

Original Research Article

Icariin protects myocardial cells in spontaneously hypertensive rats by inhibiting mitochondrial and endoplasmic reticulum stress-related pathways

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Abstract

Purpose: To investigate the protective effect of icariin (ICA) on myocardial cells in spontaneously hypertensive rats (SHRs), and the mechanism involved.

Methods: Twenty-four SPF-grade, 12-week-old SHRs were randomly assigned to model group and ICA group, with 12 rats/group. There were 12 Wistar-Kyoto (WKY) rats (aged 12 weeks) in the control group. Rats in ICA group were given ICA suspension (40 mg/kg) through gavage, daily for 3 months, while model rats were given equivalent volume of double distilled water (in place of ICA suspension) via gavage for 12 weeks. Blood pressure, cardiac function, left ventricular (LV) mass index, myocardial morphology and apoptosis-related protein levels were determined and compared among the four groups.

Results: The myocardial cells were hypertrophic and disorderly arranged, with widened intercellular spaces. Besides, there were increases in protein expression levels of p53, caspase 3, Bok, Bax, GRP78, p-PERK, ATF-4, CHOP and DR5, while Bcl-2 protein was down-regulated. In contrast, the levels of these indicators in the ICA group were significantly better than those in the model group ($p < 0.05$).

Conclusion: ICA reduces blood pressure in rats, but improves cardiac function and cardiomyocyte morphology by decreasing apoptosis in cardiomyocytes through down-regulation of mitochondrial and endoplasmic reticulum (ER) stress-related apoptosis pathways. Thus, icariin may be suitable for the treatment of hypertension; however, clinical trials need to be undertaken first.

Keywords: Icariin, Hypertension, Apoptosis, Mitochondria, Endoplasmic reticulum stress, PERK, Bcl-2

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INTRODUCTION

Hypertension is one of the main factors that predispose to heart failure, the incidence of which has been on the increase in recent years,

accounting for about 40 % of deaths associated with cardiovascular and cerebrovascular diseases [1]. Studies have shown that the incidence of hypertension in China in the age range of 35-74 years was about 27 % in 2000,

and it was projected that the incidence will reach about 40 % by 2024 [2]. Ventricular remodeling is one of the frequent complications seen in hypertensive patients. At the early stage, there is myocardial thickening which is an adaptation used to maintain normal functions. At the later stage, increased myocardial hardness and decreased compliance occur, leading to other dysfunctions such as arrhythmia and heart failure. Therefore, it is crucial to prevent and treat ventricular remodeling in patients with hypertension [3,4]. Cardiomyocyte apoptosis is the cytological basis of ventricular remodeling in hypertensive patients. Studies have shown that cardiomyocyte apoptosis pathway mediated by mitochondrial regulation and ER stress plays an important role in ventricular remodeling [5,6].

Pharmacological studies have confirmed that ICA exerts anti-inflammatory, immuno-regulatory and cardiovascular and cerebrovascular functions [7]. It regulates hypotension and inflammation during ventricular remodeling. However, not much is known on the effect of ICA on myocardial cell apoptosis, and the associated mechanism. Therefore, this research was aimed at investigating the influence of ICA on myocardial cell apoptosis in a hypertensive rat model, and the underlying mechanism. This was with a view to laying an experimental foundation for preventing ventricular remodeling in patients with hypertension in clinical practice.

EXPERIMENTAL

Animals

A total of 24 male SPF and SHR rats aged 12 weeks, and 12 SPF Wistar-Kyoto Rats (WKY) also aged 12 weeks, served as experimental animals. They were obtained from Beijing Weitong Lihua Lab. Animal Technol. Co. Ltd.

Ethical approval

This animal study received approval from the Ethics Authority of Jingmen Traditional Chinese Medical Hospital (approval no. 20220319), and was conducted according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [8].

Main instruments and reagents

The instruments and drugs/reagents used, and their suppliers (in parenthesis) were: non-invasive rat tail sphygmomanometer (Kent Scientific CODA, Kent Scientific, USA); centrifuge (Eppendorf 5417R, Eppendorf, Germany); fluorescence quantitative PCR (CFX

Connect, Bio-rad, USA); electrophoresis apparatus (Mini-Protean3, Bio-RAD, USA); (Vevo2100, Visual Sonics, Canada), and -80 °C refrigerator (Qingdao Haier, China). The others were ICA (Nanjing Zelang Pharmaceutical, batch no. FY17420615, China); RIPA lysing buffer (Beijing Solebo, China); PVDF membrane (Millipore, USA), and BCA protein concentration determination kit (Jiangsu Biyuntian, China).

Preparation of solutions

(i) *ICA suspension*: ICA powder was weighed in an analytical balance, dissolved in appropriate amount of double-distilled water (ddH₂O) and shaken thoroughly before each trial.

(ii) *2 % pentobarbital injection*: 2.0 g of pentobarbital was weighed in an electronic balance, dissolved in ddH₂O and made up to a volume of 100 mL.

Animal grouping and treatments

All rats were acclimatized to the laboratory environment for 1 week. The 24 SHR rats were assigned to model and ICA groups, with 12 rats per group, while the 12 Wistar Kyoto rats served as blank group. Rats in ICA group were given ICA suspension (0.04 g/kg) through gavage, daily for 3 months. The other rat groups received equivalent volume of ddH₂O in place of ICA, *via* intragastric administration for 12 weeks.

Assessment of parameters/indices

Blood pressure

At the end of treatment every week, rat tail sphygmomanometer was used to record the systolic blood pressure of the rats in a quiet and dark environment when the animals were calm.

Cardiac function

At the end of intervention, 2 % pentobarbital was intraperitoneally injected into each rat (dose = 0.2 mL/100 g). Under anesthesia, the dorsal hair of each rat was shaved off, and the rat was fixed on an ultrasound test table. The LV heart cycle of each rat was monitored and recorded by applying coupling agents to the thoracic cavity. The LV activity of rats was determined using Vevo2100 animal ultrasound system.

LV mass

Rat weights in each group were recorded. At the same time, after measurement of cardiac function, the thoracic cavity of each rat was

sliced open, and cardiac tissue was cut out, rinsed in cold 0.90 % NaCl solution, and the LV was separated and weighed. Then, left ventricular index LV) was calculated using Eq 1.

$$LVI (\%) = \frac{\text{Left ventricular weight}}{\text{Body weight}} \times 100 \dots\dots (1)$$

Myocardial morphology

The LV tissues were put in 4 % neutral HCHO fixative for 2 days, followed by paraffin-embedding, sectioning, H & E staining, and TUNEL staining. The myocardial sections were examined for morphological changes under a light microscope. Image Pro Plus software was applied for computing apoptosis (A) as indicated in Eq 2.

$$A (\%) = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells}} \times 100 \dots\dots (2)$$

At the same time, left ventricular tissue was cut into sections of 1 mm³ in size and placed in 2.5 % glutaraldehyde fixative solution. After fixation for 48 h, morphological changes in myocardium were examined under electron microscopy.

Myocardial protein expression levels of Bcl-2, p53, Caspase3, Bok, Bax, GRP78, PERK, ATF-4, CHOP and DR5

Total protein was extracted from 200 mg of frozen myocardial tissue from each rat, using 1 mL of RIPA buffer containing 10 µL of PMSF and protein phosphatase inhibitor. The homogenate which was prepared on ice, was left for 30 min, after which it was centrifuged at 12000 rpm at 4°C for 20 min. The concentration of protein in the lysate was determined with BCA method. Then, equal amounts of protein (40 µg/10µL) were subjected to SDS-polyacrylamide gel

electrophoresis, followed by transfer to PVDF membranes using Bio-Rad Mini trans-blot system. The membranes were blocked by incubation with 5 % protein blocking solution at room temperature for 2.5 h, followed by rinsing thrice in TBST, each for 10 min.

Then, the membranes were incubated overnight at 4°C with primary antibodies for Bcl-2 (1:2000), P53 (1:2000), Caspase3 (1:1000), Bok (1:2000), Bax (1:500), PERK (1:1000), GRP78 (1:500), P-perk (1:1000), ATF-4 (1:800), DR5 (1:1000) and CHOP (1:1000). Thereafter, the membranes were rinsed 3 times using TBST, followed by incubation with HRP-labeled rabbit anti-rat 2° immunoglobulin (1:2000) at laboratory temperature for 1.5h. The blots were subjected to ski luminant ECL. Bio-Rad CCD imaging system was used for imaging, while Quantity One software was used for grayscale analysis.

Statistical analysis

The SPSS version 20.0 software was used for data collation and statistical analysis. Measurement data are expressed as mean ± standard deviation (SD). Differences amongst groups were determined using ANOVA. Values of *p* < 0.05 indicate that the differences are statistically significant.

RESULTS

Blood pressure after intervention

The results showed that, compared with blank control group, there were marked increases in systolic blood pressure of rats in the model and ICA groups, with higher systolic blood pressure in model group than in ICA group (*p* < 0.05). These data are presented in Table 1.

Table 1: Comparison of blood pressure in each group after intervention (mean ± SD, mmHg, n = 12)

Group	Blank control	Model	ICA
Week 13	111.75±12.71	151.25±17.90*	141.09±13.40*#
Week 14	116.09±11.73	156.42±18.04*	143.64±13.10*#
Week 15	116.80±10.37	159.35±18.79*	144.80±13.52*#
Week 16	110.49±11.22	162.65±18.42*	146.71±13.81*#
Week 17	118.42±11.65	165.87±18.67*	147.98±14.04*#
Week 18	114.90±13.09	167.32±19.03*	150.16±14.72*#
Week 19	121.35±12.41	168.93±19.32*	152.06±14.90*#
Week 20	119.37±12.53	173.52±19.70*	154.75±15.30*#
Week 21	112.70±11.57	179.03±19.65*	157.20±15.69*#
Week 22	120.74±12.52	188.22±19.92*	159.72±16.17*#
Week 23	117.95±11.37	198.42±18.98*	160.34±16.82*#
Week 24	126.79±13.03	203.80±19.69*	162.67±17.26*#

**P* < 0.05, vs blank control; #*p* < 0.05, vs model

Cardiac functions

Compared with rats in the blank control group, there were lower ultrasound results, and lower stroke volume and ejection fraction in rats in the model and the ICA groups. However, stroke volume and ejection fraction were significantly lower in the model group than the corresponding values in the ICA group ($p < 0.05$). These results are shown in Table 2.

Table 2: Values of stroke volume and ejection fraction in the 3 groups

Group	Stroke volume (μL)	Ejection fraction (%)
Blank control	272.65 \pm 29.42	61.12 \pm 9.58
Model	124.26 \pm 22.21*	37.72 \pm 6.85*
ICA	222.09 \pm 26.40*#	50.15 \pm 7.51*#

Data are mean \pm SD (n = 12). * $P < 0.05$, vs blank control; # $p < 0.05$, vs model

Left ventricular (LV) mass index

There were significant increases in LV mass index of rats in model and ICA groups, when compared to corresponding values in rats in the blank control group ($p < 0.05$). However, LV mass index was markedly higher in model rats than in ICA rats ($p < 0.05$; Table 3).

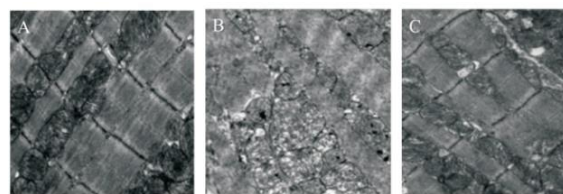
Table 3: Left ventricular mass index values after intervention

Group	Left ventricular mass index
Blank control	0.232 \pm 0.009
Model	0.314 \pm 0.010*
ICA	0.263 \pm 0.009*#

* $P < 0.05$, vs blank control; # $p < 0.05$, vs model

Ultrastructural changes in myocardial cells

Transmission electron microscopy showed that the z-line and transverse lines of myocardial cells in blank control group were neatly arranged, and the mitochondrial membrane structure was intact, with no obvious mitochondrial lesions. In contrast, myocardial cells in the model group were damaged. The visual fields revealed mitochondrial swelling, proliferation, mitochondrial crest disappearance, blurred z-line and transverse stripes, and irregular arrangement of myocardial cells. The degree of damage to myocardial cells in ICA group was less than that in model group. Thus, ICA mitigated mitochondrial damage.



Protein expression levels of Bcl-2, p53, Caspase3, Bok, Bax, GRP78, P-perk, ATF-4, CHOP and DR5 in the various groups

The protein levels of p53, Caspase3, Bok and Bax in model group and ICA group were up-regulated, relative to blank control rats, but their expression levels in model rats were up-regulated, relative to ICA rat values. However, protein expressions of Bcl-2 in model and ICA groups were down-regulated, relative to blank control rats, and the Bcl-2 level in model rats was lower than that in ICA rats ($p < 0.05$; Table 4).

Table 4: Gray values of target proteins and GAPDH

Group	Blank control	Model	ICA
n	12	12	12
P53	1.35 \pm 0.12	2.37 \pm 0.19*	1.69 \pm 0.11*#
Bcl-2	1.41 \pm 0.08	0.79 \pm 1.68*	1.12 \pm 0.09*#
Bax	1.13 \pm 0.06	1.78 \pm 0.07*	1.42 \pm 0.07*#
Bok	1.10 \pm 0.12	1.67 \pm 0.16*	1.39 \pm 0.10*#
Caspase3	0.56 \pm 0.04	1.04 \pm 0.20*	0.78 \pm 0.70*#
GRP78	0.26 \pm 0.05	0.47 \pm 0.07*	0.19 \pm 0.07*#
p-PERK	0.67 \pm 0.09	0.96 \pm 0.12*	0.56 \pm 0.09*#
ATF-4	0.47 \pm 0.05	0.67 \pm 0.08*	0.39 \pm 0.05*#
CHOP	0.41 \pm 0.07	0.72 \pm 0.07*	0.47 \pm 0.05*#
DR5	0.69 \pm 0.12	0.91 \pm 0.12*	0.62 \pm 0.09*#

* $P < 0.05$, vs blank control; # $p < 0.05$, vs model

DISCUSSION

Cardiovascular disease is one of the deadliest ailments in China, and it poses a serious threat to human health. High blood pressure is amongst the frequently diagnosed cardiovascular illnesses in clinical practice, and it is closely related to pathogenesis of many cardiovascular and cerebrovascular diseases such as stroke, myocardial infarction and heart failure [9]. Studies have shown that the incidence of hypertension in China is about 30 %, and only about 27 % of patients have normalized blood pressure after treatment, indicating that the diagnosis and treatment of hypertension in China do not result in optimal outcomes [10]. In patients with hypertension, the heart undergoes adaptive functional and structural changes to meet the challenges of work overload. At the initial stage, left ventricular hypertrophy and diastolic function are the main manifestations, which ultimately result in decreased myocardial contractility, and eventually to heart failure [11,12].

Spontaneously hypertensive rats (SHRs) are a breed of rats with stable expression of inherited hypertension which is manifested as increased heart weight at 4 weeks of age, with cardiovascular complications gradually appearing in subsequent weeks of age. The degree of hypertension increases with maturity of the rats, and it reaches peak blood pressure at about 24 weeks. Thus, SHR is an ideal animal model for carrying out research on hypertension-induced heart disease [13,14].

Epimedium, a plant genus in the berberis family, improves cardiovascular and cerebrovascular functions. Its main bioactive component is the flavonoid ICA. Studies have shown that ICA exerts pharmacological effects such as alleviation of vascular endothelial injury, down-regulation of oxidative stress response level, improvement of nervous system function, and induction of mesenchymal stem cell differentiation [15]. It has also been reported that ICA alleviated left ventricular remodeling and mitigated myocardial fibrosis through various pathways [16]. Based on these findings, this study investigated the effect of ICA on hypertension-induced heart disease, as well as its method of action, so as to evolve a scientific basis for therapy of hypertensive heart disease.

The results of this study showed that the blood pressure of rats in ICA group was significantly lower than that in model group, suggesting that ICA effectively reduced the blood pressure of SHRs. Ultrasonic instrument was used to determine the effect of elevated cardiac load on left ventricular structure of rats. The results showed higher degree of left ventricular wall thickening, lower ventricular stroke volume and lower ejection fraction in model rats, while these parameters were improved in rats in the ICA group. Moreover, the left ventricular mass index (LVMI) was lower in ICA group than in the model group. Rat myocardial cells in the ICA group were arranged more orderly, and apoptosis ratio was lower, relative to model group. Therefore, ICA inhibited myocardial cell apoptosis, mitigated pathological lesions in myocardial cells, and reduced hypertension-induced myocardial hypertrophy, thereby improving cardiac function.

Mitochondria are organelles with double-membrane structure. They are centers that generate energy from aerobic metabolism. Mitochondria are also regarded as the energy factory of cells. The structural integrity of the mitochondrion reflects its functional status [17]. Transmission electron microscopy (TEM) showed that the model group had mitochondrial swelling and disappearance of mitochondrial

crest which were accompanied by disordered z-line and transverse striae, while the degree of mitochondrial damage in ICA rats was significantly decreased, relative to the model rats, suggesting that ICA exerted a protective effect on the mitochondria. Results from TUNEL staining showed reduction in the apoptosis of cardiomyocytes in ICA rats, suggesting that ICA might play an anti-apoptotic role through mitochondrion-related apoptosis pathway. The Bcl-2, p53, Bok and Bax proteins are all attached to the mitochondrial membrane, and they are closely related to apoptosis [18].

The P53 protein is generated by the tumor suppressor gene p53 which is involved in regulation of mitochondrial autophagy and apoptosis. When p53 protein is activated by apoptotic signal, Bax and Bok proteins are activated by up-regulation of apoptotic modulators, while the expression of Bcl-2 protein is inhibited [19]. Mitochondrial membrane permeability is positively correlated with protein levels of Bax and Bok, and their functions are inhibited by Bcl-2. When Bcl-2 is down-regulated by p53, the protein levels of Bax and Bok are increased, resulting in increased permeability of mitochondrial membrane. This results in release of cytochrome C which enters the cytoplasm via the PT channels, and it activates Caspase-3 in cytoplasm. Apoptosis is induced by separation of ZINC finger structure of DNA, up-regulation of endonuclease activity, and cleavage of nucleosome DNA.

The protein expressions of p53, Bok, Bax and Caspase3 were up-regulated in the model group, while the protein levels of Bcl-2 were down-regulated, suggesting increased level of apoptosis in the hypertensive rats. In contrast, there were lower proteins of p53, Bok, Bax and Caspase3 in ICA rats than in model rats, while the protein level of Bcl-2 was higher than that in the model group. These results suggest that ICA mitigated hypertension-induced myocardial cell apoptosis through regulation of the mitochondria-related apoptosis pathway.

It is known that ER is an intracellular organelle involved in several vital processes such as polypeptide folding, transformation of synthesized proteins, removal of misfolded proteins, as well as lipid biosynthesis. It plays an important role in maintaining cardiac myocyte ion homeostasis and myocardial contraction. Studies have shown that ischemia and hypoxia, oxidative stress, viral infection, and ion disorders result in accumulation of misfolded proteins and unfolded proteins in ER, leading to ER stress [20]. Under normal conditions, appropriate levels of ER

stress ameliorate the pathological damage caused by protein accumulation by up-regulating degradation responses, thereby protecting cells. However, excessive ER stress easily leads to dysregulation of cardiac autophagy and apoptosis, thereby affecting cardiac function and structure [21].

The PERK pathway is one of the ER signaling pathways that regulate apoptosis [22]. Physiologically, PERK is attached to the ER, and it is inactivated in this state. However, when ER stress occurs, PERK dissociates from GRP78 and becomes activated. When PERK is phosphorylated, it activates ATF-4, which in turn, up-regulates CHOP. In turn, CHOP induces apoptosis by activating its receptor DR5 protein. The data obtained in the present research suggest that the ER apoptosis route was activated in hypertensive rats. Moreover, the results suggest that ICA may down-regulate apoptosis by regulating the PERK ER stress pathway, thereby protecting the cells from apoptosis.

CONCLUSION

The findings of this study show that ICA reduced the level of apoptosis in cardiomyocytes in SHR and improved the morphology of cardiomyocytes by down-regulating mitochondrial and ER stress-related apoptotic pathways, thereby decreasing blood pressure and improving cardiac function. Thus, carin is a potentially suitable agent for the management of hypertension.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Siyu Guan designed the study, supervised the data collection, and analyzed the data. Wen Li interpreted the data and prepared the manuscript for publication. Luhong Li supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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