Pharmacological mechanism and apoptosis effect of baicalein in protecting myocardial ischemia reperfusion injury in rats

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Abstract: Baicalein is one of the main active ingredients of Scutellaria baicalensis Georgi. Baicalein has many good biological activities, such as anti-inflammatory, antioxidant and anti-apoptosis. The protective effect of baicalein on myocardial ischemia and reperfusion injury was studied. The results showed that baicalein could decrease the content of MDA (malondialdehyde) and MPO (myeloperoxidase) in serum of rats and increase the level of SOD (superoxide dismutase), which was significantly different from the model group (P<0.05). The results showed that baicalein could enhance the antioxidant capacity and alleviate neutrophil mediated inflammatory injury. Compared with the model group, the SOD activity of baicalein low concentration group (25mg/kg) increased significantly (3.47-0.28). The content of MDA in myocardium of rats with high concentration of baicalein (50mg/kg) decreased significantly (425.87±19.24), whereas he GSH/GSSG ratio increased significantly (30.28±0.48), P<0.05. High concentration of baicalein preconditioning can significantly reduce the release of CK (creatine kinase) and LDH (lactate dehydrogenase) induced by myocardial ischemia/reperfusion injury, reduce the rate of myocardial infarction and reduce the rate of myocardial apoptosis.

Keywords: Baicalein, SOD activity, apoptosis rate, inflammation, oxidative stress.

INTRODUCTION

In recent years, the incidence of ischemic cardiomyopathy is increasing. According to the WHO (World Health Organization) report, ischemic heart disease has become the leading cause of death in humans (Amin et al., 2017). In most cases, if the ischemic myocardium is perfused with blood in time, the myocardium can be perfected and repaired to a certain extent (Bagatini et al., 2011). However, sometimes this kind of reperfusion not only can not restore cardiac function, but also can lead to myocardial dysfunction and structural damage. This phenomenon is called myocardial ischemia/reperfusion Injury (MIRI) in which serious arrhythmias, massive myocardial necrosis, and cardiac rupture and death occur (Chitourou et al., 2015; Bergmann et al., 2016). MIRI is a complex and multifactorial pathophysiological process. At present, the mechanism of MIRI has not been fully elucidated. It is generally believed that the outbreak of free radicals, calcium overload, myocardial energy metabolism disorders, endothelial cell dysfunction, neutrophil infiltration, cell apoptosis and mitochondrial damage are important reasons for the development of MIRI (Li et al., 2017). MIRI can occur in heart transplantation, arterial bypass grafting, thrombolytic therapy, recanalization after myocardial infarction, percutaneous transluminal coronary angioplasty, cardiopulmonary cerebral resuscitation, cardiopulmonary bypass surgery and shock. Therefore, MIRI will be a major clinical problem, and finding new therapeutic strategies and targets for MIRI is the key to solve this clinical problem (Purkayastha et al., 2015).

MD2 (Myeloid Differentiation-2) is an important auxiliary protein of TLR-4 (toll-like receptor). Its basic function is to assist LPS (lipopolysaccharide) to bind to TLR-4 and activate the inflammatory signal pathway of LPS-TLR4. Scutellaria baicalensis is a traditional Chinese medicine with a long history (Li et al., 2015). Baicalein is one of the main active ingredients of Scutellaria baicalensis Georgi (Karen et al., 2017). Recent studies have shown that baicalein alleviates inflammation by inhibiting TLR4-mediated NF-kappa B and MAPK signaling pathways in LPS-induced mastitis in mice (Ajami et al., 2016). It is precisely because baicalein has a good anti-inflammatory and antioxidant effect, so baicalein is important tissue and organ ischemia/reperfusion injury has been reported (Purkayastha et al., 2015). Baicalein not only protects MIRI through mitochondrial oxidation, but also reduces liver and kidney injury induced by MIRI (Bartzatt et al., 2010). Although the protective effect of baicalein on MIRI has been confirmed, the target of baicalein on MIRI remains unclear. The hypothesis is that baicalein protects MIRI by anti-inflammatory, anti-oxidative and targeting MD2 protein.

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MATERIALS AND METHODS

Experimental animal
In this experiment, 40 healthy adult male SD rats were selected. Body weight 250-300g is provided by Jinan Central Hospital Laboratory Animal Center. The control of feeding environment should meet the following requirements: temperature 18-26 C, relative humidity 40-70%, noise below 85 dB, ammonia concentration below 20 PPM, ventilation 8-12 times per hour. There should always be enough fresh and dry feed in the hopper of the rat box. For the group box, feed should be added two days a week, and other time can be added at any time according to the situation. The animal experiment program has been approved by the experimental animal ethics committee, which conforms to the principles of animal protection, animal welfare and ethics, and conforms to the relevant provisions of the national laboratory animal welfare ethics, No.JCHLPF/16SD.

Drugs and reagents
Baicalein was provided by Institute of Traditional Chinese Medicine with a purity of 98%. The suspension was prepared with 0.5% CMC sodium before the experiment. Lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), myeloperoxidase (MPO) and Coomassie leucocyanin quantitative kits, etc.

Method
SD male rats were randomly divided into 4 groups according to body weight, 10 in each group. They were sham operation group, model group, low dose baicalein group (25 mg/kg), high dose baicalein group (50 mg/kg).

Sham operation group: Silk thread was used to penetrate the artery of rats, but the silk thread was not ligated and was left open for 75 minutes. In model group: Silk thread was used to penetrate the artery of mice, stabilize for 10 minutes and ligate the blood vessel. After 30 minutes, the small plastic tube was removed and reperfused for 45 minutes. The operation of baicalein in low dose group and high dose group was same as that in model group. The model group and sham operation group were given 0.5% CMC sodium by intragastric administration 40 minutes after administration.

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (0.35g/kg), then fixed in supine position and inserted needle-shaped electrode subcutaneously into limbs to monitor standard limb lead II ECG. Cut the skin from the middle of the neck, separate the trachea, intubate the trachea, connect the miniature artificial respirator for artificial respiration (breathing rate 60 times / min, tidal volume 20 ml / kg), open the chest along the left edge of the sternum between the third and fourth ribs, carefully cut the pericardium, expose the heart. The left coronary vein at the junction of left atrial appendage and pulmonary cone was used as a marker. A 0.6 cm long plastic tube with a diameter of 0.1 cm was placed in the center of the suture line. The small plastic tube was compressed on the left anterior descending coronary artery to facilitate occlusion and preservation. Restore blood flow. After ligation for 30 minutes, the small plastic tube was removed and reperfused for 45 minutes. According to the following grouping, ST segment elevation and T wave elevation were used as the successful markers of ligation. The elevated ST segment decreased and T wave gradually recovered to confirm the success of reperfusion. At the end of reperfusion, the abdominal cavity was opened quickly, the abdominal veins were exposed, and the venous blood was collected for 5 ml and then centrifuged for 10 minutes at ~3500 rpm. Immediately after taking blood, open the chest and take out the heart, wash the blood stains in the precooled normal saline, take the myocardial tissue of the ischemic area of the apex, and fix it in 10% formalin solution for pathological observation. The serum LDH (lactate dehydrogenase), SOD (superoxide dismutase), MDA (malondialdehyde) and MPO (myeloperoxidase) levels were determined by enzyme labeling instrument in strict accordance with the instructions. The myocardium was fixed for more than 24 hours and embedded in paraffin. The myocardial necrosis was observed under ordinary light microscope.

Detection of cardiomyocyte apoptosis by TUNEL
(1) Dewaxing in xylene for 5-10 minutes. Replace with fresh xylene and dewaxing for 5-10 minutes. Absolute alcohol for 5 minutes. 90% ethanol for 2 minutes. 70% ethanol for 2 minutes, distilled water for 2 minutes.
(2) Dropping 20 UG g/ml without DNase protease K, 20-37 C C for 15-30 minutes.
(3) PBS was washed for 3 times. Note: the protease K must be cleaned in this step, otherwise it will interfere with subsequent marker reaction.
(4) Preparation of TUNEL detection solution.
(5) Myocardial tissue was dripped with TUNEL solution and then sealed.
(6) Myocardial tissue apoptosis was detected under a microscope.

STATISTICAL ANALYSIS
SPSS 21.0 and Graph Pad 6.0 Software were used to
analyze the data. The measurement data were expressed as mean±standard deviation (x±s). There was a significant difference between the two groups in one-way ANOVA (p < 0.05).

**Fig. 1: Infarct size**

![Infarct size](image)

**Fig. 2: Cardiomyocyte apoptosis, CK-MB and LDH**

![Apoptosis and Enzyme Activities](image)

**RESULTS**

**Effects of baicalein on myocardial ischemia reperfusion in rats**

The levels of LDH, SOD, MDA and MPO in the model group were significantly higher than those in the sham operation group (P <0.001), indicating that the myocardial ischemia reperfusion model was successful. There was a significant difference in LDH between the model group and the baicalein group (P<0.05). The serum MDA and MPO in the model group were significantly higher than those in the sham operation group and SOD was significantly lower than that in the sham operation group (P<0.01), indicating that myocardial ischemia-reperfusion injury resulted in a significant decrease in tissue antioxidant capacity, activation of neutrophils and increased release of granules. Baicalein group could decrease the content of MDA and MPO in serum and increase the level of SOD, which was significantly different from the model group (P < 0.05), indicating that baicalein could improve the antioxidant capacity and alleviate neutrophil-mediated inflammatory injury, as shown in table 1.

**Effects of baicalein on the activity of CK and LDH in coronary effluent of isolated rat heart**

The activities of LDH and CK in the effluent of isolated coronary arteries of rats in normal group were very low, and the activities of LDH and CK in the effluent of isolated coronary arteries remained basically constant at 10 min before reperfusion, 20 min and 40 min after reperfusion. Compared with the normal group, the LDH and CK activities in the coronary effluent of the model group increased significantly after reperfusion for 20 and 40 minutes (P < 0.01). Compared with the model group, the LDH and CK activities in the coronary effluent of rats with low concentration of baicalein after reperfusion for 20 and 40 minutes were significantly decreased (P < 0.05), but there was no significant change in the high concentration group (P > 0.05). The results showed that high concentration of baicalein could inhibit the release of LDH and CK induced by myocardial injury, as shown in table 2.

**Effects of baicalein on SOD activity, MDA content and GSH/GSSG ratio in myocardium**

Compared with the normal group, the SOD activity and MDA content in the isolated cardiac myocardium of the model group decreased significantly, and the GSH / GSSG ratio decreased significantly (P<0.01). Compared with the model group, the SOD activity in myocardium of rats with low concentration of baicalein was significantly increased (3.47±0.28), in high concentration group, the content of MDA in myocardium of isolated heart was significantly reduced (425.87±19.24). The ratio of GSH/GSSG increased significantly (30.28±0.48) (P<0.05) and the results were shown in table 3.

**Baicalein reduces infarct size after myocardial ischemia/reperfusion in mice**

After TTC/ Evance Blue staining, the normal myocardium was stained blue-black, the infarcted area was stained white, and the non-infarcted area was stained red. There was no myocardial ischemia and infarction in the sham group, and myocardial infarction occurred in the model group (I/R group). The infarction area of low concentration baicalein group was significantly smaller than that of the model group. The infarction area of high concentration baicalein group was further reduced.

**Baicalein alleviated myocardial apoptosis, CK-MB and LDH after myocardial ischemia/reperfusion injury in mice**

After one-step TUNEL staining, there was no obvious apoptosis in myocardial tissue of sham-operation group. The number of apoptotic cells in model group (I/R group) was significantly increased. The number of apoptotic cells
Pharmacological mechanism and apoptosis effect of baicalein in protecting myocardial ischemia reperfusion injury

Table 1: Effects of baicalein on serum levels of LDH, SOD, MDA and MPO

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>LDH(U/L)</th>
<th>SOD(U/ml)</th>
<th>MDA(nmol/ml)</th>
<th>MPO(U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>10</td>
<td>6127.7±213.5</td>
<td>151.4±3.5</td>
<td>6.14±0.3</td>
<td>21.5±6.5</td>
</tr>
<tr>
<td>Model</td>
<td>10</td>
<td>7456.0±205.6</td>
<td>137.7±7.2</td>
<td>7.96±0.8</td>
<td>45.6±14.1</td>
</tr>
<tr>
<td>Baicalein (25mg/kg)</td>
<td>10</td>
<td>7068.2±324.5</td>
<td>148.1±4.2</td>
<td>6.02±0.2</td>
<td>33.8±11.4</td>
</tr>
<tr>
<td>Baicalein (50mg/kg)</td>
<td>10</td>
<td>7148.2±317.4</td>
<td>154.3±7.6</td>
<td>7.13±0.8</td>
<td>22.7±6.3</td>
</tr>
</tbody>
</table>

Table 2: The activity of LDH and CK in the coronary outflow of isolated rat heart was detected

<table>
<thead>
<tr>
<th>Detection index</th>
<th>Group</th>
<th>Before stopping irrigation, 10min</th>
<th>Reperfusion 20min</th>
<th>Reperfusion 40min</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH</td>
<td>Sham</td>
<td>17.85±4.24</td>
<td>17.19±3.86</td>
<td>16.42±7.12</td>
</tr>
<tr>
<td></td>
<td>Model</td>
<td>17.36±6.35</td>
<td>63.20±4.17</td>
<td>57.35±8.17</td>
</tr>
<tr>
<td></td>
<td>Baicalein (25mg/kg)</td>
<td>18.24±3.24</td>
<td>45.12±2.45</td>
<td>34.71±4.46</td>
</tr>
<tr>
<td></td>
<td>Baicalein (50mg/kg)</td>
<td>17.17±5.76</td>
<td>21.45±6.17</td>
<td>24.45±7.37</td>
</tr>
<tr>
<td>CK</td>
<td>Sham</td>
<td>26.34±9.12</td>
<td>25.28±5.54</td>
<td>25.63±8.60</td>
</tr>
<tr>
<td></td>
<td>Model</td>
<td>22.65±7.13</td>
<td>367.04±15.23</td>
<td>113.87±13.085</td>
</tr>
<tr>
<td></td>
<td>Baicalein (25mg/kg)</td>
<td>22.38±4.58</td>
<td>276.17±16.57</td>
<td>85.12±11.46</td>
</tr>
<tr>
<td></td>
<td>Baicalein (50mg/kg)</td>
<td>18.60±12.23</td>
<td>236.54±15.14</td>
<td>70.48±17.75</td>
</tr>
</tbody>
</table>

Table 3: Detection results of SOD activity, MDA content and GSH/GSSG ratio in cardiac muscle tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD, U/mgprot</th>
<th>MDA, μmol/kgprot</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>8.13±0.48</td>
<td>279.67±23.12</td>
<td>37.12±0.54</td>
</tr>
<tr>
<td>Model</td>
<td>1.05±0.35</td>
<td>701.54±27.18</td>
<td>17.28±0.26</td>
</tr>
<tr>
<td>Baicalein (25mg/kg)</td>
<td>3.47±0.28</td>
<td>516.64±23.48</td>
<td>23.15±1.37</td>
</tr>
<tr>
<td>Baicalein (50mg/kg)</td>
<td>5.17±0.12</td>
<td>425.87±19.24</td>
<td>30.28±0.48</td>
</tr>
</tbody>
</table>

in low baicalein concentration group (25 mg/kg) was decreased compared with model group. The number of apoptotic cells in high baicalein concentration group (50 mg/kg) was further decreased. These data indicate that baicalein can reduce the apoptosis of myocardial cells after ischemia/reperfusion injury, and has obvious protective effect on myocardial cells after ischemia/reperfusion injury. The elevation of CK-MB and LDH in blood can reflect the degree of myocardial tissue (or cells) injury after ischemia/reperfusion injury, which further indicates that baicalein can protect myocardial tissue from ischemia/reperfusion injury.

**Effects of baicalein on myocardial pathomorphology in rats with myocardial ischemia-reperfusion**

The myocardial fibers in sham-operated group were well arranged, no degeneration, necrosis, swelling and rupture, and no other obvious abnormalities were observed. In model group, degeneration and necrosis of myocardial cells, swelling and rupture of myocardial fibers, infiltration of inflammatory cells in interstitium, and bleeding were observed. The myocardial lesion in baicalein group was alleviated, which was between model group and sham-operated group. Between.

**DISCUSSION**

MIRI is a complex and multifactorial pathophysiological process. At present, the mechanism of MIRI has not been fully elucidated (Karen et al., 2017). It is generally believed that the outbreak of free radicals, calcium overload, myocardial energy metabolism disorders, endothelial cell dysfunction, neutrophil infiltration, cell apoptosis and mitochondrial damage are important reasons for the development of MIRI (Cormier et al., 2012). *Scutellaria baicaleansis* is a traditional Chinese medicine with a long history. Baicalein is one of the main active ingredients of *Scutellaria baicaleansis*. Baicalein has many biological characteristics, such as anti-inflammatory, anti apoptosis, anti-oxidation, anti thrombosis, anticancer, and so on (Gao et al., 2010; Danir et al., 2017). Because baicalein has a good anti-inflammatory and anti-oxidative effect, there are more reports on the protection of baicalein against ischemia/reperfusion injury of important tissues and organs (Toko et al., 2010). Recent studies have shown that baicalein alleviated hepatic ischemia-reperfusion injury by inducing autophagy (Fardeau et al., 2014). Baicalein preconditioning also protects the liver from ischemia/reperfusion injury by inhibiting the activation of NF-kappa B; 12/15-lipoxygenase inhibitor baicalein reduces the expression of PPAR gamma and nuclear metastasis induced by cerebral ischemia/reperfusion (Thygesen et al., 2012). The anti-tumor effect of *Scutellaria baicaleansis* involves different tumor models. The effects of effective
components of Scutellaria baicalensis were studied from the aspects of immunity, enzyme activity and inhibition of tumor cells (Jubie et al., 2012). The apoptosis rate of prostate cancer cell DU145 was detected by flow cytometry and the expression of apoptosis-related proteins was detected by immunohistochemical staining. The results showed that baicalin could induce apoptosis of prostate cancer cells, inhibit the proliferation of cancer cells, and had a direct anti-tumor effect (Ji et al., 2015; Karen et al., 2017). Many studies have confirmed that baicalin can induce apoptosis of gastric cancer, liver cancer and lung adenocarcinoma cells, but the mechanism is still unclear (Jubie et al., 2012; Li et al., 2013). In addition, studies have shown that baicalin can also inhibit the proliferation of primary or secondary malignant glioma cells, can assist other chemotherapeutic drugs for anti-cancer treatment (Nguyen et al., 2011).

The role of free radicals and intracellular calcium overload are two important links in the pathogenesis of ischemia-reperfusion injury (Patel et al., 2009). The changes of left ventricular function were measured by left ventricular catheterization 10 minutes before coronary artery ligation (Sakamoto et al., 2006). The results showed that baicalin had protective effect on left ventricular function in rats with myocardial ischemia reperfusion injury (Patel et al., 2010). Injection of baicalin via femoral vein can significantly decrease the content of MDA and increase the activity of SOD and GSH-Px in myocardium of rats with ischemia-reperfusion injury, suggesting that baicalin has protective effect on myocardium after reperfusion injury. It can be related to lipid peroxidation induced by oxygen free radicals. During myocardial ischemia-reperfusion in rats, baicaline can protect the ischemia-reperfusion myocardium by inhibiting cardiomyocyte apoptosis and inflammatory cell infiltration (Ceylan et al., 2016).

In this study, we found that MD2 knockout reduced myocardial infarct size, plasma levels of CK-MB and LDH, inflammatory response and oxidative stress induced by ischemia/reperfusion, endoplasmic reticulum stress and apoptosis-related protein expression. These data fully demonstrate that MD2 participates in the pathological process of myocardial ischemia/reperfusion injury and inhibits the activation of MD2 protein to alleviate myocardial ischemia/reperfusion injury (Yigitkanli et al., 2013). Under myocardial ischemia/reperfusion conditions, MD2 knockout resulted in decreased endoplasmic reticulum stress and apoptosis-related protein expression induced by myocardial ischemia/reperfusion (Zhang et al., 2015). The mechanism is whether the expression of ER stress and apoptosis-related proteins is directly affected by MD2 knockout or the expression of ER stress and apoptosis-related proteins is decreased by MD2 knockout, anti-inflammatory and anti-oxidation.

CONCLUSION

Significant myocardial dysfunction was observed after myocardial ischemia/reperfusion injury, resulting in changes in the release of some myocardial isoenzymes (such as CK, LDH) and myocardial infarction, as well as myocardial apoptosis. Some of the phenomena following myocardial ischemia/reperfusion injury in this study are consistent with those reported in many other studies. High concentration of baicalein preconditioning can significantly reduce the release of CK and LDH induced by myocardial ischemia/reperfusion injury, reduce the rate of myocardial infarction and reduce the rate of myocardial apoptosis. MIRI is a complex and multifactorial pathophysiologic process. No single pathway or single drug can completely clarify or treat myocardial ischemia/reperfusion injury. The combination of multiple pathways and multiple drugs may achieve greater success in clinical prevention and treatment of myocardial ischemia/reperfusion injury.

REFERENCES


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