasoactive molecules such as endothelin are released during ischemia and reperfusion, and these molecules cause significant damage ranging from endothelial dysfunction and edema to multi-organ failure (Kuzu *et al.,*1998; Souza *et al.,* 2000; Borjesson *et al.,* 2002). Therefore, there is a significant correlation between ischemia and reperfusion time and the severity of I/R injury. In a study on lower extremity I/R, the authors found the rate of necrosis in the muscle tissue to be 21% at the 4th hour of ischemia, 61% at the 5th hour and 92% after the 5th hour (Petrasek *et al.,* 1994). While necrosis was detected in 46% at the 4th hour of ischemia, the rate was observed as 70% after 4.5 hours. A close relationship was also found between muscle necrosis and decreased ATP stores. While glycogen and creatine phosphate decrease in ischemic muscle tissue, myonecrosis formation is lower at this stage. Afterwards, muscle necrosis rapidly increases with the reduction of ATP. After 6 hours of muscle ischemia, 80% decrease in ATP stores and the necrosis of all muscle tissue are observed (Hayes *et al.,* 1988; Skjeldal *et al.,* 1993). However, it has been shown in various studies that antioxidants used before I/R significantly reduce the severity of injury (Elmalı *et al.,* 2007; Curek *et al.,* 2010; Akdemir *et al.,* 2016). In this study, 125 mg/kg AST was administered through oral gavage daily for preventive purposes 7 days before facilitating I/R, and after 2 hours of ischemia and 2 hours of reperfusion, degenerative and necrotic changes were observed in the muscles from moderate to severe histopathologically in the entire I/R group. Mild to moderate degenerative and necrotic changes were seen in only two cases in the I/R+AST group.

Free radicals formed during oxidative stress caused by I/R, the contraction of muscle fibers and deterioration of the internal structure in some muscle fibers, degeneration, and necrosis cause a chain of pathological events such as vascular endothelial cell damage and increased microvascular permeability

(Ozyurt *et al.,* 2007). In this respect, the existing literature on I/R injury has characterized it as a chain of events very similar to acute inflammatory processes (Carden and Granger, 2000). In I/R model studies similar to this study (Akar *et al.,* 2001), histopathologically increased inflammatory cell (PMNL) infiltration in the muscles after ischemia and reperfusion, segmental necrosis, loss of striation, nuclear centralization, hemorrhage, and intercellular and interfascicular edema have been observed, and there have been some findings such as dimensional changes and an increase in connective tissue. Even when the cause of acute ischemic conditions is eliminated, and reperfusion is provided in I/R cases, the risk of mortality and morbidity may continue. In fact, these risks increase in cases where surgical intervention is delayed. Even if reperfusion in the extremity is fully achieved, a chain of events begins that will lead to extremity loss, acute kidney and respiratory failure, dysfunction in tissues such as the heart, intestine, brain and spleen. Despite the advances in surgical techniques for the surgical removal of acute extremity ischemia, it has not been possible to completely get rid of these undesirable events, and studies on this topic are continuing. Naturally, in order to find methods and drugs to prevent this situation, it is first necessary to reveal the causes and the development of events that start this situation. Studies are still ongoing on various pharmacological and therapeutic strategies to reduce reperfusion injury. For this purpose, studies have been carried out on substances such as vitamins C and E, superoxide dismutase, mannitol, allopurinol, and deferoxamine (Enkaya *et al.,*1999). Similarly, in a study examining the effects of quercetin on I/R injury, neutrophil infiltration was observed between and around muscle fibers in the I/R group, internal structures were impaired in some muscle fibers, and these damages were largely prevented in the group where I/R and quercetin were administered together. It was reported that the histological structure in the IR and quercetin group was almost the same as the one in the control group (Arıcı, 2006). In another study by Chau *et al*. (2014), in which the effect of mitoquinone combined with apocynin on myocardial ischemia-reperfusion injury was investigated, it was found that in the group given apocynin together with mitoquinone, it significantly reduced I/R injury compared to the I/R group. It was emphasized that the ventricular contractile force was higher. Elmalı *et al*. (2007) reported that resveratrol had a significant protective effect against I/R damage in skeletal muscles due to its strong antioxidant effect, in the study where they evaluated polymorphic nucleated leukocyte infiltration in muscle tissues, edema, dimensional changes in muscle fibers, and segmental necrosis using resveratrol. In another study investigating the protective efficacy of leflunomide in an experimentally created lower extremity I/R injury in rats (Gürsul, 2012), the filamentous arrangement of collagen fibers was disrupted in the epimysium of the skeletal muscle tissue of the rats in the I/R group, and a very intense connective tissue cell increase occurred between the collagen fibers. It was emphasized that there was an intense leukocyte migration from the blood vessels to the epimysium and perimysium, as well as mastocyte cells, dilatation of the vessels and a deterioration in the wall structure of the arteries, and a spongiotic appearance in the smooth muscle cells that form the tunica media layer of the muscular arteries. With other histopathological findings such as a decrease in lipid peroxidation, an increase in antioxidant enzyme activities, a decrease in intramuscular leukocyte infiltration with leflunomide application, it was stated that the use of antioxidants before surgical interventions using tourniquets can reduce or prevent oxidative muscle damage that may occur due to I/R. Astaxanthin, a powerful antioxidant, was used in this study, which was carried out considering the aim of minimizing reperfusion injury after acute ischemia. In the histopathological examination of the muscle tissues of the rats in the I/R group, despite the obvious histopathological findings such as loss of striation in the longitudinal sections of the muscle fibers, separation of the muscle fibers, intercellular and interfascicular edema, perivascular and interfibrillar inflammatory cell infiltration, degeneration in myocytes and coagulation necrosis were determined. It was observed that these findings decreased significantly in the I/R +AST group and even showed a histological appearance almost the same as the control group. This situation was similar to the studies of the researchers mentioned above and their findings in the experimental groups where various antioxidants were used to prevent I/R injury.

Conclusion: The results of this study supported the view that astaxanthin can exert a protective effect against skeletal muscle injury caused by I/R in rats. It was concluded that astaxanthin has an antioxidant role in the formation of this protective effect, but more extensive experimental studies are needed for its clinical use.

Ethical Considerations: The study was approved by the Local Ethics Committee (23.06.2016-2016/04) for Animal Experiments.

Declaration of Interest: All authors declare that there are no conflicts of interest.

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Author Contributions: A. Uyar: Conceptualization, methodology, investigation and data curation, writing-original draft preparation, writing-review and editing. (25%).

H. T. Akkoyun: Conceptualization, methodology, investigation and data curation, writing-original draft preparation, writing-review and editing. (25%).
A. Ş. Bengu: Formal analysis and validation, statistical analysis, writing-review and editing. (10%).
M. B. Akkoyun: Formal analysis and validation, writing-review and editing. (10%).
Ö. F. Keleş: Technical support, formal analysis and validation. (10%).
T. Atçali: Technical support, formal analysis and validation. (10%).
Ş. Melek: Technical support, formal analysis and validation. (5%).
T. Yaman: Formal analysis and validation. (5%).
All authors have read and agreed to the published version of the manuscript.

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