Safflor yellow A protects neonatal rat cardiomyocytes against anoxia/reoxygenation injury in vitro

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Aim: To investigate the effects of safflor yellow A (SYA), a flavonoid extracted from Carthamus tinctorius L, on cultured rat cardiomyocytes exposed to anoxia/reoxygenation (A/R).

Methods: Primary cultured neonatal rat cardiomyocytes were exposed to anoxia for 3 h followed by reoxygenation for 6 h. The cell viability was measured using MTT assay. The releases of lactate dehydrogenase (LDH) and creatine kinase (CK), level of malondialdehyde (MDA), and activities of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were analyzed. Hoechst 33258 staining and changes in Bcl-2/Bax ratio and caspase 3 activity were used to examine A/R-induced apoptosis.

Results: The A/R exposure markedly decreased the viability of cardiomyocytes, suppressed the activities of SOD, GSH, CAT and GSH-Px, and Bcl-2 protein expression. Meanwhile, the A/R exposure markedly increased the release of LDH and CK, and MDA production in the cardiomyocytes, and increased the rate of apoptosis, caspase 3 activity, Bax protein expression. Pretreatment with SYA (40, 60 and 80 nmol/L) concentration-dependently blocked the A/R-induced changes in the cardiomyocytes. Pretreatment of the cardiomyocytes with the antioxidant N-acetylcysteine (NAC, 200 μmol/L) produced protective effects that were comparable to those caused by SYA (80 nmol/L).

Conclusion: SYA protects cultured rat cardiomyocytes against A/R injury, maybe via inhibiting cellular oxidative stress and apoptosis.

Keywords: safflor yellow A; N-acetylcysteine; antioxidant; cardiomyocyte; anoxia/reoxygenation; oxidative stress; apoptosis

Introduction

In developed countries, cardiovascular diseases are the major cause of disability and mortality[1]. Myocardial ischemia and reperfusion is a major source of cardiomyocyte injury within the context of a host of clinical pathologies[2]. Ischemia/reperfusion (I/R) injury includes a series of events that may occur together or separately: reperfusion arrhythmias, myocardial stunning in “reversible mechanical dysfunction”, microvascular damage and cell death[3–7]. Oxidative stress and apoptosis have been reported to play significant roles in the progression of I/R[8–11]. Inhibition of oxidative stress and/or a direct intervention in apoptotic pathways at the membrane, at the mitochondria or at nuclear activation sites could provide molecular targets for potential therapeutic treatments[12]. The search for the most effective therapeutic drugs for I/R injury has long presented a problem. In the past few decades, there has been an increase in the use of complementary treatments, such as herbal remedies, in the treatment of diseases in the whole world. Many traditional plants have been reported to be useful for the control of problems due to ischemia and associated pathologies[13].

Carthamus tinctorius L, a member of the Compositae, has been used as a Chinese medicine in the clinic to treat cardiovascular disease with anti-myocardial ischemia[14, 15]. The chemical constituents in Carthamus tinctorius L are reported to include lignans[16], flavonoids[17], triterpene alcohols[18], and polysaccharides[19]. Research shows that the main effective constituent of Carthamus tinctorius L is safflower yellow (SY), which contains several related flavonoids. SY consists of hydroxysafflor yellow A (HSYA), safflor yellow A (SYA), safflower yellow B (SYB), and other chemicals[20–24]. Many studies have demonstrated that SY possesses various physiological and pharmacological activities, including anticoagulant, antioxidant, antiapoptotic and calcium antagonist. However, the component responsible for these protective effects remains...
unknown.

Like SYB and HSYA, SYA (Figure 1), a natural compound from the flower of *Carthamus tinctorius* L. is a water-soluble monomer of SY. Increasing evidence demonstrates that SYB and HSYA provide myocardial protection by acting at multiple target points, such as via antioxidant and antiapoptotic activity \(^{25-28}\), but there is little research on the effect of SYA on myocardial preservation. The aim of our study is to investigate whether SYA is an effective component of *Carthamus tinctorius* L with cardioprotective action.

![Figure 1. Chemical structure of safflor yellow A. Molecular formula: C\(_{27}H_{29}O_{15}\); molecular weight: 593.15.](image)

**Materials and methods**

**Materials**
SYA (purity >98%) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological products (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from GIBCO (Life Technologies, Grand Island, NY, USA), and fetal bovine serum (FBS) was provided by Hangzhou Sijiqing Biological Engineering Materials Co Ltd (Hangzhou, China). N-acetylcysteine (NAC), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide], type I collagenase and Western blot reagents were purchased from Sigma (St Louis, MO, USA). The kits for the determination of LDH, CK, MDA, CAT, SOD, and GSH were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Anti-β-actin, anti-Bax, and anti-Bcl-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The fluorescent kit for Hoechst 33258 was obtained from Roche (Mannheim, Germany). The caspase 3 assay kit was purchased from Chemicon International Inc (Temecula, CA, USA).

**Animals**

All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health, and the local Animal Ethics Committee approved all procedures. This study used 1- to 3-d-old Sprague-Dawley rats (gender was not determined).

**Primary cardiomyocyte culture**

Isolation of cardiac myocytes was performed as previously described \(^{29}\). Briefly, the hearts were harvested and placed in PBS. Then, the hearts were minced and dissociated with 1 mg/mL type I collagenase. Cardiomyocytes were enriched by preplating for 90 min to eliminate fibroblasts. More than 90% of the cells were identified as cardiac myocytes by immunocytochemistry with anti-sarcomeric β-actin (Sigma). The cells were then cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin and maintained in 5% CO\(_2\) at 37°C. The cells were seeded at a density of 4×10\(^5\) cells/mL in 96-well microplates for MTT assays or at a density of 3×10\(^3\) cells/mL in 6-well microplates for LDH, CK, MDA activity assays and antioxidant enzyme assays, and for the measurement of other biochemical indicators. The culture medium was changed every second day. After 72 h, cultured cardiomyocytes were used in subsequent experiments. Cardiac myocytes were randomly divided into six groups: 1) a control group without any treatment; 2) a model group, which was cultured under anoxic conditions for 3 h and then under reoxygenation conditions for 6 h; 3) a NAC treatment group, which was pretreated with 200 μmol/L NAC for 24 h before anoxia/reoxygenation (A/R) injury; 4) SYA-40 treatment group, which were pretreated with SYA at concentration of 40 nmol/L for 24 h before A/R injury; 5) SYA-60 treatment group, which were pretreated with SYA at concentration of 60 nmol/L for 24 h before A/R injury; 6) SYA-80 treatment group, which were pretreated with SYA at concentration of 80 nmol/L for 24 h before A/R injury.

**In vitro A/R injury model**

The *in vitro* model of A/R used in the present study was similar to the model previously described by Koyama *et al* \(^{30}\). To mimic an A/R model (via anoxia in the medium), cells were placed in a hypoxia solution and incubated for 3 h at 37°C in a hypoxic chamber (HEPA CELL 100, Germany) filled with 95% N\(_2\) and 5% CO\(_2\). The hypoxia solution contains (in mmol/L: NaCl 98.5, KCl 10, MgSO\(_4\) 1.2, CaCl\(_2\) 1.0, HEPES 20, and sodium lactate 40, at pH 6.8, 37°C). For A/R studies, the medium was then replaced by maintenance medium during the reoxygenation period, and cells were cultured for 6 h under normal conditions.

**Cell viability assays**

Cell viability was determined by MTT assay. Cells were seeded at a density of 4×10\(^4\) cells/well in 96-well plates. After treatment, 20 μL of the MTT solution (5 mg/mL) was added to each well, resulting in a final concentration of 5 mg/mL, and kept for 4 h at 37°C. Afterward, the medium was removed and DMSO (150 mL) was added to each well. The optical density (OD) was determined spectrophotometrically at 490 nm with a microplate reader (Infinite M200 PRO, Switzerland), and the cell survival ratio was expressed as a percentage of the control.
LDH and CK activities assays
Many experiments have reported that LDH and CK are two reliable markers of cardiac myocyte I/R injury\[^{31, 32}\]. After the A/R procedure, cell culture medium was collected to determine LDH and CK levels by measuring the conversion of pyruvate to lactate and creatine to phosphagen, respectively. The activities of LDH and CK were analyzed with microplates at 450 nm.

Measurement of MDA activity
MDA is a product of lipid peroxidation that can cause cross-linking polymerization of proteins, nucleic acids and some macromolecules, so the amount of MDA often reflects the degree of lipid peroxidation\[^{33, 34}\]. Culture medium was collected 6 h post reoxygenation, transferred to clean epoxy epoxide (EP) tubes and stored at -80 °C. Thiobbituric acid reactive substances were assessed by measuring the MDA concentration at 532 nm with the thiobbituric acid method, which is based on the reaction of MDA with thiobbituric acid to form a stable chromophoric product.

Determination of cellular antioxidant enzyme activities
Determination of the activities of antioxidant enzymes was conducted according to the instructions provided in the assay kits (Jiancheng Bioengineering Institute, Nanjing, China). In brief, after 6 h of reoxygenation, the cells were washed twice in PBS, resuspended in 1 mL of 0.1 mol/L phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged at 4000 rounds per minute for 10 min at 4 °C, and the supernatants were collected after centrifugation for subsequent tests. GSH activity was assayed at 420 nm and calculated on the basis of a GSH calibration curve. SOD activity was assayed at 560 nm on the basis of its ability to inhibit the oxidation of hydroxylamine by the superoxide anion from the xanthine oxidase system. GSH-Px activity was measured at 412 nm on the basis of the rate of oxidation of reduced glutathione to oxidized glutathione by H\(_2\)O\(_2\) under the catalysis of GSH-Px. The principles of the assay for catalase are based on the determination of the H\(_2\)O\(_2\) decomposition rate at 240 nm. The protein content in the cell lysate was determined with Coomassie blue staining solution.

Morphological assessment and quantification of apoptotic myocytes
Hoechst 33258 staining, which allows apoptotic and normal cells to be clearly distinguished on the basis of nuclear morphology (chromatin condensation and fragmentation), was used for quantification of apoptotic myocytes. Briefly, cells were washed in ice-cold PBS, fixed in 10% neutral buffered formalin for 10 min at room temperature, and washed again in ice-cold PBS. Then, the cells were exposed to Hoechst 33258 (2 μg/mL in PBS) and incubated for 20 min at room temperature. Cells were then washed three times in PBS and examined under a fluorescence microscope with an appropriate filter. The percentage of apoptotic cells displaying chromatin condensation and nuclear fragmentation was determined. Apoptosis in cardiomyocytes was quantified by the number of apoptotic nuclei in the total number of nuclei in 10 continuous microscopic fields under ×400 magnification by using the following formula: apoptosis index=(apoptotic nuclei/total nuclei)×100%.

Caspase 3 activity
Caspase 3 is an important component of the final pathway leading to cell death. Its activity was measured using a fluorometric assay kit according to the manufacturer’s protocol. In brief, 4×10\(^5\) control or A/R treated cells were lysed in 50 μL ice-cold lysis buffer, placed on ice for 30 min, and then centrifuged at 10 000×g for 5 min at 4 °C. The protein concentrations in the supernatants were quantified with the Coomassie blue staining solution. Reaction buffer (50 μL) and caspase 3 substrate (5 μL) were added to the cell lysates. After incubation at 37 °C for 4 h, the fluorescence was measured on a microplate reader with excitation at 400 nm.

Western blotting analysis
For Western blot, equal amounts of protein lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. The gels were blotted onto a nitrocellulose membrane and incubated with the indicated antibodies. Blots were developed by ECL according to the manufacturer’s instructions. β-Actin was used as a loading control.

Statistical analysis
All values were expressed as the mean±SD. A one-way analysis of variance followed by Bonferroni’s correction was carried out to test for any differences between the mean values of groups. A P value <0.05 was considered to be significant.

Results
SYA protected cells from A/R-induced cell viability loss
Cell viability was shown in Figure 2 using the MTT assay. We first examined the viability of cardiomyocytes after treatment with SYA (0–200 nmol/L). SYA at concentrations below 100 nmol/L did not significantly affect cell viability, so three concentrations (40, 60, and 80 nmol/L) were selected as the working concentrations. A/R caused a significant decrease in cell viability (P<0.01); the results in Figure 2B indicate that SYA protected cells against injury in a dose-dependent manner.

Effect of SYA on LDH and CK activities
The release of LDH and CK was used as an index of cardiomyocyte injury. LDH and CK release in the model group was higher than that in the control group (P<0.01), and significant decreases in LDH and CK activities were observed after pretreatment with SYA (40, 60, and 80 nmol/L) or NAC (200 μmol/L) compared with the model group (P<0.01) (Figure 3). These results indicate that SYA is a potent cardioprotective agent against A/R injury.
Effect of SYA on MDA content
The concentration of MDA in the culture media was measured as a biochemical marker for lipid peroxidation. Cardiomyocytes subjected to A/R showed an increase in MDA content compared with untreated cells. Treatment with SYA (60 and 80 nmol/L) or NAC (200 μmol/L) produced a significant reduction in MDA content in cardiomyocytes undergoing A/R \((P<0.01)\) (Figure 4).

Effect of SYA on antioxidant enzyme activities
GSH, glutathione peroxidase (GSH-Px), SOD, and catalase are important antioxidant enzymes, and the activities of these four enzymes were examined in this study. When the cardiomyocytes were damaged, the activities of these enzymes (GSH, GSH-Px, catalase, and SOD) were depressed compared with their activities in normal cells \((P<0.01)\). These changes demonstrated that the antioxidant system was disrupted by A/R. Treatment with SYA or NAC significantly increased the activities of the antioxidant enzymes \((P<0.01)\) and protect cardiomyocytes from oxidative damage (Figure 5).

Effect of SYA on apoptosis
Nuclear morphological change was observed by Hoechst 33258 staining, which illustrated that the control cells exhibited uniformly dispersed chromatin, normal organelles and intact cell membranes. After A/R injury, myocytes featured typical characteristics of apoptosis, including shrinkage of the nuclei, chromatin condensation and the appearance of a
few apoptotic bodies. However, with SYA pretreatment, the morphological changes were significantly attenuated, and the number of cells with nuclear condensation and fragmentation was significantly decreased ($P < 0.01$) (as shown in Figure 6).

**Effect of SYA on caspase 3 activity**

To further characterize the inhibitory effect of SYA on myocardial cell apoptosis, we examined whether SYA could inhibit caspase 3 activity. In this study, caspase 3 activity was triggered by A/R ($P < 0.01$) and could be inhibited by SYA pretreatment in a dose-dependent manner (Figure 7).

**Effect of SYA on Bcl-2 and Bax expression**

As shown in Figure 8, A/R potently inhibited Bcl-2 expression and promoted Bax expression so that the Bcl-2/Bax ratio was reduced in A/R-injured cardiomyocytes compared with control ($P < 0.01$); however, SYA reversed the effects of A/R on Bcl-2 and Bax expression, increasing the Bcl-2/Bax ratio in a dose-dependent manner ($P < 0.01$).

**Discussion**

*Carthamus tinctorius* L (safflower) is a widely used herb in the traditional medical systems of China. The major constituents of safflower are flavonoids, a group of polyhydroxy phenols. Flavonoids are good antioxidants, at least in vitro\(^{36}\). There is ample evidence to indicate the beneficial effects of flavonoids on ischemic reperfused hearts through in vitro application (when added to perfusates) or when administered to blood\(^{36-41}\), which could be of use in acute ischemia/reperfusion situations, such as for heart surgeries and transplants. SYA, a flavonoid, is a natural compound from the flower of the safflower plant, and there are few previous reports on its pharmacological activity. In this study, we investigated the effect of SYA on oxidative damage and apoptosis induced by A/R in cardiomyocytes. We utilized primary cardiomyocytes subjected to A/R in a hypoxic solution to simulate A/R injury in order to examine the mechanisms of SYA-mediated protection of cardiomyocytes against A/R injury. We used NAC, which has been reported to have antioxidant properties\(^{42-44}\), as the positive control to investigate the possible mechanism for SYA’s protection of cardiomyocytes against A/R injury.

We monitored the cell survival rate through MTT assay. After 6 h of reperfusion, the percentage cell survival was decreased in the model group, and SYA protected cells in a dose-dependent manner. Three concentrations of SYA without toxic effect were chosen in the following experiments. LDH and CK are expressed constitutively in mammalian cells and are abundant in endochylema. They cannot transit through the cytoplasmic membrane in the normal physiological state, but when a cell is damaged or dead, LDH and CK are released from the cell\(^{45, 46}\). Therefore, LDH activity and CK activity in the culture media represent the extent of cell injury induced by A/R. Our results showed that LDH activity and CK activity were significantly decreased following SYA treatment (vs model, $P < 0.01$), implying that SYA has the ability to protect cardiomyocytes from A/R induced injury.
It is well-known that oxidative stress has been largely implicated in the pathogenesis of myocardial ischemia/reperfusion injury\(^{47, 48}\). Reactive oxygen species (ROS) may interact degradatively with cellular components, including nucleic acids, proteins, and lipids, to compromise structure and function\(^{49}\). In the heart, ROS-induced abnormalities include cytotoxicity, cardiac stunning, arrhythmias, alterations of \(\text{Ca}^{2+}\) homeostasis, and intracellular ATP depletion\(^{50, 51}\). Cellular defense against free radical injury is mediated by several potent antioxidant enzymes, including SOD, catalase, and GSH-Px, all of which physiologically reduce ROS levels\(^{52, 53}\). As one of the peroxidized products of polyunsaturated fatty acids, MDA concentration in cells reflects the degree of lipid peroxide and indirectly reflects the production of intracellular ROS. SOD has the ability to transform intracellular superoxide anions to \(\text{H}_2\text{O}_2\), and the formed \(\text{H}_2\text{O}_2\) is subsequently scavenged by catalase and GSH-Px through enzymatic reactions. Catalase is an enzyme located in the peroxisome and very efficiently promotes the conversion of \(\text{H}_2\text{O}_2\) to \(\text{H}_2\text{O}\) and \(\text{O}_2\)\(^{54}\). GSH-Px acts in conjunction with the tripeptide glutathione (GSH), which is present in cells in high (micromolar) concentrations. GSH-Px decomposes peroxides to water while simultaneously oxidizing GSH\(^{55}\).

Based on previous studies, we investigated whether the protection of SYA was attributed to its antioxidative activity. We determined MDA content and the activity of cellular antioxidants, including the activities of catalase, GSH-Px and SOD and the content of GSH. Our results clearly demonstrated that SYA decreased cellular MDA content and enhanced antioxidant enzyme activity and increased antioxidant agent content. These findings suggested that the cardioprotection of SYA could be partly attributed to enhanced antioxidant capacity in cardiomyocytes.
Accumulating evidence indicates that apoptosis, a gene controlled programmed cell death pathway, contributes significantly to post-ischemic cardiomyocyte death, suggesting that therapeutic interventions that inhibit apoptotic cell death may attenuate I/R-induced heart injury[58]. Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and the formation of apoptotic bodies[52]. In this work, an antiapoptotic effect was confirmed by Hoechst 33258 staining, and the results strongly suggested that SYA might exert a protective effect against apoptosis in response to A/R.

Further investigation was performed, focusing on the possible mechanisms involved in the antiapoptosis effect of SYA. Previous studies have indicated that an increase in proapoptotic Bax family proteins and a decrease in antiapoptotic Bcl-2 family proteins are involved in apoptosis[57, 58]. Thus, the ratio of Bcl-2 to Bax may be a critical factor in the cellular threshold for apoptosis[59-63]. The caspase cascade also plays a key role in apoptosis: the activation of caspase 3 results in cleavage of cytoskeletal and nuclear proteins and nucleosomal fragmentation of DNA[64].

Our data showed that A/R increased caspase 3 activity and Bax expression and that it inhibited Bcl-2 synthesis in neonatal rat cardiomyocytes so that the balance between Bcl-2 and Bax was broken. As a result, the cells fell into programmed cell death. SYA inhibited cell apoptosis by suppressing caspase 3 activity and Bax expression and increasing Bcl-2 synthesis, maintaining the Bcl-2/Bax balance. These results indicated that alleviating apoptosis during A/R might be one of the mechanisms of the cardioprotective effect of SYA. At the same time, the changes may be associated with an increase in antioxidant capacity and a decrease in MDA content. However, whether the alleviation of apoptosis was caused by enhanced antioxidant activity remains to be determined.

In conclusion, we cultured neonatal rat cardiomyocytes and demonstrated that SYA induced protection against A/R injury, as measured by the survival rate of cells and the leakage of LDH and CK into the culture media. SYA displays antioxidant activity by enhancing the activity of catalase, GSH-Px, and SOD and by elevating the level of GSH. Its antioxidant ability at 80 nmol/L may be as good as that at 200 μmol/L NAC. We also found that SYA had antiapoptotic activity. However, it is not clear whether the mechanisms are mutually independent or part of a cascade event; further study of the detailed mechanisms is now in progress. In this paper, we discussed the myocardial protection mediated by SYA and its promise as a therapeutic intervention for the treatment of myocardial ischemia. This study is a preliminary investigation of SYA, and much more work is required to develop it into a new drug. It also should be emphasized that the effective dose is close to the minimum toxic concentration, and safety should be tested in subsequent research.

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Author contribution
Jia-lin DUAN, Yue GUAN, Ying YIN, and Guo WEI designed the research; Jing-wen WANG, Jia CUI, Dan ZHOU, Yan-rong ZHU, and Wei QUAN performed the research; Jia-lin DUAN, Yue GUAN, Ying YIN, and Guo WEI analyzed the data; Jialin DUAN wrote the paper; Ai-dong WEN and Miao-miao XI revised the paper.

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