Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Geniposide from *Gardenia jasminoides* var. *radicans* Makino Attenuates Myocardial Injury in Spontaneously Hypertensive Rats via Regulating Apoptotic and Energy Metabolism Signalling Pathway

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Introduction: Hypertension is closely related to myocardial injury. Long-term hypertension can cause myocardial injury. Therefore, it is very important to find drugs to treat myocardial injury caused by hypertension. The aim of present study is to investigate the effects and mechanisms of geniposide on myocardial injuries in spontaneously hypertensive rats (SHR) and H9c2 cells induced by NaCl solution.

Materials and Methods: Male Wistar–Kyoto (WKY) and SHR rats were given different doses of geniposide (25 mg/kg/d or 50 mg/kg/d) or distilled water for three consecutive weeks. Meanwhile, an H9c2 cell line-injury model was established using a solution of 150 μmol/L NaCl for 8 h. The cardiac function and related indexes of rats were detected.

Results: The results showed that geniposide decreased the levels of COI and COIII, which promoted the phosphorylation of AMPK (p-AMPK) and enhanced the energy metabolism pathway. Geniposide improved myocardial apoptosis by regulating apoptotic proteins (p38, BAX and Bcl-2). Finally, heart function was regulated, and the markers of myocardial injury were decreased. Geniposide increased the viability of H9c2 cells treated with the NaCl solution and decreased the rate of apoptosis by regulating the levels of apoptotic proteins. Geniposide could activate energy metabolism signalling pathway (AMPK/SirT1/FOXO1) and reduce H9c2 cell apoptosis.

Conclusion: Our results showed that the mechanisms by which geniposide improves myocardial injury in SHR may be through regulating the energy metabolism signalling pathway (AMPK/SirT1/FOXO1) and improving myocardial apoptosis by regulating apoptotic proteins.

Keywords: geniposide, SHR, myocardial injury, apoptosis, energy metabolism

Introduction

Hypertension is one of the most common diseases in cardiology. Long-term hypertension increases cardiac pressure and volume load. Increased pressure load results in heightened stress of ventricular wall and the sarcomere during the contractile period, which results in myocardial hypertrophy.[1] Fibrosis occurs once there is an accumulation of collagen that exceeds 20%, which then leads to myocardial diastolic and systolic dysfunction and heart failure.[2] The pathological development

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of myocardial hypertrophy, fibrosis and heart failure leads to a gradual increase in injury to myocardial tissue. Therefore, it is important to identify drugs to treat hypertension-induced myocardial injury and to clarify their mechanisms.

Gardenia jasminoides Ellis (GJE) has obvious antihypertensive effect.[3] The fruit of the rubiaceae plant *Gardenia jasminoides* var. *radicans* Makino (GJRM) has been regarded as a forgery of the fruit of GJE. The main active components of GJRM are similar to those of GJE.[4] A systematic study of the chemical constituents of GJRM was previously carried out in our laboratory, which yielded a large amount of geniposide (Figure 1).[4] We studied the efficacy of the compound and accidentally found that it could improve the cardiac function of spontaneously hypertensive rats (SHR). Then, we explored whether geniposide can improve myocardial injury in SHR and the mechanism.

Materials and Methods

Animals

Wistar male rats (180–200 g), male SHR and Wistar-Kyoto(WKY) rats (8 weeks old) weighing approximately 190 g were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. under the animal license number: SCXK (Beijing) 2016–0011. The rats were given free access to rodent chow and drinking water in an SPF-grade animal facility and were housed at 23 ± 2 °C with a 12-h light/dark cycle. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Henan University of Chinese Medicine, and experiments were approved by the Animal Ethics Committee of Henan University of Chinese Medicine.

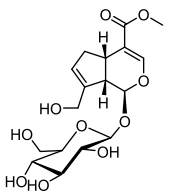

Figure 1 Chemical structure of geniposide.

Drugs

Bumetanide (BMTN, BM170801) was provided by the Guilin Pharmaceutical Co., Ltd. Bumetanide is a diuretic that has obvious effects on patients with heart failure with fluid retention.[5] At the same time, previous studies have found that geniposide also has a good diuretic effect.[6] Therefore, bumetanide was chosen as the pharmacodynamic control drug for geniposide. *Gardenia jasminoides* var. *radicans* Makino was collected from Tanghe County, Nanyang City, Henan Province, China. Geniposide (CAS:24512-63-8) was provided by Sigma-Aldrich at a purity of 98%. Compound C (HY-13418A) was purchased from the MCE Company.

Preparation of GJRM

One kilogram of GJRM was extracted twice with 95% ethanol for 1 h each and filtered through four layers of gauze. The filtrate was concentrated and freeze-dried to obtain a 95% total ethanol extract of GJRM. The extraction rate was approximately 27.5%. The 95% ethanol extract of GJRM was tested by high-performance liquid chromatography (Figure 2). The instrument is a Waters 2695 separation module. The column is HS C18. The mobile phase used water (solvent A) and methanol (solvent B). The conditions are 0–15 minutes (10% B–30% B), 15–25 minutes (30% B–40% B), and 25–30 minutes (40% B–60% B), the flow rate was 0.8 mL/min.[6]

Animal Grouping and Administration

The 180–200 g male WKY and SHR SPF-grade rats were divided into 6 groups according to their body weight: WKY group (WKY, n = 6), SHR group (SHR, n = 6), SHR and bumetanide group (BMTN, 5 mg/kg/d, n = 6), SHR and 95% total ethanol extract of GJRM group (GJRM, 360 mg/kg/d, n = 6), SHR and geniposide low-dose group (Geni-L: 25 mg/kg/d, n=8), and high-dose group (Geni-H: 50 mg/kg/d, n = 6). The rats in each group were given 10 mL/kg of medicine by ig once a day for 3 weeks. The WKY group and the SHR group were given the same dose of distilled water. After administration, heart function was measured. A solution of 10% chloral hydrate (0.3 mL/100 g body weight) was injected intraperitoneally. Blood samples were immediately taken from the abdominal aorta. The rat heart was removed, weighed, and frozen in liquid nitrogen prior to being stored at −80 °C. The heart was stained by HE staining and Image J software was used to analyze the average area of cardiomyocytes.

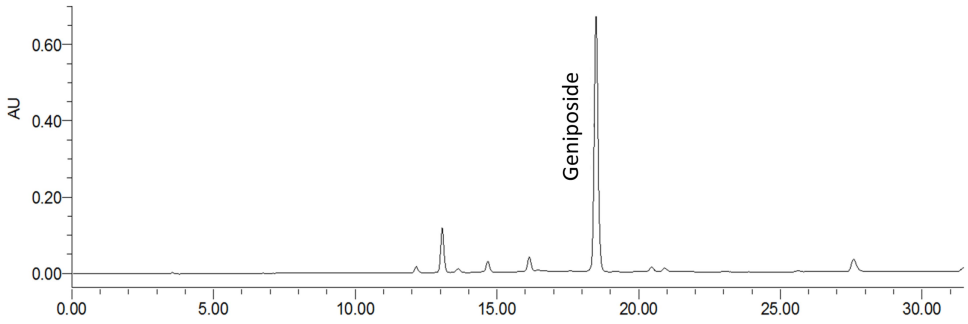

Figure 2 HPLC profiles of 95% ethanol extract of GJRM.

Detection of Cardiac Function by Echocardiography

The left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were measured by an ultrasonic cardiograph (Sonosite, USA). The rats were anaesthetized. The hair of rat's anterior heart region was removed, and the chest region was coated with a layer of coupling agent prior to placing the ultrasonic probe in the precordial region. An M-mode echocardiography that was guided by a parasternal left ventricular short-axis two-dimensional echocardiography was used to measure the middle section of the left ventricle three times for each group. The mean values were then analysed.

Enzyme-Linked Immunosorbent Assay

The levels of α-SMA (E-EL-R2583c, Elabscience, China) and H-FABP (E-EL-R0871c, Elabscience, China) in the serum of rats were detected the double-antibody sandwich for ELISA method. Either the sample or the standard was added and bound to the corresponding antibody on the carrier. The biotinylated antibody was then added and bound specifically to the antigen, which was tested on the carrier. Horseradish peroxidase-labelled avidin and biotin were then combined to form a specific immune complex that developed the colour. The OD values were detected.

Immunofluorescence Assay

After soaking the rat heart in formalin for two days, the heart was sectioned and placed in a constant-temperature incubator at 70 °C. We then dewaxed and rehydrated the tissue and repaired the antigen. After the sheep serum was added, the antibody of p-AMPK was incubated overnight at 4 °C. The secondary antibody was then added and incubated for 30 min and preserved at 4 °C in a dark container. The immunostaining was then visualized and recorded on a laser-scanning confocal microscope. Three representative regions were selected for each slice. Image-pro Plus 6.0 software was used to analyse each slice. The cumulative optical density (IOD) and pixel (AREA) of the tissue were obtained. The average optical density values (AO value) were then calculated.

Western Blot

PMSF and lysate (1:1000, P00136, Beyotime, China) were added to the hearts of each group of rats. The homogenate was centrifuged 5 min at 4 °C and 12,000 g. The supernatant was extracted, and the protein was quantified by the BCA method (CW00145, Solarbio, China). The protein was separated by polyacrylamide gel electrophoresis, and the gel containing the target protein was isolated. The PVDF membrane (Millipore, Billerica, MA, USA) was imprinted with the protein by the semi-dry method. The PVDF membrane was rinsed by PBS and then sealed with 5% skimmed-milk powder for 1 h. The antibody of p38 (1:1000, GR3181256-9, Abcam), p-p38 (1:1000, 4511S, CST), Bcl-2 (1:1000, ab32124, Abcam), BAX (1:1000, GR3180247-10, Abcam), p-AMPK (1:1000, GR208507-48, Abcam) and β-actin (1:1000, 10004156, Proteintech) were incubated overnight at 4 °C. The PVDF membrane was washed with PBST, then placed in the diluted fluorescent secondary antibody (1:20,000, C80911-11 and C80816-16, LI-COR) solution for 1 h, and washed with PBST. The ODYSSEY CLx double-infrared laser imaging system (Odyssey, USA) was used to analyse the protein bands at 680 nm and 800, and protein expression was analysed using the fluorescence intensity. The protein bands were analysed using Image Studio Ver 5.2 software.

Real-Time PCR (RT-qPCR)

The RNA was extracted with a total RNA extraction kit (R1200-100T). The extracted samples were detected by a limited-protein accounting analyser, and the RNA was reverse-transcribed into cDNA using the Hiscript II 1st Strand cDNA Synthesis Kit (R223-01, Vazyme, China). Finally, the QuantiNova^{TM} SYBR Green PCR Kit (711-02/03, QIAGEN, Germany) was used to measure the fluorescence via quantitative PCR (Applied Biosystems, USA). The cycle threshold (Ct) value of each sample was measured by RT-qPCR and the change in Ct values for each group was analysed. The primer sequences are shown in Table 1.

Cell Culture

The rat cardiomyocyte cell line H9c2 was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences.

H9c2 cells were cultured in DMEM (Gibco, USA) containing 10% foetal bovine serum (Zhejiang Tianhang Biotechnology, China), 100 U/mL penicillin and 100 g/mL streptomycin. The cells were cultured in a CO_2 constant-temperature incubator with a 5% volume fraction at 37 °C.

Cell Vitality

The H9c2 cells were distributed in a 96-well plate at a density of 5×10^4 cell/mL. After 12 h of culture in the incubator, the normal control group (NC) was given complete medium while the other groups were cultured in media containing the following additives: the model group (M) was treated with a NaCl solution of 150 μmol/L; the BMTN was treated with 5 μmol/L bumetanide; and the geniposide groups were treated geniposide with 0.1 μmol/L (Geni-0.1), 0.5 μmol/L (Geni-0.5) or 5 μmol/L (Geni-5). After 8 h of drug treatment, 20 μL/well of MTT was added, and the cells were cultured for 4 h. The culture medium was removed and DMSO was added for 10 min before measuring the colour in the enzyme marker range of 490 nm.

In-Cell Western Blot

The formaldehyde solution was added to immobilize the cells for 20 min. The solution of 0.1% Triton was added to permeabilize the cells by gently shaking for 5 min and repeating the process 5 times. After adding 5% skim milk, the samples were incubated at room temperature for 1.5 h. The samples were incubated with the antibody of p38 (1:100, GR3181256-9, Abcam), p-p38 (1:100, 4511S, CST), Bcl-2 (1:100, ab32124, Abcam), BAX (1:1000, GR3180247-10, Abcam), SirT1 (1:100, 1F3, CST), FOXO1 (1:100, C29H4, CST), AMPK (1:100, 10929-2-AP, Proteintech), p-AMPK (1:100, GR208507-48, Abcam) and GAPDH (1:100, E12-062, EnoGene) at 4 °C overnight. The samples were then washed with PBST and placed into the diluted fluorescent secondary antibody solution for 1 h before being washed once more with PBST. The ODYSSEY CLx double-infrared laser imaging system was used to analyse the protein. The protein expression was quantified using the fluorescent intensity. The proteins were analysed using Image Studio Ver 5.2 software.

Seahorse XF

A mixture of sodium pyruvate, L-glutamine and a 100 mL base medium (Agilent, China) was heated to 37 °C. After adjusting the pH to 7.45 with NaOH, the liquid was filtered through a 0.22 μm membrane. The Seahorse XF Calibrant was then added to the XFe24 Flux Assay Kit (103015-100, Agilent, China) and placed in a CO_2-free 37 °C incubator overnight.

The H9c2 cells were placed in an XF24 cell culture plate at a density of 1×10^5 cells/mL in a volume of 100 μL/well. After the cells had adhered to the well, the medium was supplemented with 150 μL/well and cultured overnight. The cells were then washed twice with XF detection solution. The detection solution was added to a final volume of 500 μL/well. The cells were placed in a 37 °C CO_2-free incubator for 1 h. The drug that is

Table 1 List of Primers and Their Sequences Used in This Study

Gene	Forward Primer	Reverse Primer
COI	CAGGCTGGTGTGATGGGATT	CACCTCGTTCTCCAGCCTTT
COIII	GTGAAACTGGTGAACGTGGC	GGGACCTGGATGTCCACTTG
ANP	CTGGGACCCCTCCGATAGAT	GTCAATCCTACCCCCGAAGC
BNP	TCCTTAATCTGTCGCCGCTG	GGCGCTGTCTTGAGACCTAA
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

Table 2 The Effects of Geniposide on Cardiac Functions in Rats ($\bar{x}$ ± s, n = 6)

Groups	LVEDD (cm)	LVESD (cm)	LVEF (%)	LVFS (%)	Heart Indices
WKY	0.42±0.07	0.21±0.01	88.45±2.38	63.26±3.83	0.0034±0.00021
SHR	0.53±0.04*	0.26±0.03*	80.76±4.29**	52.68±5.19	0.0040±0.00035**
BMTN	0.43±0.06$^{\#}$	0.22±0.01$^{\#}$	84.71±3.13	60.64±5.53	0.0039±0.00047
GJRM	0.43±0.04$^{\#}$	0.21±0.01$^{\#\#}$	84.98±2.45	69.64±7.21$^{\#}$	0.0039±0.00034
Geni-L	0.43±0.05$^{\#}$	0.21±0.01$^{\#}$	83.79±2.03	56.42±2.79	0.0037±0.00025$^{\#\#}$
Geni-H	0.43±0.01$^{\#}$	0.18±0.02$^{\#\#}$	84.57±4.52	68.55±9.15$^{\#}$	0.0036±0.00019$^{\#\#}$

Notes: The effects of geniposide on cardiac functions in rats. LVEDD and LVESD were measured by ultrasonic cardiograph. $\mathrm{LVEF} = \frac{100\times \mathrm{LVEDD}^2 - 85\times \mathrm{LVESD}^2}{\mathrm{LVEDD}^2}$. $\mathrm{LVFS} = \frac{(\mathrm{LVEDD}-\mathrm{LVESD})\times 100}{\mathrm{LVEDD}}$. The values are expressed as the means ± SD. **P<0.01, *P<0.05 compared with WKY; $^{\#\#}P$<0.01, $^{\#}P$<0.05 compared with SHR.
Abbreviations: LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; LVFS, left ventricular fraction shortening.

included in the mitochondrial pressure test box of the XF cells was then prepared. The hydrated test plate was removed and the standard protocol was used to add the treatment to the well. The XF controller was then configured manually and the plate was placed on the test board to be assayed (Agilent, China). The data were generated by the Seahorse XF instrument and were analysed by a report generator provided by the manufacturer.

Flow Cytometry

The cells were digested with EDTA-free trypsin. The cells were centrifuged and the supernatant was discarded. The cells were then resuspended by adding PBS, and 100 μL of the cell suspension was added to the flow tube. After adding fluorescein isothiocyanate (FITC) and propidium iodide (PI) dyes (8176893, BD Biosciences, USA) and the suspension was shielded from light for 15 min. A binding buffer was added to the suspension, which was transferred into the instrument to detect the cells by flow cytometry (FACSAria; BD Biosciences, USA).

Statistical Analysis

The data were analyzed by SPSS 18.0 software and compared with each other by one-way analysis of variance (one-way ANOVA). Experimental data are expressed as mean ± SD. P<0.05 indicates that the difference is significant, and P<0.01 indicates that the difference is extremely significant. Perform at least three parallel experiments in each trial.

Results

Effect of Geniposide on Cardiac Functions in Rats

The cardiac functions can directly reflect the state of the heart. The results of the echocardiography showed that compared with the WKY group, the levels of the LVEDD, LVESD, and the heart indices in the SHR group increased significantly (P<0.05 or P<0.01), while the levels of LVSD and LVFS decreased (P<0.05). Geniposide could decrease the levels of LVEDD, LVESD, and the heart indices (P<0.05 or P<0.01), improve the systolic function of the left ventricle and increase the level of LVEF and LVFS (P<0.05). This result suggested that geniposide could improve cardiac functions in hypertensive rats (Table 2).

Effect of Geniposide on Myocardial Injury Markers in Rats

Myocardial injury is accompanied by an increase in α-SMA and H-FABP.[7,8] At the same time, the synthesis and secretion of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are important indicators of myocardial injury.[9] The levels of α-SMA and H-FABP in the serum, ANP and BNP in heart of the SHR group were significantly higher than the WKY group (P<0.01). After the administration of geniposide, the levels of α-SMA, H-FABP, ANP and BNP decreased significantly (P<0.01). These results suggested that geniposide could improve cardiac function and reduce myocardial injury markers in the SHR rats (Figure 3A–D).

Effects of Geniposide on Cardiac Structure in Rats

HE staining was used to detect the pathological changes of cardiac structure in rats. The results showed that compared with WKY rats, cardiomyocytes in SHR rats had hypertrophy, blurred cell edge and deformation of cell structure. Geniposide ameliorated myocardial injury in SHR rats. These results showed that geniposide could improve myocardial hypertrophy in SHR rats (Figure 3E and F).

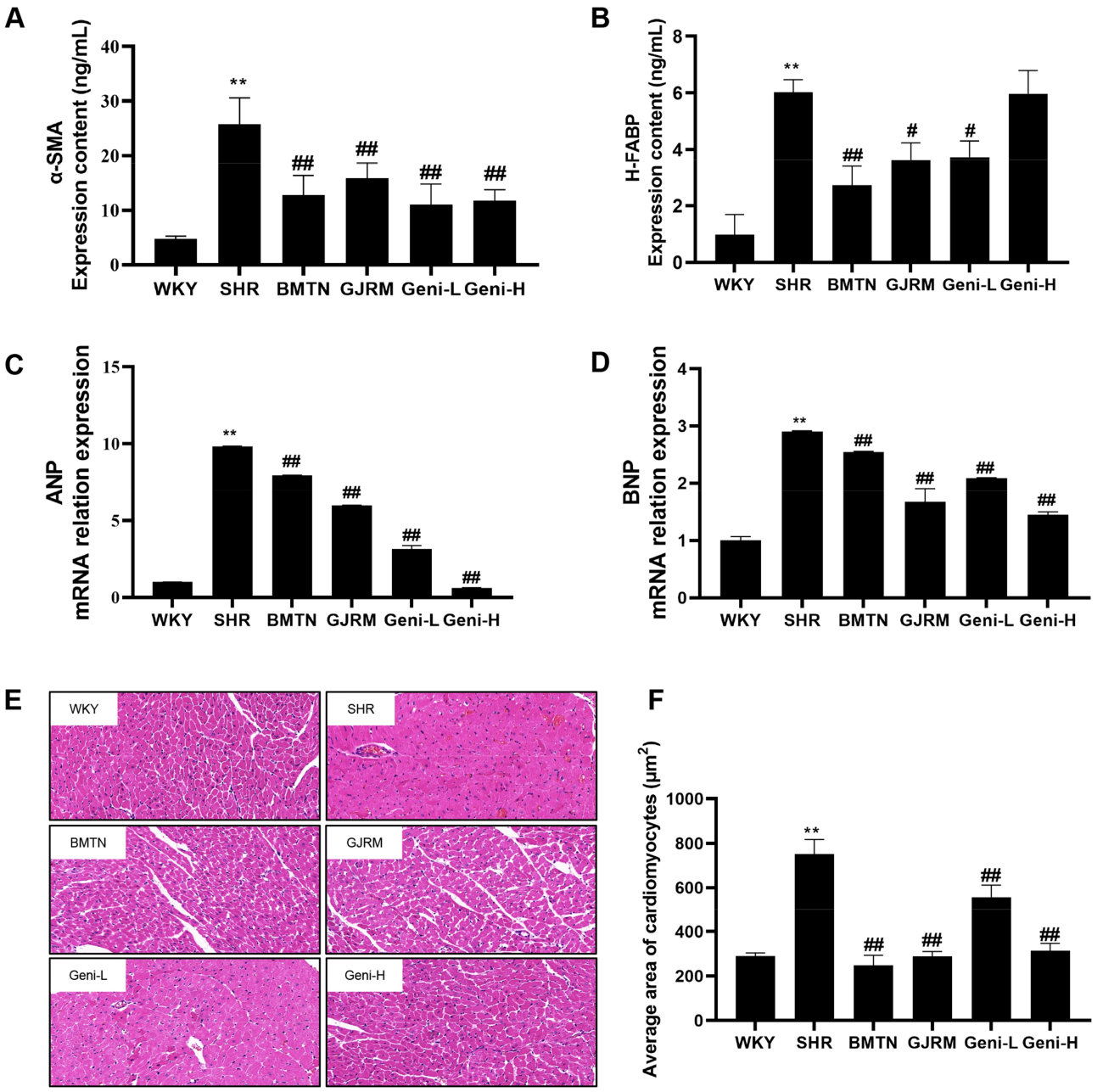

Figure 3 Effect of geniposide on myocardial injury markers and cardiac structure in rats. (**A**) α-SMA expression content in the serum. (**B**) H-FABP expression content in the serum. (**C**) mRNA levels of ANP in rat hearts. (**D**) mRNA levels of BNP in rat hearts. (**E**) Cardiac structure. (**F**) Average area of cardiomyocytes (μm^2). Values are expressed as the means ± SD (n = 6, $^{**}P<0.01$ compared with WKY; $^{\#\#}P<0.01$, $^{\#}P<0.05$ compared with SHR).

Abbreviations: α-SMA, α smooth muscle actin; H-FABP, heart fatty acid-binding protein; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; SHR, spontaneously hypertensive rats; BMTN, treated with bumetanide (5 mg/kg/d); GJRM, treated with *Gardenia jasminoides* var. *radicans* Makino (360 mg/kg/d); Geni-L, treated with geniposide (25 mg/kg/d); Geni-H, treated with geniposide (50 mg/kg/d).

Effects of Geniposide on Apoptosis and Energy Metabolism-Related Factors in Rat Heart

The above results showed that geniposide could improve the heart function of hypertensive rats. Maintaining normal physiological function of the heart requires a lot of energy. The body's energy metabolism is closely related to the heart. To explore the mechanisms of how geniposide affects hypertension-induced myocardial injury, we measured apoptosis and energy metabolism-related proteins. The expression of p-p38 and BAX proteins in the SHR group was significantly higher than that of the WKY group

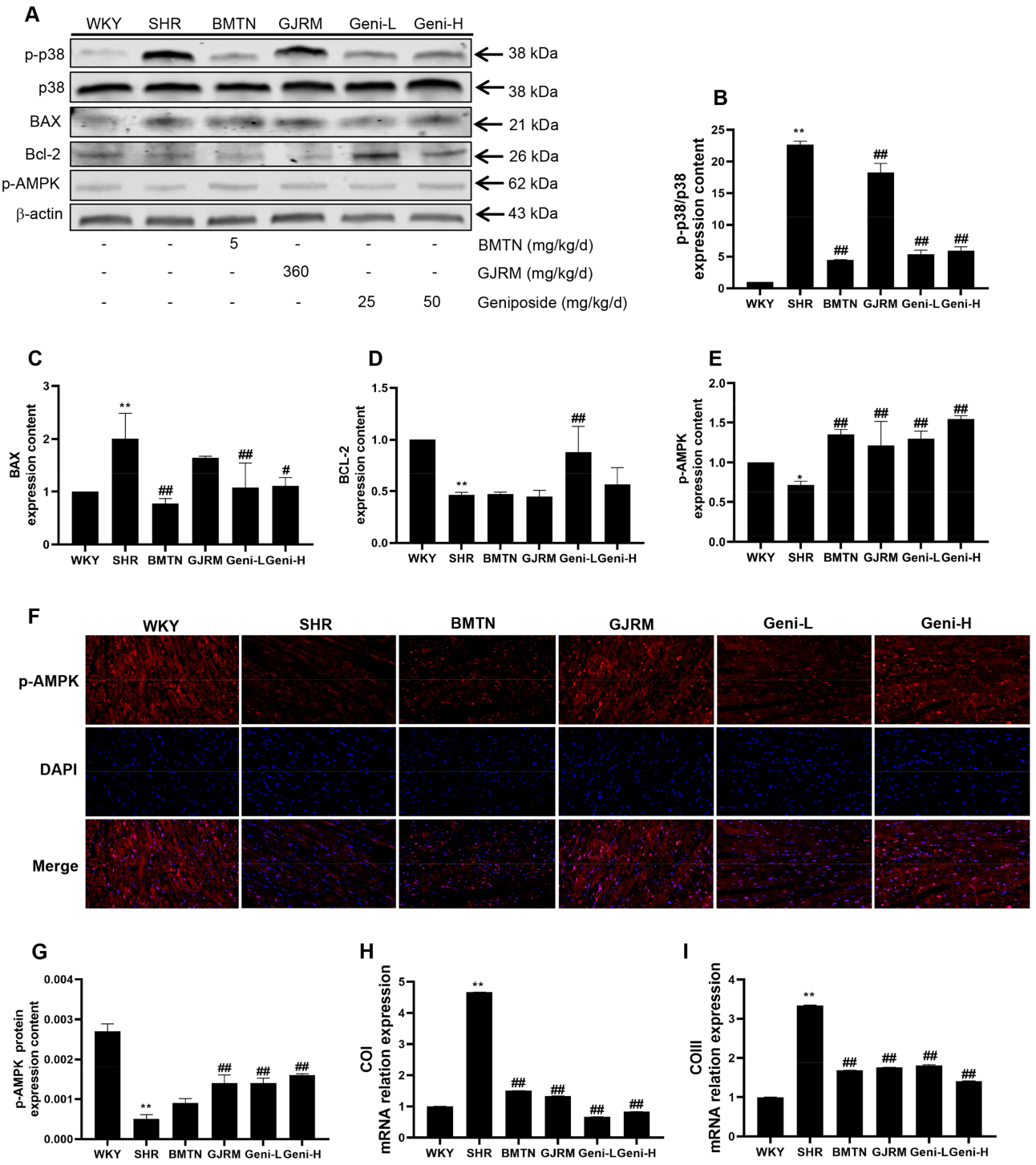

Figure 4 Effects of geniposide on apoptosis and energy metabolism-related proteins in rat heart. (**A**) The expressions of p-p38, p38, BAX, Bcl-2, and p-AMPK determined by Western blot. (**B**) Quantification of p-p38/p38 expression levels. (**C**) Quantification of BAX expression levels. (**D**) Quantification of Bcl-2 expression levels. (**E**) Quantification of p-AMPK expression levels. (**F**) The expressions of p-AMPK determined by immunofluorescence assays. (**G**) Quantification of p-AMPK expression levels. (**H**) mRNA levels of COI in rat heart. (**I**) mRNA levels of COIII in rat hearts. Values are expressed as the means ± SD (n = 3, $^{**}P<0.01$, $^{*}P<0.05$ compared with WKY; $^{\#\#}P<0.01$, $^{\#}P<0.05$ compared with SHR).

Abbreviations: p-p38, phosphorylation of p38; BAX, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma-2; p-AMPK, phosphorylation of adenosine 5′-monophosphate (AMP)-activated protein kinase; COI, cytochrome c oxidase subunit I; COIII, cytochrome c oxidase subunit III; SHR, spontaneously hypertensive rats; BMTN, treated with bumetanide (5 mg/kg/d); GJRM, treated with *Gardenia jasminoides* var. *radicans* Makino (360 mg/kg/d); Geni-L, treated with geniposide (25 mg/kg/d); Geni-H, treated with geniposide (50 mg/kg/d).

(P<0.01), and the expression of Bcl-2 and p-AMPK proteins was significantly lower than the WKY group (P<0.01). These results suggested that geniposide may reduce myocardial injury in SHR by reducing apoptosis and regulating the expression of energy metabolism-related proteins (Figure 4A–D).

Energy metabolism mainly occurs in the mitochondria.[10] The mRNA levels of COI and COIII in the rat hearts were determined by RT-qPCR. The results showed that the mRNA levels in the hearts of the SHR rats were significantly higher than those of the WKY rats (P<0.01). After the administration of geniposide, the mRNA levels were significantly decreased (P<0.01). These results suggested that geniposide could decrease the gene overexpression of COI and COIII, decrease the overproduction of ATP, and increase the ratio of AMP/ATP. Then, AMPK pathway was activated, which regulates energy metabolism (Figure 4E–I).

Detection of Cell Viability by MTT

To further explore the mechanism by which geniposide improves hypertension-induced myocardial injury, we used H9c2 cells to simulate the hyperosmotic environment of the heart under hypertension. When the concentration of extracellular Na^+ is higher than that in the cell, the osmotic pressure increases, which leads to the cardiac myocyte enlargement and gradual apoptosis.[11]

The results showed that cell viability in the model group was significantly lower than that in the normal group (P<0.01). After the cells were treated with geniposide at different concentrations, their viability increased (P<0.01), and the most effective concentration of geniposide was determined to be 0.5 μmol/L. These results suggested that geniposide could improve the viability of H9c2 cells that were stimulated with high levels of NaCl solution (Figure 5).

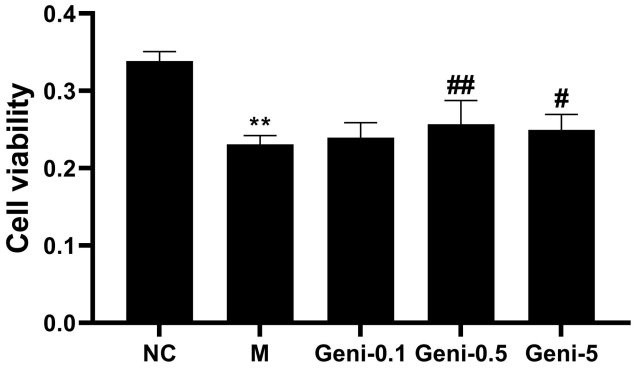

Figure 5 The effects of geniposide on cell viability. Values are expressed as the means ± SD. (n = 6, $^{**}P$<0.01 compared with NC; $^{\#\#}P$<0.01, $^{\#}P$<0.05 compared with M).

Abbreviations: NC, the normal control group; M, treated with NaCl (150 μmol/L); Geni-0.1, treated with geniposide (0.1 μmol/L); Geni-0.5, treated with geniposide (0.5 μmol/L); Geni-5, treated with geniposide (5 μmol/L).

Effects of Geniposide on Apoptosis and Energy Metabolism-Related Proteins in H9c2 Cells

The levels of apoptosis-related proteins (p-p38, p38, BAX and Bcl-2) and energy metabolism-related proteins (p-AMPK, AMPK, FOXO1, and SirT1) in the H9c2 cells were detected by in-cell Western blot. The results showed that the expression of the apoptosis-related proteins p-p38/p38 and BAX was upregulated while the expression of the Bcl-2 was downregulated in the model group (P<0.01). Meanwhile, the expression of the energy metabolism-related proteins p-AMPK/AMPK, FOXO1 and SirT1 was significantly decreased in the model group (P<0.01). Geniposide could reduce apoptosis in H9c2 cells by inhibiting the activation of the apoptosis-related proteins p38 and BAX, and upregulating the expression of the Bcl-2 protein (P<0.05 or P<0.01). At the same time, geniposide upregulated the expression of energy metabolism-related proteins, such as p-AMPK/AMPK, FOXO1 and SirT1 (Figure 6A–C).

Effects of Geniposide on ATP Production in H9c2 Cells

COI and COIII affect the activation of AMPK by regulating changes in ATP, so we measured the amount of ATP in the cells. The production of ATP in the mitochondria of H9c2 cells was detected by Seahorse XF. The results showed that ATP production in the model group increased significantly (P<0.05), and the ATP production decreased significantly after geniposide administration (P<0.05). These results suggested that geniposide could increase the ratio of AMP/ATP by reducing ATP production and then activating the energy metabolism signalling pathway (AMPK/SirT1/FOXO1) to reduce H9c2 cell injury (Figure 6D and E).

Effects of Inhibition of AMPK on Energy Metabolism Pathway and Apoptosis in H9c2 Cells

To investigate whether geniposide could regulate energy metabolism and apoptosis through AMPK pathway and

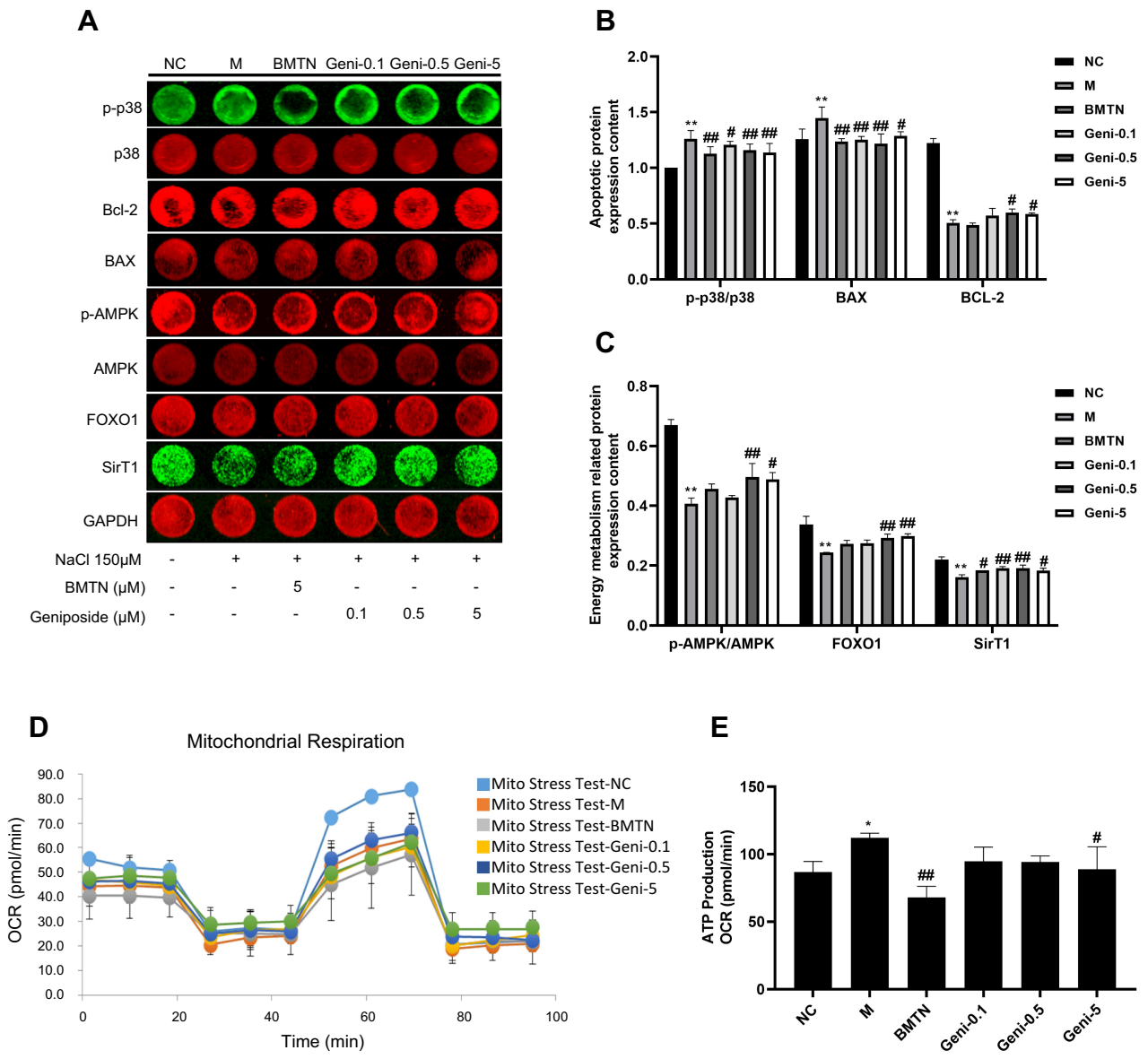

Figure 6 Effects of geniposide on apoptosis and energy metabolism-related proteins in H9c2 cells. (**A**) The expressions of p-p38, p38, BAX, Bcl-2, p-AMPK, AMPK, FOXO1 and SirT1 determined by In-cell Western blot. (**B**) Quantification of p-p38/p38, BAX and Bcl-2 expression levels. (**C**) Quantification of p-AMPK/AMPK, FOXO1 and SirT1 expression levels. (**D**) The OCR of mitochondrial respiration. (**E**) ATP production OCR. Values are expressed as the means ± SD (n = 3, $^{**}P<0.01$, $^{*}P<0.05$ compared with NC; $^{\#\#}P<0.01$, $^{\#}P<0.05$ compared with M).

Abbreviations: p-p38, phosphorylation of p38; BAX, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma-2; AMPK, adenosine 5′-monophosphate (AMP)-activated protein kinase; p-AMPK, phosphorylation of adenosine 5′-monophosphate (AMP)-activated protein kinase; FOXO1, forkhead box protein O1; SirT1, sirtuin 1; OCR, O_2 consumption rate; ATP, adenosine triphosphate; NC, the normal control group; M, treated with NaCl (150 μmol/L); BMTN, treated with bumetanide (5 μmol/L); Geni-0.1, treated with geniposide (0.1 μmol/L); Geni-0.5, treated with geniposide (0.5 μmol/L); Geni-5, treated with geniposide (5 μmol/L).

attenuate myocardial injuries in rats, we inhibited the AMPK protein with Compound C and measured levels of its downstream proteins and the degree of apoptosis. Compound C (2 μmol/L), which is an AMPK inhibitor, was added to the Geni-0.5 group and cultured for 8 h to detect changes in the AMPK/SirT1/FOXO1 signalling pathway and apoptosis. The results showed decreased expression of p-AMPK and its downstream proteins (SirT1 and FOXO1) in the model group ($P<0.05$ or $P<0.01$) and significant activation of the AMPK/SirT1/FOXO1 signalling pathway after geniposide administration ($P<0.05$ or $P<0.01$). After being treated with the AMPK inhibitor, the geniposide-activated energy metabolism protein pathway was eliminated ($P<0.05$ or

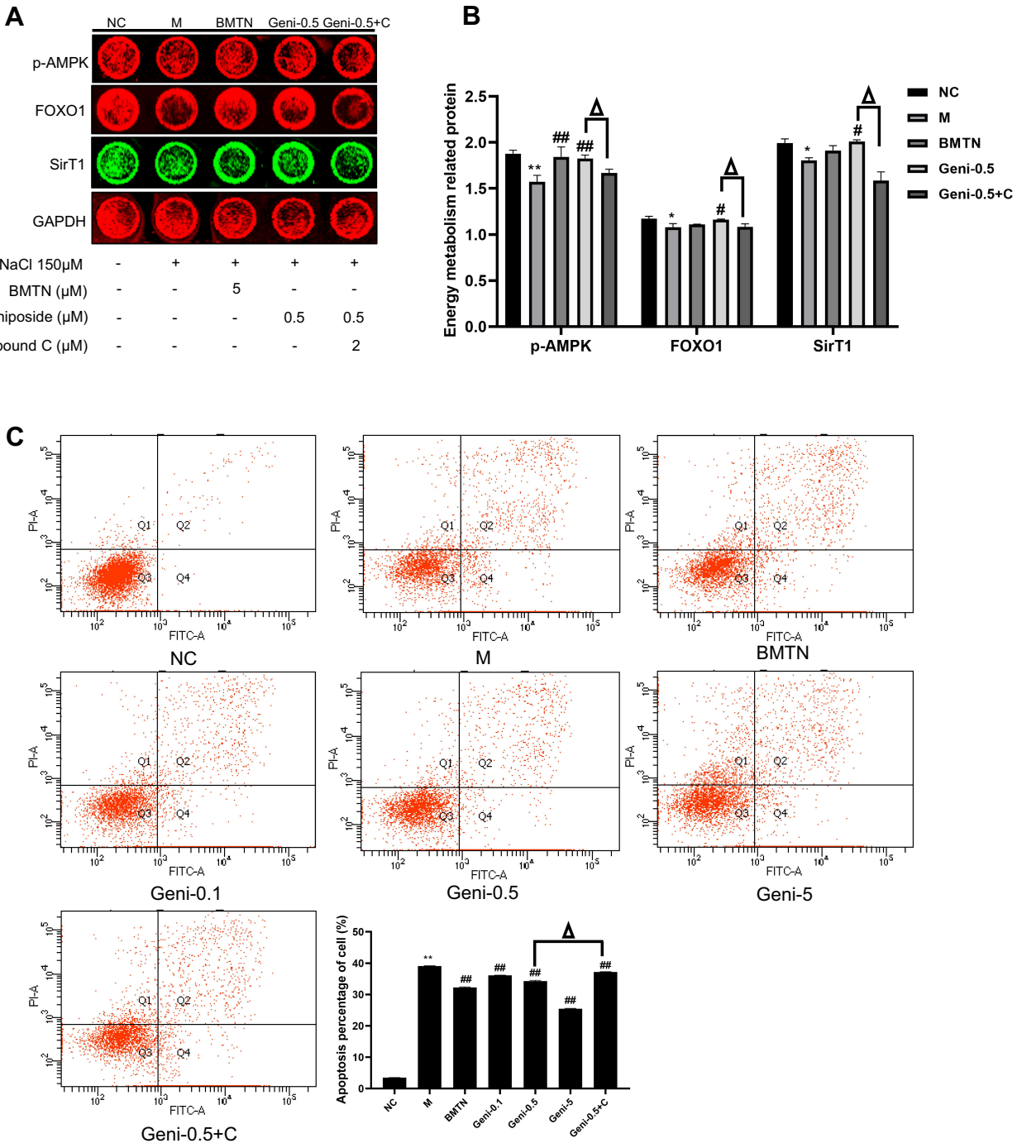

Figure 7 Effects of geniposide on the AMPK pathway and its downstream proteins in H9c2 cells. (**A**) The expressions of p-AMPK, FOXO1 and SirT1 determined by In-cell Western blot. (**B**) Quantification of p-AMPK, FOXO1 and SirT1 expression levels. (**C**) The apoptosis rate of H9c2 cells determined by flow cytometry. Values are expressed as the means ± SD (n = 3, $^{**}P<0.01$, $^{*}P<0.05$ compared with NC; $^{\#\#}P<0.01$, $^{\#}P<0.05$ compared with M; $^{\Delta}P<0.05$ represents the significant difference between the Geni-0.5 group and the Geni-0.5 + C group).

Abbreviations: p-AMPK, phosphorylation of adenosine 5′-monophosphate (AMP)-activated protein kinase; FOXO1, forkhead box protein O1; SirT1, sirtuin 1; NC, the normal control group; M, treated with NaCl (150 μmol/L); BMTN, treated with bumetanide (5 μmol/L); Geni-0.1, treated with geniposide (0.1 μmol/L); Geni-0.5, treated with geniposide (0.5 μmol/L); Geni-5, treated with geniposide (5 μmol/L); Geni-0.5+C, treated with geniposide (0.5 μmol/L) and Compound C (2μmol/L).

$P<0.01$). These results suggested that geniposide regulates the energy metabolism signalling pathway (AMPK/SirT1/FOXO1) in the H9c2 cells (Figure 7A and B).

Apoptosis was detected by flow cytometry. The results showed that the apoptosis rate of H9c2 cells significantly increased after stimulation with high levels of NaCl

solution for 8 h ($P<0.01$), and the apoptosis rate of H9c2 cells was significantly decreased after treatment with geniposide ($P<0.01$). Compound C, which is an AMPK inhibitor, diminished the protection provided by geniposide for the H9c2 cells. These results suggested that geniposide could reduce the rate of apoptosis in H9c2 cells by activating AMPK pathway and attenuate H9c2 injury (Figure 7C).

Discussion

The heart is one of the most frequently damaged organs in patients with hypertension and has been the subject of extensive research. When blood pressure increases, it is necessary to maintain sufficient contractility to keep the heart working and to sustain sufficient blood flow. LVEF, LVFS, LVEDD and LVESD are common indexes of heart function.[12] LVEDD and LVESD reflect the left ventricular end-diastolic and end-systolic heart state.[13] Impaired heart function can increase the myocardial load and lead to a pathological widening of the inner diameter. When heart function is impaired, LVEF is significantly decreased, and the total output of the heart is decreased.[14] As our results showed, the levels of LVEDD, LVESD and the heart index in the SHR group were significantly higher than those of the WKY group, while the levels of LVEF and LVFS decreased. These observations indicated that there was increased left ventricular load, increased ejection volume and impaired cardiac function in the SHR group. To address this condition, our results showed that geniposide could reduce the functional load of the heart and alleviate cardiac injury. The effect of geniposide in improving heart function was similar to bumetanide.

It was previously reported that H-FABP rapidly diffused into the plasma after an SHR myocardial injury and could be used as a marker of early myocardial injury.[15] The proliferation and phenotypic transformation of cardiac fibroblasts are important mediators involved in remodelling after myocardial injury. Myofibroblasts express α-SMA, which when combined with the appearance of stress fibres, is a reliable biomarker of the myofibroblast phenotype.[16] ANP has been the best marker to identify heart failure.[17] Previous studies have shown that plasma levels of ANP and BNP increase in proportion to the severity of the cardiac dysfunction.[18] Similarly, elevated ANP and BNP have been identified in patients with various cardiovascular diseases.[19] Our results showed that geniposide could significantly reduce the amount of H-FABP, α-SMA, ANP, BNP, and inflammatory factors (Figure 1S), which indicates that it could attenuate myocardial injury. The effect of geniposide in attenuating myocardial injury was similar to bumetanide.

Myocardial injury is usually accompanied by cardiomyocyte apoptosis.[20–22] Cell apoptosis is an active and programmed process of cell death that is under the control of the self-gene.[23,24] Apoptosis of cardiomyocytes leads to their loss and cardiac dysfunction.[25,26] p38MAPK plays an important role in regulating the physiological processes of cells, including proliferation, differentiation and apoptosis.[27] Several studies have shown that p38 MAPK plays an important role in different aspects of cardiogenesis, such as the regulation of cardiomyocyte differentiation and apoptosis. p38 MAPK is closely related to myocardial hypertrophy, myocardial contractility, and inflammation.[28–31] Furthermore, the Bcl-2 protein family plays an important role in the apoptosis pathway.[32] This protein family can be divided into its anti-apoptotic member (Bcl-2) and its pro-apoptotic member (BAX).[33] Many studies have shown that an increase in Bcl-2 and a decrease in BAX determine whether the cell survives or undergoes apoptosis.[34] Our results showed that geniposide could decrease the expression of p-p38/p38 and BAX proteins, increase the expression of the Bcl-2 protein, and the apoptosis was decreased. Therefore, geniposide may inhibit cardiomyocyte apoptosis and improve myocardial injury through the regulation of p38 pathway. In reducing cardiomyocyte apoptosis by regulating p38/Bcl-2/BAX pathway, geniposide had a similar effect to bumetanide.

At the same time, the heart is an important organ that provides energy to the body. Myocardial hypertrophy caused by hypertension includes not only the hypertrophy of myocardial parenchymal cells and the changes of structure and function in cell but also the proliferation of myocardial interstitial cells and the abnormal accumulation of collagen fiber. In the pathogenesis of hypertension, compensatory myocardial hypertrophy occurs, causing abnormal energy metabolism.[35] Cytochrome oxidase (COX) is strongly associated with mitochondrial function and cellular energy production. The three largest subunits in COX are COI, COII and COIII. COX must be assembled to form a completed active molecule. COI and COIV can initiate assembly. COIII and other subunits can form a subcomplex intermediate first, and then combine with COI and COIV to form a complete COX.[36] COX catalyses the redox reaction using cytochrome and oxygen as substrates, which is then coupled with proton transport across the inner mitochondrial membrane and

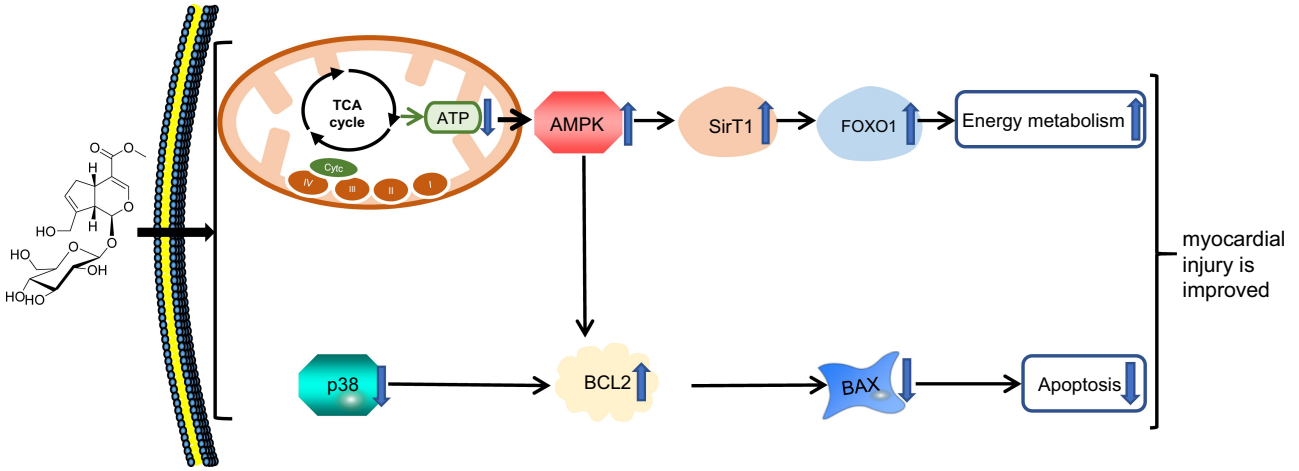

Figure 8 Signaling pathway of geniposide in improving myocardial injury.
Abbreviations: ATP, adenosine triphosphate; Cytc, cytochrome c; AMPK, adenosine 5′-monophosphate (AMP)-activated protein kinase; Bcl-2, B-cell lymphoma-2; BAX, Bcl-2 associated X protein; SirT1, sirtuin 1; FOXO1, forkhead box protein O1.

results in proton-electrochemical gradients that drive the synthesis of ATP, known as the "energy currency" of organism.[37,38] The excessive release of ATP promotes the inflammatory response of hypertension.[39] AMPK can sense the increase in the intracellular AMP/ATP ratio and become activated. As an energy regulator, AMP-activated protein kinase (AMPK) plays an important role in the regulation of energy metabolism.[40] AMPK maintains the body's energy homeostasis by regulating the balance of the energy generation and consumption processes.[41] AMPK enhances the activity of SirT1, which in turn can lead to the deacetylation of the transcription factor forkhead box protein O1 (FOXO1), thus regulating energy metabolism.[42,43] AMPK signalling pathway could also exert anti-apoptotic effects by increasing the ratio of Bcl-2/BAX.[44] Our results showed that geniposide could decrease the gene overexpression of COI, COIII, and the overproduction of ATP while increasing the ratio of AMP/ATP and activating the AMPK/SirT1/FOXO1 pathway. We also showed that AMPK inhibitors could block the activation of this pathway in H9c2 cells and increase the rate of apoptosis. Therefore, geniposide could regulate the energy metabolism pathway (AMPK/SirT1/FOXO1) and reduce cardiomyocyte apoptosis. In attenuating myocardial injury by regulating the energy metabolism signalling pathway (AMPK/SirT1/FOXO1), bumetanide only had some effects on AMPK protein. Therefore, bumetanide may attenuate myocardial injury of SHR by regulating other signal pathways related to AMPK.

In conclusion, geniposide is a potential drug for the treatment of myocardial injury caused by hypertension. Geniposide attenuates myocardial injury induced by spontaneous hypertension possibly by regulating the energy metabolism signalling pathway (AMPK/SirT1/FOXO1), and by regulating p38/Bcl-2/BAX pathway to reduce myocardial apoptosis. The concrete illustration is shown in Figure 8.

Conclusion

In conclusion, geniposide attenuates myocardial injury induced by spontaneous hypertension possibly by regulating the energy metabolism signalling pathway (AMPK/SirT1/FOXO1), and by regulating p38/Bcl-2/BAX pathway to reduce myocardial apoptosis.

Acknowledgments

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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