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Total flavonoids from *Rosa Laevigata* Michx fruit attenuates hydrogen peroxide induced injury in human umbilical vein endothelial cells

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ABSTRACT

The aim of the present study was to evaluate the protective effect and possible mechanism of the total flavonoids (TFs) from *Rosa Laevigata* Michx fruit (RLMF) against hydrogen peroxide (H_2O_2) induced damage in human umbilical vein endothelial cells (HUVECs). The cell injury caused by H_2O_2 was protected by pretreatment with the TFs for 1 h. Compared with the model group, the TFs decreased S phase cells, suppressed nuclear morphological damage, inhibited the collapse of mitochondrial membrane potentials ($\Delta\Psi m$), attenuated excessive reactive oxygen species generation, reduced glutathione depletion, impacted the mitochondrial morphology change, decreased caspase-3, -9 activities, and decreased fragmented DNA. Further mechanism investigation showed that the TFs could increase the protein expressions of Procaspase-3, Bcl-2, and decrease the expressions of Bak, Bax, Bid and p53. Generally, the TFs from RLMF is an effective natural product for the treatment of cardiovascular and cerebrovascular diseases.

1. Introduction

Cardiovascular and cerebrovascular diseases (CCVD) are the leading causes of death all over the world. According to the data from the World Health Organization (WHO), the number of deaths caused by CCVD will reach to 23.6 million by 2030 (WHO, 2009). Many risk factors such as age, sex, smoking, oxidative damage, high blood pressure, hypercholesterolemia and diabetes mellitus can all lead to CCVD (Baena Díez et al., 2005). Among these, oxidative damage is one of the most important factors (Lanteri et al., 2007), which is involved in various pathologic disorders associated with ischemia/hypoxia and inflammation. At present, great deals of research and effort have been taken to find new and effective drugs to block oxidative damage, which is necessary and critically important for treatment of CCVD.

Traditional Chinese medicine (TCM) has the advantages of low toxicity and high effectiveness, which has been used in China for thousands of years (Li et al., 2010). Nowadays, screening active compounds from natural sources against oxidative stimulus for treatment of cardiovascular diseases has been widely investigated (Fariba et al., 2009). Flavonoids, one kind of low molecular weight polyphenolic substances, widely exist in many medicinal plants (Okuda et al., 1992). Pharmacological studies have shown that flavonoids have anti-oxidant, anti-carcinogenic, anti-diabetics and anti-cancer activities (Williams et al., 2004).

Rosa laevigata Michx, a famous medicinal plant, belongs to the family of *Rosaceae*. Pharmacological researches have demonstrated that the fruit of *R. laevigata* Michx has the capability to treat chronic cough, frequent micturition, hyperpiesia and dermatogic diseases (Gao et al., 2010; China Pharmacopoeia Committee, 2005). The main active components of this plant are considered to be polysaccharose, saponins and flavonoids (Zhao et al., 2003). In our previous study, the total flavonoids (TFs) from *R. laevigata* Michx fruit (RLMF) have been investigated, and we found that the prepared TFs had excellent hepatoprotective effect against paracetamol-induced liver injury (Liu et al., 2011a,b) and antioxidant activities (Liu et al., 2010). Furthermore, a 90-day subchronic toxicity study of the TFs has been investigated in our previous study and the dose of 500 mg/kg/day for male and female rats was selected as the no-observed-adverse-effect level (Zhang et al., 2012). Until now, there is no report about the activity of the TFs from RLMF against oxidative damage related to CCVD.

Therefore, the aim of the present paper was to evaluate the protective effect of the TFs from RLMF against H_2O_2 induced oxidant damage in HUVECs, and further to investigate the possible mechanism. Our findings demonstrate that the TFs from RLMF is an effective natural product for attenuating injury caused by H_2O_2, which could be developed as a new natural medicine for treatment of CCVD.

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2. Materials and methods

2.1. Reagents

Glutathione (GSH) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Cell Cycle and Apoptosis Analysis Kits, Nitric oxide (NO) Assay Kit, Caspase-3 and -9 activities Assay Kits, Reactive Oxygen Species Assay Kit, lysis buffer and Biotech Plus were all purchased from Beyotime Institute of Biotechnology (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), In situ Cell Death Detection Kit, 2-(6-amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester (Rhoda-mine123) and propidium iodide (PI) were purchased from Roche Diagnostics (Mannheim, Germany). 4,6-Diamidino-2-phenylindole (DAPI), H_2O_2, Tris and SDS were purchased from Sigma (St. Louis, MO, USA). FBS and 1640 were purchased from THERMO (Beijing, China). Antibodies against Procaspase-3, Bcl-2, Bak, Bax, Bid, p53, GAPDH, horseradish peroxidase-conjugated goat anti-rabbit IgG, and horseradish peroxidase-conjugated goat anti-mouse IgG were supplied by Proteintech Group, Inc., Chicago, USA.

2.2. Tested total flavonoids (TFs)

The raw material was purchased from Yunnan Qiancaoyuan Pharmaceutical Company Co., Ltd. (Yunnan, China), in April 2008, and identified to be the dry fruit of *R. laevigata* Michx by Dr. Yunpeng Diao (College of Pharmacy, Dalian Medical University, Dalian, China). A voucher specimen (DLMU, JYZ080412) was deposited in the Herbarium of the College of Pharmacy, Dalian Medicinal University (Dalian, China). The total flavonoids (TFs) from *R. laevigata* Michx fruit was isolated by the method reported in our previous study (Liu et al., 2011a,b). The content and chemical constituents of the prepared TFs were also investigated (Zhang et al., 2012). Then, the dry powders were preserved in a refrigerator for subsequent experiments.

2.3. Endothelial cells culture

Human umbilical vein endothelial cells (HUVECs) were provided from American type culture collection (ATCC) (Washington, DC, USA). The cells were cultured in 1640 medium heat-inactivated FBS (10%, v/v), and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For all experiments, HUVECs were grown to 80–90% confluence and then pretreated with designated agents for 60 min prior to H_2O_2 exposure in fresh medium.

2.4. Cell viability assay

Cell viability was estimated by MTT assay. In brief, HUVECs were seeded on a 96-well plate (2×10^4 cells/well). Twenty-four hours after plating, the cells were pretreated with the TFs (26, 52, 104 μg/ml), then 100 μM H_2O_2 was added into the plate and cultured at 37 °C for an additional 2 h. Ten microliters of MTT stock solution (5 mg/ml in PBS without phenol red in the dark and filtered through a 0.2 μm filter before use) was then added into each well to attain a total reaction volume of 160 μl. After incubating for 4 h at 37 °C, 100 μl three-joint fluid (10% SDS + 5% Isobutanol and one drop HCl) was added to each well to dissolve the formazan crystals. Then the plates were gently shaken for 1 min and determined by a microplate reader (Thermo, USA) at 570 nm. The morphology of the cells was evaluated by inverted microscope (TS100, Nikon, Japan).

2.5. Cell cycle assay

After treatment, HUVECs growing in 6-well plate (2×10^5 cells/well) were harvested and determined using the Cell Cycle and Apoptosis Analysis Kits according to the manufacturer's protocols. Then the cell cycle was quantified by a flow cytometer (FACSCalibur, BECTON DICKINSON, USA).

2.6. DAPI staining

For morphological assessment of nuclei, after being treated, the cells in 6-well plate were washed twice with PBS and stained with DAPI (1 μg/ml) solution for 10 min at 37 °C, then washed twice with PBS. Finally, the stained cells in the plate were imaged by inverted fluorescence microscopy (CKX41, OLYMPUS, Japan).

2.7. Determination of mitochondrial membrane potential ($\Delta\Psi m$)

Rhodamine123, the uptake and retention of the cationic fluorescent dye, was used for measuring $\Delta\Psi$m. The treated cells were collected and washed three times with PBS, and centrifuged at 100*g* for 10 min. Then, the cell pellet was re-suspended in 1 ml of PBS containing 10 μg/L rhodamine123 and incubated at 37 °C in an incubator for 40 min. The stained HUVECs were separated and washed three times with PBS, then re-suspended in 1 ml PBS. The $\Delta\Psi$m was measured fluorimetrically using a fluorescence spectrophotometer (F-7000, HITACHI, Japan) at the wavelengths of 490 nm for excitation and 520 nm for emission. The images of the stained cells in 6-well plate were analyzed by inverted fluorescent microscopy (CKX41, OLYMPUS, Japan).

2.8. Transmission electron microscopy (TEM)

The treated HUVECs were fixed overnight at 4 °C in 2% glutaraldehyde. The cells were rinsed three times (each for 15 min) in 0.1 M sodium cacodylate buffer, and then fixed for 2 h in 1% osmium tetroxide in buffer. Then the slides were dehydrated in gradient ethanol solutions (25%, 50%, 75%, 95%, 100%, 100%; 15 min each). After that, the slides were successively infiltrated with propylene oxide for 20 min. the mixture of propylene oxide and embedded solution (1:1) for 1 h at 37 °C, and pure embedded solution for 2 h. The slides were placed into the bottom of the capsules, and the excess contain solution was drained from the contain solution-filled slide-duplicating capsules. The sections were infiltrated overnight at 37 °C and polymerized in an oven at 60 °C for 48 h. Regions of interest were excised with a glass scribe and mounted for ultramicrotomy. Then, the ultramicrotomies were stained and imaged with an electron microscope (JEM-2000EX, JEDL, Japan).

2.9. Measurement of reactive oxygen species (ROS) production

The level of intracellular ROS was examined using DCFH-DA. HUVECs in 6-well plate (2×10^5 cells/well) were treated and determined using the Reactive Oxygen Species Assay Kit according to the manufacturer's protocol. The fluorescence of DCF was detected by using a spectrofluorophotometer at 488 nm for excitation and 525 nm for emission. The images of the stained cells in 6-well plate were analyzed by inverted fluorescent microscopy (CKX41, OLYMPUS, Japan).

2.10. Measurement of the intracellular GSH

Levels of intracellular GSH were determined by a spectrophotometer (U-3010, HITACHI, Japan). After treatment, the culture medium from the plate was discarded and the cells were washed with PBS. Then the cells were removed, collected and centrifuged at 1000*g* for 5 min at 4 °C. Cell homogenate was prepared in PBS and centrifuged at 10,000*g* for 10 min at 4 °C. GSH level in the supernatant was determined according to supplied manual. Protein content was measured based on the method described in the report (Kurien and Hal Scofield, 2006) and bovine serum albumin (BSA) was used as the standard.

2.11. NO release assay

HUVECs were cultured and treated. Then, the supernatant was collected from plates and the concentration of NO was measured using Nitric oxide (NO) assay kit. The assay was carried out according to the guidelines of the kit.

2.12. Caspase-3 and -9 activities assay

HUVECs were cultured and treated. Then the activities of caspase-3 and caspase-9 were determined using the caspase-3 and caspase-9 activities assay kits according to the manufacturer's protocols. The samples were measured with a microplate reader at 405 nm and the activities were expressed as percentage of enzyme activity compared with control. All the experiments were carried out in triplicate.

2.13. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay

Apoptosis was assessed by a modification of the TUNEL method. After being treated, the cells in 6-well plate were fixed in freshly prepared 4% Paraformaldehyde in PBS (pH 7.4) for 20 min. Then the cells were rinsed twice with PBS for 10 min, and then incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate, freshly prepared) for 2 min on ice. The cells were washed twice with PBS again. The broken DNA ends of the cells were labeled with TdT and fluorescein-dUTP in a moist chamber at 37 °C for 60 min. Then the TUNEL reaction solution was removed and the cells were washed three times with PBS. The apoptotic cells were observed and photographed by a fluorescence microscope (CKX41, OLYMPUS, Japan).

2.14. Western blot analysis

The cell culture medium of the 6-well plate was removed, and the cells were extracted by a cold lysis buffer (RIPA, 200 μM PMSF) for 15 min at 4 °C, then the mixtures were centrifuged at 12,000*g* for 5 min and the total protein was obtained. Then, the protein content was determined by Coomassie brilliant blue G. Western blotting assays were performed as follows: protein (5 mg/mL) was denatured by mixing with an equal volume of 2× sample loading buffer and then boiling at 100 °C for 5 min (Kurien et al., 2006). An aliquot (20 μL, containing 50 μg protein) of the supernatant was fractionated by an electrophoresis on 10–15% SDS–PAGE and transferred onto a PVDF membrane. After blocking non-specific binding sites for 3 h with 5% non-fat milk in TTBS (TBS with Tween 20), the membranes were individually incubated for overnight with human anti-procaspase-3 (1: 500 dilution), human anti-bcl-2 (1: 500 dilution), human anti-bax (1: 500 dilution), human anti-p53 (1: 500 dilution), or mouse anti-GAPDH (1: 1000 dilution). Then the blots