

# Huoxue Qianyang Qutan recipe attenuates Ang II-induced cardiomyocyte hypertrophy by regulating reactive oxygen species production

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**Abstract.** Continuous and irreversible cardiac hypertrophy can induce cardiac maladaptation and cardiac remodeling, resulting in increased risk of developing cardiovascular diseases. The present study was conducted to investigate the therapeutic effect of Huoxue Qianyang Qutan recipe (HQQR) on angiotensin II (Ang II)-induced cardiomyocyte hypertrophy. Primary cardiomyocytes were isolated from the cardiac tissue of neonatal rats, followed by flow cytometry detection to confirm the proportion of primary cardiomyocytes. Cell Counting Kit-8 assay and immunofluorescence detection were performed to examine the effect of Ang II and HQQR on cardiomyocyte hypertrophy. Reactive oxygen species (ROS) and a series of metabolic indicators were quantified to investigate the effect of HQQR on Ang II-induced cardiomyocyte hypertrophy. Mitochondrial electron transport chain complex activity and related coding gene expression were determined to explore the effect of HQQR on mitochondrial function. HQQR significantly inhibited Ang II-induced cardiomyocyte hypertrophy and restored Ang II-induced ROS accumulation, metabolic indicators, and membrane potential levels. HQQR also regulated the mitochondrial function related to the sirtuin 1 pathway in Ang II-induced cardiomyocytes by increasing the activity of the mitochondrial electron transport chain complex and affecting the expression of genes encoding mitochondrial electron transport chain complex subunits. HQQR

could alleviate Ang II-induced cardiomyocyte hypertrophy by modulating oxidative stress, accumulating ROS and increasing mitochondrial electron transport chain activity.

## Introduction

Cardiovascular diseases (CVDs) are a group of heart and blood vessel disorders that cause ~17.5 million deaths each year and have become the leading cause of death worldwide (1). Cardiac hypertrophy (CH) is an adaptive reaction of cardiomyocytes to pressure overload and neurohumoral and cytokine abnormalities (2), which are manifested by increased size of cardiomyocytes and contractile protein synthesis (3,4). However, continuous pathological stress overload results in irreversible CH and induces cardiac maladaptation and cardiac remodeling (5), thereby increasing the risk of developing various CVDs such as arrhythmia, myocardial ischemia, heart failure and sudden death.

Accumulating studies have demonstrated that, in myocardial remodeling, the content of angiotensin II (Ang II) is directly proportional to the degree of myocardial hypertrophy (6-8). Ang II directly participates in the development of myocardial hypertrophy by generating reactive oxygen species (ROS), reducing membrane potential and inducing mitochondrial dysfunction (6-8). Mitochondria are critical in energy metabolism, signal transduction and cell proliferation of cardiomyocytes (9). Mitochondrial dysfunctions are the major cause of CH, which include mitochondrial ROS increase, electron transport chain protein damage, mitochondrial DNA (mtDNA) damage and a decrease in metabolic capacity (10-12). Therefore, reducing the content of Ang II and maintaining mitochondrial function in cardiomyocytes are potential strategies for the treatment of pathological CH.

Traditional Chinese medicine (TCM) has a history of >2,000 years. It is widely used in Eastern Asia to prevent or treat a multitude of diseases, including CVDs (13). Tanshinones, berberine, matrine/oxymatrine, qiliqiangxin and *Radix puerariae* are representative compounds that can prevent or alleviate pathological CH and cardiac remodeling (14-17). Huoxue Qianyang Qutan recipe (HQQR) is a TCM compound consisting of *Salviae miltiorrhizae*, Stone

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Cassia, *Ligusticum chuanxiong*, *Uncaria angustifolia*, mulberry parasite, hawthorn and corn whisker. A previous study demonstrated that, in rats with obesity and hypertension (OBH), HQQR could alleviate mitochondrial dysfunction and left ventricular hypertrophy by modulating the sirtuin 1 (SIRT1)/peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) deacetylation pathway *in vivo* (18). Furthermore, one of our previous studies revealed that the TCM Huoxue Qianyang decoction synergized with the activating transcription factor 6/CHOP endoplasmic reticulum stress signaling pathway and improved heart remodeling in rats with OBH (19). However, the therapeutic effect and the specific molecular mechanisms underlying the action of HQQR in Ang II-induced cardiomyocyte hypertrophy remain unclear.

Therefore, the present study was conducted to investigate the effect of HQQR on Ang II-induced cardiomyocyte hypertrophy in isolated primary cardiomyocytes, and to evaluate the role of HQQR in mitochondrial function to explore its underlying mechanism.

## Materials and methods

**Animals and drugs.** All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at Yueyang Hospital of Integrated Traditional Chinese and Western Medicine Affiliated to Shanghai University of Traditional Chinese Medicine (approval no. 18922), according to the principles outlined in the National Institutes of Health Guidelines for Care and Use of Laboratory Animals.

Pregnant Sprague Dawley rats (n=2; female; 10 weeks old; 260–270 g) purchased from the Vital River Laboratory Animal Technology Co., Ltd. [animal license number, SCXK (Beijing)] were fed *ad libitum* in an animal facility room with a 12-h light/dark cycle at a temperature of 20–22°C, with ~50% humidity. After pregnant rats gave birth, neonatal rats (within 24 h of birth; 5–6 g) were euthanized using CO<sub>2</sub> inhalation at a flow rate of 1.2 l/min, which displaces 30% of the cage volume per min, followed with further cervical dislocation.

Valsartan capsules (Novartis International AG) were dissolved in 100% DMSO as 10 mmol/l stock solutions and stored at -80°C before use. The compound stock solution was diluted in cell culture medium (DMEM medium with 10% FBS and 1% double-antibiotic) to a final concentration of 10  $\mu$ mol/l (20). HQQR, consisting of *Salvia miltiorrhiza*, Stone Cassia, *Ligusticum chuanxiong*, *Uncaria angustifolia*, corn whisker, mulberry parasite and hawthorn (5:10:3:5:10:5:5), was decocted and dried according to a common protocol (18).

**Isolation and culture of rat primary cardiomyocytes.** Primary cardiomyocytes were isolated from the cardiac tissue of neonatal rats (within 24 h of birth) as previously described (21). Briefly, the hearts of the neonatal rats were collected, and the atrium of each heart was put into 5-ml sterile centrifuge tubes to be fully cut into small pieces. After full digestion with 0.4% Collagenase IV (cat. no. C4-BIOC; Sigma-Aldrich; Merck KGaA) and 0.05% trypsin-EDTA (cat. no. T1300; Beijing Solarbio Science & Technology Co.,

Ltd.) in 37°C constant temperature water bath for 20 min, the cell suspension was centrifuged at room temperature for 10 min at 1,000  $\times$  g. After multiple digestion, the cell suspensions were filtered through a 100-mesh filter and mixed, and the cells were cultured at 37°C in DMEM medium (cat. no. SH30243.01; Hyclone; Cytiva) with 10% FBS (cat. no. 16000-044, Gibco; Thermo Fisher Scientific, Inc.) and 1% double-antibiotic (penicillin-streptomycin; cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.) in a 37°C, 5% CO<sub>2</sub> incubator. Next, the isolated cells were subjected to flow cytometry detection of troponin I (troponin I, TnI; 1:50; cat. no. ab196384; Abcam) to identify the purity of primary cardiomyocytes. The morphology of cardiomyocytes was observed, and pictures were captured with a light microscope (magnification,  $\times$ 200).

**Experimental procedure.** Cardiomyocytes were divided into the following five groups (5 $\times$ 10<sup>5</sup> cells; 6-well plate): i) Vehicle (solvent group, DMSO); ii) Ang II + vehicle; iii) Ang II + 0.2 mg/ml HQQR; iv) Ang II + 0.5 mg/ml HQQR; and v) Ang II + valsartan. Ang II (cat. no. 4474-91-3) was purchased from Beijing Solarbio Science & Technology Co., Ltd., and the corresponding concentration (1  $\mu$ mol/l) was prepared according to the manufacturer's instructions. Ang II and HQQR were added at the same time.

**Fluorescent immunostaining.** Cell suspensions containing 1 $\times$ 10<sup>5</sup> primary rat cardiomyocytes were added to a 24-well plate and treated with Ang II, Ang II + 0.2 mg/ml HQQR, Ang II + 0.5 mg/ml HQQR, Ang II + 10  $\mu$ mol/l valsartan, or Ang II + ROS scavenger (1 mM; cat. no. S6205; Selleck Chemicals) for 24 h. Then, slides containing experimental primary cardiomyocytes were harvested, fixed with 4% formaldehyde for 30 min at room temperature, and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. After blocking with 1% bovine serum albumin (BSA; cat. no. A8010; Beijing Solarbio Science & Technology Co., Ltd.) in PBS for 30 min at room temperature, the slides were incubated with anti- $\alpha$ -actinin antibody (1:200; cat. no. ab68194; Abcam) and anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; 1:500; ab32575; Abcam) overnight at 4°C in a wet-box. Subsequently, the slides were incubated with Alexa Fluor 555-labeled donkey anti-rabbit IgG (H + L) antibody (1:500; cat. no. A0453; Beyotime Institute of Biotechnology) for 1 h at room temperature in a wet-box before staining the nuclei with DAPI-containing media for 15 min at room temperature (1:500; cat. no. C1002; Beyotime Institute of Biotechnology). Pictures were taken using a fluorescence microscope (Nikon Corporation).

**Cell Counting Kit-8 (CCK-8) assay.** CCK-8 assay was performed in accordance with the manufacturer's kit protocol (Beyotime Institute of Biotechnology). Cell suspensions containing 3 $\times$ 10<sup>3</sup> primary rat cardiomyocytes were added to a 96-well plate. The cells were treated with 1  $\mu$ mol/l Ang II (22) and gradient concentrations of HQQR (0, 0.05, 0.1, 0.2, 0.5 and 1 mg/ml) or valsartan (10  $\mu$ mol/l) after 24 h of incubation. Finally, 10  $\mu$ l CCK-8 solution were added into each well, and the cells were incubated in an incubator at 37°C with 5% CO<sub>2</sub> for 1 h. Cell proliferation at 0, 12, 24 and 48 h was detected by recording the optical density at 450 nm (OD<sub>450</sub>).

Table I. Primers for reverse transcription-quantitative PCR analysis.

Gene	Forward primer (5' to 3')	Reverse Primer (5' to 3')
ANP	GGGCTTCTTCCTCTTCCTG	TCTGAGACGGGTTGACTTCC
BNP	TAGCCAGTCTCCAGAACAATCC	ACCTCAGCCCGTCACAGC
β-MHC	TGACAACGCCTATCAGTACATG	CCTGGGGTCTGGTCCTTC
Sirt1	GGTTAGGTGGCGAGTATGC	TATGAAGAGGTGTTGGTGGC
Nrf1	ACCCAAGCATTACGGACC	CAGTACCAACCTGGATGAGC
Tfam	CGCATAACCCTCGCCTGTC	GTTCTGAAACTTTTGCATCTGG
Ndufa13	CGGGGACTGTCGGGATAC	GGAGGAGTGGCATGAGGG
SDHB	TACAAATCCATTGAGCCCTATC	GCACTCATAAATCCGTCCAG
COX IV	GGCGTGACTACCCCTTGC	CTCATTGGTGCCCTTGTTTC
COX1	AACTAGGACAACCAGGAGCAC	AATCATTAGCGGCACAAGC
ATPase 6	GAGCCGTAATTCTAGGTTTCC	ATTGTAGCGGTTGGTGGG
Ppargc1a	AACCGCAGTCGCAACATG	GGAGGAGTCGTGGGAGGAG
mtDNA	AGCTCCAGCTTTTGTTC	CTTCCGGTTCGTATGTTGTG
GAPDH	GGAGTCTACTGGCGTCTTAC	ATGAGCCCTTCCACGATGC

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; Ppargc1a, peroxisome proliferator-activated receptor-gamma coactivator-1α; Nrf1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor A; Ndufa13, NADH:ubiquinone oxidoreductase subunit A13; SDHB, succinate dehydrogenase complex iron sulfur subunit B; COX IV, cytochrome *c* oxidase subunit IV; COX1, anti-cyclooxygenase 1; Sirt1, sirtuin 1; mtDNA, mitochondrial DNA.

**Reverse transcription-quantitative PCR.** Total RNA was extracted from primary rat cardiomyocytes using TRIzol® reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Complementary DNA was synthesized using the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and under the following conditions: 37°C for 60 min; 85°C for 5 min; 4°C for 5 min. mRNA levels were quantified using SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and under the following thermocycling conditions: 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 45 sec. Relative mRNA levels were obtained using the 2<sup>-ΔΔC<sub>q</sub></sup> method (23). GAPDH was used as an internal control for normalization. The primer sequences are listed in Table I.

**Western blot analysis.** The total protein of primary rat cardiomyocytes was extracted using RIPA buffer, which contained a protease and phosphatase inhibitor (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.). Protein was then quantified using a BCA kit (cat. no. PICPI23223; Thermo Fisher Scientific, Inc.). Subsequently, proteins (25 μg per lane) were separated using 10% SDS-PAGE and transferred to PVDF membranes. After blocking in 5% non-fat milk for 1 h at room temperature, the protein bands were incubated with primary antibodies overnight at 4°C, followed by incubation with the corresponding secondary antibodies [anti-rabbit (cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology) or anti-mouse (cat. no. A0216; 1:1,000; Beyotime Institute of Biotechnology) labeled with HRP] at 37°C for 1 h. After a 5-min incubation with Immobilon Western chemiluminescent HRP substrate (cat. no. WBKLS0100; MilliporeSigma) in the dark, the protein

bands were visually measured using a chemiluminescent imaging system (Tanon-5200, Tanon Science and Technology Co., Ltd.), and then quantified using ImageJ software (v1.8.0.112; National Institutes of Health). Anti-SIRT1 (cat. no. ab110304; 1:1,000), anti-PGC-1α (cat. no. ab54481; 1:2,000), anti-nuclear respiratory factor 1 (NRF1; cat. no. ab175932; 1:5,000), anti-mitochondrial transcription factor A (Tfam; cat. no. ab131607; 1:2,000), anti-NADH:ubiquinone oxidoreductase subunit A13 (NDUFA13; cat. no. ab110240; 1:1,000), anti-succinate dehydrogenase complex iron sulfur subunit B (SDHB; cat. no. ab178423; 1:5,000), anti-cytochrome *c* oxidase subunit IV (COX IV; cat. no. ab16056; 1:500), anti-cyclooxygenase 1 (COX1; cat. no. ab695; 1:1,000), anti-ATPase 6 (cat. no. ab192423; 1:500), anti-brain natriuretic peptide (BNP; cat. no. ab239510; 1:2,000), anti-Bax (cat. no. ab32503; 1:5,000), anti-Bcl-2 (cat. no. ab196495; 1:1,000) antibodies were obtained from Abcam, anti-cleaved caspase 3 (cat. no. 9661; 1:1,000), anti-caspase 3 (cat. no. 9662; 1:1,000) were obtained from CST, anti-atrial natriuretic peptide (ANP; cat. no. RQ4453; 0.5 μg/ml) antibody was obtained from NSJ Bioreagents, anti-β-myosin heavy chain (β-MHC; cat. no. MA1-26180; 0.5 μg/ml) antibody was obtained from Invitrogen; Thermo Fisher Scientific, Inc., and anti-GAPDH as a loading control (cat. no. 60004-1-IG; 1:5,000) was obtained from ProteinTech Group, Inc. The antibodies were diluted in PBST (containing 0.05% Tween-20).

**ROS detection.** ROS accumulation in cardiomyocytes was quantified using the Active Oxygen Detection kit (cat. no. S0033; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. After treatment with 1 μmol/l Ang II and HQQR (0.2 and 0.5 mg/ml) or valsartan (10 μmol/l), DCFH-DA probe solution was prepared and used for incubation with the harvested cell samples at 37°C for 30 min in the dark. The cells

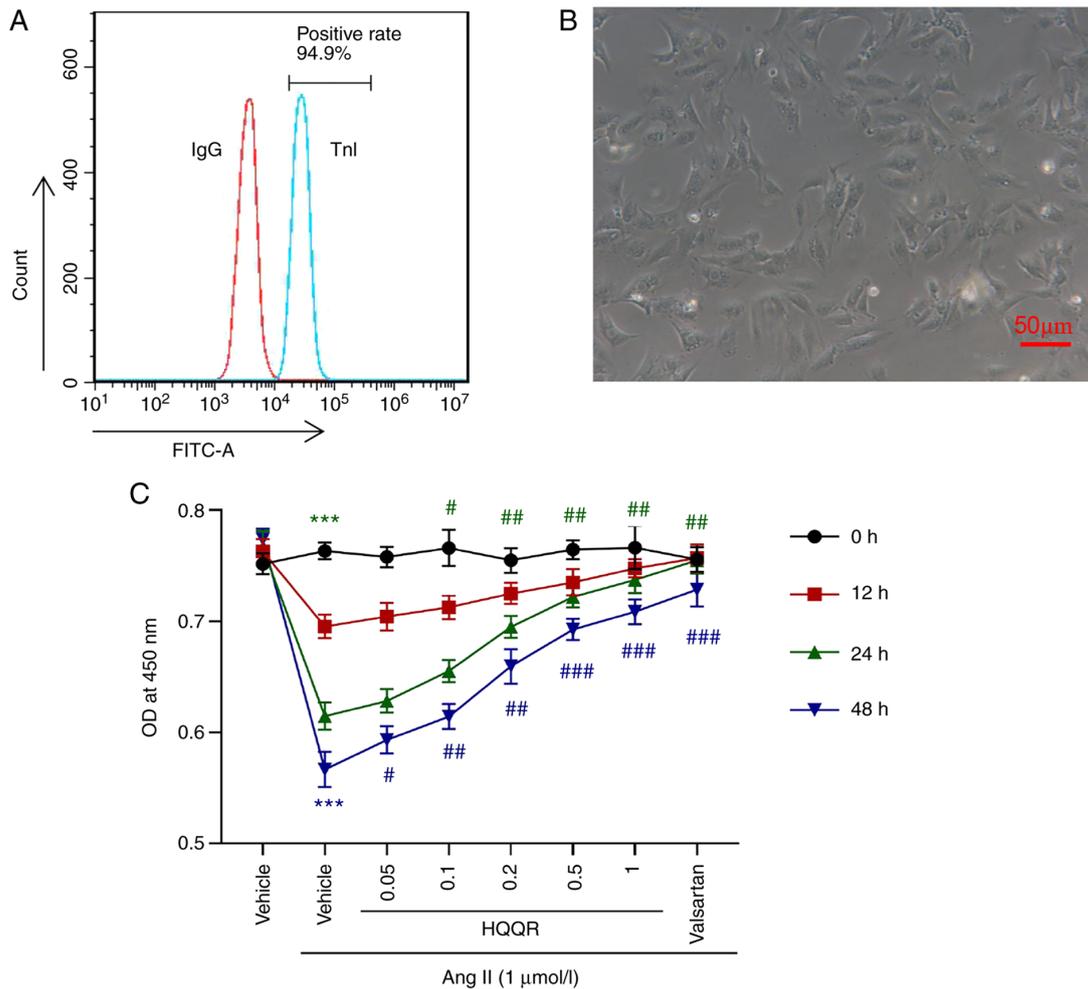


Figure 1. HQQR attenuates the inhibitory effect of Ang II on rat cardiomyocyte proliferation. (A) TnI was detected using flow cytometry analysis to identify the percentage of primary cardiomyocytes. (B) Image of isolated primary rat cardiomyocytes (magnification, x200; scale bar, 50  $\mu$ m). (C) Cell Counting Kit-8 assays were used to examine the proliferation of primary cardiomyocytes at 0, 12, 24 and 48 h after treatment with Ang II (1  $\mu$ mol/l) and HQQR powder (0, 0.05, 0.1, 0.2, 0.5 and 1 mg/ml). Vehicle and valsartan were used as negative and positive controls, respectively. \*\*\* $P$ <0.001 vs. the vehicle group; # $P$ <0.05, ## $P$ <0.01 and ### $P$ <0.001 vs. the Ang II + vehicle group. HQQR, Huoxue Qianyang Qutan recipe; Ang II, angiotensin II; TnI, troponin I; OD, optical density.

and solution were gently inverted every 10 min. After washing three times with DMEM without serum, the samples were examined via flow cytometry (Accuri C6; BD Biosciences) using FlowJo software (v10.0.7r2; Tree Star, Inc.).

**Biochemical detection.** Primary rat cardiomyocytes were treated with 1  $\mu$ mol/l Ang II and HQQR (0.2 and 0.5 mg/ml) or valsartan (10  $\mu$ mol/l) for 24 h, and then, the cells were collected, and the supernatant was kept. The levels of ATP and protein carbonyl, and the activities of glutathione peroxidase (GSH-PX), catalase (CAT), malondialdehyde (MDA) and superoxide dismutase (SOD) were respectively detected using the BCA (cat. no. A045-3), ATP (cat. no. A095), protein carbonyl (cat. no. A087), GSH-PX (cat. no. A005), CAT (cat. no. A007-1), MDA (cat. no. A003-2) and SOD (cat. no. A001-1) kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions, and measured using a visible spectrophotometer. The activities of the mitochondrial complexes I-IV and V were respectively detected using the mitochondrial complex I (cat. no. BC0515), II (cat. no. BC3235), III (cat. no. BC3245), IV (cat. no. BC0945) and V (cat. no. BC1445) Activity Test kits (Beijing

Solarbio Science & Technology Co., Ltd.) and measured using a UV spectrophotometer.

**Mitochondrial potential assay.** Alterations in mitochondrial membrane potential were evaluated using the Mitochondrial Membrane Potential Detection kit with JC-1 (cat. no. C2006; Beyotime Institute of Biotechnology). According to the manufacturer's instructions, the cell suspension was prepared and detected using a flow cytometer (Accuri C6; BD Biosciences) via the software of Flow Jo (v10.0.7r2 version). JC-1 is an ideal fluorescent probe widely used to detect mitochondrial membrane potential. In high mitochondrial membrane potential, JC-1 aggregates in the matrix of mitochondria and forms polymer (J-aggregates), which produces red fluorescence. When the mitochondrial membrane potential is low, JC-1 cannot aggregate in the matrix of mitochondria. At this time, JC-1 is a monomer and can produce green fluorescence.

**Cell apoptosis.** Primary rat cardiomyocyte cells after 24 h treatment with Ang II, Ang II + 0.2 mg/ml HQQR, Ang II +

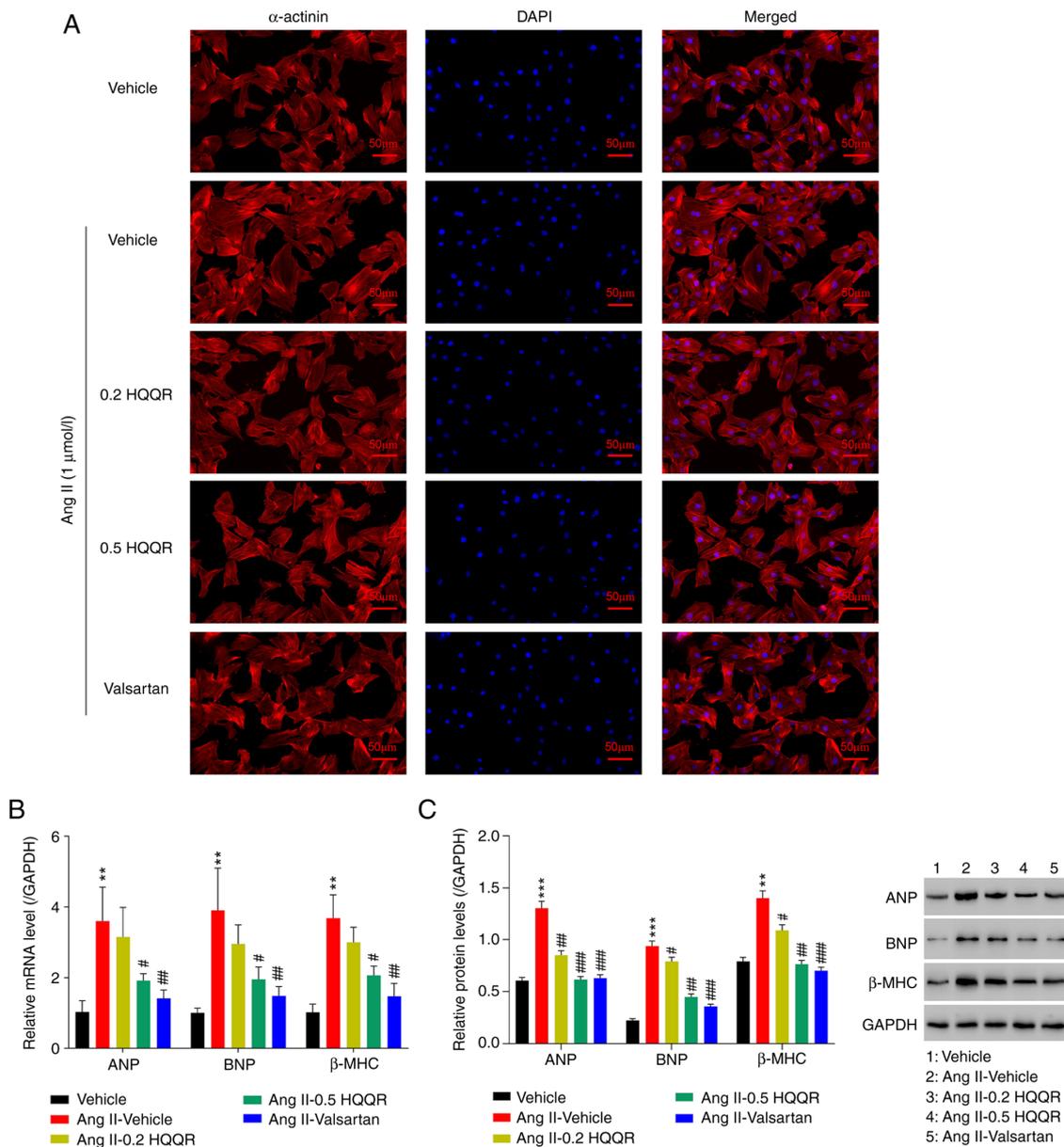


Figure 2. HQQR improves Ang II-induced cardiomyocyte hypertrophy. (A) Immunofluorescence experiments were performed using anti- $\alpha$ -actinin antibodies to analyze cardiomyocyte hypertrophy (magnification,  $\times 200$ ; scale bar,  $50 \mu\text{m}$ ). (B) mRNA expression levels of the myocardial hypertrophy markers ANP, BNP and  $\beta$ -MHC were examined using reverse transcription-quantitative PCR. (C) Protein expression levels of the myocardial hypertrophy markers ANP, BNP and  $\beta$ -MHC were examined using western blotting. \*\* $P < 0.01$  and \*\*\*\* $P < 0.0001$  vs. the vehicle group; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. the Ang II + vehicle group. HQQR, Huoxue Qianyang Qutan recipe; Ang II, angiotensin II; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide;  $\beta$ -MHC,  $\beta$ -major histocompatibility complex.

0.5 mg/ml HQQR, Ang II +  $10 \mu\text{mol/l}$  valsartan were stained using Annexin V Apoptosis kit (C1062; Beyotime Institute of Biotechnology) according to the manufacturer's instructions and a Beckman CytoFLEX Flow Cytometer (Beckman Coulter, Inc.) with FlowJo software (v10.0.7r2; Tree Star, Inc.) was used to analyze apoptosis.

**Statistical analysis.** Statistical analysis was conducted using GraphPad Prism 7.0 software (GraphPad Software, Inc.). Data are presented as the mean  $\pm$  SD. One-way ANOVA followed by Tukey's post hoc test was applied for comparisons between groups. Results are representative of at least three repeated experiments.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*HQQR attenuates the inhibitory effect of Ang II on rat cardiomyocyte proliferation.* To investigate the effect of HQQR on Ang II-induced cardiomyocyte hypertrophy, rat primary cardiomyocytes were isolated. After detection using TnI, the results of flow cytometry analysis confirmed that  $\sim 95\%$  of the isolated cells were primary cardiomyocytes (Fig. 1A). Microscopic observations (light microscope) also indicated that the isolated cell morphology was consistent with the morphological characteristics of primary cardiomyocytes (Fig. 1B), including a short columnar form with generally only one nucleus being mostly located in the middle of the cell, while the cell shape is ellipse- or

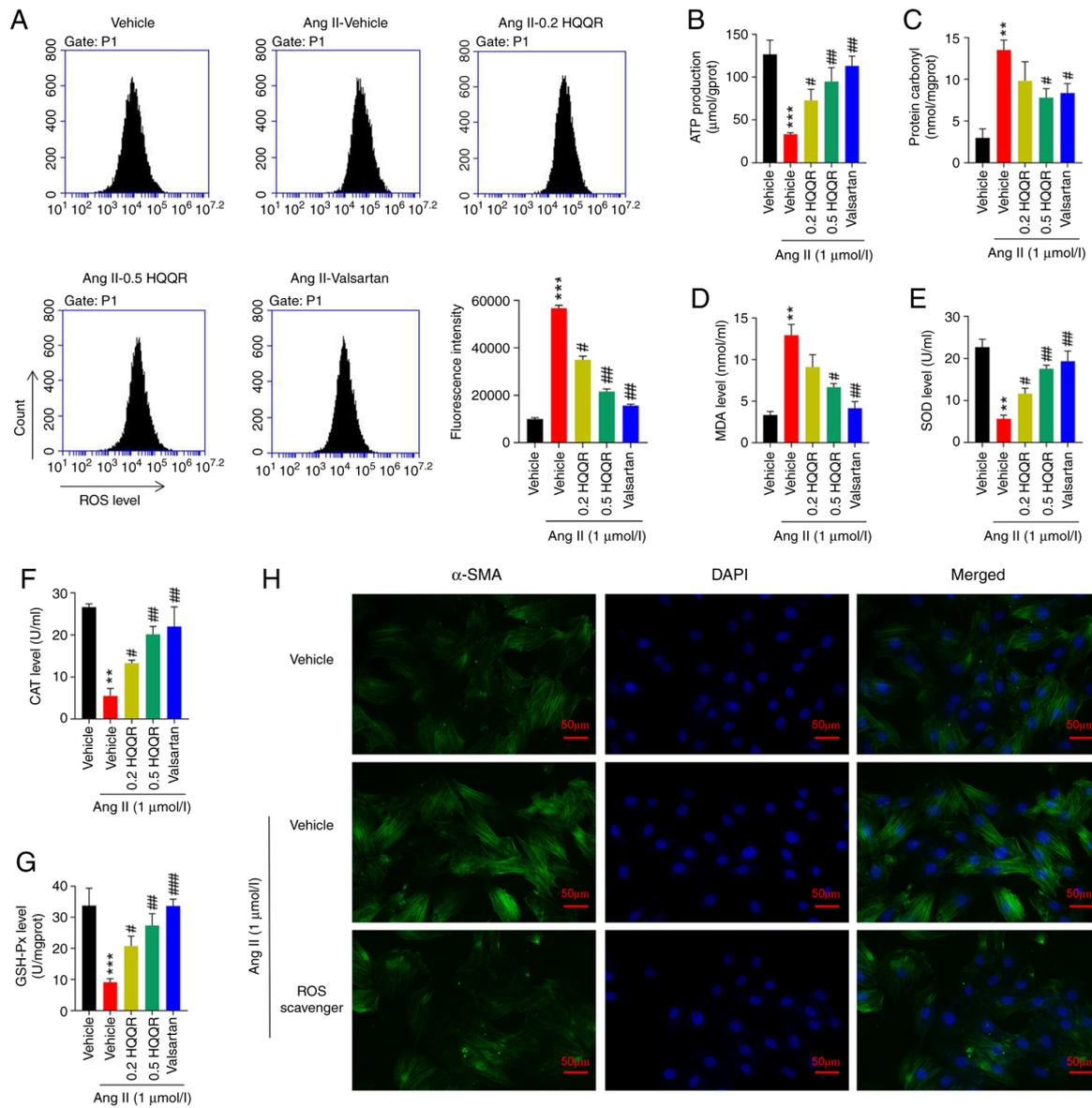


Figure 3. HQQR alleviates AngII-induced cardiomyocyte hypertrophy by modulating ROS accumulation. (A) Flow cytometry analysis to detect the changes in ROS accumulation. Levels of (B) ATP and (C) protein carbonyl content, and activities of (D) MDA, (E) SOD, (F) CAT and (G) GSH-Px were quantified. (H) Immunofluorescence experiments were performed using anti- $\alpha$ -SMA antibodies to analyze cardiomyocyte hypertrophy (magnification,  $\times 200$ ; scale bar,  $50 \mu\text{m}$ ). \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. the vehicle group; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. the Ang II + vehicle group. HQQR, Huoxue Qianyang Qutan recipe; Ang II, angiotensin II; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH-PX, glutathione peroxidase;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

rectangle-like, and its long axis is in the same direction as in myofibrils. The cytotoxicity of HQQR was detected using a CCK-8 assay (Fig. S1), the results of which revealed that 0.2 and 0.5 mg/ml HQQR had no cytotoxicity and the 24 h after treatment had the best effect. Cell proliferation experiments revealed that, compared with normally cultured (medium without other treatment) primary cardiomyocytes, Ang II ( $1 \mu\text{mol/l}$ ) treatment significantly inhibited the proliferation of primary cardiomyocytes, which was markedly abolished by incubation with valsartan ( $10 \mu\text{mol/l}$ ), the antagonist of angiotensin receptor used as a positive control. The addition of HQQR solution ameliorated the inhibitory effect of Ang II on primary cardiomyocyte proliferation in a dose-dependent manner (Fig. 1C). On the basis of the present results, Ang II treatment for 24 h followed by 0.2 and 0.5 mg/ml HQQR concentrations were selected as treatment conditions for

subsequent experiments. Valsartan was used as a positive control.

#### HQQR improves AngII-induced cardiomyocyte hypertrophy.

To further examine the role of HQQR in Ang II-induced cardiomyocyte hypertrophy, primary cardiomyocytes were divided into the following five groups: i) Vehicle; ii) Ang II ( $1 \mu\text{mol/l}$ ) + vehicle; iii) Ang II ( $1 \mu\text{mol/l}$ ) + HQQR (0.2 mg/ml); iv) Ang II ( $1 \mu\text{mol/l}$ ) + HQQR (0.5 mg/ml); and v) valsartan + Ang II ( $1 \mu\text{mol/l}$ ). The immunofluorescence of  $\alpha$ -actinin revealed that, compared with that in the vehicle group, cardiomyocyte hypertrophy was observed in the Ang II-treated group, indicating that Ang II induced cardiomyocyte hypertrophy. However, treatment with HQQR could significantly inhibit Ang II-induced cardiomyocyte hypertrophy (Fig. 2A) and reduce the Ang II-induced expression of the

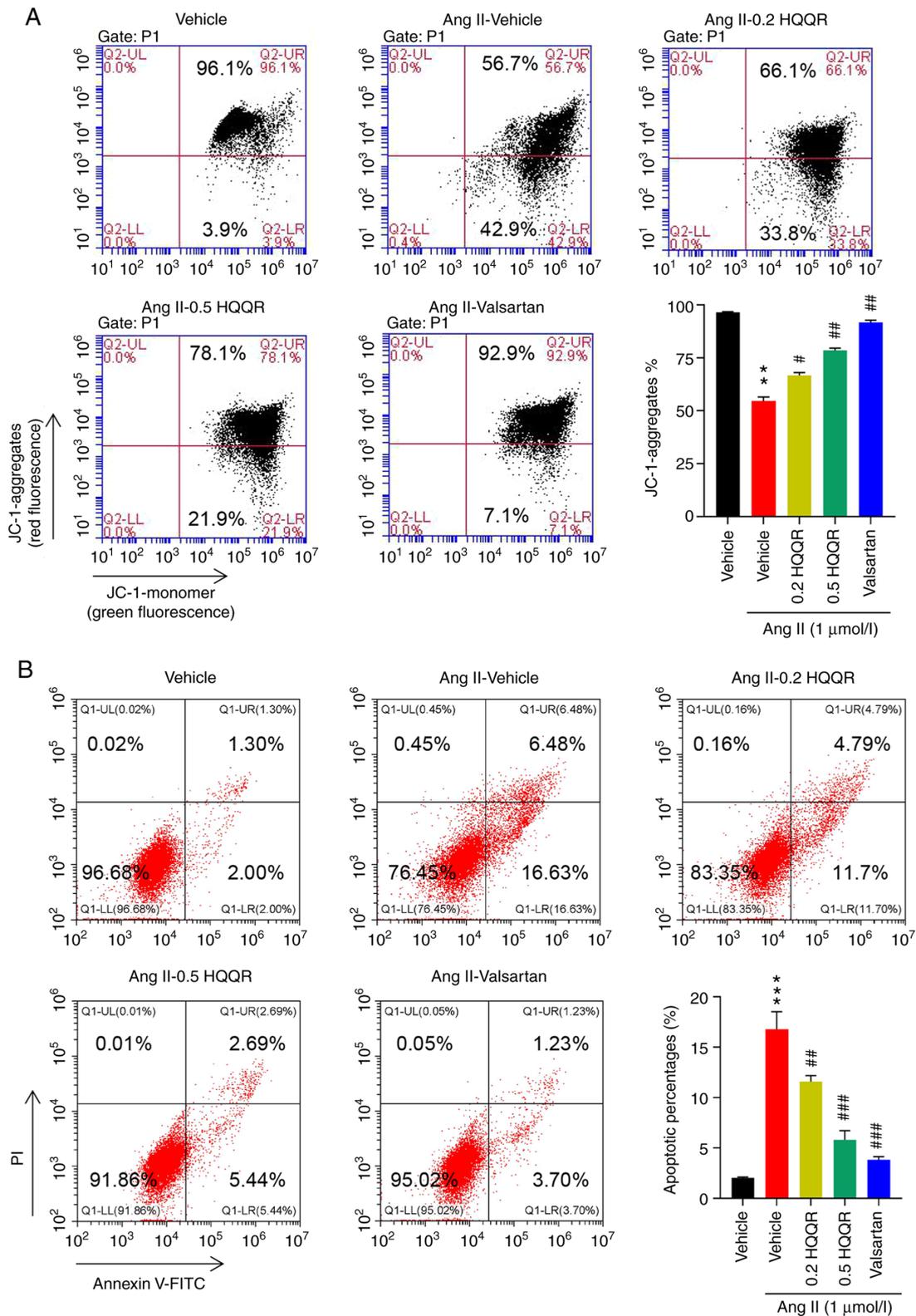


Figure 4. HQQR reverts cardiomyocyte hypertrophy-induced membrane potential reduction and apoptosis increase. (A) Flow cytometry analysis to detect the changes in membrane potential. (B) Flow cytometry analysis to detect the changes in cell apoptosis. \*\*P<0.01 and \*\*\*P<0.001 vs. the vehicle group; #P<0.05, ##P<0.01 and ###P<0.001 vs. the Ang II + vehicle group. HQQR, Huoxue Qianyang Qutan recipe; Ang II, angiotensin II.

myocardial hypertrophy markers ANP, BNP and  $\beta$ -MHC (Fig. 2B and C). Valsartan was used as a positive control.

*HQQR alleviates AngII-induced cardiomyocyte hypertrophy by modulating ROS accumulation.* Ang II treatment

markedly enhanced ROS accumulation, whereas treatment with HQQR significantly inhibited ROS accumulation in Ang II-treated primary cardiomyocytes, in a dose-dependent manner (Fig. 3A). HQQR could also rescue the effect of Ang II treatment on the levels of MDA, SOD, ATP, CAT,

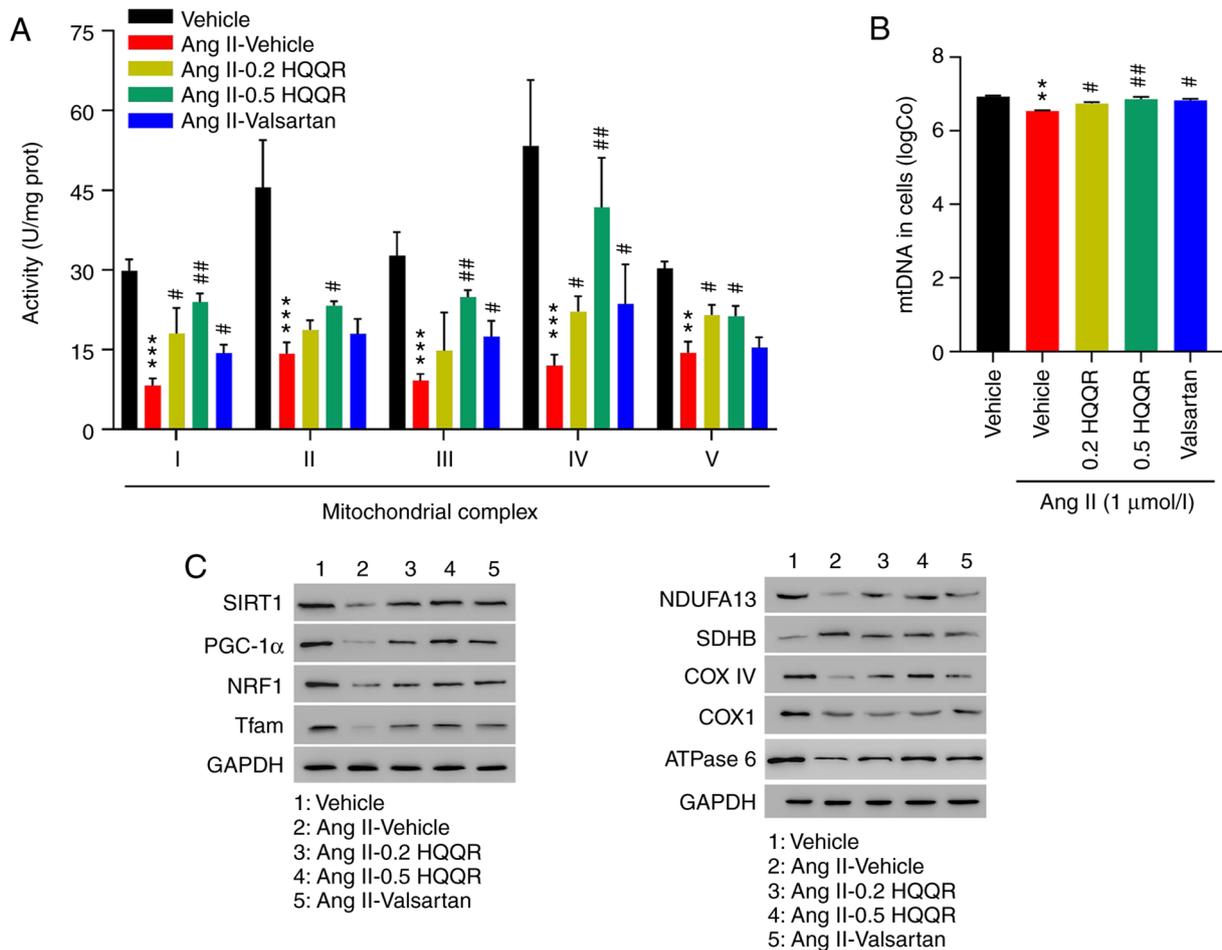


Figure 5. HQQR increases the activity of mitochondrial electron transport chain complexes in Ang II-treated rat cardiomyocytes. (A) The activities of the mitochondrial complexes I-IV and V were determined. (B) The level of mtDNA was quantified using RT-qPCR. (C) Protein levels of SIRT1, PGC-1 $\alpha$ , NRF1, Tfam, NDUFA13, SDHB, COX IV, COX1 and ATPase 6 in primary cardiomyocytes were examined by western blotting. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. the vehicle group; # $P < 0.05$  and ## $P < 0.01$  vs. the Ang II + vehicle group. HQQR, Huoxue Qianyang Qutan recipe; Ang II, angiotensin II; SIRT1, sirtuin 1; mtDNA, mitochondrial DNA; RT-qPCR, reverse transcription-quantitative PCR; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor-gamma coactivator-1 $\alpha$ ; NRF1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor A; NDUFA13, NADH:ubiquinone oxidoreductase subunit A13; SDHB, succinate dehydrogenase complex iron sulfur subunit B; COX IV, cytochrome *c* oxidase subunit IV; COX1, anti-cyclooxygenase 1; RT-qPCR, reverse transcription-quantitative PCR.

GSH-Px and protein carbonyl in primary cardiomyocytes (Fig. 3B-G). Consistent with these results, the immunofluorescence of  $\alpha$ -SMA revealed that ROS was directly involved in AngII-induced cardiomyocyte hypertrophy, and that the ROS scavenger alleviated AngII-induced cardiomyocyte hypertrophy (Fig. 3H). The present findings suggested that HQQR treatment could alleviate Ang II-induced cardiomyocyte hypertrophy, potentially by modulating ROS accumulation. Valsartan was used as a positive control.

*HQQR reverts cardiomyocyte hypertrophy-induced membrane potential reduction and apoptosis increase.* Ang II treatment notably reduced mitochondrial membrane potential (Fig. 4A) and increased cell apoptosis (Fig. 4B). In Ang II-stimulated cardiomyocytes, treatment with HQQR significantly reversed the hypertrophy-induced membrane potential reduction and apoptosis in a dose-dependent manner. Moreover, Ang II induced the expression of apoptosis-related proteins Bax and cleaved caspase 3, which were significantly decreased upon HQQR treatment, while anti-apoptotic Bcl-2

was significantly decreased by Ang-II and increased by HQQR (Fig. S2). Valsartan was used as a positive control.

*HQQR increases the activity of mitochondrial electron transport chain complexes in Ang II-treated rat cardiomyocytes.* To further explore the mechanism by which HQQR affects Ang II-induced cardiomyocyte hypertrophy, the activities of the mitochondrial complexes I-IV and V were studied 24 h after treatment. As presented in Fig. 5A, Ang II markedly inhibited the activity of the mitochondrial complexes I-IV and V, whereas the addition of HQQR or valsartan partially rescued their activities. Furthermore, HQQR or valsartan treatment markedly inhibited Ang II-induced mtDNA leakage (Fig. 5B). The mRNA and protein expressions of the mitochondrial complex and involved in mitochondrial biogenesis were subsequently evaluated. As indicated in Figs. 5C and S3, Ang II treatment reduced both mRNA and protein levels of SIRT1, PGC-1 $\alpha$ , NRF1, Tfam, NDUFA13, COX IV, COX1 and ATPase 6, and elevated the expression of SDHB. These effects were notably

rescued by treatment with HQQR or valsartan, which was used as a positive control.

## Discussion

CH is generally considered to be a major inducer of several types of CVDs (24). There is a need to explore effective therapeutic targets with low toxicity for the clinical treatment of CH and CVDs. Because of its low toxicity, TCM has been used to treat chronic diseases in East Asia throughout history. Several types of TCM have been demonstrated to be effective for CH treatment by affecting multiple signaling pathways or physiological activities. For instance, Bu-Shen-Jiang-Ya decoction suppressed left ventricular hypertrophy by regulating the extracellular signal-regulated kinase signaling pathway in spontaneously hypertensive rats (25). Moreover, QiShenYiQi pills ameliorated fatigue-induced CH through the regulation of energy metabolism (26). Qiliqiangxin was also found to attenuate phenylephrine-induced CH by upregulating peroxisome proliferator-activated receptor  $\gamma$  and PGC-1 $\alpha$  (27). Combined with the role of HQQR (18,19) in hypertensive rats, these findings provide evidence that TCM or a combination therapy of TCM and western medicine has the potential to treat CH. It is necessary to investigate TCM targeting CH treatment further, along with the potential mechanisms to prevent drug resistance and improve therapeutic effectiveness.

In the present study, the CCK-8 and immunofluorescence experiments confirmed that Ang II could induce CH (6). Functional analysis of HQQR in the treatment of CH revealed that HQQR significantly alleviated Ang II-induced CH in primary cardiomyocytes, thereby suggesting the significance of HQQR in CH treatment.

The mechanism by which HQQR abolished Ang II-induced CH was investigated. Mitochondria are double-membrane organelles that support a variety of physiological activities, including energy production, cell death and oxidative metabolism (9). Mitochondrial dysfunction has been implicated in the occurrence and development of cardiomyocyte hypertrophy (28). Mitochondrial biogenesis is essential for sustaining mitochondrial function and is related to ROS production (29). Although acute or mild oxidative stress can trigger mitochondrial biogenesis due to the involvement of mitochondrial quality control (30), the present study reported that Ang II treatment promoted the accumulation of ROS in cardiomyocytes and induced the disturbance of mitochondrial biogenesis, which may further result in cardiomyocyte injury and hypertrophy. HQQR treatment attenuated oxidative stress and restored the levels of mitochondrial transcription factors in cardiomyocytes. The inhibition of ROS accumulation and the normal activity of mitochondrial electron transport chain are crucial for alleviating mitochondrial-related diseases (10). After Ang II-induced ROS production, ROS activate several critical signaling pathways related to cell death and fibrosis, such as the mitogen-activated protein kinase pathway, the extracellular regulated protein kinase signaling pathway, protein kinase C signaling and NF- $\kappa$ B; these signaling pathways participate in the process of CH (31-33). Therefore, drugs that directly or indirectly target ROS production might

inhibit the occurrence or development of CH. As anticipated, HQQR treatment significantly reduced the accumulation of ROS and the indicators of oxidative stress, and elevated the levels of scavenging system indicators, such as SOD, CAT and GSH-PX, thus suggesting that HQQR partially prevented Ang II-induced CH by reducing the accumulation of ROS and repressing oxidative stress in myocardial cells. Ang II reduced the activity of the mitochondrial electron transport chain and suppressed the expression of mitochondrial complex subunits in cardiomyocytes; however, this impact was significantly reverted by HQQR treatment, indicating that HQQR could also exert its therapeutic effect by maintaining normal electron transfer.

HQQR treatment increased the ATP content in cardiomyocytes and elevated the levels of NRF1 and Tfam, which are critical for mitochondrial biogenesis. SIRT1 is a deacetylase that plays a key role in cell proliferation, differentiation, senescence, apoptosis and metabolism (34). The SIRT1/PGC-1 $\alpha$  pathway contributes to mitochondrial dysfunction and mitochondrial biogenesis (35,36). Inhibition of PGC-1 $\alpha$  or PGC-1 $\alpha$  deacetylation significantly abolishes the process leading to CH (27). The present study indicated that HQQR rescued Ang II-inhibited SIRT1 and PGC-1 $\alpha$  expression, thus implying that HQQR protected cardiomyocytes from the development of CH by enhancing the SIRT1/PGC-1 $\alpha$  regulatory pathway. It can be speculated that HQQR improves mitochondrial biosynthesis, leading to the reduction of oxidative stress.

Currently, there are relatively few studies on the efficacy of HQQR in CVDs. The present study provided evidence for the protective role of HQQR in CH, enriching the strategies for CH treatment. Furthermore, our previous study (18) confirmed the effect of HQQR in glucolipid metabolism and blood pressure regulation, thereby demonstrating the potential of HQQR in treating metabolic diseases and CVDs.

However, the effect of HQQR on the development of CH was only investigated at the cellular level. Therefore, *in vivo* experiments are required to further confirm the therapeutic effect of HQQR on CH and further explore underlying molecular mechanisms to expand the clinical application of HQQR.

The present study provided evidence that Ang II treatment could induce CH in primary cardiomyocytes, and that HQQR could significantly prevent Ang II-induced CH by preventing mitochondrial dysfunction and oxidative stress, reducing ROS accumulation and protecting the electron transport chain. Although additional studies are required to further verify the function and mechanisms of HQQR in the development of CH, the present study implied that HQQR could be considered a novel approach for CH treatment.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Authors' contributions

DF conceived and designed the study. MG, LY, BL, JW, XZ, JL and ZD performed the experiments. DF wrote the manuscript. DF and MG have confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

All the experiments conducted in the present study were approved by the Ethics Committee of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine Affiliated to Shanghai University of Traditional Chinese Medicine (approval no. 18922, Shanghai, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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