

## Original Article

# Protective effect of apigenin against myocardium injury in ischemia reperfusion rats by stimulating JAK2/STAT3 signal pathway

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Received March 27, 2017; Accepted December 1, 2017; Epub January 15, 2018; Published January 30, 2018

**Abstract:** The present study aimed to assess the effects and mechanisms of apigenin in the rat model of myocardial ischemia reperfusion injury. The rat hearts were exposed to the left anterior descending coronary artery (LAD) ligation for 30 min followed by 1 h of reperfusion. In the rat of myocardial ischemia/reperfusion (I/R), it was found that apigenin pretreatment reduced myocardial infarct size, improved the heart rate, and decreased creatine kinase (CK) and lactate dehydrogenase (LDH) levels in coronary flow. This pretreatment also increased catalase (CAT), superoxide dismutase (SOD) activities, but decreased glutathione (GSH), malondialdehyde (MDA) levels. Further more, we determined that apigenin can attenuate serum interleukin-6 (IL-6), interleukin-1 beta (IL-1 $\beta$ ) and tumour necrosis factor (TNF- $\alpha$ ) levels. The enhanced phosphorylation of JAK2 and STAT3 was further strengthened by apigenin (2, 4 mg/kg/) in a dose-dependent manner. These results presented here demonstrated that apigenin intake might reduce the risk of coronary heart disease by stimulating JAK2/STAT3 signal pathway, decreasing oxidative damage.

**Keywords:** Apigenin, antioxidant, signal pathway

## Introduction

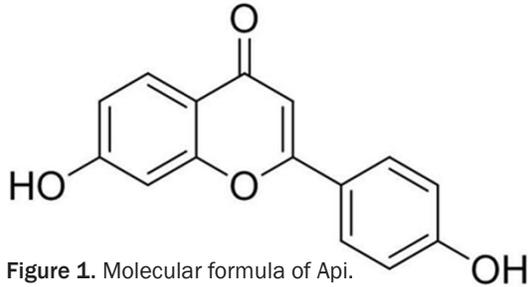
Myocardial ischemia-reperfusion is the leading cause for the events of cardiovascular disease, and is considered as a major contributor to the morbidity and mortality associated with coronary occlusion. The myocardial damage caused by ischemia-reperfusion injury constitutes the primary pathological manifestation of coronary artery disease. It results from the interaction between the substances that accumulate during ischemia and those that are delivered on reperfusion. The level of this damage can range from a small insult resulting in limited myocardial damage to a large injury culminating in myocyte death. Importantly, major ischemia-reperfusion injury to the heart can result in permanent disability or death [1-3].

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway transduces cellular signals from the plasma membrane to the nucleus and plays a vital role in mediating cardioprotection against ischemia/reperfusion injury. Several studies have dem-

onstrated that the Janus kinase 2 (JAK2)/STAT3 signaling pathway is significant in mediating an inflammatory response [4-6]. STAT3 is positioned downstream of JAK2 and can be activated via the phosphorylation of serine (Ser) 727 and tyrosine (Tyr) 705. Once activated, STAT3 is translocated into the nucleus to regulate target genes.

Apigenin (4,5,7-trihydroxyflavone or Api) (as shown in **Figure 1**) is a non-mutagenic flavone subclass of flavonoids exhibiting low levels of toxicity, which is isolated from the leaves of *Apiumgraveolens L. var. dulce* DC (a traditional Chinese medicinal herb). Api is also present in a variety of shrubs, vegetables, plants, fruits and herbs, a number of which are widely marketed as dietary and herbal supplements [7-9]. Previous studies have demonstrated that Api possesses a wide range of biological activities, including anticarcinogenic, antiviral, antibacterial, antioxidant and anti-inflammatory effects [10-13]. Following the ingestion of Api with food, Api becomes widely distributed in various tissues and provides several protective effects. A previous study demonstrated that Api protects

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**Figure 1.** Molecular formula of Api.

the endothelium-dependent relaxation of the rat aorta against oxidative stress [14]. In addition, the intake of Api-rich foods can significantly increase the levels of antioxidant enzymes *in vivo* [15], and Api is correlated with a reduced incidence of cardiovascular disease [16]. Since little is known about the effects of Api on JAK2 and STAT3, we performed this study to determine whether Api protected against myocardial ischemia/reperfusion injury via the JAK2/STAT3 signaling pathway.

### Materials and methods

#### Materials

Api was provided by the National Institutes for the Control of Pharmaceutical and Biological Products (Beijing, China), and its purity was identified by high performance liquid chromatography (HPLC). Lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA), superoxide dismutase (SOD) and superoxide generation assay kits were purchased from the Institute of Jiancheng Bioengineering (Nanjing, China). The enzyme-linked immunosorbent assay (ELISA) kits for determination of IL-6, IL-1 $\beta$  and TNF- $\alpha$  were produced by Nanjing KeyGEN Biotech. The primary antibodies against JAK2, p-JAK2, STAT3, p-STAT3, caspase-3, Bcl-2, Bax, and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

#### Experimental protocol

Sprague-Dawley rats (10 rats in each group) were purchased from Shanghai Slac Laboratory Animal Ltd. (Shanghai, China). All animals were housed with free access to water and food with a 12 h light/dark cycle at the constant temperature. Rats were acclimated for 7 days before any experimental procedures. The I/R injury animal model was established by the left anterior descending (LAD) coronary artery ligation

for 30 min followed by 1 h reperfusion. Briefly, after being anesthetized with a 30 mg/kg pentobarbital sodium intraperitoneally, rats were ventilated with a positive pressure respirator at a stroke volume of 12 mL/kg and a rate of 60 strokes per minute with 95% O<sub>2</sub> and 5% CO<sub>2</sub> throughout the experiment. The rat heart was exposed through a left thoracotomy. Then, the LAD was ligated 2-3 mm from its origin and loosened to simulate I/R rat model (ischemia for 30 min and reperfusion for 1 h). Rats were randomly apportioned in equal numbers to four experimental groups: (1) sham group; (2) model group and (3) I/R + Api (2 mg/kg) group, I/R + Api (4 mg/kg) group. In Api treatment groups, rats were administered with Api (2 or 4 mg/kg) by oral gavage for 20 d before IR operation.

#### Evaluation of myocardial infarct area

The slipknot around the LAD coronary artery was retied at the end of the reperfusion, and 1 mL of 1% Evans blue dye was injected into the aortic artery. The heart was then quickly excised and frozen at -80°C. After that, the frozen heart was sliced transversally into 1-mm thick sections and then incubated at 37°C for 30 min in 2% TTC solution as described in our previous studies. Then, digital images were captured and analyzed. The sizes of the areas of infarct (INF) and areas at risk (AAR) were measured digitally by using Image-ProPlus software.

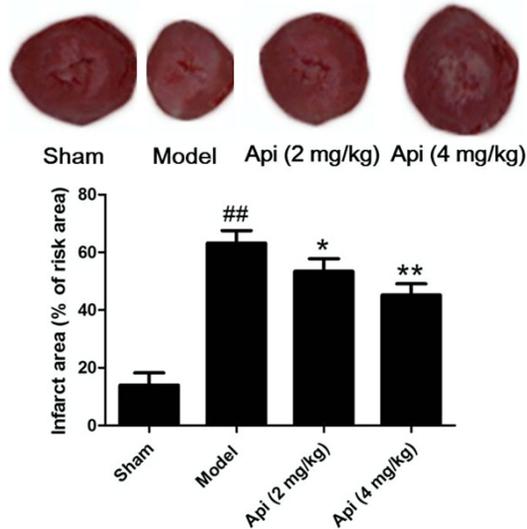
#### Histopathologic examination of heart

To investigate the effects of Api on the protecting from the myocardial ischemia-reperfusion injury, hematoxylin-eosin staining (HE) was performed on heart tissues. 5  $\mu$ m sections were obtained, stained with HE, and observed the pathological changes in the heart tissues under a light microscopy and photomicrographs were taken.

#### Determination of creatine kinase (CK) and lactate dehydrogenase (LDH)

After the end of the reperfusion, blood samples (0.5 mL) were drawn and total serum lactate dehydrogenase (LDH) and creatine kinase (CK) activities were measured spectrophotometrically in a blinded manner. All kits for measuring LDH and CK activities were purchased from the Institute of Jiancheng Bioengineering (Nanjing, China).

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**Figure 2.** Effect of Api on myocardial damage in rats subjected to MI/R. TTC staining to assess the extent of myocardial necrosis. Bars represent the percent of ischemic area at risk in hearts. ## $P < 0.01$  compared with the sham group; \* $P < 0.05$ , \*\* $P < 0.01$  compared with the model group.

### Detection of oxidative stress biomarkers

SOD activity, MDA level, CAT activity, and GSH level in heart homogenates were determined according to the instructions recommended by the manufacturers.

### Detections of TNF- $\alpha$ , IL-6 and IL-1 $\beta$ in serum by ELISA

The levels of inflammatory cytokines, such as IL-6, IL-1 $\beta$  and TNF- $\alpha$  in the serum samples were quantified using specific ELISA kits for rat according to the manufacturers' instructions.

### Western blotting

The heart tissue was chopped into pieces and homogenized in ice cold RIPA (Radio Immunoprecipitation Assay) buffer, containing protease inhibitor. The supernatant was collected after centrifugation at 12,000 rpm for 20 min. The protein concentration was determined by using the BCA (bicinchoninic acid) protein assay kit. Protein extracts were loaded and separated by an SDS (sodium dodecylsulfate)-polyacrylamide gel electrophoresis, and the resolved proteins were transferred onto PVDF membranes. The membranes were blocked with 5% skim milk in Trisbuffer saline and incubated at 4°C overnight with separate primary antibodies,

anti-Caspase-3 (1:1000), anti-Bax (1:1000), anti-Bcl-2 (1:1000), anti-JAK2 (1:500), anti-p-JAK2 (1:500), anti-STAT3 (1:1000), anti-p-STAT3 (1:1000) and anti- $\beta$ -actin (1:1000). After washing three times with Tris-buffered saline-Tween-20, the membranes were incubated with secondary antibody (1:12,000) for 1.5 h at room temperature. The bands were visualized by using enhanced chemiluminescence detection reagents and a gel imaging system.

### Statistical analysis

All values were expressed as the mean  $\pm$  S.D. and analyzed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test using SPSS version 13.0 software; a  $P$ -value of less than 0.05 was considered significant and  $P < 0.01$  was considered to be statistically very significant.

## Results

### Api reduced the myocardial infarction area induced by I/R

Myocardial infarct area was significantly increased in model group compared with the sham group. In contrast, this effect was markedly diminished by pretreatment with Api, particularly at the high dose (**Figure 2**).

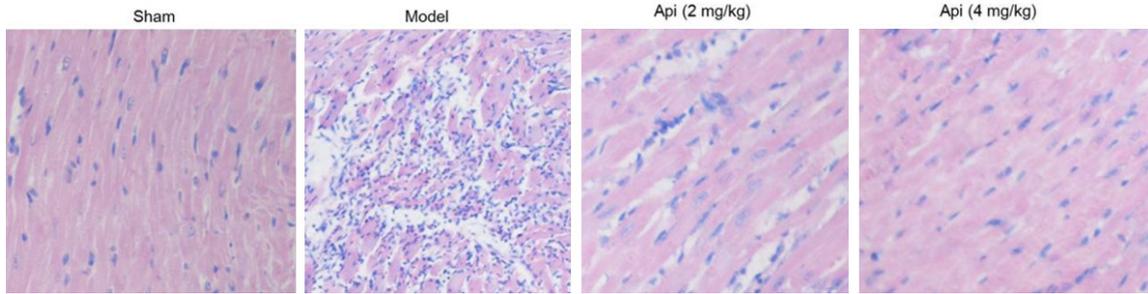
### Api improved myocardial structure turbulence induced by I/R Injury

Optical micrographs of rat myocardial structures are shown in **Figure 3**. The myocardial membrane damage and infiltration of inflammatory cells were observed in the myocardial structures of model group as compared to those of sham control group. Moreover, compared with the model group, the group pretreated with Api showed marked improvement evidenced by reduced degree of myonecrosis, edema, infiltration of inflammatory cells, and lesser vacuolar changes compared to the model group. The results displayed that Api could attenuate the histopathological condition in myocardial tissue.

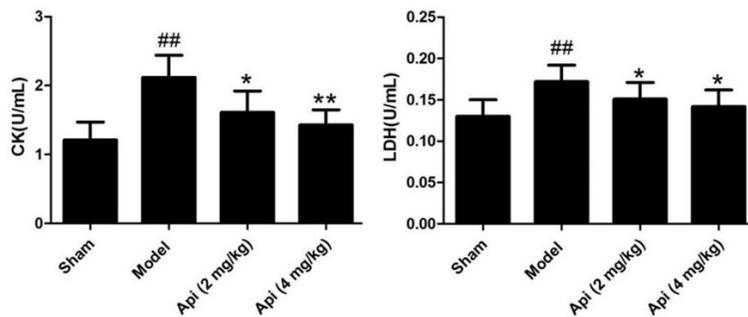
### Api reduced LDH, CK release after I/R injury in rats

To examine the effects of Api on I/R-induced injury, the present study measured the extent

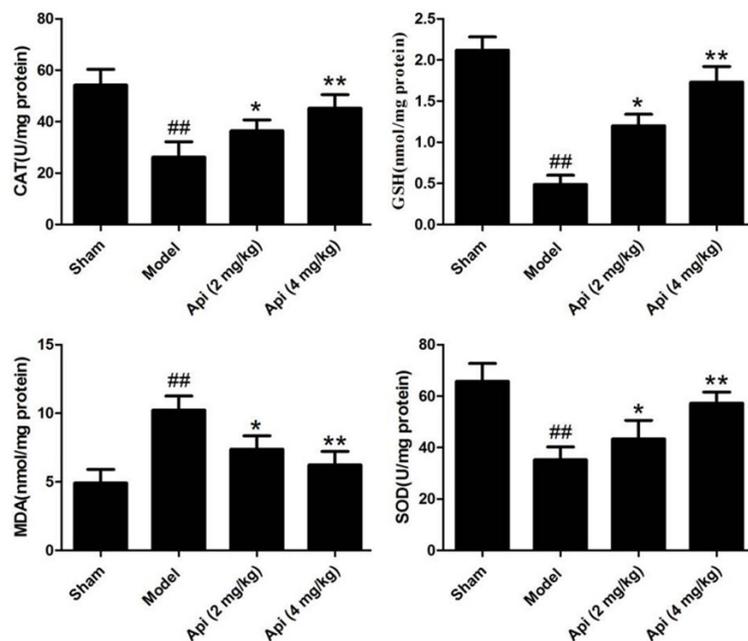
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**Figure 3.** Effects of Api on myocardial histology ( $\times 400$ ).



**Figure 4.** Effect of Api treatment on serum CK and LDH levels in rats subjected to myocardial ischemia-reperfusion. Compared with sham: <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$ ; Compared with model: <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$ .



**Figure 5.** Effect of Api treatment on CAT activity, SOD activity, MDA level, and GSH level. <sup>##</sup> $P < 0.01$  compared with the sham group; <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$  compared with the model group.

of LDH and CK levels. I/R markedly increased the extent of CK and LDH (**Figure 4**). Treatment

of the rats with Api significantly decreased the levels of LDH, compared with the model group. In addition, Api also significantly decreased the levels of CK in a dose-dependent manner.

*Api ameliorated oxidative stress of myocardial tissues induced by I/R injury*

Treatment of the rats with Api decreased the level of MDA, increased the activities of SOD and CAT, as well as an increased GSH level in a dose-dependent manner compared with the model group as illustrated in **Figure 5**.

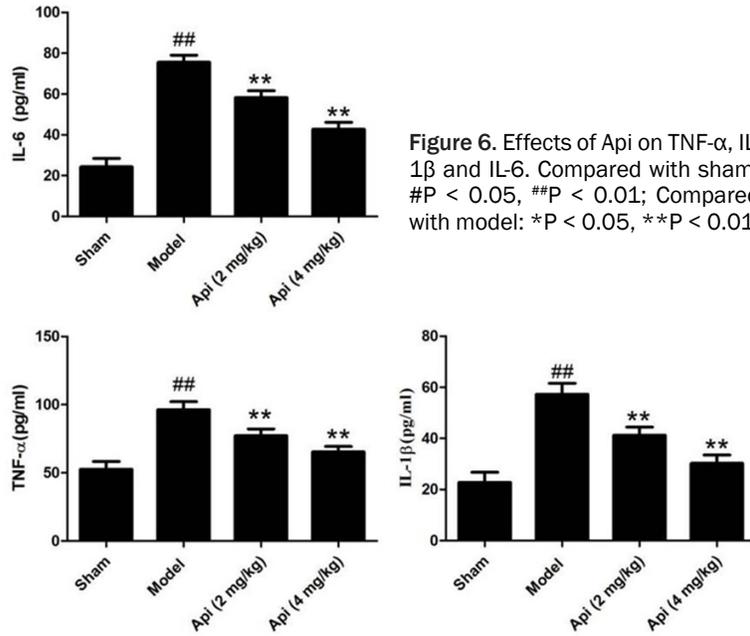
*Api decreased TNF- $\alpha$ , IL-6 and IL-1 $\beta$  serum levels*

As seen in **Figure 6**, Api treatment effectively decreased serum TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels compared with the model group.

*Api decreased Bax and Caspase-3, and increased Bcl-2 levels*

IR significantly increased the myocardial expression of Bax, and Caspases-3 proteins and decreased the Bcl2 expression when compared to sham control rats. Api effectively reduced the myocardial expression of Bax and Caspase-3, and increased the Bcl2 level (**Figure 7**). As shown in

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**Figure 6.** Effects of Api on TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Compared with sham: #P < 0.05, ##P < 0.01; Compared with model: \*P < 0.05, \*\*P < 0.01.

**Figure 7,** the phosphorylations of JAK2 and STAT3 were increased obviously after IR compared to sham control group. In addition, the enhanced phosphorylation of STAT3 but not STAT1 was further strengthened by Api in a dose-dependent manner. By contrast, total JAK2 and STAT3 proteins expression did not obviously vary between groups.

### Discussion

In the present study, the results demonstrated that preconditioning with Api remarkably improved the I/R-induced cardiac injury through inhibiting inflammation and relieving oxidative stress, whereas Api affected the pathway of JAK2/STAT3. Moreover, the treatment of Api, which reduced the myocardial infarct size, may work as a cardioprotective agent.

A previous study demonstrated that an increase in LDH is observed following an increase in infarct size in the heart. The increase in infarct size is also accompanied by increased levels of CK [17]. In the present study, treatment with Api treatment resulted in a decrease in myocardial infarct size. In all the Api-treated groups, the serum levels of LDH and CK were reduced, which suggested that Api had cardioprotective effects. Several studies have reported that I/R-induced oxidative stress increases the production of MDA and inhibits the activity of SOD in the heart [17-19]. In addition, studies have

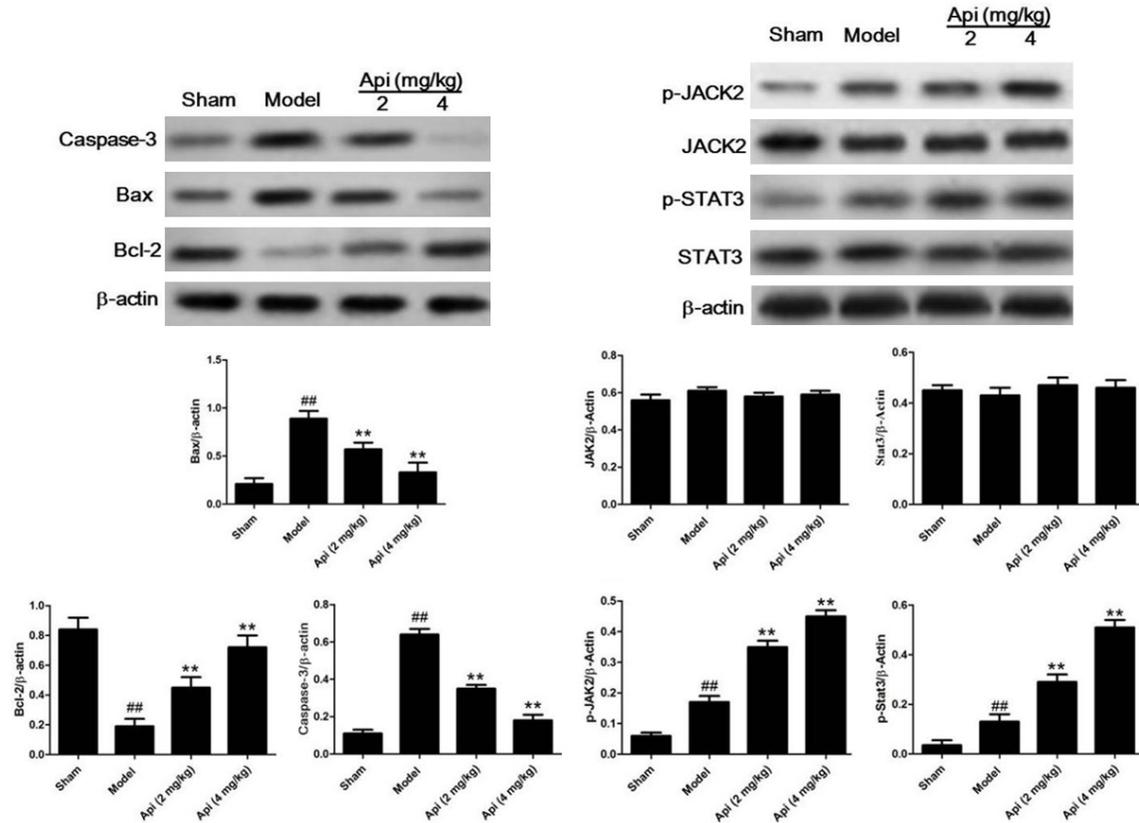
reported that Api decreases the content of MDA and improves the activity of SOD in brain and intestine following I/R [20-21]. The present study demonstrated that I/R up regulated the content of MDA and down regulated the activity of SOD. Cytokines, a heterogeneous group of proteins, have been associated with the inflammatory response in the progress of ischemia/reperfusion injury [22]. Api preconditioning significantly reversed the response, suggesting the anti-inflammation properties of Api were involved in its cardioprotective effect in the I/R rats. Taken these results together with the experiment data, it was suggested that

the protective ability of Api against ischemia/reperfusion injury was exerted by means of mediating reactive oxygen species.

Caspase-3 is a key terminal molecular regulated apoptosis in cellular apoptosis pathways. Bcl-2 is essential to the process of apoptosis because it suppresses the initiation of the cell death process; moreover, Bax gene is the first identified pro-apoptotic member of the Bcl-2 protein family [23]. In this study, the expression changes of Bcl-2 and Bax induced by Api treatment showed a signaling mechanism of anti-apoptotic effect of Api. In this study, we showed that Api intake attenuates myocardial injury by inhibiting intrinsic apoptotic cascades in MIR rats.

In the study, we focused on the potential role of JAK2-STAT3 pathway in Api-induced protection. This is because inactivation of STAT3 or deletion of STAT3 appears to be a key event in the diminution of cardioprotection in response to various physiological stresses including I/R [24-26]. Moreover, it has been shown that mice with a cardiomyocyte-restricted deletion of STAT3 develop spontaneous heart failure in response to stress. Taken together, the current findings demonstrate that Api exerts antioxidative and antiapoptotic effects on MI, which lead to the improved myocardial function and attenuated heart damage. Our results show that Api induced phosphorylation of JAK2 and STAT3,

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**Figure 7.** Effect of Api treatment on myocardium Caspase-3, Bax, Bcl-2, JAK2, p-JAK2, STAT3 and p-STAT3 protein expression. ##P < 0.01 compared with the sham group; \*P < 0.05, \*\*P < 0.01 compared with the model group.

with decreasing Bax and Caspase-3, and increasing Bcl-2 levels. These studies provide novel mechanistic insights into Api-induced cardioprotection, which may help in expanding the therapeutic utility of this drug in limiting myocardial infarction and apoptosis following I/R injury, in addition to its current use in drug-eluting stents to reduce coronary restenosis.

### Disclosure of conflict of interest

None.

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