

Bauhinia championii flavone inhibits apoptosis and autophagy via the PI3K/Akt pathway in myocardial ischemia/reperfusion injury in rats

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Abstract: This study aimed to determine the effects of *Bauhinia championii* flavone (BCF) on myocardial ischemia/reperfusion injury (MI/RI) in rats and to explore potential mechanisms. The MI/RI model in rats was established by ligating the left anterior descending coronary artery for 30 minutes, then reperusing for 3 hours. BCF at 20 mg/kg was given 20 minutes prior to ischemia via sublingual intravenous injection, with 24 µg/kg phosphoinositide 3-kinase inhibitor (PI3K; wortmannin) as a control. The creatine kinase-MB and nitric oxide content were assessed by colorimetry. The levels of mitochondrial permeability transition pores and tumor necrosis factor alpha were determined by an enzyme-linked immunosorbent assay. Cardiomyocyte apoptosis was detected by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Additionally, the expression of PI3K, endothelial nitric oxide synthase, caspase-3, and Beclin1 was analyzed by fluorescence quantitative polymerase chain reaction and Western blotting, respectively. Akt and microtubule-associated protein 1 light chain 3-II protein levels were also evaluated. Pretreatment with BCF significantly decreased the levels of creatine kinase-MB, tumor necrosis factor alpha, and mitochondrial permeability transition pores, but increased the nitric oxide content. Furthermore, BCF inhibited apoptosis, downregulated caspase-3, Beclin1, and microtubule-associated protein 1 light chain 3-II, upregulated PI3K, and increased the protein levels of phosphorylated Akt and endothelial nitric oxide synthase. However, all of the previously mentioned effects of BCF were blocked when BCF was coadministered with wortmannin. In conclusion, these observations indicated that BCF has cardioprotective effects against MI/RI by reducing cell apoptosis and excessive autophagy, which might be related to the activation of the PI3K/Akt signaling pathway.

Keywords: *Bauhinia championii* flavone, myocardial ischemia/reperfusion injury, apoptosis, autophagy, PI3K/Akt

Introduction

Ischemic heart disease is a major cause of mortality and disability and is commonly associated with acute coronary artery blockage.¹ Thrombolysis or primary percutaneous coronary intervention are the common therapeutic strategies in the clinic, but subsequent reperfusion induces further cardiac dysfunction including myocardial stunning, arrhythmia, and myocardial cell death, which is known as myocardial ischemia/reperfusion injury (MI/RI).² The reduction of MI/RI is important in the prevention and treatment of ischemic heart disease.

Recent studies have showed that myocardial cell death is the main reason for the poor prognosis of patients with myocardial infarction. Apoptosis and autophagy are two principal pathways for programmed cell death, and thus may be important in ischemic RI.^{3,4}

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Apoptosis occurs when DNA damage is unrecoverable and can be induced via the opening of mitochondrial permeability transition pore (MPTP) accompanied by the release of pro-death protein.⁵ Caspase-3 is a key effector of the mitochondrial apoptotic pathway. Autophagy is an evolutionarily conserved lysosome-dependent process that results in the degradation of long-lived proteins and damaged organelles.⁶ It is widely accepted that Beclin1-mediated autophagy/apoptosis and the mammalian target of rapamycin (mTOR)-mediated autophagy/mTOR mutual feedback signaling are the two classical pathways of autophagy.^{7,8} Although some autophagy is essential for maintaining cell functioning, excessive autophagy leads to the progressive consumption of cellular constituents and subsequently to autophagic cell death.⁹ Research has shown that autophagy is a “double-edged sword” in MI/RI: a slight increase of autophagy during ischemia inhibited apoptosis and promoted cell survival, but an abnormal increase in autophagy during the reperfusion period aggravated myocardial injury.¹⁰ Beclin1 and microtubule-associated protein 1 light chain 3-II (LC3-II) are two important markers of autophagosomes, which are upregulated during the reperfusion period and signify ongoing autophagy and cellular damage.¹¹

The phosphoinositide 3-kinase/serine/threonine protein kinase (PI3K/Akt) pathway is an important antiapoptosis/proliferation signaling pathway that plays a key role in normal cellular functioning, including proliferation, adhesion, migration, invasion, energy metabolism, protein synthesis, and prosurvival.¹² Activated Akt is a downstream effector of PI3K, which can inhibit apoptosis by regulating multiple targets such as MPTP, ATPase, tumor necrosis factor alpha (TNF- α), endothelial nitric oxide synthase (eNOS), the Bcl-2 family proteins, and NF- κ B.¹³ PI3K regulates the autophagy function of the “double-edged sword” by upstream effects on Beclin1 and mTOR, thus comprising a PI3K–Beclin1–mTOR autophagy regulatory network.^{7,14} Many studies have shown that the activation of the PI3K/Akt signaling pathway could suppress apoptosis and excessive autophagy and protect the heart against MI/RI.^{14–16}

Bauhinia championii (Benth.) Benth. is a traditional Chinese medicinal herb and functional food that is widely distributed in the Guangxi Province of People’s Republic of China.¹⁷ Previous studies have suggested that extracts of its stems promote blood circulation, remove blood stasis, and possess anti-inflammatory, antioxidative, and antiplatelet aggregative effects.^{18,19} *Bauhinia championii* flavone (BCF) is the primary active component of the stem extract. Our previous studies have demonstrated that BCF scavenges free radicals, improves myocardial energy metabolism, reduces

inflammation and myocardial injury, inhibits apoptosis and enhances the cardiocyte survival rate in hypoxia/reoxygenation injury,^{20,21} as well as alleviating pituitrin-induced acute myocardial injury in vivo.²² However, the molecular mechanism of BCF cardioprotection on MI/RI has not been clearly elucidated.

Accordingly, this study used wortmannin (WM), a PI3K/Akt pathway inhibitor, as a control drug to determine whether the protective effects of BCF on MI/RI mediated the activation of the PI3K/Akt signaling pathway and to further explore the impact of BCF on the upstream and downstream targets of the PI3K/Akt pathway. Elucidation of the mechanisms of BCF-induced cardioprotection might have clinical implications for the development of new target drugs.

Materials and methods

Animals

The experimental procedures and protocols were approved by the Ethics Committee for the Experimental Use of Animals at Guangxi Medical University (Guangxi, People’s Republic of China) on May 15, 2014 (number 20140515-06) and carried out in accordance with their guidelines. Male Sprague–Dawley rats (40), weighing 250–280 g, were purchased from the Guangxi Medical University Laboratory Animal Center, Nanning, People’s Republic of China, (certificate number SCXK [Gui] 2014-0003). The rats were housed under standard conditions (20°C–25°C, 50%–60% humidity, with a 12 hours light-dark cycle) and were given standard rodent chow and free access to water.

Drugs and reagents

BCF was obtained from the Department of Pharmacology of Guilin Medical University, Guilin, Guangxi, People’s Republic of China.²³ With rutin as a reference substance, the total flavonoid content of BCF was 82% (Figure S1). BCF was diluted with saline to an appropriate concentration as needed. Creatine kinase-MB (CK-MB) and nitric oxide (NO) detection kits were obtained from the Jiancheng Bioengineering Institute (Nanjing, People’s Republic of China). MPTP, TNF- α , and enzyme-linked immunosorbent assay kits were purchased from Chenglin Biological Technology Co. Ltd. (Beijing, People’s Republic of China). A terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) apoptosis detection kit was purchased from Roche Diagnostics (Mannheim, Germany). Primers were purchased from Takara Biotechnology Co. Ltd. (Dalian, People’s Republic of China). WM was obtained from Sigma-Aldrich Co. (St Louis, MO, USA). All antibodies were purchased from

Cell Signaling Technology Inc. (Danvers, MA, USA). The extraction and isolation of BCF and each chemical structure of BCF constituents are in the Supplementary material.

Experimental design

After surgery, the rats were divided randomly into five groups (n=8): 1) a sham-operated group in which animals underwent a similar procedure without ligation of the coronary artery; 2) the model group in which the MI/RI model was established by ligating the left anterior descending coronary artery (LADCA) for 30 minutes, then reperfusing for 3 hours; 3) MI/RI rats pretreated with BCF (20 mg/kg); 4) MI/RI rats pretreated with WM (24 µg/kg); 5) MI/RI rats pretreated with BCF + WM. The animals were treated with BCF at 20 mg/kg and/or WM at 24 µg/kg. The drugs were given 20 minutes prior to ischemia via sublingual intravenous injection and the sham-operated and model group received the same volume of saline, respectively.

General surgical procedure

Rats were anesthetized with 20% ethyl carbamate (5 mL/kg) and placed in the supine position. The MI/RI operation was performed 20 minutes after administration. The chest was opened through the fourth intercostal space, and the ribs were gently retracted to expose the heart. After cutting the pericardium, the LADCA was positioned between the left atrial appendage and the pulmonary conus. The LADCA was ligated using a 4-0 silk suture. Using a Small Animal Ventilator (Alcott Biotech CO., Ltd., Shanghai, People's Republic of China), oxygen was supplied through the trachea with a respiration rate of 70 per minute, a respiration-to-expiration ratio of 1:2, and a tidal volume of 50 mL/kg body weight. The LADCA was ligated for 30 minutes and then released to allow reperfusion for 3 hours. Subcutaneous electrodes were inserted into the rat's limbs and an electrocardiogram was monitored continuously throughout the MI/RI procedure with a MS4000 biological signal record and analysis system. The model was considered successfully established when the ST-segment elevated during the MI period and dropped at least 50% during the reperfusion period.²⁴

Measurement of CK-MB activity and NO content in serum

After reperfusion, blood samples were collected and centrifuged at 3,000× rpm for 15 minutes. Serum CK-MB and NO levels were measured by a colorimetric method using commercial kits (Jiancheng Bioengineering Institute), according to the manufacturer's protocols.

Determination of MPTP and TNF-α levels

Blood samples from the abdominal aorta were collected after reperfusion. Serum levels of TNF-α and MPTP were determined according to the methods described in the manual for the enzyme-linked immunosorbent assay kits (Chenglin Biological Technology Co. Ltd., Beijing, People's Republic of China).

TUNEL assay

After reperfusion, the heart was removed as previously described.²⁵ Cardiomyocyte apoptosis was determined using an In Situ Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Briefly, tissue sections were washed in phosphate-buffered saline and then fixed in a 4% paraformaldehyde solution before incubation in 20 µg/mL proteinase K for 15 minutes. After washing with phosphate-buffered saline, the tissue sections were immersed in the TUNEL reaction mixture for 1 hour at 37°C in a humid chamber. The reaction was terminated by transferring the slides to a 2× sodium citrate saline solution. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide. Finally, streptavidin horseradish peroxidase was bound to the biotinylated nucleotides and the peroxidase activity was visualized in each section by the application of the stable chromogen, diaminobenzidine. In this method, the apoptotic nuclei were stained dark brown. Normal nuclei were stained blue with hematoxylin. Five sections from each myocardial sample were randomly selected and ten microscopic fields per section (BX53F microscope; Olympus Corporation, Tokyo, Japan) were evaluated by two independent double-blind observers. In each field, the nuclei were counted and the percentage of TUNEL-positive nuclei was calculated.

RNA extraction and fluorescence quantitative polymerase chain reaction analysis

Total RNA was extracted by using RNAiso Plus kits (Takara Biotechnology Co., Ltd). RNA quality was assessed by electrophoresis on 1% agarose gels based on the integrity of the 28S, 18S, and 5S bands after ethidium bromide staining. cDNA was synthesized from RNA with a PrimeScript™ RT reagent Kit with a cDNA Eraser (Takara Biotechnology Co., Ltd), according to the manufacturer's instructions. Fluorescence quantitative polymerase chain reaction (FQ-PCR) was performed on a ABI Prism 7300 real-time thermocycler (Applied Biosystems, Foster City, CA, USA). For FQ-PCR, each reaction was run in triplicate and contained the following:

10.0 μ L of Premix Ex Taq II (Tli RNaseH Plus, 2 \times Conc., Takara Biotechnology Co., Ltd), 0.8 μ L of forward primer and reverse primer (2.5 μ M), 0.4 μ L of ROX Reference Dye (50 \times Conc., Takara Biotechnology Co., Ltd), and 100 ng of cDNA template, in a final reaction volume of 20 μ L.

After a preincubation step at 95°C for 30 seconds to activate DNA polymerase, PCR products were amplified in 40 denaturation cycles at 95°C for 5 seconds and annealed at 60°C for 31 seconds. After amplification, melting curves were generated in the range 65°C–95°C with increments of 0.5°C every 5 seconds. The relative expression level of each target gene was normalized by using the comparative cycle threshold (CT) method with glyceraldehyde-3-phosphate dehydrogenase as an endogenous reference. The amount of gene expression was normalized by the $2^{-\Delta\Delta CT}$ relative quantitative method. The information for the PCR primers is given in Table 1.

Western blotting

At the end of reperfusion, a piece of ~70 mg of myocardial tissue was taken from the infarct area of the left ventricle. After homogenization and protein quantification, equal amounts of protein from each sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride-plus membranes. After blocking with 5% bovine serum albumin, the membranes were incubated overnight at 4°C with the following primary antibodies: PI3K (p110 α , 1:1,000), Akt (1:2,000), phospho-Akt (Ser473, 1:1,000), eNOS (1:2,000), phospho-eNOS (Ser1177, 1:1,000), Beclin1 (1:1,000), LC3-II (1:1,000), caspase-3

(1:1,000), and glyceraldehyde-3-phosphate dehydrogenase (1:500). Then, the membranes were washed three times in Tris-buffered saline with 0.1% Tween 20 and incubated with the corresponding secondary antibody (1:5,000) conjugated to horseradish peroxidase at room temperature for 2 hours. Finally, the membranes were washed three times in Tris-buffered saline with 0.1% Tween 20. Relative densitometry was performed by using a computerized software package (NIH Image 1.63 software, Image J 3.0; National Institutes of Health, Bethesda, MA, USA).

Statistical analysis

Each sample was assayed in triplicate. The results were averaged and expressed as the mean \pm standard deviation and data were evaluated using the Sigma Stat (version 21.0) statistical analysis program (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance followed by Bonferroni's multiple comparison test was used for statistical analysis. *P*-values <0.05 were considered statistically significant.

Results

Effect of BCF on the CK-MB and NO content in serum

As a consequence of MI/RI, the serum CK-MB level was significantly higher in the model group than in the sham group ($P < 0.01$) and was reduced remarkably by BCF pretreatment ($P < 0.05$). In addition, the NO content was conspicuously elevated in the BCF group compared to the model group ($P < 0.05$). However, in the presence of WM, a specific PI3K/Akt pathway inhibitor, the effects of BCF were almost completely abolished ($P < 0.05$), while WM alone had no significant effect on the serum levels of CK-MB and NO compared with the model group ($P > 0.05$) (Figure 1).

Effect of BCF on TNF- α and MPTP levels

As shown in Figure 2, rats exposed to MI/RI had higher serum concentrations of TNF- α and MPTP than those in the sham group ($P < 0.05$ or $P < 0.01$). As expected, pretreatment with BCF significantly inhibited the elevation of TNF- α and MPTP ($P < 0.05$), but WM reversed these changes ($P < 0.05$). Additionally, the levels of TNF- α and MPTP were not significantly different among the WM, BCF + WM, and model groups ($P > 0.05$) (Figure 2).

Effect of BCF on cardiomyocyte apoptosis

TUNEL staining showed that apoptosis was absent in the sham group. Conversely, the model group exhibited

Table 1 Description of FQ-PCR primers

| Gene | Sequence |
|------------------|-----------------------------|
| <i>PI3K</i> | |
| Sense | 5'GCATCAGTGGCTCAAGGACAAG3' |
| Antisense | 5'CAAGATAAAGGTTGCCACGCAGT3' |
| <i>eNOS</i> | |
| Sense | 5'CTGTGTGACCCTCACCGATACAA3' |
| Antisense | 5'AGCACAGCCACGTTAATTTCCA3' |
| <i>Caspase-3</i> | |
| Sense | 5'GAGACAGACAGTGGAACTGACG3' |
| Antisense | 5'GGCGCAAAGTGACTGGATGA3' |
| <i>Beclin1</i> | |
| Sense | 5'GAAACTGGACACGAGCTTCAAGA3' |
| Antisense | 5'ACCATCCTGGCGAGTTTCAATA3' |
| <i>GAPDH</i> | |
| Sense | 5'GGCACAGTCAAGGCTGAGAATG3' |
| Antisense | 5'ATGGTGGTGAAGACGCCAGTA3' |

Abbreviations: eNOS, endothelial nitric oxide synthase; FQ-PCR, fluorescence quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3K, phosphoinositide 3-kinase.

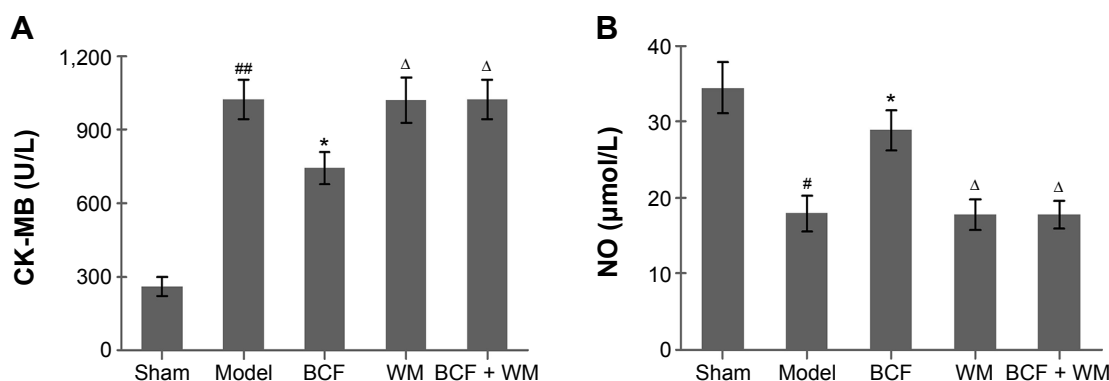


Figure 1 Effect of BCF on the CK-MB and NO content in serum.

Notes: Values of (A) CK-MB and (B) NO are expressed as the mean \pm SD ($n=8$, each group), respectively. ^{*} $P<0.05$ vs model group; [#] $P<0.05$; ^{##} $P<0.01$ vs sham group; ^Δ $P<0.05$ vs BCF group.

Abbreviations: BCF, *Bauhinia championii* flavone; CK-MB, creatine kinase-MB; NO, nitric oxide; SD, standard deviation; WM, wortmannin.

severe tissue damage that appeared to dramatically increase the number of TUNEL-positive cells ($P<0.01$ vs sham). Interestingly, pretreatment with BCF resulted in a marked reduction of TUNEL-positive cells compared with the model group ($P<0.01$) but this effect was largely alleviated after coadministering WM ($P<0.01$). There was no significant difference in the TUNEL-positive cell number among the BCF + WM, WM, and the model group ($P>0.05$) (Figure 3).

Effect of BCF on the messenger RNA expressions of PI3K, eNOS, caspase-3, and Beclin I

Obvious decreases in *PI3K* and *eNOS* messenger RNA (mRNA) levels, and increases in *caspase-3* and *Beclin1* mRNA levels were detected in the model group ($P<0.05$ or $P<0.01$). Compared with the model group, BCF preconditioning markedly upregulated *PI3K* and *eNOS*, but

downregulated *caspase-3* and *Beclin1* expression ($P<0.05$ or $P<0.01$). However, these effects of BCF were reversed by the addition of WM ($P<0.05$ or $P<0.01$). Thus, no significant difference among the BCF + WM, WM, and model groups with regard to these genes was detected ($P>0.05$) (Figure 4).

Effect of BCF on the expression of caspase-3, Beclin I, and LC3-II protein

As shown in Figure 5, the expression of caspase-3, Beclin1, and LC3-II proteins in the model group was remarkably higher compared with the sham group ($P<0.01$). However, the increased expression of caspase-3, Beclin1, and LC3-II proteins was effectively inhibited by BCF ($P<0.01$). Furthermore, coadministration of WM abrogated the above effects of BCF ($P<0.01$); no significant difference was found in the expression of these proteins among the BCF + WM, WM, and model groups ($P>0.05$).

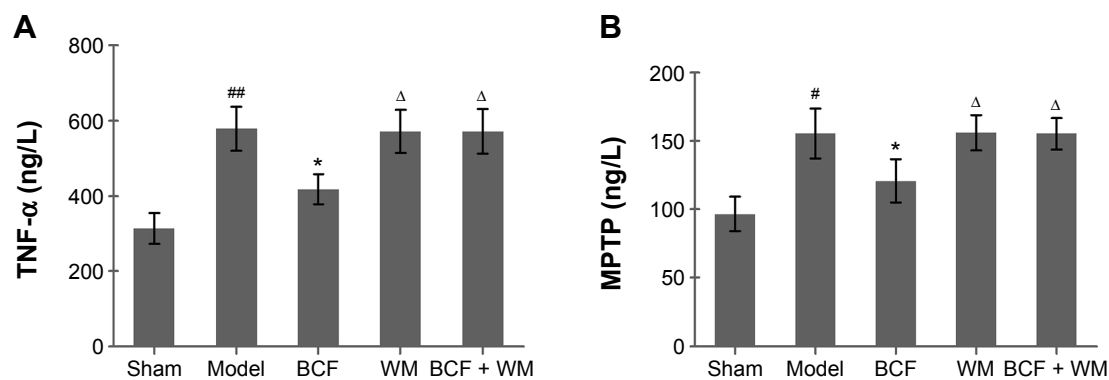


Figure 2 Effect of BCF on the levels of TNF- α and MPTP.

Notes: Statistical results from ELISA for (A) TNF- α , and (B) MPTP. Values are expressed as the mean \pm SD ($n=8$, each group). ^{*} $P<0.05$ vs model group; [#] $P<0.05$; ^{##} $P<0.01$ vs sham group; ^Δ $P<0.05$ vs BCF group.

Abbreviations: BCF, *Bauhinia championii* flavone; ELISA, enzyme-linked immunosorbent assay; MPTP, mitochondrial permeability transition pores; SD, standard deviation; TNF- α , tumor necrosis factor alpha; WM, wortmannin.

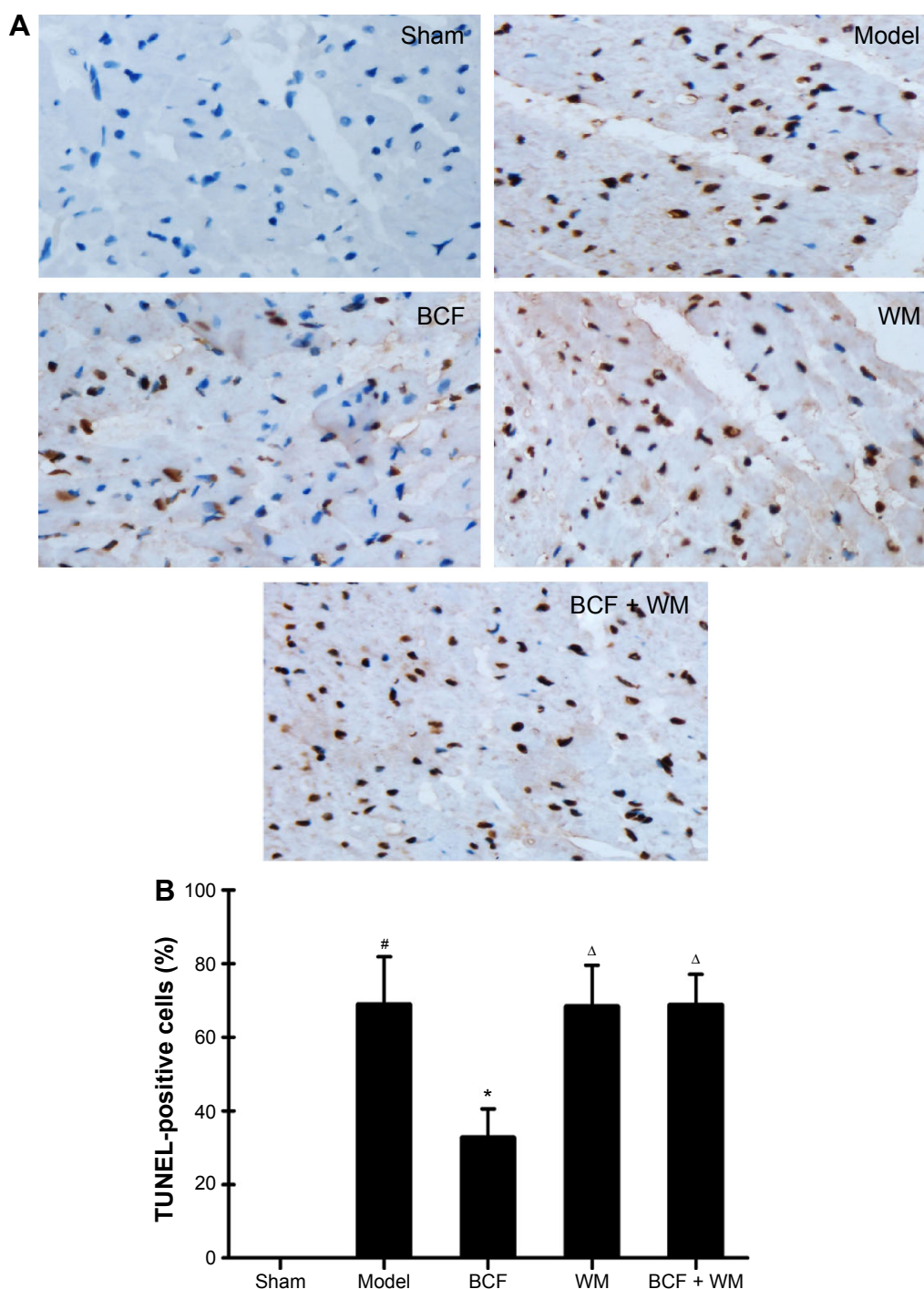


Figure 3 Effect of BCF on myocardial apoptosis.

Notes: (A) Representative photomicrographs of TUNEL staining ($\times 400$). Apoptotic cardiomyocyte nuclei appear brown stained whereas normal nuclei appear blue. (B) Quantitative analysis of percentage of TUNEL-positive cells. Values represent mean \pm SD ($n=8$, each group). $^*P<0.01$ vs model group; $^{\#}P<0.01$ vs sham group; $^{\Delta}P<0.01$ vs BCF group.

Abbreviations: BCF, *Bauhinia championii* flavone; SD, standard deviation; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; WM, wortmannin.

Effects of BCF on the expression of PI3K, Akt, and eNOS proteins

In order to elucidate the role of the PI3K/Akt signaling pathway in the protective effect of BCF on MI/RI, the expression of PI3K, Akt, and eNOS proteins was further investigated

(Figures 6 and 7). Compared to the sham group, a significant decrease in the levels of PI3K, phosphorylated Akt (p-Akt), and phosphorylated eNOS (p-eNOS) was noted in the model group ($P<0.01$) but a remarkable increase in these proteins occurred in the BCF group ($P<0.05$). The upregulating

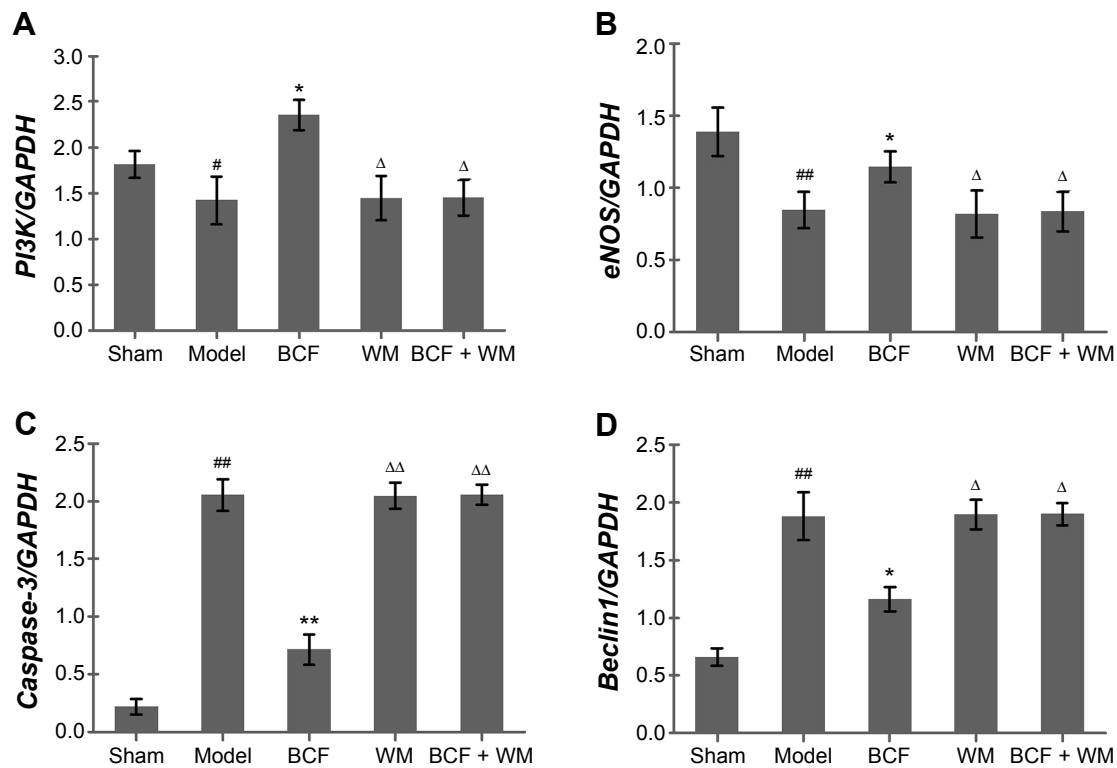


Figure 4 Effect of BCF on mRNA expression of *PI3K*, *eNOS*, *caspase-3*, and *Beclin1* in rats.

Notes: The relative levels of **(A)** *PI3K*, **(B)** *eNOS*, **(C)** *caspase-3*, and **(D)** *Beclin1* mRNA were assessed by FQ-PCR. Results were normalized to *GAPDH*. Values represent mean \pm SD (n=8, each group). * $P < 0.05$, ** $P < 0.01$ vs model group; # $P < 0.05$, ## $P < 0.01$ vs sham group; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$ vs BCF group.

Abbreviations: BCF, *Bauhinia championii* flavone; *eNOS*, endothelial nitric oxide synthase; FQ-PCR, fluorescence quantitative polymerase chain reaction; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; *PI3K*, phosphoinositide 3-kinase; SD, standard deviation; WM, wortmannin.

effect of BCF was blocked by the coadministration of WM ($P < 0.05$), while the protein levels of total-Akt and total-eNOS remained unchanged in all groups.

Discussion

It is well known that MI/RI causes a high proportion of cardiac dysfunction and heart failure and that no effective treatment is available to prevent this damage.² In this study, we demonstrated that BCF had a protective effect on MI/RI via the inhibition of apoptosis and excessive autophagy in a PI3K/Akt-dependent manner.

Accumulating evidence suggests that myocardial apoptosis is initiated shortly after ischemia, is amplified by reperfusion and partially contributes to cardiomyocyte death during MI/RI.²⁶ Therefore, blocking the apoptotic process may reduce the loss of contractile cells, minimizing MI/RI.²⁷ In mammalian cells, apoptosis can occur by activating mitochondria or extrinsic apoptotic pathways.²⁸ A key target in the mitochondrial apoptosis pathway, MPTP is a large, nonselective conductance pore located in the inner mitochondrial membrane. Opening of mPTP can cause mitochondrial swelling and the subsequent rupture of the outer membrane, activate

the caspase family proteins and release pro-apoptotic proteins leading to apoptotic cell death.²⁹ Research has shown that MI/RI promotes MPTP opening especially during reperfusion, mainly by increasing Ca^{2+} in the intracellular matrix and by increasing the levels of reactive oxygen species.²⁹ Our previous studies have demonstrated that BCF could elevate ATPase activity, inhibit calcium sensing receptor mRNA expression, scavenge oxygen free radicals, enhance antioxidative capability, and inhibit apoptosis in an MI model.^{21,22} On the other hand, p-Akt can activate eNOS and/or the production of NO to prevent the opening of MPTP, thereby inhibiting apoptosis.³⁰ As a pivotal modulator of biological systems, NO is involved in the mitochondrial pathway of apoptosis and plays an important role in cardioprotection.³¹ Three members of the NO synthase (NOS) family are responsible for the synthesis of NO, including neuronal NOS, inducible NOS, and eNOS. The major source of cardiac NO generated by eNOS can exert a potent anti-inflammatory and antiapoptotic effect in MI/RI.³² Our data demonstrated that pretreatment with BCF significantly upregulated phosphorylated eNOS and p-Akt protein expression, increased the NO content, and decreased the MPTP level. These findings suggested that the

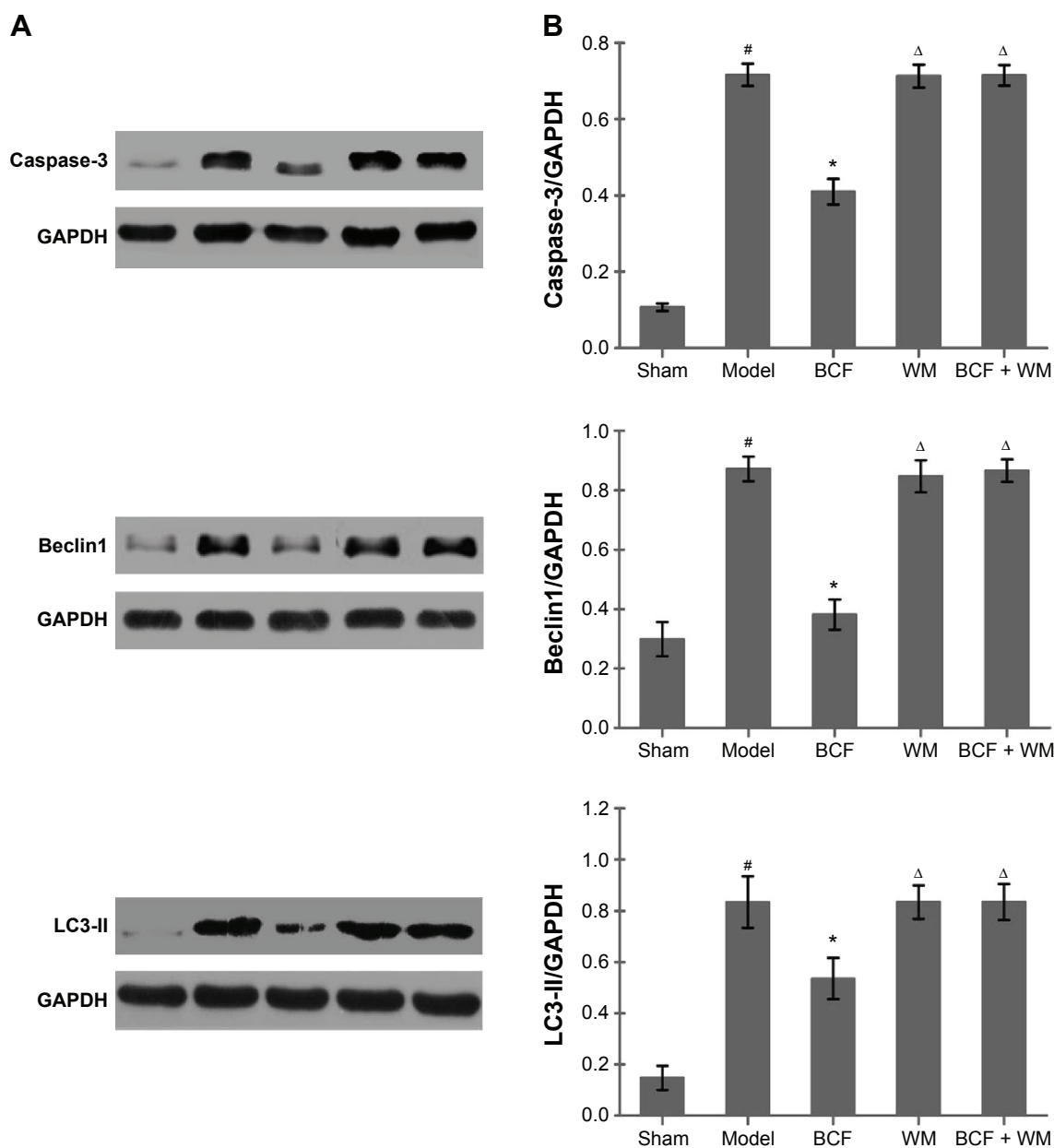


Figure 5 BCF decreases myocardial caspase-3, Beclin I, and LC3-II expression.

Notes: (A) Representative graphs for the expression of caspase-3, Beclin I, and LC3-II proteins as determined by Western blot. (B) quantitative analysis demonstrated the levels of caspase-3, Beclin I, and LC3-II. Results were normalized to GAPDH. Values represent the mean \pm SD (n=4, each group). * $P < 0.01$ vs model group; [#] $P < 0.01$ vs sham group; ^Δ $P < 0.01$ vs BCF group.

Abbreviations: BCF, *Bauhinia championii* flavone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC3-II, microtubule-associated protein I light chain 3-II; SD, standard deviation; WM, wortmannin.

inhibition of MPTP opening in the myocardium might be at least partially involved in the antiapoptosis mechanisms of BCF in MI/RI.

TNF- α may bind to a TNF-receptor and induce apoptosis via activation of the extrinsic apoptotic pathway.²⁸ Many reports have demonstrated that TNF- α is involved in cardiomyocyte apoptosis, as indicated by a constantly high concentration.³³ The results in this study also showed a remarkably higher concentration of TNF- α in the model

group than that in the sham group, whereas pretreatment of BCF decreased the concentration of TNF- α .

The caspase family is another pathway that induces apoptosis. Caspase-3 is a pivotal protease that directly leads to apoptotic cell disintegration, cell protein dissolution, and enzyme and DNA degradation.³⁴ The PI3K/Akt signaling pathway is a classical pro-survival and antiapoptosis pathway.¹³ As a key component of the RI salvage kinase pathway, PI3K/Akt is involved in cardiac protection against MI/RI.

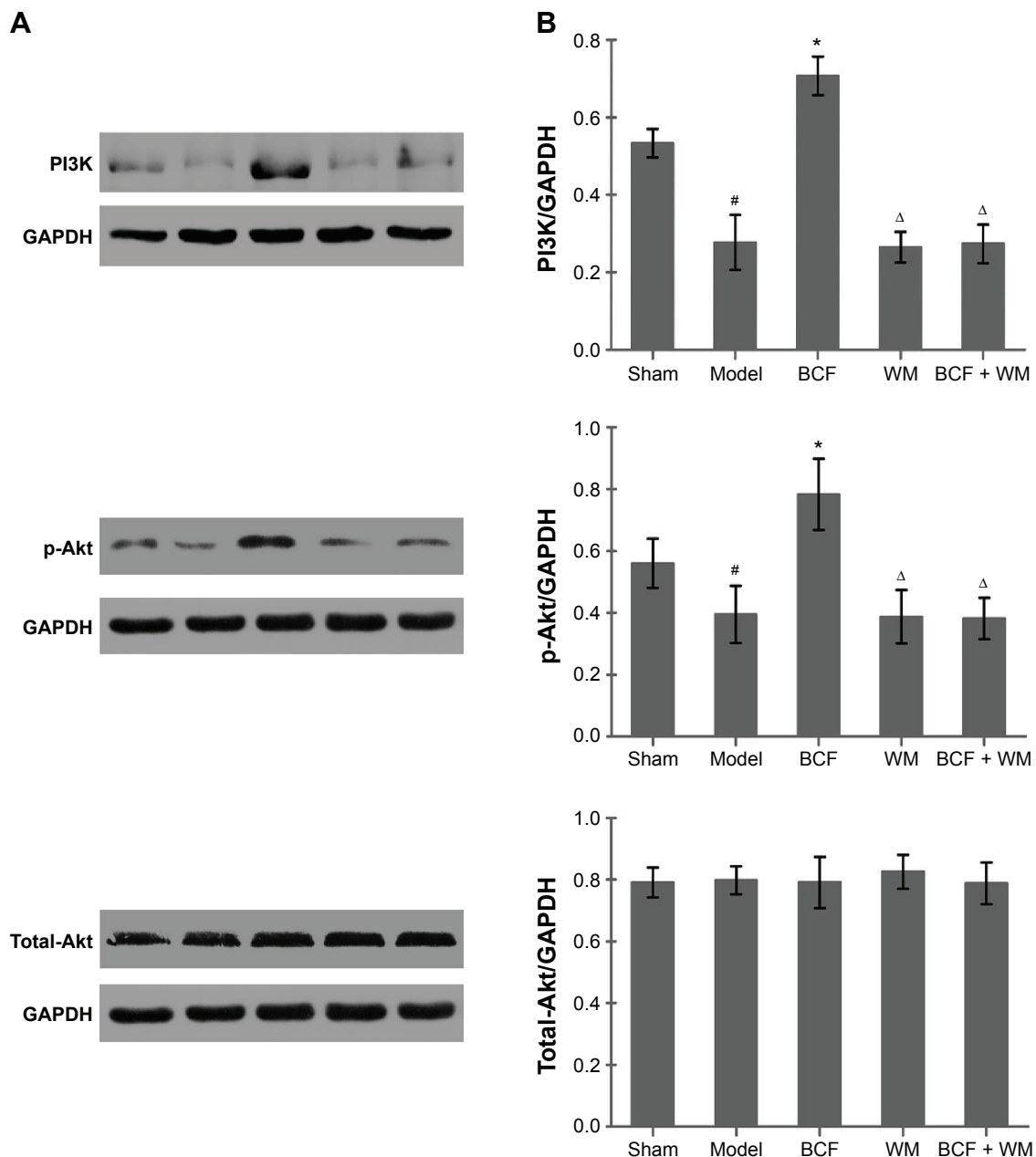


Figure 6 BCF regulates PI3K/Akt activation.

Notes: (A) Representative graphs for PI3K, p-Akt, and total-Akt protein expression by Western blot; (B) quantitative analysis demonstrated the levels of PI3K, p-Akt, and total-Akt. Results were normalized to GAPDH. Values represent mean \pm SD (n=4, each group). * P <0.05 vs model group; [#] P <0.01 vs sham group; ^Δ P <0.05 vs BCF group.

Abbreviations: BCF, *Bauhinia championii* flavone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; p-Akt, phosphorylated Akt; PI3K, phosphoinositide 3-kinase; SD, standard deviation; WM, wortmannin.

Activation of the PI3K/Akt signaling pathway leads to Akt phosphorylation at the Ser473 locus, producing p-Akt, and stimulates downstream targets such as MPTP, TNF- α , NF- κ B, eNOS, the Bcl-2 family proteins, and the caspase family proteins; this then regulates apoptosis, adjusts transcription factors, affects metabolism, enhances cardiomyocyte survival, and reduces the morbidity and mortality of MI/RI.^{30,35-37} As shown in Figures 5 and 6, compared to the model group, BCF pretreatment significantly increased the

expression of PI3K and p-Akt, and decreased the expression of caspase-3. Additionally, BCF reduced the MI marker CK-MB activity (Figure 1). Therefore, we deduced that BCF might protect ischemia of the myocardium by inhibiting apoptosis, via the PI3K/Akt activation.

In addition to apoptosis, autophagy is another major factor leading to cell death in MI/RI, and LC3-II is a recognized marker of autophagy.¹⁰ Autophagy that occurs during the ischemia phase is beneficial; however, excessive

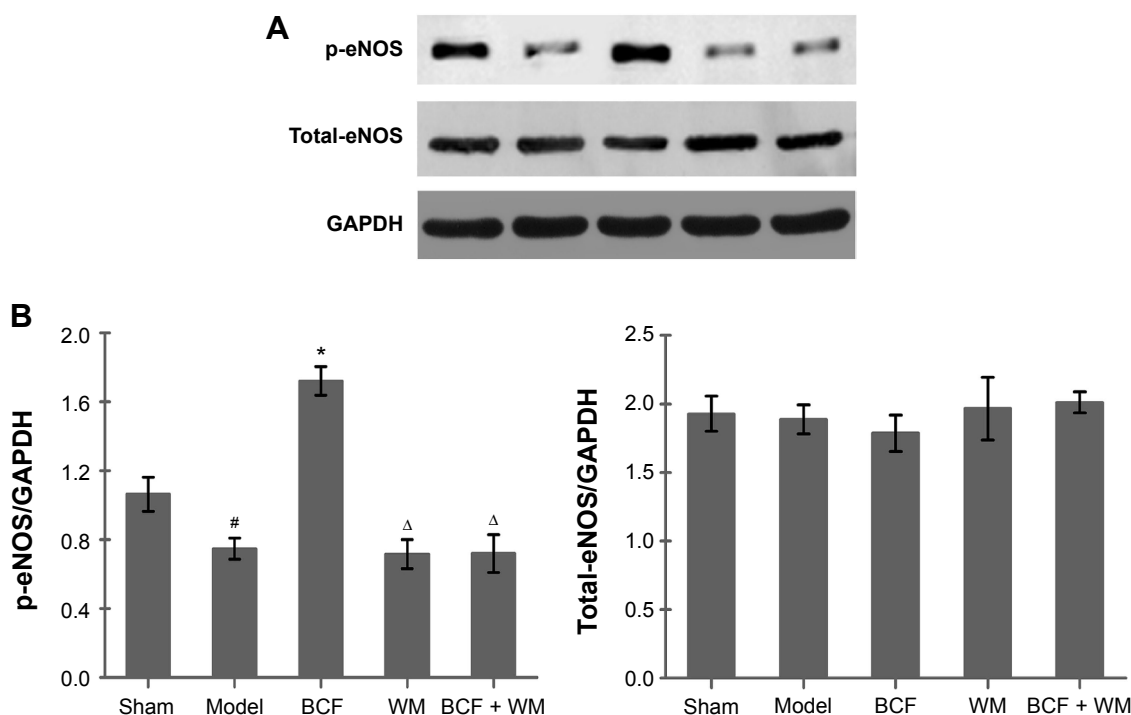


Figure 7 BCF increases eNOS phosphorylation in response to MI/RI.

Notes: (A) Representative graphs for the expression of total-eNOS and p-eNOS proteins as determined by Western blot; (B) quantitative analysis demonstrated the levels of total-eNOS and p-eNOS. Results were normalized to GAPDH. Values represent mean \pm SD (n=4, each group). * P <0.05 vs model group; [#] P <0.01 vs sham group; ^Δ P <0.05 vs BCF group.

Abbreviations: BCF, *Bauhinia championii* flavone; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MI/RI, myocardial ischemia/reperfusion injury; p-eNOS, phosphorylated eNOS; SD, standard deviation; WM, wortmannin.

autophagy during reperfusion induces a progressive consumption of cellular constituents and leads ultimately to autophagic cell death.¹⁰ Beclin1 is a homologue of the protein product of yeast autophagy gene 6 (*Atg6*) that binds to class III PI3Ks, participates in autophagosome formation, and further mediates the localization of other autophagy proteins to the preautophagosomal membrane.³⁸ Many studies have shown that the autophagy-promoting activity of Beclin1 is suppressed by antiapoptotic members of the Bcl-2 family via direct binding, which means that Beclin1-mediated autophagy can be negatively regulated by Bcl-2, thus indicating that cross-talk exists between autophagy and apoptosis.³⁹ Our previous study demonstrated that BCF could inhibit apoptosis by upregulating Bcl-2 expression.²¹ In this study, we observed that BCF pretreatment upregulated PI3K expression and downregulated Beclin1 expression, suggesting that BCF might simultaneously inhibit apoptosis and excessive autophagy via the modulation of Beclin1/Bcl-2 expression. However, further studies are required to elucidate the mechanisms underlying this phenomenon.

In particular, the coadministration of WM reversed the effects of BCF. We also noticed that compared to the

model group, pretreatment with BCF enhanced the protein expression of p-Akt but the total-Akt was not notably changed. As a specific inhibitor of the PI3K/Akt pathway, WM blocked Akt phosphorylation, further preventing BCF from affecting downstream targets. Thus, we can infer that the PI3K/Akt pathway is involved in the mediation of the cardioprotection of BCF in MI/RI.

Conclusion

This study indicated for the first time that BCF alleviated MI/RI in rats, possibly by inhibiting apoptosis and excessive autophagy, and the cardioprotection required the activation of the PI3K/Akt pathway.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version

to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

The extraction and isolation of *Bauhinia championii* flavone

Dried *Bauhinia championii* (Benth.) Benth. (10 kg) plants were crushed, and extracted three times with 78 L 70% ethanol for 72 hours each time. The filtered solvents were combined and concentrated under reduced pressure to yield 1.7 L of crude extract. After washing with 3 L of petroleum ether to remove pigments, the crude extracts were further partitioned with 4.5 L of ethyl acetate. The ethyl acetate layer was concentrated and dried under a vacuum to obtain a dry residue (100 g). This dry residue was fractionated by column chromatography using a silica gel H-packed column (10×120 cm) with a gradient mixture of chloroform and methyl alcohol (0%–100% methyl alcohol, 2,500 mL each fraction). Then, all the fractions were combined and thin layer chromatography analysis was performed, yielding eight fractions (Fractions 1–8). The antioxidative activities of all prefractionations were evaluated as previously reported¹ in order to select for the further purified constituents. Fraction 2 (ie, *Bauhinia championii* flavones [BCF]) showed a strong antioxidative effect. With rutin as

a reference substance, the total flavonoid content of BCF was 82%.

Fraction 2 (2.3 g) was further fractionated by column chromatography (Agilent ZORBAX SB-C18, 250×10 mm, 5 μm) and eight subfractions were obtained as follows: Fraction 2-1 (18.5 mg): mobile phase: 20% methyl alcohol at 3 mL/minute; Fraction 2-2 (4.8 mg): mobile phase: 23% methyl alcohol at 3 mL/minute; Fraction 2-3 (4.2 mg) and Fraction 2-8 (6.6 mg): mobile phase: 18% methyl alcohol at 3 mL/minute; Fraction 2-4 (25.2 mg): mobile phase: 20% methyl alcohol at 3 mL/minute; Fraction 2-5 (4.1 mg) and Fraction 2-6 (4.7 mg): mobile phase: 23% methyl alcohol at 3 mL/minute; Fraction 2-7 (6.3 mg): mobile phase: 16% methyl alcohol at 3 mL/minute.

Eight chemical constituents were purified from BCF for the first time: catechin (**1**), (-)-epicatechin (**2**), catechin-3-*O*- α -L-rhamnopyranoside (**3**), (-)-epigallo-catechin-3-*O*-gallate (**4**), dimethyl(R)-hexahydroxydiphenoate (**5**), 4-hydroxy-3-methoxyphenyl-1-*O*-(6'-*O*-galloyl)- β -D-glucopyranoside (**6**), 3,5-dimethoxy-4-hydroxyphenyl-1-*O*- β -D-(6'-*O*-galloyl)-glucopyranoside (**7**), rocymsin A (3,4-dihydroxyphenethyl alcohol 4-*O*-D-(6''-*O*-galloyl)-glucopyranoside) (**8**).

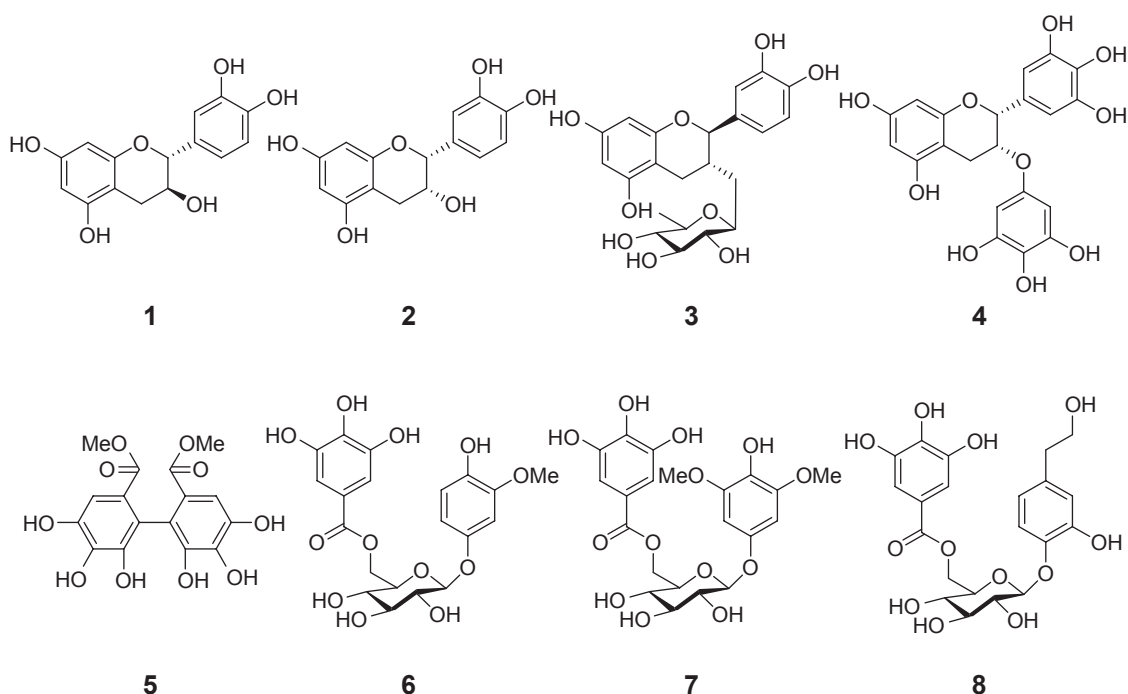


Figure S1 The chemical structure of BCF constituents.
Abbreviation: BCF, *Bauhinia championii* flavone.

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