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Protective Effects of Crocin on Experimental Gastrocnemius Muscle Ischemia/Reperfusion Model in Rat

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Abstract: The aim of the present study was to define whether Crocin, has a regulatory effect againstexperimental gastrocnemius muscle ischemia/reperfusion injury model in rats.A total of 32 Sprague-Dawley male, 200-250g rats were randomly divided into four groups: Control, Crocin (60mg/kg/i.p), I/R and I/R+Crocin groups. All rats were anesthetized with xylazine and ketamine. Except for the control and Crocin group, the left lower limbs of the other 2 groups were applied to 2 hours of ischemia and 2 hours of reperfusion with tourniquet. SOD, MDA, GSH, CAT, GPx, MPO, NO, IMA, AOPP ve PON levels were measured in the plasma; the levels of SOD, MDA, GSH, CAT and GPx were determined in gastrocnemius muscle. Increases in SOD, GSH, CAT, GPx, AOPP and PON levels, decreases in the MDA, MPO, NO and IMA levels were significant in the Crocin groups according to the I/R group. These differences were statistically significant. The results of this study support the possibility that Crocin may play a protective role against skeletal muscle injury caused by I/R in rats by reducing oxidative stress. Crocin can be safely used for ischemia/reperfusion injury.

Keywords: Crocin, Ischemia, Rat, Oxidative stress, Reperfusion

Introduction

Ischemia is one of the most widespread damage that are mostly caused by decreased blood flow to an organ various reasons such as hypovolemia, transplantation, thrombosis, surgical procedures, energy production stopping and lack of oxygen and nutrients that occurs in vascular substrates of the tissues (Robbins et al., 2012). While reconstituting blood flow to tissues in an ischemia may contribute to their survival, it may also cause simultaneous damage during reperfusion. This is known as ischemia-reperfusion (I/R) injury.

Cellular damage is a special and widespread clinical event after reperfusion of beforehand viable ischemic tissues in lower extremity. The mechanism of ischemia-reperfusion is multifactorial and involves divergent biological mechanisms, such as ion accumulation, immune activation and the formation of toxic reactive oxygen species (ROS) defined as free radicals (Turer and Hill 2010; Kilic et al, 2017). ROS are the most important key molecules in the reperfusion injury and toxic substances produced in various clinical conditions (Hensley et al., 2000; Braunersreuther and Jaquet, 2012). Oxidative stress is known as a disturbance between the antioxidant and prooxidant balance resulting in cell injury by oxidation of lipids, proteins and DNA (Garcia-de-la-Asuncion et al., 2012). In tourniquet-related surgery or major vascular surgery procedures involving ischemia-reperfusion phases, For this reason, it is not possible to distinguish between oxidative stress caused by surgical procedures and oxidative stress caused by ischemia-reperfusion (Green et al., 1994). Hence, in order to investigate the effect of a possible intervention on the response elicited by ischemia- reperfusion solely, it would require a model where the influencing factors of surgery and anesthesia were eliminated.

Crocin can be isolated in pure form the saffron extract and directly crystallized (Khayatnouri et al., 2011). Saffron, the spice contain many chemical substances like carbohydrates, minerals, mucilage, vitamins (especially riboflavin and thiamin) and pigments including crocin, anthocyanin, carotene, lycopene, and zigzantin (Assimopoulou et al., 2005; Fernández, 2006). Crocin has also shown various pharmacological activities such as antioxidant, anticancer, radical scavenging and genoprotective (Aung etal., 2007;

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Hosseinzadeh et al., 2008; Hosseinzadeh et al., 2009). Crocin as anti-tumor functions has got a special place in pharmaceutics (Nair et al., 1995). According to crocin effects and by attention to so far any article on has not been reported protective effect of crocin against ischemia/reperfusion, therefore, the present study was conducted to analyze the protective effect of crocin on the damage induced by ischemia/reperfusion.

The aim of this study was to investigate the effect of crocin on gastrocnemius muscle I/R injury, which may happen often after the tourniquet method.

Materials and Methods

Chemicals

Crocin and all of other chemicals used were of analytical level and were bought from the Sigma Chemical Co. (St. Louis, MO, USA)

Animals

The experimental protocol were conducted according to the ethical norms approved by the Ethic Committee of Experimental Animal Teaching and Researcher Center (No: 29.11.2017, 36643897-000-E.1700331423-174). Rats were obtained from the Medical and Experimental Application and Research Center (ATADEM), Erzurum, Turkey. Thirty two Sprague–Dawley male rats weighing between 200–250 g were used in this experimental study. Throughout the animal experiments were processed following the internationally accepted ethical guidelines for the care of laboratory animals. Prior to the experiments, rats were accommodated in the standard laboratory conditions with a temperature-controlled room 21^0C (+/- 2) with natural light and dark cycle (12 / 12 hours light:dark) and humidity (55+/-5%) were fed and water adlibitium, in order to adapt to the laboratory conditions. Before from the study, animals were fasted overnight, but were allowed free access to water. All rats were anesthetized with xylazine (8 mg / kg) and ketamine (60 mg / kg). Except for the control and crocin 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 2 groups were applied to 2 hours of ischemia and 2 hours of later tourniquate was released and reperfusion was initiated. Reperfusion was verified by edema, the extremity return to normal temperature, receiving pulse and the extremity colour changed to pink. Six animals were used for each group of study. Rats were divided into the following groups:

Control Group (C): 0.5 mL 5% DMSO was administered by intraperitoneal one times applied 30 minutes before ischemia. Blood samples were obtained from the aorta abdominalis under anesthesia for biochemical analyzes. Tissue specimens were taken under anesthesia from the left extremity.

Crocin Group: Crocin 60mg/kg/i.p. (It was dissolved in 5% DMSO).

Ischemia/Reperfusion Group (I/R): Under the anesthesia, 2 hours tourniquet was applied to the lower extremity (low temperature and cyanotic claw marks the occurrence of ischemia). Subsequently, the tourniquets were opened and reperfusion was applied for 2 hours (the pinking of the claws and the increase in temperature indicate reperfusion) and afterwards, the abdomen was opened with midline incision, and blood samples for biochemical analysis were taken from Aorta abdominalis. Tissue samples were taken from the left extremity.

I/R+Crocin Group: Prior 30 minutes to ischemia, rats were given Crocin 60 mg/kg/i.p. (one times) (It was dissolved in 5% DMSO) by intraperitoneal. After 30 minutes, under anesthesia, 2 hours tourniquet was applied to the lower extremity. Subsequently, the tourniquets were opened and reperfusion was applied for 2 hours, and afterwards, blood and tissue samples were taken as they were in the sham group.

Biochemical Analyzes in Plasma and Gastrocnemius Muscle

Whole blood was collected into lithium heparinised tubes from *Aorta abdominalis*. Plasma was obtained from these whole blood samples by centrifugation (3000 rpm for 10 min) and used for the determination of the biochemical parameters. The gastrocnemius muscle tissues were homogenized in a Qiagen TissueLyserII using a buffer of 1.15% KCl to obtain 1:10 (w/v) 0.1 M phosphate buffer (pH 7.4) to obtain a 1:10 (w/v) homogenate.

Superoxide dismutase (SOD) (Sun ve ark, 1988), malondialdehyde (MDA) (Yoshioka ve ark, 1979), glutathione (GSH) (Tietze, 1969), catalase (CAT) (Goth, 1991), glutathione peroxidase (GPx) (Matkovics ve ark, 1988), myeloperoxidase (MPO) (MPO Instant ELISA kit, eBioscience, Vienna, Austria), nitric oxide (NO) (Green et al., 1982), ischemia modified albümin (IMA) (Bar-Or et al., 2001), advanced oxidation protein product (AOPP) (Immunodiagnostic AG kit, Bensheim, Germany and paraoxonase (PON) (Renault et al., 2006) in plasma; SOD (Sun ve ark, 1988), MDA (Placer ve ark,), GSH, CAT (Goth, 1991) and GPx (Matkovics ve ark, 1988) levels were analyzed in the gastrocnemius muscle. The protein concentration was also measured in the supernatant according to the method of Lowry et al. (1951). These levels were measured with Biotek ELISA Reader (Bio Tek µQuant MQX200 Elisa reader/USA).

Statistical Analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) followed using SPSS software package, version 20.00. 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 (SE) for each group.

Results and Discussion

The levels of SOD, MDA, GSH, CAT, GPx, MPO, NO, IMA, AOPP and PON in the plasma samples (Table 1) and SOD, MDA, GSH, CAT and GPx in the gastrocnemius muscle tissue (Table 2) were measured all groups in this study. Plasma and tissue SOD, GSH, CAT, GPx and plasma PON levels were significantly lower in the I/R group than the other groups but plasma MDA and tissue MDA levels, MPO, NO, IMA and AOPP levels were significantly higher in the I/R group than in the other groups p<0.001.

Table 1. The effects of Crocin on plasma biochemical parameters (ANOVA)

GROUPS	SOD (EU/mL)	MDA (mmol/L)	GSH (mmol/L)	CAT (kU/L)	GPx (U/mL)	MPO (ng/mL)	NO (µmol/L)	IMA (ΔABSU.un)	AOPP (µmol/L)	PON (IU/mg. protein)
C	16.67 ±0.12a	19.08 ±0.51b	2.77	158.94 ±8.94a	0.26 ±0.01ab	3.63 ±0.07b	13.71 ±0.68c	0.35±0.00b	0.003 ±0.00b	144.66 ±0.82
Crosin	16.77 ±0.14a	18.58 ±0.55b	2.77 ±0.06a	173.28 ±4.86a	0.28 ±0.01a	3.56 ±0.10b	12.54 ±0.69c	0.34 ±0.00b	0.003 ±0.00b	145.76 ±0.56
I/R	15.58 ±0.25b	26.80 ±1.08a	2.30 ±0.02c	91.63 ±2.46c	0.21 ±0.00c	7.57 ±0.06a	31.59 ±0.37a	0.68 ±0.04a	0.005 ±0.00a	67.04 ±0.60
I/R+Crosin	16.25 ±0.14a	20.95 ±0.86b	2.53 ±0.07b	128.31 ±3.24b	0.25 ±0.01b	3.78 ±0.02b	17.39 ±0.54b	0.37 ±0.00b	0.003± 0.00b	139.86 ±0.95
P	***	***	***	***	***	***	***	***	***	***

Data are expressed as means±SEM (n=6). Values in a row with different superscripts differ significantly (***p<0.001)

The levels of plasma and tissue SOD, GSH, CAT, GPx and plasma PON levels were significantly increased in the I/R+Crocin group according to I/R group (P<0.001). The levels of plasma MDA and tissue MDA levels, MPO, NO, IMA and AOPP levels were significantly decreased in the I/R+Crocin group according to I/R group (P<0.001).

Table 2. The effects of crocin on gastrocnemius muscle tissue biochemical parameters (ANOVA)

GROUPS	SOD (EU/mL)	MDA (mmol/L)	GSH (mmol/L)	CAT (kU/L)	GPx (U/mL)
C	26.73±0.29^{a}	18.42±0.23^{b}	2.13±0.04^{a}	225.68±1.49^{a}	0.015±0.01^{ab}
Crosin	26.54±0.48^{a}	18.16±0.35^{b}	2.14±0.03^{a}	231.34±5.16^{a}	0.016±0.00^{a}
I/R	17.81±0.66^{b}	66.93±0.96^{a}	1.87±0.02^{c}	150.82±9.89^{c}	0.013±0.00^{c}
I/R+Crosin	25.12±0.27^{a}	18.80±0.17^{b}	2.10±0.01^{b}	187.29±9.54^{b}	0.014±0.00^{b}
P	***	***	***	***	***

Data are expressed as means±SEM (n=6). Values in a row with different superscripts differ significantly (***p<0.001)

The results of this study confirm the findings of thestudy by Ertekin & Apaydin Yildirim, 2018, in which decreased antioxidant parameter in I/R group. The antioxidant properties of crocin have been shown to decrease gastrocnemius muscle tissue damage and oxidative stress in I/R injury.

Conclusion

This study showed that crocin can protect of gastrocnemius tissue I/R injury in rat treated which may happen often after the tourniquet method. The antioxidant effects and reducing oxidative stress of crocin can be this positive impact. Crocin can be safely used for ischemia/reperfusion injury. According to crocin effects any article on hasn't reported protective effect against gastrocnemius muscle I/R injury. Further studies are required on this subject.

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