Scutellarin benzyl ester partially secured the ischemic injury by its anti-apoptosis mechanism in cardiomyocytes of neonatal rats

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Objective: To investigate the protective effects of scutellarin benzyl ester on neonatal rats’ cardiomyocytes injured by ischemia and its anti-apoptosis mechanism.

Methods: The cardiomyocytes in primary culture were prepared from ventricular tissue of 1- to 3-day-old Sprague-Dawley rats and the cells in good condition were assigned to five groups: control group, ischemic model group and three scutellarin benzyl ester groups (doses of 100, 50 and 25 μmol/L, respectively). The model of ischemic injury was established in the primary culture of cardiomyocytes under glucose-free anoxic condition. After ischemia for 6 h, the metabolic ability of the cells was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay and the activity of lactate dehydrogenase (LDH) in the media was measured by biochemistry approaches. The nuclear damage was revealed by Hoechst-propidium iodide staining. The percentage of apoptotic cells was monitored by flow cytometry. The expression levels of cytochrome c and caspase-3 mRNAs and proteins were determined by reverse transcription-polymerase chain reaction and Western blotting, respectively.

Results: After exposure to ischemic condition, the cell viability of the model group was degraded compared with that of the control group (P<0.01) and scutellarin benzyl ester (high and medium doses) could attenuate the loss of cell viability induced by ischemia (P<0.01 and P<0.05). In addition, each dose of scutellarin benzyl ester could significantly reduce the release of LDH from cardiomyocytes injured by ischemia (P<0.01). In morphology, the injured
nuclei presented significant changes such as condensation of chromatin, and shrinkage and fragmentation of nuclei, which could be attenuated remarkably by pretreatment with scutellarin benzyl ester. Furthermore, scutellarin benzyl ester could significantly decrease the percentage of apoptosis induced by ischemia (P < 0.01) and inhibit the increased expression levels of cytochrome c and caspase-3 mRNAs and proteins (P < 0.01).

**Conclusion:** Scutellarin benzyl ester has a remarkable protective effect against myocardial ischemic injury and the protective mechanism may associate with its anti-apoptosis effect by inhibiting cytochrome c release and caspase-3 activation.

**Keywords:** *Erigeron brevicaus*; scutellarin benzyl ester; prodrugs; myocytes; cardiac; ischemia; apoptosis; rats

Scutellarin is well known as the major active component of Chinese herbal medicine *Erigeron brevicaus* (Vant) Hand-Mazz that has been generally used in clinical practice to treat cerebrovascular and cardiovascular diseases, such as stroke, myocardial ischemia and myocardial infarction. It has been shown that scutellarin has a protective effect against ischemia or ischemia-reperfusion injury and its mechanism may be associated with antioxidation, anti-apoptosis and inhibition of Ca²⁺ inflow[1, 2]. In addition, a recent study has provided the experimental evidence that scutellarin could be a potential drug for promoting angiogenesis[3]. Although great progress has been made in the field of pharmacological action of scutellarin, scutellarin has obvious drawbacks on solubility for low hydrophilicity and lipophilicity resulting in its poor oral bioavailability. For this reason, it is difficult for scutellarin to produce marked effect as an oral drug in clinical practice. In order to solve the problem, many researchers have paid much attention to the development of scutellarin derivatees. As reported, ester formation can greatly increase lipophilicity, improve membrane permeability and promote oral absorption of drugs[4]. Therefore, scutellarin benzyl ester was synthesized by our research group and the results of our early studies showed that its lipophilicity increased to a large extent compared with scutellarin and its anti-ischemic effect was also better than scutellarin[5]. Based on previous studies, the objective of this study was to investigate the protective effect of scutellarin benzyl ester on neonatal rats' cardiomyocytes injured by ischemia and to explore its anti-apoptosis mechanism.

**1 Materials and methods**

**1.1 Drugs and reagents** Scutellarin (purity > 90%) was purchased from Nanjing Zelang Medical Technology Co. Ltd. (Jiangsu, China). As described previously[4], scutellarin benzyl ester was synthesized as follows. Scutellarin was transformed to sodium scutellarin (scutellarin 1.1 mmol, sodium bicarbonate (NaHCO₃) 1.1 mmol, water bath at 50 °C for 0.5 h; yields: 97.4%). The suspension of sodium scutellarin in N, N-dimethylformamide was mixed with benzyl bromide and catalyst KI at 80 °C for about 2 h while the reaction was monitored by thin layer chromatography (yield: 17.7%). The melting point of scutellarin benzyl ester was 104 to 105 °C.

Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM/F12) supplemented with penicillin G (100 U/mL), streptomycin (100 ng/mL) and 10% fetal bovine serum (FBS) was Gibco® product (Invitrogen, California, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Beijing Dingguo Biotech Co. Ltd. (Beijing, China). The kits of lactate dehydrogenase (LDH) and Hoechst-propidium iodide (PI) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). The RNA kit (AMV) version 3.0 was purchased from TaKaRa Biotechnology (Dalian) Co. Ltd.
(Liaoning, China). The caspase-3 antibody was purchased from Santa Cruz (California, USA).

1.2 Experimental animals and grouping Neonatal Sprague-Dawley (SD) rats of any gender (specific pathogen-free, Certificate No. SCK-2002-001) were supplied by the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). The rats were housed at 23 °C temperature and 40% humidity. The cardiomyocytes in primary culture were randomly divided into 5 groups: control group, ischemic model group and three scutellarin benzyl ester groups (doses of 100, 50 and 25 μmol/L, respectively) with 6 wells in each group.

1.3 Primary neonatal rats’ cardiomyocytes culture The cardiomyocytes in primary culture were prepared from ventricular tissue of 1- to 3-day-old SD rats. Briefly, the ventricles of rat heart were isolated aseptically, minced in chilled Ca²⁺/Mg²⁺-free and Hanks’ solution, cut into small pieces and then digested with 0.25% trypsin at 37 °C. After digestion, each supernatant except the first one was collected at an interval of 10 min and centrifuged at 110 × g for 10 min. The precipitation was then re-suspended and supplemented with 10% FBS and the cell suspension was filtered. After cultivation about 70 min for purification, the non-attached cells were seeded at a concentration of 7 × 10⁵ in 5% CO₂ at 37 °C. The following studies were performed after the cells were cultured for 4 d.

1.4 Establishment of ischemic model The culture medium was replaced with the D-Hank’s buffer solution bubbled with 95% nitrogen (N₂)/5% CO₂. The cells were exposed to 95% N₂/5% CO₂ in an air-tight incubator at 37 °C for 6 h; the oxygen partial pressure in the incubator was adjusted to less than 1%. Simultaneously, the cells in the culture control were incubated at 37 °C in a 5% CO₂ humidified incubator. Prior to ischemia, the cells in treatment groups were treated with different doses of scutellarin benzyl ester (100, 50 and 25 μmol/L).

1.5 Cell viability assay Cell viability was determined by MTT assay. Briefly, cells on the 96-well culture plates at 3 × 10⁴ per well were incubated for 4 d. After exposure to ischemic condition, the MTT solution (20 μL, 5 g/L) was added into each well (final concentration 0.5 g/L) and then the mixtures were incubated in 5% CO₂ at 37 °C for 4 h. Subsequently the medium in each well was replaced with dimethyl sulfoxide (150 μL) and the amount of MTT formazan was quantified by the absorbance at 490 nm using an enzyme-labeled meter (Bio-Rad 680, California, USA).

1.6 Determination of LDH release Briefly, the cells on the 24-well culture plates at 3 × 10⁴ per well were incubated for 4 d. After exposure to ischemic condition, the LDH activity in the cultured supernatant was measured by using a spectrophotometer (Shimadzu UV-1601, Kyoto, Japan) on the base of the enzyme conversion of lactate. The activity of LDH was quantified by the absorbance at 440 nm according to the specification.

1.7 Hoechst-PI staining for assessment of apoptosis Briefly, after exposure to ischemic condition, the cells were digested and centrifuged at 110 × g for 10 min. The precipitation was suspended with phosphate buffer solution (PBS, 0.01 mol/L) and centrifuged at 110 × g for 10 min. The cells precipitation suspended with PBS solution (0.01 mol/L) were added with the mixture of Hoechst33258/PI and incubated for 10 min at 37 °C. After a final rinse with PBS (0.01 mol/L), the morphology changes of nuclear were observed by a fluorescence microscope (Nikon E800, Tokyo, Japan).

1.8 Flow cytometry for apoptosis assay After exposure to ischemic condition, the cells were digested and suspended in PBS solution (0.01 mol/L). The suspension was centrifuged at 110 × g for 10 min and the precipitations were fixed in 70% alcohol (−20 °C) overnight. The cells were washed with PBS solution (0.01 mol/L) and then incubated in PI solution at 4 °C for 30 min and finally were analyzed by a flow cytometer (Elite Esp, California, USA).

1.9 Reverse transcription-polymerase chain reaction analysis The total RNA was extracted from cardiomyocytes by TRizol reagent and its concentration was determined by the absorbance at 260 nm. The upstream primer of cytochrome c was 5'-GGCTTCTCCTACACTCATGAA-3' and the downstream primer was 5'-CCGAAACAGATTGCTTTTCC-3'. The upstream primer of caspase-3 was 5'-AGATACAGCAGAAAAAGGAGCATG-3' and the downstream primer was 5'-TCAAGCAGTGCTCGCAACACGTA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal RNA loading control with the upstream primer 5'-TGAAGTGTCGGAGTCTACACGGATTG-3' and the downstream primer 5'-CACCAACCTGGAGTACCAGGTCA-3'. The cDNA was amplified under the following cycle conditions: 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 58 °C for 45 s and 72 °C for 1 min and a final extension at 72 °C for 10 min. The polymerase chain reaction (PCR) products were determined by 1.5% agarose gel stained with ethidium bromide and analyzed by image analysis system. The semi-quantitative determination for cytochrome c and caspase-3 mRNAs was expressed as a ratio to GAPDH mRNA.

1.10 Western blot analysis The cells were collected and lysed in the buffer (50 mmol/L tris (hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), 150 mmol/L sodium chloride (NaCl), 40 mmol/L sodium fluoride (NaF), 5 mmol/L ethylene diamine tetraacetic acid (EDTA), 2.175 mmol/L sodium orthovanadate, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1% aprotinin and 1 mmol/L phenylm-
ethylsulfonyl fluoride). The lysate was centrifuged at 12,000 x g for 10 min at 4°C and the supernatant was collected. The protein concentration was measured by Lowry’s method[7]. The protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After transfer, the membranes were blocked with 5% fat-free milk for 1 h and then incubated in primary antibodies at a 1:1000 (v/v, volume ratio) dilution at 4°C overnight. The membranes were then washed with Tris-HCl buffer solution and TBS-Tween (TBST) solution, and incubated in horseradish peroxidase-conjugated secondary antibodies. And finally the immunodetection was carried out by staining with dianinobenzidine. The densities of bands were scanned and quantified by an image analysis system (GelPro 4.5, New York, USA). Cytochrome c and caspase-3 proteins were determined as a ratio to β-actin.

1.11 Statistical analysis Values were presented as mean ± standard deviation. Statistical differences were evaluated by one-way analysis of variance followed by the least significant difference (LSD)-t test. P < 0.05 was considered significant.

2 Results

2.1 Effects of scutellarin benzyl ester on cell viability

After exposure to ischemic condition, the cell viability of the model group decreased markedly compared with that of the control group (P < 0.01). High and medium dose of scutellarin benzyl ester could significantly attenuate the loss of cell viability induced by ischemia (P < 0.01 and P < 0.05). There was no statistical difference of cell viability between the low-dose scutellarin benzyl ester group and the model group (Table 1).

2.2 Effects of scutellarin benzyl ester on LDH release

After exposure to ischemic condition, the LDH release of the model group increased remarkably compared with that of the control group (P < 0.01). Each dose of scutellarin benzyl ester could significantly reduce the release of LDH induced by ischemia (P < 0.01) (Table 2).

Table 1 Effects of scutellarin benzyl ester on cell viability (mean ± standard deviation)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>OD value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>0.51 ± 0.054</td>
</tr>
<tr>
<td>Model</td>
<td>5</td>
<td>0.21 ± 0.025 **</td>
</tr>
<tr>
<td>High-dose scutellarin benzyl ester (100 μmol/L)</td>
<td>5</td>
<td>0.28 ± 0.032 ** ΔΔ</td>
</tr>
<tr>
<td>Medium-dose scutellarin benzyl ester (50 μmol/L)</td>
<td>5</td>
<td>0.23 ± 0.032 ** Δ</td>
</tr>
<tr>
<td>Low-dose scutellarin benzyl ester (25 μmol/L)</td>
<td>5</td>
<td>0.21 ± 0.021 ** ΔΔ</td>
</tr>
</tbody>
</table>

** P < 0.01, vs control group; ΔΔ P < 0.01, vs model group. OD: optical density.

Table 2 Effects of scutellarin benzyl ester on LDH release (mean ± standard deviation, U/L)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>78.8 ± 12.8</td>
</tr>
<tr>
<td>Model</td>
<td>5</td>
<td>386.1 ± 30.7 **</td>
</tr>
<tr>
<td>High-dose scutellarin benzyl ester (100 μmol/L)</td>
<td>5</td>
<td>54.2 ± 22.9 ΔΔ</td>
</tr>
<tr>
<td>Medium-dose scutellarin benzyl ester (50 μmol/L)</td>
<td>5</td>
<td>101.4 ± 22.7 ΔΔ</td>
</tr>
<tr>
<td>Low-dose scutellarin benzyl ester (25 μmol/L)</td>
<td>5</td>
<td>141.3 ± 23.1 ** ΔΔ</td>
</tr>
</tbody>
</table>

** P < 0.01, vs control group; ΔΔ P < 0.01, vs model group. LDH: lactate dehydrogenase.

2.3 Effects of scutellarin benzyl ester on ischemia-induced apoptosis of cardiomyocytes

2.3.1 Nuclear damages revealed by Hoechst-PI staining After exposure to ischemic condition, the injured nuclei exhibited typical characteristics of apoptosis such as condensation of chromatin and shrinkage and fragmentation of the nuclei. The morphology changes of injured nuclei could be attenuated remarkably by pretreatment with scutellarin benzyl ester (Figure 1).

2.3.2 Percent apoptosis revealed by flow cytometry After exposure to ischemic condition, the percentage of apoptotic cells of the model group increased remarkably compared with that of the control group (P < 0.01) and about 20% of the cells of the model group were arrested in G1/M phase. Scutellarin benzyl ester could significantly decrease the percentage of apoptosis induced by ischemia (P < 0.01) and depress the arrested population (Figure 2, Table 3).

Figure 1 Changes of nuclei tested by Hoechst-prodium iodide staining (Microscopy, ×400)
A: Control; B: Model; C: Scutellarin benzyl ester (100 μmol/L).
Table 3  Changes of apoptosis percentage tested by flow cytometry

(mean±standard deviation, %)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Apoptosis percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>4.52±0.29</td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>30.14±1.89**</td>
</tr>
<tr>
<td>High-dose scutellarin benzyl ester (100 μmol/L)</td>
<td>6</td>
<td>18.93±1.45** △△</td>
</tr>
<tr>
<td>Medium-dose scutellarin benzyl ester (50 μmol/L)</td>
<td>6</td>
<td>22.10±1.27** △△</td>
</tr>
<tr>
<td>Low-dose scutellarin benzyl ester (25 μmol/L)</td>
<td>6</td>
<td>29.80±2.12** △△</td>
</tr>
</tbody>
</table>

** * P<0.01, vs control group; △△ P<0.01, vs model group.

2.3.3 Changes in cytochrome c release and caspase-3 activation

To investigate the inhibitory effect of scutellarin benzyl ester on apoptosis induced by ischemia, the expressions of cytochrome c and caspase-3 mRNAs and proteins were examined by RT-PCR and Western blotting. The results showed that the expressions of cytochrome c and caspase-3 of the model group increased remarkably compared with those of the control group (P<0.01) and scutellarin benzyl ester could significantly inhibit the increased expressions of cytochrome c and caspase-3 mRNA and protein levels induced by ischemia (P<0.01) (Figures 3 to 6).

Figure 3  Changes in the expression of cytochrome c mRNA tested by RT-PCR

A: Control group; B: Model group; C: Scutellarin benzyl ester (100 μmol/L) group; D: Scutellarin benzyl ester (50 μmol/L) group; E: Scutellarin benzyl ester (25 μmol/L) group. ** * P<0.01, vs control group; △△ P<0.01, vs model group. RT-PCR: reverse transcription-polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Figure 4  Changes in the expression of caspase-3 mRNA tested by RT-PCR

A: Control group; B: Model group; C: Scutellarin benzyl ester (100 μmol/L) group; D: Scutellarin benzyl ester (50 μmol/L) group; E: Scutellarin benzyl ester (25 μmol/L) group. ** * P<0.01, vs control group; △△ P<0.01, vs model group. RT-PCR: reverse transcription-polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Figure 5  Changes in the expression of cytochrome c protein tested by Western blotting

A: Control group; B: Model group; C: Scutellarin benzyl ester (100 μmol/L) group; D: Scutellarin benzyl ester (50 μmol/L) group; E: Scutellarin benzyl ester (25 μmol/L) group. ** * P<0.01, vs control group; △△ P<0.01, vs model group.
cardiomyocytes were isolated and placed in glucose-free D-Hank’s solution bubbled with 95% N₂/5% CO₂ for 6 h, responding with a reduction in contractile activity. This study revealed that ischemia for 6 h may significantly decrease the cell viability and increase cardiomyocytes apoptosis as evidenced by MTT assay and elevated LDH release in the culture medium, condensed and fragmented chromatin in the nuclei and the increased percentage of apoptotic cells. However, pretreatment with different doses of scutellarin benzyl ester may greatly decrease cell viability loss in a dose-dependent manner, reduce LDH release, attenuate morphology changes of the injured nuclei and reduce percentage of the apoptotic cells. These results strongly suggested that scutellarin benzyl ester has a protective effect against ischemic injury and can inhibit cardiomyocytes apoptosis in response to hypoxia.

Based on the obtained results that scutellarin benzyl ester may protect cardiomyocytes against apoptosis in ischemic injury, further investigation was performed with focus on the possible molecular mechanism involved in the anti-apoptosis effect of scutellarin benzyl ester. Apoptosis, the programmed cell death, is an early and significant event in the process of myocardial ischemia and the mitochondria plays a critical role in apoptosis\textsuperscript{12, 13}. It is well accepted that the cellular pathway of apoptosis is associated with cytochrome c release from mitochondria followed by reactions of caspase proteases and finally with DNA fragmentation\textsuperscript{14}. Moreover, caspase activation may cause the release of cytochrome c in turn by inducing mitochondria permeability transition pore opening. In the cascade reactions, caspase-3 is considered the crucial molecule and its activation finally induces the occurrence of apoptosis\textsuperscript{15}. In this study, it was found that 6 h of ischemia caused cytochrome c release and subsequently the activation of caspase-3, evidenced by the increased expression levels of cytochrome c and caspase-3 mRNAs and proteins. However, pretreatment with scutellarin benzyl ester could significantly inhibit the release of cytochrome c from mitochondria to cytoplasm and caspase-3 activity, thus preventing the apoptotic process during ischemia.

In conclusion, scutellarin benzyl ester has a remarkable protective effect against myocardial ischemic injury and the protective mechanism may associate with its anti-apoptosis effect by inhibiting cytochrome c release and caspase-3 activation.

4 Acknowledgments

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5 Competing interests

The authors declare that they have no competing interests.

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灯盏乙素苄酯衍生物对乳鼠心肌细胞
缺血损伤的影响及其抗凋亡作用机制

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目的：探讨灯盏乙素苄酯衍生物对乳鼠心肌细胞缺血损伤的保护作用及抗凋亡机制。
方法：体外原代培养 SD 雌鼠的心肌细胞，将猪心状态良好且生长密度无明显差异的心肌细胞随机分为 5 组：正常对照组、缺血模型组、灯盏乙素苄酯衍生物组（高、中、低剂量分别为 100、50、25 μmol/L，缺血造模前加入干预药物）。通过葡萄糖剥夺模型体外模拟心肌缺血损伤，缺血 6 h 后，通过 MTT 法测定各组心肌细胞的代谢活力，采用生化手段检测各实验组培养基中乳酸脱氢酶活性。采用 Hoechst-碘化丙啶双染法观察各组心肌细胞核的形态改变，采用流式细胞术测定各组心肌细胞凋亡率，通过逆转录聚合酶链反应技术检测各组细胞的细胞色素 c 和 caspase-3 在 mRNA 水平的表达。通过蛋白质印迹技术检测各组细胞的细胞色素 c 和 caspase-3 在蛋白水平的表达。
结果：与正常对照组相比，缺血后模型组心肌细胞的代谢活力显著下降（P<0.01）。与模型组相比，灯盏乙素苄酯衍生物可显著减轻缺血损伤心肌细胞代谢活力的丧失（高剂量 P<0.01，中剂量 P<0.05）；各剂量灯盏乙素苄酯衍生物可显著减少缺血损伤心肌细胞乳酸脱氢酶的外溢（P<0.01）；形态学方面，灯盏乙素苄酯衍生物可有效减轻缺血损伤心肌细胞的染色质凝集、细胞核固缩等凋亡形态学改变；灯盏乙素苄酯衍生物能明显降低缺血损伤心肌细胞的凋亡百分率（P<0.01）；灯盏乙素苄酯衍生物可显著抑制缺血损伤心肌细胞的细胞色素 c 和 caspase-3 在 mRNA 及蛋白水平的表达（P<0.01）。
结论：灯盏乙素苄酯衍生物对体外培养心肌细胞的缺血损伤具有显著的保护作用。这种保护作用的机制可能是通过抑制细胞色素 c 释放和 caspase-3 活化从而阻断细胞凋亡的途径而实现的。
关键词：灯盏细辛；灯盏乙素苄酯；药物前体；心肌细胞；心肌；缺血；细胞凋亡；大鼠