Effect of puerarin on action potential and sodium channel activation in human hypertrophic cardiomyocytes

**Running title:** Puerarin on action potential and sodium channel

Yu-hui Lin¹,#, Xiao-Bin Ni²,#, Jian-wu Zhang³, Cai-wen Ou⁴, Xiao-qing He¹, Wen-jun Dai¹, Xi-ming Chen¹,* Min-sheng Chen⁴,*

¹The Third Affiliated Hospital of Guangzhou Medical University

²Shantou Central Hospital

³Nanfang Hospital, Southern Medical University

⁴Zhujiang Hospital, Southern Medical University

#These authors contributed equally to this work.

**Corresponding author**

Xi-ming Chen

Department of Cardiovascular Medicine, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou Guangdong 510150 China

Tel: 020-81292119

Email: gysycxm2019@163.com

Min-sheng Chen

Address: Department of Cardiology Heart Center, Zhujiang Hospital Southern Medical University, Guangzhou Guangdong 510280 China

Tel: 020-61648022
Email: gzminsheng@vip.163.com

**The word count:** 3554

**The figure/table count:** 8

**The table count:** 1

**Conflict of interest:** The authors declare that they have no conflict of interest.

**Data availability**
The datasets generated and analyzed during the present study are available from the corresponding author upon reasonable request.

**Author contributions**
Yu-hui Lin and Xiao-Bin Ni performed most of the investigation and data analysis and wrote the manuscript;
Jian-wu Zhang and Cai-wen Ou contributed to cell culture;
Xiao-qing He and Wen-jun Dai contributed to cell patch clamp experiments;
Xi-ming Chen and Min-sheng Chen contributed to interpretation of the data and analyses.
All authors have read and approved the manuscript.
Abstract

OBJECTIVE: To study the effect of puerarin on electrophysiology using a hypertrophic cardiomyocyte (HC) model.

MATERIALS AND METHODS: Human urine epithelial cells were used to generate the HC model (hiPSC-CM). Cardiomyocyte hypertrophy was induced by applying 10 nM endothelin-1 (ET-1). The effects of puerarin pre-treatment (PPr) and post-treatment (PPo) on action potential, sodium current (I_{Na}) activation and inactivation, and recovery following I_{Na} inactivation were tested using patch clamp electrophysiology.

RESULTS: Depolarization to repolarization 50% time (APD50) and repolarization 30% time (APD30) were significantly prolonged in the PPo and PPr groups compared to the controls. However, there were no significant differences in the action potential depolarization amplitude (APA) or the maximum depolarization velocity (Vmax) in phase 0. The PPr group had a slightly shortened APD90, and an extended APD50 and APD30, but did not exhibit any significant changes in stage A of APA and Vmax. The PPo group did not exhibit any significant changes in I_{Na}, while 12 hours of PPr improved I_{Na}. However, puerarin did not significantly affect the activation, inactivation, or recovery of the sodium channel.

CONCLUSIONS: Cardiomyocyte hypertrophy significantly decreased the Vmax of the action potential and the peak density of I_{Na}. PPr inhibited the decrease of Vmax and increased the peak density of I_{Na}. Thus, puerarin could be used to stabilize the electrophysiological properties of hypertrophic cardiomyocytes and reduce arrhythmias.

Key words: Induced pluripotent stem cells; Cardiomyocytes; Puerarin; Ion channel; Action potential
Abbreviations

APA: depolarization amplitude; BNP: brain natriuretic peptide; BR: beating rate; CEHCDCM: CardioEasy human cardiomyocyte differentiation complete medium; CEHMMMM: CardioEasy human myocardium maintenance medium; CEHMPCM: CardioEasy human myocardial purified complete medium; CI: cell index; dv/dt Max or Vmax: maximal depolarization velocity; ET-1: endothelin-1; HC: hypertrophic cardiomyocyte; hiPSCs: human induced pluripotent stem cells; hiPSC-CMs: hiPSC-cardiomyocytes; INa: sodium currents; JNK: Jun N-terminal kinase; KLM4: Kruppel-like factor 4; MAPK: mitogen-activated protein kinase; MYH6: α-cardiac myosin heavy chain; MYL2: myosin light chain 2; NKX2-5: NK2 homeobox 5; OCT4: octamer-binding transcription factor 4; PKC: protein kinase C; PLA2: phospholipases A2; PLC: phospholipase C; Ppo: puerarin post-treatment; PPr: puerarin pre-treatment; RP: resting potential; RTCA: real-time label-free cell analyzer; SOX2: sex determining region Y-box 2; TNNT2: troponin T2; Vmax: maximum depolarization velocity
Introduction

Cardiac hypertrophy refers to abnormal cardiac enlargement or thickness of the cardiac muscle. Cardiac hypertrophy is a compensatory mechanism that can be caused by physiological (exercise) or pathophysiological (hypertension or valvular disease) changes, resulting in altered cardiomyocyte size and extracellular matrix production. Prolonged pathophysiologic hypertrophy can damage cardiomyocyte elasticity, potentially resulting in heart failure. Cardiomyocyte decompensation, as well as decreased systolic and diastolic function, can also alter cardiomyocyte electrophysiological activity, thereby inducing the onset of arrhythmia.

Endothelin-1 (ET-1) is a twenty-one amino acid polypeptide that is a potent vasoconstrictor secreted by vascular endothelial cells and cardiomyocytes. Recent studies have shown that ET-1 levels in the myocardium are higher compared to levels in the circulation. ET-1 also varies compared to other myocardium hormones, such as angiotensin II, isoproterenol, growth hormone, and insulin-like growth factor-1. Thus, ET-1 is more likely to be a local regulator than a systemic hormone. ET-1 induces hypertrophic responses by activating fetal gene expression and promoting protein synthesis and accumulation to increase cell surface area and cell size. The signaling pathway induced by ET-1 in cardiac hypertrophy involves activation of phospholipase C (PLC), protein kinase C (PKC), phospholipase A2 (PLA2), Jun N-terminal kinase (JNK), and mitogen-activated protein kinase (MAPK).

Puerarin is an isoflavone extracted from the root of the leguminous creeper Pueraria (Radix puerariae) and can also be found in several plants and herbs, especially in the kudzu plant. Puerarin has multiple pharmacological properties and has been used to treat cardiovascular disorders, such as...
myocardial infarction and hypertensive angina pectoris\textsuperscript{7}. Puerarin confers cardio protection by inhibiting inward rectifier potassium current and inhibiting the L-type calcium channel\textsuperscript{8-10}.

Adult somatic cells have been successfully induced into pluripotent stem cells (hiPSCs), which are derived from autologous cells\textsuperscript{11-13}, and can be differentiated into many cell types, including cardiomyocytes (hiPSC-CMs)\textsuperscript{14}. hiPSC-CMs display various pharmacological and electrophysiological properties of human cardiomyocytes, including the ability to generate action potentials and respond to anti-arrhythmia drugs\textsuperscript{15, 16}. Previous studies have primarily used human embryonic kidney cells, Xenopus oocytes, and Chinese hamster ovary (CHO) cells for ion channel studies. However, these heterologous expression systems often lack the macromolecular complexes required to form ion channels in human cardiomyocytes. The integrity of the ion channel structure is essential for normal electrophysiological activity. The electrophysiological properties of the myocardium in transgenic animals are also different from those in human myocardium. However, hiPSC-CMs can genocopy and phenocopy properties of human hereditary heart disease. Therefore, given the difficulty in obtaining human ventricular myocytes, hiPSC-CMs are a good model to study human myocardial electrophysiological activity\textsuperscript{17}.

In view of the significant increase in the incidence of arrhythmia after cardiac hypertrophy, there are limited therapeutic options to prevent or treat arrhythmias. Therefore, we investigated the effects of puerarin on action potential geneation and sodium channel activity in ET-1-induced hypertrophic cardiomyocytes.

Material and methods
Ethics statement

Urine was obtained from a healthy donor who was informed of the study purpose and provided written consent. This study was authorized by the Ethics Committee of the Medical Faculty at Mannhein, The Third Affiliated Hospital of Guangzhou Medical University.

Generation of hiPSCs

hiPSCs were prepared from primary human epithelial cells obtained from the urine of the healthy donor using the Sendai virus (Invitrogen, Groningen, The Netherlands), which carries reprogramming factors [sex determining region Y-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4), Kruppel-like factor 4 (KLF4) and c-MYC], as previously described. To verify if the isolated hiPSCs were pluripotent, we stained the cells with the immunofluorescence marker Alkaline Phosphatase and measured teratoma formation.

hiPSC-CM differentiation

The hiPSCs were cultured in an incubator with 5% CO2 at 37°C in CardioEasy Human Cardiomyocyte Differentiation Complete Medium I (CEHCDCM) (CELLAPY, Beijing, China) for 48 hours, followed by CHCD complete medium III (CELLAPY, Beijing, China) for 24 hours, and then CEHCDCM II (CELLAPY, Beijing, China) for 48 hours. Differentiation started once the confluence rate of hiPSC-CMs reached 90%. The non-cardiomyocytes gradually died in the CardioEasy Human Myocardial Purified Complete Medium (CEHMPCM) (CELLAPY, Beijing, China), purifying the differentiated cultured cardiomyocytes (hiPSC-CMs). Following hiPSC differentiation, the culture medium was replaced for further testing.

Identification of myocardial specific markers
α-actinin (Cell Signaling, Danvers, MA, USA) and troponin T2 (TNNT2) (Abeam, Cambridge, UK) expression in hiPSC-CMs were detected using immunofluorescence. Expression of OCT4, NANOG, NK2 homeobox 5 (NKX2-5), myosin light chain 2 (MYL2), α-cardiac myosin heavy chain (MYH6), and MYH7 in the differentiated hiPSC-CMs were measured using RT-PCR. Briefly, cellular genomic RNA and cDNA were prepared using the Cell to cDNA kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Corresponding primers were designed, and RT-PCR was performed according to the manufacturer’s protocol.

**Cardiomyocyte hypertrophy model**

hiPSC-CMs were uniformly seeded into 24-well plates and divided into either the control or hypertrophic cardiomyocyte (HC) group. For cardiomyocyte hypertrophy induction, hiPSC-CMs were incubated with 10 nM ET-1 for 24 hours. A group of cells was also treated with 10 μm of the ET-1 inhibitor Bosentan (Selleck, USA, Cat: S305102) 24 hours following ET-1 incubation. The cell culture medium was changed every 24 hours. Expression of brain natriuretic peptide (BNP) (Abcam, UK, Cat: ab236101) and α-actinin (Cell Signaling, USA, Cat: #3134S) was detected using immunofluorescence. The ratio of MYH7/MYH6T in the HC group was determined using real-time PCR. Briefly, total RNA was extracted, and the corresponding cDNAs were reverse transcribed according to the instruction provided by the GoScript™ Reverse Transcription System kit (Promega; Madison, Wisconsin, USA). MYH7 and MYH6 expression were detected using real-time fluorescent quantitative PCR using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The mean RT-qPCR threshold (Ct) values of the reference gene (GAPDH) and target genes (MYH7 and MYH6) was calculated, and the ratio of MYH7/MYH6 was estimated. The primers were designed as follows: GAPDH-F: 5'-
ACAGCAACAGGGTGGTGAC-3'; GAPDH-R: 5'-TTTGAGGGTGCAGCGAACTT-3';
MYH6-F: 5'-CCGATGACAAGGAAGAGTTG-3'; MYH6-R: 5'-ATCTTGTCGAACCTGGTGG-3'; MYH7-F: 5'-AGTTCAACGCCTCAAAGAG-3'; MYH7-R: 5'-TCTGCCAGGTTGTCTTGTTC-3'.

Safety evaluation of puerarin on cardiomyocytes

The culture medium was aspirated from the matrix coated 96-well platinum plates (E-Plate 96 form ACEA Biosciences, San Diego, CA, USA) and then we added 50 μL of myocardial medium. Cells were cultured at 5% CO₂ at 37°C and then processed using real-time label-free cell analysis (RTCA) with the xCELLigence® RTCA MP instrument (ACEA Biosciences) to determine the baseline volume. Cardiomyocytes were seeded at 30,000 cells/well onto the pretreated 96-well platinum plates. The medium was changed every other day until the cells reached steady state (~3 - 4 days). Different concentrations of puerarin were then added to the wells. RCTA was continuously performed for 48 hours.

Patch-clamp

Action potential measurement

A capillary glass tube (Sutter Instruments, Novato, CA, USA) was stretched to form a recording electrode using a microelectrode puller (Sutter Instruments). The MP285 Microelectrode Manipulator (Sutter Instruments) was operated under an AE31 TrinocularAE30 Inverted Microscope (Motic, BC, Canada) to expose the recording electrode to the cells, and vacuum suction was applied to form a GΩ seal. Once the GΩ seal was formed, rapid capacitance
compensation was performed. The negative pressure was continued, and the cell membrane was suctioned for whole cell recording. We compensated for the slow capacitance and recorded the film capacitance and series resistance. No leakage compensation was applied, and changes in the action potential parameters were recorded.

The current recording scheme for action potential measurement was as follows: the current recorder [EPC-10 USB amplifier (HEKA)] was switched to the current clamp mode once the whole cell was sealed, and the cellular membrane current was clamped at 0 pA. The current was gradually decreased in 100 pA increments and recording continued until the action potential was excited. The test drug was then added, and the current was continuously recorded for 3 minutes. All data were stored in the PatchMaster (HEKA, Lambrecht, Germany) software. The extracellular solution used for patch clamping consisted of 140 mM NaCl, 10 mM HEPES, 3.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 1.25 mM NaH₂PO₄, at pH 7.4. The pipette solution used for patch clamping consisted of 140 mM K-gluconate, 5 mM NaCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 1 mM EGTA, and 2 mM Mg-ATP, at pH 7.2 (KOH adjusted).

**Sodium ion channel measurement**

IV-curve: The cell membrane potential was initially maintained at -90 mV, and then stepped from -120 mV to 100 mV in 5 mV step increments. Activation-curve: The cell membrane potential was initially maintained at -90 mV and then stepped to -120 mV for 200 ms. The cell membrane potential was then stepped from -80 mV to 100 mV in 5 mV step increments to detect the activation state of the channel. Inactivation-curve: The cell membrane potential was initially maintained at -90 mV, then stepped from -120 mV to 40 mV in 5mV step increments for 1,000
ms, and then continuously stepped to 0 mV for 50 ms.

**Sodium current detection**

Recovery-curve: The cell membrane potential was initially maintained at -90 mV, then stepped to -10 mV for 250 ms to inactivate the sodium current, then stepped to -90 mV to restore the sodium current. Finally, the cell membrane potential was stepped to -10 mV for 250 ms to detect the sodium current.

During the recordings of sodium current, the pipette solution contained 120 mM CsCl, 1 mM MgCl$_2$, 10 mM HEPES, 4 mM Mg-ATP, 10 mM EGTA, 0.3 mM Na$_2$-GTP, at pH 7.2 (adjusted with CsOH). The extracellular solution consisted of 140 mM NaCl, 3.5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 10 mM glucose, 1.25 mM NaH$_2$PO$_4$ 1, 0.1 mM CdCl, 1 mM 4AP, at pH 7.4 (adjusted with NaOH).

**Cell groups**

The experiment was divided into five groups: control group, HC group (10 nM ET-1 treatment for 24 hours), puerarin control group (200 μg/mL treatment for 12 hours), puerarin pre-treatment group (PPr) (pre-treatment with 200 μg/mL puerarin for 12 hour followed by 10 nM ET-1 induced cardiac hypertrophy), and puerarin post-treatment (PPo) group (10 nM ET-1 induced cardiac hypertrophy, followed by 200 μg/mL puerarin for 12 hours). Eight cells were independently tested in each group during the recording period, and all electrophysiological experiments were performed at 32 - 35°C.
**Statistical analysis**

Numerical data are presented as mean ± standard error of the mean (SEM). Comparisons were performed using an unpaired Student *t*-test (two-tails) and one-way ANOVA followed by Tukey’s post-test. Each data point was repeated from 8 - 10 independent samples. A *P* value < 0.05 was considered significant.

**Results**

**Differentiation and purification of hiPSC-CMs**

hiPSCs cultured with CEHDCM I for 48 hours displayed typical morphological deformation, and a large number of mesodermal cells migrated out from the clones (Figure 1A). After culturing the cells with CEHDCM III for 24 hours, the mesoderm cells continued to grow and increased in density (Figure 1B). After incubation with CEHDCM II for 48 hours, a large number of mesoderm cells differentiated into cardiomyocytes, displaying specific cardiomyocyte morphology and functional properties (Figure 1C). The differentiated cardiomyocytes were purified with CEH MPCM, while the non-cardiomyocytes gradually died (Figure 1D). The differentiated cardiomyocytes were maintained in CardioEasy Human Myocardium Maintenance Medium (CEHMMM) for 24 hours. The final purity of the differentiated cardiomyocytes exceeded 95% (Figure 1E). A timeline for hiPSC-CMs culture is shown in Figure 1F.

The differentiated cardiomyocytes were then identified using immunofluorescence detection of cardiomyocyte markers α-actinin and TNNT2. The cells stained positively for both α-actinin and TNNT2 (Figure 2A). Real-time PCR showed that the pluripotency-related genes NANOG and OCT4 were expressed in the hiPSCs; however, cardiomyocyte-related genes, including
NKX2-5, MYL2, MYH6, and MYH7, were not detected. Compared to the hiPSCs, all of the cardiomyocytes-related genes (NKX2-5, MYL2, MYH6, and MYH7), but not the pluripotency genes (OCT4, NANOG), were expressed in the differentiated cardiomyocytes (hiPSCs-CMs) (Figure 2B).

**Preparation of HC model**

After induction of the hiPSCs with 10 nM ET-1 for 24 hours, the cell morphology was indicative of hypertrophic cardiomyocytes and BNP and α-actinin expression was increased (Figure 3). Quantitative analysis of the fluorescence images showed that the expression of proBNP and α-actinin in the ET-1 group was significantly higher compared to the control group (P < 0.05). After treatment with Bosentan, the expression of proBNP and α-actinin was reduced compared to the ET-1 group (P < 0.05); however, the expression of these proteins was still higher compared to the control group (P < 0.05) (Figure 4). In addition, the MYH7/MYH6 ratio was significantly increased in the ET-1 treated cells compared to the control hiPSCs (P = 0.0422) (Figure 5). We found that the ET-1-treated hiPSCs differentiated into HCs (hiPSC-CMs), indicating that the cardiac hypertrophy model was successfully established. hiPSC-CM differentiation was ET-1 dependent since pre-treatment with Bosentan (an ET-1 antagonist) prevented hiPSC-CM differentiation (Figure 3).

**Safety evaluation of puerarin in cardiomyocytes**

We evaluated the influence of serial concentrations of puerarin on cell index (CI) and beating rate (BR) of the hiPSC-CM using RTCA. We found that puerarin did not cause significant cytotoxicity in the hiPSC-CMs at the ~200 μg/mL. No significant difference in CI
was observed between the different puerarin and negative control (0 μg/mL) groups at each time point. BR slowed after 12 hours of continuous observation. However, there was no correlation between functional effects and puerarin concentration (Figure 5). The highest concentration (200 μg/mL) of puerarin was 10 times greater than that normally found in the blood after 4 hours of clinical application. Since this concentration did not cause any cytotoxic effect on hiPSC-CMs, we selected this concentration for use in subsequent experiments.

**Puerarin effects on cardiomyocyte action potential**

The HC and the PPo groups had slightly prolonged depolarization at 90% of the repolarization time (APD90), significantly elongated depolarization started at 50% of the repolarization time (APD50), and 30% of the repolarization time (APD30). In addition, treatment extended the overall time course of the cardiomyocyte action potential. There was no significant difference in the depolarization amplitude (APA) of the hiPSC-CMs action potential in phase 0 among the HC and PPo groups. The maximal depolarization velocity (dv/dt Max or Vmax) in phase 0 was significantly reduced. The resting potential (RP) of the hi-PSC-CMs was slightly reduced and the frequency of spontaneous pulsation of the cells was accelerated. The puerarin and the PPr groups had a slightly shortened APD90, but prolonged APD50 and APD30, which slightly curtailed during the course of the full action potential. APA was not significantly different among the groups and there was no effect on Vmax and RP, but the spontaneous beat frequency of the cardiomyocytes slowed (Figure 6).

**Kinetic influence of puerarin on the sodium ion channel**
The I-V curve and sodium ion channel peak current density column were recorded in the control, HC, puerarin, PPr, and PPo groups. The original records of the $I_{\text{NA}}$ are shown in Figure 7. A typical current trace of the sodium channel is shown in Figure 8. Compared with the control group (-371.7 ± 97.76), the peak current density of the sodium channel in the HC group (-178.5 ± 38.57) was significantly decreased, indicating that the sodium channel was inhibited by ET-1. There were no significant sodium channel effects in the puerarin group (-357.9 ± 48.76).

Meanwhile, $I_{\text{NA}}$ did not significantly improve in the PPo group (-205.6 ± 16.56) compared to the HC group. However, PPr (-265.0 ± 45.14) did improve the $I_{\text{NA}}$ compared to the HC group. The recorded I-V curves and the current density histograms are presented in Figure 7A and 7B, respectively. With regards to the sodium channel activation-curve, the half-activation voltage (V50) and the slope of the curve (Slope) were not significantly different among the groups (Figure 7C). There were no significant differences in the V50 and Slope in the sodium channel inactivation – curve among the groups (Figure 7D). There were no significant differences in the sodium channel recovery time (Tau) among the groups (Figure 7-E). Comparison of the activation curve, inactivation curve V50, Slope, and recovery curve (Tau) between each group are shown in Table 1.

Discussion

Here, we demonstrated the ability to differentiate hiPSCs into cardiomyocytes (hiPSC-CMs) using a series of CEHCDCM cell culture mediums. The typical characteristics of the hiPSC-CMs were confirmed by: 1) immunofluorescence staining of cardiomyocyte markers, α-actinin and
TNNT2; 2) comparing expression of pluripotency-related genes (OCT4 and NANOG) and negative expression of cardiomyocyte-related genes (NKX2-5, MYL2, MYH6 and MYH7); 3) measuring spontaneous cardiomyocyte beats 10 days following differentiation onset; and 4) recording action potentials in the spontaneous beating cells.

Inherently, there are significant differences between experimental animal model cardiomyocytes and human cardiomyocytes. For instance, the number of beats per minute in the human myocardium are significantly different compared to rat, mouse, and rabbit myocardium. Our hiPSC-CM model displayed standard electrophysiological and biochemical properties of normal human cardiomyocytes, which can be stimulated by electrical excitation. Therefore, hiPSC-CMs are a good cellular model to investigate a variety of biochemical and electrophysiological properties in cardiomyocytes.

The cardiomyocyte hypertrophy model can be induced by angiotensin II, isoproterenol, growth hormone, insulin-like growth factor-1, and ET-1. Among them, isoproterenol is a synthetic chemical that is not naturally produced in the human body, while angiotensin II is a circulating hormone that does not increase significantly in the local myocardium. Growth hormone and insulin-like growth factor-1 can induce cardiac hypertrophy in vitro and in animal experiments, yet its concentration in humans is not high enough to cause cardiac hypertrophy. ET-1 can be synthesized by stimulated vascular endothelial cells and cardiomyocytes. ET-1 can accumulate in myocardial tissue through autocrine or paracrine mechanisms, leading to a series of pathophysiologial processes, such as cardiac hypertrophy. Therefore, the ET-1-induced HC model that we used in this study most closely mimicked the pathological state of human cardiac hypertrophy and is therefore an ideal model for drug testing.

Puerarin has been shown to influence ion channels in animal cardiomyocytes by inhibiting
the inward rectifier potassium channel\textsuperscript{8}, activating the calcium-sensitive potassium channel\textsuperscript{9}, and blocking the calcium channel\textsuperscript{10}. Most studies investigating the activity of puerarin on myocardial ion channels have generally been performed in animal-derived cardiomyocytes. Thus, the effects of puerarin on ion channels in human cardiomyocytes have not been reported. Experiments directly performed in human cardiomyocytes could closely represent human pathophysiology and therefore would be more valuable for developing future clinical applications to treat arrhythmias.

The patch clamp technique entails creating a tight seal between a microelectrode and a cellular membrane which allows one to record the electrical activity of ion channels with voltage or current clamping. In 1980, Dr. Sigwrth and Dr. Neher used a vacuum suction on the recording electrode to generate a high-resistance seal of 10-100 G\(\Omega\) between the cell membrane and microelectrode. This method greatly improved the patch clamp technique, which provided a reliable and sensitive technology to investigate ion channel function\textsuperscript{21}. Therefore, we used the whole-cell patch clamp method to study the action potential and sodium current of hypertrophic cardiomyocytes. The recorded action potentials of the hiPSC-CMs in this study were divided into five periods with patterns identical to those of other reported human ventricular myocardium action potentials\textsuperscript{22}. The results showed that the APD50 and APD30 of HCs and the overall action potential duration were prolonged compared to normal cardiomyocytes. The APA in phase 0 did not change; however, the V\textsubscript{max} was significantly reduced. PPo did not eliminate or reduce the above effects. Although the PPr group had no effect on APD50 and APD30 in HCs, it did improve V\textsubscript{max} in phase 0. Since the depolarization of ventricular myocytes in phase 0 is closely related to the influx of sodium ions, we next focused on the parameters of sodium ion channels.
The functional states of ion channels can be classified into resting state, activated state, and inactive state. In the resting state, the ion channel is closed. The ion channel will enter the active state from the resting state when the membrane potential changes, and at this moment the ion can pass through the cell membrane. This ion influx will change the membrane potential and cause the ion channel to enter the inactive state, which in turn will close the ion influx through the cell membrane. Along with constant depolarization, the ion channel re-enters the resting state. To determine the effect of puerarin treatment on sodium channels in HCs, we assessed the I-V curve, activation curve, inactivation curve, and recovery curve after inactivation of the sodium channel. Among these, the I-V curve reflects the influence of external factors on ion selectivity and rectification characteristics of the channel. The activation curve, the inactivation curve, and the post-inactivation recovery curve comprise the ion channel voltage-dependent gating characteristics. During myocardial depolarization, sodium ions flow through the sodium ion channel into the cell along the chemical gradient, which is the main inward current that plays a crucial role in the rapid rise of the membrane potential and the action potential. The gene encoding the sodium channel of human cardiomyocytes arises from the third pair of chromosomes, called SCN5A, and its abnormal expression can lead to congenital long QT syndrome, Brugada syndrome, and tachyarrhythmia. Our experimental data indicated that the peak density of $I_{Na}$ decreased after myocardial hypertrophy, but the shape of the I-V curve remained unchanged, suggesting that the ion channel selectivity and rectification characteristics did not change. $I_{Na}$ is closely related to phase 0 of the action potential, and the decrease in current density is an important underlying cause of the decrease in $V_{max}$. This change can lead to impaired impulse conduction and the formation of excitatory re-entry arrhythmias. We found that PPO did not increase the peak density of $I_{Na}$; however, PPr elevated the peak density of $I_{Na}$.
Therefore, PPr can increase the rate of action potentials in the 0 phase and accelerate sodium ion inflow and impulse conduction to prevent excitatory re-entry arrhythmias. In addition, PPr stabilized the electrophysiological properties of cardiomyocytes and reduced the heterogeneity between cardiomyocytes. The preventive activity of puerarin on myocardial depolarization and repolarization dispersion reduced the incidence of re-entry and delayed depolarization, as well as decreased the occurrence and progression of ventricular hypertrophy arrhythmia. Interestingly, there was no significant effect on the activation, inactivation, and recovery of the sodium channel among the groups, indicating that the voltage-dependent gating characteristics of the sodium ion channel did not significantly change.

**Conclusion**

A human hypertrophic cardiomyocyte model was successfully obtained. Patch-clamp study indicated that pre-treatment with puerarin can improve the depolarization rate of the hypertrophic cardiomyocytes by increasing the peak density of INa current. This result suggested that puerarin has a certain preventive effect on re-entrant excitation arrhythmia as well as positive significance on the electrophysiological stability of hypertrophic cardiomyocytes.

**Acknowledgments**

This project was supported by the National Natural Foundation of China (U1501222, 31771060, 31700844), Guangdong Medical Research Foundation (2018 A2018563, 2018 A2018097).

**Data availability**
The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.
References

1. Haider AW, Larson MG, Benjamin EJ, Levy D. Increased left ventricular mass and hypertrophy are associated with increased risk for sudden death. J Am Coll Cardiol 1998; 32: 1454-1459.

2. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988; 332: 411-415.

3. Russell FD, Molenaar P. The human heart endothelin system: ET-1 synthesis, storage, release and effect. Trends Pharmacol Sci 2000; 21: 353-359.

4. Ito H, Hirata Y, Hiroe M, Tsujino M, Adachi S, Takamoto T, Nitta M, Taniguchi K, Marumo F. Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. Circ Res 1991; 69: 209-215.

5. Barac YD, Zeevi-Levin N, Yaniv G, Reiter I, Milman F, Shilkrut M, Coleman R, Abassi Z, Binah O. The 1,4,5-inositol trisphosphate pathway is a key component in Fas-mediated hypertrophy in neonatal rat ventricular myocytes. Cardiovasc Res 2005; 68: 75-86.

6. Yeung DK, Leung SW, Xu YC, Vanhoutte PM, Man RY. Puerarin, an isoflavonoid derived from Radix puerariae, potentiates endothelium-independent relaxation via the cyclic AMP pathway in porcine coronary artery. Eur J Pharmacol 2006; 552: 105-111.

7. Zhou YX, Zhang H, Peng C. Puerarin: a review of pharmacological effects. Phytother Res 2014; 28: 961-975.

8. Zhang H, Zhang L, Zhang Q, Yang X, Yu J, Shun S, Wu Y, Zeng Q, Wang T. Puerarin: a novel antagonist to inward rectifier potassium channel (IK1). Mol Cell Biochem 2011; 352: 117-123.
9. Gao Q, Yang B, Ye ZG, Wang J, Bruce IC, Xia Q. Opening the calcium-activated potassium channel participates in the cardioprotective effect of puerarin. Eur J Pharmacol 2007; 574: 179-184.

10. Qian Y, Li Z, Huang L, Han X, Sun J, Zhou H, Liu Z. Blocking effect of puerarin on calcium channel in isolated guinea pig ventricular myocytes. Chin Med J (Engl) 1999; 112: 787-789.

11. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007; 131: 861-872.

12. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126: 663-676.

13. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, II, Thomson JA. Induced pluripotent stem cell lines derived from human somatic cells. Science 2007; 318: 1917-1920.

14. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA, Kamp TJ. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res 2009; 104: e30-41.

15. Germanguz I, Sedan O, Zeevi-Levin N, Shtrichman R, Barak E, Ziskind A, Eliyahu S, Meiry G, Amit M, Itskovitz-Eldor J, Binah O. Molecular characterization and functional properties of cardiomyocytes derived from human inducible pluripotent stem cells. J Cell Mol Med 2011; 15: 38-51.

16. Ma J, Guo L, Fiene SJ, Anson BD, Thomson JA, Kamp TJ, Kolaja KL, Swanson BJ, January CT. High purity human-induced pluripotent stem cell-derived cardiomyocytes:
electrophysiological properties of action potentials and ionic currents. Am J Physiol Heart Circ Physiol 2011; 301: H2006-2017.

17. Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, Winterstern A, Feldman O, Gepstein A, Arbel G, Hammerman H, Boulos M, Gepstein L. Modelling the long QT syndrome with induced pluripotent stem cells. Nature 2011; 471: 225-229.

18. Zhou T, Benda C, Duzinger S, Huang Y, Li X, Li Y, Guo X, Cao G, Chen S, Hao L, Chan YC, Ng KM, Ho JC, Wieser M, Wu J, Redl H, Tse HF, Grillari J, Grillari-Voglauer R, Pei D, Esteban MA. Generation of induced pluripotent stem cells from urine. J Am Soc Nephrol 2011; 22: 1221-1228.

19. Lin YH, Chen XM, Zhang JW, He XQ, Dai WJ, Chen MS. Preclinical study on induction of pluripotent stem cells from urine of dilated cardiomyopathy patients. Eur Rev Med Pharmacol Sci 2016; 20: 1450-1457.

20. Cittadini A, Stromer H, Katz SE, Clark R, Moses AC, Morgan JP, Douglas PS. Differential cardiac effects of growth hormone and insulin-like growth factor-1 in the rat. A combined in vivo and in vitro evaluation. Circulation 1996; 93: 800-809.

21. Sigworth FJ, Neher E. Single Na+ channel currents observed in cultured rat muscle cells. Nature 1980; 287: 447-449.

22. Honda M, Kiyokawa J, Tabo M, Inoue T. Electrophysiological characterization of cardiomyocytes derived from human induced pluripotent stem cells. J Pharmacol Sci 2011; 117: 149-159.

23. Fux JE, Mehta A, Moffat J, Spafford JD. Eukaryotic Voltage-Gated Sodium Channels: On Their Origins, Asymmetries, Losses, Diversification and Adaptations. Front Physiol 2018; 9: 1406.
24. Patterson E, Scherlag BJ, Lazzara R. Rapid inward current in ischemically-injured subepicardial myocytes bordering myocardial infarction. J Cardiovasc Electrophysiol 1993; 4: 9-
Figure legends

Figure 1. Differentiation and purification of hiPSC-CMs. A. After culturing with CEHCDCM I medium for 48 hours, the hiPSCs showed typical deformation (short arrow) and a large number of mesodermal cells migrated out from the clones (long arrow). Scale bar, 300 μm.

B. Following incubation with CEHCDCM III for 24 hours, the mesoderm cells continued to grow and increase in density. Scale bar, 300 μm. C. After incubation with CEHCDCM II for another 48 hours, a large number of mesodermal cells differentiated into cardiomyocytes (hiPSC-CMs, with typical myocardial morphology) (short arrow) and began to beat (long arrow). Scale bar, 300 μm. D. The differentiated hiPSC-CMs were purified in CEHMPCM, and the non-cardiomyocytes began to die. Scale bar, 100 μm. E: The differentiated hiPSC-CMs were stable after incubation with CEHMMM for 24 hours. Scale bar, 100 μm. F. Figure shown a schedule of cardiomyocytes (hiPSC-CMs) differentiation from hiPSCs. CEHCDCM, CEHCDCM I, CEHCDCM II, and CEHCDCM III were different differentiation mediums purchased from CELLAPY (Beijing, China). D1, D3, D5, and D7 represented day 1,3, 5, and 7, respectively.

Figure 2. Identification of the differentiated hiPSC-CMs. A. Immunofluorescence staining of hiPSC-CMs with α-actinin (green, right-up) and TNNT2 (red, left-down) antibodies. Nuclei were stained using DAPI (blue, up-left). Scale bar, 25 μm. B. Gene expression of pluripotency-
related genes (OCT4 and NANOG) and myocardial specific genes (NKX2-5, MYL2, MYH6 and MYH7) was measured using RT-PCR. GAPDH expression was used as the reference gene.

**Figure 3. Immunofluorescence staining of the ET-1-induced cardiomyocytes (hiPSC-CM).**

A. After induction of hiPSCs with 10 nM ET-1 (middle panels) for 24 h, expression of BNP (red) and α-actinin (green) were enhanced, and the cell shape was increased compared to the non-treated cells (up panels) and Bosentan (ET-1 antagonist) treated cells (lower panels). Scale bar, 40 μm. B. The levels of proBNP and α-actinin were significantly increased in the ET-1 group compared to the control group. However, Bosentan treatment reduced the expression of proBNP and α-actinin; however, both were still expressed at lower levels compared to the control group. *: $P < 0.05$ compared with control group.

**Figure 4. Alteration of MYH7/MYH6 ratio after ET-1 induction.** ET-1 induction significantly increased the ratio of MYH7/MYH6 in the hiPSC-CMs compared to the non-ET-1 treated cells (native) as measured by RT-PCR.

**Figure 5. Toxic effect of serial concentrations of puerarin on cardiomyocytes.** A. Cell survival (CI) was detected under the indicated treatment concentrations of puerarin from 0 to 40 hours. B. Statistical analysis of the CI data in the groups treated with different concentration of puerarin at 0, 1, 2, 4, 8, 12, and 24 hours. C. The beating rate (BR) was recorded under the indicated treatment concentrations of puerarin from 0 to 40 hours. D. Statistical analysis of the BR data in the groups treated with different concentration of puerarin at 0, 1, 2, 4, 8, 12, and 24
hours. N.S: $P > 0.05$.

**Figure 6. Activity of puerarin on action potential.** Action potentials were recorded in the indicated groups. APA: action potential phase 0 depolarization amplitude; APD30: depolarization start to repolarization 30% time; APD50: depolarization start to repolarization 50% time; APD90: depolarization start to repolarization 90% time; $dv/dt$ Max (Vmax): maximum depolarization velocity in phase 0; HR: number of beats per minute in myocardium; RP: resting membrane potential; Action potential: action potential map of hiPSC-CMs. *: $P < 0.05$ compared to the control group. N.S: $P > 0.05$.

**Figure 7. Electrophysiology measurements of the sodium ion channel.**

A. IV curves: cell membrane potential in the indicated groups was initially maintained at -90 mV, and then gradually stepped from -120 to -100 mV in 5 mV step intervals. B: The average of the peak currents in each group. C: Activation curves of sodium channel: cell membrane potential in the indicated groups was initially maintained at -90 mV, and gradually stepped to -120 mV for 200 ms, and then stepped from -80 mV to 100 mV in 5 mV step intervals to detect the activation state of the channel. D: Inactivation curves of sodium channel: cell membrane potential in the indicated groups was initially maintained at -90 mV, and then gradually stepped from -120 to 40 mV for 1,000 ms, then stepped to 0 mV for 50 ms in 5 mV step intervals to detect sodium current. E: Recovery curves of sodium channel: cell membrane potential in the indicated group was initially maintained at -90 mV, and then gradually stepped to -10 mV for 250 ms to inactivate sodium current, then stepped to -90 mV for different durations to recover the sodium current, and then finally stepped to -10 mV for 250 ms to detect the sodium current. Groups: control (●).
myocardial hypertrophy (▲), puerarin (■), puerarin postconditioning (▼), and puerarin preconditioning (♦). *: $P < 0.05$ or NS: $P > 0.05$ compared with the control group.

**Fig. 8. Typical current trace of sodium channel**

Cellular membrane potential was maintained at -90mV, and then increased at 5mV interval in the control, puerarin, ET-1, puerarin preconditioning, and puerarin postconditioning group, respectively. Once the cellular membrane potentials stepped to 100mV, the current mode of iNa was recorded.
Table 1. Average data of activation, inactivation, and recovery curves of each group

|                  | Control                  | Hypertrophy cardiomyocyte | Puerarin post-treatment | Puerarin pre-treatment |
|------------------|--------------------------|---------------------------|-------------------------|------------------------|
| Act. Curv V50    | -34.40±0.69              | -42.22±1.61               | -39.68±1.01             | -32.34±0.55            |
| Act. Curv Slope  | 4.54±0.61                | 6.74±1.47                 | 5.91±0.91               | 4.36±0.48              |
| Inact. Curv V50  | -81.90±0.60              | -80.66±0.52               | -80.22±0.43             | -77.51±0.24            |
| Inact. Curv Slope| 9.09±0.53                | 8.56±0.46                 | 8.10±0.38               | 7.45±0.21              |
| Rec. Curv Ta     | 8.869 to 11.39           | 9.023 to 10.49            | 6.326 to 7.156          | 6.529 to 7.336         |

Act. Curv: Activation curve; Inact. Curv: Inactivation curve; Rec. Curv: Recovery curve; V50 and Slope are expressed as mean ± standard deviation; Tau is expressed in 95% confidence interval. The activation curve, V50, Slope of the inactivation curve, and the Tau of the recovery curve of each group were not statistically different compared to the control group.
A. Transform of Normalized Cell Index

B. Cell index

C. Transform of Normalized Beating Rate

D. Beating Rate
