Extract of *Scutellaria baicalensis* Georgi Root Exerts Protection Against Myocardial Ischemia-Reperfusion Injury in Rats

Enoch Chan,* Xing-Xian Liu,* De-Jian Guo,† Yiu-Wa Kwan,** George Pak-Heng Leung,‡ Simon Ming-Yuen Lee§ and Shun-Wan Chan*

*State Key Laboratory of Chinese Medicine and Molecular Pharmacology
Department of Applied Biology and Chemical Technology
The Hong Kong Polytechnic University, Hong Kong

†Institute of Vascular Medicine
School of Biomedical Sciences, Faculty of Medicine
The Chinese University of Hong Kong, Hong Kong

‡Department of Pharmacology and Pharmacy
The University of Hong Kong, Hong Kong

§Institute of Chinese Medical Sciences
University of Macau, Av. Padre Tomas Pereira S. J.
Taipa, Macau, China

Abstract: Ischemic heart disease is a major cause of death in the world. Common therapies, such as primary coronary angioplasty and thrombolysis, are applied to restore blood supply to the heart, limit infarct size and reduce mortality. However, the restoration of blood supply would generate reactive oxygen species in damaged sites of the myocardium, intensifying the damage to the cardiac tissues. *Radix Scutellariae baicalensis* (Huangqin) is a well-known herb in traditional Chinese medicine with high antioxidant power. In this study, extract of the dry root of *Scutellaria baicalensis* Georgi (Sb) was confirmed to have a high content of flavonoids and phenolic compounds. The cardioprotective effects of the Sb extracts (3, 30 and 300 mg/kg) were evaluated in myocardial ischemia-reperfusion injured rats. The results showed that animals that had received five-day pretreatment of the Sb extract (30 mg/kg) had a significant reduction in myocardial infarct size and a marked increase in the activity of catalase in the liver. The Sb extract could additionally enhance acetylcholine-induced vasorelaxation. It was proposed that the Sb extract exerted its cardioprotection by stimulating the catalase activity and improving vascular elasticity.

Correspondence to: Dr. Shun-Wan Chan, State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, P. R. China. Tel: (+852) 3400-8718, Fax: (+852) 2364-9932, E-mail: bcswchan@polyu.edu.hk
Introduction

Ischemic heart disease is a major cause of death and disability in the world (World Health Organization, 2008). Primary reperfusion therapies such as primary coronary angioplasty and thrombolysis are the first-line treatments for ischemic heart disease, since immediate restoration of blood flow to ischemic myocardium limits infarct size and reduces mortality. Paradoxically, reperfusion may intensify the damage of the myocardium by introducing more oxygen-derived free radicals to the heart (Yellon and Hausenloy, 2007; Buja, 2005).

The generation of reactive oxygen species (ROS) during the reintroduction of blood flow to a previously ischemic area is one of the main mechanisms underlying myocardial reperfusion injury. Damage occurs when endogenous free radical scavenging mechanisms are overwhelmed or are themselves adversely affected by the ischemic insult. After reperfusion, neutrophils are activated and their accumulation occurs in the damaged part of the myocardium. Neutrophils release ROS, proteolytic enzymes, and inflammatory mediators that further amplify the infiltration of neutrophils into the damaged myocardium (Jordan et al., 1999; Moens et al., 2005; Welbourn et al., 1991). ROS in the restored bloodstream contribute greatly to cardiac tissue injury by reacting with lipids and amino acids to damage cell membranes. The loss of membrane integrity would result in necrotic cell death (Moens et al., 2005; Bolli and Marban, 1999). The major sources of ROS are NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) and myeloperoxidase in leukocytes, xanthine oxidase in the myocardium, and mitochondrial electron transport chain of cardiomyocytes after ischemia-reperfusion (Akhlaghi and Bandy, 2009; Babior, 2004; Landmesser et al., 2006). Since oxidative stress plays a key role in myocardial ischemia-reperfusion injury, the administration of agents with a high antioxidant capacity could be a possible treatment against myocardial ischemia-reperfusion injury.

Radix Scutellariae baicalensis, also known as “huangqin” in traditional Chinese medicine, is one of the most well-known herbs used in traditional Chinese medicine and complementary medicine in some other countries. It has been used for the treatment of hyperlipidemia, atherosclerosis, hypertension, dysentery and the common cold (Li-Weber, 2009). A recent report from our group suggested that the extract of Radix Scutellariae baicalensis has significant antioxidant capacity (Chan et al., 2010). Therefore, the administration of the herbal extract might improve ventricular function after ischemia-reperfusion.

In this study, the water extract of the dry root of Scutellaria baicalensis Georgi (Sb) was analyzed by measuring its total flavonoid and phenolic content. The protective effect of the Sb extract was studied using an animal model in which the animals’ hearts were subjected to ischemia-reperfusion by surgery. The activity of three common antioxidant enzymes in the liver, catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), was measured. In addition, the effect of the Sb extract on acetylcholine-induced vasorelaxation in isolated aortic rings was also studied.
Materials and Methods

Chemicals

The Sb water extract (extract ratio 5:1) and quercetin (98%) was purchased from Nanjing Zelang Medical Technological Co. Ltd. (Nanjing, China). Acetylcholine (ACH), gallic acid and phenylephrine (PE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Determination of Total Flavonoid and Phenolic Contents

The total flavonoid content was determined by the aluminum chloride colorimetric method (Lin and Tang, 2007). Briefly, the dry extract of the herb (0.1 g) was dissolved in 1 ml deionized water. This solution (150 μl) was mixed with 450 μl of 95% alcohol, 30 μl of 10% aluminum chloride hexahydrate (AlCl₃ · 6H₂O), 30 μl of 1M potassium acetate (CH₃COOK), and 840 μl of deionized water. After incubating at room temperature for 30 min, the reaction mixture’s absorbance was measured at 415 nm against a deionized water blank on a Spectronic 20 Genesys spectrophotometer (Spectronic Instruments, Inc, Rochester NY, USA). Quercetin was chosen as a standard. Using a seven-point standard curve (0–250 mg/l), the levels of total flavonoid content in the extract were determined in triplicate. The data were expressed as mg quercetin equivalents (QE)/g of dry powder.

Total phenolic contents of Sb extract were determined by the Folin-Ciocalteu method (Lin and Tang, 2007). Briefly, the dry extract of the herb (0.1 g) was dissolved in 1 ml deionized water. This solution (30 μl) was mixed with 840 μl of deionized water, 600 μl of 2% sodium carbonate (Na₂CO₃), and 30 μl of 2N Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO, USA). After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750 nm against a deionized water blank on a Spectronic 20 Genesys spectrophotometer. Gallic acid (GA) was chosen as a standard. Using a seven-point standard curve (0–300 mg/l), the levels of total phenolic contents in the extract were determined in triplicate. The data were expressed as mg gallic acid equivalents (GAE)/g of dry powder.

Treatment of Animals

Male Sprague-Dawley rats (250 ± 5 g) were randomly divided into six groups: (1) Control: treated with 10 ml/kg deionized water per day; (2) treated with 3 mg/kg of the Sb extract per day; (3) treated with 30 mg/kg of the Sb extract per day; (4) treated with 300 mg/kg of the Sb extract per day; (5) treated with 30 mg/kg of ascorbic acid per day; and (6) treated with 300 mg/kg of ascorbic acid per day. The extract of Sb and ascorbic acid were dissolved in deionized water at concentrations of 0.3, 3 and 30 mg/ml for the dosage of 3, 30 and 300 mg/kg, respectively. Each animal received intragastric injection of 10 ml/kg of the solutions daily for five days before surgical induction of myocardial ischemia-reperfusion injury.
Surgical Procedures on Animals

One hour after the last intragastric injection of the Sb extract, the rat was anesthetized with pentobarbital (40 mg/kg) through intraperitoneal injection. The trachea was cannulated for artificial respiration. Artificial respiration started immediately with air at a rate of ~60 beats/min to maintain normal pCO$_2$ and pO$_2$. Heart rate was monitored with a lead aVF electrocardiogram (ECG). The chest was opened by thoracotomy, followed by sectioning of the fourth and fifth ribs. The pericardium was incised afterwards. A silk suture was quickly placed under the left coronary artery. The artery was occluded by tying it with a small nylon string, thus subjecting the heart to ischemia. The animal was allowed to recover. After 30 min, reperfusion was achieved by removing the nylon string. The animal was then allowed to recover for 60 min before being sacrificed. The experimental protocol was conducted under the animal license issued by the Health Department of the Government of the Hong Kong SAR and the Animal Subjects Ethics Sub-committee of The Hong Kong Polytechnic University. All procedures were consistent with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and the principles outlined in the Declaration of Helsinki. Every effort was made to limit animal suffering and to limit the number of animals used in the study.

Measurement of Infarct Area

After the animal was sacrificed, the heart was quickly removed, frozen and cut into 2 mm transverse slices. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC) in pH 7.4 buffer at 37°C for 20 min. TTC stains living tissue deep red (TTC positive) while necrotic tissue appears tan in color (TTC negative). The TTC-positive and TTC-negative areas were digitally captured and measured by Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA, USA). The infarct area ($n = 3–6$ per group) was defined as the percentage of the total TTC-negative area over the total ventricle areas measured.

Assessment of Activities of Antioxidant Enzymes in Rat Liver

Liver isolated from each animal was quickly washed in saline and stored at −80°C. Total protein was extracted from the liver tissue. The activities of total superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were assessed using the SOD, CAT and GPx activity kits purchased from Nanjing KeyGEN Biotech. Co. Ltd. (Nanjing, China).

Isolation of Thoracic Aorta from Rats

Male Sprague-Dawley rats of 250 ± 5 g were killed by cervical dislocation and their thoracic aortas were quickly excised. Their aortas were then placed into Krebs-Ringer buffer consisting of 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 25 mM NaHCO$_3$, 1 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, and 11 mM D-glucose and fatty tissues and connective tissues were removed. All aorta rings were cut into 2–3 mm long sections.
Assessment of the Effect of Scutellaria baicalensis Georgi Extract on Acetylcholine-Induced Relaxation in Isolated Rat Aorta

Aorta rings were fixed onto two parallel hooks maintained in 5 ml water-jacketed organ baths containing Krebs-Ringer buffer, with constant aeration with gas mixture of 95% O₂ and 5% CO₂ and maintained at 37°C. Their isometric tensions were monitored. The resting tension was gradually increased to reach 1.0 g. In each turn of tension adjustment, the old Krebs-Ringer buffer in the organ baths was replaced with fresh drug-free one and after 10 min of equilibration, tension was adjusted and aorta rings were allowed to equilibrate for 10 min. KCl (40 mM) was used to sensitize the aortic rings. After a plateau was reached, the buffer in the baths was replaced with fresh drug-free one again. The sensitization process was repeated until the KCl-induced contractile responses were reproducible in two consecutive cycles. The isometric tensions of aorta rings were monitored and recorded by isometric force-displacement MLT1030/D transducers (AD Instruments, Australia) connected with a PowerLab data acquisition system (AD Instruments, Australia) (Li et al., 2009).

Integrity of endothelium in the aorta rings was tested by contracting the aortic rings with PE (1 mM). After a plateau was reached, ACh (1 mM) was added. If ACh could relax the aortic rings to up to 55% of the maximal contraction induced by 1 μM of PE, the rings would be regarded as endothelium-intact. After testing of endothelium integrity, the organ baths were washed with drug-free Krebs-Ringer buffer for 20 min until the aortic rings were fully relaxed.

In order to investigate the effect of the Sb extract on the vascular elasticity, aortic rings were pre-treated with either 1 or 10 μg/ml Sb extract, and then pre-contracted with PE (1 μM). After the contractile response had reached a plateau, cumulative concentrations of ACh were added into the organ baths. The vasorelaxant effect of ACh and extracts was assessed as any inhibition on full contractile response induced by PE (1 μM).

Statistical Analysis

Data were expressed as means ± standard error of the mean (S.E.M.), and n denotes the number of replications for each data point. Relaxations (%) were expressed as the percentage of the PE (1 μM)-induced contraction. GraphPad Prism 5.01 (San Diego, California, USA) was used for curve fitting and the estimation of the magnitude of maximum relaxation (E_max). Comparison of parameters among various groups was made with one-way analysis of variance, followed by Dunnett’s test for multiple comparisons among groups. In all cases, p < 0.05 means that there is a significant difference between two groups. All statistical analysis tests were performed using GraphPad Prism 5.01 for Windows.

Results and Discussions

Total Flavonoid and Phenolic Contents

Flavonoid and phenolic compounds are major antioxidants found in plants and medicinal herbs. The results indicated that there were significant amounts of flavonoid and phenolic
compounds in the Sb extract. Total flavonoid content in the Sb extract was 22.87 ± 0.92 mg QE/g of dry extract; while total phenolic content was 38.97 ± 2.12 mg GAE/g of dry extract. Therefore, flavonoids and total phenolic compounds accounted for approximately 2% and 3.9%, respectively, by mass in the dry extract.

Protective Effect of the Extract Against Myocardial Ischemia-Reperfusion Injury

As shown in Fig. 1, myocardial infarct size expressed as the percentage of total area after the surgical procedure increased up to 46.83 ± 5.28% in the control group. Extract of Sb in the dosages from 3 to 300 mg/kg gave a U-shaped suppression on the infarct size. The infarct area was significantly lower in rats with five-day pretreatment of 30 mg/kg extract of Sb (15.23 ± 2.46%, p < 0.05) than that of the control. However, neither five-day
pretreatment of 3 mg/kg extract of Sb nor that of 300 mg/kg extract of Sb could significantly reduce the infarct sizes in rats, with infarct areas equal to 38.87 ± 2.84% and 37.00 ± 8.81%, respectively. Ascorbic acid did not give significant protective effects as the Sb extract. The infarct areas in rats fed with 30 mg/kg and 300 mg/kg of ascorbic acid were 46.27 ± 9.03% and 35.59 ± 9.99%, respectively. In summary, only a five-day pretreatment of 30 mg/kg extract of Sb could exert a significant protective effect against myocardial ischemia-reperfusion injury in the current experimental setting.

The failure of ascorbic acid (both 30 and 300 mg/kg) to protect the heart from myocardial ischemia-reperfusion injury confirmed that direct scavenging free radicals may not be an effective strategy to defend against myocardial ischemia-reperfusion injury (Bellows et al., 1995; Marczin et al., 2003). Moreover, the mechanism by which Sb extract exerts protection against myocardial ischemia-reperfusion injury may not be simply due to its ability to directly scavenge free radicals. The Sb extract may exert its protective effect by other mechanism(s).

**Effect of Sb Extract on Antioxidant Defense System**

To elucidate the mechanism of the cardioprotective effect of the Sb extract, its effect on the antioxidant defense system was studied by measuring the activities of liver SOD, CAT and GPx in rats pretreated with different dosages of the Sb extract or ascorbic acid. SOD and GPx activities in rat liver were not significantly affected by the various treatments (Figs. 2A and 2C). However, there was a significant increase (p < 0.05) of CAT activity in the livers of rats pretreated with 30 mg/kg (but not 3 or 300 mg/kg) of the Sb extract (Fig. 2B). This inversed U-shaped increase in liver CAT activity through the dose range of the Sb extract (3 to 30 mg/kg) was similar to that observed in the suppression on the infarct size in the heart of the rats with myocardial ischemia-reperfusion injury. Interestingly, there was a decrease (p < 0.05) of liver CAT activity in rats pretreated with 30 and 300 mg/kg ascorbic acid to 0.49 ± 0.06-fold and 0.48 ± 0.10-fold of the control, respectively. This may be due to the potent effect of ascorbic acid in increasing circulating antioxidants. CAT is an antioxidant enzyme that converts H$_2$O$_2$ into H$_2$O and its activity is regulated by its substrate H$_2$O.$\!$ When cellular concentration of H$_2$O$_2$ increases, CAT is phosphorylated and activated by redox-sensitive tyrosine kinases (Thomas et al., 2008). It is possible the daily administration of ascorbic acid decreased the cytosolic level of H$_2$O$_2$ so that the liver CAT was not activated.

In this study, five-day pretreatment with the Sb extract led to an increase of the CAT activity, a result consistent with another study in which the administration of baicalin, a common flavonoid in Sb, had increased the expression of antioxidant enzymes SOD, CAT and GPx in streptozotocin-induced diabetic rats (Waisundara et al., 2009). In fact, flavonoids can bind to the heme moiety or other domains of CAT, leading to either activation or inhibition of CAT (Doronicheva et al., 2007) or increase in the mRNA level of the gene encoding CAT (Kampkötter et al., 2008). Since the Sb extract has a high flavonoid content, it is reasonable to speculate that the protective effect of the Sb extract against myocardial ischemia-reperfusion injury may be related to the ability of its flavonoids to enhance the
CAT activity or up-regulate CAT expression or both. Further studies on molecular signaling pathways are needed to explain the role of flavonoids in the Sb extract on the protection against myocardial ischemia-reperfusion injury.

Assessment of the Effect of Sb Extract on Ach-Induced Relaxation in Isolated Rat Aorta

To evaluate the possibility of the Sb extract acting directly on vascular tone, the ACh-induced relaxation of isolated rat aortic rings pretreated with various concentrations of Sb
extract (0, 1 or 10 µg/ml) was compared (Fig. 3). Results showed that pretreatment with Sb extract (1 or 10 µg/ml) could potentiate the vasodilation effect of ACh in rat aortic rings (Fig. 3A). Pretreatment with Sb extract (1 µg/ml) had increased $E_{\text{max}}$ from 45.90 ± 1.90% to 96.03 ± 3.89%; while treatment with Sb extract (10 µg/ml) had increased $E_{\text{max}}$ to 103.0 ± 10.26% (Fig. 3B). Therefore, Sb extract could significantly increase the aortic rings’ sensitivity toward ACh ($p < 0.05$). This effect may account for the protection of Sb extract against myocardial ischemia-reperfusion injury, as the dilatation of other branches of coronary artery could undoubtedly help the re-establishment of blood flow in post-ischemic hearts.

Because of the high flavonoid content in the Sb extract, it was speculated that flavonoids from the Sb extract could directly inhibit the activity of NADPH oxidase to produce superoxide anions in vascular endothelial cells (Steffen et al., 2008). This in turn would protect the NO released from the endothelial cells from the superoxide anions’ action to form peroxynitrite, an inactive form of NO (Benito et al., 2002).

Apart from the aforementioned antioxidant properties of flavonoid compounds, other cardiovascular beneficial mechanisms have been shown to be related to the flavonoid and phenolic compounds. Both clinical and experimental studies indicate that these compounds could improve the ability of endothelial cells to control vascular tone (Olszanecki et al., 2002; Schini-Kerth et al., 2010). Experiments with isolated arteries have shown that flavonoid and phenolic compounds cause NO-mediated endothelium-dependent relaxations and increase the endothelial formation of NO (Schini-Kerth et al., 2010; Wu et al., 2010). The Sb extract contains high content of flavonoid and phenolic compounds, which could enhance ACh-induced relaxation by increasing the bioavailability of NO. To increase the bioavailability of NO, flavonoids may up-regulate the activity of endothelial nitric oxide
synthase (eNOS) by increasing intracellular Ca$^{2+}$ in endothelial cells, leading to increased activity of eNOS (Olszanecki et al., 2005), or increased expression of eNOS (Sanchez et al., 2006).

In addition, flavonoids may inhibit Ca$^{2+}$ influx to vascular smooth muscle cells, or activate voltage-gated K$^+$ channels on vascular smooth muscle cells to cause vasodilation (Ajay et al., 2003; Calderone et al., 2004). Similar effects on Ca$^{2+}$ movement in the myocardium may prevent the damaging event of a Ca$^{2+}$ overload after ischemia-reperfusion (Akhlaghi and Bandy, 2009).

Taking all of the findings together, the Sb extract could limit the damage induced by ischemia-reperfusion by multiple mechanisms through its flavonoid and phenolic components. Further studies are needed to understand the detailed mechanisms involved in the cardiovascular beneficial effects of the Sb extract on myocardial ischemia-reperfusion injury.

In conclusion, it was found that the extract of Sb has a significant protective effect against myocardial ischemia-reperfusion injury. The herbal extract was likely to have exerted the protection by its stimulatory effect on the CAT activity in the liver and vasorelaxation response on the blood vessel, aiding the re-establishment of blood supply to myocardium. Thus, Radix Scutellaria baicalensis could be considered as a potential source of drugs against ischemia-reperfusion injury. However, more investigations at the molecular level are needed to identify the heart protection mechanism of Radix Scutellaria baicalensis from ischemia-reperfusion injury.

Acknowledgments

This research project was financially supported by the Shenzhen Municipal Key Laboratory Advancement Program, Shenzhen, People’s Republic of China, as well as the Health and Health Services Research Fund, Food and Health Bureau, Hong Kong SAR, People’s Republic of China (Ref. number: 08090471). Our special thanks are due to Ms. Josephine Hong-Man Leung and Ms. Siu-Hung Tsui for proofreading and providing critical comments on the manuscript.

References


