Determination of tanshinone II A in *Salvia miltiorrhiza* Bunge extract and its effect on ischemia-reperfusion injury

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To establish an HPLC method for determination of tanshinone II A in *Salvia miltiorrhiza* Bunge extract, and observe the protective effect of *Salvia miltiorrhiza* extract on myocardial ischemia-reperfusion injury in rats. Column used is a Diamonsil C₁₈, mobile phase is methanol-water (75:25), and detection wavelength is 270 nm. Ischemia-reperfusion injury model is replicated, and the size of myocardial infarction area is observed, as well as myocardial pathological changes under light microscope (LM) and transmission electron microscope (TEM). Tanshinone II A shows a good linearity (r=0.9999) within 0.074~0.37 µg, and its recovery is 99.17% (RSD 1.76%, n=6). In *Salvia miltiorrhiza* extract group, myocardial infarction area shrinks markedly, and the degree of myocardial cell degeneration and necrosis, and ultrastructural morphological changes in myocardial cells are significantly reduced under LM and TEM. The method established is simple and accurate. *Salvia miltiorrhiza* extract has a protective effect on myocardial ischemia-reperfusion injury in rats.

**Keywords:** HPLC, Tanshinone II A, Content determination, Myocardial Ischemia-reperfusion injury, Myocardial protection

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*Salvia miltiorrhiza* Bunge is the dried roots and rhizomes of *Salvia miltiorrhiza* Bge. in the genus *Salvia* of the family Lamiaceae, which is mainly grown in China and Japan. *Salvia miltiorrhiza* is bitter, slightly cold, and has stasis removing, pain relieving, blood circulation activating, meridian stimulating, heart clearing and vexation eliminating actions. *Salvia miltiorrhiza* contains a variety of chemical constituents, major constituents include diterpene quinones and phenolic acids; there are also a large amount of volatile oil constituents. Pharmacological studies have found that *Salvia miltiorrhiza* has anti-cancer, anti-bacterial and anti-oxidant effects; in addition, it can also dilate coronary artery, increase coronary blood flow, improve microcirculation, and protect the heart. *Salvia miltiorrhiza* has very significant effects on cardiovascular system; such actions are of important significance to the protection against myocardial ischemia-reperfusion injury.

Myocardial ischemia-reperfusion injury often occurs during intracoronary thrombolysis, intracoronary balloon angioplasty, open heart cardiopulmonary bypass surgery and other related procedures after acute myocardial infarction. Its mechanism is very complex, which is currently believed to be associated with intracellular Ca²⁺ overload after reperfusion, oxygen free radical overgeneration, microvascular endothelial cell injury, interaction between vascular endothelial cells and leukocytes/platelets, etc. In recent years, some progress has been made in the research on prevention and treatment of myocardial ischemia-reperfusion injury by Chinese medicine; basic studies have demonstrated that Chinese medicine *Salvia miltiorrhiza* has a protective effect on ischemia-reperfusion injury through multiple ways. In this study, *Salvia miltiorrhiza* extract is obtained by extraction of *Salvia miltiorrhiza*, tanshinone II A content in the extract is determined by HPLC, as well as the effects of the extract on myocardial ischemia-reperfusion injury in rats, the efficacy of the extract is evaluated, and the protective mechanism of the extract on ischemia-reperfusion myocardium is explored, in order to enhance people's awareness and clinical application of Chinese medicine in prevention and treatment of myocardial ischemia-reperfusion injury.
Materials

Instruments
Waters 510 HPLC, 486 detector, 740 integrator, Waters automatic gradient mixer.

Reagents
Tanshinone II A reference substance (purchased from the National Institute for the Control of Pharmaceutical and Biological Products, batch No. 201403-9641); crude Salvia miltiorrhiza drug was purchased from Chengdu Hengda Tianqi Pharmaceuticals, which was identified by Professor Wang Enmin at the Capital University of Chinese Medicine as the dried roots and rhizomes of Salvia miltiorrhiza Bge. in the genus Salvia of the family Lamiaceae. Methanol was HPLC grade, water was purified water, and all other reagents were of analytical grade.

Extraction method
Two Kg of crude Salvia miltiorrhiza drug was taken, and extracted under reflux twice with 5 L of 95% ethanol. After filtration, the filtrate was subjected to column chromatography on MCI (microporous resin), followed by collection of 30% ethanol eluent. The eluent was recovered under reduced pressure to give the extract, which was then freeze-dried to yield a dry extract for later use.

Experimental animals
Healthy male SD rats, weighing (210–20) gm, provided by the SPF Laboratory Animal Center of Nanjing Medical University.

Methods and results
Chromatographic conditions
Diamonsil C18 column (4.6 mm×250 mm, 5 µm); mobile phase: methanol-water (75:25); detection wavelength: 270 nm; column temperature: 30°C; injection volume: 10 µL. Number of theoretical plates was not less than 4,000 calculated based on the chromatographic peak of tanshinone II A. Under the above chromatographic conditions, tanshinone II A was well separated from other constituents (Fig. 1).

Preparation of reference solution
Appropriate amount of tanshinone II A reference substance was accurately weighed, placed in a stoppered flask, and added with methanol to prepare a 3.7 mg/mL tanshinone II A reference solution.

Preparation of test solution
About 0.2 gm of the crude Salvia miltiorrhiza drug powder (passed through a 50 mesh sieve) was accurately weighed, placed in a stoppered conical flask, added precisely with 25 mL of methanol, weighed, and heated under reflux for 1 hr, allowed to cool, then weighed again. After the lost weight was replenished with ethanol, the solution was shaken well, filtered, and the subsequent filtrate was collected as the test solution.

Methodological evaluation
Evaluation of linearity
2, 4, 6, 8 and 10 µL of tanshinone II A reference solutions were precisely drawn, and injected under the above chromatographic conditions, respectively. Peak areas were measured, and the regression equation was obtained with reference volume as the independent variable, and peak area as the dependent variable as 
\[ Y = 63257.24X - 3254.19 \] 
\( r = 0.9998 \), which indicated good linearity of tanshinone IIA within a 0.074–0.37 µg range.
**Precision test**

The same test solution was successively injected seven times, and the RSD of peak area integral of tanshinone II A was determined to be 0.52%, which indicated good precision of the instrument.

**Stability test**

Test solution was prepared as per the method under "Preparation of test solution", and injected for HPLC determination 0, 2, 4, 8, 12 and 24 hrs after test solution preparation, respectively. The RSD of peak area integral of tanshinone II A was determined to be 0.61%, indicating that the test solution was stable within 24 hrs.

**Reproducibility test**

Crude *Salvia miltiorrhiza* drug with the same batch number was taken, prepared into 6 parallel test solutions according to the methods under "Extraction method" and "Preparation of test solution", and injected into the HPLC for sample content determination. The results revealed an RSD of 3.27%, indicating good reproducibility of the sample preparation method.

**Recovery test**

0.1 gm of *Salvia miltiorrhiza* extract of known content was taken in 6 aliquots, accurately weighed, added separately with 23 mg of tanshinone II A reference substance, and prepared into test solutions according to the method under "Preparation of test solution", and determined, followed by calculation of recovery. The results revealed that the average recovery (n=6) of tanshinone II A was 99.17%, with a RSD of 1.76%, indicating good accuracy of the method (Table 1).

**Sample determination results**

0.2 gm of crude *Salvia miltiorrhiza* drugs with 6 different batch numbers were accurately weighed in two parallels, and prepared into test solutions according to the methods under "Extraction method" and "Preparation of test solution". Peak areas were measured, and contents were calculated, the results are shown in Table 2.

**Animal model and grouping**

Rats were intraperitoneally anesthetized with 20% urethane (1.0 gm/kg), and endotracheally intubated for artificial respiration. Left thoracotomy was performed at the fourth intercostal space, pericardium was cut open, myocardium was exposed, and non-invasive rounded 6/0 suture was placed 2 mm below the initial segment of left anterior descending coronary artery for later use. Coronary artery was ligated; 011 mV ST elevation in ECG leads or T-wave towering and darkened myocardium were considered the signs of successful ligation. After ligating for 60 min, ligature was released for reperfusion for 240 min. 15 SD rats were randomly divided into five groups, namely sham operation group, ischemia-reperfusion group and *Salvia miltiorrhiza* extract group (10 mg/kg). Drug intervention group was administered by intravenous injection through the right internal jugular vein 10 min before ischemia. Sham operation and ischemia-reperfusion groups were given equivalent volume of saline at the same time. After completion of the experiment, blood was sampled from common carotid artery, and serum was separated and stored in a -20° refrigerator for later testing. Then hearts were quickly removed. Another 15 SD rats were grouped and made into animal model as above; their myocardia were removed for NBT staining to measure the myocardial infarct size.

**Observation indices**

**Measurement of myocardial infarction size**

Rat hearts were removed, washed with iced saline, and weighed. Myocardia were cut into 5 parts from the ligature, and stained with 0.125% NBT at room temperature for 15 min, non-infarcted zone was stained blue, while infarcted zone was grey white. The myocardia in the infarcted zone were cut off, weighed, and the size of myocardial infarction was represented by the percentage of infarcted myocardial wet weight in total myocardial wet weight.
Histopathological observation of myocardia

The myocardia were fixed in 10% formalin solution, embedded in paraffin, then sectioned routinely, and HE stained. Pathological changes were observed under light microscope.

Ultrastructural Pathological observation of myocardia

After removal of hearts, normal and ischemic myocardia each 1 mm³ in size were quickly excised, placed in 4°C 3% glutaraldehyde, and prepared into electron microscopy samples routinely. Changes in myocardial ultrastructure were observed under LIBRA 120 TEM.

Statistical processing

All data were processed using SPSS statistical software package, and expressed as mean ± standard deviation (x ± s). Comparison among groups was performed by one-way ANOVA, and pair wise comparison was done by Student-Newman-Keuls q test.

Results

Comparison of myocardial infarction size after ischemia-reperfusion injury between groups is shown in Table 3. *Salvia miltiorrhiza* extract could reduce the myocardial infarction size, and the difference was significant compared with the ischemia-reperfusion group (P<0.01).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Infarction zone wet weight (gm)</th>
<th>Myocardial wet weight (gm)</th>
<th>Myocardial infarction size (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemia-reperfusion</td>
<td>8</td>
<td>0.31±0.02</td>
<td>0.65±0.02</td>
<td>47.69±2.31</td>
</tr>
<tr>
<td><em>Salvia miltiorrhiza</em></td>
<td>8</td>
<td>0.24±0.02</td>
<td>0.66±0.02</td>
<td>36.36±3.63*</td>
</tr>
</tbody>
</table>

Note: comparison with the ischemia-reperfusion group, * P<0.01.

Pathological observation under light microscope

In the sham operation group, myocardial fibers were arranged regularly, without myocardial degeneration or necrosis. In the ischemia-reperfusion injury group, acidophilic change and vacuolar degeneration of myocardial cells were observed, and myocardial fibers were irregularly arranged, with contraction band formation. Myocardial inter fibrillar hemorrhage and focal myocardial necrosis were noted. The *Salvia miltiorrhiza* extract group showed more orderly arrangement of myocardial fibers, with occasional acidophilic change and vacuolar degeneration.

Obseration of myocardial ultrastructure

Normal control group: myofibrils were arranged orderly, and mitochondrial structure was normal (Fig. 2). Ischemia-reperfusion group: cell membranes shrank, and were disrupted partially. Nuclei were irregular, chromatins were marginalized, heterochromatins increased, mitochondrial cristae were indistinct and partly disappeared, and a large number of vacuoles were observed between mitochondria. Myofibrils were arranged irregularly, patchy, and homogenized; sarcomeres were indistinct, and light and dark bands were blurred (Fig. 3). In the *Salvia miltiorrhiza* extract group, lesions were significantly reduced as
compared with the ischemia-reperfusion group. Myofibrils were arranged quite orderly, and mitochondrial cristae were smooth. Dense granules were observed occasionally (Fig. 4).

Discussion

*Salvia miltiorrhiza* has some protective effect against ischemia-reperfusion injury, in which tanshinone II A is main active constituent. However, tanshinone II A content is low in crude *Salvia miltiorrhiza* drug, which is less than 1%. Therefore, the extraction of crude *Salvia miltiorrhiza* drug and improvement of tanshinone II A content are of important significance to the activity research of *Salvia miltiorrhiza* extract. The results of this study find that tanshinone II A content in *Salvia miltiorrhiza* extract is about 23.12%, achieving the goal of improving tanshinone II A content. To ensure the efficacy, further establishment of limits on content of *Salvia miltiorrhiza* extract is vitally necessary.

Free radical production, cellular Ca$^{2+}$ overload and lipid peroxidation induced therefrom are one of the important aspects of ischemia-reperfusion injury. To restore normal cell function, the search for effective oxygen free radical scavengers and Ca$^{2+}$ antagonists has become an important direction of research on ischemia-reperfusion injury protective drugs. Pathological observation in this experiment finds that the rats are subjected to the same degree of ischemia-reperfusion injury; after pretreatment with *Salvia miltiorrhiza* extract, the size of myocardial infarction caused by acute ischemia-reperfusion was significantly reduced in rats. The differences were statistically significant for all pretreatment groups as compared with the ischemia-reperfusion model group (P<0.01). Light microscopy showed alleviated lesions, regularly arranged myocardial fibers, and less common acidophilic change or vacuolar degeneration in the *Salvia miltiorrhiza* extract pretreatment groups. Electron microscopic myocardial ultrastructure showed markedly reduced pathological changes in rats in the *Salvia miltiorrhiza* extract pretreatment groups, with only mild swelling of mitochondria; cristae and myofibrils were arranged regularly, which were almost normal. In the ischemia-reperfusion model group, mitochondria were apparently swelled; cristae and myofibrils were arranged irregularly, and fractured; sarcomeres were indistinct; and the differences were significant.

The above pathological changes further illustrate the protective effect of *Salvia miltiorrhiza* extract on acute myocardial ischemia-reperfusion injury in rats. Its protective mechanism may be associated with the following factors: (1) Expansion of coronary artery, reduction of vascular resistance, and increase of ischemia-reperfusion blood flow. (2) Inhibition of myocardial inflammatory cell infiltration, anticoagulation, and inhibition of coronary artery spasm caused by TXA2 and 5-HT release due to platelet aggregation and activation through glucocorticoid-like effects, reduction of blood viscosity, improvement of myocardial nutritional blood flow, and supply of sufficient blood and nutrient to ischemic myocardial cells. (3) Improvement of myocardial cell metabolism, promotion of myocardial mitochondrial function recovery, and increase of myocardial cellular ATP production, storage and utilization. (4) Prevention of Ca$^{2+}$ influx, increase of sarcoplasmic reticulum Ca$^{2+}$ uptake, reduction of cytoplasmic free Ca$^{2+}$ concentration, and blockage of Ca$^{2+}$ overload-induced aggravation of myocardial cell injury through Ca$^{2+}$ antagonist-like effects.

References