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Original Research Article

Protective effect of crocin on chronic heart failure and its mechanism of action

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Abstract

Purpose: *To explore the mechanism of action of crocin in rat chronic heart failure (CHF).*
Methods: One hundred male Sprague Dawley (SD) rats were used to establish CHF rat model by the abdominal aorta constriction method. They were equally randomized into either the model control group (injected with distilled water), crocin low, medium, and high dose groups (daily administered 0.05, 0.1, and 0.75 g/kg of crocin, respectively), or positive control group (daily administration of benazepril hydrochloride), and normal control (without treatment). Parameters evaluated include heart function, inflammatory index changes, and oxidative stress damage.
Results: *The crocin low, medium, and high dose groups and the positive control group had significantly better cardiac function indices versus the model control group ($p < 0.05$). High-dose crocin resulted in significantly lower levels of inflammatory factors than a low or medium dose ($p < 0.05$). Rats that received a medium or high dose of crocin showed significantly increased activity of myocardial antioxidant enzymes, and reduced malondialdehyde (MDA) and reactive oxygen species (ROS) content when compared to those given low doses of crocin ($p < 0.05$). Protein expressions of Bax-activated caspase-3, and NF-kB decreased significantly with increase in crocin dosage. A high dose of crocin produced a significantly lower apoptotic rate of cardiomyocytes, sodium–calcium exchanger (NCX) level and higher content of sarcoplasmic reticulum calcium pump 2a (SERCA2a) compared with low- and medium-doses.*
Conclusion: *Crocin protects myocardial tissue and enhances ventricular diastolic function of CHF rats through down-regulation of NCX expression and up-regulation of SERCA2a expression. Further studies using clinical CHF models to categorize and analyze crocin-related cellular pathways will be required.*

Keywords: *Crocin, Chronic heart failure, Protective effect, Mechanism of action*

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INTRODUCTION

Chronic heart failure (CHF) is considered a critical public health issue worldwide owing to its high mortality and prevalence [1]. To date, the global figure of heart failure patients has exceeded 20 million, and a 25 % increase in its prevalence by 2030 has been estimated [2]. After cardiac arrest, airway ventilation is required in cardiopulmonary resuscitation (CPR) following the restoration of spontaneous circulation (ROSC) [3]. The current treatment for CHF

centers on symptom alleviation and prognosis improvement. However, the overall treatment efficacy remains unsatisfactory, posing a tremendous mental and financial burden to patients [4-6]. β-Blockers, angiotensin receptor blockers, and diuretics are commonly used drugs for the management of CHF with established therapeutic effects. Nonetheless, patients exhibit poor treatment compliance, and recurrence cases have been frequently reported after drug discontinuation [7-9]. There exists an urgent need for the exploration of new CHF treatments. Crocin is the main component of the traditional Chinese medicine (TCM) (saffron) with the effect of "activating blood and removing blood stasis, dispelling stagnation and dispersing nodules", which could enhance intracellular oxygen-free radical metabolism, inhibit inflammation, and improve blood circulation [10-11]. Moreover, pharmacological research has found [12] that crocin improved circulation and inhibited cell apoptosis. Accordingly, the current study was conducted to explore the mechanism of crocin on CHF rats.

EXPERIMENTAL

Animals

One hundred male Sprague-Dawley (SD) rats weighing 200 – 230 g and aged, 8 weeks were used. They were strictly fed as per the animal feeding guidelines under natural light, at a room temperature of 24 – 27 ℃. During the experiment, the rats were given adaptive feeding for 1 week, followed by the preparation of the CHF rat model by the abdominal aorta constriction method [13], which reduced the diameter of the abdominal aorta of the rat by 40 – 50 %. They were randomized into five groups containing 20 rats per group. The groups were classified into; the model control group, low-dose crocin group, medium-dose crocin group, high-dose crocin group, or positive drugs control group, and another 20 rats of the same age were used as normal controls. This experiment was reviewed by The First Affiliated Hospital of Hebei North University Bioethics Committee (approval no. 2018-205-21), and conducted as per the protocol of the Association for Assessment and Accreditation of Laboratory Animal Care, International [14].

Drugs

Crocin (Xi'an Prius Bioengineering Co, Ltd, purity ≥ 99.9 %); Benazepril hydrochloride (Shanghai Xinya Pharmaceutical Minhang Co. Ltd; National Medicine Standard: H20044840; Specification: 10 mg)

Treatments

The low-dose, medium-dose, and high-dose groups received 0.05, 0.075, and 0.1 g/kg of crocin, respectively. The positive control group received Benazepril hydrochloride (0.9 mg/kg), the model control group received distilled water of the same volume, and the normal control group was left untreated. The rats were intra-gastrically administered the treatments daily for four consecutive weeks.

Evaluation of parameters/indices

Hematoxylin and eosin (H&E) staining

This was used for myocardial tissue cytopathological examination, transmission electron microscopy (Guangzhou Jinjian Laboratory Technology Co. Ltd) was used to observe the ultrastructure of myocardial cells and mitochondrial organelles, and the left ventricular mass index (LVMI), collagen volume fraction (CVF), left ventricular posterior wall diameter in diastole (LVPWD), left ventricular posterior wall diameter in systole (LVPWs), left ventricular internal diameter at end-diastole (LVIDd), left ventricular end-systolic diameter (LVIDs), left ventricular fraction shortening (LVFS), left ventricular ejection fraction (LVEF) were recorded.

Enzyme levels and inflammatory factors

The serum angiotensin II (Ang-II), B-type brain natriuretic peptide (BNP), cardiac troponin 1 (cTn1), and serum levels of myocardial enzymes, namely, creatine phosphokinase (CPK), lactic dehydrogenase (LDH), aspartate aminotransferase (AST), were determined. C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) levels, myocardial tissue antioxidant enzymes (superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase (CAT) activity, malondialdehyde (MDA), reactive oxygen free radical (ROS) content, myocardial tissue sodium-potassium pump (Na^+-K^+-ATPase), Ca^{2+}-ATPase activity and ATP content, free Ca^{2+} concentration, and expression of bcl-2, Bax, activated caspase-3, and NF-kB protein were determined, and bcl-2/Bax was calculated.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The method was used to determine the myocardial cell apoptosis rate of the rats, which were anesthetized using 3 % sodium pentobarbital through intraperitoneal injection,

followed by the collection of left ventricular myocardial tissue. After washing, the tissue was fixed with 10 % formaldehyde, dehydrated for 24 h, and prepared into paraffin sections with a thickness of 3 - 4 μm, and the apoptotic nuclei were labeled with TUNEL. The number of apoptotic cells was observed in a 200-fold field of view to calculate the apoptosis rate (A) shown in Eq 1.

$$A (\%) = (Ac/Tc)100 \quad \text{..... (1)}$$

Where Ac = the number of apoptotic cells and Tc = the number of total cells

Western blot

The Western blot was adopted to determine the level of Na/Ca exchanger (NCX) and sarcoplasmic reticulum calcium pump 2a (SERCA2a) in the myocardial tissue of each group of rats. Rats' vascular smooth muscle cell lines (RVSMCs) (5×10^5 per well) were inoculated in 10 cm dishes, and total protein was extracted from each group of cells using protein lysate on ice and quantified. The proteins were transferred to the PVDF membrane after SDS-PAGE electrophoresis and sealed with 5 % skimmed milk for 2 h. The primary antibody (1:2000) was added and incubated overnight at 4 ℃, followed by the addition of the secondary antibody (1:5000) for 2 h at room temperature. Photographs were taken after chromatography.

Statistical analysis

Statistical Package for the Social sciences (SPSS) 23.0 was employed for data analyses, and GraphPad Prism 7 (GraphPad Software, San Diego, USA) was used for graph plotting. Measurement data not conforming to normal distribution were transformed for normality. Count data were represented by {n (%)} and analyzed by the chi-square test. Measurement data were represented by mean ± standard deviation (SD) and analyzed by the variance-analysis and post hoc testing. Statistical significance was indicated by $p < 0.05$.

RESULTS

Cardiac function of the rats

The crocin low, medium, and high dose groups and the positive control group were associated with significantly better cardiac function indices when compared to the model control group ($p < 0.05$). The cardiac function indices among different doses of crocin were comparable (Table 1).

Inflammatory factor levels

The serum concentration of CRP, TNF-α, and lL-6 were highest in the model control group and lowest in the normal control group (all $p < 0.05$). A high dose of crocin resulted in significantly lower inflammatory factor levels than a low or medium-dose ($p < 0.05$), while those of the high-dose group were comparable with the positive control group ($p > 0.05$) (Table 2).

Antioxidant enzyme activity and MAD and ROS contents

The plasma concentrations of MDA and ROS were highest in the model controls and lowest in the normal controls ($p < 0.05$). Rats with medium or high doses of crocin showed increased activity of myocardial antioxidant enzymes and reduced MDA and ROS content versus those given a low dose of crocin ($p < 0.05$), while those of positive controls or rats with a high dose of crocin were similar ($p > 0.05$).

The model controls exhibited the lowest serum concentrations of SOD, GSHPx, and CAT, which were the highest in the normal controls ($p < 0.05$). Rats with a high dose of crocin showed concentrations of SOD, GSHPx, and CAT than the model controls and those with a low- and medium-dose of crocin ($p < 0.05$), and were similar to the positive controls ($p > 0.05$; Table 3).

Na^+-K^+-ATPase, and Ca^{2+}-ATPase activities, ATP content, and free Ca^{2+} concentrations

The plasma concentrations of Na^+-K^+-ATPase, ATP, and free Ca^{2+} were highest in the model controls and lowest in the normal controls ($p < 0.05$). Rats with a high-dose crocin showed lower plasma concentrations of Na^+-K^+-ATPase, ATP, and free Ca^{2+} than the model controls and those with a low- and medium-dose of crocin ($p < 0.05$), while they were similar to the positive control group ($p > 0.05$).

The expression of Ca^{2+}-ATPase activity was lowest in the model controls and highest in the normal controls ($p < 0.05$). A high dosage of crocin resulted in higher Ca^{2+}-ATPase activity than the model control treatment and a low- and medium-dose of crocin ($p < 0.05$), while the results were similar to the positive control group ($p > 0.05$; Table 4).

Table 1: Comparison of cardiac function indices of rats in each group (mean ± SD; n = 20)

Group	LVMI (mg/g)	CVF (%)	LVPWD (mm)	LVAWs (mm)	LVPWd (mm)	LVIDs (mm)	LVFS (%)	LVEF (%)
Model control	3.46±0.32[b]	22.15±0.53[b]	2.96±0.23[b]	2.32±0.34[b]	0.37±0.23	7.28±0.25[b]	19.82±4.26[b]	40.15±5.27[b]
Crocin low-dose	3.02±0.23[a]	19.20±2.16[a]	2.73±0.16 [a]	3.42±0.46 [a]	0.64 ±0.18 [a]	5.05±0.21[a]	36.38±4.36[a]	53.18±6.28[a]
Crocin medium dose	2.86±0.21[a]	15.26±2.34[a]	2.35±0.13[a]	3.91±0.36[a]	0.82±0.23[a]	4.85±0.23[a]	42.36±5.28[a]	57.62±5.48[a]
Crocin high-dose	2.42±0.15[a]	12.35±2.07[a]	2.03±0.07 [a]	4.17±0.42 [a]	0.96±0.24 [a]	4.05±0.17[a]	47.28±5.71[a]	62.36±5.86[a]
Positive control	2.03±0.13[a]	9.25±2.15[a]	1.89±0.12[a]	4.51±0.53[a]	1.36±0.21[a]	3.25±0.24[a]	53.27±4.72[a]	68.92±6.27 [a]
Normal control	1.74±0.08[a]	5.26±0.67 [a]	1.73±0.06 [a]	4.73±0.67 [a]	1.62±0.17[a]	2.17±0.15[a]	58.36±4.35[a]	84.37±5.25[a]

Note: Compared with the model control group, [a]$P < 0.05$; compared with the normal control group, [b]$P < 0.05$

Table 2: Comparison of serum inflammatory factor levels in rats (mean ± SD; n = 20)

Group	CRP (ng/mL)	TNF-α (pg/mL)	IL-6 (pg/mL)
Model control	175.27±22.17[b]	42.17±4.72[b]	263.16±21.63[b]
Crocin low-dose	160.25±21.26[ac]	25.24±2.68[ac]	175.28±22.05[ac]
Crocin medium dose	154.27±18.29[acd]	21.61±2.34[acd]	162.17±21.26[acd]
Crocin high-dose group	148.29±17.25[ade]	17.27±1.25[ade]	154.28±17.28[ade]
Positive control	147.67±18.92[a]	17.18±1.67[a]	152.35±15.27[a]
Normal control	136.28±16.28	10.24±0.51	135.27±17.28

Note: a indicates $p < 0.05$ when compared with the model group. b indicates $p < 0.05$ when compared with the normal control group. c indicates $p < 0.05$ when compared with the positive control group. d indicates $p < 0.05$ when compared with the crocin low-dose group. e indicates $p < 0.05$ when compared with the crocin medium-dose group

Table 3: Comparison of antioxidant enzyme activity and MAD and ROS content in the myocardium of rats in each group (mean ± SD; n = 20)

Group	SOD (U/mL)	GSHPx (U/mL)	CAT (U/mg)	MDA (nmol/mg)	ROS (U/mL)
Model control	46.28±5.18[b]	409.23±42.37[b]	2.63±0.36[b]	28.95±2.14[b]	4.31±0.72[b]
Crocin low-dose	66.28±4.27[ac]	606.28±24.18[ac]	5.05±0.45[ac]	24.36±2.16[ac]	3.49±0.63[ac]
Crocin medium dose	71.16±4.31[acd]	623.17±28.73[acd]	5.34±0.56[acd]	20.04±2.11[acd]	3.06±0.51[acd]
Crocin high-dose	82.62±4.27[ade]	650.17±32.08[ade]	5.83±0.64[ade]	16.27±2.03[ade]	2.46±0.48[ade]
Positive control	83.27±4.86[a]	652.26±35.48[a]	6.07±0.69[a]	15.26±1.78[a]	2.37±0.42[a]
Normal control	88.62±5.37	694.26±38.18	7.13±0.84	10.92±1.85	1.87±0.46

Note: a indicates $p < 0.05$ when compared with the model group. b indicates $p < 0.05$ when compared with the normal control group. c indicates $p < 0.05$ when compared with the positive control group. d indicates $p < 0.05$ when compared with the crocin low-dose group. e indicates $p < 0.05$ when compared with the crocin medium-dose group

Table 4: Comparison of Na^+-K^+-ATPase, Ca^{2+}-ATPase activity, ATP content and free Ca^{2+} concentration in myocardial tissue of rats in each group (mean ± SD; n = 20)

Group	Na^+-K^+-ATPase (pg/mL)	Ca^{2+}-ATPase activity (mmoL/g)	ATP (pg/mL)	Free Ca^{2+}(nmol/L)
Model control	1823.23±82.16[b]	1.21±0.54[b]	32.18±3.26[b]	28.81±3.47[b]
Crocin low-dose	1346.72±71.25[ac]	1.78±0.65[ac]	29.17±3.46[ac]	24.17±3.28[ac]
Crocin medium dose	1258.29±91.67[acd]	2.32±0.70[acd]	24.37±3.27[acd]	20.36±3.46[acd]
Crocin high-dose	1164.27±93.47[ade]	3.38±0.69[ade]	16.62±3.65[ade]	14.32±3.82[ade]
Positive control	1163.26±92.35[a]	3.42±0.73[a]	16.47±3.41[a]	14.25±3.26[a]
Normal control	1035.23±87.26	3.93±0.68	12.36±3.27	11.26±3.27

Note: a indicates $p < 0.05$ when compared with the model group. b indicates $p < 0.05$ when compared with the normal control group. c indicates $p < 0.05$ when compared with the positive control group. d indicates $p < 0.05$ when compared with the crocin low-dose group. e indicates $p < 0.05$ when compared with the crocin medium-dose group

Bcl-2, Bax, activated caspase-3, NF-kB and bcl-2/Bax ratio

The model controls exhibited the highest expression levels of Bax, activated caspase-3, and NF-kB protein, which had and lowest expression in the normal controls ($p < 0.05$). Their expressions were lower in rats with high-dose crocin than the model controls and rats with low-dose and medium-dose treatment ($p < 0.05$),

Table 5: Comparison of the expression levels of bcl-2, Bax, activated caspase-3, and NF-kB protein in each group of rats (mean ± SD; n = 20)

Group	bcl-2	Bax	activated caspase-3	NF-kB protein
Model control	0.96±0.25[b]	0.97±0.18[b]	2.35±0.26[b]	2.24±0.23[b]
Crocin low-dose	1.25±0.37[ac]	0.45±0.15[ac]	1.51±0.24[ac]	1.83±0.25[ac]
Crocin medium dose	1.36±0.44[acd]	0.36±0.11[acd]	1.42±0.21[acd]	1.53±0.23[acd]
Crocin high-dose	1.45±0.38[ade]	0.28±0.07[ade]	1.34±0.24[ade]	1.42±0.23[ade]
Positive control	1.48±0.32[a]	0.22±0.05[a]	1.28±0.16[a]	1.23±0.17[a]
Normal control	1.76±0.36	0.13±0.04	1.02±0.25	1.12±0.14

Note: [a]$P < 0.05$ when compared with the model group; [b]$p < 0.05$ when compared with the normal control group; [c]$p < 0.05$ when compared with the positive control group; [d]$p < 0.05$ when compared with the crocin low-dose group; [e]$p < 0.05$ when compared with crocin medium-dose group

but were similar to the positive controls ($p > 0.05$). The lowest expression of bcl-2 was observed in the model controls and highest in the normal controls ($p < 0.05$). A high-dose crocin produced higher expression levels of Bax, activated caspase-3, and NF-kB protein than the model controls, a low- and medium-dose crocin ($p < 0.05$), but the results were similar to the positive controls ($p > 0.05$) (Table 5).

Apoptosis of rat cardiomyocytes

The normal controls showed a lower apoptosis rate of cardiomyocytes than the model controls ($p < 0.001$). The apoptosis rate of cardiomyocytes in the positive controls and the high-dose crocin-treated rats was significantly lower than that of rats with a low and medium dose of crocin ($p < 0.001$; Figure 1).

NCX level and SERCA2a protein content

The model control group showed higher NCX levels and lower SERCA2a levels than the other groups ($p < 0.001$). A high dose of crocin resulted in higher NCX levels and lower SERCA2a levels versus a low or medium dose of crocin ($p < 0.001$) (Figure 2).

DISCUSSION

Chronic heart failure is caused by heart disease and is a major cause of death in patients with cardiovascular illnesses [15]. Clinical treatment mainly involves drugs such as diuretics, vasodilators, or positive inotropic agents. Notwithstanding their cardiac function benefits, the long-term prognosis is unfavorable [16]. Traditional Chinese medicine features benefits such as multiple pathways and targets in the treatment of CHF and provides significant improvements in cardiac function and quality of life of patients [17]. Crocin is a monomer compound extracted from the TCM herb saffron. It has been demonstrated that in hemorrhagic shock rats, saffron significantly reduced serum levels of TNF-α and interleukin-6 and elevated concentrations of interleukin-10. Prior research has reported the promising efficacy of the drug in alleviating cardiac and cerebral ischemia and hypoxia. However, few reports focused on the protective effect of crocin on CHF. This study used the abdominal aortic stenosis method to establish a rat model for the first time and intervened with different doses of crocin, aiming to explore the protective effect of crocin on CHF and its mechanism of action by observing and analyzing the efficacy of crocin on heart function, oxidative stress damage and energy metabolism of model rats. Clinical research [18] has revealed compromised calcium ion homeostasis in the cardiomyocytes and reduced intracellular calcium

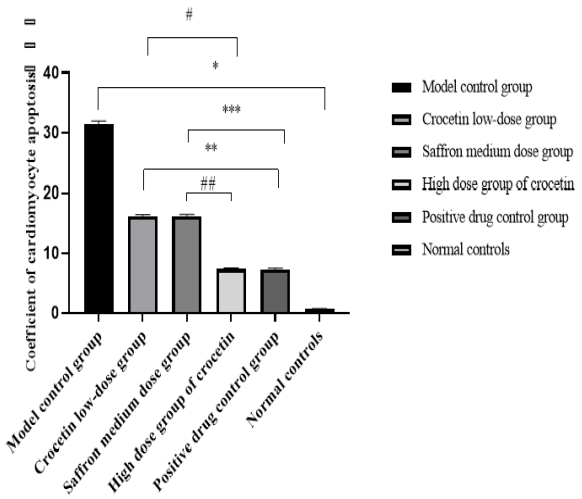

Figure 1: Comparison of the apoptotic rate of rat cardiomyocytes in each group (mean ± SD; n = 20). *$P < 0.001$ in the apoptosis rate of cardiomyocytes between the model control group and the normal control group (t = 288.8); **$p < 0.001$ in the apoptotic rate of cardiomyocytes between the low-dose crocin group and the positive control group (t = 102.881); ***$p < 0.001$ in the apoptotic rate of cardiomyocytes between the medium-dose crocin group and the positive control group (t = 120.677); [#]indicates $p < 0.001$ in the apoptotic rate of cardiomyocytes between the low-dose crocin group and the high-dose group (t = 112.811); [##]$p < 0.001$ in the apoptotic rate of cardiomyocytes in the medium-dose crocin group and the high-dose group (t = 137.834)

ion concentration during CHF progression, which is attributed to the alterations in the SERCA2a protein and NCX on the surface of the myocardial cell membrane. It has been reported that the enhanced Na^+/Ca^{2+} exchange activity of NCX could counteract the declined function of SECRA to upregulate Ca^{2+} and enhance myocardial function [19]. Therefore, the

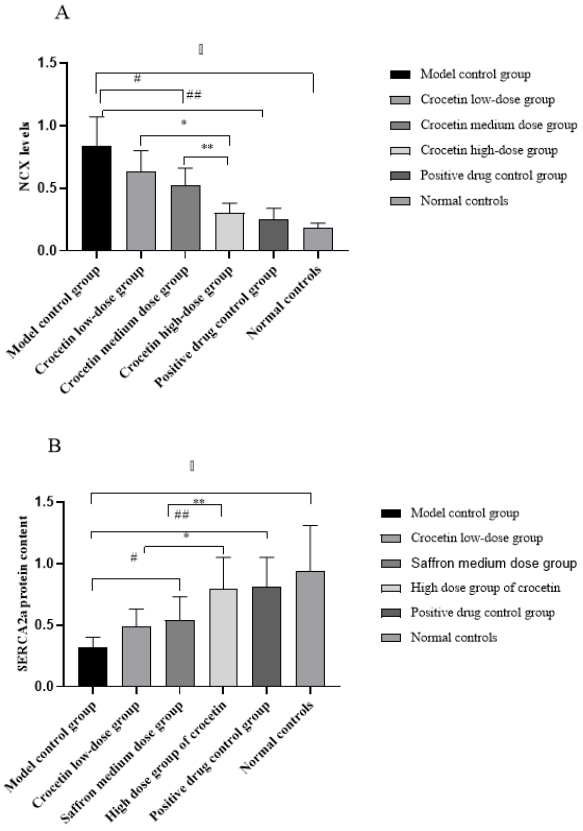

Figure 2: Comparison of (A) NCX and (B) SERCA2a protein content in myocardial tissue of rats in each group (mean ± SD; n = 20); $^{\triangle}p < 0.001$ in NCX content between the model control group and the normal control rats (t = 12.643 (A), 7.325 (B)); $^{\#}p < 0.001$ in NCX content between the model control group and the crocin medium-dose group (t = 5.315 (A), 4.772 (B)); $^{\#\#}p < 0.001$ in NCX content between the model control group and the positive group (t = 7.255 (A), 8.662 (B)); $^{*}p < 0.001$ in NCX content between the low-dose crocin group and the high-dose crocin group (t = 5.713 (A), 4.543 (B)); $^{**}p < 0.001$ in NCX content between the medium-dose crocin group and the high-dose crocin group (t = 3.606 (A), 3.472 (B))

inconsistency of NCX and SECRA with the reduction of the intracellular calcium ion concentration may result in an imbalance of calcium ions in the myocardial cells and myocardial function impairment. With the in-depth study of CHF, inhibition of oxidative stress damage is of great significance to improve the prognosis. Crocin is the main active component of saffron crocus extract that inhibits inflammation, oxidative stress, and anti-apoptosis, with established efficacy in hemorrhagic shock. Herein, the apoptosis of myocardial cells was determined by TUNEL staining, and the highest apoptotic rate of myocardial cells was found in the model controls. No significant difference was identified between the high-dose group and the positive controls, but a significant difference was observed as compared to the rats with a low- and medium-dose of crocin, indicating that high-dose crocin effectively ameliorated myocardial cell apoptosis of CHF model rats and enhanced their cardiac function versus benazepril hydrochloride. Furthermore, studies have reported that crocin yields a significantly stronger capability to scavenge hydroxyl radicals and superoxide radicals than vitamin E and β-hydroxytheophylline. Injection of crocin into myocardial infarction model mice can reduce myocardial infarction size and improve cardiac function. Therefore, it is speculated that crocin could promote vascular expansion or regeneration by boosting the activity of HIF-1α and VEGF protein and improve the tolerance of myocardial cells to hypoxia. It also increases the body's ability to transport oxygen and strengthens the adaptive survival of hypoxia, thereby mitigating hypoxia-induced myocardial injury. Moreover, the NCX concentration of rats with high-dose crocin was lower than those with low- and medium-dose crocin, while the protein content of SERCA2a was higher than those with low- and medium-dose crocin, confirming abnormalities in the balance of calcium ions in cardiomyocytes of CHF rats.

Limitations of this study

This was an animal study, and abdominal aortic coarctation was used for modeling. The model status was significantly different from clinical heart failure. In addition, this study only focused on common indicators of heart failure and did not categorize and analyze crocin-related cellular pathways.

CONCLUSION

High-dose crocin promotes the concentration of calcium ions in cardiomyocytes to stabilize by regulating NCX and SERCA2a channel protein, thereby enhancing myocardial contractility and contraction, reducing myocardial cell apoptosis, and improving prognosis. Further investigations using clinical CHF models to categorize and analyze crocin-related cellular pathways will be required to validate the findings of this study.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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