Ghrelin protects the myocardium with hypoxia/reoxygenation treatment through upregulating the expression of growth hormone, growth hormone secretagogue receptor and insulin-like growth factor-1, and promoting the phosphorylation of protein kinase B

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Abstract. Ghrelin is an endogenous ligand of growth hormone (GH) secretagogue receptor (GHSR) and has a number of biological effects, including heart protection. The present study aimed to reveal the positive effect of ghrelin on myocardium with hypoxia/reoxygenation (H/R) treatment and the involved molecular mechanisms. Successful construction of lentiviral expression vector (ghrelin-pLVX-Puro) was confirmed by colony polymerase chain reaction (PCR) verification. Primary rat cardiac myocytes were isolated and identified by immunofluorescence staining. Existence of red fluorescence of α-sarcomeric actinin indicated the successful isolation. Following ghrelin transfection and H/R treatment, primary cells were divided into four groups: Control, H/R, empty (empty pLVX-Puro + H/R) and ghrelin (ghrelin-pLVX-Puro + H/R). Cell viability and apoptosis were evaluated by Cell Counting Kit-8 (CCK-8) and Hoechst staining, respectively. The cell viability in the ghrelin group was significantly higher than that in the empty control group (P<0.05). The apoptosis rate in the ghrelin group was significantly lower than that in the empty control group (P<0.05). An *ex vivo* rat cardiac perfusion model was established. Following ghrelin incubation and H/R treatment, *ex vivo* myocardium was divided into four groups: Control, sham, H/R and ghrelin (ghrelin + H/R). Immunohistochemical analysis demonstrated that ghrelin increased the integrity of cardiac myocytes, and decreased shrinkage and apoptosis. mRNA and protein expression levels of GH, GHSR, insulin-like growth factor-1 (IGF-1), protein kinase B (Akt), phosphorylated Akt (p-Akt) were determined by reverse transcription (RT)-PCR, western blot analysis and immunohistochemical analysis. Ghrelin upregulated the mRNA and protein expression levels of GH, GHSR and IGF-1, and increased the ratio of p-Akt to Akt protein level (p-Akt/Akt) in cardiac myocytes and myocardial tissues with H/R treatment. In conclusion, ghrelin protected the myocardium with H/R treatment through upregulating the expression of GH, GHSR and IGF-1, and promoting the phosphorylation of Akt. This would provide promising insights into the treatment of hypoxic myocardial injury by ghrelin.

Introduction

In light of social continuous improvement, rapid economic development and aggravation of population aging, the number of patients with cardiovascular disease has increased annually and cardiovascular disease has become an important factor that threatens human health (1). The tolerance of myocardial cells to hypoxia injury is poor, and hypoxia-ischemia can lead to abnormal cardiac electric activity, necrosis of myocardial cells and cell apoptosis, which may induce various cardiovascular diseases (2-4). At present, there is no known radical cure. The normal physiological function of the heart can be maintained only by pharmacological remission and stent implantation. Therefore, it is urgent to develop novel treatment methods to improve and repair the damaged myocardial cells, eventually achieving a radical cure.

Ghrelin is a specific growth hormone (GH) secretagogue (GHS) containing 28 amino acid residues, which was identified in 1999 (5). It is an endogenous ligand for the GHS receptor (GHSR). GHSR abundantly exists in the cardiovascular system. The expression of GHSR mRNA is also detected in human

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atrial and ventricular myocytes. Ghrelin combines with a G protein coupled receptor to regulate GH (6-8). Ghrelin and its receptors are widely distributed in all tissues and organs, and have numerous biological effects, including increasing appetite, promoting fat accumulation and protecting the heart (8,9). In particular, as a vasoactive peptide, it has a number of protective and repair effects on the cardiovascular system (8,9). A number of studies have indicated that in addition to GHSR, ghrelin may have other unknown receptors in the cardiovascular system and may work independently of GH (10-13). The present study investigated the effect of ghrelin on myocardial repair and function through investigating the expression of ghrelin in the myocardial cells to provide solid support for the treatment of hypoxic myocardial injury by ghrelin.

Ghrelin has been known to exert positive effects in cardiovascular protection and repair. Although based on a cardiopulmonary bypass (CPB) study, Cao *et al* (14) reported that cardioprotective effects elicited by ghrelin may contribute toward the inhibition of inflammatory response through the protein kinase B (Akt) activated pathway, this is only one of a number of theories regarding the mechanism of ghrelin and there remains a great possibility of other mechanisms of action. Insulin-like growth factor-1 (IGF-1) is a type of active polypeptide that is necessary for GH to produce a physiological effect. IGF-1 has functions of regulating physiological and pathological states of the heart, relaxing blood vessels, decreasing vascular resistance and increasing cardiac blood flow (12). Therefore, the expression of IGF-1 is an important indicator of the normal function of myocardial cells and tissues. Akt is a kind of Ser/Thr protein kinase and serves an important role in cell survival and apoptosis (15). Akt is a significant component of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway that is a classic signaling pathway serving an important role in numerous physiological and pathological processes, including cell survival, differentiation, growth and apoptosis via regulating gene expression (16). Akt is generally overexpressed in damaged or apoptotic cells, thereby promoting the PI3K/Akt signaling pathway to aggravate cell apoptosis. By contrast, Akt will be phosphorylated in normal cells to inhibit its activity, such that the level of phosphorylated Akt (p-Akt) will be upregulated and the cells or tissues are improved and repaired (17,18).

Therefore, the present study further revealed the molecular mechanism of ghrelin expression improving and repairing cardiac myocytes and myocardium by investigating GH, GHSR, IGF-1, Akt and p-Akt targets associated with cardiomyocyte metabolism and apoptosis. This would also provide promising insights into the treatment of hypoxic myocardial injury by ghrelin.

Materials and methods

Materials and animals. BSA blocking buffer (5%), typsin-EDTA (0.25%) and type II collagenase were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). 3,3'-Diaminobenzidine (DAB) developing kit (cat. no. CW0125), TRIzol reagent (cat. no. CW0580S), Ultrapure RNA extraction kit (cat. no. CW0581M), HiFiScript cDNA synthesis kit (cat. no. CW2569M), UltraSYBR mixture (cat. no. CW0957M) and 2X Taq MasterMix were obtained

from CWbio Co., Ltd. (Beijing, China). DMEM/F12 (1:1) and Lipofectamine 3000 reagent were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Fetal bovine serum (FBS; cat. no. SKU 04-007-1A) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Hoechst apoptosis kit was purchased from Beyotime Institute of Biotechnology (Haimen, China). Endo-free plasmid mini kit II (cat. no. D6950-01) was obtained from Omega Bio-Tek, Inc. (Norcross, GA, USA). TIANgel midi purification kit (cat. no. DP209) was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). pLVX-Puro vector (VECT231322) was purchased from Huayueyang Bio (Beijing, China). pUC57 plasmid was purchased from BioVector NTCC, Inc. (Beijing, China). Ghrelin-pUC57 plasmid was routinely cloned in our laboratory. Mouse anti-GH antibody (cat. no. ab9821; dilution, 1:1,200), rabbit anti-IGF-1 antibody (cat. no. ab182408; dilution, 1:1,000), rabbit anti-Akt antibody (cat. no. ab81283; dilution, 1:1,000) and rabbit anti-p-Akt antibody (cat. no. ab38449; dilution, 1:1,000) were purchased from Abcam (Cambridge, MA, USA). Mouse anti-β-actin antibody (cat. no. TA-09; dilution, 1:2,000), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L; cat. no. ZB-2305; dilution, 1:2,000) and HRP-conjugated goat anti-rabbit IgG (H+L; cat. no. ZB-2301; dilution, 1:2,000) were obtained from OriGene Technologies, Inc. (Beijing, China). Rabbit anti-GH antibody (cat. no. bs-0467R), rabbit anti-GHSR antibody (cat. no. bs-11529R; dilution, 1:1,200), rabbit anti-IGF-1 receptor antibody (cat. no. bs-0227R), rabbit anti-Akt antibody (cat. no. bs-0115R), rabbit anti-α-sarcomeric actinin antibody (cat. no. bs-10367R) and ghrelin (cat. no. bs-0467P) were from BIOSS (Beijing, China). HRP-conjugated polymer anti-rabbit IgG (cat. no. SV0002) was obtained from Boster Biological Technology (Pleasanton, CA, USA).

A total of 28 specific pathogen-free (SPF) male Sprague Dawley (SD) rats (aged 8 weeks; weight, 300±30 g) and 8 SPF neonatal SD rats (4 male and 4 female; aged 1-3 days) were obtained from Hunan Slac JD Laboratory Animal Co., Ltd. [License SCXK(XIANG)2016-0002; Hunan, China]. The rats were housed with *ad libitum* access to water and food in an environment of 20-26˚C, 40-70% relative humidity and a 12/12 h light/dark cycle. The study protocol was reviewed and approved by the Ethics Committee of Children's Hospital of Suzhou University (approval no. 2016LW009).

Construction of ghrelin expression vector. Ghrelin-pUC57 plasmid was digested by incubating with *Bam*HI and *Eco*RI at 37˚C for 2 h. The enzyme digestion system (50.0 μl) contained 10X Tango buffer 5.0 μl, ghrelin-pUC57 (1 μg) 4.0 μl, *Bam*HI (10 U) 1.0 μl, *Eco*RI (10 U) 1.0 μl and ddH$_2$O 39.0 μl. Next, the product was separated by 1% agarose gel electrophoresis. The gel was visualized on an electrophoresis imaging system (Tanon1600; Tanon Science and Technology Co., Ltd., Shanghai, China). Target fragment (363 bp) was harvested quickly under the imaging system to prevent the destruction of the gene fragments by ultraviolet radiation. DNA was recovered from the harvested gel with a gel extraction kit according to the manufacturer's protocol.

pLVX-Puro vector was digested with *Bam*HI and *Eco*RI at 37˚C for 2 h. The enzyme digestion system (10.0 μl) was composed of 10X Tango buffer 1.0 μl, pLVX-Puro vector

(0.5 μg) 2.0 μl, *Bam*HI (10 U) 1.0 μl, *Eco*RI (10 U) 1.0 μl and ddH$_2$O 5.0 μl. The product was also separated by 1% agarose gel electrophoresis. The gel was visualized on an electrophoresis imaging system (Tanon1600; Tanon Science and Technology Co., Ltd.). Target fragment (8,102 bp) was harvested and recovered using a gel purification kit according to the manufacturer's protocols.

The ghrelin gene was ligated to pLVX-Puro vector in a 20 μl system (linear vector 4.0 μl, target gene 1.0 μl, 10X ligation buffer 2.0 μl, T4 DNA ligase (5 U/μl) 0.3 μl and ddH$_2$O 12.7 μl) at 22˚C for 3 h. Next, the ligated product was transformed into DH5α. The transformation product was coated on an LB plate containing 100 μg/ml ampicillin and incubated at 37˚C for 22 h.

Two single colonies were selected from the ghrelin-pLVX-Puro plate following overnight incubation to perform colony polymerase chain reaction (PCR) verification. The PCR amplification system (20.0 μl) was comprised of 2X Taq MasterMix 10.0 μl, template 1.0 μl, forward primer (10 μm) 1.0 μl, reverse primer (10 μm) 1.0 μl and ddH$_2$O 7.0 μl. Sequences of the forward and reverse primers were: 5'-CACGCTGTTTTG ACCTCCAT-3' and 5'-GGATGTGGAATGTGTGCGAG-3'. PCR parameters were as follows: Pre-denaturation at 94˚C for 2 min, denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, elongation at 72˚C for 45 sec (30 cycles of denaturation to elongation), sufficient elongation at 72˚C for 10 min. The PCR product was separated by 1% agarose gel electrophoresis. The gel was visualized on an electrophoresis imaging system (Tanon1600; Tanon Science and Technology Co., Ltd.).

The ghrelin-pLVX-Puro colonies were incubated in LB broth containing 100 μg/ml ampicillin overnight. The ghrelin-pLVX-Puro vectors were extracted using the plasmid extraction kit according to the manufacturer's protocols for the subsequent experiments.

Isolation and identification of primary neonatal rat cardiac myocytes. A total of 8 neonatal SD rats (aged 1-3 days) were anaesthetized by inhaling isoflurane (2%) and were sacrificed by cervical dislocation. Following the heart being isolated, the apex cordis was obtained using scissors and immersed in precooled PBS. The apex cordis was washed three times with PBS to remove residual blood and uniformly cut into ~1 mm^3 fragments in 0.1% trypsin. The apex cordis fragments were trypsinized in 0.1% trypsin at 37˚C for 6 min. Following continuous agitating and natural sedimentation, the supernatant was removed. Next, the tissues were trypsinized in a mixture of 0.08% trypsin and 0.05% type II collagenase at 37˚C for 5 min. During this 5 min, agitating was conducted for 3 min. The supernatant was collected into another pre-cooled centrifuge tube and an equal volume of DMEM/F12 containing 10% FBS was added to terminate the trypsinization. The aforementioned trypsinization steps were repeated 3-4 times. All the trypsinized mixture was filtered into centrifuge tubes through a 200-mesh sieve. The filtrate was centrifuged at 800 x g at 4˚C for 8 min and the pellets were resuspended in 20% DMEM/F12. Following transfer to culture plates, cells were cultured at 37˚C in 5% CO$_2$.

Cells were washed with PBS three times for 3 min each time and fixed in 4% paraformaldehyde at room temperature for 15 min. Following washing with PBS again, cells were incubated in 0.5% Triton X-100 at room temperature for 20 min. Following washing with PBS, cells were incubated in 5% BSA buffer at 37˚C for 30 min. The BSA buffer was removed and diluted rabbit anti-α-sarcomeric actinin antibody (1:300) was added at 4˚C overnight. Cells were washed with PBS and incubated in secondary antibody buffer (1:200) at 37˚C for 30 min. Following washing, cells were stained in 4',6-diamidino-2-phenylindole (DAPI) solution for 5 min in the dark. The remaining DAPI was removed by PBS washing. Finally, the culture dish was mounted with 20% glycerin and visualized under a fluorescence microscope (magnification, x200).

Cell transfection and hypoxia/reoxygenation (H/R) treatment. Cells were divided into four groups: Control, H/R, empty (empty pLVX-Puro plasmid + H/R) and ghrelin (ghrelin-pLVX-Puro plasmid + H/R). The plasmid was mixed with Lipofectamine 3000 reagent according to the manufacturer's protocol. When cell confluence reached 80%, cells were incubated with a mixture of plasmid and Lipofectamine 3000 at 37˚C and 5% CO$_2$ for 4 days. Subsequently, hypoxia (4 h) and reoxygenation (1 h) treatments were performed. Cells in the control group did not undergo any treatments, including transfection or H/R. H/R treatment is a common method used to establish the model of myocardial injury (19).

Cell viability. At 24, 48 and 72 h after the aforementioned treatments, Cell Counting Kit-8 (CCK-8) reagent (10 μl) was added to each well and then cells were cultured at 37˚C in 5% CO$_2$ for 4 h. The absorbance was determined at 550 nm on a microplate reader. The cell viability was calculated accordingly.

Cell apoptosis. Cells were seeded into the 6-well plate where coverslips had been laid. Once the confluence reached ~80%, the treatments, including transfection and/or H/R were conducted. The culture medium was discarded later and the cells were incubated in 4% paraformaldehyde at room temperature for 10 min. Subsequently, the cells were washed twice with PBS for 3 min each time. Hoechst 33258 (0.5 ml) was added to each well for 5 min. The cells were washed twice with PBS again. A drop of antifading mounting medium (S2100; Beijing Solarbio Science & Technology Co., Ltd.) was added to the slide and the slide was then covered with the coverslip carrying the cells. Eventually, the slide was visualized under a fluorescence microscope (magnification, x100; IX51; Olympus Corporation, Tokyo, Japan) with excitation at 350 nm and emission at 460 nm.

Rat cardiac perfusion tests ex vivo. K-H buffer (600 ml) was added to the reservoir of a Langendorff cardiac perfusion system (Beijing, Zhishuduobao Biological Technology, Beijing, China) and the temperature was adjusted to 37˚C. Next, a gas mixture of 95% O$_2$-5% CO$_2$ was aerated for 30 min. A total of 28 SD rats (aged 8 weeks) were anesthetized by intraperitoneal injection of 1% pentobarbital sodium at a dosage of 45 mg/kg. Immediately, the heart was placed in the reservoir containing oxygenic K-H buffer at 37˚C. Other tissues around the heart were removed and the remaining blood in the atria and ventricles was extruded by gently squeezing the heart with

cotton swabs. Retrograde perfusion was performed from the aortic cannula. The heart was fixed with 4-0 sutures. Coronary ischemia and reperfusion were controlled by the switch of perfusion pathway. The flow rate of the perfusate for balancing was ~15 ml/min.

Tests were divided into four groups: Control, sham, H/R and ghrelin (ghrelin + H/R) (n=7). The untreated hearts served as the control. In the sham group, the balancing perfusion was conducted for 20 min. In the H/R group, following balancing for 20 min, improved Thomas II cardioplegic solution was perfused for 3 min to induce cardiac arrest and then the perfusion was stopped for 30 min. Subsequently, oxygenic K-H buffer was perfused again for 2 h to induce cardioversion. In the ghrelin group, following balancing for 20 min, ghrelin (5 mg/l) was perfused for 15 min and then the normal aerobic perfusion was restored for 15 min. Subsequently, the H/R treatment was performed as demonstrated in the H/R group.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from multiple primary cardiac myocytes and *ex vivo* myocardial tissues following various treatments using TRIzol reagent according to the manufacturer's protocol. The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm. Next, the RNA was reverse transcribed to cDNA using a HiFiScript cDNA synthesis kit according to the manufacturer's protocol. The reverse transcription system (20 μl) was comprised of dNTP Mix (4 μl), primer Mix (2 μl), RNA template (7 μl), 5X RT Buffer (4 μl), dithiothreitol (DTT, 2 μl) and HiFiScript (1 μl). Sequences of the primers, which were synthesized by General Biosystems (Anhui, China), are presented in Table I. The PCR system (25 μl) comprised RNase free dH$_2$O (9.5 μl), cDNA/DNA (1 μl), forward primer (1 μl), reverse primer (1 μl) and 2X UltraSYBR Mixture (12.5 μl). Reaction parameters were as follows: Pre-denaturation at 95˚C for 10 min, denaturation at 95˚C for 10 sec, annealing at 58.5˚C for 30 sec and elongation for 30 sec at 72˚C, for 40 cycles. Dissociation curve was analyzed as follows: 15 sec at 95˚C, 1 min at 58.5˚C, 15 sec at 95˚C, 15 sec at 58.5˚C and 15 sec at 58.5˚C, and measured stepwise from 95˚C, every 0.5˚C. It was finally evaluated on a RT-PCR detection system (CFX Connect™; Bio-Rad, Laboratories, Inc., Hercules, CA, USA). β-actin served as an internal control and the expression level relative to β-actin was calculated using $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. Following various treatments, primary cardiac myocytes were incubated in radioimmunoprecipitation assay (RIPA) lysis buffer in an ice bath for 15 min and sonicated in an ice bath for another 15 min. Following various treatments, *ex vivo* myocardial tissues were ground repeatedly in RIPA lysis buffer on ice and sonicated for 15 min. The two types of lysates were centrifuged at 10,000 x g and 4˚C for 10 min. The supernatant was collected and mixed with PBS. The mixture was boiled for 5 min and then centrifuged at 10,000 x g for 5 min (cells) or 10 min (tissues). The supernatant was collected to prepare total protein. The concentration was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Haimen, China). Next, protein (20 μg per lane) was loaded to perform SDS-PAGE on 10% gels. The protein was condensed at 60 V and separated at

Table I. Sequences of the primers in reverse transcription-polymerase chain reaction.

Primer	Sequence (5'-3')	Length (bp)
GH		
Forward	CTGTTTGCCAATGCTGTGC	19
Reverse	GCTGTCCCTCGGGAATGTA	19
GHSR		
Forward	CTTCTGCCTCACTGTGCTCTA	21
Reverse	GCATCTTCACTGTCTGCTTGT	21
IGF-1		
Forward	GCACTCTGCTTGCTCACCT	19
Reverse	CATCCACAATGCCCGTCT	18
Akt		
Forward	GGCATCTTCTCCTTCCAGC	19
Reverse	AGAGTTCCTCCACCACCGT	19
β-actin		
Forward	AGGGAAATCGTGCGTGAC	18
Reverse	ATACCCAGGAAGGAAGGCT	19

GH, growth hormone; GHSR, growth hormone secretagogue receptor; IGF-1, insulin-like growth factor-1; Akt, protein kinase B.

80 V. Polyvinylidene difluoride membranes were activated by absolute methanol at room temperature for 15 sec. Membrane transfer was conducted for β-actin and GHSR at 300 mA for 1.5 h, for IGF-1 at 300 mA for 2 h, for GH at 200 mA for 50 min, and for Akt at 200 mA for 1 h. Next, it was blocked in 5% BSA buffer at room temperature overnight. The membrane was subsequently incubated in primary antibody buffer at 4˚C for 3 h. It was rinsed three times for 10 min each time and incubated in secondary antibody buffer at room temperature for 2 h. It was rinsed three times for 10 min each time. Following chemiluminescent substrate being added, the membrane was exposed on an imaging system (ChemiDoc XRS+, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Blots were semi-quantitatively analyzed with a Quantity one software (v4.62; Bio-Rad Laboratories, Inc.). β-actin served as the internal control.

Immunohistochemical analysis. Following various treatments, *ex vivo* myocardial tissues were collected, fixed in 4% paraformaldehyde at room temperature for 30 min, embedded with paraffin and cut into slices (thickness, 4 μm). Following heating at 65˚C for 2 h, the slices were incubated in xylene for 10 min and in fresh xylene for another 10 min. Subsequently, the slices were immersed successively in 100% ethanol, 100 ethanol, 95 ethanol, 80% ethanol and water each for 5 min. The slices were later incubated in citrate buffer in a box and heated to automatic air release in a pressure cooker. After 2 min, the slices were removed and naturally cooled. Following the citrate buffer being removed and the slices being eluted with PBS, the slices were incubated in 3% fresh hydrogen peroxide in a wet box for 10 min at room temperature. Subsequently, the slices were washed in PBS three times for 5 min each
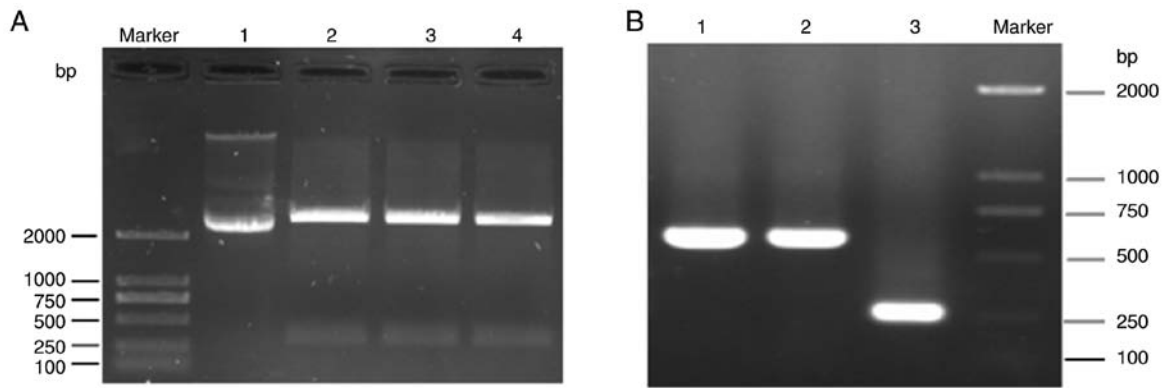
Figure 1. Electrophoretograms of ghrelin and its expression vector. (A) The electrophoretogram of the enzyme digestion product from ghrelin-pUC57 plasmid. The target bands of ghrelin (363 bp) were observed in the lanes of the ghrelin-pUC57 plasmid following incubation with *Bam*HI and *Eco*RI. 1, ghrelin-pUC57; 2-4, ghrelin-pUC57/*Eco*RI/*Bam*HI. (B) The electrophoretogram of colony PCR products. The isolated ghrelin from the ghrelin-pUC57 plasmid was ligated to pLVX-Puro vector and two single colonies containing the ghrelin- pLVX-Puro vector were verified by PCR. 1-2, ghrelin-pLVX-Puro; 3, pLVX-Puro; PCR, polymerase chain reaction.

time and 5% BSA was added dropwise onto the slices at 37˚C for 30 min. The excess blocking buffer around the tissue was absorbed with absorbent papers. Diluted primary antibodies (all 1:500) were dropwise added onto each slice. Following incubation at 4˚C overnight in a wet box, the slices were removed for 45 min at room temperature and then washed in PBS three times for 5 min each time. Diluted secondary antibody (1:200) was added dropwise onto the slices, which were subsequently incubated at room temperature for 30 min. The slices were rinsed and developed in DAB for 5-10 min followed by rinsing with PBS for 1 min. The slices were then counterstained with hematoxylin at room temperature for 3 min, differentiated in 1% hydrochloric alcohol, blued, rinsed, dehydrated, transparentized, mounted and examined under a fluorescence microscope (magnification, x200; CKX31; Olympus Corporation).

Statistical analysis. Each experiment was repeated three times. Data are expressed as the mean ± standard deviation (SD; n=7). Following confirmation of normal distribution by the Kolmogorov-Smirnov test, statistical differences among different groups were analyzed by analysis of variance followed by least significant difference post hoc test using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Construction of ghrelin expression vector. The electrophoretogram of the enzyme digestion product from ghrelin-pUC57 plasmid is shown in Fig. 1A. There was no band between 250 and 500 bp in the lane of the ghrelin-pUC57 plasmid without enzyme digestion. However, the target bands of ghrelin (363 bp) were observed in the lanes of the ghrelin-pUC57 plasmid following incubation with *Bam*HI and *Eco*RI.

The isolated ghrelin from the ghrelin-pUC57 plasmid was ligated to a pLVX-Puro vector, and two single colonies containing the ghrelin-pLVX-Puro vector were verified by PCR. In the electrophoretogram of colony PCR verification, the target genes were identified, which demonstrated that the

two colonies were positive colonies (Fig. 1B). This confirmed the successful construction of the ghrelin-pLVX-Puro vector.

Isolation and identification of primary neonatal rat cardiac myocytes. The immunofluorescent staining of primary neonatal rat cardiac myocytes was shown in Fig. 2. α-sarcomeric actinin was a specific protein of cardiac myocytes. Red and blue fluorescence represented the α-sarcomeric actinin and the cell nuclei, respectively. It was demonstrated that all the isolated cells had red α-sarcomeric actinin. It was indicated that the primary neonatal rat cardiac myocytes were successfully isolated and cultured.

Cell viability. Fig. 3 demonstrates the viability of primary neonatal rat cardiac myocytes in various groups (control, H/R, empty and ghrelin) at 24, 48 and 72 h after treatment (if any), which was examined by CCK-8 assay. Compared with the control group, the viabilities in other three groups were significantly reduced ($P < 0.05$), suggesting the inhibition of cell growth by H/R treatment. There was no significant difference in the cell viability between the H/R and empty groups. The empty pLVX-Puro vector did not promote cell proliferation. However, the cell viability in the ghrelin group was significantly higher than that in the empty group ($P < 0.05$), indicating that ghrelin was capable of improving the viability of primary neonatal rat cardiac myocytes.

Cell apoptosis. Fig. 4 demonstrates the apoptosis of primary neonatal rat cardiac myocytes in various groups (control, H/R, empty and ghrelin), which was evaluated by Hoechst staining. Compared with the control group, the apoptosis rates in the other three groups were significantly increased ($P < 0.05$), suggesting the promotion of cell apoptosis by H/R treatment. The H/R and empty groups exhibited similar apoptosis rates, demonstrating that the empty pLVX-Puro vector had no effect on cell apoptosis. However, the apoptosis rate in the ghrelin group was significantly lower than that in the empty group ($P < 0.05$), indicating that ghrelin was able to suppress the apoptosis of primary neonatal rat cardiac myocytes and repair the hypoxic cardiac myocytes.
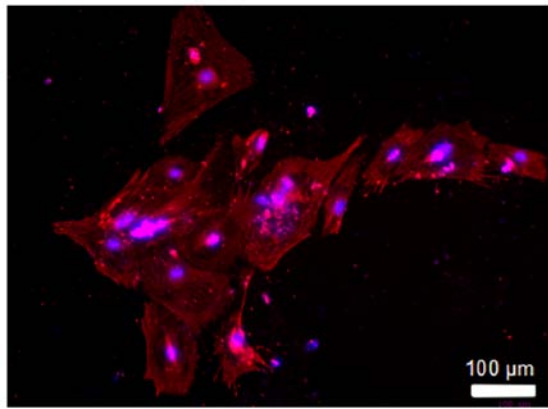
Figure 2. Immunofluorescent staining of primary neonatal rat cardiac myocytes. Red and blue fluorescence represented the α-sarcomeric actinin and the cell nuclei, respectively.
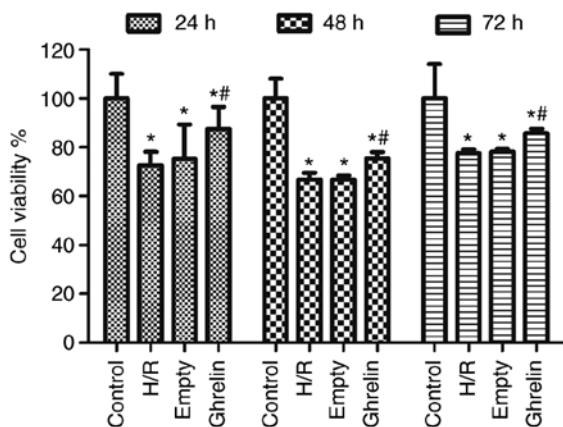

Figure 3. Viability of primary neonatal rat cardiac myocytes in various groups [control, H/R, empty (empty pLVX-Puro plasmid + H/R) and ghrelin (ghrelin-pLVX-Puro plasmid + H/R)] at 24, 48 and 72 h after treatment (if any), which was examined by Cell Counting Kit-8 assay. *P<0.05 vs. the control group; #P<0.05 vs. the empty group. H/R, hypoxia/reoxygenation.
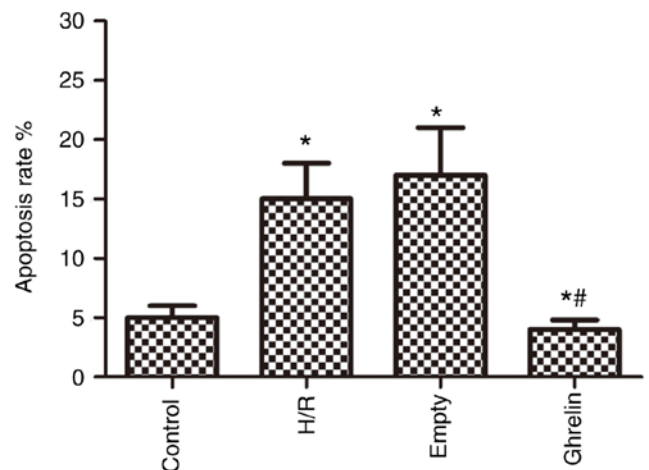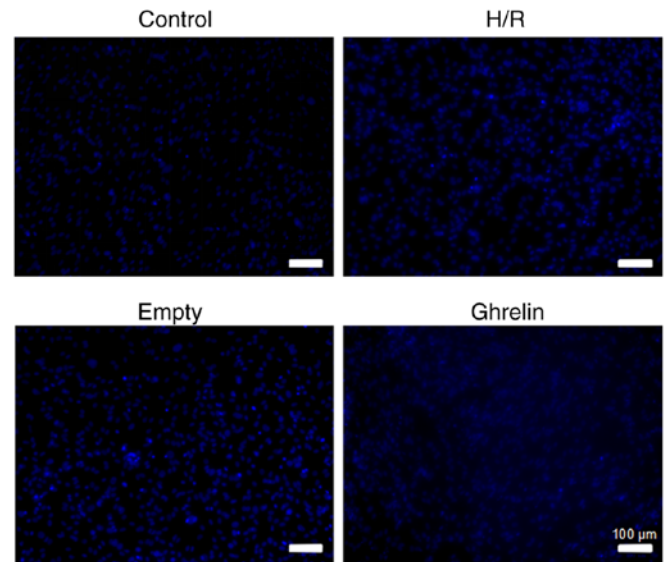

Figure 4. Apoptosis of primary neonatal rat cardiac myocytes in various groups [control, H/R, empty (empty pLVX-Puro plasmid + H/R) and ghrelin (ghrelin-pLVX-Puro plasmid + H/R)], which was evaluated by Hoechst staining. *P<0.05 vs. the control group; #P<0.05 vs. the empty group. H/R, hypoxia/reoxygenation.

Levels of GH, GHSR, IGF-1, Akt and p-Akt in primary cardiac myocytes following various treatments. The mRNA levels of GH, GHSR, IGF-1 and Akt in primary cardiac myocytes in various groups (control, H/R, empty and ghrelin), which were determined by RT-PCR, are presented in Fig. 5A. The protein expression levels of GH, GHSR, IGF-1, Akt and p-Akt in primary cardiac myocytes in various groups (control, H/R, empty and ghrelin), which were evaluated by western blot analysis, are presented in Fig. 5B. Compared with the control group, the mRNA and protein levels of GH, GHSR and IGF-1 in the other three groups were significantly decreased (P<0.05), suggesting the downregulation of GH, GHSR and IGF-1 in primary cardiac myocytes by H/R treatment. Similar mRNA and protein levels of GH, GHSR and IGF-1 were discovered between the H/R and empty groups, demonstrating that the empty pLVX-Puro vector did not affect the expression of GH, GHSR and IGF-1 in primary cardiac myocytes. Notably, the mRNA and protein levels of GH, GHSR and IGF-1 in the ghrelin group were significantly higher than those in the empty group (P<0.05), indicating that ghrelin could upregulate the expression of GH, GHSR and IGF-1 in primary cardiac

myocytes. It was demonstrated that the mRNA and protein expression levels of Akt were similar among the four groups. It was implied that ghrelin transfection and H/R treatment did not influence the expression of Akt in primary cardiac myocytes. However, compared with the control group, the ratios of p-Akt to Akt protein expression (p-Akt/Akt) in the other three groups were significantly decreased (P<0.05). The ratio of p-Akt/Akt was similar between the H/R and empty groups. Compared with the empty group, the ghrelin transfection in the ghrelin group significantly increased the ratio of p-Akt/Akt (P<0.05).

Levels of GH, GHSR, IGF-1, Akt and p-Akt in myocardial tissues following various treatments. The mRNA expression levels of GH, GHSR, IGF-1 and Akt in myocardial tissues in various groups (control, sham, H/R and ghrelin) determined by RT-PCR are shown in Fig. 6A. The protein expression levels of GH, GHSR, IGF-1, Akt and p-Akt in myocardial tissues in various groups (control, sham, H/R and ghrelin) evaluated by western blot analysis were demonstrated in Fig. 6B. Compared with the control group, the mRNA and protein expression
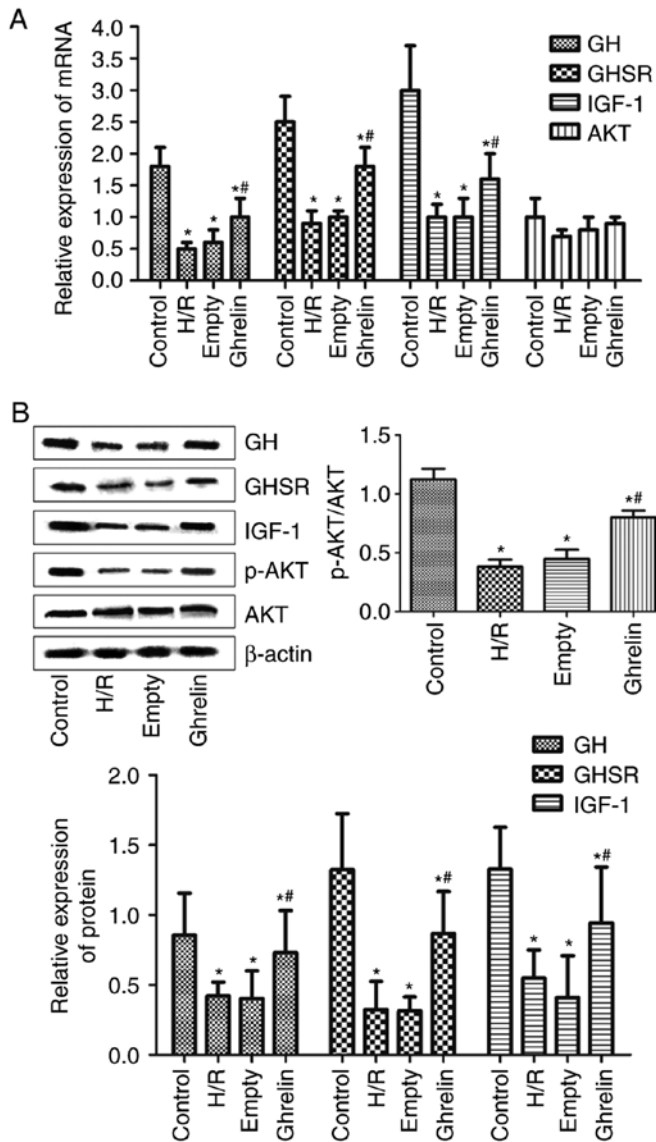
Figure 5. The expression levels of GH, GHSR, IGF-1, Akt and p-Akt in primary cardiac myocytes in various groups. (A) The mRNA expression levels of GH, GHSR, IGF-1 and Akt in primary cardiac myocytes in various groups [control, H/R, empty (empty pLVX-Puro plasmid + H/R) and ghrelin (ghrelin-pLVX-Puro plasmid + H/R)], which was determined by reverse transcription-polymerase chain reaction. (B) The protein expression levels of GH, GHSR, IGF-1, Akt and p-Akt in primary cardiac myocytes in various groups, which was evaluated by western blot analysis. GH, growth hormone; GHSR, growth hormone secretagogue receptor; IGF-1, insulin-like growth factor-1; Akt, protein kinase B; p-Akt, phosphorylated Akt. *P<0.05 vs. the control group; #P<0.05 vs. the empty group. H/R, hypoxia/reoxygenation.

Figure 6. (A) The mRNA expression levels of GH, GHSR, IGF-1 and Akt in myocardial tissues in various groups [control, sham, H/R and ghrelin (ghrelin + H/R)], which was determined by reverse transcription-polymerase chain reaction. (B) The protein expression levels of GH, GHSR, IGF-1, Akt and p-Akt in myocardial tissues in various groups, which was evaluated by western blot analysis. GH, growth hormone; GHSR, growth hormone secretagogue receptor; IGF-1, insulin-like growth factor-1; Akt, protein kinase B; p-Akt, phosphorylated Akt; H/R, hypoxia/reoxygenation. *P<0.05 vs. the control group; #P<0.05 vs. the H/R group.

levels of GH, GHSR and IGF-1 in the H/R and ghrelin groups were significantly decreased (P<0.05), suggesting the down-regulation of GH, GHSR and IGF-1 in myocardial tissues by H/R treatment. Similar mRNA and protein expression levels of GH, GHSR and IGF-1 were observed between the control and sham groups. Notably, the mRNA and protein levels of GH, GHSR and IGF-1 in the ghrelin group were significantly higher than those in the H/R group (P<0.05), indicating that ghrelin could upregulate the expression of GH, GHSR and IGF-1 in myocardial tissues. The mRNA and protein expression levels of Akt were revealed to be similar among the four groups. It was implied that ghrelin and H/R treatment did not
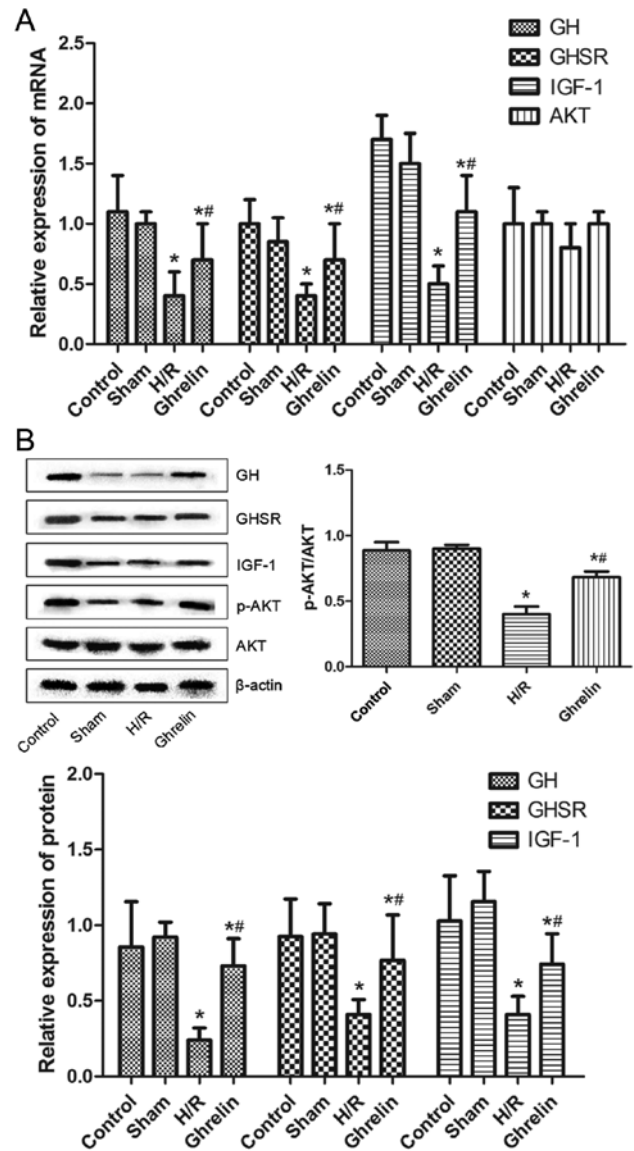
influence the expression of Akt in myocardial tissues. However, compared with the control group, the ratios of p-Akt/Akt in the H/R and ghrelin groups were significantly decreased (P<0.05). The ratio of p-Akt/Akt was similar between the control and sham groups. Compared to the H/R group, the ghrelin group exhibited a notably larger ratio of p-Akt/Akt (P<0.05).

Immunohistochemical analysis of GH, GHSR, IGF-1 and Akt in myocardial tissues following various treatments. Fig. 7 depicts the immunohistochemical staining images of GH, GHSR, IGF-1 and Akt in myocardial tissues in various groups (control, sham, H/R and ghrelin). The corresponding protein and the nuclei are stained brown and bluish violet, respectively. The expression levels of GH, GHSR, IGF-1 and
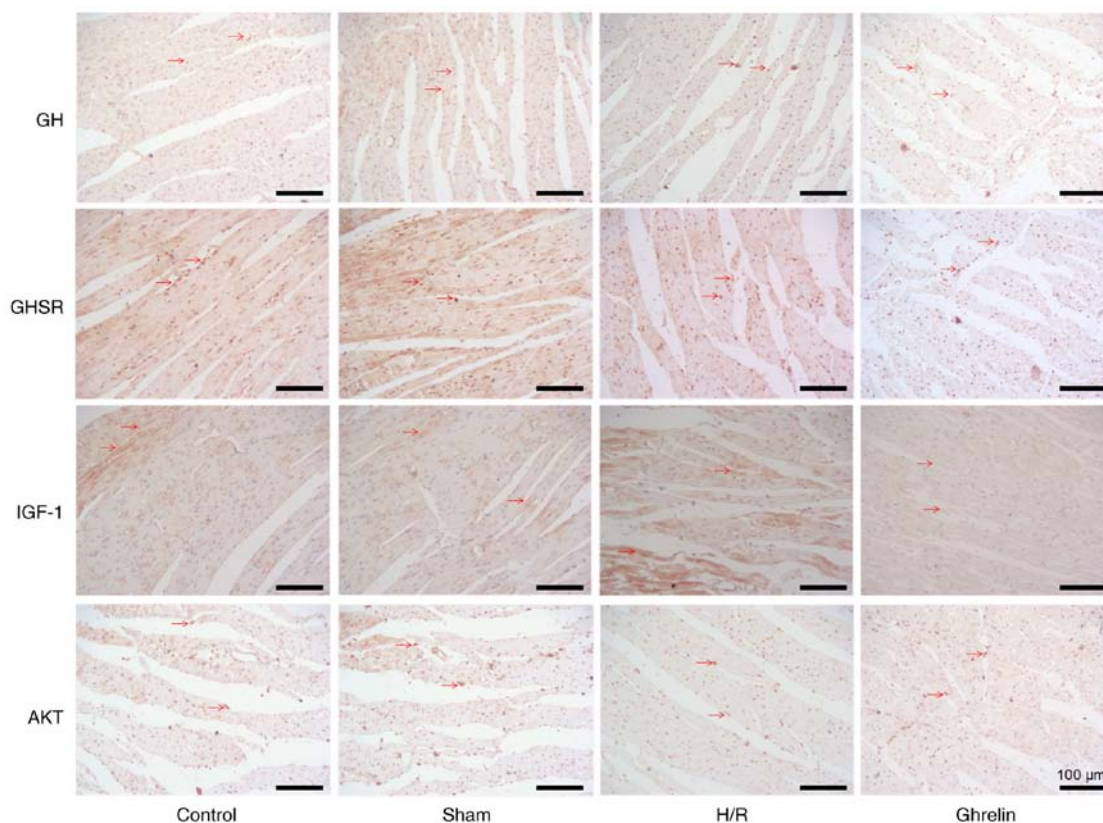
Figure 7. The immunohistochemical staining images of GH, GHSR, IGF-1 and Akt in myocardial tissues in various groups [control, sham, H/R and ghrelin (ghrelin+H/R)]. The corresponding protein and the nuclei are shown by brown (arrows) and bluish violet, respectively. GH, growth hormone; GHSR, growth hormone secretagogue receptor; IGF-1, insulin-like growth factor-1; Akt, protein kinase B; H/R, hypoxia/reoxygenation. Magnification, x200.

Akt in the H/R and ghrelin groups were intuitively lower than those in the control group. This demonstrated that the myocardial tissues following H/R treatment were damaged. However, the expression level of brown protein in the ghrelin group was notably higher than that in the H/R group, particularly for GH and GHSR. Furthermore, there were varying degrees of rupture, shrinkage and irregular appearance of the myocardial tissues in the H/R group, and this phenomenon was particularly evident in the IGF-1 protein group. Although there remained certain injuries of the myocardial tissues in the ghrelin group, this was markedly improved compared with the H/R group. Ghrelin enhanced the integrity of cardiac myocytes, and reduced shrinkage and apoptosis.

Discussion

The reduction of coronary blood flow due to various reasons results in insufficient supply of myocardial oxygen and the reduced elimination of metabolic products; therefore, this clinical condition is known as myocardial hypoxia (21). The majority of cardiac diseases can cause myocardial ischemia and hypoxia, but no radical cure is currently available in clinic. Therefore, to the best of our knowledge, the present study was the first to reveal the improving effect of ghrelin on hypoxic myocardium and the involved molecular mechanisms through constructing primary neonatal rat cardiac myocytes transfected with ghrelin lentiviral expression vector, and evaluating the subsequent cell viability and apoptosis, as well as the expression of associated genes at the cell and tissue levels.

Primary neonatal rat cardiac myocytes were isolated and the immunofluorescent staining of α-sarcomeric actinin proved that the isolated cells were the target cells. Improved cellular activity could be obtained through the cell characterization using the isolated primary cells, and this was consistent with the characterization of subsequent *ex vivo* myocardial tissues. This could more accurately reflect the repair effect of ghrelin on the myocardium at the cell and tissue levels.

CCK-8 and Hoechst assays demonstrated that ghrelin could inhibit the apoptosis of hypoxic cardiac myocytes, and that it had a protective and repair effect on hypoxic cardiac myocytes, which was in agreement with the reported heart protection function of ghrelin (22,23).

Apoptosis is regulated by intracellular apoptosis regulating proteins, which are divided into two categories: Apoptotic protein and anti-apoptotic protein (24-26). The relative balance between apoptotic protein and anti-apoptotic protein following a series of stimuli or injuries determines whether the cell is alive or apoptotic (27). RT-PCR and western blot analysis were conducted to evaluate the expression of five genes, including GH, GHSR, IGF-1, Akt and p-Akt in primary cardiac myocytes following H/R treatment. The present study demonstrated that ghrelin transfection upregulated the expression of GH, GHSR and IGF-1 at the mRNA and protein levels. Furthermore, ghrelin transfection could elevate the ratio of p-Akt/Akt. It was suggested that ghrelin promoted the phosphorylation of Akt, inhibited the activity of Akt (28), and upregulated the expression of GH, GHSR and IGF-1, consequently enhancing the viability and suppressing the apoptosis

of cardiac myocytes. Additionally, the PI3K/Akt signaling pathway may be inhibited following the phosphorylation of Akt, such that the cardiac myocytes may be repaired (17,18). Ghrelin inhibited the expression of apoptotic proteins and promoted the expression of anti-apoptotic proteins in neonatal rat cardiac myocytes, thereby inhibiting the apoptosis of cardiac myocytes following heart failure and resisting ventricular remodeling; therefore, this may be a mechanism of the anti-apoptotic effect of ghrelin (28,29).

Furthermore, a rat cardiac perfusion model was established *ex vivo* and the expression of GH, GHSR, IGF-1, Akt and p-Akt in the myocardial tissues following H/R treatment was investigated by RT-PCR, western blot analysis and immunohistochemical analysis. Immunohistochemical results demonstrated that ghrelin enhanced the integrity of cardiac myocytes and reduced shrinkage and apoptosis. These results suggested that ghrelin may protect and repair the myocardium through upregulating the expression of GH, GHSR and IGF-1, and ghrelin promoted the phosphorylation of Akt and inhibited the activity of Akt in myocardial tissues, consequently alleviating the injury of myocardial tissues. This was the same as the results at the cell level. Immunohistochemical analysis of myocardial tissues was performed, but cardiac function was not analyzed. A negative control was not included in the immunohistochemical analysis. These were limitations to the present study.

Ghrelin can improve the cardiac output, left ventricular ejection fraction and change rate of left ventricular maximum pressure, and inhibit left ventricular hypertrophy in rats with chronic heart failure (30). Ghrelin is a natural endogenous ligand for GHSR and is also considered to be a powerful stimulant for the release of GH. Certain studies have demonstrated that the main role of ghrelin in cardiac myocytes is to promote the expression of non-functional GHSR (31). Another report also indicated that, in addition to GHSR, ghrelin may have other unknown receptors in the cardiac repair system (32). According to the results of RT-PCR and western blot analysis, ghrelin promoted the expression of GHSR and GH, and repaired hypoxic cardiac myocytes. Therefore, the possible mechanism was that ghrelin upregulated the expression of GHSR through binding with GHSR, and subsequently GHSR stimulated the overexpression of GH. GH is one of the hormones synthesized and secreted in the adenohypophysis, which has a positive function in the treatment of cardiovascular disease (33). It has been demonstrated that it can stimulate the central nervous system and local organs to produce IGF-1 through autocrine and paracrine mechanisms, which can directly or indirectly influence cardiac tissues, enhance cardiac contractility, reduce cardiac load, improve cardiac function and postpone cardiomyocyte apoptosis (34,35).

Cao *et al* (14) reported that the cardioprotective effects elicited by ghrelin may contribute toward the inhibition of inflammatory response through the Akt activated pathway. The basic difference between this previous paper and the present study was in the different animal models. This previous paper used a CPB model, while the present study used a model of myocardial injury caused by H/R (14). This was the first time to investigate the therapeutic effect of ghrelin on the model of myocardial injury induced by H/R. Furthermore, in the present study, the cells used in the *in vitro* cell experiments were isolated from the neonatal rats in the same batch of rats used in the animal experiments *ex vivo*. Homologous cells and

animal materials were selected for the cell and animal experiments to conduct similar H/R treatments, which made the results of the present study more accurate and reliable.

In conclusion, to the best of our knowledge, the present study was the first to reveal that ghrelin protected the primary cardiac myocytes and the myocardium *ex vivo* with H/R treatment through upregulating the expression of GH, GHSR and IGF-1, promoting the phosphorylation of Akt, and inhibiting the activity of Akt. These results may provide novel insight into the understanding of the mechanisms of ghrelin in cardiovascular protection and repair, and provide promising guidance for the clinical application of ghrelin. One limitation of the present study was that inflammation was not investigated. Additionally, a ghrelin blocker group will be added in future studies.

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Availability of data and materials

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

Authors' contributions

YL, ZC and GG designed study and wrote the manuscript. YangL, YanlingL and GL collected and analyzed the data. All authors performed the study.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Children's Hospital of Suzhou University (Suzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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