



Original article

Puerarin attenuates pressure overload-induced cardiac hypertrophy



Yuan Yuan (PhD)^{a,b,1}, Jing Zong (PhD)^{a,b,1}, Heng Zhou (PhD)^{a,b}, Zhou-Yan Bian (PhD)^{a,b}, Wei Deng (PhD)^{a,b}, Jia Dai (PhD)^{a,b}, Hua-Wen Gan (MD)^{a,b}, Zheng Yang (PhD)^{a,b}, Hongliang Li (PhD)^{a,b}, Qi-Zhu Tang (PhD)^{a,b,*}

^a Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, China

^b Cardiovascular Research Institute of Wuhan University, Wuhan, China

ARTICLE INFO

Article history:

Received 16 January 2013

Received in revised form 22 April 2013

Accepted 9 June 2013

Available online 29 July 2013

Keywords:

Aortic coarctation

Hypertrophy

Pharmacology

ABSTRACT

Background: Puerarin is the most abundant isoflavonoid in kudzu root. It has been used to treat angina pectoris and myocardial infarction clinically. However, little is known about the effect of puerarin on cardiac hypertrophy.

Methods: Aortic banding (AB) was performed to induce cardiac hypertrophy in mice. Puerarin premixed in diets was administered to mice after one week of AB. Echocardiography and catheter-based measurements of hemodynamic parameters were performed at 7 weeks after starting puerarin treatment (8 weeks post-surgery). The extent of cardiac hypertrophy was also evaluated by pathological and molecular analyses of heart samples. Cardiomyocyte apoptosis was assessed by measuring Bax and Bcl-2 protein expression and terminal deoxynucleotidyl transferase dUTP nick end labeling staining. In addition, the inhibitory effect of puerarin (1 μM, 5 μM, 10 μM, 20 μM, 40 μM) on mRNA expression of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) in Ang II (1 μM)-stimulated H9c2 cells was investigated using quantitative real-time reverse transcription-polymerase chain reaction.

Results: Echocardiography and catheter-based measurements of hemodynamic parameters at 7 weeks revealed the amelioration of systolic and diastolic abnormalities. Puerarin also decreased cardiac fibrosis in AB mice. Moreover, the beneficial effect of puerarin was associated with the normalization in gene expression of hypertrophic and fibrotic markers. Further studies showed that pressure overload significantly induced the activation of phosphoinositide 3-kinase (PI3K)/Akt signaling and c-Jun N-terminal kinase (JNK) signaling, which was blocked by puerarin treatment. Cardiomyocyte apoptosis and induction of Bax in response to AB were suppressed by puerarin. Furthermore, the increased mRNA expression of ANP and BNP induced by Ang II (1 μM) was restrained to a different extent by different concentrations of puerarin.

Conclusion: Puerarin may have an ability to retard the progression of cardiac hypertrophy and apoptosis which is probably mediated by the blockade of PI3K/Akt and JNK signaling pathways.

© 2013 Japanese College of Cardiology. Published by Elsevier Ltd. All rights reserved.

Introduction

Pathological cardiac hypertrophy is classically considered as a condition at the boundary between the normal and the progressively failing heart [1]. Preventing or reducing pathological cardiac hypertrophy is an independent therapeutic goal, and might serve to prevent or postpone the progression of heart failure [2]. Current pharmacotherapies for heart failure have proven to be beneficial in improving the quality of life for heart failure patients without

remarkably reducing mortality rates [3]. The high mortality rates of heart failure may reflect the complexity of hypertrophic processes that contribute to heart failure and the difficulty in reversing cardiac hypertrophy with current pharmacotherapies [4]. The future challenge will be to find new pharmacological agents that target the underlying pathophysiological processes, including relevant signaling pathways and cardiac fibrosis, which lead to progressive myocardial dysfunction and unfavorable remodeling, and thus improve the long-term clinical outcomes of patients with heart failure [5].

Puerarin (7,4'-dihydroxy-8-β-D-glucosylisoflavone, C₂₁H₂₀O₉) [6], the major bioactive constituent in kudzu root, is used widely in China for the treatment of cardiovascular diseases and diabetes [7]. In addition, previous studies suggest that puerarin possesses anti-oxidant [8], anti-platelet [9], anti-inflammatory [10], anti-arrhythmic [11], and anti-apoptotic properties [12]. It is also

* Corresponding author at: Department of Cardiology, Renmin Hospital of Wuhan University, Cardiovascular Research Institute, Wuhan University at Jiefang Road 238, Wuhan 430060, China. Tel.: +86 27 88073385; fax: +86 27 88042292.

E-mail address: qztang@whu.edu.cn (Q.-Z. Tang).

¹ These authors contributed equally to the work.

reported that puerarin could promote neovascularization in the myocardium of rats suffering from heart failure induced by coronary artery ligation [13]. Two recent studies indicated that puerarin may have the ability to reduce cardiac expression of transforming growth factor $\beta 1$ (TGF $\beta 1$) in isoprenaline-treated mice or spontaneous hypertensive rats [14,15]. However, the effect of puerarin on cardiac hypertrophy and the related signaling mechanisms still needs to be clarified. In this study, we performed aortic banding (AB) to induce cardiac hypertrophy in mice and found out that puerarin may have an ability to retard the progression of cardiac hypertrophy induced by pressure overload by targeting phosphoinositide 3-kinase (PI3K)/Akt signaling and c-Jun N-terminal kinase (JNK) signaling pathways.

Materials and methods

Chemicals

Puerarin (98% purity as determined by high-performance liquid chromatography analysis) was purchased from Shanghai Winherb Medical S&T Development Co. Ltd. (Shanghai, China).

Animals

Male C57/BL6 mice (23.5–27.5 g, 8–10 weeks old) used in this study were purchased from the Institute of Laboratory Animal Science, CAMS&PUMC (Beijing, China), and housed with controlled temperature and humidity under a 12-h light–dark cycle with free access to food and water in the Cardiovascular Research Institute of Wuhan University (Wuhan, China). The animals were allowed to acclimatize to the laboratory environment for at least one week, then randomly assigned to either a sham surgery or an AB group without or with puerarin treatment (diets containing about 65 mg/kg body weight/day) started one week after surgery and maintained for a further 7 weeks. The dose of puerarin was designed according to the literature [14]. The administration of puerarin was performed according to previous studies [16,17]. Food consumption was monitored once a week and was found to be identical in all groups studied. The four groups were named as sham + puerarin, AB + puerarin, sham + vehicle and AB + vehicle respectively. AB was performed as described previously [18]. Eight weeks after surgery, animals were subjected to final echocardiography and catheter-based measurements of hemodynamic parameters prior to sacrifice. All of the experiments were approved by institutional guidelines of the Animal Care and Use Committee of Renmin Hospital of Wuhan University and performed in accordance with the Guide for the Care of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All surgeries and analyses were performed in a blinded fashion for all groups.

Echocardiography

Animals were subjected to echocardiographic analyses before surgery as well as 1 and 8 weeks after surgery. Echocardiography was performed in anesthetized (1.5% isoflurane) mice using a Mylab 30CV (Esaoete S.P.A, Genoa, Italy) equipped with a 10-MHz linear array ultrasound transducer. The left ventricle (LV) dimensions were assessed in parasternal short-axis view during systole or diastole. LV end-systolic diameter (LVESD), LV end-diastolic diameter (LVEDD), end-diastolic ventricular septal thickness (IVSd), and left ventricular posterior wall thickness (LVPWd) were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level.

Catheter-based measurements of hemodynamic parameters

For hemodynamic measurements, mice were anesthetized with 1.5% isoflurane, and a microtip catheter transducer (SPR-839, Millar Instruments, Houston, TX, USA) was inserted into the left ventricle via the right carotid artery. The signals were recorded using a Millar Pressure-Volume System (MPVS-400, Millar Instruments), and the heart rate (HR), end-diastolic pressure (EDP), end-systolic pressure (ESP), maximal rate of pressure development (dp/dt max), and minimal rate of pressure decay (dp/dt min) were analyzed using the PVAN data analysis software (Millar Instruments).

Histological analysis

Hearts were removed, arrested in diastole with 10% KCl, weighed, fixed by perfusion with 10% formalin, and embedded in paraffin. Hearts were cut transversely close to the apex to visualize the left and right ventricles. Sections were cut of 4–5 μ m and mounted onto slides. Hematoxylin and eosin (H&E) and picosirius red (PSR) staining were performed for histological analysis. Tissue sections were visualized by light microscopy. The sections were stained for myocyte cross-sectional area with fluorescein isothiocyanate-labeled wheat germ agglutinin (Invitrogen, Carlsbad, CA, USA) to visualize membranes and 4',6-diamidino-2-phenyl-indole (DAPI) to visualize nuclei. A single myocyte was measured with a quantitative digital image analysis system (Image Pro-Plus, version 6.0; Media Cybernetics, Bethesda, MD, USA). Between 100 and 200 myocytes in the left ventricles were outlined in each group.

Quantitative real-time reverse transcription-polymerase chain reaction

To examine the relative mRNA expression of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), β -myosin heavy polypeptide (β -MHC), sarcoplasmic reticulum calcium ATPase 2 alpha (SERCA2 α), connective tissue growth factor (CTGF), collagen I, collagen III, fibronectin, and TGF $\beta 1$, RNA was collected from LV tissue using TRIzol (Invitrogen, 15596-026), and reverse transcribed into cDNA for real-time polymerase chain reaction (PCR) analysis using oligo (DT) primers and the Transcriptor First Strand cDNA Synthesis Kit (04896866001, Roche, Basel, Switzerland). cDNA was synthesized from 2 μ g of total RNA. The PCR amplifications were quantified using a LightCycler 480 SYBR Green 1 Master Mix (Roche, 04707516001) and the results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

Western blotting

Western blotting was conducted to determine the activation state of PI3K/Akt signaling and mitogen-activated protein kinase (MAPK) signaling. Protein amounts from all samples were assessed using the BCA-kit (23227, Thermo Fisher Scientific, Waltham, MA, USA) followed by protein concentration normalization prior to all Western blot experiments. Protein samples (50 μ g) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and then transferred to Immobilon-FL transfer membrane (IPFL00010, Millipore, Billerica, MA, USA). The membrane was blocked with 5% milk in Tris-buffered saline Tween-20 (TBST) for 3 h and then incubated overnight at 4°C with indicated primary antibodies. Antibodies against the following proteins were purchased from Cell Signaling Technology (Danvers, MA, USA): phospho-PI3K p85^{Tyr458}/p55^{Tyr199} (#4228), PI3K p85 (#4257), phospho-Akt^{Ser473} (#4060), Akt (#4691), phospho-mammalian target of rapamycin

(mTOR)^{Ser2448} (#2971), mTOR (#2983), phospho-forkhead box O1 (FoxO1)^{Ser256} (#9461), FoxO1 (#2880), phospho-FoxO3a^{Ser318/321} (#9465), FoxO3a (#2497), phospho-glycogen synthase kinase 3 β (GSK3 β)^{Ser9} (#9322), GSK3 β (#9315), phospho-extracellular signal-regulated kinase (ERK)1/2^{Thr202/Tyr204} (#4370), ERK1/2 (#4695), phospho-p38^{Thr180/Tyr182} (#4511), p38 (#9212), phospho-JNK1/2^{Thr183/Tyr185} (#4668), JNK1/2 (#9285), Bax (#2772), Bcl-2 (#2870). The antibody for GAPDH (MB001) was purchased from Bioworld Technology, St Paul, MN, USA. The blots were scanned by a two-color infrared imaging system (Odyssey, LI-COR, Lincoln, NE, USA). Specific protein expression levels were normalized to GAPDH protein for total cell lysates.

Assessment of apoptosis

Apoptosis was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining according to the protocol of ApopTag[®] Plus Fluorescein In Situ Apoptosis Detection Kit (S7111, Chemicon, Temecula, CA, USA). Briefly, 4 μ m thick paraffin-embedded sections were prepared from mouse hearts of each group and deparaffinized sequentially. The sections were stained with TUNEL reagents and α -actinin, and the nuclei were stained by DAPI. The numbers of apoptotic and total cells were counted, and the percentages of apoptotic cells were calculated.

Cell culture

The H9c2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (C11995, GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO, 10099), penicillin (100 U/ml) and streptomycin (100 mg/ml) (GIBCO, 15140) in humidified CO₂ incubator (SANYO 18M, Osaka, Japan) with 5% CO₂ at 37 °C. Cells at exponential growth were dissociated with 0.25% trypsin (GIBCO, 25200) and were seeded in six-well culture plates at a seeding density of 1×10^6 /well before being incubated for 24 h. Then cells were cultured with serum-free DMEM for another 12 h. Puerarin (82435, Sigma, St Louis, MO, USA) was dissolved in dimethyl sulfoxide (Sigma, D2650) at a concentration of 40 mmol/L. Ang II (Sigma, A9525) (1 μ M) in the presence or absence of different concentrations of puerarin (1 μ M, 5 μ M, 10 μ M, 20 μ M, 40 μ M) were added to the medium and the cells were incubated for 24 h. Then total RNA was extracted from the H9c2 cells. The mRNA expression of ANP and BNP was examined by quantitative real-time PCR.

Statistical analysis

Data were analyzed with a one-way ANOVA test followed by a post hoc Tukey test. Values were expressed as the mean \pm SEM. Echocardiographic data were analyzed using two-way ANOVA with repeated measures and a post hoc Tukey's test. *p*-Values of <0.05 were considered significant.

Results

Puerarin protected against cardiac hypertrophy

We determined whether puerarin administration started from 1 week after AB surgery attenuated the indices of cardiac remodeling and heart failure. Mice subjected to AB surgery exhibited obvious cardiac hypertrophy as evidenced by increased cardiac mass, myocyte cross sectional area (CSA), heart weight/body weight (HW/BW), heart weight/tibial length (HW/TL), lung weight/body weight (LW/BW), and lung weight/tibial length (LW/TL) at the end

of 8 weeks compared to the sham group. However, 7 weeks of puerarin administration attenuated these hypertrophic responses (Fig. 1A and B). The induction of hypertrophic markers including ANP, BNP, and β -MHC after AB surgery was also significantly blunted in puerarin-treated mice; moreover, decreases in the expression of α -MHC and SERCA2 α were normalized in puerarin-treated mice (Fig. 1C).

Puerarin improved the impaired cardiac function after AB

The protective effect of puerarin against AB-induced left ventricular dysfunction was assessed by echocardiography before surgery, 1 week after surgery but before starting puerarin treatment, and finally 8 weeks after surgery. As shown in Fig. 2, animals subjected to 1 week of AB suffered mild chamber diameter increase and LV dysfunction, and the LVEDd, LVEsD, EF, and fractional shortening (FS) were at the same level between the AB+vehicle group and the AB+puerarin group. Eight weeks after AB, animals exhibited significantly increased chamber diameter and depressed EF and FS, and puerarin administration resulted in attenuation of cardiac dilation and improved LV function. Catheter-based hemodynamic measurements were performed at the end of the study to further assess LV systolic and diastolic function. Eight weeks of AB resulted in significantly increased diastolic blood pressure and decreased systolic function and diastolic function, animals treated with puerarin demonstrated normalization of these hemodynamic parameters including EDP, dP/dt max, and dP/dt min (Fig. 3). There were no significant differences in HR between any groups.

Puerarin attenuated pressure overload-induced cardiac fibrosis

Fibrosis is an integral feature of LV hypertrophy and heart failure. We performed PSR staining on cardiac tissue sections to determine the development of interstitial fibrosis in mice of each group. Dramatic interstitial fibrosis was observed in mice subjected to AB surgery, which was attenuated by puerarin treatment (Fig. 4A and B). We also examined the changes in expression of myocardial pro-fibrotic genes at the end of the 8 weeks post surgery. As shown in Fig. 4C, the pressure overload-induced expression of CTGF, collagen I, collagen III, fibronectin, and TGF β 1 was normalized by puerarin.

Puerarin inhibited the activation of PI3K/Akt and JNK signaling pathways after AB

To investigate the molecular mechanisms by which puerarin mediates its anti-hypertrophic effect, we examined the activation state of PI3K/Akt and JNK signaling. Pressure overload increased the phosphorylations of PI3K, Akt, mTOR, GSK3 β , FoxO1, and FoxO3a at 8 weeks after surgery. Interestingly, puerarin treatment significantly attenuated these increases as observed in Fig. 5A and B. Similarly, upregulation of P-JNK1/2 was blunted by puerarin treatment (Fig. 5C and D). However, puerarin did not affect the protein levels of P-ERK1/2 and P-p38 (Fig. 5C and D).

Puerarin inhibits cardiomyocyte apoptosis in response to chronic pressure overload

Paraffin-embedded sections of hearts from each group were co-stained for α -actinin and TUNEL to determine the rate of cardiomyocyte apoptosis. A noticeable increase in cardiomyocyte apoptosis was observed in mice at 8 weeks after AB, and puerarin treatment reduced AB-induced cardiomyocyte apoptosis (Fig. 6A).

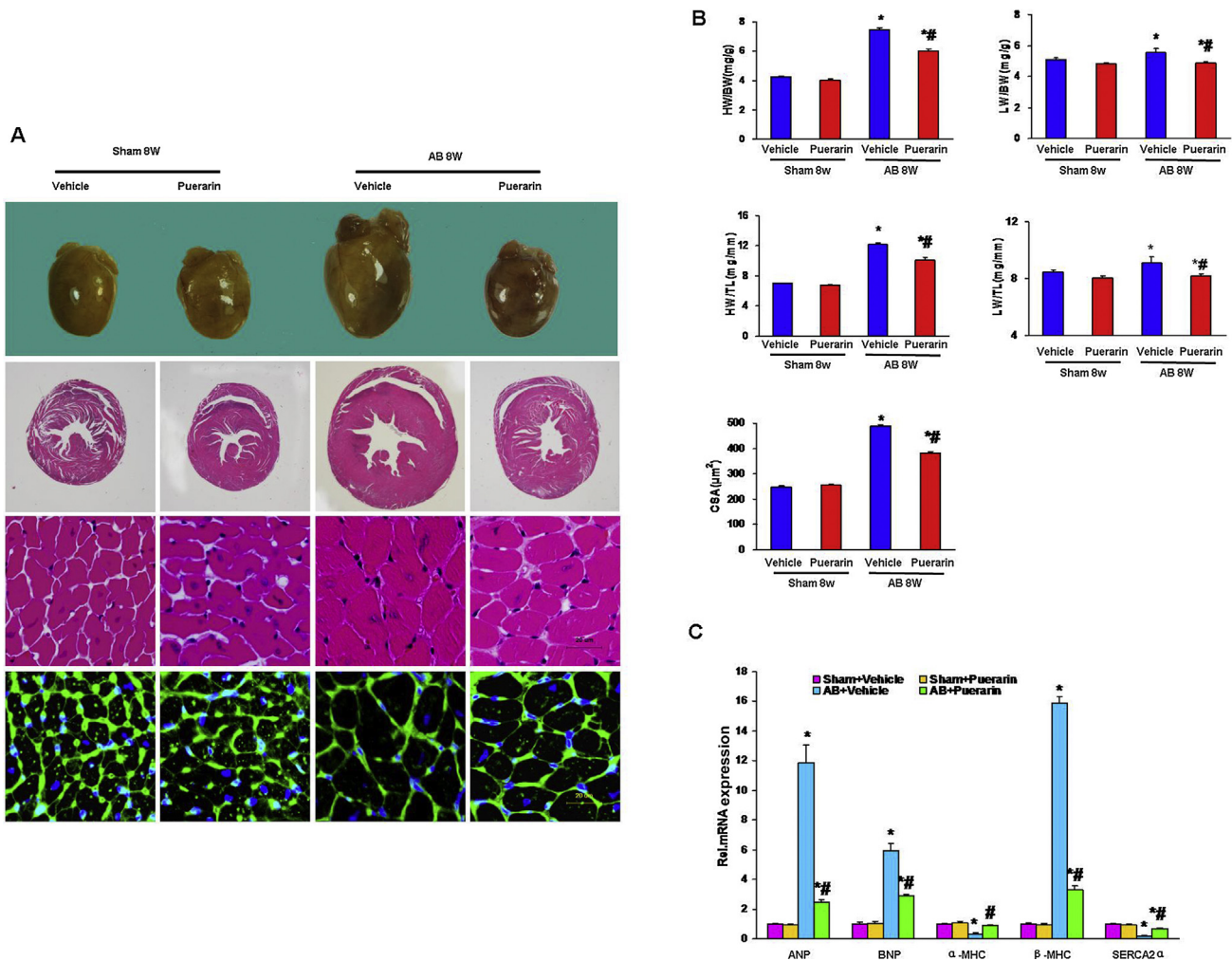


Fig. 1. Puerarin protected against cardiac hypertrophy. (A) Gross hearts, hematoxylin and eosin staining and fluorescein isothiocyanate-labeled wheat germ agglutinin staining of sham and aortic banding (AB) mice at 8 weeks post surgery. (B) Statistical results of the heart weight/body weight (HW/BW) ratio, lung weight/body weight (LW/BW) ratio, heart weight/tibial length (HW/TL) ratio, lung weight/tibial length (LW/TL) ratio, and myocyte cross-sectional areas (CSA) of indicated groups. (C) Expression of transcripts for atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), α -myosin heavy polypeptide (MHC), β -MHC, and sarcoplasmic reticulum calcium ATPase 2 alpha (SERCA2 α) induced by AB were determined by reverse transcription-polymerase chain reaction analysis ($n=6$). * $p < 0.05$ as compared with the corresponding sham group. # $p < 0.05$ vs AB+vehicle group.

Furthermore, puerarin decreased the ratio of Bax/Bcl-2 protein expression in mice hearts after AB (Fig. 6B).

Inhibitory effect of puerarin on the mRNA expression of ANP and BNP in Ang II- (1 μ M) stimulated H9c2 cells

Treatment of H9c2 cardiomyocytes with Ang II (1 μ M, 24h) up-regulated the mRNA expression of the hypertrophic markers including ANP and BNP. The induction of ANP and BNP in response to Ang II (1 μ M) was restrained to different extents by different concentrations of puerarin (1 μ M, 5 μ M, 10 μ M, 20 μ M, 40 μ M) (Fig. 7). It indicated that treatment with puerarin led to a significant reduction of the mRNA expression of the hypertrophic markers ANP and BNP in a concentration-dependent manner.

Discussion

Previous studies have provided support for the traditional uses of kudzu root on cardiovascular, cerebrovascular, and endocrine systems including diabetes and its complications. Puerarin is a pure extract and the major bioactive constituent of kudzu root [7]. In

this study, we found that 8 weeks after AB, mice developed cardiac hypertrophy and fibrosis characterized by increased HW/BW ratio, HW/TL ratio, cross-section area of cardiomyocytes, collagen accumulation, expression of hypertrophic and fibrotic markers in myocardium, leading to LV relative dilation and dysfunction. Cardiomyocyte apoptosis and induction of Bax in response to AB were suppressed by puerarin. Furthermore, the increased mRNA expression of ANP and BNP induced by Ang II (1 μ M) was restrained to different extents by different concentrations of puerarin. Puerarin treatment retarded the development of cardiac hypertrophy, fibrosis, and apoptosis in mice subjected to pressure overload associated with the regulation on PI3K/Akt and JNK pathways.

Much of the recent work has focused on the antipyretic [6], anti-inflammatory [10], anti-oxidative [8], anti-apoptosis [12], and estrogen-like biological activities of puerarin [19]. For more than 2000 years, kudzu root has been used as a herbal medicine for the treatment of several diseases including fever, diabetes, and cardiovascular diseases [7]. As the major bioactive constituent in kudzu root, puerarin injection has been widely used to treat coronary heart disease and angina pectoris in clinical practice [20]. However, it is not clear whether puerarin can retard the process of

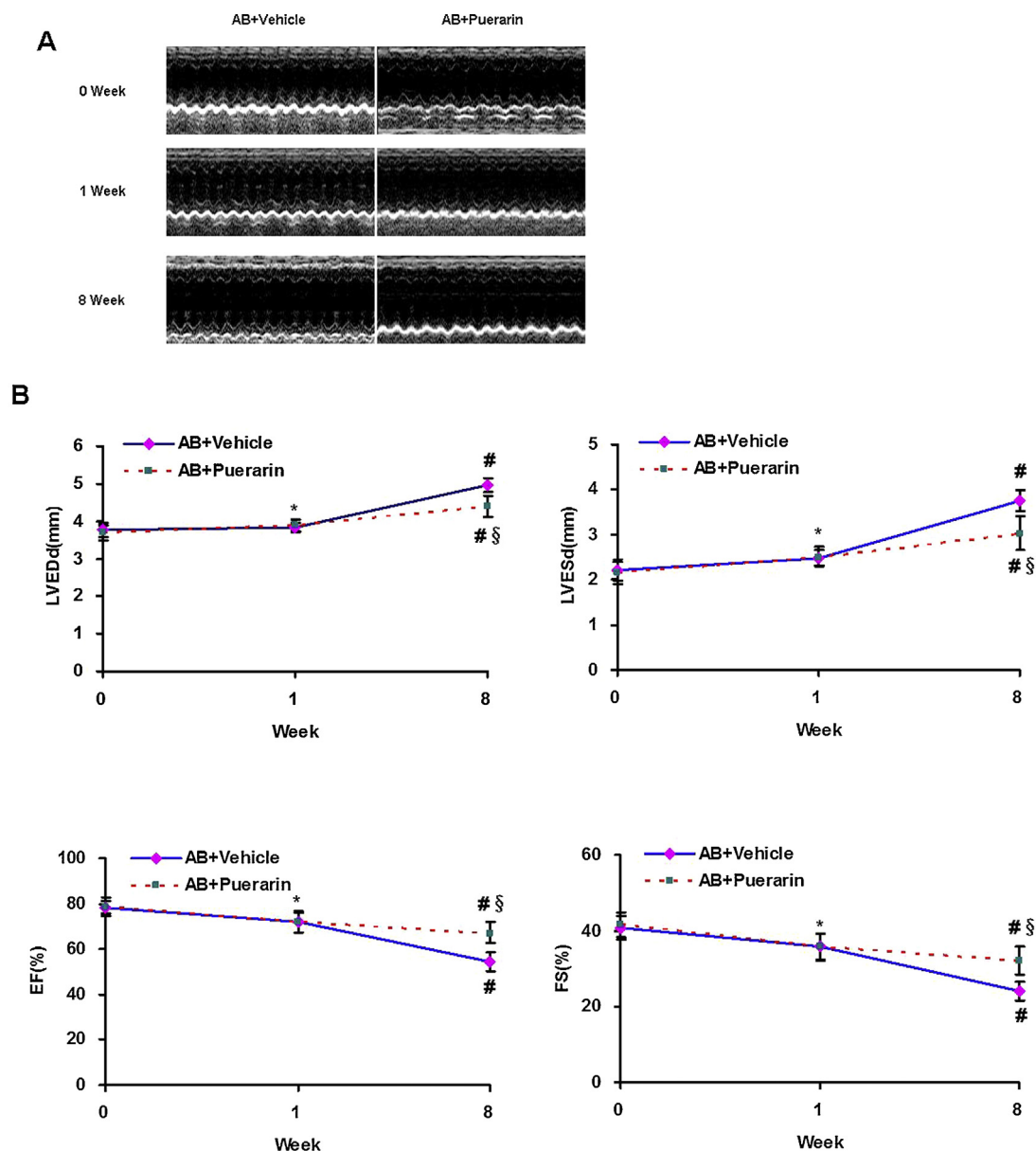


Fig. 2. Evidence of attenuation of aortic banding (AB)-induced left ventricular dysfunction by serial echocardiography. (A) Representative M-mode images of AB + vehicle and AB + puerarin groups. (B) Puerarin-attenuated AB-induced increased left ventricular (LV) diameters including LV end-systolic diameter (LVESd) and LV end-diastolic diameter (LVEDd), and also attenuated AB-induced changes in ejection fraction (EF) and fractional shortening (FS). Echocardiography was performed before surgery (0 week), 1 week after surgery (1 week), and at the end of the study (8 weeks) ($n=9-10$). Puerarin administration was started from 1 week after AB surgery. * $p < 0.05$ vs week 0, # $p < 0.05$ vs week 1, § $p < 0.05$ vs AB + vehicle group.

cardiac hypertrophy. Accordingly, our study addresses the potential of puerarin in protecting against cardiac hypertrophy in mice subjected to pressure overload.

Pressure overload exerts a mechanical stress on the ventricles and can trigger not only cardiac hypertrophy but also fibrosis [21]. Increased cardiac collagen deposition is an important character of fibrosis [22]. It was reported that puerarin could reverse chemical-induced liver fibrosis in experimental rats [23]. In this study, we found that puerarin administration retarded the development of fibrosis in pressure overloaded hearts. The mRNA expression of procollagen type I and III, the two major collagen types in the heart, was reported to be increased in pressure overloaded heart tissue of mice [24]. It was also found that myocardial fibrosis was mediated through an interaction between the tumor necrosis factor

alpha (TNF α) and TGF β pathways that led to increased expression of fibrillar collagens [25]. Moreover, CTGF and fibronectin are pro-fibrotic markers of fibrosis [26,27]. As shown here, puerarin could negatively regulate CTGF, collagen I, collagen III, fibronectin, and TGF β 1 production in response to pressure overload. Puerarin's ability to normalize expression of pro-fibrotic genes reflects the fact that puerarin may also be effective in retarding extracellular remodeling in post pressure overload myocardium although this needs to be confirmed by additional studies examining molecular mechanisms of the fibrotic process.

We assessed the possible underlying mechanisms for puerarin to retard cardiac hypertrophy and found that protein expression of P-P13K, P-Akt, P-GSK3 β , P-mTOR, P-FoxO1, P-FoxO3a, and P-JNK increased significantly in hearts of mice from AB + vehicle

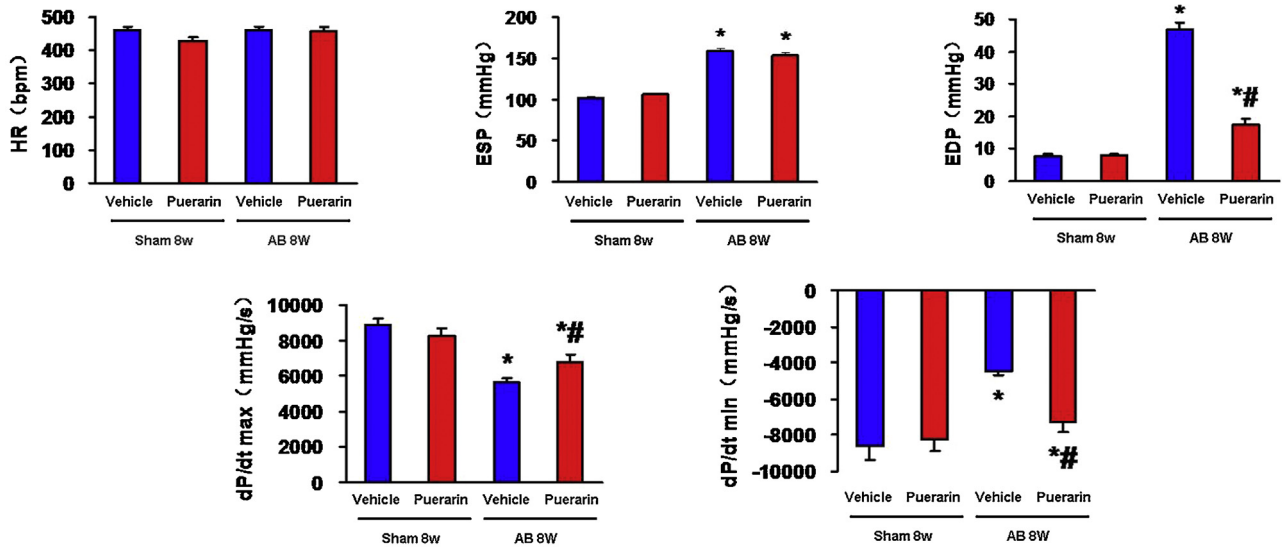


Fig. 3. Normalization of hemodynamic parameters by puerarin. * $p < 0.05$ as compared with the corresponding sham group. # $p < 0.05$ vs AB + vehicle group. AB, aortic banding; HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; dP/dt max, maximal rate of pressure development; dP/dt min, minimal rate of pressure decay.

group at 8 weeks after AB. Daily treatment with puerarin inhibited the phosphorylations of PI3K, Akt, GSK3 β , mTOR, FoxO1, FoxO3a, and JNK induced by pressure overload. Our findings indicate that puerarin's ability to retard cardiac remodeling and heart failure is likely mediated by the amelioration of the activation of PI3K/Akt and JNK signaling pathways. It was thought that life-long inhibition of PI3K might promote long-term heart benefits [28], and PI3K/Akt and JNK signaling pathways have been thought to be intimately related to cardiac hypertrophy and heart failure.

Studies demonstrated that engagement of the p85 SH2 domains of PI3K by pTyr relieved the p85-mediated inhibition of p110 isoforms, resulted in the activation of the PI3Ks p110 α , p110 δ , and probably also p110 β , and always led to the activation of Akt [29]. It was also reported that the short-term activation of Akt promoted physiological hypertrophy and protection from myocardial injury, whereas, the long-term activation caused pathological hypertrophy and heart failure [30]. GSK3 β , downstream of Akt, is inactivated by phosphorylation of serine9 during hypertrophic

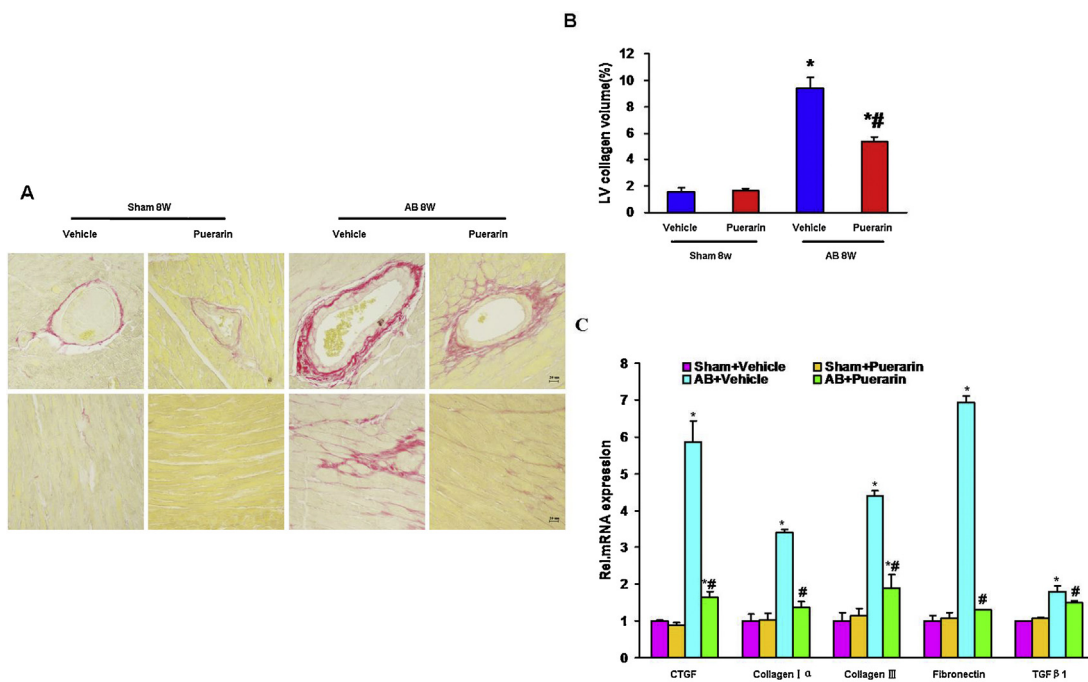


Fig. 4. Puerarin-attenuated fibrosis in pressure-overload hearts. (A) Histological sections of the left ventricle in indicated group were stained for picrosirius red. (B) Fibrotic areas from histological sections were quantified using an image-analysis system. (C) The mRNA expression of connective tissue growth factor (CTGF), collagen I α , collagen III, fibronectin, and transforming growth factor β 1 (TGF β 1) in the myocardium were obtained from indicated groups using reverse transcription-polymerase chain reaction analysis. * $p < 0.05$ compared with the corresponding sham group. # $p < 0.05$ vs AB + vehicle group. AB, aortic banding.

conditions [31], has been shown to be a negative regulator of cardiomyocyte hypertrophy [32]. Another downstream target of Akt which is involved in cardiac hypertrophy is mTOR [33,34]. Furthermore, cumulative Akt activation leads to inactivation of forkhead transcription factors, which are negative regulators of cardiac hypertrophy [35,36]. We also found that MAPK signaling was activated in pressure-overloaded hearts, which was consistent with previous studies [37,38]. Interestingly, puerarin blunted activation of JNK without affecting ERK or p38 activity *in vivo*. As one of the four best characterized MAPK subfamilies, JNK has been implicated in promoting cardiac remodeling downstream of various pathways [39,40].

FoxO3a is a member of the forkhead family of transcription factors and an important substrate of Akt. One important function of FoxO3a is to induce apoptosis [41]. In our study, puerarin inhibited cardiomyocyte apoptosis. Meanwhile, reduced phosphorylation of FoxO was identified in puerarin-treated hearts. It seemed confusing. However, in contrast to the FoxO-inhibitory actions of Akt, JNK is one of the kinases that are known to activate FoxO [42]. Chaanine et al. investigated the relationship between JNK and Akt and they found that JNK signaling dominated and overrode Akt signaling, activated FoxO3a in cardiac hypertrophy and heart failure, and

further promoted mitochondrial-induced apoptosis. It was consistent with our findings [43].

Therefore, inhibition of PI3K/Akt and JNK signaling by puerarin may play a role in retarding the development of pressure overload-induced alterations in cardiac structure and function in the aortic banding mice model.

Our results based on findings in *in vivo* studies suggest that amelioration of PI3K/Akt and JNK-dependent processes represents a viable mechanistic basis for the reversal of hypertrophy and reinforce the concept that targeting the long-term activation of some signaling pathways in cardiac hypertrophy and heart failure may represent a key approach toward developing effective therapeutic strategies although it is recognized that some signaling pathways may be cardiac protective when short-term activated and other potential intracellular targets for reversal of hypertrophy likely exist. The *in vitro* studies indicate that treatment with puerarin led to a significant reduction of the mRNA expression of the hypertrophy marker genes ANP and BNP in Ang II (1 μ M)-stimulated H9c2 cells in a concentration-dependent manner. Further studies targeting the direct effect of puerarin on cardiac myocyte hypertrophy may still be needed. We did not evaluate the toxicology of puerarin in this study, for it has a relatively low toxicity profile [7].

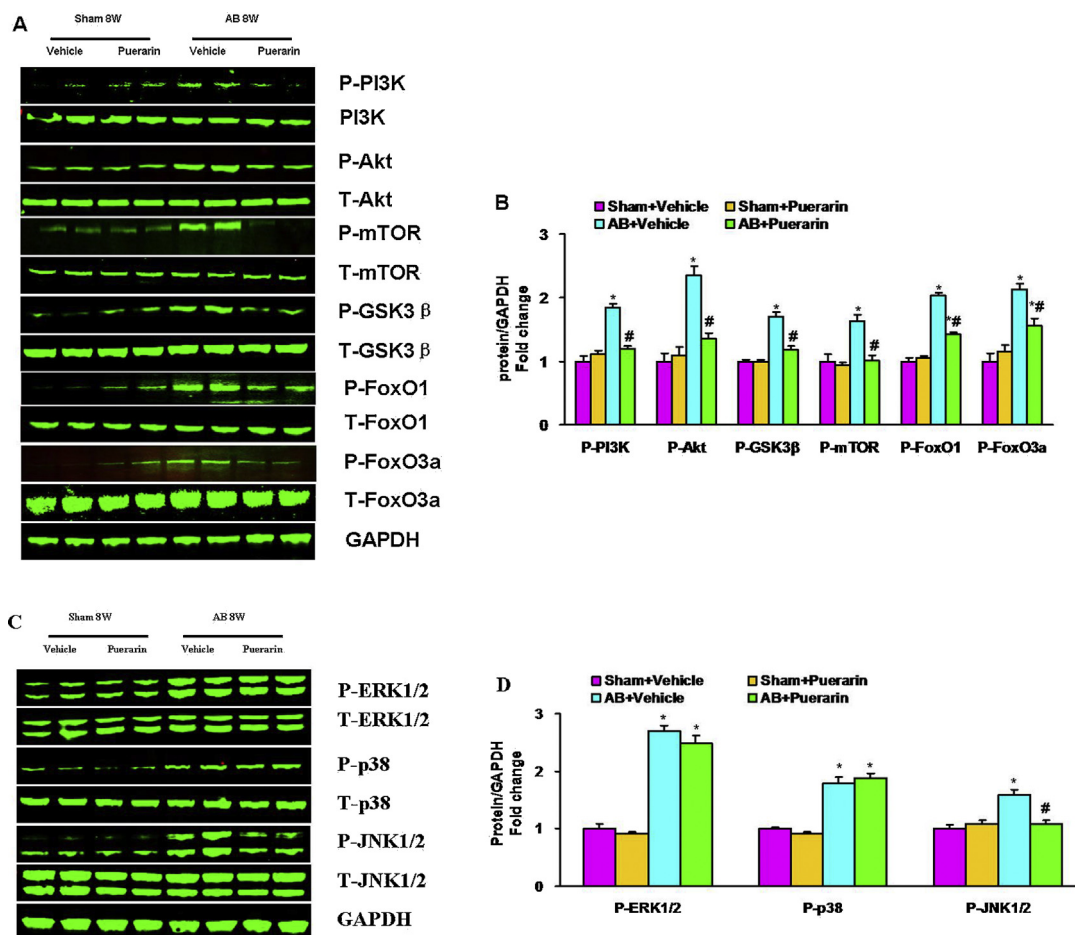


Fig. 5. Effects of puerarin on the PI3K/Akt and JNK signaling pathways. (A) Representative blots of P-phosphoinositide 3-kinase (PI3K), P-Akt, P-mammalian target of rapamycin (mTOR), P-glycogen synthase kinase 3 β (GSK3 β), P-forkhead box O1 (FoxO1), and P-FoxO3a in the heart tissues of mice in the indicated groups ($n=6$). (B) Comparison of expression among the indicated groups. (C) and (D) Mitogen-activated protein kinase (MAPK) expression based on Western blot assay. (C) Representative blots of P-extracellular signal-regulated kinase (ERK)1/2, P-p38, P-c-Jun N-terminal kinase (JNK)1/2 in the heart tissues of mice in the indicated groups ($n=6$). (D) Quantitative results. * $p < 0.05$ as compared with the corresponding sham group. # $p < 0.05$ vs AB + vehicle group. AB, aortic banding; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

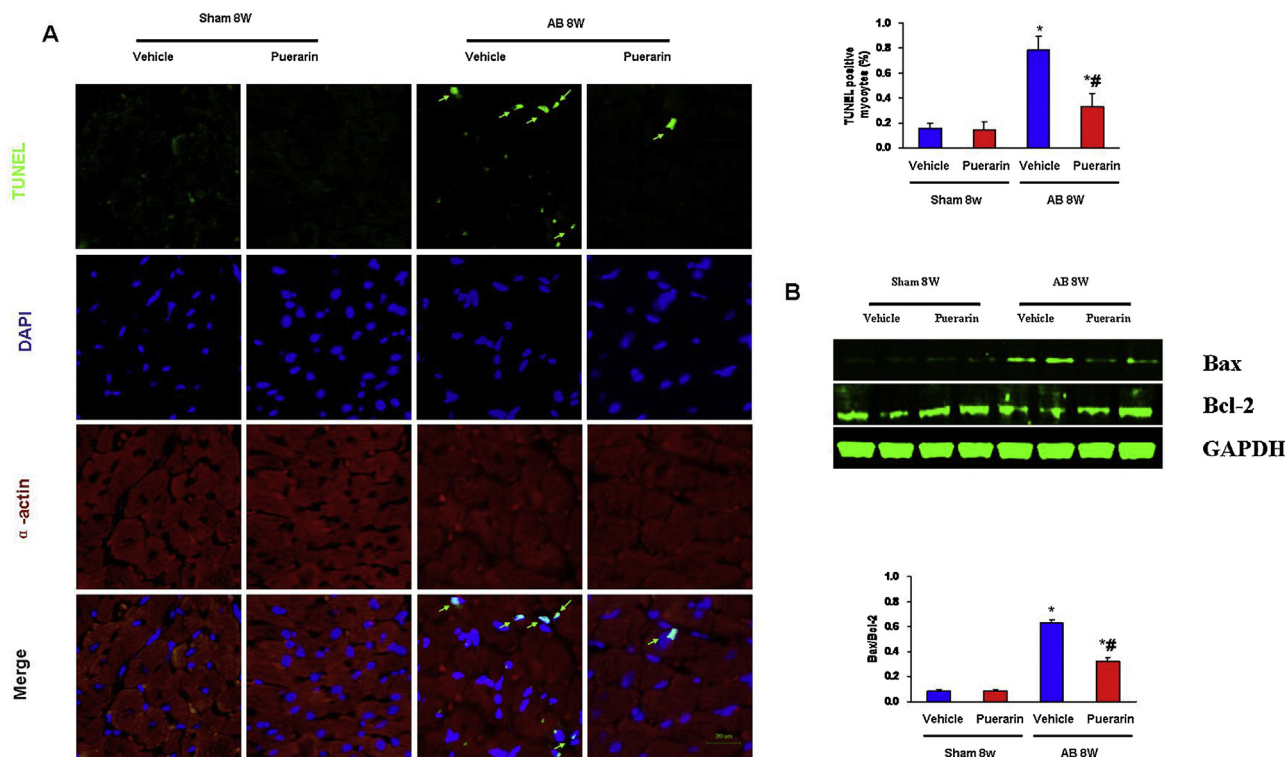


Fig. 6. Puerarin inhibits cardiomyocyte apoptosis in response to chronic pressure overload. (A) Representative images and quantification results of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in a section of mice hearts. (B) Cardiac expression of Bax and Bcl-2 determined by Western blotting analysis. * $p < 0.05$ as compared with the corresponding sham group. # $p < 0.05$ vs AB + vehicle group. DAPI, 4',6-diamidino-2-phenyl-indole; AB, aortic banding; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

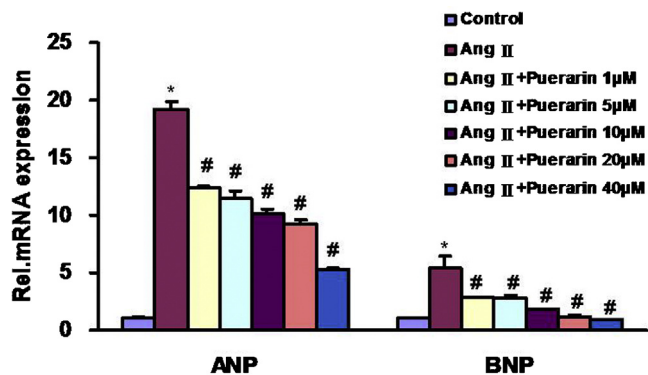


Fig. 7. Inhibitory effect of puerarin on the mRNA expression of atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) in Ang II (1 μ M) stimulated H9c2 cells. * $p < 0.05$ vs control group. # $p < 0.05$ vs Ang II group.

Conclusion

Chronic oral treatment with puerarin retarded the progression of cardiac hypertrophy and improved cardiac function in pressure overloaded mice. This cardioprotective effect of puerarin was related to its inhibition of PI3K/Akt and JNK signaling. Puerarin may offer a potentially effective and relatively safe approach to retard the processes of cardiac hypertrophy and heart failure particularly in combination with other treatment modalities.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (81000036 and 81000095) and the Fundamental Research Funds for the Central Universities of China (2012302020212 and 2012302020211).

References

- [1] Kutsuzawa D, Arimoto T, Watanabe T, Shishido T, Miyamoto T, Miyashita T, Takahashi H, Niizeki T, Takeishi Y, Kubota I. Ongoing myocardial damage in patients with heart failure and preserved ejection fraction. *J Cardiol* 2012;60:454–61.
- [2] Gjesdal O, Bluemke DA, Lima JA. Cardiac remodeling at the population level – risk factors, screening, and outcomes. *Nat Rev Cardiol* 2011;8:673–85.
- [3] Rouleau JL. New and emerging drugs and device therapies for chronic heart failure in patients with systolic ventricular dysfunction. *Can J Cardiol* 2011;27:296–301.
- [4] Hellawell JL, Margulies KB. Myocardial reverse remodeling. *Cardiovasc Ther* 2012;30:172–81.
- [5] Tamargo J, Lopez-Sendon J. Novel therapeutic targets for the treatment of heart failure. *Nat Rev Drug Discov* 2011;10:536–55.
- [6] Yao XJ, Yin JA, Xia YF, Wei ZF, Luo YB, Liu M, Feleder C, Dai Y. Puerarin exerts antipyretic effect on lipopolysaccharide-induced fever in rats involving inhibition of pyrogen production from macrophages. *J Ethnopharmacol* 2012;141:322–30.
- [7] Wong KH, Li GQ, Li KM, Razmovski-Naumovski V, Chan K. Kudzu root: traditional uses and potential medicinal benefits in diabetes and cardiovascular diseases. *J Ethnopharmacol* 2011;134:584–607.
- [8] Liu CM, Ma JQ, Sun YZ. Protective role of puerarin on lead-induced alterations of the hepatic glutathione antioxidant system and hyperlipidemia in rats. *Food Chem Toxicol* 2011;49:3119–27.
- [9] Choo MK, Park EK, Yoon HK, Kim DH. Antithrombotic and antiallergic activities of daidzein, a metabolite of puerarin and daidzin produced by human intestinal microflora. *Biol Pharm Bull* 2002;25:1328–32.
- [10] Huang F, Liu K, Du H, Kou J, Liu B. Puerarin attenuates endothelial insulin resistance through inhibition of inflammatory response in an IKK β /IRS-1-dependent manner. *Biochimie* 2012;94:1143–50.

- [11] Zhang H, Zhang L, Zhang Q, Yang X, Yu J, Shun S, Wu Y, Zeng Q, Wang T. Puerarin: a novel antagonist to inward rectifier potassium channel (IK1). *Mol Cell Biochem* 2011;352:117–23.
- [12] Liu CM, Ma JQ, Sun YZ. Puerarin protects rat kidney from lead-induced apoptosis by modulating the PI3K/Akt/eNOS pathway. *Toxicol Appl Pharmacol* 2012;258:330–42.
- [13] He H, Shi M, Yang J, Zeng X, Qiao H, Wu L, Li L. The correlation between angiogenesis and abnormal expression of SERCA2a, phospholamban and the endothelin pathway in heart failure, and improvement by puerarin. *Phytother Res* 2008;22:948–56.
- [14] Zhang NB, Huang ZG, Cui WD, Ding BP. Effects of puerarin on expression of cardiac Smad3 and Smad7 mRNA in spontaneously hypertensive rat. *J Ethnopharmacol* 2011;138:737–40.
- [15] Chen R, Xue J, Xie M. Puerarin prevents isoprenaline-induced myocardial fibrosis in mice by reduction of myocardial TGF-beta1 expression. *J Nutr Biochem* 2012;23:1080–5.
- [16] Tanaka T, Tang H, Yu F, Michihara S, Uzawa Y, Zaima N, Moriyama T, Kawamura Y. Kudzu (*Pueraria lobata*) vine ethanol extracts improve ovariectomy-induced bone loss in female mice. *J Agric Food Chem* 2011;59:13230–7.
- [17] Shu C, Chen C, Zhang DP, Guo H, Zhou H, Zong J, Bian Z, Dong X, Dai J, Zhang Y, Tang Q. Gastrodin protects against cardiac hypertrophy and fibrosis. *Mol Cell Biochem* 2012;359:9–16.
- [18] Yan L, Wei X, Tang QZ, Feng J, Zhang Y, Liu C, Bian ZY, Zhang LF, Chen M, Bai X, Wang AB, Fasset J, Chen Y, He YW, Yang Q, et al. Cardiac-specific mindin overexpression attenuates cardiac hypertrophy via blocking AKT/GSK3beta and TGF-beta1-Smad signalling. *Cardiovasc Res* 2011;92:85–94.
- [19] Tang XL, Liu XJ, Tian Q, Zhang W. Dynamic oxidative stress and DNA damage induced by oestrogen deficiency and protective effects of puerarin and 17 beta-oestradiol in ovariectomized rats. *Basic Clin Pharmacol Toxicol* 2012;111:87–91.
- [20] Wang Q, Wu T, Chen X, Ni J, Duan X, Zheng J, Qiao J, Zhou L, Wei J. Puerarin injection for unstable angina pectoris. *Cochrane Database Syst Rev* 2006;CD004196.
- [21] Fan D, Takawale A, Lee J, Kassiri Z. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. *Fibrogenesis Tissue Repair* 2012;5:15.
- [22] de Jong S, van Veen TA, de Bakker JM, Vos MA, van Rijen HV. Biomarkers of myocardial fibrosis. *J Cardiovasc Pharmacol* 2011;57:522–35.
- [23] Zhang S, Ji G, Liu J. Reversal of chemical-induced liver fibrosis in Wistar rats by puerarin. *J Nutr Biochem* 2006;17:485–91.
- [24] Zhou H, Bian ZY, Zong J, Deng W, Yan L, Shen DF, Guo H, Dai J, Yuan Y, Zhang R, Lin YF, Hu X, Li H, Tang QZ. Stem cell antigen 1 protects against cardiac hypertrophy and fibrosis after pressure overload. *Hypertension* 2012;60:802–9.
- [25] Kassiri Z, Defamie V, Hariri M, Oudit GY, Anthwal S, Dawood F, Liu P, Khokha R. Simultaneous transforming growth factor beta-tumor necrosis factor activation and cross-talk cause aberrant remodeling response and myocardial fibrosis in Timp3-deficient heart. *J Biol Chem* 2009;284:29893–904.
- [26] Koitabashi N, Arai M, Kogure S, Niwano K, Watanabe A, Aoki Y, Maeno T, Nishida T, Kubota S, Takigawa M, Kurabayashi M. Increased connective tissue growth factor relative to brain natriuretic peptide as a determinant of myocardial fibrosis. *Hypertension* 2007;49:1120–7.
- [27] Meems LM, Cannon MV, Mahmud H, Voors AA, van Gilst WH, Sillje HH, Ruifrok WP, de Boer RA. The vitamin D receptor activator paricalcitol prevents fibrosis and diastolic dysfunction in a murine model of pressure overload. *J Steroid Biochem Mol Biol* 2012;132:282–9.
- [28] Shioi T, Inuzuka Y. Aging as a substrate of heart failure. *J Cardiol* 2012;60:423–8.
- [29] Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. *Nat Rev Mol Cell Biol* 2010;11:329–41.
- [30] Chaanine AH, Hajjar RJ. AKT signalling in the failing heart. *Eur J Heart Fail* 2011;13:825–9.
- [31] Badorff C, Ruetten H, Mueller S, Stahmer M, Gehring D, Jung F, Ihling C, Zeiher AM, Dimmeler S. Fas receptor signaling inhibits glycogen synthase kinase 3 beta and induces cardiac hypertrophy following pressure overload. *J Clin Invest* 2002;109:373–81.
- [32] Haq S, Choukroun G, Kang ZB, Ranu H, Matsui T, Rosenzweig A, Molkenin JD, Alessandrini A, Woodgett J, Hajjar R, Michael A, Force T. Glycogen synthase kinase-3beta is a negative regulator of cardiomyocyte hypertrophy. *J Cell Biol* 2000;151:117–30.
- [33] Kurdi M, Booz GW. Three 4-letter words of hypertension-related cardiac hypertrophy: TRPC, mTOR, and HDAC. *J Mol Cell Cardiol* 2011;50:964–71.
- [34] Shioi T, McMullen JR, Tarnavski O, Converso K, Sherwood MC, Manning WJ, Izumo S. Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation* 2003;107:1664–70.
- [35] Skurk C, Izumiya Y, Maatz H, Razeghi P, Shiojima I, Sandri M, Sato K, Zeng L, Schiekofer S, Pimentel D, Lecker S, Taegtmeier H, Goldberg AL, Walsh K. The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. *J Biol Chem* 2005;280:20814–23.
- [36] Ronnebaum SM, Patterson C. The FoxO family in cardiac function and dysfunction. *Annu Rev Physiol* 2010;72:81–94.
- [37] Cai WF, Zhang XW, Yan HM, Ma YG, Wang XX, Yan J, Xin BM, Lv XX, Wang QQ, Wang ZY, Yang HZ, Hu ZW. Intracellular or extracellular heat shock protein 70 differentially regulates cardiac remodelling in pressure overload mice. *Cardiovasc Res* 2010;88:140–9.
- [38] Esposito G, Prasad SV, Rapacciuolo A, Mao L, Koch WJ, Rockman HA. Cardiac overexpression of a G(q) inhibitor blocks induction of extracellular signal-regulated kinase and c-Jun NH(2)-terminal kinase activity in in vivo pressure overload. *Circulation* 2001;103:1453–8.
- [39] Choudhary R, Palm-Leis A, Scott 3rd RC, Guleria RS, Rachut E, Baker KM, Pan J. All-trans retinoic acid prevents development of cardiac remodeling in aortic banded rats by inhibiting the renin-angiotensin system. *Am J Physiol Heart Circ Physiol* 2008;294:H633–44.
- [40] Fan GC, Yuan Q, Song G, Wang Y, Chen G, Qian J, Zhou X, Lee YJ, Ashraf M, Kranias EG. Small heat-shock protein Hsp20 attenuates beta-agonist-mediated cardiac remodeling through apoptosis signal-regulating kinase 1. *Circ Res* 2006;99:1233–42.
- [41] Wu C, Jin B, Chen L, Zhuo D, Zhang Z, Gong K, Mao Z. MiR-30d induces apoptosis and is regulated by the Akt/FOXO pathway in renal cell carcinoma. *Cell Signal* 2013;25:1212–21.
- [42] Ferdous A, Battiprolu PK, Ni YG, Rothermel BA, Hill JA. FoxO, autophagy, and cardiac remodeling. *J Cardiovasc Transl Res* 2010;3:355–64.
- [43] Chaanine AH, Jeong D, Liang L, Chemaly ER, Fish K, Gordon RE, Hajjar RJ. JNK modulates FOXO3a for the expression of the mitochondrial death and mitophagy marker BNIP3 in pathological hypertrophy and in heart failure. *Cell Death Dis* 2012;3:265.