

Original Research Article

Phycocyanin relieves myocardial ischemia-reperfusion injury in rats by inhibiting oxidative stress

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Abstract

Purpose: To investigate the effect of phycocyanin on myocardial ischemia-reperfusion injury, and the possible mechanisms involved.

Methods: Twenty-four Sprague-Dawley (SD) rats were randomly divided into Sham group (only threading without ligation), IRI group (myocardial ischemia-reperfusion injury group) and phycocyanin group (phycocyanin pretreatment + myocardial ischemia-reperfusion injury group). The heart was harvested and cardiomyocytes were isolated. Colorimetry was used to determine the contents of cardiomyocyte serum creatine phospho-MB (CK-MB), lactate dehydrogenase (LDH) and malondialdehyde (MDA), and the activities of total antioxidant capacity (T-AOC), catalase (CAT), glutathione (GSH), total superoxide dismutase (SOD) and other related oxidative stress indicators. Furthermore, apoptosis was evaluated using TUNEL staining. Protein levels of cardiac factor E2 related factor 2 (Nrf2), heme oxygenase-1 (HO-1), human NADPH dehydrogenase 1 (NQO1) and nuclear factor- κ B (NF- κ B) were evaluated by Western blot and immunohistochemistry.

Results: Compared with the myocardial IRI group, the contents of CK-MB, LDH, MDA and ROS in the treated group were significantly decreased ($p < 0.05$), but the activities of SOD, GSH, SOD, CAT, and T-AOC in the myocardial tissues were significantly enhanced ($p < 0.05$). Moreover, the pathological changes in myocardial tissue were significantly reduced. In addition, the expression levels of Nrf2, HO-1 and NQO-1 were significantly up-regulated after phycocyanin pretreatment, while expression of NF- κ B was significantly down-regulated ($p < 0.05$).

Conclusion: Phycocyanin improves myocardial anti-oxidative stress via activation of Nrf2 signaling pathway, and also protects rats from myocardial ischemia-reperfusion injury by reducing inflammatory response via inhibition of NF- κ B signaling pathway.

Keywords: Phycocyanin, Nrf2 signaling pathway, NF- κ B signaling pathway, Ischemia-reperfusion injury, Myocardial injury

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INTRODUCTION

In recent years, with the promotion and application of shock therapy, thrombolytic therapy, cardiac surgery and organ

transplantation, ischemia-reperfusion injury has been of immense concern to scholars [1]. Coronary heart disease is one of the major diseases that cause human death. World Health Organization (WHO) reported that in 2011, the

number of people who died of coronary heart disease accounted for 12.8 % of all deaths [2,3]. Thrombolytic therapy, percutaneous transluminal coronary angioplasty, and coronary artery bypass grafting can rapidly restore the blood perfusion and oxygen supply to ischemic myocardium [3,4]. Reperfusion is a "double-edged sword". It restores blood and oxygen supply to the heart muscle, but it also worsens the injured ultrastructure, metabolic and functional damage, and even causes irreversible myocardial ischemia-reperfusion injury (MIRI) [5]. The MIRI attenuates the benefits of ischemia-reperfusion and it is one of the key issues to be solved in the treatment of coronary heart disease. Therefore, the search for a suitable method of prevention and treatment is what scientists need to solve.

Ischemia-reperfusion injury is associated with disorders in energy metabolism, oxygen free radical injury, calcium overload and cell apoptosis. Myocardial ischemia and reperfusion can induce increase of oxygen free radical and oxidative stress [6,7]. The reactive oxygen species (ROS) produced in oxidative stress plays a key role in the development of MIRI [8]. Researchers generally believe that Nrf2 plays an important role in the protection of MIRI. At present, most of the known antioxidant enzymes and the related enzymes on the biosynthesis route of non-enzymatic small molecule antioxidants are regulated by Nrf2, so Nrf2 is also known as the most critical regulator of the organism's redox balance state [9]. Under physiological conditions, the production and clearance of oxygen free radicals in the body are kept in a dynamic balance [10]. Preliminary experiments (data not shown) on the rat MIRI model showed that MIRI caused a significant increase in ROS in the heart tissue of mice and was positively correlated to serum creatine phosphate phospho-MB (CK-MB), lactate dehydrogenase (LDH), and other functional and pathological indicators. The CK-MB and LDH are the two of the most commonly used markers of myocardial injury. At the same time, it was found that the expressions of Nrf2 and downstream anti-oxidation proteins was significantly up-regulated in mouse proximal tubular cells during MIRI [9]. In view of the pathological features of ischemia-reperfusion and the current molecular mechanisms of ischemia-reperfusion injury, drug intervention to reduce MIRI has become one of the starting points for many studies.

The influence of phycocyanin on the occurrence and development of organ damage caused by toxins and ischemia has attracted the attention of scholars in various fields at home and abroad

[11-13]. They found that phycocyanin has a protective effect on ischemic reperfusion injured liver by reducing the oxidative stress level and increasing the substance against oxidative stress. Therefore, in this study, *in vitro* experiments were used to investigate the effect of phycocyanin on IRI-induced myocardial injury and the underlying mechanism, which provided important preclinical evidence and laid a solid foundation for clinical applications in the use of phycocyanin to treat and prevent the progression of toxic myocardial injury.

EXPERIMENTAL

Chemicals and reagents

The following instruments and reagents were used in the present study: SAR-830/P small animal ventilator (IITC, USA); Biopac MPI50 monitoring system (Biopac Systems, Goleta, CA, USA); Digital camera DSC-T10 (Sony Corporation, Tokyo, Japan). Phycocyanin (Sinopec Shanghai Reagents Co., Ltd. Shanghai, China); DAPI (4,6-Bipyridin-2-Phenylhydrazone) (Sigma, St. Louis, MO, USA); Malondialdehyde (MDA) test kit for total antioxidant capacity (T-AOC) Detection Kit, Catalase (CAT) Assay Kit, Reduced Glutathione (GSH) Assay Kit, Total Superoxide Dismutase (SOD) Assay Kit, Serum Creatine Phosphorus TB-MB and lactate dehydrogenase (LDH) test kits (Nanjing Institute of Biomedical Engineering, Nanjing, China); One-step TUNEL cell apoptosis test kit (Biyuntian Biotechnology Co., Ltd. Shanghai, China); and Ordinary balance, electronic thermometer; 721 spectrophotometer (Shanghai Analytical Instrument Factory, Shanghai, China).

Animals and study protocol

This study was approved by the Animal Ethics Committee of The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University Animal Center, and followed international guidelines for animal studies. Twenty-four adult Sprague Dawley (SD) rats (obtained from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. Beijing, China) weighing 200 ± 20 g were housed in a temperature-controlled room and stayed up for 12 h each day for free eating and drinking.

The Sprague Dawley (SD) rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg), and supinely fixed on the operating table. After tracheal intubation, manual mechanical ventilation, electrocardiogram monitoring, the left internal jugular vein catheter was applied on them. After

inserting the right carotid artery catheter, the arterial blood pressure was monitored. The skin on the left side of the sternum was cut, and the chest was opened from the fourth intercostal space to expose the heart and cut the purse. The anterior descending branch of the left coronary artery was ligated with Prolene No. 6 line. The myocardium under the ligature became dark red, and the T wave of the electrocardiogram was clearly high-pointed, indicating a successful ligation.

The rats were divided into 3 different groups with 8 rats per group viz: sham group; myocardial ischemia-reperfusion injury group (IRI group); Phycocyanin preconditioning + myocardial ischemia / reperfusion injury group (phycocyanin group). At the commencement of reperfusion, 10 mL of diluted phycocyanin was added to the blood reservoir; all rats with the exception of those in the Sham group experienced 45-min ischemia and 120-min reperfusion. Hemodynamic parameters were observed. Four hearts were obtained from each group, and Hematoxylin and Eosin (H & E) staining and TUNEL staining were used to detect apoptosis. The remaining 4 hearts were used to determine changes in oxidative stress markers and related genes in the Nrf2 signaling pathway, and changes in body weight and daily activities were monitored during the entire dose period. All rats were sacrificed by intranasal venous blood sampling after anesthesia.

Measurement of myocardial infarction area

After 24 h of myocardial ischemia and reperfusion in rats, 3 % Evans Blue was injected into the left ventricle and then the heart was removed. Ischemic myocardium was identified (normal myocardium is blue and ischemic myocardium is white). After rinsing with saline, the myocardium was placed in a refrigerator at 20 °C. Starting from the opening of the coronary artery, 5 pieces of equal thickness were cut from the apex of the heart and the thickness was 5 µm. One percent (1 %) chlorinated triphenyl tetrazolium phosphate buffer (pH 7.4) was placed in a 37 °C water bath for 15 min. Three divisions appeared: non-infarcted area (red stained), infarcted area (non-red stained, white), dangerous area (blue stained), and the ischemic area referred to the non-dangerous area (red stained + white). The myocardial infarct size was measured with Image ProPlus 6.0 software.

Assessment of cardiomyocyte function

At the end of the experiment, 2 mL blood sample was taken. After the blood was coagulated for 30

min, the supernatant was centrifuged at 3500g. The corresponding kits were used to determine the levels of serum creatine phosphonium citrate MB (CK-MB) and lactate dehydrogenase (LDH) in each group (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histological examination

Myocardial tissues from each treatment group were obtained and subjected to coronal cutting. Hematoxylin and eosin staining were performed on 10 % formalin-fixed and paraffin-embedded myocardium. Histological changes were assessed using semi-quantitative detection of necrosis of the myocardial tubules. The evaluator blindly chose 5 fields of each specimen and took the average value as the lesion score of each specimen.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Apoptosis of cardiomyocytes was quantified using terminal deoxynucleotidyl transferase-mediated dUTP *in situ* blunt-end labeling (TUNEL). Cardiomyocyte *in situ* apoptosis detection methods are briefly described as follows. Two hours after the reperfusion, the ischemic myocardium tissue was taken, fixed, embedded, and sectioned. Apoptotic cells were labeled and detected by an *in situ* apoptosis kit assay program. Under fluorescent microscope, TUNEL staining showed that the apoptotic cardiomyocytes were green. The DAPI staining showed blue myocytes. Cells were observed and counted under fluorescence microscopy. Five fields per slice, were counted and the number of apoptotic cells in 150 - 200 cardiomyocytes per field was recorded. The percentage of TUNEL-positive cells as an apoptosis index (AI) was calculated as shown in Eqn 1.

$$\text{Apoptotic index (AI)} = \frac{\text{Number of apoptotic cells}}{\text{Total number of counted cells}} \times 100\% \dots (1)$$

Biochemical measurements

Three hundred milligram of tissue blocks was rinsed in ice-cold saline. And the blood was removed, dried the filtered. The tissues were weighed, and then placed in a 5 mL beaker with saline. The volume was 9 times that of the tissue block. The tissues were homogenized on ice until no macroscopic tissue particles were observed. The homogenate (0.1 mL) was taken for measurement of malondialdehyde (MDA), total antioxidant capacity (T-AOC), catalase (CAT), reduced glutathione (GSH), total superoxide dismutase (SOD), and reactive oxygen species

(ROS). Reagents were prepared according to kit instruction. Finally, the absorbance and protein content of each tube were determined.

Immunohistochemical staining

After dewaxing and hydrating the wax block, 50 μ L of primary anti-rat Nrf2 and NF- κ B (1:100) was added and allowed to stand for 1 h. After washing 3 times in phosphate buffered saline (PBS) for 5 min each time, 40 - 50 μ L of secondary antibody was added dropwise and allowed to stand for 1 h. The section was stained with diaminobenzidine (DAB) for 5 - 10 min, hematoxylin for 2 min, differentiated with hydrochloric acid, and then rinsed with deionized water 10 - 15 min. After the section was dehydrated and mounted, the section was observed under a microscope.

Western blot analysis

The transfected cells were lysed using a cell lysis buffer, and shaken on ice for 30 minutes, and then centrifuged at 14,000g for 15 min. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted proteins were separated on a 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After the electrophoresis, the membranes were incubated with the primary antibodies Nrf2, Ho-1, NQO-1, NF- κ B and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then incubated with the secondary antibodies. All the antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Statistical analysis

The SPSS statistical analysis software (version 26.0) was used for data analysis. Experimental data are expressed as mean \pm standard error of the mean (SEM), multiple groups were compared using One-way ANOVA test, followed by Post Hoc Test (Least Significant Difference). $P < 0.05$ was considered statistically significant.

RESULTS

Phycocyanin improved myocardial function in ischemia and reperfusion injury

The expression levels of CK-MB in the peripheral blood of rats in the IRI group and the phycocyanin-pretreated group was significantly higher than that in the Sham group (Figure 1 A)

($p < 0.05$). In addition, the expression of LDH in rat peripheral blood was significantly different between Sham group and IRI group, and there was also a significant difference between phycocyanin-pretreated group and other two groups (Figure 1 B) ($p < 0.05$). This shows that the expression levels of CK-MB and LDH in phycocyanin protection group were better than those in the IRI group, but they did not return to normal levels.

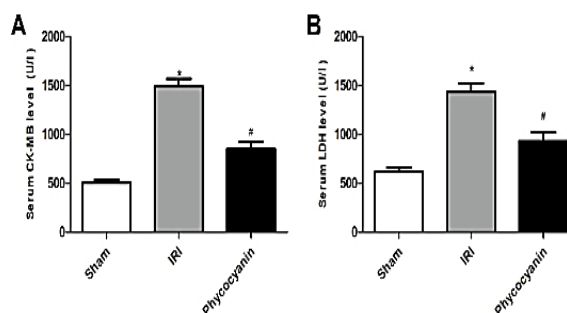


Figure 1: Phycocyanin conserved myocardial function in ischemia and reperfusion injury, (A) Serum CK-MB level of Sham group, IRI group and phycocyanin-treated group, (B) Serum LDH level of blood sample collected from the different management groups (* $p < 0.05$, vs. Sham group; # $p < 0.05$, vs. IRI group)

Phycocyanin preserved myocardial histologic architecture and mitigates neutrophil infiltration

The myocardial infarction area of rats in IRI group was significantly higher than that in sham group. Compared with the IRI group, the area of myocardial infarction after phycocyanin pretreatment was reduced ($p < 0.05$) (Figure 2 A). It was observed under the microscope, that rat myocardial cells in Sham group are regular and well-ordered, with only a small amount of focal infiltration of inflammatory cells. In the IRI group, the myocardial cells were arranged disorderly, with more inflammatory cells infiltrating and some of the blood vessels showed pink protein mucus exudation and inflammatory cell infiltration. However, after the addition of phycocyanin, normal myocardial microstructure still presented, and some pink blood vessels showed mucous membrane exudation with inconspicuous inflammatory cell infiltration.

Phycocyanin decreased cell apoptosis and enhanced cell proliferation

Compared with Sham group, the number of TUNEL-positive cells in the IRI group increased significantly. However, TUNEL-positive cells in the myocardial tissue sections were significantly reduced after adding phycocyanin protective drugs ($p < 0.05$) (Figure 2 B).

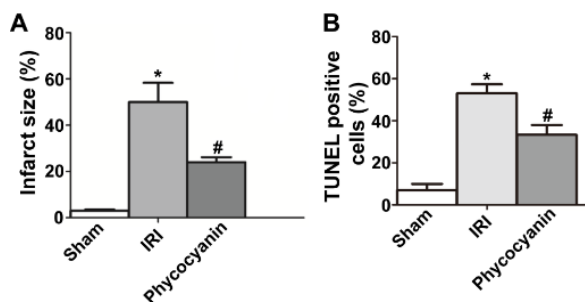


Figure 2: Phycocyanin prevent myocardial ischemia reperfusion injury in cardiac morphology, (A) Infarct size as a percentage of total volume, (B) Quantification of TUNEL-positive cells by average number of 5 HPF in different groups. (* $p < 0.05$, vs. Sham group; # $p < 0.05$, vs. IRI group)

Phycocyanin decreased ROS production and tissue impairment

Compared with the IRI group, the lipid peroxidation index (MDA), was also markedly reduced in the myocardium of the phycocyanin-protected group (Figure 3 A). In addition, compared with the Sham group, IRI significantly impaired the anti-oxidation ability of the myocardium, and increased the production of ROS (Figure 3 B). The administration of phycocyanin restored the markers of antioxidant activity in the myocardial injury tissues, including GSH, SOD, CAT and T-AOC (Figure 3 C - F).

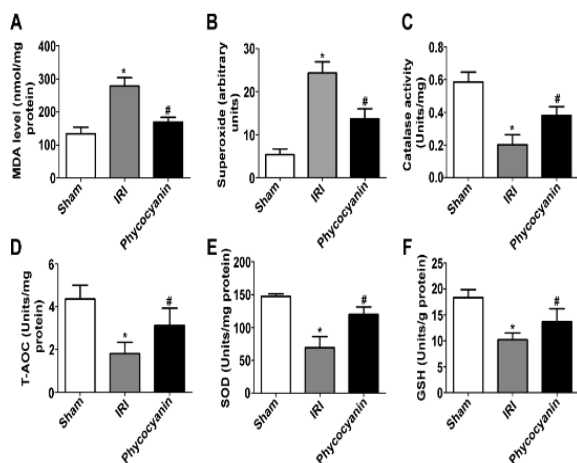


Figure 3: Phycocyanin attenuated oxidative stress injury, (A) Content of malondialdehyde (MDA) in myocardial tissues. (B) Density of ROS was reported as arbitrary units per millimeter square field, (C) Content of catalase activity in myocardial tissues, (D) Content of total antioxidant capacity (T-AOC) in myocardial tissues, (E) Content of superoxide dismutase (SOD) in myocardial tissues, (F) Content of glutathione (GSH) in myocardial tissues, (* $p < 0.05$, vs. Sham group; # $p < 0.05$, vs. IRI group)

Phycocyanin upregulated the expressions Nrf2 and Nrf2 downstream gene

In order to further understand the protective mechanism of phycocyanin on IRI-induced myocardial injury, rat myocardial tissues in sham, IRI and phycocyanin protection groups were collected. Immunohistochemical staining revealed that phycocyanin significantly increased the expression level of Nrf2, while reducing the expression of NF- κ B (Figure 4 A and B). Subsequently, proteins in the cytoplasm and nucleus were extracted from each group. After treatment with phycocyanin, the expression of Nrf2 in nucleus was significantly higher than that in the Sham group and the IRI group. Western blot results showed that the nuclear translocation of Nrf2 in the phycocyanin-protected group was also remarkably higher than that in the Sham group and the IRI model group (Figure 4 C). In addition, Western blot showed that after phycocyanin treatment, the expression of NF- κ B in the nucleus was significantly lower than that in IRI model group (Figure 4 D). At the same time, Nrf2 and several downstream genes were significantly up-regulated at the protein level. Western blot analysis showed that HO-1 and NQO-1 in the phycocyanin-protected group were dramatically enhanced when compared with the Sham and IRI groups (Figure 4 E and F). The above results indicated that phycocyanin significantly increased the anti-inflammatory and anti-oxidation abilities of ischemic myocardium.

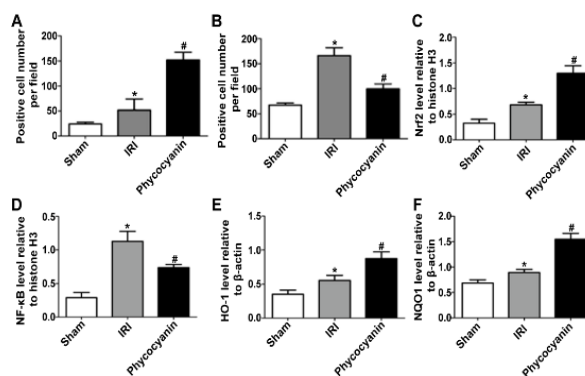


Figure 4: Phycocyanin supplementation enhances Nrf2 nuclear translocation, and increases HO-1 and NQO-1 protein expression, (A) Nrf2 and NF- κ B expression levels in Sham, I/R injury and I/R injury treated with phycocyanin rats in immunohistochemical staining. Statistical analyses of IHC results in Nrf2 expression levels in the different groups. (B) Statistical analyses of IHC results in NF- κ B expression levels in the three groups; (C-D) Protein expression levels of Nrf2 and NF- κ B determined by densitometric analysis and normalized to histone H3 signal; (E-F) Protein expression levels of HO-1 and NQO1 in different groups, with β -actin as a protein standard (* $p < 0.05$, vs. Sham group; # $p < 0.05$, vs. IRI group)

DISCUSSION

Early recovery from ischemic myocardial hemoperfusion is a fundamental measure required to prevent and treat myocardial ischemia injury. However, after the restoration of blood perfusion, some patients or animal's ischemic myocardium will cause further damage to heart ultrastructure and function, which is called myocardial ischemia-reperfusion injury [3-5]. In the IRI group, the myocardial cells were arranged in an disorderly manner, with more inflammatory cells infiltrating, and some of the blood vessels showed pink protein mucus exudation and inflammatory cell infiltration. Many studies have shown that phycocyanin has an antioxidative effect on ischemic reperfusion injured liver, because it can significantly reduce the oxidative stress level and increase the substances against oxidative stress [11-13].

In the present study, phycocyanin was used to pretreat myocardial IRI rats, and to observe the protective effect of phycocyanin on acute myocardial ischemia-reperfusion injury. The results showed that the expression levels of CK-MB and LDH in phycocyanin protection group were better than those in the IRI group, indicating that phycocyanin improved myocardial function in ischemia and reperfusion injury, preserved myocardial histologic architecture and mitigated neutrophil infiltration.

Oxidative stress caused by pathological overproduction of reactive oxygen species and reactive nitrogen species (ROS, RNS) plays an important role in the development of renal ischemia-reperfusion injury [14]. Oxidative stress caused by increased oxygen free radicals during ischemia-reperfusion injury is a key event [15-17]. In addition, excess ROS induced inflammatory body formation, autoimmune activation and platelet activation, which have further damaging consequences [17-19]. Naturally, it is a viable option for IRI therapy because it strengthens the cell's antioxidant defense system to fight oxidative stress. In this experiment, compared with the Sham group, the IRI group showed significant histopathological changes, higher oxidative stress levels and lower levels of antioxidant capacity. This indicated that there was obvious oxidative stress in the myocardial ischemia-reperfusion injury, which reduced the anti-oxidation ability of the tissue, causing damage to cells.

This study showed that the administration of phycocyanin obviously restored the markers of antioxidant activity in the myocardial injury tissues, including GSH, SOD, CAT and T-AOC.

Interestingly, the normal myocardial microstructure could still be observed in the rats pretreated with phycocyanin. Whether the treatment of phycocyanin could alleviate the symptoms after the emergence of these microstructures will require further investigation.

Studies have shown that Nrf2 is an important nuclear transcription factor for cell defense against oxidative stress. After binding to anti-oxidation response elements (ARE) in the nucleus, Nrf2 can regulate the expression of multiple downstream antioxidant genes [10,11]. Phycocyanin has recently been successfully synthesized as a potent Nrf2 inducer, and other *in vitro* experiments have confirmed its biological function of anti-oxidation and anti-apoptosis. In this study, the expression of Nrf2 was low while NF- κ B was highly expressed in the IRI group. However, the expression of NF- κ B was decreased in the phycocyanin group when compared with the IRI group, indicating that ROS generated by IRI could activate NF- κ B.

However, phycocyanin inhibited the activation of NF- κ B by activating Nrf2 to reduce the inflammatory response induced by myocardial IRI, which was consistent with the role of phycocyanin in alleviating inflammation in other ischemia-reperfusion injured tissues. The preliminary conclusion was therefore that phycocyanin may exert protective effect on myocardial cells by activating Nrf2 and inhibiting NF- κ B. However, the underlying mechanism of phycocyanin on Nrf2 needs to be further studied.

CONCLUSION

The findings of this study suggest that phycocyanin reduces the pathological damage induced by myocardial IRI, and inhibits oxidative stress as well as inflammatory response. In addition, phycocyanin may exert protective effect on myocardial cells by activating Nrf2 and inhibiting NF- κ B. However, only the effect of phycocyanin on myocardial IRI was preliminarily discussed. Thus, the specific mechanism needs further investigation.

DECLARATIONS

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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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