PDE5 inhibitor protects the mitochondrial function of hypoxic myocardial cells

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Abstract. Protective effect of phosphodiesterase 5 (PDE5) inhibitor sildenafil on hypoxic injury of isolated myocardial cells and its mechanism of action were investigated. Myocardial cells of neonatal mice were isolated, cultured and divided into blank, control, and PDE5 inhibitor group. Cells in the control and the PDE5 inhibitor group were treated with hypoxia and serum deprivation for 6 h to simulate the myocardial ischemia process *in vivo*, while those in the PDE5 inhibitor group were treated with 1 μmol/l sildenafil. The cell viability was detected via Cell Counting kit-8 (CCK-8), the cytotoxicity was detected via lactate dehydrogenase release assay, and the apoptosis level was detected via flow cytometry, Hoechst staining and caspase-3 activity assay. Moreover, changes in mitochondrial membrane potential of myocardial cells were detected via JC-1 staining and flow cytometry, fluorescein adenosine triphosphate (ATP) assay kit was used to detect the production of ATP in myocardial cells, and reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the level of Sirt3 messenger ribonucleic acid (mRNA) in myocardial cells. Finally, the expression and changes of Sirt3, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and acetylated PGC-1α were detected via western blot analysis. After hypoxia treatment, the cell viability was decreased, the cytotoxic effect was enhanced, the percentage of apoptosis was increased, the activity of apoptosis-related protein was increased, the mitochondrial membrane potential was decreased, the production of ATP was reduced, the expression of Sirt3 was decreased, and the acetylation level of PGC-1α was increased. However, after pretreatment with sildenafil, the damage to membrane potential of myocardial cells was significantly alleviated, and the production of ATP was increased. At the same time, myocardial cell apoptosis was decreased, Sirt3 expression was increased and PGC-1α acetylation was decreased. PDE5 inhibitor inhibits apoptosis of hypoxic myocardial cells through protecting mitochondrial function.

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Introduction

The treatment of cardiovascular diseases, especially ischemic heart disease, has been greatly improved, but heart failure, as the end-stage manifestation of various cardiovascular diseases, is the main cause of high mortality and disability rates of cardiovascular diseases (1). Despite the great progress made in the treatment of heart failure, the current treatment still fails to control the progression and death of heart failure to the greatest extent (2). In recent years, it has been gradually realized that myocardial cell metabolism plays an important role in the occurrence and development of congestive heart failure (3). Studies have found that myocardial energy metabolism disorder is the main reason for the occurrence and development of heart failure. In congestive heart failure, the damage to mitochondrial structure is increased, mitochondrial dysfunction leads to reduced adenosine triphosphate (ATP) production, and insufficient energy supply increases myocardial cell apoptosis. Increased myocardial cell apoptosis is the main mechanism of myocardial remodeling during congestive heart failure (4).

Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a co-activator of many transcription factors in the energy metabolism pathway, which plays a crucial role in energy metabolic balance (5). Therefore, PGC-1α is regarded as a molecular switch that regulates mitochondrial energy metabolism. Studies have shown that PGC-1α plays an important role in adaptive thermogenesis, mitochondrial generation, β-oxidation of fatty acids, hepatic gluconeogenesis and other processes (6). The activity of PGC-1α is modified by multiple post-transcriptional translations, while the regulation of acetylation level is affected by a variety of sirtuins. Sirt3 is an energy regulatory molecule expressed in both mitochondria and nuclei, which, as a member of the sirtuin family, has deacetylation effect (7). However, some studies have also found that Sirt3 can not only regulate the ATP production in myocardial cells, but also hinder the development of myocardial hypertrophy and heart failure (8).

Phosphodiesterase 5 (PDE5) inhibitor is a clinically-approved mature drug for the treatment of pulmonary hypertension and

male sexual dysfunction. In recent years, a large number of clinical and laboratory experiments have been performed to study the protective effect of PDE5 inhibitor on cardiovascular diseases. PDE5 inhibitor can fight against myocardial ischemia-reperfusion injury, and inhibit myocardial remodeling in heart failure with stress overload (9). Upregulating the expression of PGC-1α in heart failure with stress overload and improving mitochondrial function can reduce myocardial cell apoptosis after myocardial infarction and improve cardiac function (10). The cardiovascular protective effect of PDE5 inhibitor is achieved by increasing the cyclic guanosine monophosphate (cGMP) level in cells (11). However, the expression of PGC-1α and Sirt3 in heart failure after myocardial infarction and the effect of PDE5 inhibitor on mitochondrial energy metabolism in heart failure after myocardial infarction remain unclear.

Materials and methods

Experimental materials. Neonatal mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Cell Counting kit-8 (CCK-8), lactate dehydrogenase (LDH), ATP, caspase-3 and JC-1 assay kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Primary rabbit polyclonal PGC-1α antibody (cat. no. ab54481; dilution, 1:500); rabbit polyclonal Sirt3 antibody (cat. no. ab86671; dilution, 1:500); rabbit polyclonal β-actin antibody (cat. no. ab8227; dilution, 1:1,000) and secondary goat anti-rabbit (HRP) IgG antibody (cat. no. ab6721; dilution, 1:2,000) were all purchased from Abcam (Cambridge, MA, USA).

This study was approved by the Animal Ethics Committee of Shandong Provincial Third Hospital Animal Center (Jinan, China).

Cell isolation, culture and treatment. The ventricular tissues of 1-3-day-old C57BL/6 neonatal mice were taken, cut into 0.5-1 mm^3 tissue blocks and digested with trypsin containing ethylene diamine tetraacetic acid (EDTA). The digestion process was repeated, and the supernatant was collected into a centrifuge tube after each digestion. An equal volume of Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 5% fetal bovine serum and 10% horse serum was added to terminate the digestion, followed by centrifugation at 850 x g at 4˚C for 5 min. The supernatant in the centrifuge tube was removed after centrifugation, and cells were blown away using DMEM/F12 medium containing 5% fetal bovine serum and 10% horse serum for standby application. Undigested tissue blocks were filtered and removed via the 200-mesh nylon mesh, and cells were inoculated into a 25 cm^2 culture flask and cultured in an incubator with 5% CO_2 and saturated humidity at 37˚C. Non-myocardial cells were separated via differential adhesion (culture flask wall) method. Bromodeoxyuridine (BrdU) in a final concentration of 0.1 mm/l was added into the culture solution to inhibit fibroblast proliferation, and double antibodies (100 U/ml penicillin and 100 U/ml streptomycin) were added to prevent bacterial contamination, followed by culture under 5% CO_2 at 37˚C for 3 days. The isolated myocardial cells were taken and added into serum-free medium for hypoxia for 24 h in a hypoxia tank (the oxygen partial pressure was <0.1%) to establish the hypoxia model.

CCK-8 assay. Cells in the logarithmic growth phase were digested, collected and adjusted into cell suspension at a concentration of $1x10^5$/ml. The suspension was inoculated into a 96-well plate (100 µl per well), 3 repeated wells were set in the experiment, and the blank control was also set. After inoculation overnight, it was confirmed via microscopic observation that cells adhered well to the wall. After grouping and treatment, 20 µl methyl thiazolyl tetrazolium (MTT) was added into each well, followed by culture at 37˚C for 4 h. Then the supernatant was carefully discarded, and 150 µl dimethyl sulphoxide (DMSO) was added into each well and mixed evenly. The optical density (OD) value of each well was detected at a wavelength of 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was repeated 3 times.

LDH assay. Myocardial cells in the logarithmic growth phase were digested, collected and adjusted into cell suspension at a concentration of $1x10^5$/ml. The suspension was inoculated into the 96-well plate (100 µl per well), 3 repeated wells were set in the experiment, and the blank control was also set. After cell adherence, the diluted silibinin was added to make the final concentration 0, 25, 50, 100, 150 and 200 µM. Cells in the culture plate were incubated in the incubator with 5% CO_2 at 37˚C for 24 h. The supernatant was taken (20 µl per well) and added with the corresponding reagent according to instructions of the kit. The mixture was mixed evenly and placed at room temperature for 3 min, followed by zero setting using 440 nm double distilled water and detection of OD value using the microplate reader (Bio-Rad Laboratories, Inc.). Unit definition: After 1,000 ml culture solution reacted with the substrate at 37˚C for 15 min, 1 gmolpyruvic acid produced in the reaction system was regarded as 1 unit. The LDH content in the medium was calculated using the formula.

Flow cytometry. BGC823 (cat. no. BNCC337689; BeNa Culture Collection, Beijing, China; http://www.bnbio.com/) cells were inoculated into a 6-well plate ($5x10^5$/ml) overnight. Silibinin solution at a final concentration of 0, 50, 100 and 200 µM was added, respectively, and cells were incubated in the incubator with 5% CO_2 and saturated humidity at 37˚C for 24 h, digested with trypsin and collected, followed by centrifugation at 850 x g and 4˚C for 4 min. Then cells were collected, and the medium was abandoned. Centrifuged cells were washed twice with cold phosphate-buffered saline (PBS), and resuspended using 200 µl binding buffer at a concentration of approximately $1x10^6$/ml. A total of 10 µl annexin V-fluorescein isothiocyanate (FITC) was added into the cell suspension and gently mixed evenly, followed by incubation in the dark at room temperature for 15 min. A total of 5 µl propidium iodide (PI) was added and gently mixed evenly, followed by detection using a flow cytometer within 1 h. MitoProbe™ $DiOC_2$ (3) Assay kit (cat. no. M34150; ThermoFisher Scientific; Waltham, MA, USA) was used for flow cytometry. Data were obtained and analyzed using the CellQuest professional software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Data were obtained and analyzed using the CellQuest professional software. The experiment was repeated 3 times.

Hoechst staining. The detection of apoptosis was displayed using the Hoechst staining (KeyGen Biotech Co., Ltd.,

Nanjing, China). BGC823 cells were seeded in 6-well plates. After hypoxia for 4-6 h, NRVMs were stained with Hoechst for 15 min and washed by PBS for 5 min 3 times. Paraformaldehyde (4%) (Beyotime Institute of Biotechnology, Shanghai, China) was used for the fixative for 10 min at 4 ˚C. Cell observation used fluorescence microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Detection of caspase-3 activity. After gradient dilution of standard sample using the standard sample diluent, 100 μl standard sample in each concentration was taken and added into the 96-well plate. The absorbance value at a wavelength of 405 nm (A405) of each well was detected using the microplate reader (Bio-Rad Laboratories, Inc.) and the standard curve was drawn. First, test buffer and protein sample were added and mixed evenly in the 96-well plate, and incubated in the incubator at 37˚C for 60 min. At the same time, a small number of protein samples were taken to determine the protein concentration using the bicinchoninic acid (BCA) method. Five repeated wells were set for each sample. A405 of each well was detected using the microplate reader (Bio-Rad Laboratories, Inc.). According to A405 of standard sample in each concentration, the standard curve was drawn. The caspase-3 activity in each sample was calculated according to the standard curve, and the average was taken.

JC-1 mitochondrial membrane potential detection. Cells are collected after treatment, and resuspended in 0.5 ml cell culture medium that could contain serum. A total of 0.5 ml JC-1 staining working solution was added, and mixed several times evenly, followed by incubation in a dark place in the incubator at 37˚C for 30 min and centrifugation at 500 x g and 4˚C. Then the supernatant was discarded, and cells were washed with 1X JC-1 staining buffer twice, and resuspended using 1X JC-1 staining buffer, followed by centrifugation at 500 x g and 4˚C and precipitation, and the supernatant was discarded. The above washing step was repeated once. The fluorescence intensity of cells was detected using the flow cytometer.

Fluorescein ATP detection. Cells in each group were lysed on ice for 30 min. The protein in each group was collected into an Eppendorf (EP) tube using a cell scraper. After centrifugation at 1,050 x g at 4˚C for 10 min, the supernatant was taken to quantify the protein via BCA. The ATP content was determined according to instructions of the kit. The sample and reagent were added into the 96-well plate, fully mixed for 2 sec, and quickly placed into the fluorescence microplate for fluorescence detection for 10 sec. The relative level of ATP in each group was calculated based on the fluorescence amount measured.

Polymerase chain reaction (PCR). Cells in each group were collected after treatment, and the total ribonucleic acid (RNA) was extracted using TRIzol. After the concentration of sample was measured, the reverse transcription system was added for reverse transcription reaction. The first 40 cycles were used to synthesize complementary deoxyribonucleic acid (cDNA), and conditions of reverse transcription reaction were set for PCR amplification. Fluorescence signals were collected in real time after each cycle, and amplification and solubility curves were recorded.

Table I. Viability and cytotoxicity of hypoxic myocardial cells in 2 groups.

Groups	Blank	Control	Sirtuins
Cell viability (%)	100%	58.44±4.41[a]	81.74±4.2[b]
LDH (fold of blank)	1	4.2±0.7[a]	2.3±0.4[b]

[a]P<0.05 vs. blank group, [b]P<0.05 vs. control group. LDH, lactate dehydrogenase.

Western blot analysis. Cells in each group were taken and washed twice with D-Hank's solution, and D-Hank's solution was sucked dry with absorbent paper. A total of 150 μl pre-cooled lysis buffer was added in each group and cells were lysed on ice for 30 min. The protein in each group was collected into an EP tube using the cell scraper, followed by centrifugation at 10,500 x g at 4˚C. The supernatant was taken and transferred into a new EP tube. After the protein concentration was determined using the BCA method, 5X loading buffer was added and mixed evenly, and the protein was heated at 100˚C for 6 min. A total of 30 μl protein was added into the loading wells of separation gel (10%) and spacer gel (5%), followed by electrophoresis in the electrophoretic buffer solution under appropriate voltage. After electrophoresis, gel closely contacted with the polyvinylidene fluoride (PVDF) membrane, followed by membrane transfer in transfer buffer at 0˚C under constant voltage at 100 V for 60 min. Then the PVDF membrane was sealed in 5% skim milk powder at room temperature for 1 h, cut according to the molecular weight and sealed in the primary antibody in a refrigerator at 4˚C overnight. The next day, the PVDF membrane was taken, rinsed with Tris-buffered saline with Tween-20 (TBST), and added with the secondary antibody immunoglobulin G (IgG) (1:5,000) for incubation at room temperature for 1 h. After incubation, the membrane was rinsed again with TBST, and the image was developed using the Tanon 5200 immunofluorescence imaging system (Tanon Science & Technology Co., Ltd., Shanghai, China), followed by calculation of gray-scale.

Statistical analysis. Experimental data were presented as mean ± standard deviation. SPSS 16.0 SPSS, Inc., Chicago, IL, USA) statistical software was used for statistical analysis. One-way analysis of variance (ANOVA) followed by post hoc test (Least Significant Difference) was used for the intergroup comparison, and t-test was used for the comparison between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

PDE5 inhibitor reduced cytotoxicity of hypoxic myocardial cells. In this study, CCK-8 assay was used to detect changes in cell viability. In the experiment, cells were divided into blank group, control group and PDE5 inhibitor group. Results showed that the viability of hypoxic myocardial cells in the control group was decreased significantly (58.44±4.41%; P<0.05) compared with that in the blank group, while that in the PDE5 inhibitor

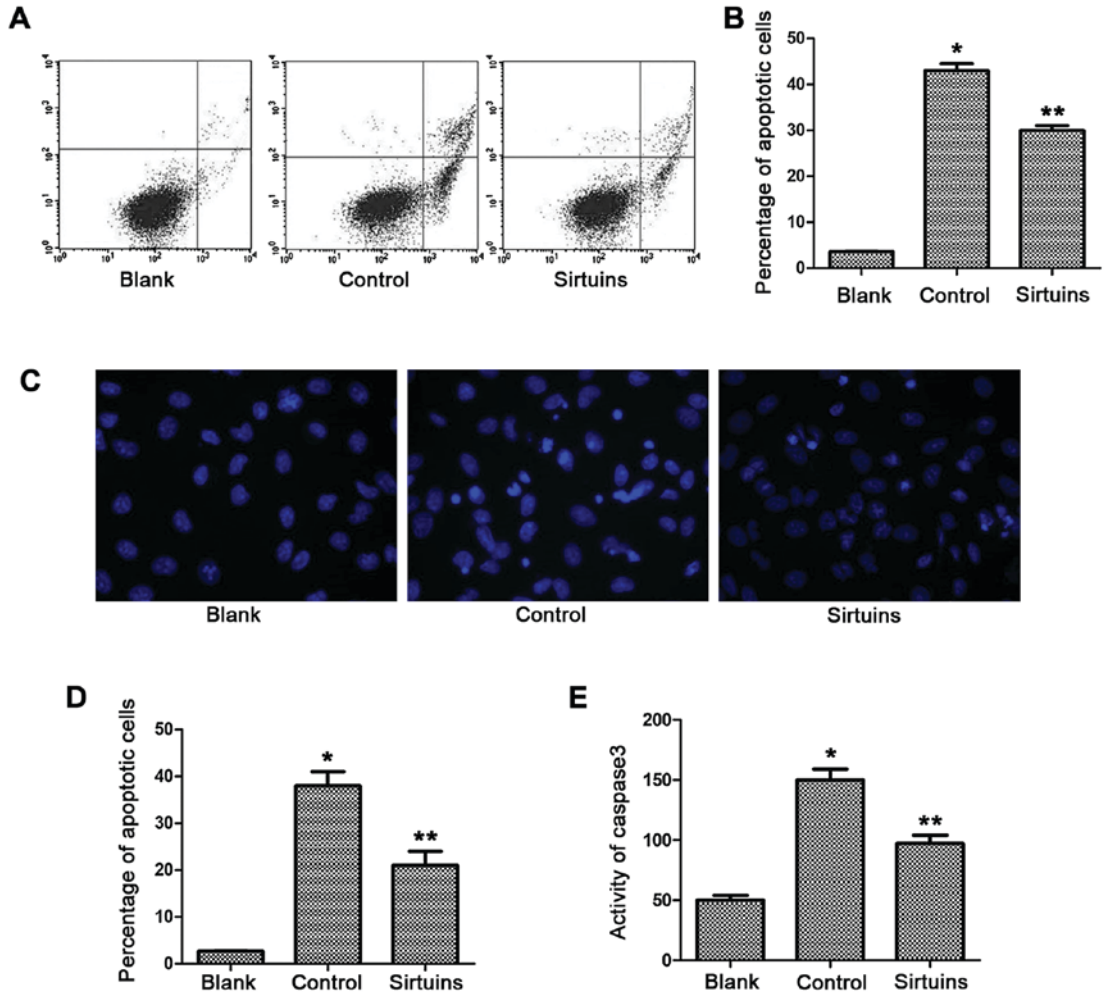

Figure 1. Detection of apoptosis level in each group. (A) Detection of proportion of apoptotic cells in blank, control and sirtuins groups via flow cytometry. (B) Statistical results of proportion of early apoptotic cells in three groups. (C) Detection of number of cells with karyopyknosis and karyorrhexis in blank, control and inhibitor groups via Hoechst staining. (D) Statistical results of number of cells with karyopyknosis and karyorrhexis in three groups. (E) Detection of caspase-3 activity via enzyme-linked immunosorbent assay (ELISA). *P<0.05 vs. blank group, **P<0.05 vs. control group.

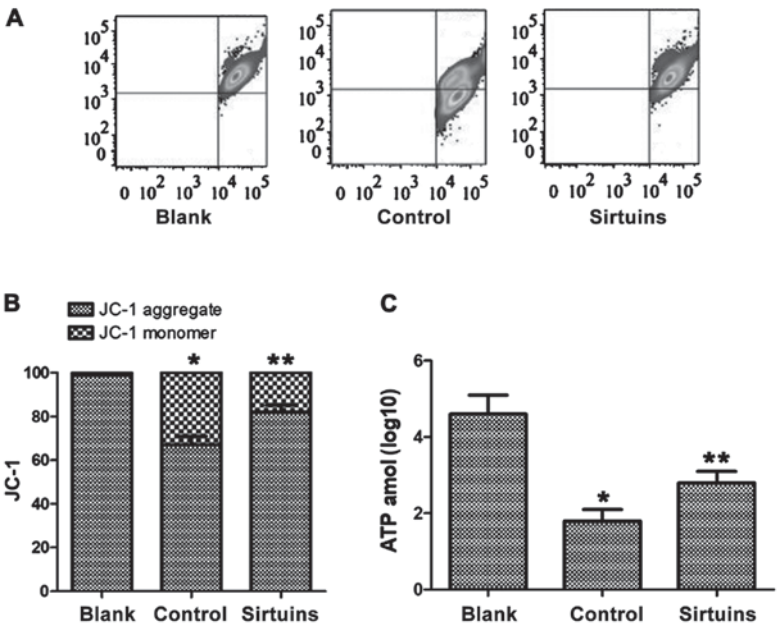

Figure 2. (A) Detection of mitochondrial membrane potential fluorescence levels in blank, control and sirtuins groups via flow cytometry. (B) Statistical results of fluorescence levels in three groups. (C) Detection of energy production in blank, control and sirtuins groups via ATP. *P<0.05 vs. blank group, **P<0.05 vs. control group.

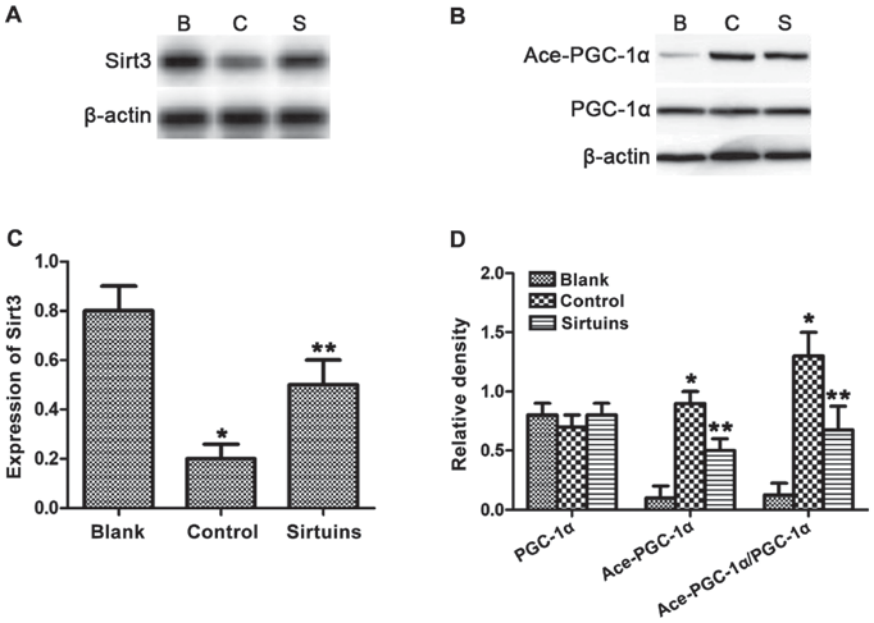

Figure 3. (A) Detection of Sirt3 in blank, control and sirtuins groups via western blot analysis. (B) Detection of PGC-1α and acetylated PGC-1α in each group via western blot analysis. (C) Detection of Sirt3 expression in each group via gray-scale scan. (D) Detection of PGC-1α and acetylated PGC-1α in each group via gray-scale scan. *P<0.05 vs. blank group, **P<0.05 vs. control group.

group was significantly increased (81.74±4.2%; P<0.05) compared with that in the control group. The cytotoxic effect was detected via LDH assay. Results showed that the cytotoxic effect of hypoxia in the control group was larger, and the LDH release was significant compared with that in the blank group (P<0.05). The cytotoxic effect of hypoxia in the PDE5 inhibitor group was reduced, and the change was significant compared with that in the control group (P<0.05) (Table I).

PDE5 inhibitor inhibited apoptosis of hypoxic myocardial cells. Apoptosis level of myocardial cells was detected via flow cytometry, Hoechst staining and caspase-3 activity assay. Results showed that the proportion of early apoptotic cells in the control group was significantly increased compared with that in the blank group (42.8±2.4 vs. 2.6±0.2%; P<0.05) (Fig. 1A and B). Hoechst staining was used to mark the proportion of apoptotic cells with karyopyknosis and karyorrhexis, and results revealed that the proportion of apoptotic myocardial cells after hypoxia was increased compared with that in the blank group (38.5±3.9 vs. 4.43±0.6%; P<0.05) (Fig. 1C and D). PDE5 inhibitor could reduce the early apoptosis induced by hypoxia. Results of flow cytometry showed that there was a significant difference in apoptosis compared with that in the control group (30.44±2.7 vs. 42.8±2.4%; P<0.05), and the proportion of cells with karyopyknosis and karyorrhexis was also significantly decreased (21.8±4.1 vs. 38.5±3.9%; P<0.05) (data not shown). The activity of caspase-3 in the control group was increased compared with that in the blank group, and the caspase-3 activity was reduced after addition of PDE5 inhibitor (P<0.05) (Fig. 1E).

PDE5 inhibitor protected mitochondria of hypoxic myocardial cells. Changes in mitochondrial membrane potential in each group of cells were detected via JC-1 staining. Results of JC-1 fluorescence staining showed that the mitochondrial membrane potential of myocardial cells in the control group was decreased obviously. ATP assay showed that ATP synthesis was blocked in myocardial cells after hypoxia, and the ATP content in cells was decreased significantly compared with that in the blank group (Fig. 2). PDE5 inhibitor could protect the mitochondrial membrane potential, ensure the normal function of mitochondria and increase the ATP content in cells, showing significant differences from the control group (Fig. 2).

PDE5 inhibitor could regulate Sirt3 and PGC-1α acetylation. The content of PDE5a, Sirt3, PGC-1α and acetylated PGC-1α in each group of cells was detected via western blot analysis. Results revealed that the expression of Sirt3 was decreased in myocardial cells after hypoxia, whereas PDE5 inhibitor could reverse such a change induced by hypoxia (Fig. 3A and C). The expression level of acetylated PGC-1α was increased compared with that in the blank group, whereas PDE5 inhibitor could significantly reduce PGC-1α acetylation (Fig. 3B and D).

Discussion

After myocardial ischemia, mitochondrial dysfunction and myocardial energy metabolism disorder are the main factors leading to myocardial cell apoptosis, as well as the main factors of myocardial remodeling after myocardial ischemia. Myocardial remodeling can result in further decline in ischemic cardiac function, and constant decrease.

PGC-1α is a co-activator of many transcription factors in the energy metabolism pathway, which plays a crucial role in energy metabolic balance. Moreover, PGC-1α can promote expression of related genes in mitochondrial biogenesis and mitochondrial respiratory function through increasing the capacities of mitochondrial fatty acid oxidation and oxidative phosphorylation (12). PGC-1α is highly expressed in the heart and exerts an important protective effect on the cardiovascular

system. When PGC-1α is acetylated, it will be transformed into a protein molecule that lacks activity. Sirtuin family is a protein family, and Sirt3, as a member of the sirtuin family, is the only protein associated with longevity (13).

Recent studies have shown that PDE5 is highly expressed in patients with heart failure, and its expression in myocardium of left ventricle of patients with heart failure is 4-5 times that in normal myocardium (14). PDE5a-specific inhibitor sildenafil can inhibit the decomposition of cGMP, so that a variety of growth pathways are inactivated, thus preventing the development of heart failure and myocardial hypertrophy caused by pressure overload (15). In animal models, sildenafil can alleviate the myocardial ischemia-reperfusion injury and reduce the myocardial infarct area.

In this study, it was found that after hypoxia of myocardial cells, the cell viability was significantly decreased, the cytotoxic effect was enhanced, the proportion of early apoptotic cells was increased, the number of cells with karyopyknosis and karyorrhexis was significantly increased, the activity of apoptosis-related protein caspase-3 was increased, the mitochondrial function was decreased obviously, the Sirt3 expression was decreased significantly, and the PGC-1α acetylation level was increased. After addition of PDE5 inhibitor, cell injury caused by hypoxia was significantly alleviated, apoptosis was reduced, mitochondrial function was protected, Sirt3 protein expression was induced and PGC-1α acetylation level was decreased. Results suggested that PDE5 inhibitor can increase Sirt3 expression, reduce PGE-1α acetylation, and protect mitochondrial function, thus protecting hypoxic myocardial cells from apoptosis.

In conclusion, PDE5 inhibitor inhibits PGC-1α acetylation and protects mitochondrial function via increasing Sirt3 expression, thus inhibiting apoptosis of hypoxic myocardial cells.

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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

HJ and YY designed the study and performed the experiments, HJ and ZG raised the animals, HJ and ZG collected the data, HJ and YY analyzed the data, HJ and YY prepared the manuscript. All authors read and approved the final study.

Ethics approval and consent to participate

This study was approved by the Animal Ethics Committee of Shandong Provincial Third Hospital Animal Center (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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