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# Lentivirus mediated interference of Caspase-3 expression ameliorates the heart function on rats with acute myocardial infarction

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**Abstract. – AIM: To explore the heart protection effects by RNA interference of Caspase-3 on rat acute myocardial infarction (AMI).**

**MATERIALS AND METHODS: 45 SD rats were randomly divided into sham group, AMI group and Caspase-3-siRNA group. Animal model of AMI was established by ligation of anterior descending branch (LAD). Rats of sham operation group and AMI group were administered with lentivirus harboring empty vector into myocardial tissue. Rats of Caspase-3-siRNA group were administered with lentivirus harboring Caspase-3 RNA interference vector. After 72 hours, the heart function were evaluated by echocardiogram analysis. Then the rats were sacrificed and the myocardial tissue were collected. The expression level of Caspase-3 mRNA and protein in each group were analyzed by RT-PCR and Western blot, respectively. Moreover, the infarct size was analyzed by triphenyltetrazolium chloride (TTC) staining and the apoptosis index of myocardial cells were analyzed by TUNEL assay.**

**RESULTS: Compared with the sham operation group, the expression levels of Caspase-3 mRNA and protein were significantly upregulated ($p < 0.05$) and the Caspase-3 activity was enhanced ($p < 0.05$) in AMI group and Caspase-3-siRNA group. However, the Caspase-3-siRNA group showed lower expression levels of Caspase-3 mRNA/protein and weaker Caspase-3 activity, compared with AMI group ($p < 0.05$). Furthermore, the apoptosis index and the infarction range of myocardial cells were enhanced in AMI group and Caspase-3-siRNA group ($p < 0.05$). The apoptosis index and infarction range of myocardial cells in Caspase-3-siRNA group were decreased ($p < 0.05$). Interestingly, the Left Ventricular End-Diastolic dimension (LVEDd) and the Left Ventricular End-Systolic diameter (LVESd) in AMI group and Caspase-3-siRNA group were improved ($p < 0.05$), but the Ejection Fraction (EF) and Fractional Shortening (FS) in AMI group and Caspase-3-siRNA group were reduced ($p < 0.05$). All the LVEDd, LVESd, EF and FS in Caspase-3-siRNA group significantly improved than that of AMI group ($p < 0.05$).**

**CONCLUSIONS: The downregulation of Caspase-3 mediated by lentivirus interference can decrease the infarct size of myocardial tissue and the apoptosis index of myocardial cells. It also can improve heart function in rats with acute myocardial infarction.**

*Key Words:*
Caspase-3, RNA interference, Acute myocardial infarction, Heart function.

## Introduction

Despite of the impressive strides in diagnosis and management over the past three decades, acute myocardial infarction (AMI) is still a major public health issue and becoming an increasingly problem in both developed and developing countries[1,2]. A lot of animal and clinical researches have proven that many severe pathological mechanisms, such as oxidative stress, ischemia, hypoxia damage and reperfusion, are triggered the apoptosis of myocardium after AMI. Since the apoptosis of myocardium plays a critical role in ventricular remodeling and heart failure in the process of AMI[3,4], it is noticeable to emphasize that to decrease the apoptosis of myocardium is important for the AMI recovery. In mechanism, the apoptosis myocardium is caused by a serial of Caspase cascade response, especially the Caspase-3. As a cooperation protein expressed on cell surface, Caspase-3 involves in apoptosis processes of both cell-surface pathway and mitochondrial pathway[5,6]. Plenty of clinical researches have found a significantly increased Caspase-3 expression levels and protein activity in patients with acute myocardial infarction myocardial, indicating there is a certain relationship between Caspase-3 and AMI[7]. With this hypothesis, some evidences have been shown in animal AMI model that the Caspase-3 inhibitor could alleviate the



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myocardium apoptosis by preventing the reperfusion injury, and even it is benefit to the heart function ultimately[8,9]. However, due to its poor specificity, Caspase inhibitor which is related to variant of reaction ligands could hardly tell what the exact function of Caspase in AMI. Many studies demonstrated that the expression of Caspase-3 is significantly elevated in myocardium tissue during AMI, indicating that Caspase-3 might be a meaningful biomarker for the AMI diagnosis and treatment[10,11]. To investigate the effect of Caspase-3 in AMI, we carried out RNA interfering to down-regulate the expression of Caspase-3 in rat myocardium tissue in this study.

## Materials and Methods

### Experimental Animals

SD rats weighted from 180 to 200 g were purchased from Comparative Medicine Center, Yangzhou University. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of People's Hospital of Zhengzhou.

### Establishment of the Myocardial Infarction Rat Model

45 rats were randomly divided into three groups: sham group, AMI group and Caspase-3-siRNA group. All the rats were anesthetized with pentobarbital, and then were performed thoracotomy. For the sham group, rats were not treated with LAD ligation. AMI group and Caspase-3 interference rats were operated for ligation of LAD. A visible pale wall on the left anterior ventricle indicated the success of model building. Meanwhile, poly-leads physiological detector displayed a ST segment elevation and (or) a T-wave elevation on ECG standard lead II[12]. After surgery, the sham group and AMI group were intramyocardially injected 100 μl ($1\times10^7$ PFU) lentivirus without carrying Caspase-3 cDNA. In addition, Caspase-3 interference group was injected the same amount lentivirus carrying Caspase-3 cDNA. Then sutured the incision, and penicillin was applied for resistance to infection. Rats were sacrificed at 48 hour after operation, collected myocardial tissue for the following analysis.

### RT-PCR

Sacrificed the rat and collected 0.2 g heart tissue in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA). Added 200 μl trichloromethane followed by shaking tube vigorously. Incubated on ice and centrifuged at 15,000 g for 15 min. Transferred the aqueous phase of the sample into a new tube and added equal volume of isopropanol to the aqueous phase. Incubated the mixture for 30 min on ice to RNA precipitation. Centrifuged at 15,000 g for 15 min. Removed the supernatant from the tube, leaving only the RNA pellet. Washed the pellet with 1 ml of 75% precool ethanol twice. Centrifuged at 10,000 g for 10 min. Resuspended the RNA pellet in DEPC-treated water. Diluted sample in RNase-free water, and measured absorbance at 260 nm and 280 nm by spectrophotometer. Reversed RNA to cDNA with reverse transcription reagent (Takara, Dalian, China). Designed rat Caspase-3 qPCR primer: Forward: 5'-GGTATTGAGACAGACAGTGG-3'; Reverse: 5'- CATGGGATCTGTTTCTTTGC-3'. Mixed the all these reagent for PCR: 2×SYBR Green general qPCR Master Mix (Roche, Basel, Switzerland) 10 μl, Forward and Reverse primer (10 μmmol/L) 1 μl each, cDNA 1 μl added water until the total reaction volume reached to 20 μl. Added 20 μl mixed PCR reaction system into each designed well, and the conditions were as follows: 95°C denaturation for 5 min, 95°C for 15 s, 60°C for 1 min for 30 cycles.

### Western Blot

Added 1g rat heart tissue into 300 μl tissue lysis buffer with 3 μl proteinase inhibitor. Homogenated tissue on ice for 30 min. Centrifuged the mixture at 15,000 g for 15 min. Harvested the supernatant. The protein concentration was determined by using the BCA protein assay kit (Takara, Dalian, China). Added 4×loading buffer and boiled for 30 min. Loaded the sample on 12% SDS-PAGE gel for electrophoresis. The initial voltage was 80V, and reset it to 12V when the bromophenol blue reached the separating gel. After electrophoresis, transferred the protein to PVEF membrane. Blocked the samples with 5% milk and incubated with Caspase-3 antibody (Abcam, Cambridge, UK) overnight. Washed with PBST three times. The membranes were incubated with the corresponding HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Enhanced chemiluminescence reagents were used for detection.

The autographed films were scanned and processed with imaging system. The densitometric value of each protein band was normalized to housekeeping proteins -actin. The relative quantitation expression level of protein was calculated by gray calculation software.

### Caspase-3 Protein Activity Assay

Added 100 μl tissue lysis buffer per 3-10 mg tissue. Homogenated the pellet on ice and then transferred the mixture into a 1.5 ml EP tube followed by lysis on ice for another 5 min. Centrifuged at 16,000-20,000 g at 4°C for 15 min. Transferred the supernatant to a precool EP tube. According to the instruction of the Caspase-3 activity test kit (Beyotime Biotech, Nantong, China), incubated the mixture of sample and reagent at 37°C for 120 min. Examed the absorptive value of Caspase-3 protein at $A_{405}$ nm. The Caspase-3 activity was calculated by the standard curve and activity curve.

### TUNEL Assay

The slide of heart tissue were dried for 5 min before being fixed with 4% paraformaldehyde for 20 min at room temperature and then repaired the antigen. Washed with phosphate buffered saline and tween (PBST) for three times. The following steps were strictly guided by TUNEL analysis kit (Roche, Basel, Switzerland). As the result, the apoptotic nuclei were labeled with green fluorescence and observed under fluorescence microscope. Under the 400 × microscope, randomly picked 10 views at the infarct region and board region. Calculated the percentage of apoptosis cells as the index of apoptosis level of the heart tissue.

### TTC (Triphenyltetrazolium Chloride) Assay

2 mm section slide of the heart tissue was stained in 1% TTC phosphate buffer (Senbeijia Bio. Co, Nanjing, China) at 37°C for 20 min. The normal tissue appeared red and the infarct tissue was white. Isolated the infarct tissue under the dissection scope and then weighted both red and white part. Calculated the percentage of infarct issue as the infarct area of the heart tissue.

### Heart Function Test

After the anesthesia with 1% Sodium pentobarbital, performed rat ultrasonic cardiogram with 12 MHz detector through the chest. M-ultrasound cardiogram & echocardiogram was carried out to show the Left Ventricular End-Diastolic dimension (LVEDd), Left Ventricular End-Systolic dimension (LVESd), Ejection Fraction (EF) and Fraction Shortening (FS).

### Statistical Analysis

All data were expressed as means ± SD for each group and compared using Student's unpaired t-test by SPSS 17.0 (SPSS Inc, Chicago, IL, USA). Comparisons between multiple measurement data were analyzed with ANOVA method, comparisons of intragroup data were analyzed with LSD method. Test results were reported as 2-tailed $p$ values, where $p < 0.05$ was considered statistically significant.

## Results

### Effect of the Caspase-3 Interference

All the rats in sham group, AMI group and Caspase-3-siRNA group were sacrificed at 48 h after interference. The results of qPCR and Western blot showed that the mRNA and protein expression levels of Caspase-3-siRNA group in AMI group were significantly decreased, the difference was statistically significant ($p < 0.05$) (Figure 1A, B and C). Caspase-3 activity analysis showed that the enzyme activity in AMI and Caspase-3-siRNA groups were significantly higher than the sham group ($p < 0.05$). However, compared with the AMI group, the activity of Caspase-3 was significantly decreased ($p < 0.05$) (Figure 1D).

### The Analysis of Apoptosis in Three Groups

Compared with sham group, the infarct index and infarct area of both AMI and Caspase-3-siRNA groups were obviously larger ($p < 0.05$). But the infarct index and infarct area of Caspase-3-siRNA group were even lower than the AMI group ($p < 0.05$) (Figure 2).

### The Heart Function of the Three Groups

Apoptosis level of myocardium directly affects the heart function. To explore the apoptosis level in these three groups, the ultrasonic cardiogram demonstrated that LVEDd and LVESd in both AMI and Caspase-3-siRNA groups were significantly reduced, meanwhile the EF and FS was significantly increased, compared to sham group. Furthermore, LVEDd and LVESd in the Caspase-3-siRNA group were lower than AMI group, while EF and FS were increased significantly, compared with the AMI group ($p < 0.05$) (Figure 3).

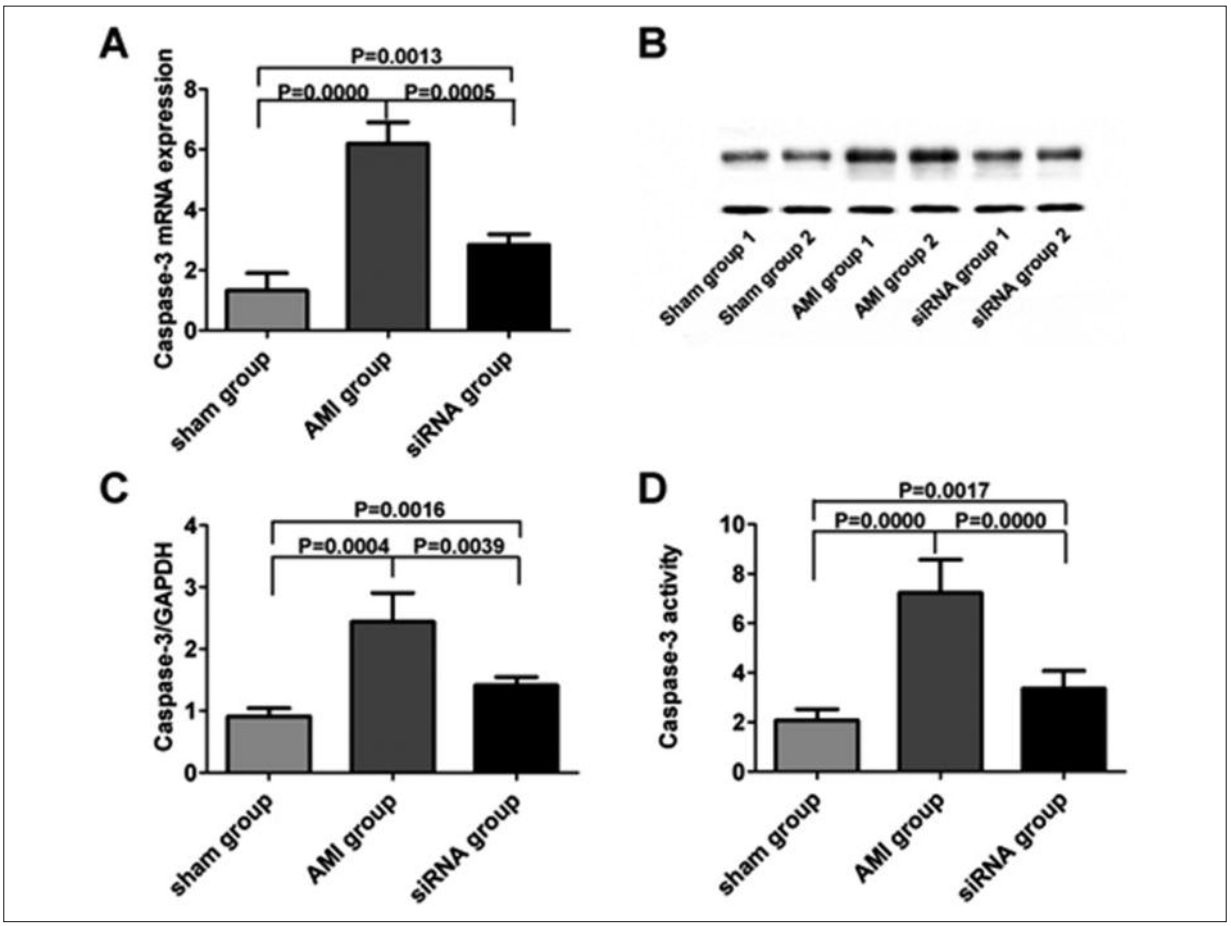


**Figure 1.** Comparison of Caspase-3 mRNA, protein and activity in sham group, AMI group and siRNA group. ***A,*** Caspase-3 mRNA level in sham group, AMI group and siRNA group by real time PCR. ***B,*** Caspase-3 protein level in sham group, AMI group and siRNA group by Western blot. ***C,*** Quantitative analysis of Caspase-3 protein level in sham group, AMI group and siRNA group by Western blot. ***D,*** Comparison of Caspase-3 activity in sham group, AMI group and siRNA group.

## Discussion

Since its high mortality and sudden onset, acute myocardial infarction has become a serious impact on human health. At present, the recommended treatment of acute myocardial infarction are thrombolysis, anti-oxidation, and correcting electrolytes disorder. But recent studies have shown that myocardial infarction caused myocardial ischemia and thrombolytic tissue hypoxia can lead to myocardial reperfusion injury, which leads to the apoptosis of myocardium cells. The loss of myocardium can further deteriorate myocardial injury, and accelerate the development of the disease[13,14]. There is few of report about the clinical usage of apoptosis inhibitors. The *in vitro* and *in vivo* experiments using apoptosis inhibitors can significantly improve the myocardial apoptosis in AMI, as well as heart function. Therefore, it encourages us to looking for the specific target of myocardial apoptosis during AMI.

Caspase-3 is the major regulator in the downstream of apoptosis signaling pathway. Either extracellular receptor mediated apoptosis or intracellular mitochondrial mediated apoptosis ultimately induce the cell death through activating substrate degradation by Caspase-3 and/or Caspase-7, etc[15-18]. Therefore, this study explored the effect of Caspase-3, which is potential target for inhibition of apoptosis. It also can inhibits the apoptosis of myocardial cell and improve the cardiac function. As a mature genetic technology for gene expression, lentiviral-mediated RNA interference of Caspase-3 was well designed in this study. It showed a high infection effect and specific targeting, indicating that RNA interference is a powerful delivery system for our study[19,20].

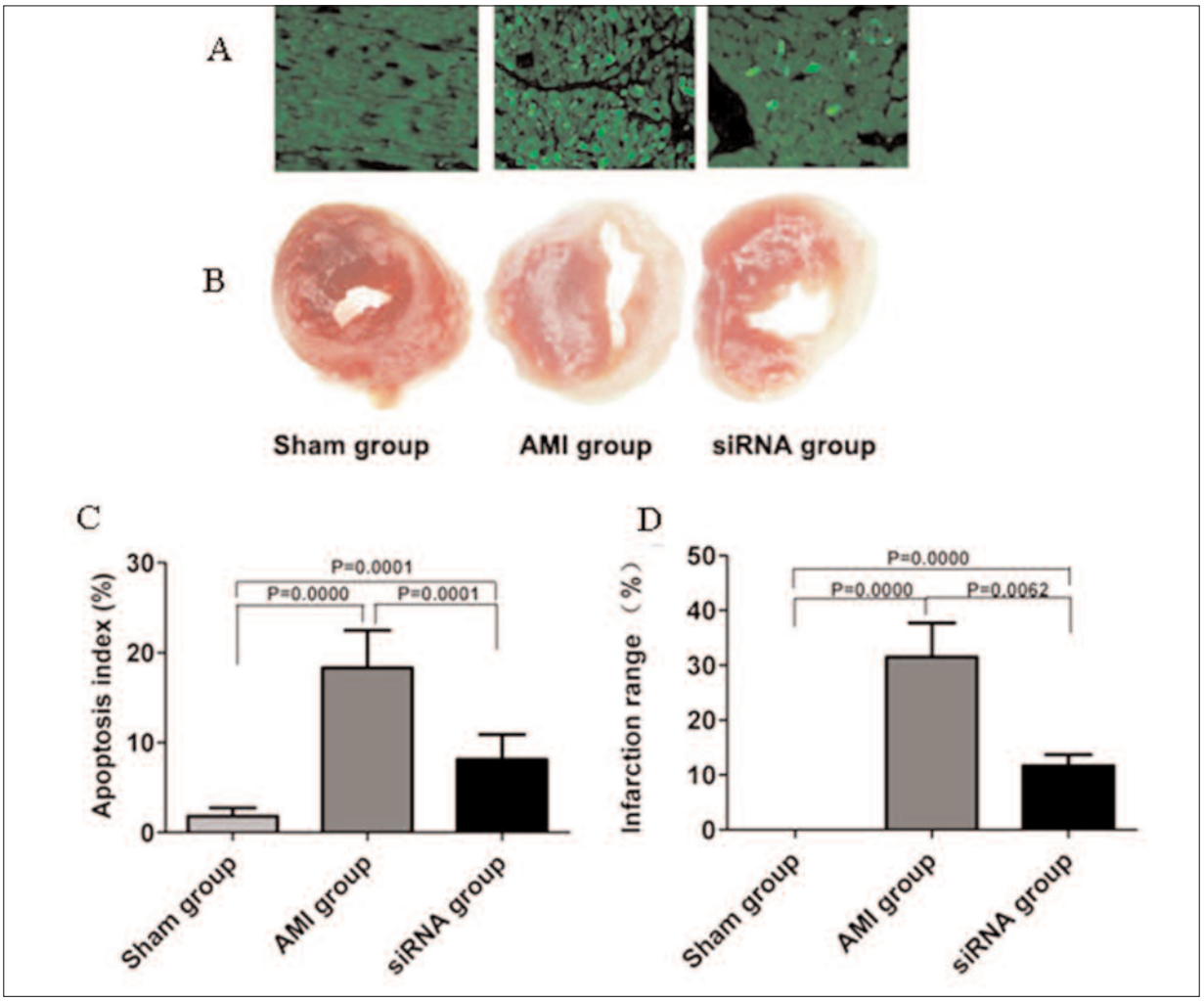


**Figure 2.** Comparison of apoptosis index and infarction range in sham group, AMI group and siRNA group. ***A,*** TUNEL staining in sham group, AMI group and siRNA group. ***B,*** TTC staining in sham group, AMI group and siRNA group. ***C,*** Quantitative analysis of apoptosis index in sham group, AMI group and siRNA group. ***D,*** Quantitative analysis of infarction range in sham group, AMI group and siRNA group.

After injecting lentivirus carrying Caspase-3 interference into the myocardial tissue, real-time quantitative PCR and Western blot analysis showed that the mRNA and protein expression levels of Caspase-3 decreased significantly in infarct myocardial tissue, indicating that lentivirus-mediated Caspase-3 downregulation could stably and enduringly repress the transcription of Caspase-3 in myocardial tissue. As a form of zymogen in normal cytoplasm, Caspase-3 is activated due to the apoptosis induction, then forming the big and small subunits. Ultimately the activated Caspase-3 plays a biological role in variant signal pathways[21]. This study analyzed the activity of Caspase-3. The suppressed Caspase-3 gene showed a significantly lower protein biological activity in rat model of myocardial infarction, implying that silence of Caspase-3 effectively blocks the apoptotic signaling pathway; thereby, inhibits the Caspase-3 activity[22].

## Conclusions

Lentiviral-mediated silence of Caspase-3 in myocardial tissue reduces the expression of Caspase-3. Moreover, this method inhibits myocardial apoptosis and benefits to myocardial infarction. It significantly improves heart function, and provides a theoretical basis of drug screen for clinical anti-apoptotic therapy in AMI.

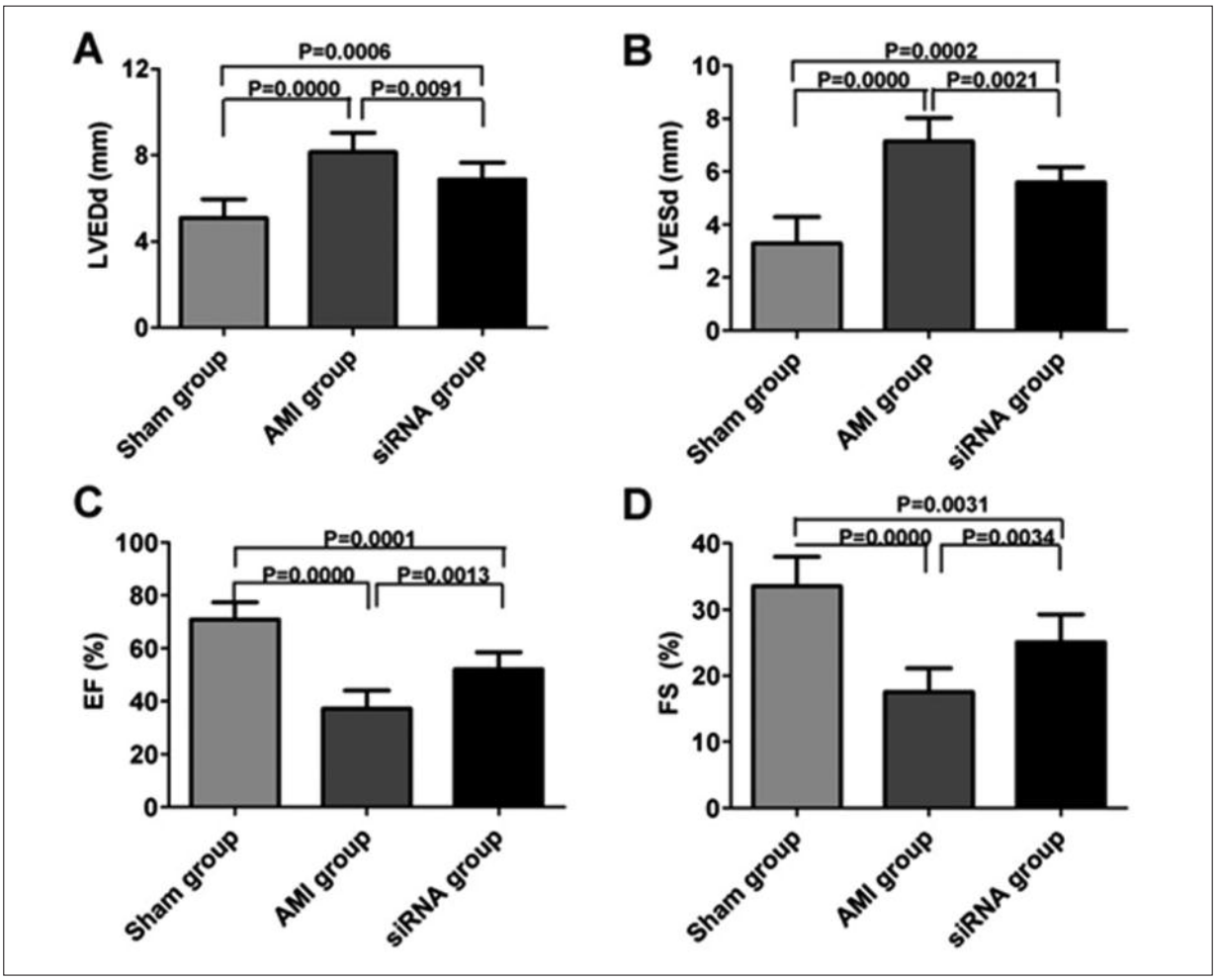


**Figure 3.** Comparison of cardiac function in sham group, AMI group and siRNA group. ***A,*** Comparison of LVEDd level in sham group, AMI group and siRNA group. ***B,*** Comparison of LVESd level in sham group, AMI group and siRNA group. ***C,*** Comparison of EF level in sham group, AMI group and siRNA group. ***D,*** Comparison of FS level in sham group, AMI group and siRNA group.

## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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