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Short Communication

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Effects of Rutin from Leaves and Flowers of Buckwheat (*Fagopyrum esculentum* Moench.) on Angiotensin II–induced Hypertrophy of Cardiac Myocytes and Proliferation of Fibroblasts

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SUMMARY. Rutin was isolated from dried leaves and flowers of buckwheat (*Fagopyrum esculentum* Moench.). The effects of rutin on angiotensin II–induced hypertrophy of cultured cardiac myocytes and proliferation of cardiac fibroblasts of neonatal rats were evaluated by analyzing the cell surface area, measuring the protein synthesis rate through ^{3}H-leucine incorporation, and the MTT method. Rutin (0.8 to 8.0 mg/l) exhibited a strong inhibition on the hypertrophy and proliferation. The results suggested that rutin from buckwheat flowers and leaves might have protective effects on cardiac hypertrophy.

INTRODUCTION

Cardiac hypertrophy is a common complication of cardiovascular system diseases and an independent risk factor giving rise to augmented mortality for cardiovascular diseases, which often results in congestive heart failure, serious cardiac dysrhythmia and significant artery branch disruption. As to the treatment of cardiac hypertrophy, immunosuppressant cyclosporine A was generally proved to be effective [1-5], but the toxicities to kidney cells had limited its clinical application. Many studies [6] discovered that flavanoids could protect cardial and cerebral ischemia, and cardiac arrhythmia. Therefore, the study and development of new drugs especially those from natural resources with few side effects for cardiovascular diseases, has special significance.

Buckwheat, *Fagopyrum esculentum* Moench. [syn: *F. sagittatum* Gilib; *Polygonum fagopyrum* L.], is a crop for food as well as medicine. It is native to China and planted widely especially in the north. Though buckwheat seeds are widely used as health food, few studies about buckwheat flowers and leaves are reported. To our best knowledge, the whole plant of buckwheat is rich in flavonoids, and rutin is in the majority [7], which exists mostly in the flowers especially flower buds followed by leaves. Frost comes quite early in the north (Inner Mongolia), so that lots of buckwheat plants are discarded for can't fruit in autumn. Subsequent work was carried out to isolate rutin from buckwheat flowers and leaves, and assess its effects on cardiac hypertrophy with cultured cells.

MATERIALS AND METHODS

Plant materials

Buckwheat, flowers and leaves collected in late autumn from Ku Lun, Inner Mongolia (China), was identified by the Department of Natural Medicinal Chemistry, North China Coal Medical College, Tangshan, China. A voucher specimen is deposited in the Department of Pharmacology, North China Coal Medical College, Tangshan, China.

Isolation and preparation of rutin

The air-dried flowers and leaves were grounded, refluxed for 30 min with water (three

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times). The combined water extracts were concentrated and evaporated to proper volume, filtered to remove insoluble precipitate. The filtrate was dealed with separating agents, cooled down, centrifugated to semi-solid, and dried in an oven at 70 °C to obtain a powder extract. The extract was dissolved with absolute ethanol to remove the impurities by filtration, then appropriate amount of water was added in, and the ethanol was evaporated. The filtrate was concentrated and cooled down to obtain the crude rutin. The refined rutin (yield 5.26%) was obtained through further extraction from crude rutin by MeOH. The extracted rutin with a purity of 99.8% authenticated by HPLC was identified by its color, shape, melting point and the HCl-Mg reaction. The structure was the same as the standardized rutin by coupling the UV spectra and Agilent 1100 MS, and the data were identical with those in literature [8].

Cell culture

Primary cardiac myocytes and fibroblasts were prepared from 1 to 3-day-old Wistar rats hearts following the sequential enzymatic digestion method described previously with some modification [9]. Briefly, the hearts were taken out, the ventricles were separated and minced into approximately 1 mm^3 pieces and digested sequentially with 0.1 % trypsin for 5-8 min and 0.07% collagenase II three times for 10 min each time. The cells were collected and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 60 min at 37 °C in a 5% CO_2 humidified incubator. The fibroblasts had already attached to the culture flasks while the myocytes remained floating. The suspension was moved to another flask and incubated for 48h with 0.1mmol/l 5'-Brdu to inhibit the growth of nonmyocytes. The attached fibroblasts were cultured in 10% FBS DMEM.

Induction of hypertrophy and proliferation

Hypertrophy of cardiac myocytes and proliferation of fibroblasts were induced by adding 10^{-7} mol/l Ang II, a known promoting growth factor, to serum-free medium.

Experimental groups

The primarily cultured cardiac myocytes and the third generation fibroblasts (purified by passage culture) were synchronized with serum-free DMEM for 24 h as they grew to the proper density, then both types of cells were grouped as follows: a) Control group: cultured in serum-free DMEM: b) Model group: cultured in serum-free DMEM supplemented with Ang II; c) Rutin I group: cultured in serum-free DMEM supplemented with 0.8 mg/l rutin 30 min prior to Ang II; d) Rutin II group: Cultured in serum-free DMEM supplemented with 4.0 mg/l rutin 30 min prior to Ang II; d) Rutin III group: Cultured in serum-free DMEM supplemented with 8.0 mg/l rutin 30 min prior to Ang II.

Cell surface area assay

Cardiac myocytes were dealed with drugs for 7 days (drug supplemented every 24 h, media changed every 48 h), then the surface area was determined. In brief, the cells were fallen off by 0.25% trypsin, suspended with 10% FBS DMEM, and photographed. Totally, surface area of 100 cells in each group, from 5-10 random eyeshots of 2-3 containers, were analyzed by MoticMed System 6.0A medical science image analysis system to give the mean value (μm^2 for unit).

^{3}H-Leu incorporation assay

Primarily cultured cardiac myocytes were seeded in 96-well plate (3×10^5 cells/ml, 200 μl per hole), interfered with drugs, then ^{3}H-Leu incorporation quantity was determined to reflect protein synthesis rate. Briefly, cells were grouped and dealed with drugs for 24 h following the method mentioned above, then co-incubated with ^{3}H-Leu (1 μCi/ml) for another 24 h, washed thoroughly with D-Hanks, harvested onto fiberglass filter membrane, washed with 10% trichloracetic acid and dehydrated alcohol. The torrefied filter membrane was put in liquid scintillation cup containing 4ml scintillation fluid, and intensity of radioactivity was measured by liquid scintillation counter (the results expressed as cpm/well).

Proliferation assay

Cardiac fibroblasts were treated with various concentrations of rutin for 24 h, and fibroblast viability was determined by testing mitochondrial enzyme function according to the colorimetric methyl thiazolyl tetrazolium (MTT) method. Briefly, 20,000 fibroblasts were subcultured into a 96-well culture plate 24 h prior to synchronization, and dealed with drugs for another 24 h; then, treated with MTT (20 μl, 5mg/ml) 4 h before the experiment was terminated. The supernatant was removed and DMSO (150 μl) was added to dissolve formazan; then, the absorbance, which reflects the proliferation of fi-

broblasts, was measured by automatic microplate reader at 490 nm (results expressed as A_{490nm}). The growth inhibition ratios (GIR) were calculated to evaluate the effects of different concerntration rutin. All procedures were approved by the Ethical Committee for Laboratory Animals of the North China Coal Medical College (Tangshan) and were performed in accordance with the Principles in Animal Care and Use of the Committee.

Statistical analysis

Data were expressed as mean ± standard deviation (S.D). Statistical significances were analyzed using the ANOVA test. A value of $P < 0.05$ was considered significant. GIR were calculated according to the following equation: GIR(%) = $(A_{model}-A_{rutin})/A_{model} \times 100\%$, where A is the average absorbance of three experiments with 6 replicates.

RESULTS

The effects of buckwheat rutin on cardiac hypertrophy were studied with cultured cells by measuring cell surface area and ^{3}H-Leu incorporation quantity, and the MTT method.

Effects of rutin from buckwheat flowers and leaves on hypertrophy of cardiac myocytes

Surface area of cardiac myocytes in Ang II (10^{-7}mol/L) group increased significantly compared with that of control group ($P < 0.01$), however there was an obvious decrease of surface area in different concentration rutin group compared with model group and degraded 7.3% ($P < 0.05$), 24.9% ($P < 0.01$), 28.7% ($P < 0.01$) respectively in a dose-dependent manner. An obvious increase of ^{3}H-Leu incorporation in Ang II group was observed compared with that of control group ($P <0.01$). Different concentration rutin inhibited ^{3}H-Leu incorporation of cultured cardiac myocytes induced by Ang II and decreased 18.3% ($P < 0.05$), 35.9% ($P < 0.01$), 50.7% ($P < 0.01$) respectively compared with that of model group. The results showed that ^{3}H-Leu incorporation diminished gradually with the augmentation of rutin concentration in a dose -dependent manner. The results were given in Table 1.

Effects of rutin from buckwheat flowers and leaves on proliferation of cardiac fibroblasts

The results of MTT showed that Ang II could enhance the proliferation of cardiac fibroblasts, while rutin in different concentrations could obviously suppress that ($P <0.01$) and presented a dose-dependent manner (suppress rate 19.75%, 32.29%, 47.02 %). (See also Table 2).

Group	Surface area (μm²)	^{3}H-Leu incorporation (cpm/well)
Control	464.49 ± 76.95	126 ± 16.73
Model	721.48 ± 157.39 ##	284 ± 27.75 ##
Rutin I	668.69 ± 130.56 *	232 ± 24.14 *
Rutin II	542.01 ± 89.08 **	182 ± 15.81 **
Rutin III	514.36 ± 109.33 **	140 ± 24.49 **

Table 1. Effects of rutin from buckwheat flowers and leaves on cell surface area and ^{3}H-Leu incorporation of AngII- induced cultured neonatal rat cardiac myocytes. Values are expressed as mean ±S.D. ##$P <$ 0.01 in comparison to control group; * $P < 0.05$, ** $P <$ 0.01 in comparison to model group.

Group	A_{490nm}	Inhibition ratio (%)
Control	0.250 ± 0.058	…
Model	0.319 ± 0.034 ##	…
Rutin I	0.256 ± 0.022 **	19.75
Rutin II	0.216 ± 0.027 **	32.29
Rutin III	0.169 ± 0.020 **	47.02

Table 2. Proliferation activity of AngII-induced cardiac fibroblasts treated with rutin from buckwheat flowers and leaves. Values are expressed as mean ±S.D. ##P < 0.01 in comparison to control group; ** $P < 0.01$ in comparison to model group.

DISCUSSION

Cardiac hypertrophy includes hypertrophy of cardiac myocytes, proliferation of myocardial interstitial cells (mainly fibroblasts), and rebuilding of extracellular matrix. Many previous studies focused on hypertrophy of cardiac myocytes, but in recent years, people gradually learned that non-myocardial cells (accounting for 70% of the total number of cells), mainly the fibroblasts not only had supportive and protective effect on the structure of cardiac myocytes but also had autocrine and paracrine function, thus influenced the structure and function of cardiac myocytes. Therefore, researches in the field of cardiovascular had become focused on cardiac fibroblasts. In present study, the effect of rutin from buckwheat flowers and leaves on Ang II-induced cardiac fibroblasts had been studied for

the first time. More over, through the way of cell culture, influences of hemodynamics and interactions among AngII and other hormones could be ruled out. Generous investigations have manifested that AngII has growth factor like effect and is regularly used to induce cardiac hypertrophy [10-11]. In this study, Ang II caused significant increase in cell surface area and ^{3}H-Leu incorporation quantity of cardiac myocytes, and proliferation of cardiac fibroblasts in comparison to control group. Rutin significantly decreased myocyte cell surface area and protein synthesis rate, inhibited the proliferation of fibroblasts, and presented a dose-dependent manner. This indicated that rutin could inhibit cardiac hypertrophy induced by Ang II.

At present, the main raw material for extraction of rutin is buds of *Sophora japonica* L., with fewer resources. Buckwheat is a plant resistant to freezing and barren environment, and is widely planted in China, the former Soviet Union, Japan, Poland, France, Canada, and the United States etc. Therefore, it really has extensive prospects both in exploitation and application.

CONCLUSION

On the basis of results obtained, it can be concluded that buckwheat rutin possesses the activity of suppressing cardiac hypertrophy. So, it can be said that buckwheat flowers and leaves are good natural materials to develop new agent to treat cardiac hypertrophy, especially in some cold areas, minority nationality areas, and remote mountainous areas where waste will be made into treasures.

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