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ANTI-FATIGUE EFFICACY OF CROCIN IN MICE VIA REGULATION OF NRF-2/HO-1 PATHWAY-MEDIATED OXIDATIVE STRESS

EFICÁCIA ANTIFADIGA DA CROCINA EM CAMUNDONGOS POR MEIO DE REGULAÇÃO DE ESTRESSE OXIDATIVO MEDIADA PELA VIA NRF-2/HO-1

EFICACIA ANTIFATIGA DE LA CROCINA EN RATONES A TRAVÉS DE LA REGULACIÓN DEL ESTRÉS OXIDATIVO MEDIADA POR LA VÍA NRF-2/HO-1

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ABSTRACT

Introduction: Fatigue is a manifestation of sub-health status, which causes serious negative effects in daily life. The antioxidant properties of crocin have been widely investigated in many diseases. However, no correlation between crocin and fatigue was reported. Objective: To verify the anti-fatigue efficacy of crocin in swimming-induced exhaustive time. Materials and Methods: In this study, mice were treated with crocin for 28 days to assess its anti-fatigue efficacy. Exhaustive time, fatigue-relevant biochemical indices, inflammatory cytokines, energy metabolism indicators, oxidation parameters, and the NRF-2/HO-1 pathway were analyzed to explore evidence of crocin in high-intensity exercise. Results: Our research indicated that crocin dramatically extended swimming-induced exhaustive time. In strenuous swimming, crocin clearly eliminated BLA and SUN to maintain internal environment homeostasis, while it markedly improved glycogen concentrations in the muscles and liver to promote energy reserves. Moreover, crocin visibly improved the inflammatory reaction, as represented by reductions in TNF-a and IL-6, promoting endurance capacity. In the muscles, crocin noticeably enhanced SDH and Na+K+-ATP activities to improve energy metabolism in strenuous swimming. Lastly, crocin markedly improved SOD and CAT activities via the NRF-2/HO-1 pathway to defend against oxidative stress-induced fatigue. Conclusions: Crocin provides oxidation resistance and can be developed into anti-fatigue nutriments. ***Evidence level II; Comparative prospective study.***

Keywords: Exercise; Fatigue; Crocin; NRF-2; HO-1.

RESUMO

Introdução: A fadiga é uma manifestação de um estado de saúde inferior, que causa efeitos negativos graves na vida diária. As propriedades antioxidantes da crocina foram amplamente investigadas em muitas doenças. No entanto, nenhuma correlação entre crocina e fadiga foi relatada. Objetivo: Verificar a eficácia antifadiga da crocina com relação ao tempo de exaustão induzido pela natação. Materiais e métodos: Neste estudo, os camundongos foram tratados com crocina por 28 dias para avaliar sua eficácia antifadiga. Tempo de exaustão, índices bioquímicos relevantes para a fadiga, citocinas inflamatórias, indicadores do metabolismo energético, parâmetros de oxidação e a via NRF-2/HO-1 foram analisados para explorar evidências de crocina em exercícios de alta intensidade. Resultados: Nossa pesquisa indicou que a crocina estendeu notavelmente o tempo induzido pela natação até a exaustão. Em natação extenuante, a crocina eliminou claramente o BLA e o SUN para manter a homeostase do ambiente interno, ao mesmo tempo em que melhorou significativamente as concentrações de glicogênio nos músculos e no fígado para promover as reservas de energia. Além disso, a crocina melhorou visivelmente a reação inflamatória, como mostram as reduções de TNF-a e IL-6, promovendo a resistência. Nos músculos, a crocina aumentou visivelmente as atividades de SDH e Na + K ± ATP para melhorar o metabolismo energético em natação extenuante. Por último, a crocina melhorou muito as atividades de SOD e CAT por meio da via NRF-2/HO-1 quanto à defesa contra a fadiga induzida pelo estresse oxidativo. Conclusões: A crocina proporciona resistência à oxidação e pode ser usada em nutrientes antifadiga. ***Nível de evidência II; Estudo prospectivo comparativo.***

Descritores: *Exercício; Fadiga; Crocina; NRF-2; HO-1.*

RESUMEN

Introducción: La fatiga es una manifestación del mal estado de salud, que provoca graves efectos negativos en la vida cotidiana. Las propiedades antioxidantes de la crocina han sido ampliamente investigadas en muchas enfermedades. Sin embargo, no se ha reportado ninguna correlación entre la crocina y la fatiga. Objetivo: Verificar la eficacia antifatiga de la crocina con respecto al tiempo de agotamiento inducido por la natación. Materiales y métodos: En este estudio, se aplicó el tratamiento de crocina a ratones durante 28 días para evaluar su eficacia antifatiga. Se analizaron el tiempo de agotamiento, los índices bioquímicos relevantes para la fatiga, las citocinas inflamatorias, los indicadores

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del metabolismo energético, los parámetros de oxidación y la vía NRF-2/HO-1 para explorar evidencias de la crocina en ejercicios de alta intensidad. Resultados: Nuestra investigación indicó que la crocina prolongó notablemente el tiempo de agotamiento inducido por la natación. En la natación extenuante, la crocina eliminó claramente el BLA y el SUN para mantener la homeostasis del ambiente interno, al tiempo que aumentó significativamente las concentraciones de glucógeno en el músculo y el hígado para favorecer las reservas de energía. Además, la crocina mejoró visiblemente la reacción inflamatoria, como lo demuestran las reducciones de TNF-a e IL-6, promoviendo la resistencia. En los músculos, la crocina aumentó visiblemente las actividades de SDH y Na + K ± ATP para mejorar el metabolismo energético en la natación extenuante. Por último, aumentó en gran medida las actividades de SOD y CAT a través de la vía NRF-2/HO-1 en la defensa contra la fatiga inducida por el estrés oxidativo. Conclusiones: La crocina proporciona resistencia a la oxidación y puede utilizarse en nutrientes antifatiga. ***Nivel de evidencia II; Estudio prospectivo comparativo.***

Descriptores: *Ejercicio; Fatiga; crocina; NRF-2; HO-1.*

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INTRODUCTION

Fatigue is one of sub-health status. Fatigue, attracted by intense pressures physically and mentally, is divided into acute fatigue and chronic fatigue. Fatigue can be primitively regarded as inefficiency in a sense. However, long-term fatigue can bringabout chronic faciguesynroe, which is associated with a battery of acute secondary healthissue, such as neuropsychiatric disease and immune system dysfunction.[1, 2] Exercise-induced fatigue, which has been widely and formerly focused on athlete and military, has caused material adverse effect in our daily life and becomeapopularresearch, because excessive exercise with invalid modulation leads to fatigue.[3] Nowadays, an increasing number of people are being subjectedto chronic fatigue with fast-paced lifestyles.[4] Hence, to seek reasonable substances to promote exercise performance is the potent requirement of anti-fatigue.

Exercise intolerance, which is defined as disability in maintaining voluntary activity, is a form of fatigue development and progress in physiology. And oxidativestress is crucial mechanism in physiological fatigue.[5] In excessive exercise, superfluous free radicals are generated to cause oxidative injury in livingorganism.[6, 7] In previousresearches, natural agents with antioxidant, have been demonstrated to possess potential function in improvement of physiological durability.[8] Therefore, elimination of exercise-induced fatigue can be attributed to anti-oxidant activity.

Crocin, as an important active component of saffron, has been used in the treatment of a variety of disorders, such as muscle dysfunction and cardiovascular disease.[9] Increasing evidences have indicated that crocin possesses a wide range of pharmacological effects, such as anti-oxidant and anti-inflammatory.[10,11] In osteoarthritis rat, crocin possesses potential therapeutic activities for muscle dysfunction by reducing LPO and NRF-2, and increasing GSH and GSH-px.[12] Furthermore, crocin has been also widely used in the treatment of hemorrhagic shock in the experimental animal model and exerts the protection against muscle damages via partly reducing TNF-α and IL-6 in rat.[13] Those findings suggest that crocin is closelyrelated with muscle function. The aim of this study was to investigate the effect of crocin treatment on exercise-induced fatigue and to clarify the potential anti-fatigue mechanisms by discussing its effect on the antioxidant properties in muscle.

MATERIALS AND METHODS

Eighty male ICR mice (8-week old) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Changsha, China). Mice were housed in light-dark (12: 12 h) cycle with humidity (50 ± 10 %) and temperature (23 ± 2 °C). After one-week of acclimation, mice were subsequently assigned into 4 groups: (1) Control group (Con), (2) 1 mg/kg crocin (Crocin-L), (3) 20 mg/kg crocin (Crocin-M) and (4) 40 mg/kg crocin (Crocin-H). Crocin was administered by gavage for 4-week successively. The control group was treated with water equivalently. The study and all the participants were reviewed and approved by Ethics Committee of Hunan Normal University (NO. 2018162).

Forced swimming test

Time-exhaustive was tested via weight-loaded forced swimming. Thirty min after last administration, mice with a lead (5% of body weight) bathed severally in cistern (25-26°C, 30 cm deep). Exhaustive time were checked when mice could not maintain in surface for over 10 s.

Biochemical assessment

After 4-week administration, mice were forced via a ninety-min swimming without lead. After one-hour rest, mice were individually anesthetized through administration with chloral hydrate. Blood was collected by segregateing eyeball. Blood was centrifuged (1500g, 4 °C, 10 min) to trap serum. The fatigue-related factors of BLA and SUN were assessed by spectrophotometer in strenuous swimming.

Inflammatory variables determination

The variables of TNF-a and IL-6 were measured by ELISA. In this study, antibody was labeled with biotin, and incubated with avidin-peroxidase. Then, the complex chemically reacted with TMB substrate at absorbance value of 405 nm.

Glycogen, oxidant stress, energy metabolism detection

After death, tissue was immediately cut, weighed, and saved at −80 °C. In follow-up experiment, liver and muscle were ground at low-temperatureenvironment. Then, the mixture was centrifuged (4000g, 4 °C, 15 min) to measure glycogen contents. In muscle, SOD and CAT activities were tested to evaluate anti-oxidant capacity. SDH and Na+-K+-ATPase concentrations were detected to assess energy supply ratio.

mRNA gene expression

RNA extraction was derived by TRIzol in muscle. Furthermore, RNA was transformed into cDNA to calculate mRNA expression. Gene expressions were measured by real-time PCR. Primers were showed in Table 1. Gene expressions were demonstrated as relative CT via standardized β-actin

Table 1. Primers were used in this study.

Gene	Accession number		Primer sequences
NRF-2	NM_010902	Forward primer	5´-TCCGCTGCCATCAGTCAGTC-3´
		Reverse primer	5´-ATTGTGCCTTCAGCGTGCTTC-3´
HO-1	NM_010442	Forward primer	5´-TGCAGGTGATGCTGACAGAGG-3´
		Reverse primer	5´-GGGATGAGCTAGTGCTGATCTGG-3´
β-actin	NM_007393	Forward primer	5´- GATTACTGCTCTGGCTCCTAGC -3´
		Reverse primer	5´- GACTCATCGTACTCCTGCTTGC -3´

Statistical Analyses

All results were expressed as mean ± SD. The significance was demonstrated by a one-way ANOVA. $p < 0.05$ were deemed significant.

RESULTS

Body weight and organ index calculation

The performance of crocin on mice weights and main organ index were revealed in Table 2. No significant difference presented among control, crocin-L, crocin-M and crocin-H groups in both initial and terminal body weights ($p > 0.05$). Moreover, Treatment with crocin had no remarkable disadvantage on relative organs index, including heart, liver, kidney, and lung ($p > 0.05$). These results indicated crocin at diverse dosages was safe and reliable, which had no toxicity on mice during experimental study.

Exhaustive swimming time measurement

The performance of crocin on weight-loaded swimming capacity was revealed in Figure 1. The time-exhaustive of control, crocin-L and crocin-H groups were 8.90 min, 10.50 min, 14.26 min, and 19.85 min, respectively. Exhaustive times in crocin-M and crocin-H groups were markedly prolonged by 60%, 123%, respectively ($p < 0.01$). These results indicated crocin possessed anti-fatigue properties in strenuous exercise.

Biochemical parameters analysis

The performance of crocin on biochemical parameters, which were also relatedto fatigue, was revealed in Figure 2. The concentrations of BLA and SUN were observably alleviated in crocin-M and crocin-H groups compared to control group ($p < 0.01$). These results indicated crocin eliminated harmful metabolites to facilitate physical function.

Inflammatoryresponses detection

The performance of crocin on inflammatory cytokines, which were involved in immune dysregulation to evoke fatigue, was revealed in Figure 3. The concentrations of TNF-a and IL-6 were markedly mitigated in crocin-M and crocin-H groups compared to control group ($p < 0.01$, $p < 0.05$). These results indicated crocin ameliorated inflammatoryreaction to promote endurance capacities in strenuous exercise.

Table 2. Performance of crocin on weight changes.

	CON	Crocin-L	Crocin-M	Crocin-H
Initial BW(g)	19.92±1.30	19.89±1.19	19.81±1.42	19.83±1.33
Final BW(g)	31.56±1.90	31.91±2.22	30.89±2.62	31.31±2.34
Relative liver weight(%)	4.93±0.35	5.04±0.46	4.87±0.54	4.94±0.62
Relative kidney weight(%)	1.37±0.20	1.34±0.18	1.30±0.16	1.33±0.17
Relative heart weight(%)	0.59±0.05	0.58±0.05	0.57±0.06	0.56±0.04
Relative lung weight(%)	0.62±0.03	0.60±0.05	0.62±0.03	0.60±0.05

The representation of organ index is 100 g/G (n = 10 mice/group). Crocin-L, Crocin low-dose group (1 mg/kg). Crocin-M, crocin middle-dose group (20 mg/kg). Crocin-H, crocin high-dose group (40 mg/kg).

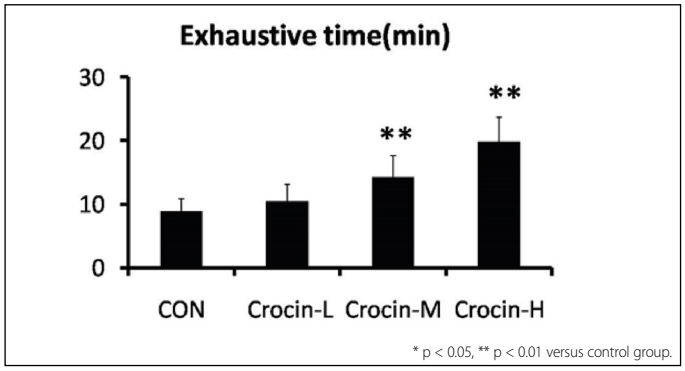

Figure 1. Performances of crocin on exhaustive times (n = 10 mice/group). Exhaustive swimming times were markedly prolonged in crocin groups compared to control group.

Glycogen contents exploration

The performance of crocin on glycogen contents, which were directly related to energy supplement in vital movement, was revealed in Figure 4. The glycogen contents in muscle and liver were notably ameliorated in crocin-M and crocin-H groups compared to control group ($p < 0.01$). These results indicated crocin improved energy reserve to promote postpone fatigue in strenuous exercise.

Energymetabolism enzymes activities test

The performance of crocin on energymetabolism, which wasassociatedwith ATP synthesis in vital activity, was revealed in Figure 5. The activities of SDH and Na+K+-ATP in muscle were observably improved in crocin-M and crocin-H groups compared to control group ($p < 0.01$, $p < 0.05$). These results indicated crocin regulated energy supplier to attenuate exercise-induced fatigue.

Anti-oxidation enzymes activities test

The performance of crocin on anti-oxidation functions, which were classic theory of anti-fatigue in strenuous exercise, was revealed in Figure 6. The anti-oxidation enzymes activities, including SOD and CAT, in muscle were markedly aggrandized in crocin-M and crocin-H groups compared to control group ($p < 0.01$). These results indicated crocin modulated antioxidant defense system to promote athleticability.

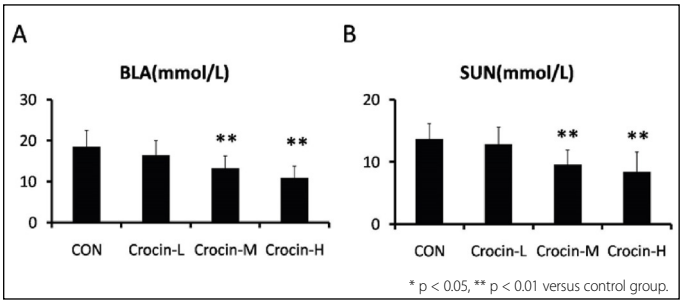

Figure 2. Performances of crocin on fatigue-related parameters (n = 10 mice/group). crocin observably alleviated (A) BLA and (B) SUN concentrations in high--intensity swimming.

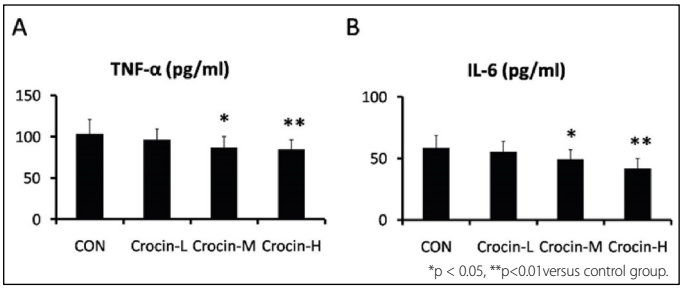

Figure 3. Performances of crocin on inflammatory cytokines (n = 10 mice/group).crocin observably alleviated (A) TNF-a and (B) IL-6 concentrations in high-intensityswimming.

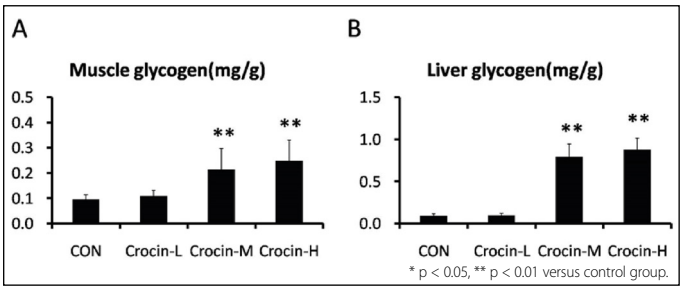

Figure 4. Performances of crocin on glycogen contents (n = 10 mice/group). crocin observably ameliorated glycogen reserve in (A) muscle (B) liver in high--intensity swimming.

Gene expressions experiment

The performance of crocin on anti-oxidant regulatory genes, which were involved in modulating anti-oxidation enzymes activities, was revealed in Figure 7. The expressions of NRF-2 and HO-1 in muscle were visibly upregulated in crocin-M and crocin-H groups compared to control group ($p < 0.01$). These results indicated crocin mediated NRF-2 / HO-1 pathway to promote anti-fatigue in strenuous exercise.

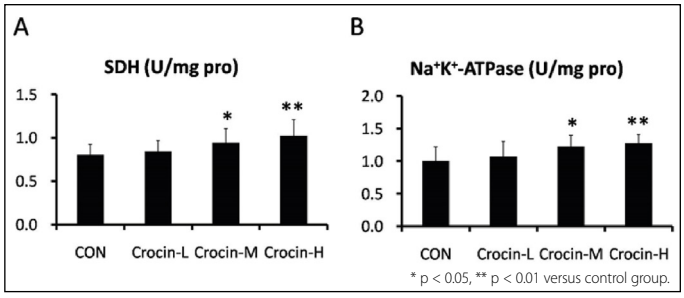

Figure 5. Performances of crocin on energy metabolism enzymes (n = 10 mice/group). crocin observably regulated (A) SDH and (B) Na+K+-ATP in muscle in high--intensity swimming.

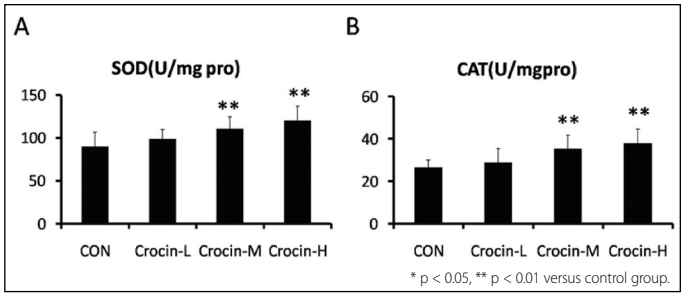

Figure 6. Performances of crocin on anti-oxidation enzymes activities (n = 10 mice/group). crocin markedly modulated (A) SOD and (B) CAT in muscle in high--intensity swimming.

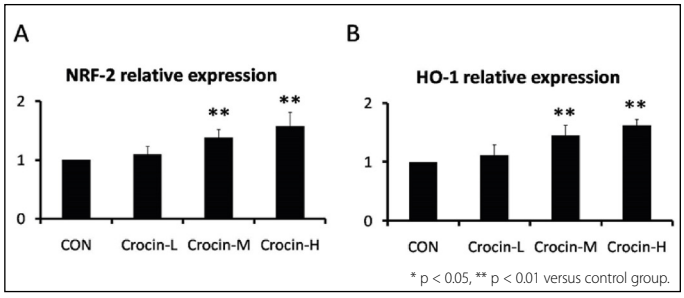

Figure 6. Performances of crocin on mRNA expression of anti-oxidant regulatory genes (n = 3 mice/group). (A) NRF-2 and (B) HO-1 gene expressions were visibly upregulated in crocin groups compared to control group.

DISCUSSION

In this study, the model of swimming to fatigue was established to demonstrate the anti-fatigue properties of crocin in mice. Previous research has proved excessive exercise gaveriseto fatigue physically and mentally, and continuance of exercise endurance was deemed as anti-fatigue indicators.[14] Treatment with crocin significantly prolonged loaded swimming time. The endurance capacities were enhanced by 60%, and 123%, respectively, in middle- and high-dosages of crocin. Moreover, crocin administration madenodifference in relative organs weight throughout whole experiment. These data indicated crocin was an efficient and healthy anti-fatigue nutriment.

There are many fatigue-related biologicalfactors, such as BLA and SUN, which were extensively used to appraise endurance capacities in strenuousexercise. LA, as byproduct of anaerobic metabolism, is generated in fatigue development and progress, when energy production can't keep up with higher-intensity exercise requirement in aerobic metabolism.[15] In addition, protein was treated assubstanceofenergy-source in high-intensityexercise. Then amassof SUN was produced in amino acid catabolism.[16] Hence, improvement of LA and SUN is regardas an effectiveway to retard fatigue and strengthen endurance capacities in high-intensityexercise. Previous report showed crocin significantly declined SUN in trunk blood to protect against renal damage in diabetic.[17] In this study, crocin observably alleviated BLA and SUN concentrations to facilitate physical performance in high-intensityswimming, which suggested crocin accelerated elimination of tiredness via moderating metabolite production in energy supplement process.

Inflammatory factors are also considered as indicators of fatigue, because organ damage often takes place during high-intensity exercise. Hence, excessive exercise can evoke immune dysregulation, which is strongly linked to fatigue induction and incapacity of body function. Previous report showed quercetin-3-O-gentiobiose signallyrelieved exhaustion exercise-induced inflammatory cytokines, such as TNF-a and IL-6, to delay fatigue.[18] What is more, crocin was proved to ameliorate cerulean-induced pancreatitis by restricting TNF-a and IL-6 expressions.[19] In this study, crocin markedly mitigated TNF-a and IL-6 levels to promote endurance capacities in high-intensity swimming, which suggested crocin allayedtiredness probably by hindrance of inflammatory cytokines accumulation.

Glycogen belongs to polysaccharides, which serves as energy storage to maintain glucose level and supplement ATP.[20] There is a negative relative between glycogen and fatigue. In other words, the stronger endurance capacity of the body, the more prominently glycogen content increases. Previous report showed unique polysaccharide markedly alleviated exercise-induced fatigue, which is partly dueto increased glycogen level in muscle and liver.[21] In this study, crocin notably ameliorated glycogen level in muscle and liver to prolong exercise tolerance, which suggested crocin mitigated physical fatigue by increasing energy reserve.

ATP is source of energy in life movement. In high-intensity exercise, many adverse metabolites, such as BLA and SUN, broke the balance of internal environment homeostasis to affect enzyme activity, which is disadvantage for ATP synthesis. Previous report showed Okra extract markedly enhanced SDH, ATP and ATPase contents to extend swimming time.[22] In addition, crocin notably recovered ATP synthesis to attenuate MPP.(+)-induced mitochondrial injury.[23] In this study, crocin observably improved SDH and Na+K+-ATP activities in muscle, which suggested crocin mitigated exercise-induced fatigue by upregulating energy supplier.

Strenuousexercise leads to superfluous ROS to evoke oxidative stress and muscle damage.[24] Hence, to scavenge ROS is a powerful way to athleticability, and antioxidant defense system is acknowledged to antagonism of oxidativestress. SOD and CAT are primary parts of antioxidant system. Previous report showed Ginsenoside Rg3 significantly alleviated high-intensity exercise-induced oxidativestress in aged rats, as evidenced by preservation of SOD and CAT activates.[25] In addition, crocin had antioxidant ability in improvement of sperm quality in paraquat-induced mouse.[26] In this study, crocin markedly aggrandized SOD and CAT activities in muscle, which suggested crocin extended exercise performance by modulating antioxidant enzymes.

To deeply clarify antioxidant effect of crocin in strenuous exercise, oxidant stress-relevant pathway genes, such as NRF-2 and HO-1, were examined in muscle. HO-1 belongs to detoxification enzyme and is involved in modulating inflammatory responses.[27] In addition, HO-1 expression is regulated by NRF-2, which is a redox-sensitive gene of antioxidant response element.[28] Hence, NRF-2/ HO-1 pathway is

deemed to be a vital target for improvement of oxidative stress-induced fatigue. Previous report showed anwulignan observably prolonged motor ability by regulating NRF-2 and HO-1expession.[29] In addition, crocin ameliorated cigarette smoke-induced oxidative damage by enhancement of NRF-2 expression.[30] In this study, crocin markedly amplified NRF-2 and HO-1 expressions in muscle, which suggested crocin relieved fatigue and fatigue-relevant muscle dysfunction by regulating antioxidant signaling.

CONCLUSIONS

In this study, crocin was proved to ameliorate excessive swimming-induced fatigue. The performance of crocin against fatigue was associated with its retardation of oxidative injury via NRF-2/HO-1 pathway. Hence, our researches demonstrate crocin can be developed into a safeandreliable nutriment to putoff fatigue.

Abbreviations

BLA: blood lactic acid; CAT: catalase; peroxidase; HO-1: heme oxygenase; IL-6: interleukin 6; NRF-2: Nuclear factor (erythroid-derived 2)-like 2; SDH: saccharopine dehydrogenase; SOD: superoxide dismutase; SUN: serum urea nitrogen; TNF-a: tumor necrosis factor.

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AUTHORS' CONTRIBUTIONS: LM: writing, statistical analysis, intellectual concept and preparation of the entire research project; LX: writing, revision and performing the researches; LS: researches, analysis of the data, and writing; PH: statistical analysis, revision, and intellectual concept.

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