Madecassoside Reduces Ischemia-Reperfusion Injury on Regional Ischemia Induced Heart Infarction in Rat

Guang-Xing BIAN, Gui-Gui LI, Yun YANG, Rui-Ting LIU, Jian-Ping REN, Li-Qing WEN, Shao-Ming GUO, and Qiu-Jun LU∗

Department of Pharmacology and Toxicology, Beijing Institute of Radiation Medicine; Beijing 100850, People’s Republic of China. Received September 19, 2007; accepted December 6, 2007; published online December 13, 2007

Madecassoside (MA), one of the principle terpenoids in Centella asiatica, has shown protect effect on isolated rat hearts and isolated cardiomyocytes against reperfusion injury in our previous studies. The aim of this study is to investigate if MA also protected against myocardial ischemia-reperfusion injury in vivo. The ischemia infarction model was established in rats. Left ventricular function was monitored during the ischemia-reperfusion period by a multi-channel recorder. After the ischemia-reperfusion process the infarcted areas were assessed. The levels of lactate dehydrogenase (LDH), creatinephosphokinase (CK), malondialdehyde (MDA), superoxide dismutase (SOD) and C-reactive protein (CRP) in serum were determined. Cardiomyocyte apoptosis was measured by terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining. Pre-treatment with MA (50, 10 mg/kg) attenuated myocardial damage characteristic of decreasing infarct size, decreasing LDH and CK release. Activities of SOD were increased and MDA level increased obviously in control group whereas pretreatment with MA blunted the decrease of SOD activity, markedly reduced the level of MDA and the activity of CRP, and relieved myocardial cell apoptosis. These results suggest that MA has the protective effect on myocardial ischemia-reperfusion injury. This protection ability possibly due to its anti-lipid peroxidation, anti-inflammation and anti-apoptosis function and the enhancement of SOD activity.

Key words madecassoside; ischemia-reperfusion; myocardial function; rat model

Myocardial infarction is a leading cause of death and disability, with a direct correlation between infarct size and prognosis. Reperfusion of ischemic tissue is necessary to terminate the processes of ischemic injury that ultimately result in infarction. However, abrupt reperfusion may be associated with severe metabolic and ionic disturbances that can provoke further tissue injury and myocardial cell death.1) Although this concept has proved controversial, there is experimental evidence that the extent of irreversible cell injury increases following reperfusion.2) Since reperfusion is the cornerstone of treatment for acute myocardial infarction, there is great interest in the development of adjunct therapies, which might attenuate reperfusion injury and thereby maximize the benefits of reperfusion.3)

Centella asiatica (Cea) is a creeping plant growing in damp places in China and other Asian countries. Madecassoside (MA), one of the principle terpenoids in Centella asiatica (Fig. 1), has been used as a wound healing agent and for the prevention of cicatrisation.4) In addition, it has been shown to promote fibroblast proliferation and collagen synthesis.5) A recent study demonstrated that MA accelerated the healing of gastric ulcers. However, whether MA could exert cardiovascular biologic effects remains unclear.

In our progress of screening for natural compounds with anti-ischemia activity, it was found that MA can protect isolated rat hearts and isolated cardiomyocytes against reperfusion injury in vitro. In this study, we investigated the effect of MA on prevention from the reperfusion injury in a rat ischemia-reperfusion model.

MATERIALS AND METHODS

Animals Adult male Wistar rats weighing 250—350 g were purchased from the Beijing Animal Center (Beijing, China) and allowed to acclimatize in the institutional animal house for more than 5 d before use, with standard rat food and water ad libitum. All animals were treated humanely, and the study protocols were in accordance with the Regulations of Good Laboratory Practice for non-clinical laboratory studies of drug issued by the State Food and Drug Administration of China.

Experimental Protocol Stock solutions of Madecassoside (ShangHai RongHe YiY ao KeJi Corp., HPLC>98%) were prepared in 0.5% carboxymethylcellulose (CMC)—saline and stored as aliquots at 4 °C.

Animals were randomly divided into six groups. Sham, sham-operated group with CMC; Control, 1 h-ischemia+2 h-reperfusion with CMC; MA2, MA10, and MA50, 1 h-ischemia+2 h-reperfusion with MA 2, 10, and 50 mg/kg/d, respectively; Nif, 1 h-ischemia+2 h-reperfusion with nifedipine 10 mg/kg/d. Sham was only been passed a silk suture through the myocardium of the artery beneath, no coronary artery occlusion.

In Vivo Surgical Preparation Sixty rats were sedated with pentobarbital sodium (35—50 mg/kg). Surgery was performed under sterile conditions. The right carotid artery and

Fig. 1. Chemical Structure of Madecassoside

© 2008 Pharmaceutical Society of Japan
femoral vein were cannulated to monitor left ventricular pressure as its first derivative (dp/dt), and a vinyl catheter was inserted into femoral artery to monitor blood pressure via a transducer attached to a multi-channel recorder (BIOPAC MP150, U.S.A.). A left lateral thoracotomy was performed in the fourth or third intercostal space, and the hearts were exposed in a pericardial cradle. A 4-0 silk suture on a small curved needle was passed through the myocardium beneath the middle segment of the large arterial branch coursing down the middle of the anterolateral surface of the left ventricle. A small vinyl flake was passed into both ends of the suture, which was then fixed by clamping the tube with a mosquito haemostat. Myocardial ischemia was confirmed by regional cyanosis of myocardial surface and electrocardiographic change. The rats underwent a 1 h occlusion of an anterolateral branch of the coronary artery, followed by a 2 h reperfusion.

**Hemodynamics and Measurement of the Left Ventricular Function**  
Hemodynamic data were obtained before ischemia (Pre-I), after 1 h-ischemia (I-1 h), after 1 h-ischemia + 1 h-reperfusion (I-1 h), and after 1 h-ischemia + 2 h-reperfusion (IR-2 h). Also at the same time-points, the left ventricular function including left ventricular systolic pressure (LVPSP), maximum rate of left ventricular pressure development (±dp/dt) and left ventricular end-diastolic pressure (LVEDP) were measured by a multi-channel recorder with BIOPAC MP150 Systems.

**Biochemical Analyses**  
Blood serum samples after ischemic-reperfusion (IR-2 h) were collected to measure the myocardial specific enzyme, including the activity of creatine phosphokinase (CK) and lactate dehydrogenase (LDH). The activity of CK and LDH were analyzed at 25 °C using commercial kits (ZhongSheng BeiKong Bio-Technology and Science Inc, Beijing, China) by Analyzer Medical Systems (SaBa-18, Roma, Italy).

**Infarct Size Assessment**  
After the completion of the infract protocol and infusion, the heart was quickly removed. Infarct size was determined by tetrazolium staining. The hearts were frozen at −20 °C for 1—2 h and 2 mm thick sections were cut from the apex to the level of the coronary suture, then incubated in 1% triphenyltetrazolium chloride (TTC) in a phosphate buffer (pH 7.4) at 37 °C for 15—20 min. In a globally ischemic heart, the whole ventricle is at risk of infarction, the area of left ventricle and the area of the infarcted tissue were measured by an independent blinded observer using planimeter. The volumes of the infarcted zone were calculated by multiplying the planimetered areas by slice thickness. Infarct volume was expressed as a percentage of left ventricular volume for each heart.

**Measurement of MDA Levels and Activity of SOD**  
Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were used as indices of oxygen free radical and lipid superoxide level. The content of MDA and activity of SOD were measured using commercial kits (JianCheng Bioengineering Institute, Nanjing, China) with a spectrophotometer (Beckman, U.S.A.).

**CRP Measurement**  
Blood serum samples after ischemic-reperfusion injury were collected to measure C-reactive protein (CRP). A rat CRP cytoscreen ELISA kit (Bionewtrans Pharmaceutical Biotechnology, U.S.A.) was used according to the manufacturer’s instructions and the resulting yellow to blue color intensity was recorded at 450 nm by a Sorin-Biomedica microplate reader (Thermo, U.S.A.).

**Apoptosis Assay**  
After reperfusion, the heart was quickly removed, washed in ice-cold PBS solution and incubated in 4% paraformaldehyde. Four rats from each group were euthanized, and tissues were sampled from the ischemic area of the left ventricle from hearts fixed by paraformaldehyde perfusion and processed as previously described. The tissues were then fixed in an automatic tissue fixing machine and embedded in paraffin. In Situ Cell Apoptosis Detection Kit I.POD (Boster, Wuhan, China) was used, which detects apoptosis-induced nuclear DNA fragmentation via a fluorescence assay assay based on terminal deoxynucleotidyl trans-ferase (TdT)-mediated dUTP nick-end labeling (TUNEL). Then 5 watch fields were chosen randomly under microscope on each section. Positive brown cells and total cells were counted by MIAAS4.0 (Medical Image Analysis System, Beijing BingYang KeJi Corp.). Apoptosis index (positive cells/total cells·100%) was used as the indicator of apoptosis.

**Statistical Analysis**  
Quantitative data are expressed as mean±S.E.M. of n observations. Data were analyzed by unpaired Student’s t-test and one-way analysis of variance (ANOVA) followed by the Dunnett’s test for multiple comparisons. Differences in the incidence of treatment groups at individual time points were analyzed by Fisher’s exact probability test. p values of less than 0.05 were considered statistically significant.

**RESULTS**

**Effects of Madecassoside on Hemodynamic and Left Ventricular Function**  
As expected, the values of heart rate (HR) during ischemia were decreased in control group as compared with baseline (before ischemia, Pre-I). Compared with control group at the same time-points, no significant differences were observed in Nifedipine (Nif) group (Table 1). Only compared with control group, HR values were slightly increased in Nif group at reperfusion process. MA caused significant changes at all concentrations after reperfusion for 2 h, and this also was observed at 50 mg/kg high concentration after reperfusion for 1 h in HR compared with the control group. But HR of all groups decreased slightly compared with baseline in the early phase of ischemia-reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Control</th>
<th>MA 50 mg/kg</th>
<th>MA 10 mg/kg</th>
<th>MA 2 mg/kg</th>
<th>Nif 10 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-I</td>
<td>362±32</td>
<td>352±40</td>
<td>356±52</td>
<td>365±34</td>
<td>380±19</td>
<td>390±19$^*$</td>
</tr>
<tr>
<td>I-1 h</td>
<td>341±43</td>
<td>327±47</td>
<td>329±33</td>
<td>322±26$^*$</td>
<td>390±25$^*$</td>
<td>380±22$^*$</td>
</tr>
<tr>
<td>IR-1 h</td>
<td>352±34</td>
<td>317±53</td>
<td>367±49$^*$</td>
<td>390±19$^*$</td>
<td>390±19$^*$</td>
<td>390±19$^*$</td>
</tr>
<tr>
<td>IR-2 h</td>
<td>361±37</td>
<td>305±41$^*$</td>
<td>351±53</td>
<td>389±22$^*$</td>
<td>390±19$^*$</td>
<td>390±19$^*$</td>
</tr>
</tbody>
</table>

As expected, heart rate (HR) during ischemia was decreased in control group as compared with Pre-I. Compared with control group, no significant differences were observed in Nifedipine (Nif) group. MA caused significant changes after reperfusion for 2 h, and this also was observed at 50 mg/kg high concentration after reperfusion for 1 h compared with the control group. Pre-I, before ischemia; I-1 h, ischemia for 1 h; IR-1 h, IR-2 h, Reperfusion for 1 h, 2 h respectively. *p<0.05 vs corresponding Pre-I; $p<0.05$ vs control group.
chemia-reperfusion injury (I-1 h and IR-1 h) in the experiment. LVSP was markedly high in Nif groups after ischemia-reperfusion injury than in control groups. Only MA of 50 mg/kg dose was evidenced by significant differences during the periods of ischemia-reperfusion injury (n=6—7). Significant differences in LVEDP were noted in Nif group compared with control group. 50 mg/kg MA group maintained a higher mean value than control group in the early phase of ischemia-reperfusion injury process. But other concentration has no impact on the LVEDP value in the progress. (C) Effect of Madecassoside on dp/dt max of rats in ischemia-reperfusion injury. dp/dtmax was slightly higher in the MA and Nif groups than in control, especially apparent at reperfusion for 2 h. But there is no significant difference between MA groups and control group. No change was observed in sham operation group at all of time-points. (n=6—7). *p<0.05 vs. sham group; # p<0.05 vs. control group; & p<0.05 vs. the baseline of the same group.

Table 2. Effect of Madecassoside on the Infarct Size of Rats in Ischemia-Reperfusion Injury (±S.D., n=10)

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Infarct size (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ventricular area (%)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>24.9±3.9</td>
</tr>
<tr>
<td>MA 50</td>
<td>50</td>
<td>16.7±3.4* #</td>
</tr>
<tr>
<td>MA 10</td>
<td>10</td>
<td>17.8±3.2* #</td>
</tr>
<tr>
<td>MA 2</td>
<td>2</td>
<td>21.7±5.1*</td>
</tr>
<tr>
<td>Nif</td>
<td>10</td>
<td>13.3±2.2*</td>
</tr>
</tbody>
</table>

Infarct volume as well as infarct size were noticeably reduced in Nif group compared with the control group. MA at high concentrations (50, 10 mg/kg) significantly deduced the infarct size after rat myocardial ischemia-reperfusion injury. *p<0.05 vs. Nif group; # p<0.05 vs. control group.

Effect on Myocardial Infarct Size Infarct volume as well as infarct size were noticeably reduced in Nif group compared with the control group as indicated in Table 2. MA at high concentrations (50, 10 mg/kg) significantly deduced the infarct size after rat myocardial ischemia-reperfusion injury. In control group, the activity of CK was also markedly higher than sham group. MA groups have decreased CK activity significantly compared with control group (Fig. 3B).

Effect on MDA and SOD In the post-ischemia reperfused myocardia of rat, the content of MDA in control markedly increased compared with sham. MA exhibited a decrease in the content of MDA compared with the Control (Fig. 4A).

SOD activity in control group and Nif group markedly decreased compared with sham group. But the SOD activity in
MA groups were even higher than in sham group and significantly higher than in control group after ischemia-reperfusion injury as shown in Fig. 4B.

Effect on CRP In control group, the activity of CRP was markedly higher than sham group. MA groups have decreased CRP activity significantly compared with control group. (n=6—7), *p<0.05 vs. sham group; #p<0.05 vs. control group.

DISCUSSION

In the ischemic myocardium, achieving reperfusion as soon as possible is essential in order to salvage cells and cardiac function. This has been clinically made possible by the coronary reperfusion, such as thrombolytic therapy, percutaneous coronary angioplasty or coronary artery by pass surgery.6,7) However, although restoration of blood flow to the jeopardized myocardial area is a prerequisite for myocardial salvage, reperfusion itself may exacerbate the injury sustained during the ischemic period. The mechanisms proposed to cause reperfusion injury include formation of oxygen free radicals, calcium over-load; neutrophils mediated myocardial and endothelial injury, progressive decline in microvascular flow to the reperfused myocardium and depletion of high-energy phosphate store.8) The pathogenesis of ischemia/reperfusion injury consists of many perplexing mechanisms, so current pharmacological approaches are limited in their ability to modify significantly the course of the disease, and offer...
incomplete and transient benefit to patients.

MA, isolated from *Centella asiatica*, has been used as a wound healing agent for the prevention of cicatrisation. In addition, it has been shown to promote fibroblast proliferation and collagen synthesis and to have antiulcer activity. In our laboratory, it is found that MA can exert anti-ischemic effect in vitro and protect isolated rat hearts and cardiomyocytes against reperfusion injury. MA also has the protective effect on vascular endothelial cell injury and can raise the coronary flow of isolated rat heart (unpublished). The results in the present study show that pre-treatment with MA as a multi-functional compound with additional pharmacological effects in vivo can prevent oxidative stress, inflammation and myocardial cell apoptosis.

MA caused no significant changes in the recorded hemodynamic profiles, such as systolic blood pressure and heart rate, compared with the Nif-treated group. These results demonstrate that MA dose not effect hemodynamic profiles differ from Nif. Myocardial enzymes can be released from the injured myocytes induced by ischemia and reperfusion. So, enzyme analysis has proved considerably valuable in the diagnosis of myocardial infarction. Alterations in myocardium specific CK and LDH have been considered as the important markers of myocardial injury. In the present study, we found that the activity of CK and LDH formation in the serum of the MA-treated hearts was significantly lower than those of control animals, suggesting that MA can protect myocardial cells. Furthermore, MA-treated hearts were resistant to ischemia-reperfusion injury as evidenced by improved post-ischemic ventricular function. All of these findings suggest that MA exerted a beneficial effect on ischemic and reperfused heart. Our study demonstrates that the treatment with Madecassoside can provide myocardial protection against reperfusion injury by decreasing infarct size.

In this study, we also investigated the potential mechanism of MA myocardial protection against reperfusion injury. Reactive oxygen species results in enhanced lipid peroxidation as indicated by an increase in MDA levels documented both in clinical and experimental studies in conditions of myocardial ischemia-reperfusion injury. In concert MDA formation in blood serum of the hearts pretreated with MA was significantly lower as compared to control animals suggesting that MA reduced free radical formation. On the other hand, MA markedly increased the activity of SOD in blood serum. The results indicate that treatment of MA can blunt a series of cascade reactions which may be responsible for myocardial damage.

Inflammation plays a role in the development of coronary heart disease and is involved in the inflammatory process linked to ischemic myocardial and necrosis. Elevation in

---

**Table 3. Effect of Madecassoside on the Apoptosis Index of Rats in Ischemia-Reperfusion Injury (±S.D., n=4)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>Apoptosis index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>—</td>
<td>0.29±0.28</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>12.7±5.4</td>
</tr>
<tr>
<td>MA 50</td>
<td>50</td>
<td>3.2±1.8*</td>
</tr>
<tr>
<td>MA 10</td>
<td>10</td>
<td>3.7±1.1*</td>
</tr>
<tr>
<td>MA 2</td>
<td>2</td>
<td>5.2±2.3</td>
</tr>
<tr>
<td>Nif 10</td>
<td>10</td>
<td>8.2±2.2</td>
</tr>
</tbody>
</table>

Apoptosis cells were noticeably increased in control group compared with the sham. MA at high concentrations (50, 10 mg/kg) significantly deduced the apoptosis cells after rat myocardial ischemia-reperfusion injury. *p<0.05 vs. control group.*

---

![Fig. 6. Representative Photo-Micrograph of *in Situ* Detection of DNA Fragment in Tissue from Rat Heart Subjected to Ischemia-Reperfusion and the Pre-treatment (×400)](image)
CRP levels seems to correlate with in-hospital and short-term adverse prognosis irrespective of the extent of myocardial damage and reflects an important role of a pre-existing inflammation for ischemia-reperfusion damage. In our study, it was found that MA can prevent the CRP increase and inhibit CRP release further as additional treatment during ischemia/reperfusion.

Cell loss through apoptosis contributes to the impairment of cardiac performance and also plays an important role in myocardial remodeling processes. Induction of apoptosis is implicated in myocardial ischemia/reperfusion injury among other cardiovascular diseases. According as staining of ventricular myocardium by TUNEL, the TUNEL-positive cells of MA group were significantly decreased. It is suggested that MA has the protective effect on ischemia-reperfusion injury, which may be related to its effect of anti-apoptosis.

Recent experiment and clinical evidence have demonstrated that drug treatment can prevent myocardial injuries. It is now well established that calcium channel blockers have cardio-protective effects, such as reduction of mortality after myocardial infarction in humans and animals, reductions of infarct size after ischemia-reperfusion injury and prevention of cardiac remodeling after myocardial infarction. Several highly specific drugs that have only one target have clearly proven the usefulness of mono-target medicine. However, clinicians are becoming increasingly convinced that modulating a multiplicity of targets can be an asset in the treatment of a multiplicity of targets can be an asset in the treatment of a ring of disorders. The preparation of dual- or multiple-ligands with reperfusion injury and the problem of the therapeutic window.

In order to elucidate the additional mechanisms by which MA can reduce myocardial apoptosis and the potential clinical implications of such actions, we need to further investigate the relationship of the effects of MA by apoptotic signals with reperfusion injury and the problem of the therapeutic window.

In conclusion, the present study suggests that MA significantly reduced the myocardial infarction induced by ischemia-reperfusion injury in rats. MA did not show a significant influence on the hemodynamic profiles including left ventricular function and heart rate in rats. MA has the protective effect on myocardial ischemia-reperfusion injury possibly through its roles of anti-oxidative, anti-inflammatory and anti-apoptosis.

Acknowledgement The authors would like to thank technician Rui-Fu Li for assistance with experiments.

REFERENCES