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Research Paper

Icariin attenuates cardiac remodelling through down-regulating myocardial apoptosis and matrix metalloproteinase activity in rats with congestive heart failure

Yao-Hong Song[a], Hui Cai[b], Ning Gu[a], Chun-Fa Qian[a], Shou-Pei Cao[a] and Zhi-Ming Zhao[b]

[a]Department of Cardiology, The Third Affiliated Hospital of Nanjing University of Traditional Chinese Medicine and [b]Department of Integrated Traditional Chinese and Western Medicine, Nanjing General Hospital of Nanjing Military Command, Nanjing, China

Abstract

Objectives In this study, the anti-heart failure effect of icariin, a natural flavonol glycoside, and the underlying mechanisms were investigated.

Methods Heart failure was induced by isoproterenol in male Sprague–Dawley rats. Matrix metalloproteinase activity was determined by gelatin zymography assay. The mRNA expression was determined by real-time PCR. The protein expression was determined by Western bolt. Mitochondria structure was examined by transmission electron microscopy.

Key findings Isoproterenol administration resulted in a severe heart failure, as shown by the increased levels of left ventricular weight index, heart rate, left ventricular end diastolic pressure, maximal rate of left ventricular pressure decline (dp/dt_{min}), decreased levels of left ventricular systolic pressure and maximal rate of left ventricular pressure rise (dp/dt_{max}). Against these, icariin dose-dependently reversed the changes of these cardiac morphometric and haemodynamic parameters. In addition, icariin significantly inhibited serum levels of tumour necrosis factor-α, noradrenaline, angiotensin II and brain natriuretic peptide in rats with congestive hear failure and improved the histological changes, including cardiocyte hypertrophy, cardiocyte degeneration, inflammatory infiltration and cardiac desmoplasia. Furthermore, the expression and activity of matrix metalloproteinase (MMP)-2 and MMP-9, which regulate collagen production, were also blocked by icariin. Moreover, myocardial apoptosis was remarkably attenuated by icariin through regulating Bcl-2/Bax axle.

Conclusions Icariin ameliorates left ventricular dysfunction and cardiac remodelling through down-regulating matrix metalloproteinase-2 and 9 activity and myocardial apoptosis in rats with congestive heart failure.

Keywords cardiac remodelling; congestive heart failure; icariin; matrix metalloproteinase; myocardial apoptosis

Introduction

Congestive heart failure (CHF) is a severe cardiovascular disease with increasing incidence and prevalence. Despite recent advances in CHF therapy, mortality remains high.[1] Therefore, new therapeutic remedies or approaches are needed to decrease morbidity and mortality in heart failure patients. Usually, CHF is invariably associated with cardiac hypertrophy, and the changes in the cardiac remodelling (shape and size of cardiocyte) are often considered responsible for cardiac dysfunction in CHF. It is worth noting that left ventricular remodelling is a dynamic response of the heart to injury and is a critical component in the development of CHF. Inhibition of cardiac remodelling is emerging as a promising novel therapeutic approach for the treatment of CHF.[2,3] Several lines of evidence both from various experimental models of CHF and from patients with different types of CHF have indicated that elevated matrix metalloproteinase (MMP) activity and myocardial apoptosis are responsible for cardiac remodelling.[4–6] Some previous studies have indicated that both angiotensin-converting enzyme inhibitors and beta-blocking agents improve cardiac function in failing hearts through attenuating changes in cardiac remodeling, such as sarcolemma, sarcoplasmic reticulum and myofibril enzyme activity.[3,7,8] It is suggested that

Correspondence: Yao-Hong Song, Department of Cardiology, The Third Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, 1 Jinling Road, Nanjing 210001, China.
E-mail: tcmantichf@126.com

cardiac remodelling is an excellent target for the development of improved drug therapy for CHF. Thus, inhibition of cardiac remodelling may be a promising novel therapeutic approach for the treatment of CHF.[9]

On the other hand, Herba Epimedii, a traditional Chinese herb, is widely used for treating various diseases including coronary heart disease, impotence and osteoporosis.[10] Epimedium is one representative of traditional Chinese medicine derived from *Shen Nong Ben Cao Jing*, a pharmaceutical classic in China. An extract of epimedium has been shown to have a protective role in experimental myocardial infarction in rats.[11] Icariin, a prenylated flavonol glycoside derived from Herba Epimedii, exhibits a variety of pharmacological actions, including tonic, aphrodisiac, antirheumatic, antidepressant, cardiovascular protective, immunomodulatory and anti-tumour activity.[12–15] It has been previously demonstrated that icariin exerts a potent osteogenic effect via activation of bone morphogenetic protein signalling.[16] Previous studies have demonstrated that icariin facilitates the directional differentiation of murine embryonic stem cells into cardiomyocytes *in vitro*.[17] However, the exact mechanism of the anti-heart failure effect of icariin remains unclear. To our knowledge, this is the first time it has been reported that icariin significantly improves left ventricular remodelling and function in rats with CHF induced by isoproterenol. The beneficial effect of icariin in animal models of CHF might be mediated at least in part by inhibition of elevated MMP-2 and MMP-9 activity, as well as myocardial apoptosis, and this effect contributed to the amelioration of CHF in clinical conditions.

Materials and Methods

Drugs and reagents

Icariin (2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone, purity >98%, chemical structure shown in Figure 1) purchased from Xi'an Tongjiang Biotechnology Co., Ltd (Xi'an, China) was dissolved at a concentration of 20 mM in 100% DMSO as a stock solution, stored at −20°C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study (all the control groups were composed of 0.1% DMSO). The following drugs and reagents were used: isoproterenol (Sigma); metoprolol tartrate (AstraZeneca); kit for determining serum tumour necrosis factor (TNF-α; General Hospital of Liberation Army, Radioimmunity Center, Nanjing, China); angiotensin II (Ang II; Beijing North Biotech Co. Ltd, Beijing, China); brain natriuretic peptide (Enzyme immunonassay goat anti-rat BNP, Adlitteram Diagnostic Laboratories, Inc., San Diego, CA, USA); ELISA kit for rat noradrenaline (norepinephrine, RapidBio Lab., Calabasas, CA, USA); 96-well culture plates (Nunclon); RPMI 1640 (Gibco BRL); new bovine serum (NBS; Hangzhou Sijiqing Co. Ltd, Hangzhou, China); anti-Bcl-2 monoclonal antibody, anti-Bax monoclonal antibody and anti-α-tubulin were from Santa Cruz Biotechnology; Cleaved caspase-3 was from Cell Signaling Technology (Boston, MA, USA). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

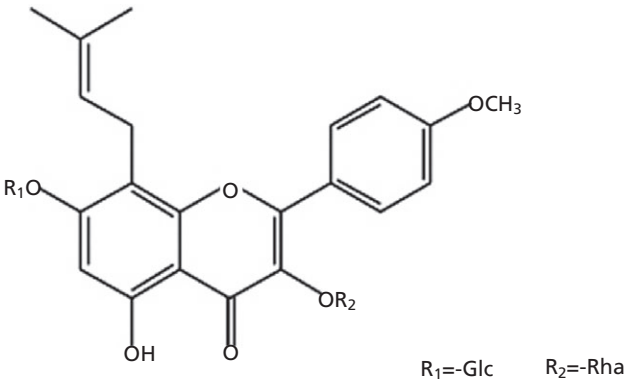

Figure 1 The chemical structure of icariin.

Animal model and echocardiography measurements

Adult, healthy, male Sprague–Dawley rats, 230–280 g, were chosen for production of experimental heart failure using a modified isoproterenol administration method.[18] Briefly, all rats received two subcutaneous injections (separated by a 24-h interval) of 170 mg isoproterenol per kilogram of body weight. Echocardiography was performed on rats four weeks after completion of the injection protocol to evaluate the degree of heart failure of rats. Rats were anaesthetized with pentobarbital sodium (35 mg/kg i.p.), then the chest was shaved and the rat was placed in the supine position. Transthoracic echocardiography was performed with a Sonos 5500 and a 7.5-MHz transducer (Hewlett-Packard). The heart was imaged in the 2-dimensional mode in short-axis view of the left ventricle at the level of the papillary muscle. All rats that had an ejection fraction >45% were excluded after the echocardiography examination. The remaining rats that had an ejection fraction <45% were randomly divided into five groups: control group ($n = 15$); metoprolol 8 mg/kg/day group ($n = 12$); icariin 10 mg/kg/day group ($n = 11$); icariin 20 mg/kg/day group ($n = 10$); icariin 40 mg/kg/day group ($n = 12$). The drugs were given intragastrically at the specified doses for eight weeks. Then, the rats were anaesthetized for measuring haemodynamic parameters, including left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), dp/dt_{max}, dp/dt_{min}. The rats were then killed with intravenous pentobarbital sodium (100 mg/kg). The hearts were removed, weighed and rinsed in ice-cold normal saline. Ventricles were separated from septum and weighed. Transmural samples from the left ventricles were processed immediately or stored in liquid nitrogen for later analysis. Animal welfare and experimental procedures were carried out strictly in accordance with the guide for the care and use of laboratory animals (National Institutes of Health, the United States) and the related ethical regulations of our hospital. All efforts were made to minimize animal's suffering and to reduce the number of animals used.

Haemodynamic measurements

After anaesthetic induction, the left carotid artery was exposed through blunt dissection and cannulated with a

saline-filled catheter. The catheter was attached to a pressure transducer and introduced into the left ventricle for measuring LVSP, LVEDP, maximal rate of left ventricular pressure rise (dp/dt_{max}) and maximal rate of left ventricular pressure decline (dp/dt_{min}). All haemodynamic data were recorded on a BL-410 biological functional experimental system (Taimeng Science and Technology Co. Ltd, Chengdu, China) and analysed by BL-NewCentury software.

Histological analysis

Formalin-fixed, paraffin-embedded ear tissue was sectioned at 5 mm in thickness, and the sections were stained with hematoxylin and eosin (H&E) and Masson, respectively. Histopathological scoring was done using a range from 0 (no change) to 4 (most severe) to evaluate congestion, oedema and inflammatory cell infiltration by a pathologist who had no prior knowledge of the induction of heart failure or other experimental data.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was extracted from frozen left ventricular tissue using Trizol reagent (Invitrogen) as described by the manufacturer. Single-stranded cDNA was synthesized from 2 μg of total RNA by reverse transcription using 0.5 μg primer of oligo$(dT)_{18}$. Then the amplification was performed using the following primers (Invitrogen, Shanghai, China): GAPDH (GenBank Access No. NM_017008) 5′-TATCGGACG CCTGGTTAC and 3′-CGTTCAAGTTGCCGTGTC, MMP-2 (GenBank Access No. NM_031054) 5′-GGAGGCACGA TTGGTCTG and 3′-TCTGGTACGCCTTTGGTT, MMP-9 (GenBank Access No. NM_031055) 5′-CTGCGTATTTCC ATTCATC and 3′-GAGATTTGGACTGGGTTCC. The PCR cycle conditions were: 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 28 cycles. After the amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide dyeing. RNA without reverse transcription did not yield any amplification; therefore, no genomic DNA contamination occurred. For quantitative real-time PCR analysis, message levels were quantified using the ABI 7000 Sequence Detection System (Applied Biosystems Inc.). Amplification was carried out in a total volume of 20 μl for 40 cycles of the same PCR condition mentioned above and product was detected using SYBR Green I dye (Molecular Probes Inc., Oregon). Samples were run in triplicate, and their relative expression was determined by normalizing expression of each target to GAPDH, and then comparing this normalized value with the normalized expression in a reference sample to calculate a fold-change value.

Gelatin zymography assay

Analysis by zymography on gelatin gel allows detection of enzymatic activity of the secreted collagenases MMP-2 and MMP-9.[19] Briefly, protein was extracted from frozen left ventricular tissue and samples containing 10 μg of protein were mixed with 10 μl sample buffer (62.5 mM Tris-HCl containing 10% glycerol, 0.00125% bromophenol blue and 12% sodium dodecyl sulfate (SDS)) without reducing agent, and they were subjected to SDS-PAGE in 5% polyacrylamide gels that were copolymerized with 2 mg/ml of gelatin at 4°C for 1 h. After electrophoresis, the gels were washed twice in the rinsing buffer (50 mM Tris-HCl containing 2.5% Triton X-100, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3) for 1 h at room temperature to remove SDS. Then, they were incubated for 36 h at 37°C in the incubation buffer (50 mM Tris-HCl containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.05% NaN_3). The gels were stained with 0.1% Coomassie Brilliant Blue R250 for 30 min, and destained for 8 h in a solution of 10% acetic acid and 10% isopropanol. The proteolytic activity was shown as clear bands (zones of gelatin degradation) against the blue background of stained gelatin.

Western blot

Protein was extracted from frozen left ventricular tissue. Protein concentration was determined by using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) and bovine serum albumin as a standard. Samples containing 30 μg of protein were loaded on 10% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane. The membrane was then incubated in blocking solution (5% dry milk in phosphate-buffered saline (PBS)/0.05% Tween 20) for 1 h at room temperature and subsequently exposed to antibodies overnight at 4°C. The next day the membrane was washed by 0.05% Tween 20 in PBS and incubated with peroxidase conjugated goat anti-mouse or goat anti-rabbit IgG for 2 h at room temperature. The transferred proteins were visualized with X-ray films.

DNA fragmentation and caspase-3 activity assay

The qualitation of DNA fragmentation was measured using the Suicide-TrackTM DNA Ladder Isolation Kit (Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions.[20] The quantitation of DNA fragmentation was determined by photometric enzyme immunoassay using the Cell Death Detection ELISAplus kit (Roche, Penzberg, Germany) following the manufacturer's instructions.[21] Activation of caspase-3 was determined using the caspase-3 colorimetric assay kit (MBL International, Woburn, MA, USA) following the manufacturer's instructions.[21]

Transmission electron microscopy

For transmission electron microscopy, the tissue were fixed for 2 h with 4% glutaraldehyde in sodium cacodylate buffer, post-fixed for 1 h with 1% osmium tetroxide, dehydrated and embedded in Epon (EMS). Thin sections adsorbed onto nickel grids were stained with 1% uranyl acetate and 0.3% lead citrate and imaged in a JEOL-1200 EX electron microscope at 80 kV. Mitochondria surfaces were assessed with the Image Tools 3.0 software.[22]

Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test and one-way analysis of variance test were used for statistical analyses of the data. All statistical analyses were conducted using SPSS 10.0 statistical software (SPSS, Chicago, IL, USA). $P < 0.05$ or <0.01 was considered statistically significant.

Results

Icariin ameliorated isoproterenol-induced congestive heart failure in rats

Cardiac morphometric and haemodynamic parameters

As shown in Table 1, isoproterenol administration resulted in a severe heart failure, as shown by the increased levels of left ventricular weight index (LVWI), heart rate (HR), LVEDP as well as maximal rate of left ventricular pressure decline (dp/dt_{min}), and by the decreased levels of LVSP and maximal rate of left ventricular pressure rise (dp/dt_{max}). Against these, icariin dose-dependently reversed the changes of these cardiac morphometric parameters and haemodynamic parameters. The positive control metoprolol also ameliorated left ventricular dysfunction.

Plasma cytokines

Icariin significantly reduced the plasma levels of TNF-α, noradrenaline, Ang II, and BNP in a dose-dependent manner in rats with CHF induced by isoproterenol (Table 2).

Histopathological changes

Icariin remarkably improved the histological changes including cardiocyte hypertrophy, cardiocyte degeneration, inflammatory infiltration and cardiac desmoplasia (Figure 2). The improvement in metoprolol-treated rats was also significant (Figure 2).

Icariin reduced MMP-2 and MMP-9 expression and left ventricular activity in rats with congestive heart failure caused by isoproterenol

As shown in Figure 3a, MMP-2 and MMP-9 mRNA expressions of left ventricles were pronouncedly increased in model rats with CHF caused by isoproterenol. Against this, icariin remarkably down-regulated MMP-2 and MMP-9 mRNA expressions in a dose-dependent manner. In addition, this result was further confirmed by real-time PCR (Figure 3b). Moreover, the activity of MMP-2 and MMP-9 was also suppressed by icariin, as shown by using gelatin zymography assay (Figure 3c).

Table 1 Cardiac morphometric and haemodynamic parameters in rats 12 weeks after isoproterenol injection treated either with normal saline, metoprolol or various doses of icariin

Parameter	Normal ($n = 8$)	Isoproterenol injection				
		Control ($n = 10$)	Metoprolol ($n = 8$)	ICA-Low ($n = 9$)	ICA-Middle ($n = 8$)	ICA-High ($n = 8$)
BW (g)	388.6 ± 15.8	310.2 ± 36.6	272.3 ± 12.4	263.9 ± 8.9	315.8 ± 9.5	275.6 ± 9.4
LVW (g)	0.84 ± 0.05	0.85 ± 0.06	0.68 ± 0.04**	0.68 ± 0.05**	0.77 ± 0.05	0.64 ± 0.04**
LVWI	2.17 ± 0.06##	2.93 ± 0.25	2.54 ± 0.14*	2.57±0.12	2.6 ± 0.16*	2.43 ± 0.11**
HR (bpm)	412.4 ± 14.3##	436.3 ± 10.8	331.5 ± 8.9**	445.5 ± 10.8	432.0 ± 17.5	368.9 ± 3.9**
LVSP (mmHg)	118.2 ± 2.3##	106.8 ± 1.8	103.4 ± 1.5	107.8 ± 2.8	113.4 ± 3.5	117.7 ± 2.1**
LVEDP (mmHg)	1.4 ± 0.7##	14.5 ± 0.8	5.7 ± 0.4**	8.8 ± 0.3**	7.9 ± 0.28**	6.4 ± 0.4**
dp/dt_{max} (mmHg/s)	3620 ± 532##	2442±454	3756 ± 448**	2651 ± 628**	2878 ± 774**	3196 ± 623**
dp/dt_{min} (mmHg/s)	–3674 ± 385##	–1960 ± 619	–2875 ± 453**	–2289 ± 479**	–2355 ± 411**	–2475 ± 429**

bpm, beats per minute; BW, body weight; Control, model rats treated with normal saline after isoproterenol injection; dp/dt_{max}, maximal rate of left ventricular pressure rise; dp/dt_{min}, maximal rate of left ventricular pressure decline; Normal, normal rats without isoproterenol injection; HR, heart rate; ICA-High, model rats treated with 10 mg/kg, 20 mg/kg and 40 mg/kg of icariin after isoproterenol injection, respectively; ICA-Low, ICA-Middle; LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; LVW, left ventricular weight; LVWI, left ventricular weight index; Metoprolol, model rats treated with 8 mg/kg of metoprolol after isoproterenol injection. Values are mean ± SEM. ##$P < 0.01$ vs control; *$P < 0.05$, **$P < 0.01$ vs control.

Table 2 Plasma cytokines parameters in rats 12 weeks after isoproterenol injection treated either with normal saline, metoprolol or various doses of icariin

Parameter	Normal ($n = 8$)	Isoproterenol injection				
		Control ($n = 10$)	Metoprolol ($n = 8$)	ICA-Low ($n = 9$)	ICA-Middle ($n = 8$)	ICA-High ($n = 8$)
TNF-α (pg/ml)	368 ± 22##	1032 ± 70	911 ± 64	579 ± 22.3**	511 ± 40**	418 ± 27.3**
Noradrenaline (ng/ml)	32.9 ± 4.6##	82.9 ± 6.9	75.2 ± 14.7	86.1 ± 14.5	69.8 ± 13.0	62.9 ± 5.24*
Ang II (pg/ml)	273.8 ± 53.5##	677.9 ± 39.2	526.7 ± 44.0*	592.7 ± 43.7	517.8 ± 44.9*	512.1 ± 67.4*
BNP (pg/ml)	44.26 ± 6.1##	232.8 ± 17.8	171.6 ± 17.0*	227.3 ± 11.6	171.5 ± 13.3*	149.3 ± 21.3*

Ang II, angiotensin II; BNP, brain natriuretic peptide; Control, model rats treated with normal saline after isoproterenol injection; ICA-High, model rats treated with 10 mg/kg, 20 mg/kg and 40 mg/kg of icariin after isoproterenol injection, respectively; ICA-Low, ICA-Middle; Metoprolol, model rats treated with 8 mg/kg of metoprolol after isoproterenol injection; Normal, normal rats without isoproterenol injection; TNF-α, tumour necrosis factor-α. Values are mean ± SEM. ##$P < 0.01$ vs control; *$P < 0.05$, **$P < 0.01$ vs control.

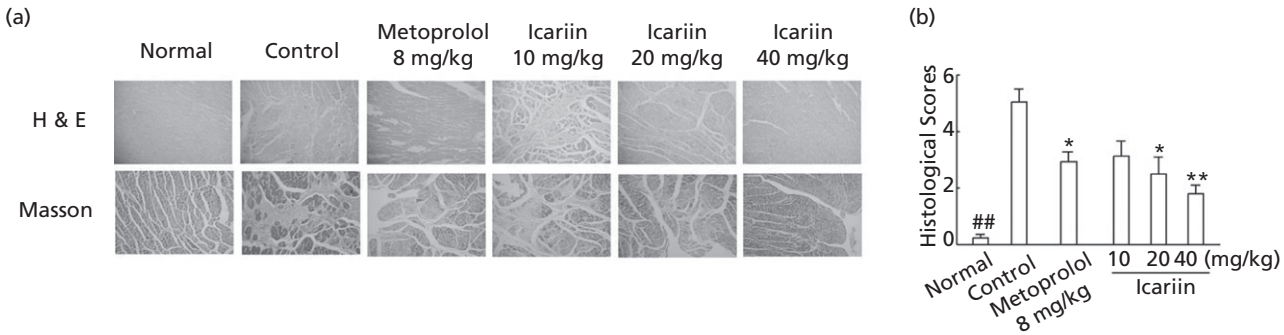

Figure 2 Effect of icariin on histopathological changes of the left ventricular remodelling in rats with congestive heart failure caused by isoproterenol. (a) Hematoxylin & Eosin (H&E) and Masson staining (original magnification ×100). Normal, normal rats; Control, model rats treated with normal saline after isoproterenol injection; Metoprolol, model rats treated with metoprolol after isoproterenol injection; Icariin, model rats treated with icariin after isoproterenol injection. (b) Left ventricular histological scoring. Each column indicates the mean ± SEM of 6–9 rats. ##$P < 0.01$ vs control; *$P < 0.05$, **$P < 0.01$ vs control.

Icariin inhibited myocardial apoptosis of the left ventricles in rats with congestive heart failure caused by isoproterenol

The protein expressions of Bax, and cleaved caspase-3 in the control group were obviously increased compared with those in the normal group, while Bcl-2 expression was deeply decreased. Icariin reversed these changes (Figure 4). In addition, icariin dose-dependently induced a significant decrease in DNA fragmentation (Figure 5a) and caspase-3 activity (Figure 5b) in myocardial tissues. Icariin also significantly ameliorated the damaged mitochondria and striated muscle of myocardial tissues (Figure 6). On histological analysis, staining by hematoxylin & eosin, the necrotic cells were not obviously seen (data not shown).

Discussion

Cardiac remodelling is a dynamic response of the heart to injury and a critical component in the development of CHF. There is increasing evidence that inhibition of cardiac remodelling may be a novel drug target for the treatment of CHF.[3] Epimedium is a traditional Chinese medicine currently being tried for treatment of cardiovascular diseases. However, little is known of the mechanism by which it exerts protective role in heart failure. In this study, we first investigated the therapeutic efficacy of icariin in rats with CHF induced by isoproterenol. We observed that icariin significantly improved left ventricular function, inhibited inflammatory cytokine production and MMP activity, and reduced myocardial apoptosis. The effect of icariin on left ventricular morphology, remodelling and function was comparable to the effect observed with the positive control β-blocking agent metoprolol, which has a beneficial effect in the treatment of heart failure.[7]

CHF in the rats, induced by isoproterenol, is accompanied by myocardial dysfunction.[18] In this study, icariin significantly improved haemodynamic parameters (LVSP, LVEDP, HR, dp/dt_{max} and dp/dt_{min}) in rats with CHF (Table 1). Importantly, histological examination of left ventricle sections from control rats without medication showed marked cardiocyte hypertrophy, cardiocyte degeneration, inflammatory infiltration and cardiac desmoplasia (Figure 1). Treatment with icariin dose-dependently reduced the extent of heart damage and especially inhibited collagen hyperplasia and left ventricular fibrosis. Cardiac remodelling in rats with CHF is characterized by interstitial and perivascular deposition of fibrillar collagen leading to cardiac muscle stiffness and left ventricular dysfunction and hence to progressive cardiac dysfunction and heart failure.[23] Fibrosis in the myocardium is a major determinant of cardiac remodelling in ischaemic cardiomyopathy.[24] In this study, icariin also dose-dependently decreased the content of hydroxyproline in left ventricles (data not shown). In line with the results from Masson staining (Figure 1), we confirmed that icariin impaired left ventricular fibrosis in rats with CHF. Thus, we demonstrated that icariin significantly improved left ventricular systolic and diastolic function and attenuated left ventricular fibrosis in rats with CHF induced by isoproterenol.

Isoproterenol-induced heart failure is thought to be a suitable model of CHF. Left ventricular dysfunction and cardiac remodelling play a crucial role in the process of CHF. It is worth noting that heart failure induced by isoproterenol results in the elevation of various plasma cytokines, including TNF-α, noradrenaline, Ang II and BNP, which are responsible for the development of CHF.[25–27] TNF-α is a cytokine that may play a role in the pathogenesis of heart failure. In patients with heart failure, increased levels of TNF-α were observed that were high enough to reduce cardiac contractility *in vitro*. The mortality of heart failure patients increases with higher levels of TNF-α. For this reason, inhibition of TNF-α appears to be a valid target for the improvement of heart failure therapy.[25] Inhibition of noradrenaline in plasma has been proved beneficial for patients with chronic heart failure.[26] Ang II, frequently elevated in CHF patients, contributes to decreased arterial distensibility.[27] BNP, a peptide hormone secreted chiefly by ventricular myocytes, plays a key role in volume homoeostasis. The plasma concentration of BNP is raised in various pathological states, especially heart failure. Many studies suggest that measurement of plasma BNP has clinical utility for excluding a diagnosis of heart failure in

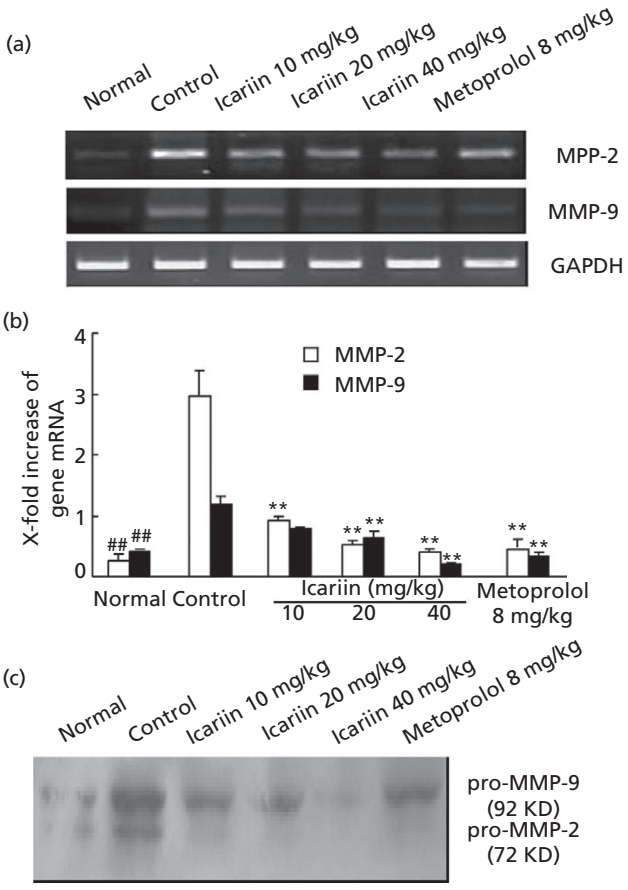

Figure 3 Effect of icariin on the message RNA expression and activity of MMP-2 and MMP-9 of the left ventricle in rats with congestive heart failure caused by isoproterenol. The drugs were given intragastrically at the specified doses for 8 weeks, then the left ventricle was subjected to assay. (a) MMP-2 and MMP-9 mRNA expression of the left ventricles in rats with congestive heart failure were examined by RT-PCR. The graph shown here is one of three different experiments. (b) MMP-2 and MMP-9 mRNA expression was examined by real-time PCR. Each column represents the mean ± SEM of three independent experiments and each experiment includes triplicate sets. ##$P < 0.01$ vs control; *$P < 0.05$, **$P < 0.01$ vs control. (c) MMP-2 and MMP-9 activity was examined by gelatin zymography assay. The graph shown here is one of three different experiments.

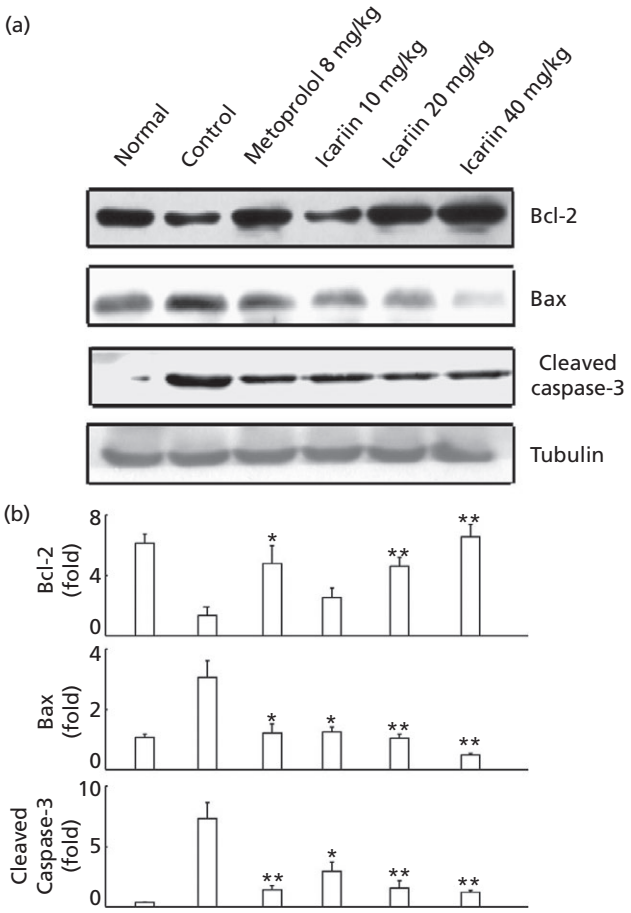

Figure 4 Effect of icariin on myocardial apoptosis of the left ventricle in rats with congestive heart failure caused by isoproterenol. The drugs were given intragastrically at the specified doses for 8 weeks, then the left ventricle was subjected to assay. Bcl-2, Bax and cleaved caspase-3 expression in the left ventricle was examined by Western blot (a). The graph shown here is one of three different experiments. (b) The statistical data of protein expression from three independent experiments. *$P < 0.05$, **$P < 0.01$ vs control.

patients with dyspnoea or fluid retention and for providing prognostic information in those with heart failure or other cardiac disease.[28] In line with the results from haemodynamic and histological examination, icariin significantly reduced the plasma levels of TNF-α, noradrenaline, Ang II and BNP in a dose-dependent manner in rats with CHF. These findings suggest icariin attenuates left ventricular dysfunction and cardiac remodelling.

MMPs are an endogenous family of proteolytic enzymes implicated in cardiac remodelling. Gene expression and gelatinolytic activity of MMPs in the left ventricles were significantly increased in experimental myocardial infarction in mice,[29] in hypertensive rats,[30,31] in rats with progressive heart failure[32] and, most importantly, in patients with heart failure.[33] Previous studies demonstrated pharmacological inhibition of MMPs resulted in suppression of cardiac remodelling in animal models of CHF.[34] In addition, there is evidence that the beneficial effects of angiotensin-converting enzyme inhibitors on cardiac remodelling is due to the inhibition of collagen synthesis, as well as influence on MMPs, particularly MMP-2.[35] Taking these findings into account, there is no doubt about MMPs' crucial role in promoting cardiac remodelling. Considering that the expression and activity of MMP-2 and MMP-9 were also blocked by icariin (Figure 2), it is therefore conceivable to assume that in our study icariin exerts an inhibitory effect on MMP-2 and MMP-9, leading to the prevention of cardiac remodelling and dysfunction.

Myocardial apoptosis has been causally linked to the pathogenesis of heart failure.[36,37] Some apoptotic proteins, including Bcl-2, Bax and caspase-3, are involved in the development of myocardial apoptosis.[36] Bcl-2 family proteins may be either pro- or anti-apoptotic, including Bcl-2, Bcl-xL, Bax, Bid *et al*. Bcl-2 itself inhibits apoptosis in

response to a wide variety of signals and over-expression of Bcl-2 can protect cardiac myocytes.[38] Bax, on the contrary, is a pro-apoptotic protein of the Bcl-2 family and a decreased Bcl-2/Bax ratio has been shown to increase the probability for myocardial cell apoptosis.[39] Caspases, a subclass of cysteine proteases that cleave substrates after aspartic acid residues, are central to the execution of apoptosis.[40] Cardiac myocytes undergo apoptosis in response to a myriad of stimuli including β-adrenergic agonists, Ang II, TNF-α and so on. Several studies have demonstrated that inhibition of myocardial apoptosis provided beneficial effects on cardiac remodelling and left ventricular function.[41–43] In our study, myocardial apoptosis was obviously induced by isoproterenol injection. Against this, icariin remarkably attenuated myocardial apoptosis through down-regulating Bax, caspase-9 and caspase-3 expression and up-regulating Bcl-2 expression in cardiocytes (Figure 3). It could be speculated that icariin reduced cardiac remodelling at least in part by inhibiting myocardial apoptosis. Previously, we have reported that ethanol extract from *Epimedium brevicornum* attenuated left ventricular dysfunction and cardiac remodelling through down-regulating MMP-2 and MMP-9 activity and myocardial apoptosis in rats with CHF. [44] However, the identity of the active compounds in the ethanol extract from *Epimedium brevicornum* remains unclear. This study aims to answer this question. To our knowledge, this is the first time reported that icariin, a natural compound from *Epimedium brevicornum* significantly improved left ventricular remodelling and function in rats with CHF.

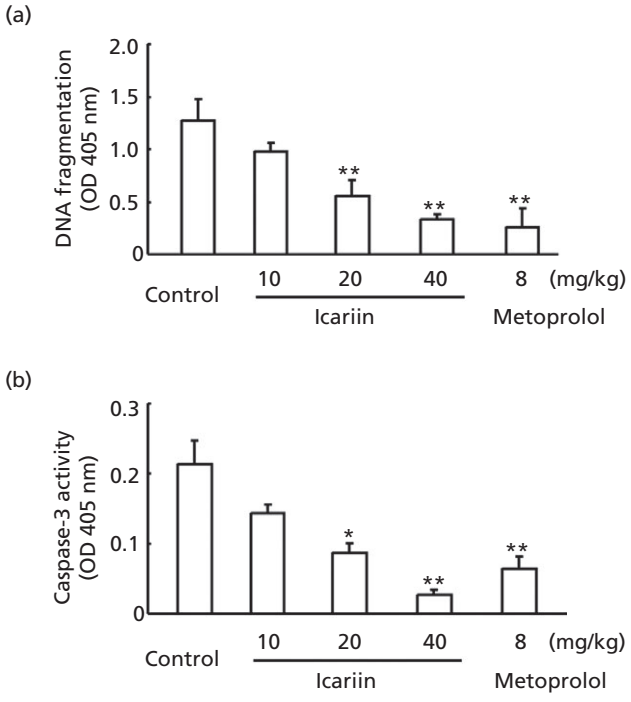

Figure 5 Effect of icariin on myocardial DNA fragmentation and caspase-3 activity of the left ventricle in rats with congestive heart failure caused by isoproterenol. The drugs were given intragastrically at the specified doses for 8 weeks, then the left ventricle was subjected to apoptosis assay. Apoptosis was evaluated by enzyme-linked immunosorbent assay (a) and caspase-3 activation (b). $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ vs control.

Conclusion

Icariin significantly improved left ventricular remodelling and function in rats with CHF induced by isoproterenol. The beneficial effect of icariin in animal models of CHF may be mediated at least in part by inhibition of elevated MMP-2 and MMP-9 activity as well as myocardial apoptosis and this effect contributes to the amelioration of CHF in clinical conditions.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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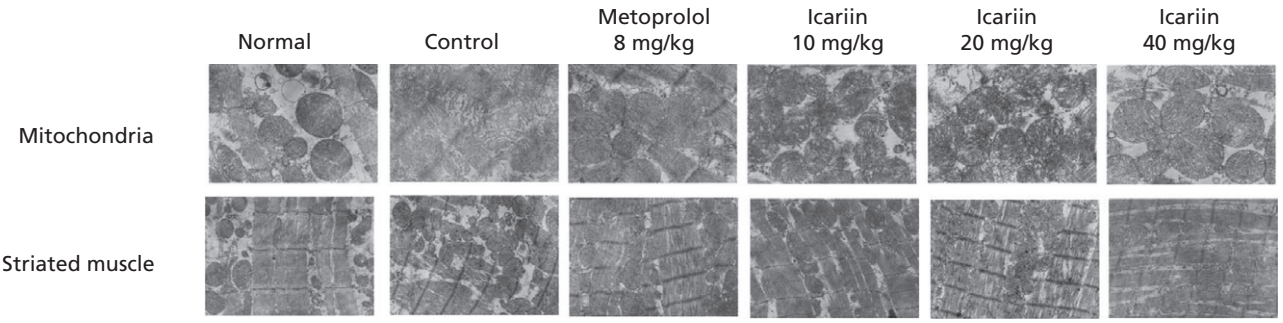

Figure 6 Effect of icariin on myocardial mitochondria and striated muscle of the left ventricle in rats with congestive heart failure caused by isoproterenol. The drugs were given intragastrically at the specified doses for 8 weeks, then the left ventricle was subjected to assay. The graph shown here is one of three different experiments.

References

1. Hartmann RW *et al*. Discovery of selective CYP11B2 (aldosterone synthase) inhibitors for the therapy of congestive heart failure and myocardial fibrosis. *Eur J Med Chem* 2003; 38: 363–366.
2. Brower GL *et al*. The relationship between myocardial extracellular matrix remodeling and ventricular function. *Eur J Cardio-Thoracic Surg* 2006; 30: 604–610.
3. Dhalla NS *et al*. Subcellular remodeling as a viable target for the treatment of congestive heart failure. *J Cardiovasc Pharmacol Ther* 2006; 11: 31–45.
4. Takai S *et al*. Inhibition of matrix metalloproteinase-9 activity by lisinopril after myocardial infarction in hamsters. *Eur J Pharmacol* 2007; 568: 231–233.
5. Morita H *et al*. Selective matrix metalloproteinase inhibition attenuates progression of left ventricular dysfunction and remodeling in dogs with chronic heart failure. *Am J Physiol Heart Circ Physiol* 2006; 290: H2522–H2527.
6. Batlle M *et al*. Down-regulation of matrix metalloproteinase-9 (MMP-9) expression in the myocardium of congestive heart failure patients. *Transplant Proc* 2007; 39: 2344–2346.
7. Bristow MR. Mechanism of action of beta-blocking agents in heart failure. *Am J Cardiol* 1997; 80: L26–L40.
8. Dixon IMC *et al*. Effect of ramipril and losartan on collagen expression in right and left heart after myocardial infarction. *Mol Cell Biochem* 1996; 165: 31–45.
9. Francis GS, Tang WHW. Early cardiac resynchronization therapy and reverse remodeling in patients with mild heart failure is it time? *Circulation* 2009; 120: 1845–1846.
10. Ye L-C, Chen J-M. Advances in study on pharmacological effects of epimedium. *Zhongguo Zhongyao Zazhi* 2001; 26: 293–295.
11. Huang X-L *et al*. Protective effect of epimedium flavonoids injection on experimental myocardial infarction rats. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 2006; 26: 68–71.
12. Pan Y *et al*. Icariin from Epimedium brevicornum attenuates chronic mild stress-induced behavioral and neuroendocrinological alterations in male Wistar rats. *Pharmacol Biochem Behav* 2007; 87: 130–140.
13. Xu HB, Huang ZQ. Icariin enhances endothelial nitric-oxide synthase expression on human endothelial cells in vitro. *Vascul Pharmacol* 2007; 47: 18–24.
14. He W *et al*. Immunoregulatory effects of the herba Epimediia glycoside icariin. *Arzneimittelforschung* 1995; 45: 910–913.
15. Yang JX *et al*. Anti-proliferative efficacy of icariin on HepG2 hepatoma and its possible mechanism of action. *Am J Chin Med* 2009; 37: 1153–1165.
16. Zhao J *et al*. Icariin induces osteogenic differentiation in vitro in a BMP- and Runx2-dependent manner. *Biochem Biophys Res Commun* 2008; 369: 444–448.
17. Ding L *et al*. Involvement of p38MAPK and reactive oxygen species in icariin-induced cardiomyocyte differentiation of murine embryonic stem cells in vitro. *Stem Cells Dev* 2008; 17: 751–760.
18. Teerlink JR *et al*. Progressive ventricular remodeling in response to diffuse isoproterenol-induced myocardial necrosis in rats. *Circ Res* 1994; 75: 105–113.
19. Torimura T *et al*. Laminin deposition to type IV collagen enhances haptotaxis, chemokinesis, and adhesion of hepatoma cells through beta 1-integrins. *J Hepatol* 2001; 35: 245–253.
20. Calvino-Fernandez M *et al*. Oxidative stress by Helicobacter pylori causes apoptosis through mitochondrial pathway in gastric epithelial cells. *Apoptosis* 2008; 13: 1267–1280.
21. Appierto V *et al*. PLAB induction in fenretinide-induced apoptosis of ovarian cancer cells occurs via a ROS-dependent mechanism involving ER stress and JNK activation. *Carcinogenesis* 2009; 30: 824–831.
22. Chauhan D *et al*. Superoxide-dependent and -independent mitochondrial signaling during apoptosis in multiple myeloma cells. *Oncogene* 2003; 22: 6296–6300.
23. Gazoti Debessa CR *et al*. Age related changes of the collagen network of the human heart. *Mech Ageing Dev* 2001; 122: 1049–1058.
24. Beltrami CA *et al*. Structural basis of end-stage failure in ischemic cardiomyopathy in humans. *Circulation* 1994; 89: 151–163.
25. Muller-Ehmsen J, Schwinger RHG. TNF and congestive heart failure: Therapeutic possibilities. *Expert Opin Ther Targets* 2004; 8: 203–209.
26. Swedberg K *et al*. Effects of sustained-release moxonidine, an imidazoline agonist on plasma norepinephrine in patients with chronic heart failure. *Circulation* 2002; 105: 1797–1803.
27. Lage SG *et al*. Angiotensin II contributes to arterial compliance in congestive heart failure. *Am J Physiol Heart Circ Physiol* 2002; 283: H1424–H1429.
28. Adams KF Jr *et al*. Biomarker guided therapy for heart failure: focus on natriuretic peptides. *Heart Fail Rev* 2009; 3: 1382–4147.
29. Rohde LE *et al*. Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. *Circulation* 1999; 99: 3063–3070.
30. Iwanaga Y *et al*. Excessive activation of matrix metalloproteinases coincides with left ventricular remodeling during transition from hypertrophy to heart failure in hypertensive rats. *J Am Coll Cardiol* 2002; 39: 1384–1391.
31. Sakata Y *et al*. Activation of matrix metalloproteinases precedes left ventricular remodeling in hypertensive heart failure rats its inhibition as a primary effect of angiotensin-converting enzyme inhibitor. *Circulation* 2004; 109: 2143–2149.
32. Peterson JT *et al*. Matrix metalloproteinase inhibition attenuates left ventricular remodeling and dysfunction in a rat model of progressive heart failure. *Circulation* 2001; 103: 2303–2309.
33. Spinale FG *et al*. A matrix metalloproteinase induction/activation system exists in the human left ventricular myocardium and is upregulated in heart failure. *Circulation* 2000; 102: 1944–1949.
34. Chancey AL *et al*. Effects of matrix metalloproteinase inhibition on ventricular remodeling due to volume overload. *Circulation* 2002; 105: 1983–1988.
35. Seeland U *et al*. Effect of ramipril and furosemide treatment on interstitial remodeling in post-infarction heart failure rat hearts. *J Mol Cell Cardiol* 2002; 34: 151–163.
36. Crow MT *et al*. The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res* 2004; 95: 957–970.
37. Kim DS *et al*. The involvement of endoplasmic reticulum stress in flavonoid-induced protection on cardiac cell death caused by ischaemia/reperfusion. *J Pharm Pharmacol* 2010; 62: 197–204.
38. Gustafsson AB, Gottlieb RA. Bcl-2 family members and apoptosis, taken to heart. *Am J Physiol Cell Physiol* 2007; 292: C45–C51.
39. Condorelli G *et al*. Increased cardiomyocyte apoptosis and changes in proapoptotic and antiapoptotic genes bax and bcl-2 during left ventricular adaptations to chronic pressure overload in the rat. *Circulation* 1999; 99: 3071–3078.
40. Thornberry NA, Lazebnik Y. Caspases: Enemies within. *Science* 1998; 281: 1312–1316.

41. Xu J *et al*. Synergism of simvastatin with losartan prevents angiotensin II-induced cardiomyocyte apoptosis in vitro. *J Pharm Pharmacol* 2009; 61: 503–510.
42. Chandrashekhar Y *et al*. Long-term caspase inhibition ameliorates apoptosis, reduces myocardial troponin-I cleavage, protects left ventricular function, and attenuates remodeling in rats with myocardial infarction. *J Am Coll Cardiol* 2004; 43: 295–301.
43. Mohanty IR *et al*. Bacopa monniera protects rat heart against ischaemia-reperfusion injury: role of key apoptotic regulatory proteins and enzymes. *J Pharm Pharmacol* 2010; 62: 1175–1184.
44. Song YH *et al*. Ethanol extract from Epimedium brevicornum attenuates left ventricular dysfunction and cardiac remodeling through down-regulating matrix metalloproteinase-2 and -9 activity and myocardial apoptosis in rats with congestive heart failure. *Int J Mol Med* 2008; 21: 117–124.