# Original Article
# Effects of target regulation of LDHA through the PDK1/Akt/mTOR pathway on myocardial apoptosis caused by ischemia/reperfusion injury in rats

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Abstract: Objective: To explore the effect and mechanism of lactate dehydrogenase A (LDHA) on myocardial apoptosis in rats with myocardial ischemia-reperfusion injury (MIRI). Methods: Thirty newborn Wistar rats were killed and dissected under aseptic condition, then their hearts were cut for primary culture of cardiomyocytes. A model of myocardial hypoxia/reoxygenation (H/R) injury was established in vitro. The mRNA and protein expression levels of LDHA in rats' primary cardiomyocytes were tested according to Real-time PCR and Western blot methods. LDHA overexpression vectors were constructed and transfected into cardiomyocytes, and were divided into 4 groups: control group, control +LDHA transfection group, H/R group and H/R+LDHA transfection group. CCK-8 method was used to detect myocardial proliferation activity in each group, flow cytometer to detect myocardial apoptosis, Western blot method to detect the effect of the LDHA overexpression on PDK1/AKt/mTOR protein expression under H/R and dual-luciferase method to identify target regulation of LDHA to PDK1. Results: Compared with control group, the proliferation activity of rats' primary cardiacmyocytes in H/R group reduced significantly (P=0.002), while the myocardial apoptosis and the expression of LDHA increased (P=0.001, P=0.000). Compared with H/R group, the overexpression of LDHA in H/R+LDHA transfection group could promote myocardial proliferation significantly (P=0.000), and inhibit apoptosis (P=0.001). And the results of Western blot suggested the expressions of AKt, mTOR and PDK1 proteins in H/R+LDHA transfection group increased significantly and the differences had statistical significance (P=0.000). Compared with control group, the results of dual-luciferase method showed the luciferase signal intensity of vectors carrying wild type PDK1 reporter gene decreased by 45%, but there was no significant changes in the luciferase signal intensity of vectors carrying mutant type PDK1 reporter gene. Conclusion: The increasing LDHA expression in cardiomyocytes could up-regulate the activity of PDK1/AKt/mTOR pathway, and inhibit myocardial apoptosis in H/R.

Keywords: Myocardial ischemia-reperfusion injury, lactate dehydrogenase A, PDK1, Akt, mTOR, apoptosis, proliferation

## Introduction

Myocardial ischemia-reperfusion injury (MIRI) is a common and important disease in cardio-vascular diseases, which is severely harmful to human health. MIRI will cause myocardial apoptosis, necrosis, aggravated damage of cardiac structure and function, myocardial stunning, malignant arrhythmia, cardiac dysfunction, etc., which seriously affect patient's reperfusion treatment and prognosis [1, 2]. At present, MIRI is still a problem in the treatment of myocardial ischemia, and scholars pay increasing attention on it. Therefore, it is of theoretical

and practical significance to investigate how to reduce or eliminate MIRI on the basis of effectively recovering coronary blood flow.

The most important factor of myocardial ischemia is hypoxia of cardiomyocytes. Mitochondrion is abundant in cardiomyocytes, and it is a main source of energy for regulating cell survival and apoptosis-promoting molecule, whose main function is energy metabolism that closely related to the pathogenesis of myocardial reperfusion injury [3]. Lactic dehydrogenase is important for glycolysis, which can catalyze the conversion of pyruvate to lactate, thereby less-

ening the amount of pyruvate in cells [4]. Subtype lactic dehydrogenase A (LDHA), plays a key role in carbohydrate metabolism. Recent studies suggested that it could regulate cell proliferation and apoptosis. Some studies indicated that in the hepatic carcinoma, pancreatic carcinoma, nasopharyngeal carcinoma or other malignant tumors, the decrease of LDHA could induce the changes of cell energy metabolism and oxidative stress, leading to cell death [5-8]. Meanwhile, the decrease of LDHA can inhibit the occurrence of breast cancer through inducing mitochondrion-mediated apoptosis by oxidative stress [9]. Studies have shown that LDHA is abnormally overexpressed in various tumors including gastric cancer, pancreatic cancer, etc., which is related to the occurrence and development of tumors [10]. However, the mechanism of LDHA in cardiomyocytes is still unknown.

PI3K/Akt signal transduction pathway also plays an important role in MIRI. The combination of PDK1 and Akt proteins triggers the activation of phosphorylated Akt, which acts on multiple relevant substrates, thereby affecting cell division, proliferation and differentiation. Myocardial ischemia could lead an up-regulation of PDK expression, which regulates the activity of mitochondrial pyruvate dehydrogenase complex to catalyze the oxidation decarboxylation of pyruvate, and then to link glycolysis to the tricarboxylic acid cycle and adenosine triphosphate (ATP). But in the study of the mitochondrial energy metabolism dysfunction in MIRI, the interaction between LDHA and PDK has not been reported.

This study aims to explore the effect and mechanism of LDHA on myocardial apoptosis in MIRI, thereby explaining the molecular mechanism of cell apoptosis caused by MIRI.

## Materials and methods

### Experimental animal

Thirty SPF newborn Wistar rats, 2 days old, were purchased from Shanghai Slac Laboratory Animal Co. Ltd.

### Main reagents and instruments

DMEM culture medium (Hyclone Co., USA), fetal bovine serum (Gibico Co., USA), flow cytometry kit (BD Co., USA), Lipo2000 and Trizol Regent (Invitrogen Co., USA), CCK-8 regent (Sigma Co.,

USA), crystal violet (Sigma Co., USA) and pC-MV-SPORT6-LDHA plasmid (Sigma Co., USA), Annexin V FITC/PI (BD Biosciences Co., USA), rabbit anti-rat LDHA, monoclonal antibody of AKt, mTOR, and PDK1 (Santa Co., USA), second antibody (goat anti-rabbit, from Beijing ZSGB Bio, China), Real-time PCR instrument (ABI Co., Ltd., Japan), automatic enzyme-labeled instrument (Redu Co., USA).

### Culture of primary cardiomyocytes

Thirty newborn Wistar rats were killed and dissected under aseptic condition, and adequately cleaned the blood inside and outside of the hearts in pre-cooled DMEM, then unwanted parts (including atrium, aorta, etc.) were cut off only leaving ventricular myocardium. Myocardial tissue was cut up into about 1 mm$^3$ and transferred into centrifuge tube with added 0.125% trypsin and 0.125% type II collagenase, then it was put into a 37°C water bath for digestion with low frequency oscillation. Myocardial tissues were repeatedly digested till complete digestion, each time for about 8 min. Centrifuged for 10 min at 1000 r/min, supernatant was abandoned and DMEM with 10% fetal bovine serum was added to suspend cells, which was transferred into culture flask and placed in the incubator at 37°C.

### Establishment of hypoxia/reoxygenation (H/R) injury modeling of primary cardiomyocytes

Serum-free and sugar-free DMEM culture medium was chosen as hypoxia medium, and it was balanced by mixed gas (95% $N_2$ and 5% $CO_2$) for more than 2 h for saturation; normal medium was discarded and immediately replaced with saturated hypoxialiquid, then cardiomyocytes were placed in the hypoxic incubator (95% $N_2$ and 5% $CO_2$) at 37°C for 3 h, which was called hypoxia modeling. After myocardial hypoxia for 3 h, replacing the culture medium with DMEM medium (containing sugar and 10% FBS), the cardiomyocytes were placed in the incubator (95% air and 5% $CO_2$) for 3 h, which was called reoxygenation modeling.

### Construction and identification of LDHA overexpression vector

According to the known human LDHA gene sequence in the GenBank, primer premier 5.0 software was used for designing and completing the primers, which are to amplify their full
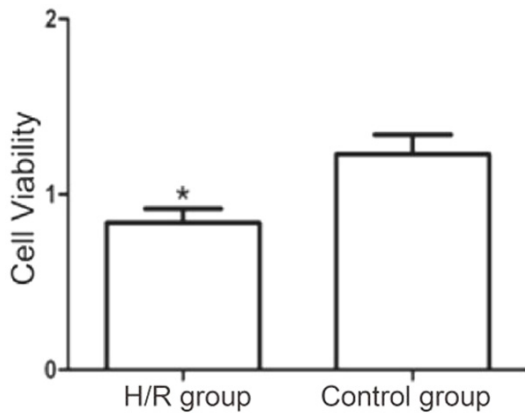
**Figure 1.** Comparison of proliferation of primary cardiomyocytes between control group and H/R group. Compared with control group, *P=0.002.
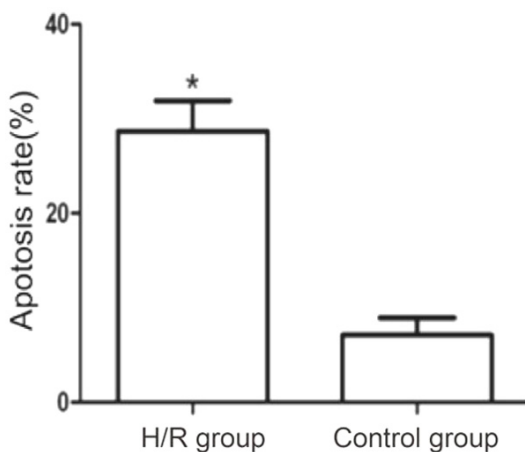


**Figure 2.** Comparison of apoptosis rate of primary cardiomyocytes between control group and H/R group. Compared with control group, *P=0.001.

coding region: F1: 5'-CAGTATGCTAGCATCGTA-GACGGCCACAATCGGT-3' and R1: 5'-AATAGCCT-CGAGTCTTTACACCCCTACCAG-3'. The Nhe I and Xho I restriction sites (underlined parts) were inserted on 5' endpoints of the primers respectively, and LDHA gene was amplified with PCR by taking plasmid clone of full-length of pCMV-SPORT6-LDHA gene as a template. PCR reaction system was as follows: 1 μL DNA templates of LDHA, 2 μL 10×PCR buffer, 1.2 μL $MgCl_2$, 0.8 μL dNTP, 0.4 μL upstream and downstream primers, 0.25 μL Taq enzymes and $ddH_2O$. Reaction condition: initial denaturation at 95°C for 3 min, degeneration at 95°C for 50 s, annealing at 56°C for 1 min, elongation at 72°C for 2 min, and a final elongation at 72°C for 10 min after 35 cycles. PCR amplification products were purified by gel electrophoresis and the tar-

get gene fragments were recovered. Using Nhe I and Xho I enzymes to cut plasmid vector pcDNA3.1 (+), then the enzyme-digested fragments were recycled. The LDHA target gene fragments and enzyme-digested vectors were ligated by $T_4$ DNA ligase, transformed into Escherichia coli DH5α competent cells and uniformly inoculated in the agaric solid medium. The clones were selected, then the sequences were identified after extracting plasmids on a small amount, and the positive clones were named as pcDNA3.1 (+)-LDHA.

*Transfection and grouping of cardiomyocytes*

Primary cardiomyocytes were randomly divided into 4 groups after 5 days of cultivation: blank control+no vector group (control group): cardiomyocytes were cultivated under normoxic condition and transferred with pcDNA3.1 (+) vector by Lipo2000; blank control +LDHA transfection group (control +LDHA group): cardiomyocytes were cultivated under normoxic condition, and transferred with pcDNA3.1 (+)-LDHA vector by Lipo2000; model+no vector group (H/R group): after being transfected by pcDNA3.1 (+) vector, primary cardiomyocytes were performed reoxygenation for 3 h after 3 h of hypoxia; model +LDHA transfection group (H/R+LDHA group): after being transfected by pcDNA3.1 (+)-LDHA vector, primary cardiomyocytes were performed reoxygenation for 3 h after 3 h of hypoxia. The cells were collected at different time points for next experiment, and the experiments were repeated 3 times in each group.

*CCK-8 method to detect cell proliferation of cardiomyocytes*

Cardiomyocytes of each group were inoculated in 96-well plates at the density of $4*10^4$/ml, and culture medium was added, then the cardiomyocytes were cultivated in 5% $CO_2$ incubator at 72°C for 72 h. Added 10 μL CCK-8 in each well, the cardiomyocytes continued to be incubated in the cell incubator for 4 h, and then the absorbance of each well was measured at 450 nm by microplate reader. The average values of 5 wells were recorded.

*Flow cytometry to detect apoptosis of cardiomyocytes*

Cardiomyocytes of each group were inoculated in 6-well plates at the density of $4*10^5$/ml and cultivated for 72 h. The cardiomyocytes collected in flow tubes after they were washed with
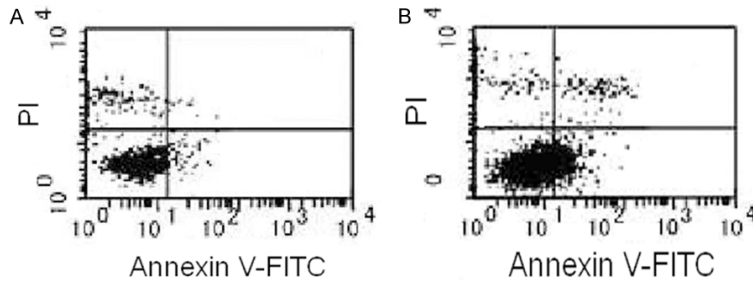
**Figure 3.** Flow cytometry to detect the myocardial apoptosis in control group and H/R group. A: Control group; B: H/R group.
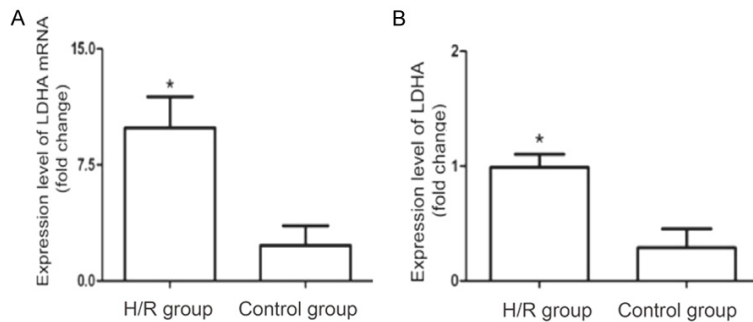


**Figure 4.** Comparison of LDHA expression of cardiomyocytes between control group and H/R group. Compared with control group, *P=0.000. A: Real-time PCR detection; B: Western blot detection.
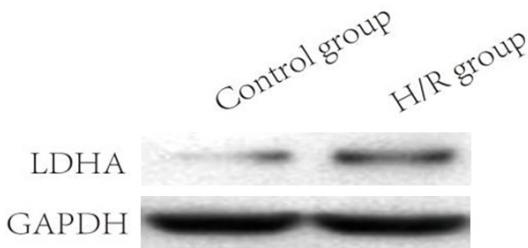


**Figure 5.** Western blot to detect the relative expressions of LDHA protein between control group and H/R group.

PBS. After the cardiomyocytes underwent double staining with Annexin V FITC/PI, the apoptosis of cardiomyocytes was detected (excitation wavelength (Ex=488 nm) and emission wavelength (Em=530 nm)).

*Real-time PCR detection*

The total myocardial RNAs in each group were extracted by Trizol reagents. The cDNAs were synthesized from the total RNAs through reverse transcriptase. The β-actin was regarded as the reference control. Upstream primer of β-actin: 5'-CATTAAGGAGAAGCTGTGCT-3', and downstream primer of β-actin: 5'-GTTGAA-

GGTAGTTTCGTGGA-3'. Upstream primer of LDHA: 5'-ATGGC-AACTCTAAAGGATCAGC-3', and downstream primer LDHA: 5'-CCAACCCCAACAACTGTAAT-CT-3'. Reaction system of 20 uL: there were 2 uL cDNAs, 0.8 uL upstream and downstream primers respectively, 10 uL 2×SYBR Green PCR Master Mix, and 7.2 uL ddH$_2$O. The reaction system was placed in Applied Biosystems 7500 quantitative PCR instrument for amplification after centrifugation. Reaction condition: initial denaturation at 95°C for 8 min, degeneration at 94°C for 20 sec, renaturation at 56°C for 20 sec, elongation at 72°C for 23 sec, 30 cycles in total. The β-actin was as the reference gene and the relative expression level of LDHA mRNA of each group was calculated by $2^{-\Delta\Delta Ct}$ method.

*Western blot detection*

Microplate reader was used to measure the protein concentration after the cardiomyocytes of each group were disintegrated. The total quantity of protein was 30 μg. After the separation by SDS-PAGE gel electrophoresis, the proteins were transferred to PVDF membrane by electroblotting. Membranes were cut according to the desired target bands and blocked at room temperature with TBS-T solution of 5% skimmed milk powder for 1 h. Then 300× rabbit anti-ratLDHA,300×AKt/400×PDK1/400×mTOR monoclonal antibody, and 200× rabbit anti-rat β-actin antibody were added respectively to the membranes and it was incubated at 4°C overnight. Then it was washed by TBS-T solution at room temperature and added goat anti-rabbit secondary antibody for 30 min-incubation. After being washed by TBS-T solution, it was reacted with chromogenic reagent, and then it was exposed and scanned. In the end, the optical densities of the target bands were calculated by Image J software.

*Dual-luciferase detection*

According to the PDK1 sequences and the predicted results of Target Scan software, the
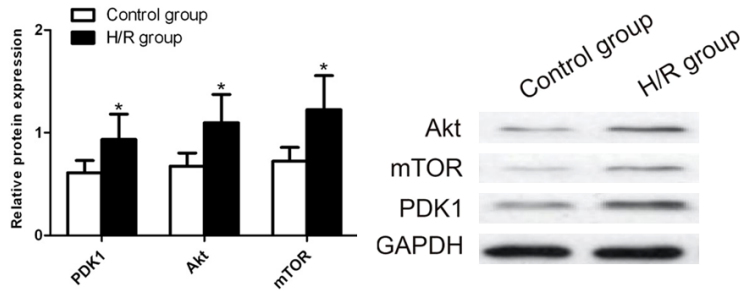
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**Figure 6.** Western blot to detect the effect of H/R on the expression of AKt, mTOR and PDK1 in cardiomyocytes between control group and H/R group. Compared with Control group, P=0.000.



**Figure 7.** Western blot to detect the expression of LDHA in transfection of cardiomyocytes with pcD-NA3.1 (+)-LDHA.

3'UTR sequence constructed by PCR amplification in the DNA sequences were cloned to the pGL-Basic reporter gene plasmid (pGL-UTR), sequenced, verified and constructed into luciferase reporter gene vectors pGL3-WT-PDK1-3'UTR and pGL3-MUT-PDK1-3'UTR. We commissioned Takara Biomedical Technology Co. Ltd. (Dalian) to complete sequencing. The Lipo2000 liposomes were used to mediate the transfection of the two plasmids and the vectors with the target sequences of LDHA gene, respectively, into the 70% confluent U251 cells (American Type Culture Collection, USA). After 48 h, the activities of fluoresceins were detected by dual-luciferase assay kit (Promega Co., USA).

*Statistical analysis*

Statistical analysis was performed by SPSS 19.0. Measurement data were expressed by mean ± standard deviation ($\bar{x} \pm s$). Pairwise measurement data comparisons were presented by t-test. Categorical data were compared by chi-square test or Fisher's exact test. P<0.05 meant that the difference was statistical significance.

## Results

*Comparison of proliferation of primary cardiomyocytes between control group and H/R group*

After 72 h-culture of primary cardiomyocytes, the OD value of primary myocardial cells in H/R group detected by microplate reader was 0.84±0.08, which was significantly lower than that in control group (1.23±0.11), and the difference was statistically significant (P=0.002). See **Figure 1**.

*Comparison of apoptosis of primary cardiomyocytes between control group and H/R group*

After 72 h-culture of primary cardiomyocytes, the apoptosis rate of primary cardiomyocytes in H/R group detected by flow cytometry was (28.7±3.23)%, which was significantly higher than that in control group (7.12±1.82)%. There was statistical difference between the two groups (P=0.001). See **Figures 2**, **3**.

*Comparison of LDHA expression of cardiomyocytes between control group and H/R group*

After 72 h-culture of primary cardiomyocytes, the LDHA expression of H/R group detected by Real-time PCR and Western blot was significantly higher than that of control group. The relative expression levels of mRNA and protein of LDHA in H/R group were (9.879±2.021) and (0.988±0.114) respectively, and those in group Control were (2.289±1.261) and (0.287±0.165) respectively. And the differences were statistically significant (both P=0.000). See **Figures 4**, **5**.

*Effects of H/R on the protein expression of AKt, mTOR and PDK1 in cardiomyocytes between control group and H/R group*

After 72 h-culture of cardiomyocytes, the results of Western blot showed that the protein expression of AKt (1.098±0.274), mTOR (1.124±0.332) and PDK1 (0.934±0.246) in H/R group increased significantly, comparing with control group. And the difference was statistically significant (all P=0.000). As shown in **Figure 6**.

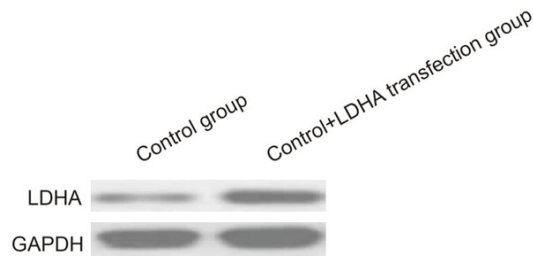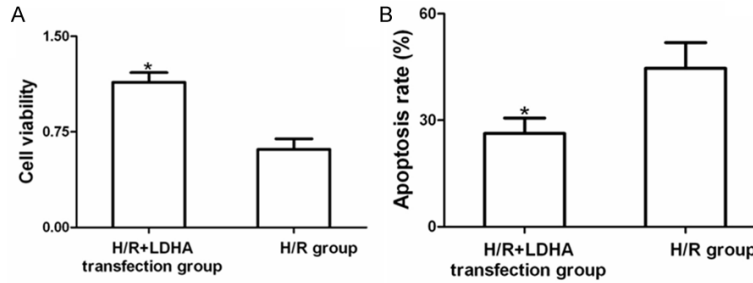**Figure 8.** Effects of LDHA overexpression on proliferation and apoptosis of primary cardiomyocytes. Compared with H/R group, *P=0.000. A: CCK-8 to detect proliferation of cardiomyocytes. B: Flow cytometry to detect apoptosis of cardiomyocytes.
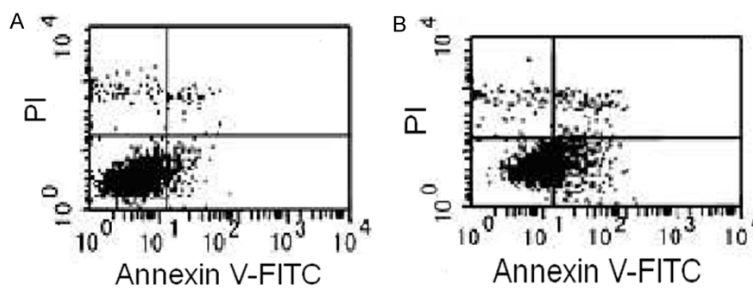


**Figure 9.** Flow cytometry to detect the effects of LDHA overexpression on apoptosis of primary cardiomyocytes under the condition of H/R. A: H/R group; B: H/R+LDHA transfection group.
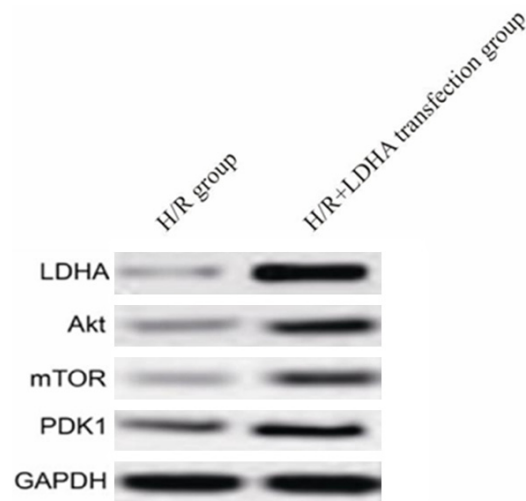


**Figure 10.** Impacts of LDHA overexpression on the expression of AKt, mTOR and PDK1 proteins under the condition of H/R.

*Effects of LDHA overexpression on proliferation and apoptosis of primary cardiomyocytes under the condition of H/R*

The expression of LDHA in cardiomyocytes was detected by Western blot after primary cardio-

myocytes being transfected with pcDNA3.1 (+)-LDHA for 48 h. The results showed that compared with control group, the expression level of LDHA in cardiomyocytes significant-ly incre-ased after being trans-fected with pcDNA3.1 (+)-LDHA. As shown in **Figure 7**.

The results of CCK-8 showed that the OD value in H/R+LDHA transfection group (1.140±0.075) was signifi-cantly higher than that in H/R group (0.612±0.084), with statistically significant differ-ence (P=0.000), which indi-cated that overexpression of LDHA significantly promoted proliferation of cardiomyocy-tes. As shown in **Figure 8A**. According to the results of flow cytometry, the apoptosis rate of cardiomyocytes in H/R+LDHA group was (26.27+4.35)%, and that in H/R group was (44.63+7.25)% with sta-tistically significant difference (P=0.000), which indicated that overexpression of LDHA could significantly inhibit apoptosis of cardiomyocytes. As shown in **Figures 8B** and **9**.

*Effects of LDHA overexpression on the expres-sion of AKt, mTOR and PDK1 proteins under the condition of H/R*

Cardiomyocytes continued to be cultured under the condition of H/R for 72 h after being trans-fected with overexpression vector of LDHA. The results of Western blot showed that compared with H/R group, the protein expression level of AKt (3.454±0.782), mTOR (3.793±1.122) and PDK1 (2.814±0.948) in H/R+LDHA transfec-tion group significantly increased with statisti-cal difference (P=0.000). See **Figures 10** and **11**.

*Dual-luciferase to detect the target regulation of LDHA on PDK1*

The results of dual-luciferase detection showed that compared with control group, after the transfection of LDHA overexpression vectors, the luciferase signal intensity of vectors carry-ing wild type PDK1 reporter gene decreased by 45%, while the luciferase signal intensity of
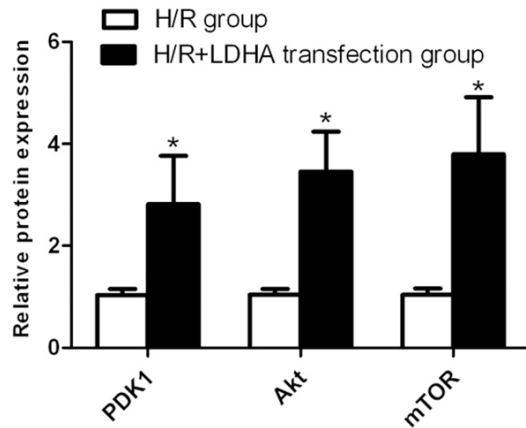
**Figure 11.** Effects of LDHA overexpression on the expression of AKt, mTOR and PDK1 proteins under the condition of H/R.
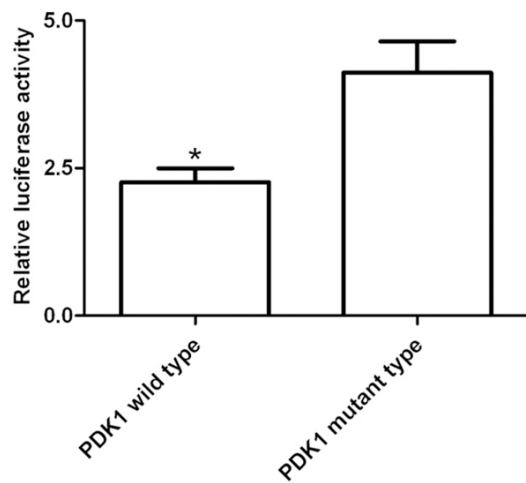


**Figure 12.** Two groups' luciferase signal intensity tests. Compared with the PDK1 mutant reporter gene vectors, *P=0.001.

vectors carrying mutant type PDK1 reporter gene had unobvious changes, which indicated that the PDK1 gene was the target of LDHA. See **Figure 12**.

## Discussion

The most effective treatment for AMI is to restore the blood supply of ischemic myocardium quickly. However, myocardial ischemia fails to reduce or eliminate the damage of ischemic cardiomyocytes but increased myocardial damage, that is, MIRI after the blood perfusion of ischemic myocardium is restored. Apoptosis plays an important role in MIRI according to some studies [11, 12]. MIRI can be reduced by

effective inhibition of myocardial apoptosis [13]. Myocardial apoptosis may be closely related to mitochondrial damage. MIRI can cause significant decrease of ATP in cardiomyocytes and the decrease of ATP/ADP ratio, which contributes to calcium overload, acidosis, mitochondrial function damage and excessive oxygen radical, thereby leading to DNA fragmentation in cardiomyocytes and inducing apoptosis. It suggested that energy metabolism dysfunction played an essential role in the occurrence of MIRI.

The energy required for metabolism of cardiomyocytes is mainly provided by glucose. Glucose metabolism is mainly through aerobic oxidation and glycolysis. In the environment of ischemia and hypoxia, as one of the key enzymes in glycolysis, LDHA can catalyze the conversion of pyruvate to lactate. At present, researches on LDHA is mainly focused on tumor. Studies have shown that most oncogenes can induce the expression of LDHA and LDHA plays a vital role in carcinogenicity of oncogenes [14, 15]. Some studies reported that the reduction of LDHA activity could reduce the incidence of gastrointestinal tumors [16, 17]. Low expression of LDHA can induce apoptosis of hepatoma cell and hepatocyte [18, 19]. And attenuation of LDHA expression can inhibit the proliferation and metastasis of prostate carcinoma cells so that the cells can be stagnant in S phase, and promote apoptosis [20]. This study mainly focused on the effect of LDHA on cardiomyocytes during the process of ischemia-reperfusion injury. The results indicated that proliferation activity of cardiomyocytes significantly decreased while the apoptosis rate significantly increased and LDHA was highly expressed in the H/R model *in vitro*, and the differences were statistically significant. LDHA overexpression can significantly promote proliferation and inhibit apoptosis of cardiomyocytes under the condition of H/R with statistical significance difference (P<0.05), which showed that LDHA played an important role in ischemia-reperfusion injury.

Currently, the mechanism of LDHA in ischemia-reperfusion injury is still unclear. AKt signaling pathway is an important signal transduction pathway that regulates cell proliferation, differentiation, and apoptosis. It can activate a variety of downstream molecules and may be involved in the occurrence of ischemia-reperfu-

sion injury [21]. mTOR is one of the downstream signaling molecules of Akt which participates in the regulation of apoptosis, growth and so on. Studies have shown that mTOR overexpression can reduce the inflammatory response and has anti-MIRI [22]. And PDK1 is an important protein on the AKT signaling pathway. The results of this study showed that LDHA overexpression could promote the expression of PDK1, Akt and mTOR in cardiomyocytes under the condition of H/R. And LDHA target regulated the expression of PDK1, which suggested that H/R might mediate or intervene cell damage by regulating LDHA/PDK1, and Akt signaling pathway.

In conclusion, this study showed that LDHA might have effects on ischemia-reperfusion injury and promote the proliferation of cardiomyocytes and inhibit apoptosis by affecting the expression of PDK1, Akt and mTOR. Signaling pathways that related to LDHA are quite complex and it needs comprehensive studies to further expound. With the increasing deepening study of molecular and cellular mechanisms, LDHA may be a therapeutic target for MIRI. However, this study has some limitations including single-center research, thus it still needs to further confirm this view through a large number of multi-center researches.

## Disclosure of conflict of interest

None.

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