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Effect of crocin on experimental gastrocnemius muscle ischemia/reperfusion injury in rat

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Abstract

Ischemia-reperfusion (IR) injury to the lower extremities causes damage to various tissues, notably the limbs. Because research in recent years have demonstrated that saffron and its components are useful in ischemic strokes, the goal of this study was to see whether Crocin (Cr), one of the active constituents in saffron, could protect the gastrocnemius muscle from IR injury. A total number of 32 Sprague-Dawley rats were randomized into four groups randomly: control, Cr, IR, and IR + Cr. Xylazine and ketamine were used to anesthetize all of the rats. The left lower limbs of the other two groups were subjected to 2 hr of ischemia and 2 hr of reperfusion with tourniquet, with the exception of the control and Cr groups. Tumor necrosis factor alfa (TNF-α), interleukin 6 (IL-6), IL-1β, total antioxidant status (TAS) and total oxidant status (TOS) levels were assessed in the blood as well as muscle IL-6, IL1β, SOD1-2, catalase (CAT) and glutathione peroxidase (GPx) expression. According to the IR group, increases in TAS levels and decreases in TNF-α, IL-6, and IL-1β levels were substantial in the Cr therapy group. Cr significantly reduced IL-6 and IL-1β mRNA expression levels in the muscle of the IR group and increased superoxide dismutases 1 (SOD1), SOD2, catalase (CAT), and GPx. Our data showed that Cr protected the gastrocnemius muscle from IR injury in rats and reduced inflammatory markers significantly. These effects of Cr might have been mediated by improved antioxidant enzyme activity, suppression of free radical generation and reduction of oxidative stress.

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Introduction

Stroke risk, an extremely dangerous to people's health, rises dramatically as they get older. By 2050, the number of persons aged 65 and up is expected to exceed 1.50 billion. As a result, numerous investigations on neuro-protective drugs for use in the treatment of neuronal damage caused by stroke are being carried out. Ischemia is one of the most common types of tissue damage caused by decreased blood flow to an organ due to a variety of factors such as hypovolemia, thrombosis, energy production ceasing and lack of oxygen, transplantation, surgical procedures and nutrition.[1,2] Peripheral artery disease is a prevalent pathological illness that is a major public health concern. It results in tissue necrosis which frequently necessitates amputation and is accompanied by inter-mittent claudication symptoms. The adverse consequences of ischemia-reperfusion (IR) are being treated with new medicines that help minimize skeletal muscle mitochondrial dysfunctions.[3,4]

Crocin (Cr) is a carotenoid that may be extracted in its purest form from *Crocus sativus* L. (saffron) extract and crystallized.[5] Saffron contains a variety of chemicals including thiamin, riboflavin, carbohydrates, amino acids, proteins, volatile oil, mucilage, gums, minerals, carotene, lycopene, zigzantin, anthocyanin, and pigments such as Cr (30.00 percent Cr, 2.50 percent volatile compounds and 5.00 - 15.00 % picrocrocin).[6-8] The Cr has anticarcino-genic, antidiabetic, gastric ulcer healing, antidepressant, antitumoral, anti-inflammatory, antioxidant, antiarthritis, antihyperlipidemic, antiatherosclerotic, metabolic syndrome management, genoprotective, memory impairment, neuroprotective, Alzheimer disease, radical scavenging, and protective brain and lung ischemic injury effects.[9-14]

According to certain research, Cr efficiently suppresses IR and its therapeutic effects by minimizing vascular and neuronal damage in various experimental IR models. According to Cr effects and based on the fact that no article on the protective impact of Cr against IR has been published thus far, the current study was done to investigate the

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protective effect of Cr on the damage caused by IR. The goal of this study was to see whether Cr could affect gastrocnemius muscle IR injury which is a common complication of the tourniquet procedure.

Materials and Methods

Animals. The experimental protocol and animal hausing were conducted according to the guidelines for ethical conduct in the care and use of animals. All experimental norms approved by the Ethic Committee of Experimental Animal Teaching and Researcher Center (Ethical approval code: No: 29.11.2017, 36643897-000-E.1700331423-174). Sprague-Dawley male rats (n = 32) were obtained from the Medical and Experimental Application and Research Center, Erzurum, Türkiye. The rats aged eight - ten weeks (weighing between 200 - 250 g) were housed under a humidity of 55.00 ± 5.00% and temperature of 21.00 ± 2.00 ˚C with a 12 hr light/dark cycle in this experimental study. Water and food were available *ad libitum*. The selected rats were divided into four random equal groups (n = 8) after training sessions.

Preparation of Cr. Crocin was purchased from Sigma-Aldrich (St. Louis, USA) as yellow powder. No mortality was observed. For preparation of intraperitoneal injection solution, it was dissolved in 5.00% dimethyle sulfoxide (DMSO; Sigma-Aldrich) using distilled water.[15]

Study design and experimental model. Before the study, animals were fasted overnight, but were allowed free access to water. All rats were anesthetized with 8.00 mg kg^{-1} xylazine (Bioveta PLC, Ivanovice na Hane, Czech Republic) and 60.00 mg kg^{-1} ketamine (Richter Pharma AG, Wels, Austria). Except for the control and Cr groups, common femoral arteries and collateral flow were occluded tightly with rubber tourniquets, the proximal of the left extremity and ischemia was confirmed by cyanosis and temperature drop in the left lower limbs of the other two groups were applied to 2 hr of ischemia and 2 hr of later tourniquate was released and reperfusion was initiated. Reperfusion was verified by edema, return of the extremity to normal temperature, apperance of pulse and the extremity colour change to pink.[15,16] Eight animals were used in each group. Rats were divided into the following groups: Control group (C): 0.50 mL 5.00% DMSO was administered intraperitoneally 30 min before ischemia.[17] Blood samples were obtained from the *Aorta abdominalis* under anesthesia for biochemical analyses. Crocin group (Cr): Crocin 60.00 mg kg^{-1} intra-peritoneally.[12,15] Ischemia/reperfusion group (IR): Under the anesthesia, 2 hr tourniquet was applied to the lower extremity (low temperature and cyanotic claw marked the occurrence of ischemia). Subsequently, the tourniquets were opened and reperfusion was applied for 2 hr (the pinking of the claws and the increase in temperature indicated reperfusion) and afterwards, the abdomen was opened with midline incision and blood samples for biochemical analyses were taken from *Aorta abdominalis*. The IR + crocin group (IR + Cr): 30 min prior to ischemia, rats were given Cr 60 60.00 mg kg^{-1} intraperitoneally. After 30 min, under anesthesia, 2 hr tourniquet was applied to the lower extremity. Subsequently, the tourniquets were opened and reperfusion was applied for 2 hr, and afterwards, blood samples were taken. We showed macroscopic picture of the gastrocnemius muscle tissues in all groups (Fig. 1).

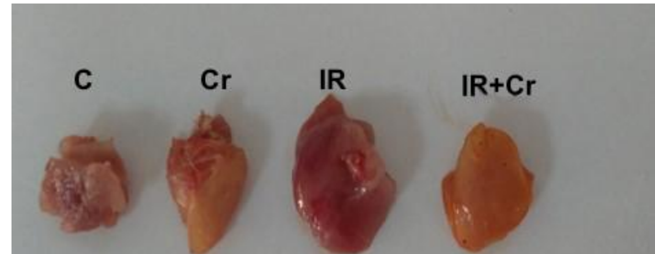

Fig. 1. Macroscopic picture of the gastrocnemius muscle tissues in all groups. C: Control group, Cr: Crocin group, IR: ischemia-reperfusion group, and IR + Cr group: Ischemia-reperfusion + crocin treatment group.

Biochemical analyses in plasma and gastrocnemius muscle tissues. Whole blood was collected into lithium heparinised tubes from *Aorta abdominalis*. Plasma was obtained from these whole blood samples by centrifugation (3,000 rpm for 10 min) and used for the determination of the biochemical parameters. The gastrocnemius muscle tissues of each animal were stabilized using RNAlater from Sigma-Aldrich and frozen at – 80.00 ˚C until RNA extraction was performed. RNA extraction was performed from the muscle samples within 24 hr following the sacrifice. The gastrocnemius muscle tissues were homogenized in a Qiagen TissueLyserII. Plasma tumor necrosis factor (TNF-α), interleukin-6 (IL-6) and IL-1β were determined using commercial enzyme-linked immunosorbent assay (ELISA) kit (Rat Tnf-α ELISA Kit Aviscera Bioscience, Santa Clara, USA; Rat IL-6 ELISA Kit Aviscera Bioscience, Santa Clara, USA ; Rat IL-1β ELISA Kit MyBioSource, San Diego, USA) according to the instructions of the manufacturer. Plasma total antioxidant status (TAS) and total oxidant status (TOS) levels were determined using commercially available kits (Rel Assay Diagnostics, Gaziantep, Türkiye). These levels were measured with Biotek microplate reader (µQuant MQX200; Bio-Tek Instruments, Winooski, USA).

Measurement of the TAS. The principle of the total antioxidant assay kit was based on the decolorization of dark blue-green-colored 2,2-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid [ABTS]) cationic radical to a colorless reduced form of ABTS by antioxidant molecules in the sample. The decolorization rate was proportional to the amount of the antioxidant molecule in the plasma sample. The assay was calibrated with a stable antioxidant

standard solution which was conventionally named as Trolox equivalent, a vitamin E analog. The data were expressed as μmol Trolox equivalents L^{-1}.[18,19]

Measurement of the TOS. The total oxidant assay kit principle was based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium and the measurement of the ferric ion by xylenol orange. Briefly, oxidants in the sample oxidized the ferrous ion–o-dianisidine complex to ferric ion. Glycerol molecule in the reaction medium was used for the prolongation of the oxidation reaction. The ferric ion formed a colored complex with xylenol orange in an acidic medium. Spectrophotometrically (μQuant MQX200; Bio-Tek Instruments), the evaluation of the color intensity was related to the total amount of oxidant molecules in the plasma sample. The assay was calibrated with hydrogen peroxide (H_2O_2) (Sigma-Aldrich) and the results were expressed in terms of micromolar hydrogen peroxide equivalent per litre (μmol H_2O_2 Eq L^{-1}).[18]

Real-time polymerase chain reaction (PCR) analysis. To investigate the effect of Cr on the levels of some proinflammatory cytokines, a realtime PCR was performed using specific PCR primers for muscle tissues on rat IL-6, IL-1β, superoxide dismutase (SOD1), SOD2, catalase (CAT) and glutathione peroxidase (GPx; Qiagen, Hilden, Germany). The gastrocnemius muscle tissues were homogenized in a Qiagen TissueLyserII using a QIAzol Lysis Reagent (Qiagen). Total RNA was extracted from the frozen homogenate samples RNeasy Mini Kit (Qiagen) and the complementary DNA (cDNA) was synthesized from 8.00 μL of the total RNA using the QuantiTect cDNA reverse transcription kit (Qiagen) according to the manufacturer's protocol. The concentration and purity of the total RNA was determined spectrophotometrically at a wavelength of 260 nm (Biotek). Quantitative realtime PCR (qPCR) cytokine and antioxidant enzyme mRNA levels were measured using quantitative realtime PCR in the Rotor Gene Q (Qiagen). Additionally, the house keeping gene (rat beta actin, Qiagen) mRNA level was determined. The values for the specific genes were normalized with the Beta actin reference gene (Qiagen). In each PCR run, the cDNA samples were amplified in three. The relative quantification of the mRNAs for IL-6 and IL-1β, SOD, CAT and GPx were performed using the 2-delta delta threshold cycle ($2^{-\Delta\Delta CT}$) method.[20]

Statistical analysis. Statistical analysis of data was done by one-way analysis of variance (ANOVA) followed using SPSS Software (version 22.00; IBM Corp., Armonk, USA). Post-hoc Tukey's test was used to compare the biochemical parameters between the groups. p-values < 0.05 were considered as significant. The results are expressed as mean ± standard error (SEM) for each group.

Results

The control groups gastrocnemius muscle had no macroscopic damage, as shown in Figure 1. Only the Cr groups gastrocnemius muscle was yellow. The IR groups gastrocnemius muscle was reddened and hemorrhagic. The total muscle area of lesions generated by IR damage was dramatically reduced after pretreatment with Cr. Cr appeared to be beneficial based on the macroscopic data. Plasma TNF-α, IL-6, IL-1β and TOS levels in the IR groups were higher than those in the IR + Cr group. This parameter level was decreased significantly in the Cr treatment group. Plasma TAS levels in the IR groups were lower than those in the IR + Cr group. TAS levels were significantly increased by administration of crocin (Table 1; $p < 0.001$). The mRNA transcript levels of IL6 and IL-1β were up-regulated in the IR group ($p < 0.001$) and these expression levels were down-regulated in the IR + Cr groups ($p < 0.001$; Fig. 2). The mRNA transcript levels of SOD1, SOD2, CAT and GPx were down-regulated in the IR group ($p < 0.001$) and these expression levels were up-regulated in the IR + Cr groups ($p < 0.001$; Fig. 3).

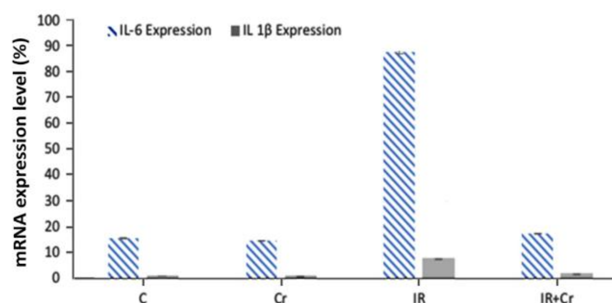

Fig. 2. Relative mRNA expression levels of rat muscle tissues IL-6 and IL-1β expression in all experimental groups. C: Control group, Cr: Crocin group, IR: Ischemia- reperfusion group and IR + Cr group: Ischemia-reperfusion+crocin treatment group. Values are expressed as mean ± SEM.

Table 1. Effect of intraperitoneal administration of crocin on the level of plasma TNF-α, IL-6, IL-1β, total antioxidant status (TAS), and total oxidant status (TOS) level in all experimental groups. Values are expressed as mean ± SEM.

Groups	TNF-α (pg mL^{-1})	IL-6 (pg mL^{-1})	IL-1β (pg mL^{-1})	TAS (mmol Trolox Eq L^{-1})	TOS (μmol H_2O_2 Eq L^{-1})
Control	134.39 ± 0.33[c]	40.02 ± 0.30[c]	44.85 ± 0.19[c]	1.33 ± 0.01[b]	3.19 ± 0.12[c]
Cr	133.66 ± 0.34[c]	39.27 ± 0.30[c]	44.07 ± 0.06[c]	1.37 ± 0.00[a]	3.05 ± 0.00[c]
IR	161.44 ± 0.61[a]	151.06 ± 1.30[a]	183.05 ± 0.89[a]	0.98 ± 0.00[d]	4.46 ± 0.03[a]
IR+Cr	139.70 ± 0.16[b]	94.77 ± 1.70[b]	54.77 ± 0.25[b]	1.27 ± 0.01[c]	3.51 ± 0.01[b]
***p*-value**	*	*	*	*	*

Cr: Crocin group, IR: Ischemia- reperfusion group and IR + Cr group: Ischemia-reperfusion+crocin treatment group.

[abcd] Values with different superscripts within one column differ significantly at $p < 0.05$.

* indicates significant differences at $p < 0.001$.

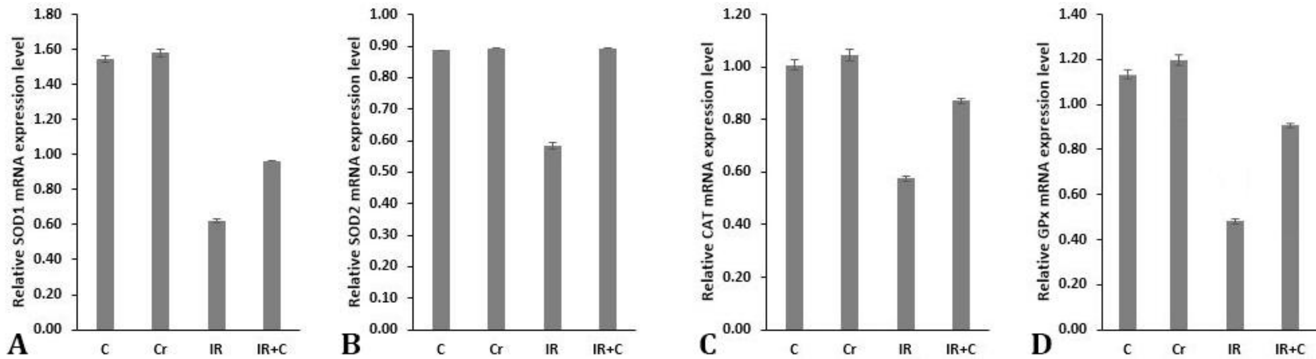

Fig. 3. Relative mRNA expression levels of antioxidant enzymes including **A)** Superoxide dismutase (SOD) -1, **B)** SOD-2, **C)** Catalase, and **D)** Glutathione peroxidase. C: Control group, Cr: Crocin group, IR: Ischemia- reperfusion group and IR + Cr group: Ischemia-reperfusion + crocin treatment group. Values are expressed as mean ± SEM.

Discussion

Crocin has been shown to protect rats from IR injury to the gastrocnemius muscle. Our data showed that treating rats with Cr could reduce cytokine levels and expression, as well as TOS level and alleviate gastrocnemius muscle IR injury by increasing TAS levels.

Blood circulation in the tissues is disrupted when a tourniquet is applied to the extremity and as a result of some changes in the tissue, cell functions are disrupted and a series of biochemical changes occur leading to cell death and damage. The use of a tourniquet for an extended period of time induces numerous metabolic reactions in the body. Despite the fact that skeletal muscle is more resistant to ischemia than other tissues, long-term tourniquet use in orthopedic surgery leads to acute compartment syndrome, reconstructive microsurgery and limb trauma with artery injury all of which result in skeletal muscle deformations and neuromuscular damage.[21]

The rise in proinflammatory cytokines such as TNF-α, IL-6, and IL-1 as well as the vast amount of reactive oxygen species have a part in the muscle damage produced by IR.[22,23] The TNF-α activates polymorphonuclear neutrophils and causes neutrophil infiltration and stimulates adhesion molecules and chemotactic factors in vascular endothelial cells allowing leukocytes to infiltrate and participate in inflammation.[24] The TNF-α, IL1, IL6 and antioxidant factors were studied in an IR model by Brüning *et al*.[25] Pro-inflammatory cytokines were found to be higher in the IR group.[25] According to Oktar *et al*. [26] the TOS value which is an indicator of all oxidant compounds was greatly elevated in IR groups, whereas, the TAS value which is an indicator of all antioxidant substances was dramatically lowered in IR groups.[26] Plasma levels and muscle gene expression were found to be in agreement with these findings.

Although the processes that produce IR damage are unknown, numerous studies have suggested that reactive oxygen species play a role in IR damage and that antioxidant enzymes guard against it.[27,28] Park *et al*. [29] observed that SOD1, SOD2, CAT, and GPx gene expressions were also lowered in the rat skeletal muscle IR model, however, Mansour *et al*. [30] found no significant change in SOD and GPx. The antioxidant enzyme activity levels of SOD and CAT were reported to be high in muscle tissue following reperfusion by Ozyurt *et al*. [31] Antioxidant enzymes such as SOD1, SOD2, CAT and GPx, whose main biological function is to protect the organism from oxidative damage, were studied for their expression levels and TAS levels. To assess the status of the antioxidant defense, we looked at the expression levels of the SOD1, SOD2, CAT and GPx antioxidant enzyme genes. IR considerably reduced the expression of SOD1, SOD2, CAT and GPx genes, whereas, Cr treatment significantly raised the expression of SOD1, SOD2, CAT and GPx genes. These findings imply that Cr may boost antioxidant enzyme levels which may help protect myocytes against IR harm. IR damage has been proven in numerous studies to reduce antioxidant enzyme levels and expression.[32] This increase in antioxidant enzyme expression has been linked to a rise in free oxygen radical generation in IR which stimulates the antioxidant defense system and causes the increase in antioxidant enzyme expression. Previous research backs up the conclusions of the current study which found that the IR group had lower antioxidant levels.[15,28,32] Except for our work on the effects of Cr on IR damage in the gastrocnemius muscle there has been no other publication.[15]

In conclusion, our study found that Cr could protect gastrocnemius tissue from IR injury in rats treated with the tourniquet method which was a common occurrence. Cr antioxidant properties and ability to reduce oxidative stress might have a good influence. Cr could be used safely to treat IR injuries. Except for our study on the effects of Cr on gastrocnemius muscle IR damage, no further articles have been located. More research is needed to understand the mechanisms behind Cr beneficial effect on gastrocnemius muscle IR damage.

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Conflict of interest

The authors report no conflicts of interest.

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