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Effect of crocin on oxidative stress in recovery from single bout of swimming exercise in rats

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Abstract. Physical exercise could cause muscle and tissue damage due to increase in the formation of free oxygen radicals during exercise. The aim of the present study was to investigate the effect of crocin on parameters associated with oxidative stress in recovery from acute swimming exercise in rats. Rats were divided into eight groups; Normal Control (NC, untreated and did not swim), Crocin Control (CC, received crocin and did not swim), Exe-1, Exe-24, Exe-48 (untreated and swam) and and Exe-Cro-1, Exe-Cro-24, Exe-Cro-48 (received crocin and swam). AST, ALP, LDH, CK, XO enzymes levels increased after swimming in untreated and crocin-treated groups, but there was a less increase in crocin-treated groups. The highest MDA levels in serum were determined in Exe-1 compared with all other groups. There was significant difference between control and exercise groups in MDA level ($p = 0.033$). In contrast, there was significant difference between control and exercise groups in GSH level ($p < 0.001$). In addition, crocin given to swimming rats significantly increased GSH levels ($p < 0.05$) and decreased MDA levels when compared with untreated exercise groups. In conclusion, crocin is able to protect liver and skeletal muscle tissue against exercise-induced oxidative damage by preventing reactive oxygen species (ROS) production.

Key words: Swimming exercise — Oxidative stress — Crocin — MDA — GSH

Introduction

Physical exercise increases reactive oxygen species (ROS) and could cause oxidative stress in muscle and various tissues (Teixeira et al. 2009). The imbalance between ROS production and antioxidant defense may increase level of oxidative stress and lead to progressive cell damage (Urso and Clarkson 2003). Exhaustive exercise-induced muscle damage is related to oxidative stress that results in the formation of ROS, oxidation of glutathione, the release of cytosolic enzymes (Sastre et al. 1992), and lipid peroxidation (Vinña et al. 2000). In addition, researchers demonstrated that ROS levels in muscle and liver tissue might increase in exhaustive exercise (Praphatsorn et al. 2010).

ROS lead to both muscle damage and inflammation (Oteiza et al. 1996). It was reported that increased antioxidant capacity might protect against exercise-induced oxidative stress after acute swimming exercise in rats (Akil et al. 2011).

Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) are the most important liver enzymes. These enzymes are used in many diseases as biomarkers of liver and skeletal muscles damage (Nuri et al. 2012). Creatine kinase (CK) is present in heart muscle, skeletal muscle and brain. Myocardial creatine kinase (CK-MB), isoenzyme of CK, is dominantly present in heart tissue. Lactate dehydrogenase (LDH) is found in

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almost all of the body's cells. All these enzymes are released from cells into the fluid portion of blood (serum) when cells are damaged or destroyed by oxidative stress (Tauler et al. 2004). The levels of CK and LDH rise in response to exercise (Vinña et al. 2000b).

Antioxidants supplementation could reduce the negative effects of exercise resulting from oxidative damage. Saffron (*Crocus sativus L.*) is used mainly as a herbal medicine or food coloring and widely grown in Iran and other countries such as India, Turkey and Greece. The major biologically active compounds of the saffron are crocin, picrocrocin, crocetin and safranal (Srivastava 2010). The results of the previous studies demonstrated that saffron extract has antitumor, anticonvulsant, antidepressant, anti-inflammatory, anti-hyperlipidemic, free radical scavenging and antioxidant effects (Hosseinzadeh et al. 2004; Asdaq and Inamdar 2010). Goyal et al. (2010) reported that crocin produced a noteworthy reduction in the activities of CK-MB isoenzyme, LDH, superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) levels in rats with isoproterenol-induced cardiotoxicity.

The aim of the present study was to investigate the effect of crocin on parameters associated with oxidative stress in recovery from acute swimming exercise in rats.

Material and Methods

Animals and experimental protocol

Forty-eight 4 months female Wistar rats (initial weight; 200 ± 20 g) were kept in a room with a 12 h light/dark inverted cycle under standardized conditions of temperature and humidity. All animals were fed water and rat chow *ad libitum*. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Inonu University Animal Ethic Committee. At the onset of the study, rats were weighted and randomly divided into eight groups each containing 6 rats.

Group 1: Normal Control (NC: untreated and did not swim)

Group 2: Crocin Control (CC: received crocin and did not swim)

Group 3: Exercise-1 (Exe-1: untreated and swam)

Group 4: Exercise-24 (Exe-24: untreated and swam)

Group 5: Exercise-48 (Exe-48: untreated and swam)

Group 6: Exercise+Crocin-1 (Exe-Cro-1: received crocin and swam)

Group 7: Exercise+Crocin-24 (Exe-Cro-24: received crocin and swam)

Group 8: Exercise+Crocin-48 (Exe-Cro-48: received crocin and swam)

Normal saline was administered in NC and untreated exercise groups (Exe-1, Exe-24 and Exe-48) and crocin (Sigma Chemical Co., St. Louis, MO, USA) dissolved in normal saline was administered in CC and treated exercise groups (Exe-Cro-1, Exe-Cro-24 and Exe-Cro-48) at a dose of 20 mg/kg body weight/day (Zheng et al. 2007). All the administrations were performed at the same time and continued for 21 days with a volume of 5 ml/kg body weight/day by gavage.

The exercise was conducted in a glass pool, which was 50 cm in depth and width, resistant to heat and had a thermostat to keep water temperature fixed at 37°C. Exercises were carried out only once for 30 min 24 h after the end of procedures (Akil et al. 2011). The experimental animals were made to swim in groups of two and NC, CC, Exe-1 and Exe-Cro-1 were sacrificed after swimming exercise. Exe-24 and Exe-Cro-24 were sacrificed 24 h after and Exe-48 and Exe-Cro-48 were sacrificed 48 h after swimming exercise under xylazine and ketamine anesthesia. Trunk blood was collected and centrifuged at 3000 rpm for 15 min to measure biochemical assays.

Biochemical analyses

Serum MDA analysis

MDA is one of the end products of lipid peroxidation that reacts with thiobarbituric acid to give a specific color absorbing at 535 nm. MDA level was assayed by using the method of Ohkawa (Ohkawa et al. 1979). 0.5 ml serum was mixed with 3 ml 1% H_3PO_4 and 1 ml 0.6% thiobarbituric acid. The mixture was heated in a boiling water bath for 45 min and extracted in 4 ml n-butanol. n-butanol was used as a blank and tetramethoxypropane was used as a standard. MDA levels were determined in a spectrophotometer (T80 UV/VIS Spectrometer, PG Instruments Ltd.), and expressed as μmol/l.

Serum GSH determination

Serum GSH levels were determined by Ellman method (Ellman 1979). Serum was mixed with trichloroacetic acid solution and centrifuged at 3000 rpm at 4°C for 20 min. GSH reacts with 5,5-dithiobis-2-nitrobenzoic acid to give a yellow product which has a maximal absorbance at 410 nm. Distilled water was used as a blank and GSH levels were expressed as μmol/l.

Serum xanthine oxidase (XO) determination

XO activity was measured by using the method of Prajda and Weber (1975). The generation of uric acid from xanthine has a maximal absorbance at 292 nm. One unit of activity was identified as μmol of uric acid formed *per* min, and serum protein concentration was determined using biuret method. XO activity was expressed as U/g protein.

Serum ALT, AST, ALP, LDH, CK and CK-MB levels were performed on Abbott Architect c 1600 automatic analyzer using commercially available kits.

Statistical Analysis

Statistical analysis was carried out using the SPSS for Windows version 13.0 (SPSS Inc., Chicago, III., USA) statistical program. The data were expressed as mean value and standard deviation (mean ± SD). Under consideration of the three independent factors (group, treatment, time) the comparisons were made by three-way analysis of variance. After Anova, pairwise comparisons were made by Bonferroni adjusted multiple comparison method. $p < 0.05$ was considered as significant.

Results

The mean liver function enzyme levels for exercise and control groups are shown in Table 1. The mean values of AST and ALP enzymes activities increased after swimming in untreated exercise group (Exe-1) compared to NC when treatment and time effects did not account. AST enzyme activity decreased significantly from 0 h to 48 h ($p < 0.001$), whereas, there were not differences from 0 h to 24 h and from 24 h to 48 h ($p > 0.05$). ALT enzyme activity was not statistically significant between groups. ALP enzyme activity in crocin-treated group (Exe-Cro-1) was lower than untreated groups ($p = 0.005$).

Table 2 presents the mean muscle enzyme levels. The mean values of LDH, CK and CK-MB levels increased after swimming compared NC when treatment and group effects did not account ($p < 0.05$). LDH, CK, CK-MB enzymes activities decreased significantly from 0 h to 48 h ($p \leq 0.003$), but there were not differences from 0 h to 24 h and from 24 h to 48 h ($p > 0.05$). Since the treatment*time interaction was not found to be significant, these results are for both crocin and no-crocin data.

The mean level of serum oxidative stress parameters are presented in Table 3. There was difference between control and exercise groups in MDA level ($p = 0.033$). Also, the highest MDA levels in serum were determined in Exe-1 compared to all other groups. The level of MDA increased in crocin-treated groups after swimming, but there was a less increase in crocin-treated groups than untreated groups. In addition, the highest XO levels in serum were measured in Exe-1 ($p < 0.05$), but this increase was less in crocin treated exercise goups. There was difference between control and exercise groups in GSH level ($p < 0.001$). However, crocin given to swimming rats significantly increased GSH levels compared to untreated exercise groups ($p < 0.05$).

Discussion

The present study was aimed at investigating the effect of crocin on parameters associated with oxidative stress in recovery from acute swimming exercise in rats. According

Table 1. The mean liver function enzyme levels

Group	Treatment	Time (h)	AST (U/l)		ALT (U/l)		ALP (U/l)	
NC^{a}	Untreated^{x}	0^{a}	132.50 ± 24.50		75.83 ± 16.62		171.33 ± 43.61	
CC^{a}	Crocin^{y}	0^{a}	112.67 ± 11.43		54.83 ± 8.77		138.33 ± 33.78	
Exe-1^{b}		0^{a}	166.17 ± 45.013		65.00 ± 11.68		174.33 ± 67.62	
Exe-24^{b}	Untreated^{x}	24^{a,b}	113.67 ± 12.94		54.17 ± 8.98		186.50 ± 46.42	
Exe-48^{b}		48^{b}	105.50 ± 15.89		54.33 ± 9.91		198.83 ± 54.41	
Exe-Cro-1^{b}		0^{a}	151.83 ± 29.39		62.17 ± 13.35		110.33 ± 36.17	
Exe-Cro-24^{b}	Crocin^{y}	24^{a,b}	130.17 ± 18.06		70.67 ± 8.43		171.67 ± 14.84	
Exe-Cro-48^{b}		48^{b}	101.67 ± 18.08		58.00 ± 10.73		131.33 ± 30.23	
Source of Variation	Group		F = 13,560	p = 0.001	F = 0,142	p = 0.708	F = 0,494	p = 0.486
	Treatment		F = 0,453	p = 0.505	F = 0,460	p = 0.501	F = 8,868	p = 0.005
	Time		F = 16,300	p < 0.001	F = 1,478	p = 0.240	F = 2,176	p = 0.127
	Group*Treatment		F = 0,077	p = 0.782	F = 3,835	p = 0.057	F = 0,760	p = 0.389
	Treatment*Time		F = 1,256	p = 0.296	F = 2,249	p = 0.119	F = 1,372	p = 0.265

Data are expressed as mean ± SD ($n = 6$). Groups: NC, untreated and did not swim; CC, received crocin and did not swim; Exe-1, Exe-24, Exe-48, untreated and swam; Exe-Cro-1, Exe-Cro-24, Exe-Cro-48, received crocin and swam. Representing statistical significance among groups; "a" and "b" letters were used for AST; "x" and "y" letters were used for ALP. The groups with different superscripts represents the statistical significance. $p < 0.05$ was considered as significant.

to Table 1, the levels of AST and ALP enzymes increased after swimming in both untreated and crocin-treated exercise groups, but this increase was lesser in crocin-treated groups than untreated exercise groups. Elevated AST and ALT levels are considered as liver damage or increased pressure on the liver (Park et al. 2000). Praphatsorn et al. (2010) demonstrated the effect of acute exercise on biochemical changes in liver and pancreas of rats. The results showed that intense exercise with 75% and 90% of maximal oxygen consumption resulted in significant increase in ALT and AST levels. Although the increase in the level of AST is in line with the results of Gaeini et al. (2013) and Praphatsorn et al. (2010). Also, our results are consistent with Ramos et al. (2013) that a single bout of swimming exercise increased in the levels of plasma AST (93%), ALT (17%), and LDH (55%).

AST, ALT and ALP enzymes exist in many organs including liver, skeletal muscles, heart tissue. However, ALT concentrotion in liver is greater than any other tissue and increase of this enzyme in plasma shows liver damage (Huang et al. 2006). According to Table 2, CK, CK-MB and LDH increased in untreated exercise group significantly at 24 h after exercise and declined at 48 h. The impact of crocin supplementation on these enzymes showed a significant recovery to almost control values at 24 h after swimming in Exe-Cro-24 group. In this study, levels of CK, CK-MB, LDH, AST, ALP were decreased significantly by crocin 48 h after exercise. Exercise-induced muscular damage occurs to increase at 8 h and 24 h following exercise (Saxton et al. 1995). Previous studies reported that CK, LDH, AST and ALT enzymes elevated due to cellular necrosis and tissue damage in skeletal muscles after strenous exercise (Nie et al. 2011). LDH and CK enzymes generate both energy and lactate, and play important roles in inflammatory conditions in muscle cells. In addition, levels of plasma LDH and CK were significantly elevated after an acute muscle injury induced by eccentric exercise (Childs et al. 2001). Also, some researchers reported that increased CK and LDH levels during physical exercise resulted in muscle membrane damage (Nosaka and Clarkson 1994).

ROS were permanently produced during metabolic processes by cells. ROS play a key role as mediators of skeletal muscle damage and inflammation after unaccustomed or strenuous exercise (Dekkers et al. 1996). Exercise-induced muscle damage also leads to inflammatory responses, which further contribute to increased level of lipid peroxidation possibly due to infiltration of macrophages to the damaged tissue (Peternelj and Coombes 2011). The antioxidant defense system consisting of enzymes such as CAT, SOD, GSH-Px, and numerous non-enzymatic antioxidants, including vitamins A, E and C, GSH, ubiquinone, and flavonoids preserve homeostasis for normal cell functions. Nevertheless, ROS can be produced at increased levels under pathophysiological conditions. Thus, ROS attack the structural molecules of cell (e.g. lipid, protein, carbohydrate and DNA), and lead to oxidative damage (Bast et al. 1991). ROS can have cytotoxic effects on the membrane phospholipids and lead to release of toxic substances such as MDA (Slatter et al.

Table 2. The mean muscle enzyme levels

Group	Treatment	Time (h)	LDH (U/l)		CK (U/l)		CK-MB (U/l)	
NC	Untreated	$0^{a,f,x}$	428.50 ± 164.28		391.67 ± 170.60		375.50 ± 94.80	
CC	Crocin	$0^{a,f,x}$	333.33 ± 81.94		382.50 ± 94.37		329.33 ± 60.41	
Exe-1		$0^{a,f,x}$	505.33 ± 326.67		604.50 ± 324.11		475.00 ± 236.19	
Exe-24	Untreated	$24^{a,b,f,g,x,y}$	437.67 ± 206.47		396.67 ± 123.41		413.17 ± 130.47	
Exe-48		$48^{b,g,y}$	217.00 ± 45.98		293.33 ± 134.88		272.50 ± 22.89	
Exe-Cro-1		$0^{a,f,x}$	362.83 ± 125.68		421.50 ± 72.95		399.50 ± 37.99	
Exe-Cro-24	Crocin	$24^{a,b,f,g,x,y}$	272.33 ± 91.49		310.17 ± 77.01		308.83 ± 83.70	
Exe-Cro-48		$48^{b,g,y}$	187.33 ± 98.98		202.33 ± 48.24		270.83 ± 79.57	
Source of Variation	Group		F = 0,619	p = 0.436	F = 4,004	p = 0.052	F = 3,429	p = 0.071
	Treatment		F = 3,843	p = 0.057	F = 2,041	p = 0.161	F = 2,275	p = 0.139
	Time		F = 6,089	p = 0.005	F = 9,002	p = 0.001	F = 6,546	p = 0.003
	Group*Treatment		F = 0,123	p = 0.728	F = 1,908	p = 0.175	F = 0,102	p = 0.751
	Treatment*Time		F = 0,578	p = 0.566	F = 0,375	p = 0.690	F = 0,668	p = 0.581

Data are expressed as mean ± SD (n = 6). Representing statistical significance among groups; "a" and "b" letters were used for LDH; "f" and "g" letters were used for CK; "x" and "y" letters were used for CK-MB. The groups with different superscripts represents the statistical significance. $p < 0.05$ was considered as significant. For abbreviations see Table 1.

2000). MDA is frequently used as biomarker of oxidative stress owing to exercise.

The majority of studies have showed that antioxidant supplementation could reduce exercise-induced oxidative stress and ROS production, accelarate recovery of muscle function and improve performance. Herein, we used crocin, one of the major compenent of saffron, to reduce intracellular ROS formation. In the present study, we observed the highest MDA levels in untreated exercise group (Exe-1), but increased MDA levels were decreased significantly by crocin in Exe-Cro-1 group. Previous studies demonstrated that single bout of exercise raised blood levels of MDA (Koska et al. 2000). In addition, Marzatico et al. (1997) observed that level of MDA elevated in following long-distance and lactacidemic performances in highly trained aerobic and sprint athletes. Our findings are in line with previous studies. In the present study, the highest MDA levels were measured in Exe-1 group, and then decreased gradually at 24 h and 48 h after swimming (Exe-24 and Exe-48 group respectively) (Table 3). Our findings were also supported with elevated CK, LDH, AST, ALT and ALP levels.

GSH, an endogenous antioxidant, is generally found in the cytosole 1–11 mM (Smith et al. 1996). GSH is also found in extracellular fluid in low concentration. When the tissues are exposed to oxidative stress, GSH is turned into its oxidized form, glutathione disulfide (GSSG). Therefore, oxidative stress results in the formation of GSSG (Sies and Akerboom 1984). Especially, reduced GSH/GSSG ratio was reduced by oxidative stress. Numerous studies reported that GSH/GSSG ratio decreased in exercise (Sastre et al. 1992). Sastre et al. (1992) found a 72% increment in GSSG levels following an acute bout of high intensity treadmill running, and GSSG levels turned back into the normal values after 1 h following testing. Our results are also in line with Sastre et al. (1992). In the present study, the lowest concentration of GSH was determined in Exe-1 group, and increased progressively at 24 h and 48 h after swimming in Exe-24 and Exe-48 groups. The effect of crocin supplementation on MDA and GSH levels showed important changes. Although MDA levels were decreased significantly by crocin, GSH levels were increased to almost control values in Exe-Cro-48 (Table 3).

XO, a potent radical forming enzyme, could be formed from xanthine dehydrogenase (XD) either reversibly or irreversibly under pathologic conditions (Hile and Nishino 1995). Hellsten et al. (1988) reported that XO is important in the formation of ROS during exercise. Some researchers reported that the inhibition of XO with allopurinol prevents damage releated to exhaustive exercise in animals (Gomez-Cabrera et al. 2005) and humans (Gomez-Cabrera et al. 2003). Allopurinol was used in these studies to inhibit XO, since it might act an antioxidant (Moorhouse et al. 1987). These studies reported that plasma XO activity increased after exercise, but allopurinol completely prevented this activity as an antioxidant. In the present study, the higest XO activity was determined in Exe-1 group, and then decreased gradually at 24 h and 48 h after swimming (Exe-24 and Exe-48 respectively) (Table 3). However, the impact of crocin supplementation on XO activity showed significant changes.

Table 3. The mean level of serum oxidative stress parameters

Group	Treatment	Time (h)	MDA (μmol/l)		GSH (μmol/l)		XO (U/g protein)	
NC^{a,f}	Untreated^{x}	0	749.1 ± 17.86		814.00 ± 235.24		4.07 ± 2.09	
CC^{a,f}	Crocin^{y}	0	726.67 ± 14.92		890.00 ± 600.91		2.78 ± 1.69	
Exe-1^{b,g}		0	867.17 ± 203.08		341.00 ± 104.00		4.54 ± 0.31	
Exe-24^{b,g}	Untreated^{x}	24	773.50 ± 52.70		510.67 ± 156.63		4.25 ± 1.33	
Exe-48^{b,g}		48	763.00 ± 33.08		517.67 ± 173.56		4.16 ± 0.41	
Exe-Cro-1^{b,g}		0	748.83 ± 30.48		516.83 ± 141.76		3.66 ± 1.27	
Exe-Cro-24^{b,g}	Crocin^{y}	24	741.50 ± 40.45		620.83 ± 114.57		3.24 ± 2.65	
Exe-Cro-48^{b,g}		48	723.67 ± 14.07		725.33 ± 283.64		2.54 ± 1.58	
Source of Variation	Group		F = 4,869	p = 0.033	F =14,426	p < 0.001	F = 1,046	p=0.313
	Treatment		F = 2,526	p = 0.120	F = 2,365	p = 0.132	F = 6,504	p=0.015
	Time		F = 2,291	p = 0.114	F = 1,583	p = 0.218	F = 0,662	p=0.521
	Group*Treatment		F = 2,276	p = 0.139	F = 0,201	p = 0.656	F = 0,095	p = 0.760
	Treatment*Time		F = 1,136	p = 0.331	F = 0,100	p = 0.905	F = 0,179	p = 0.837

Data are expressed as mean ± SD ($n = 6$). Representing statistical significance among groups; "a" and "b" letters were used for MDA; "f" and "g" letters were used for GSH; "x" and "y" letters were used for XO. The groups with different superscripts represents the statistical significance. $p < 0.05$ was considered as significant. For abbreviations see Table 1.

Especially, we observed that XO activity is nearly to control values in Exe-Cro-48.

Crocin, one of the major compenets of saffron, is a carotenoid and has the structer of crocetin di-gentiobiose ester (Ochiai et al. 2004). Crocin has a variety of pharmacological effects including improvement of learning behaviour (Abe and Saito 2000), anti-hyperlipidemic effect (Lee et al. 2005), anti-atherosclerotic effect (He et al. 2005) and anti-oxidant effect in PC-12 cells owing to increasing GSH synthesis (Ochiai et al. 2004). Carotenoids have a scavenger effect for oxygen radicals and other reactive species (Stahl and Sies 2003). The studies were proposed that crocin was one of the antioxidants to reduce intracellular ROS formation (Alavizadeh and Hosseinzadeh 2014). Thus, crocin can protect tissues against oxidative damage.

Meamarbashi and Rajabi (2014) demonstrated that 10-day supplementation with saffron significantly decreased the CK and LDH concentrations after knee eccentric exercise in male students. Hosseinzadeh et al. (2009) reported that saffron extract and its active constituents, crocin and safranal, contributed to the prevention of ischemia-reperfusion in rat skeletal muscle.

Briefly, acute swimming exercise caused oxidative stress and increased MDA levels and CK, CK-MB, LDH, AST and XO activities. In contrast, GSH levels were decreased significantly due to swimming exercise. But, crocin supplementation led to decrease in MDA levels and CK, CK-MB, LDH, AST and XO activities. Moreover, GSH levels were elevated by crocin.

In conclusion, crocin is able to protect liver and skeletal muscle tissue against exercise induced-oxidative damage by preventing ROS production.

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