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Original Article

Baicalin promotes angiogenesis of myocardial tissues in myocardial infarction rats

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Abstract: Objective: The aim of the current study was to elucidate the effects of baicalin on myocardial tissues and angiogenesis in myocardial infarction (MI). Methods: An MI model was established by ligating the anterior descending branch of the left coronary artery in rats. In this study, the rats were divided into 5 groups, including the sham group, myocardial infarction group, low-dose baicalin group, moderate-dose baicalin group, and high-dose baicalin group. Hematoxylin & eosin (HE) staining was performed to detect pathological changes in myocardial tissues. TUNEL assay was performed to detect apoptosis in myocardial cells. The current study also measured levels of creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), and lactate dehydrogenase (LDH). Aiming to understand cardioprotective mechanisms of baicalin, effects on angiogenesis and autophagy-related signaling pathways in myocardial tissues were detected using Western blot. Results: Compared with the myocardial infarction group, baicalin mitigated pathological injuries to the myocardium and decreased apoptosis in myocardial cells, in a dose-dependent manner. Measurements of myocardial enzymes showed that baicalin dose-dependently decreased levels of CK, CK-MB, and LDH in MI-induced rats. Furthermore, detection of autophagy-related signaling pathways in myocardial tissues indicated that baicalin upregulated autophagy signaling pathways, in a dose-dependent manner, promoting the release of vascular endothelial growth factor (VEG). Conclusion: Baicalin affects autophagy signaling pathways, upregulating the angiogenesis-related cytokine, VEGF. This protects the heart from MI.

Keywords: Baicalin

Introduction

Myocardial infarction (MI), one of the most common ischemic heart diseases, accounts for the death of over 7 million people every year, worldwide. It accounts for nearly 12.8% of all deaths. Additionally, MI-induced mortality cases rank first among ischemic heart diseases. Generally, MI occurs due to sudden or persistent interruption of the blood supply to the myocardium, thereby contributing to necrosis or ischemia in myocardial cells. Some Western medicines, like angiotensin converting enzyme (ACE) inhibitors, calcium channel blockers (CCB), and Angiotensin II, have been confirmed to be effective against myocardial ischemia. However, due to severe side-effects, such as cardiac depression, application of these medicines has been limited [1]. Systemic thrombolytic treatment and percutaneous coronary angioplasty (PTCA) can effectively restore reperfusion of the ischemic myocardium, reduce the MI area, and improve the prognosis of patients. However, these methods cannot prevent or reverse the death of myocardial cells, resulting in heart failure. Thus, development of novel heart-protective drugs is very important.

Baicalin, a kind of flavonoid extracted from *Scutellaria baicalensis* Georgi, has been shown to possess various pharmacological functions, including antiviral, antioxidant, and antitumor effects [2]. A recent study showed that baicalin can protect gerbils from damage from global cerebral ischemia/reperfusion through its antioxidative and anti-apoptotic properties [3]. In addition, Lin reported that pre-treatment with baicalin significantly mitigated inflammatory responses in TNF-α-induced myocardial injuries to cardiomyocytes [4]. However, protective effects of baicalin on MI in rats have not been

fully elucidated. In addition, since autophagy-related signaling pathways are critical to amelioration of ischemic damage, it was hypothesized that they may be involved in the protective effects of baicalin on the heart [5]. Thus, the current study investigated the cardio-protective effects of baicalin using an MI rat model.

Materials and methods

Animals and reagents

A total of 50 adult Wistar male rats, weighing between 250 and 300 g, were purchased from Shanghai Slaccas Experiment Animal Co., Ltd. The rats were housed at a specific-pathogen free animal center under standard conditions, with standard temperatures (22 and 24°C) and light conditions (12/12 hour light/dark cycle). Water and food were provided *ad libitum*. Baicalin (powder, M.W. 445.5, >95%, pure) was provided by Ci Yuan Biotechnology Co., Ltd. (Shaaxin, China) and was dissolved in normal saline (adjusted to pH 7.4 using NaOH).

Grouping and treatment

Fifty rats were randomized into 5 groups (n=10) using a random digit table, including the sham group (control group), myocardial infarction group, and low, moderate, and high-dose baicalin groups. Rats in the control group received normal saline (0.1 mL injected intravenously through the tail) for 7 consecutive days. Those in the myocardial infarction group received (0.1 mL) normal saline for 7 consecutive days, followed by blocking of the coronary artery. Rats in the low-, moderate-, and high-dose baicalin groups received 0.1 mL baicalin at concentrations of 50, 100, and 200 mg/kg, respectively, for 7 consecutive days. This was followed by blocking of the coronary artery. All experimental protocols were approved by the Animal Protection Committee. Animal utilization and management were in strict accordance with *Guidelines for the Care and Use of Laboratory Animals* issued by National Institute of Health (NIH).

Establishment of the rat MI model

An acute MI model was established according to previously described methods, with a minor revision [5]. Briefly, the rats were anesthetized via intraperitoneal injections of pentobarbital sodium (40 mg/kg). Under anesthesia, rats underwent mechanical ventilation using a respirator at 5 mL/min. Electrodes were then placed in the limbs and connected to the multi-lead electrophysiological recorder (Chengdu Techman Software Co., Ltd.), recording the normal electrocardiogram. At the intercostal space between the 3rd and 4th ribs, the pericardium was exposed through the thoracotomy. A 5-0 silk suture (2 mm) was used to encircle the left anterior descending coronary artery. This was followed by occlusion of the left coronary artery. Except for suturing of the coronary artery, rats in the sham group received the same treatment. Proper care was taken during this procedure to minimize suffering and the number of animals used. Finally, manifestations of local cyanosis and ST-segment elevation confirmed the successful establishment of the MI rat model.

Hematoxylin & eosin (HE) staining

Six hours following suturing of the coronary artery, the rats were anesthetized. Myocardial tissues were collected and rinsed using ice-cold normal saline. In these isolated myocardial tissues, samples were isolated from the region 2 mm away from the infarction area. Samples were then placed in 4% paraformaldehyde (PFA), embedded in paraffin, and sliced into 5-μm-thick tissue sections. Subsequently, the sections were dewaxed and hydrated for H&E staining, as previously described [6]. They were mounted on coverslips and observed under a light microscope with a magnification of 400X.

Measurement of the infarction area

Six hours following suturing of coronary artery, the heart was removed immediately and kept at -20°C for 2 hours. Sagittal sections, 2 mm thick, were then prepared by slicing the heart from the apex to the atrioventricular groove. They were incubated in 1% triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, USA) at 37°C for 30 minutes. As a result, the normal myocardium was stained with a brick-red color, while the infarcted region remained unstained. The size of the infarcted area was calculated as a percentage of the volume and weight of left chamber [7].

Cell apoptosis detection using TUNEL assay

As previously described, sections of the myocardium were dewaxed and hydrated. Apoptosis of myocardial cells was evaluated using the

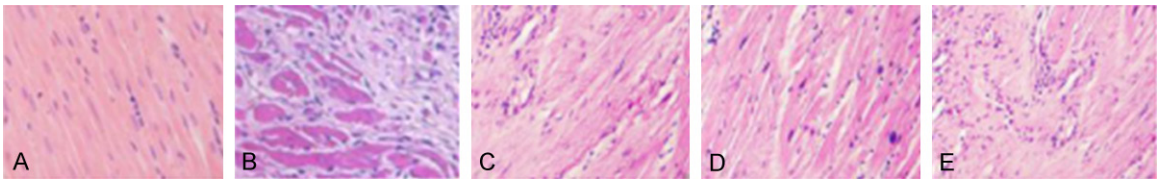

Figure 1. Cardioprotective effects of baicalin against myocardial infarction MI. H&E staining shows that baicalin can ameliorate the infiltration of inflammatory cells. A: Control group; B: Myocardial infarction group; C: Low-dose baicalin group; D: Moderate-dose baicalin group; E: High-dose baicalin group.

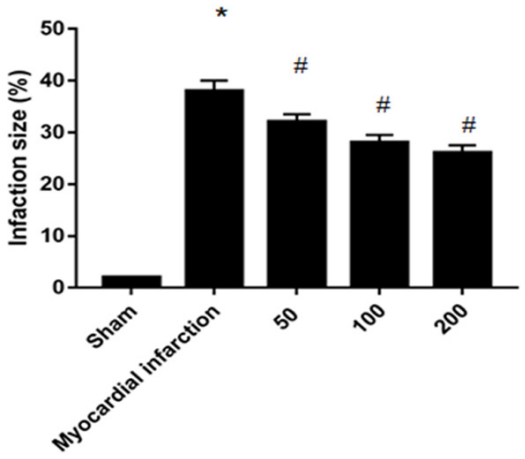

Figure 2. Effects of baicalin on infarct size of hearts in MI. Statistical analysis reveals that baicalin treatment can significantly decrease infarct size in a dose-dependent manner (*P<0.05 *vs.* the Sham group; $^{\#}$*P*<0.05 *vs.* the myocardial infarction group).

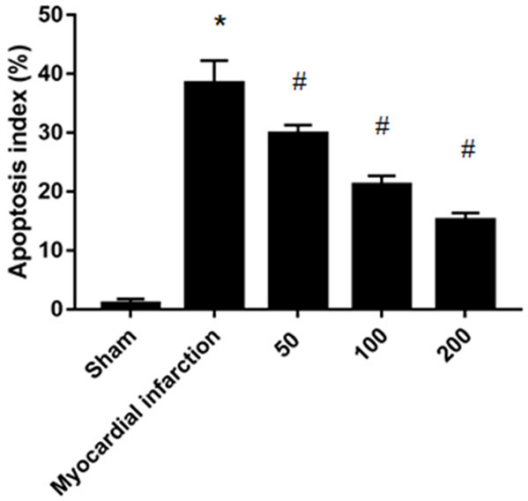

Figure 3. Baicalin effectively inhibits apoptosis in myocardial tissues. TUNEL staining shows that baicalin treatment can ameliorate apoptosis of myocardial cells in MI model rats. Statistical analysis reveals that baicalin treatment significantly decreased the percentage of infarction in a dose-dependent manner (*P<0.05 *vs.* the Sham group; $^{\#}$*P*<0.05 *vs.* the myocardial infarction group).

TUNEL apoptosis kit (Beyotime Biotechnology Institute).

Measurement of activities of serum enzymes

Six hours after occlusion of the coronary artery, blood samples were collected to identify levels of myocardium-specific enzymes, including creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), and lactate dehydrogenase (LDH). This was performed using kits provided by Nanjing Jiancheng Biotech Engineering Institute. Activities were measured using a colorimeter, according to manufacturer protocol.

Western blotting

Total proteins were isolated from myocardial tissues of the different groups, determining protein expression levels of LC3 and VEGF. Total proteins were separated using SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in 5% skimmed milk, followed by incubation with the primary monoclonal anti-LC3 and anti-VEGF antibodies. Protein expression levels were detected by enhanced chemiluminescence reagents and quantitated using Image J software. Protein levels were normalized to α-tubulin and β-actin.

Statistical analysis

Comparison of protein expression levels in myocardial tissues was conducted using non-pairwise *t*-tests (two-tail). Data are presented as mean ± standard deviation. Differences in sizes of the infarcted area and apoptosis of myocardial cells were analyzed using two-way ANOVA. Moreover, t-tests were used for comparisons between two groups among the 5 groups. *P*<0.05 indicates that differences are statistically significant. All statistical analyses were performed using GraphPad Prism 6.0 and SPSS 18.0 software.

Table 1. Effects of baicalin treatment on CK, CK-MB, and LDH activities in MI-induced rats

Group	CK (U/mL)	CK-MB (U/L)	LDH (IU/L)
Control group	0.26±0.03	80.52±7.46	1774.13±331.38
Myocardial infarction group	0.56±0.07**	187.75±9.44I**	3600.75±413.26**
Low-dose baicalin group	0.42±0.05##	113.00±8.75##	3080.25±319.96##
Moderate-dose baicalin group	0.34±0.03##	95.72±11.49##	2603.75±364.55##
High-dose baicalin group	0.29±0.03##	84.85±8.22##	2343.63±374.82##

**P<0.01 vs. control group; ##P<0.01 vs. control group.

Results

Baicalin protects rats from MI

To elucidate the protective effects of baicalin against MI, pathological changes in myocardial tissues were examined using H&E staining (**Figure 1**). In the control group, myocardial cells were arranged regularly, with clear striations. The morphology of the vessels was smooth and no apparent fibrosis was observed in the myocardium. In the myocardial infarction group, myocardial cells were faded. They were disorderly arranged with enlarged interspaces. Myocardial cells exhibited inflammatory cell infiltration, karyolysis, and severe myocardial fibrosis. In low-, moderate-, and high-dose baicalin groups, myocardium fibrosis was evidently mitigated. Infiltration of inflammatory cells was also ameliorated, along with regeneration of granulated myocardial tissues and angiogenesis.

Measurement of myocardial infarct size in MI-induced rats treated with baicalin showed a dose-dependent decrease in the percentage of infarct size, compared with the myocardial infarction group (**Figure 2**).

Baicalin evidently decreases apoptosis in myocardial tissues

Cell apoptosis is the major cause of MI. Thus, TUNEL staining assay was performed to detect cell apoptosis (**Figure 3**). It was observed that baicalin inhibited apoptosis of myocardial cells during MI.

Effects of baicalin treatment on levels of CK, CK-MB, and LDH in myocardial infarction-induced rats

In the myocardial infarction group, levels of CK, CK-MB, and LDH in serum were significantly higher than those in the sham group ($P< 0.05$). Compared with rats in the myocardial infarction group, levels of CK, CK-MB, and LDH in serum of rats in low-, moderate-, and high-dose baicalin groups were significantly decreased ($P<0.05$; **Table 1** and **Figure 4**).

Effects of baicalin on autophagy markers in MI-induced rats

This study further tested the effects of baicalin on autophagy markers in MI. As shown in **Figure 5**, baicalin upregulated autophagy, illustrated by increased LC3B-II/LC3B-I ratios in MI tissues. This exhibited the protection of myocardial tissues from infarction.

Effects of baicalin on VEGF levels in MI-induced rats

Western blotting of VEGF was performed to elucidate the cardioprotective mechanisms of baicalin in MI (**Figure 6**). Baicalin upregulated levels of VEGF in myocardial tissues, exerting protective effects against MI.

Discussion

Flavonoids are naturally occurring polyphenolic compounds known to exert a plethora of biological functions, such as anti-oxidative, anti-tumor, and anti-apoptotic effects. Accumulating evidence has suggested that flavonoids decrease the risk of coronary artery disease. Baicalin, a major flavonoid extracted from *Scutellaria baicalensis* Georgi, can protect rats with critical acute pancreatitis from heart injuries [5]. In addition, it has shown protective effects against ischemia/reperfusion injuries in cultured myocardial cells [8]. Hypoxia/reoxygenation-mediated nuclear translocation of NF-κB in cultured myocardial cells of rats was effectively reduced by baicalin treatment, indicating its cardioprotective ability [9]. In the present study, baicalin improved pathological injuries to the myocardium and reduced apoptosis of myocardial cells, thereby exerting cardioprotective effects against MI.

CK, CK-MB, and LDH, biomarkers of myocardium injuries, are believed to be critical indicators in evaluating ischemia-induced heart injuries [10]. Numerous reports have indicated that, during acute MI induced by isoprenaline,

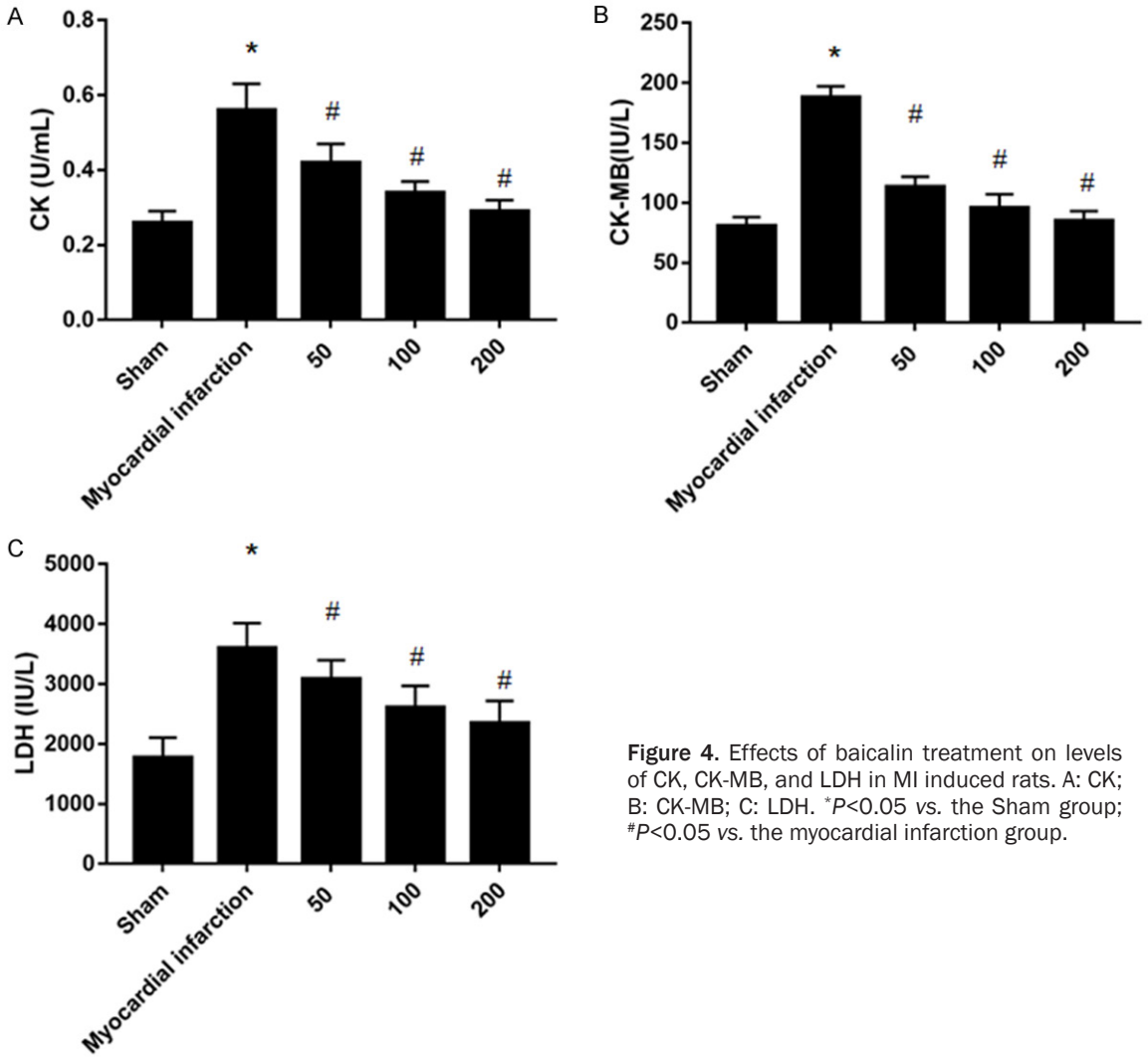

Figure 4. Effects of baicalin treatment on levels of CK, CK-MB, and LDH in MI induced rats. A: CK; B: CK-MB; C: LDH. *P<0.05 *vs.* the Sham group; #P<0.05 *vs.* the myocardial infarction group.

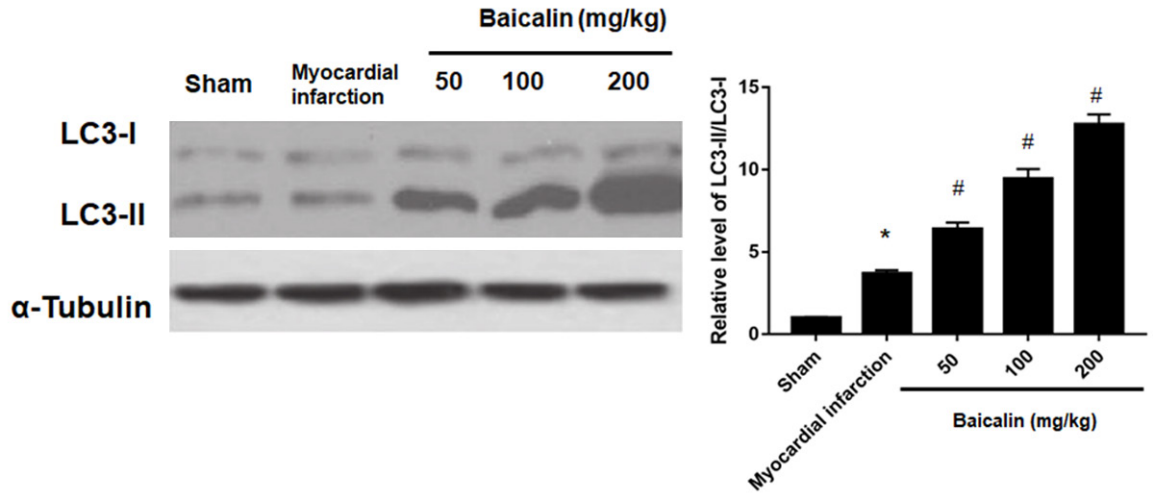

Figure 5. Effects of baicalin on autophagy markers in a rat model of MI. Representative Western blot image showing LC3-I and LC3-II levels in baicalin treated myocardial infarcted rats. Baicalin exerted protective effects on MI through upregulating autophagy in myocardial tissues in a dose-dependent manner. *P<0.05 *vs.* the Sham group; #P<0.05 *vs.* the myocardial infarction group.

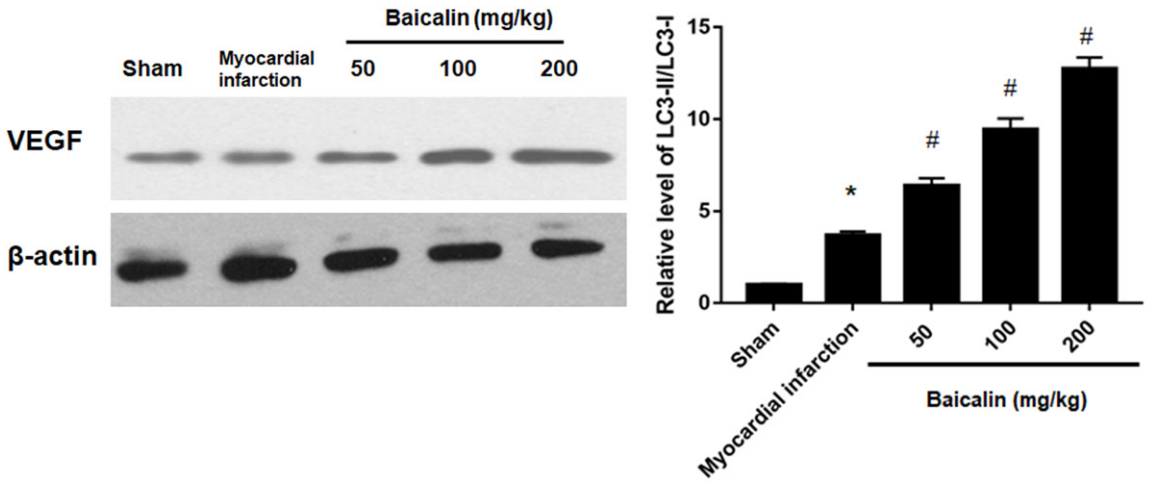

Figure 6. Effects of baicalin on VEGF protein expression in a rat model of MI. Representative Western blot image showing VEGF level in baicalin-treated myocardial infarcted rats. Baicalin showed protective effects on MI through upregulating VEGF in myocardial tissues in a dose-dependent manner. $^{*}P<0.05$ *vs.* the Sham group; $^{\#}P<0.05$ *vs.* the myocardial infarction group.

CK and CK-MB activities were increased robustly [11, 12]. Levels of CK and CK-MB serve as sensitive and specific indicators for detection of MI. Additionally, as contractile proteins, they are scarcely found in serum. However, they are specifically released during myocardial necrosis [13]. The present study showed that, in MI-induced rats, activities of CK, CK-MB, and LDH in the serum were robustly increased. In contrast, levels were acutely decreased after treatment with baicalin. Results suggest that baicalin exerts cardioprotective effects in MI-induced rats by mitigating the infiltration of inflammatory cells, decreasing the percentage of myocardial infarct size, and ameliorating injuries to myocardial cells.

Autophagy is an evolutionarily conserved process of catabolism in which the components of the cytoplasm are degraded by enveloping proteins or organelles. Thus, autophagy is important in sustaining cellular homeostasis and adapting to changing nutrient availability. Constitutive autophagy is the key to maintenance of the heart structure and function. During heart failure, upregulation of autophagy protects the myocardial cells from stress overload. However, the role of autophagy in MI has yet to be elucidated. An increase in LC3-II and concomitant decrease in LC3-I is usually an indicator of autophagy. Additionally, LC3B is the sole autophagy-related protein attached with the autophagosome. It is commonly used to visualize the autophagosome and autolysosome [14]. Therefore, LC3-II was selected as one of the key indicators in this study. It was found that, compared with the myocardial infarction group, rats receiving baicalin had an increase in LC3-II. This suggests that baicalin-mediated autophagy may be a promising target for treatment of MI.

Angiogenesis is a key compensatory mechanism in the stress response of MI, occurring under a variety of pathological and physiological conditions [15]. Many growth factors are involved in the angiogenesis process. It is a multifactorial process that includes basal membrane degradation, migration, proliferation of endothelial cells, and lumen development. Of the various factors regulating angiogenesis, VEGF is an important biological mediator of angiogenesis. A previous study showed that, in ischemic myocardial tissues, VEGF can promote migration and proliferation of endothelial cells and reduce apoptosis of endothelial cells by upregulation of eNOS and NO, along with upregulation of Notch/VEGF signaling pathways. These factors promote expression of VCAM-1 and ICAM-1 and accelerate angiogenesis [16, 17]. The current study found that baicalin increased expression of VEGF in MI, indicating that baicalin promotes angiogenesis and is involved in protection against MI.

Classic apoptotic cell death is induced through a pathway involving cleavage of PARP and procaspase-3, as well as activation of Bax [18-20].

Abnormally high expression levels of cleaved PARP, Bax, and pro-caspase-3 induced by H_2O_2 have been significantly reversed by pretreatment with baicalin [21, 22]. In addition, baicalin suppressed oxidative activity in endplate chondrocytes induced by H_2O_2, via effectively reducing levels of MDA, increasing levels of SOD, and elevating NO activities [23]. The roles of baicalin have been predominantly based on inhibiting the production of ROS, increasing intracellular antioxidants, and attenuating apoptosis [24, 25]. Results of the present study require verification in future *in vivo* investigations. However, they provide further insight into the potential benefits of baicalin for patients with MI-related diseases. In conclusion, present results show that baicalin can affect autophagy signaling pathways, upregulating the angiogenesis-related cytokine, VEGF. Baicalin may also inhibit the production of ROS, increase intracellular antioxidants, and attenuate apoptosis, thereby protecting the heart from MI.

Disclosure of conflict of interest

None.

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