Methanol Extract of Cassia mimosoides var. nomame Attenuates Myocardial Injury by Inhibition of Apoptosis in a Rat Model of Ischemia-Reperfusion

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Abstract

 Interruption of blood flow through coronary arteries and its subsequent restoration triggers the generation of a burst of reactive oxygen species (ROS), leading to myocardial cell death. In this study, we determined whether a methanol extract of Cassia mimosoides var. nomame Makino could prevent myocardial ischemia-reperfusion injury. When radical scavenging activity of the extract was measured in vitro using its α,α-diphenyl-β-picrylhydrazyl (DPPH) radical quenching ability, the extract showed an activity slightly lower than that of ascorbic acid. Three days after oral administration of the extract (400 mg/kg/day) to rats, myocardial ischemia/reperfusion injury was generated by 30 min of ligation of the left anterior descending coronary artery (LAD), followed by 3 hr reperfusion. Compared with the vehicle-treated group, administration of the extract significantly reduced infarct size (IS) (ratio of infarct area to area at risk) in the extract-treated group by 28.3%. Reduction in the cellular injury was mediated by attenuation of Bax/Bcl-2 ratio by 33.3%, inhibition of caspase-3 activation from procaspase-3 by 40%, and subsequent reduction in the number of apoptotic cells by 66.3%. These results suggest that the extract attenuates myocardial injury in a rat model of ischemia-reperfusion by scavenging ROS, including free radicals, and consequently blocking apoptotic cascades. Therefore, intake of Cassia mimosoides var. nomame Makino might be beneficial for preventing ischemic myocardial injury.

Key words: Cassia mimosoides var. nomame Makino, methanol extract, heart, ischemia-reperfusion, reactive oxygen species, apoptosis

INTRODUCTION

 Myocardial infarction (MI), a major cause of morbidity and mortality worldwide, is caused by extensive myocardial cell death, following the occlusion of coronary arteries (1). Reperfusion of the occluded arteries by angioplasty or thrombolytic drugs within 12 hours of the onset of symptoms is the approved treatment; however, the time constraint is hard to be met clinically (2). Consequently, developing protectants that can complement the reperfusion therapy will be beneficial (3). Paradoxically, however, reperfusion itself results in further cell death by apoptosis or necrosis due to increased production of reactive oxygen species (ROS) (4,5). In cardiomyocyte cell death by apoptosis, ROS trigger apoptotic cascades, including release of cytochrome c from the mitochondria, decrease of Bel-2/Bax ratio, activation of caspase 3, and, finally, cleavage of chromosomal DNA (6,7). Consequently, intake of antioxidants is one approach to eliminate ROS, leading to the prevention of ischemia-reperfusion injury (3).

Cassia mimosoides var. nomame Makino (CM) in Cassia genus (family Leguminosae) grows naturally in Korea, as well as Japan and China, the whole plant of which has been used as a food, especially a tea (8,9). In a previous study, we have shown that a methanol extract of Cassia mimosoides var. nomame Makino (CM) and an ethyl acetate fraction obtained by partitioning the extract with ethyl acetate reduced brain injury in a rat model of ischemia-reperfusion (10).

In the present study, we determined whether the methanol extract of CM could also reduce myocardial injury by inhibiting apoptosis in a rat model of ischemia-reperfusion because underlying mechanisms are similar in both ischemia-reperfusion injuries.

MATERIALS AND METHODS

Extraction of CM

 Methanol extract of the whole plant of Cassia mimosoides var. nomame Makino (HY2207) was prepared as described previously (10). Briefly, the whole plants of CM collected in Uiseong area, a county of Gyeongsangbuk-do, South Korea, were washed and dried [The voucher specimen (HY2207) was deposited at Nutrition Biochemistry Laboratory, Catholic University of Daegu, Daegu, Korea]. Then 500 g were put into an ultrasonicator (8210R-DTH, Branson Ultrasonic Corp., Danbury,
CT, USA) and extracted in 5 L methanol twice for 24 hr each at room temperature, and the extract was filtered with filter papers (Whatman No. 3, Whatman Inc., Piscataway, NJ, USA). The filtrate was vacuum-dried with a rotary evaporator [NP-1, Tokyo Rikakikai Co. (EYELA), Tokyo, Japan] with a 62 g yield, and is referred to as the extract.

**Assessment of radical scavenging activity of the extract with DPPH**

Various amounts of the extract dissolved in 20 μL dimethyl sulfoxide (DMSO) were mixed with 180 μL reaction solution containing α,α-diphenyl-β-picrylhydrazyl (DPPH) [0.15 mM DPPH dissolved in ethanol] using 96 multi-well plate. Thirty minutes after incubation of the samples at 25°C, the absorbance was measured at 540 nm with a UV-visible spectrophotometer (FLUOstar OPTIMA, BMG Labtech, Ortenberg, Germany). The experiments were repeated three times, and the absorbance was averaged. Final concentrations of the extract in the samples were 0 (negative control), 3.125, 6.25, 12.5, 25, 50 and 100 μg/mL. Ascorbic acid with the concentrations corresponding to the samples was used as a positive control. Radical scavenging activity of the extract and ascorbic acid represented by electron donating activity (EDA) was calculated with the following equation:

\[ EDA (\%) = (1 - \text{extract or ascorbic absorbance/blank absorbance}) \times 100 \]

in which extract, ascorbic acid, and blank absorbance represent absorbance of solutions containing the extract, ascorbic acid, and nothing (negative control), respectively (11,12).

**Animals**

Eight-week-old male Sprague Dawley (SD) rats were purchased from Samtaco Inc. (Osan, Korea). Experiments were carried out according to the guidelines for the animal care and use of laboratory animal protocols approved by the Institutional Animal Care and Research Advisory Committee of Catholic University, Daegu, Korea. Animals were housed with food and water available *ad libitum* under diurnal lighting conditions and in a temperature-controlled environment until the start of the experiment.

**Extract administration**

Rats were randomly assigned to one of three groups: (1) sham (n=3), (2) vehicle-treated (n=5), or (3) extract-treated group (n=6). In the extract-treated group, rats received the extract (400 mg/kg/day) with food for 3 days prior to occlusion. In the vehicle-treated group, rats received food only. In the sham group, experimental procedures were the same as those in the vehicle-treated group, except that there was no ligation.

**Rat myocardial ischemia/reperfusion model**

Myocardial infarction was produced by the left anterior descending coronary artery (LAD) ligation in male SD rats (250–300 g), as previously described by Lim et al. (13). Briefly, rats were anesthetized with intraperitoneal injections of ketamine (100 mg/kg) and xylazine (5 mg/kg). The rats were intubated and ventilated with air. The hearts were exposed by a left thoracic incision, and the LAD was ligated, about 5 mm down from aortic origin, by passing a 5-0 prolene suture (Ethicon, Somerville, NJ, USA) around LAD, followed by tightening the snare with PE-50. Occlusion of LAD was confirmed by the change of left ventricular (LV) wall to pale color. In ischemia-reperfusion experiments, the hearts were ligated for 30 min and reperfused for 3 hr by releasing the snare.

**Infarct size assessments**

Infarct size was assessed by 2,3,5-triphenyltetrazolium chloride (TTC) staining. At the end of reperfusion, the LAD was ligated by tightening the snare. Subsequently, 1 mL of 1.0% Evans blue (Sigma, St. Louis, MO, USA) was injected through the jugular vein, from which area at risk (AAR) was defined as the area showing no infiltration of Evans blue. The myocardium was then excised into four pieces about 3-mm thick, washed in phosphate-buffered saline (PBS), and cut into four transverse slices. The slices were stained in 1 mL of 2.0% TTC at 37°C for 10 min. Infarct area (IA) was defined as the area not stained with TTC. Areas of LV, AAR, and IA were determined by computerized planimetry using Image J software, from which infarct size (IS) was defined as a percentage of IA to AAR.

**TUNEL staining**

TUNEL staining was performed as previously described by Lim et al. (13). To measure DNA nicks, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining was performed to detect myocardial apoptosis, using the in situ Cell Death Detection Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer’s protocol. The heart slices were fixed in 10% formalin, embedded in paraffin, and sectioned on slides at 5-μm thickness. The sections were deparaffinized by three changes of 3 min each with xylene, followed by dehydration with 100, 95, 90, 80, and 70% ethyl alcohol (two changes, 1 min for each alcohol concentration). The sections were then successively treated with Proteinase K and 3% hydrogen peroxide for 20 and 3 min, respectively, and incubated with terminal deoxynucleotidyl transferase (TdT) and Br-
dUTP for 60 min at 37°C. For positive controls, the sections were treated with DNase I before TUNEL staining. For negative controls, TUNEL staining was performed in the absence of TdT. For counter-staining, the sections were stained with methyl green. To determine the percentage of apoptotic cells, micrographs of TUNEL-positive nuclei and methyl green-stained nuclei were captured using an Olympus microscope and counted using the Image J software (Image J version 1.43r, NIH, Bethesda, MD, USA) from 10 to 20 random fields at 400 × magnification.

Assessment of Bcl-2, Bax, and caspase-3

To assess the presence of Bcl-2, Bax, and cleaved caspase-3, immunohistochemical techniques were utilized, as described previously (13). The heart sections were incubated in PBS for 5 min, and antigens were retrieved by boiling in a microwave oven for 5 min with 10 mM citrate buffer, pH 6.0. The sections were cooled for 20 min in the buffer. Endogenous peroxidases were blocked with 3% H₂O₂ in methanol for 5 min. The sections were washed three times with PBS and blocked for 1 h in PBS containing 1% bovine serum albumin (BSA), 0.1% Triton X-100, and 5% normal goat serum. Rabbit polyclonal anti-Bcl-2, anti-Bax (1:50 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or anti-cleaved caspase-3 primary antibody (1:50 dilution, Cell Signaling, Beverly, MA, USA) was added to the 0.1% BSA solution, and the sections were incubated overnight at 4°C in a humidified chamber. A Vectastain Elite ABC kit (Vector Laboratories, Burlington, ON, Canada) was used for the staining carried out according to the manufacturer’s instructions. Color was developed by incubating the sections for 5 min in PBS containing 1 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (DAB, Roche, Mannheim, Germany) and 0.3% H₂O₂. The sections were evaluated at a 200× magnification. The intensity of DAB staining was measured using Image J software (NIH).

Statistical analysis

Values were expressed as means ± SEM. Statistical analysis for a single comparison was performed by Student’s t-test. When necessary, multiple comparisons were performed using one-way ANOVA, followed by post-hoc Tukey key analysis using SPSS software (SPSS 12.0 KO for Windows, SPSS Inc., Chicago, IL, USA). The statistical significance was considered to be p<0.05.

RESULTS

Radical scavenging activity of the extract

To determine whether the extract has antioxidant activity, radical scavenging activity of the extract was measured with DPPH. Absorbance at 540 nm decreased as concentrations of the extract and ascorbic acid, a strong antioxidant used as control, increased (data not shown). Accordingly, the electron donating ability (EDA), representing its radical scavenging activity, increased with the addition of more extract and ascorbic acid (Fig. 1). IC₅₀, concentrations representing the half maximal EDA, of the extract and ascorbic acid were 11.8 and 2 µg/mL, respectively.

Effect of the extract on infarct size

Effects of the extract on the reduction of infarct size were examined using an ischemia-reperfusion rat model (Fig. 2). Three days after the extract (400 mg/kg/day) was given with food, rats were subjected to 30 min ischemia by ligation of LAD and a subsequent 3 hr reperfusion by removal of LAD ligation. To measure the infarct size, Evans blue dye was injected to delineate the area at risk (AAR) where blood did not circulate during ischemia, and the non-ischemic region, where blood circulated during ischemia. Then, TTC staining was performed to delineate infarct area (IA) where surviving cardiomyocytes were not found after hearts were harvested and cut into four pieces. To quantify the results, infarct size (IS), the ratio of IA to AAR, was assessed for each group (Fig. 2). Administration of the extract (400 mg/kg/day) significantly reduced IS, compared with that of the vehicle (39.0±3.7 versus 54.4±4.2%, p<0.05). To confirm that IS assessed was experimentally reliable, ratios of AAR to LV, [AAR (% LV)], were
also assessed (Fig. 2). AAR (% LV) for the extract-treated group was not significantly different from that of the vehicle-treated control group (p>0.05), indicating that the surgical procedures to ligate LAD were performed consistently for each group such that the area with no blood circulation, prepared by ligation, became statistically the same. In total, these results suggest the extract was effective in attenuating myocardial infarction.

**Effect of the extract on apoptosis**

TUNEL staining, a procedure that detects nicks on DNA, was adopted to determine whether reduction of IS came from antiapoptotic effect of the extract. TUNEL staining has been successfully applied to detect apoptotic cells in mice models of myocardial infarction (14-16). The apoptotic cells in AAR were counted in two separate regions: IA and border zone (BZ) where AAR represents the sum of IA and BZ, because the pattern of apoptosis in the two regions could be different (17,18) (Fig. 3). A representative section for the extract in IA and BZ showed that the apoptotic cells in both regions were notably attenuated when the rats were treated with the extract, compared with those treated with the vehicle (Fig. 3A). To quantify the results, the ratio of apoptotic cells to total cells [Apoptotic cell (% total cells)] was assessed (Fig. 3B). The ratio was significantly attenuated in the BZ in the extract-treated group compared with vehicle-treated control group (5.8±1.0% vs. 17.2±3.2%, p<0.05). These results suggest that the extract was effective in attenuating myocardial infarction by inhibiting apoptosis.

**Effect of the extract on the activation of procaspase-3 to cleaved (active) caspase-3**

To support the results of the TUNEL staining, the amount of cleaved caspase-3 was assessed by immunohistochemistry (Fig. 4) (19,20). In the apoptotic pathways, cleaved caspase-3 (the active form of the caspase) is derived from procaspase-3 by the removal of prodomain. The caspase-3 then cleaves a restricted set of target proteins, resulting in the cell’s death by apoptosis (21, 22). Thus, increase of cleaved caspase-3 preludes the apoptotic cell death (23,24). A representative section for the extract revealed that treatment with the extract sig-

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**Fig. 2.** Effects of the extract on infarct size in rats. Following ischemia-reperfusion, Evans blue was injected through jugular vein, from which area at risk (AAR) was defined as the area not infiltrated with Evans blue. Then, the myocardium was excised into 4 pieces about 3 mm thick, and the slices were stained with TTC, from which infarct area (IA) was defined as the area not stained with TTC. Infarct size (IS) [IA (% AAR)] was expressed as a percentage of IA to AAR, and the area at risk [AAR (% LV)] was expressed as a percentage of AAR to the left ventricle mass. The numbers of rats used in vehicle- and extract-treated groups were 5 and 6, respectively. Each column represents the means±SE. p<0.05 vs. vehicle-treated control group.

**Fig. 3.** Effects of the extract on apoptosis. A. Photomicrographs of myocardial tissue sections showing TUNEL staining (400×): (a),(c) vehicle-treated control group; (b),(d) extract-treated group; (a),(b) and (c),(d) were taken from border zone (BZ) and infarct area (IA), respectively. B. Quantitative analysis of TUNEL-positive cells. The ratio (apoptotic cell number/total cell number) in AAR was assessed by TUNEL staining in two separate regions: IA and BZ where AAR represents the sum of IA and BZ. The numbers of rats used in vehicle- and extract-treated groups were 5 and 6, respectively. p<0.05 vs. vehicle-treated control group.
significantly lowered the color intensity (Fig. 4A). This was also confirmed through quantitative assessment (Fig. 4B). The amount of cleaved caspase-3 was significantly reduced in the extract-treated group, compared with that of the vehicle-treated control group (0.6±0.1% vs. 1.0±0.1%, respectively, p<0.05). These results suggest that the extract inhibits apoptosis by attenuating activation of procaspase-3 to the active caspase-3, the executioner of apoptosis.

**Effect of the extract on the expressions of Bcl-2 and Bax.**

Expression levels of Bcl-2 and Bax, located upstream of caspase-3 in the apoptotic pathways, were evaluated by immunohistochemistry. These two proteins are key regulatory components of the cell death process, in which Bcl-2 and Bax are antiapoptotic and proapoptotic, respectively (Fig. 5) (25-27). In a representative section of the extract, the expression level of Bax increased in the vehicle-treated control, compared with that in the sham group (Fig. 5A). The expression level of Bcl-2 increased in the extract-treated group, whereas that of Bax decreased, compared with those of the vehicle-treated control group. This observation was also confirmed with a quantitative assessment, for which Bcl-2/Bax ratio was used. It is this ratio, rather than the individual values, that determines whether the cells live or die (Fig. 5B) (28). The ratio in the extract-treated group increased to the value in the sham group (1.10±0.09% vs. 0.99±0.10%, p>0.05), which was significantly different from that of the vehicle-treated control group (0.66±0.04% vs. 0.99±0.10%, p<0.01). Thus, the extract can prevent the activation of procaspase-3 into cleaved caspase-3 by maintaining the Bcl-2/Bax ratio.

**DISCUSSION**

In the apoptotic pathways, ischemia, especially when coupled with reperfusion, triggers abrupt increase of reactive oxygen species (ROS), mediating the translocation of Bax into the outer mitochondrial membrane (7). The translocation of Bax stimulates the release of cytochrome c from the mitochondria, which can be inhibited by Bcl-2. The release of cytochrome c results in the activation of procaspase-3 into active caspase-3 (cleaved caspase-3).
pase-3), followed by specific DNA fragmentation of chromosomal DNA, eventually leading to apoptotic cell death (7,18,27). The results of the present study show that the methanol extract of *Cassia mimosoides var. nomame* Makino (CM) scavenges free radicals generated during ischemia-reperfusion, consequently alleviating myocardial injury by inhibiting apoptosis through the increase of the Bcl-2/Bax ratio and the subsequent inhibition of activation of procaspase-3 into active caspase-3. Indeed, many free radical scavengers or antioxidants, including herbal drugs, have a beneficial effect on reperfusion injury, resulting in the reduction of myocardial infarct size (5,29).

Bangou et al. (30) and Kuang et al. (31) also reported that methanol and 95% ethanol extracts of the whole CM plant showed high free radical scavenging activities, measured with DPPH, and high total phenolic contents. In addition, other *Cassia* species in *Cassia* genus also exhibit antioxidant activities. For example, methanol extract of seven Egyptian *Cassia* species, including *Cassia occidentalis*, revealed free radical scavenging activities (32). Therefore, it might be reasonable to assume that phenolic compounds in CM are responsible for scavenging free radicals in the rat model of myocardial ischemia-reperfusion, resulting in the inhibition of apoptosis, which ultimately leads to the reduction of myocardial injury.

**CONCLUSION**

The present study showed that the methanol extract of *Cassia mimosoides var. nomame* protected against myocardial injury in a rat model of ischemia-reperfusion by scavenging ROS, including free radicals, and consequently blocking apoptotic cascades. The extract of CM might be developed as a prophylactic food to prevent myocardial injury caused by occlusion of coronary arteries, especially for patients who had experienced previous myocardial infarction.

**ACKNOWLEDGMENTS**

This work was supported by a grant (PF06221-00) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Education, Science and Technology of Korean government (J. W. Lee).

**NOMENCLATURE**

ROS, reactive oxygen species; DPPH, α,α-diphenyl-β-picrylhydrazyl; LAD, left anterior descending coronary artery; IS, infarct size; MI, Myocardial infarction; CM, *Cassia mimosoides var. nomame* Makino; DMSO, dimethyl sulfoxide; EDA, electron donating activity; SD, Sprague Dawley; LV, left ventricular; TTC, 2,3,5-triphenyl tetrazolium chloride; AAR, area at risk; PBS, phosphate-buffered saline; IA, Infarct area; TUNEL, terminal deoxy- nucleotidyl transferase-mediated dUTP nick-end labeling; TdT, terminal deoxynucleotidyl transferase; BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine tetrahydrochloride; BZ, border zone.

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(Received May 14, 2012; Accepted September 1, 2012)