NADPH oxidase 4 contributes to oxidative stress in a mouse model of myocardial infarction

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Running title: NADPH oxidase 4 contributes to myocardial infarction

ABSTRACT

Background: Oxidative stress closely related to the progression and severity of myocardial infarction (MI). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) is one of the major enzymes that generate reactive oxygen species (ROS) in cardiovascular system. Here, we aim to elucidate the pathological role of NOX4 in MI.

Methods: MI mouse model was created by the coronary artery ligation. NOX4 was specifically knocked down in heart through intramyocardial injection of siRNA. NOX4 expression and oxidative stress indicators were determined at different time points using qRT-PCR, Western blot, and ELISA, and then analyzed by Pearson's correlation. Cardiac function was evaluated by using echocardiographic technique.

Results: NOX4 was upregulated in myocardial tissues of MI mice, which positively correlated with the elevation of oxidative stress indicators. Knockdown of NOX4 in heart significantly reduced the production of ROS and the level of oxidative stress in left ventricle tissues, which was accompanied by significant improvement of cardiac function in MI mice.

Conclusion: Selective knockdown of NOX4 in heart attenuates MI-induced oxidative stress and improves cardiac function, suggesting inhibition of NOX4/ROS axis in heart using siRNA is a potential therapeutic treatment for MI-induced cardiac dysfunction.

KEYWORDS: myocardial infarction; oxidative stress; cardiac function; NOX4

INTRODUCTION

Myocardial infarction (MI) is one of the most prevalent cardiovascular symptoms, which has become the leading contributor to the disability and sudden death all over the world [1]. The cases of MI have increased dramatically in the past three decades, which cause more than one million deaths in the United States annually [2, 3]. With the aging of the population and the increasing prevalence of obesity and diabetes globally, the importance of prevention and treatment of cardiovascular diseases will further increase.

MI is characterized as myocardial necrosis that caused by the unstable ischemic syndrome [4]. In cardiovascular system, oxidative stress is elevated accompanying with hypertrophy and heart failure [5]. MI contributes the activation of multiple deleterious cellular signals and the increase of reactive oxygen species (ROS), which promote the expression of inflammatory cytokines in endothelial cells and accelerate the infiltration of inflammatory cells to the injury region of myocardial tissue [6, 7]. In addition, multiple post-MI factors, such as myocardial ischemia, left ventricular remodeling, infarct size, hibernating myocardium, and hypertension, can affect the performance of left ventricular ejection fraction (LVEF) and left ventricular systolic dysfunction, and then impair the ventricular structure and cardiac function [8, 9]. Although many medical treatments and precaution for MI have been developed, MI still causes many deaths each year. Herein, it is urgently needed to find new targets and therapy strategies for the MI treatment.

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOXs) family is a group of enzymes that are responsible for superoxide ($O2^-$) production through transferring electrons from NADPH to molecular oxygen [10-12]. Seven NOX subtypes have been identified so far, and NOX4 is one of the most extensively distributed isoforms, which

regulates cellular activity through the modulation of protein expression under specific physio-pathological conditions, such as hypoxia, mechanical stress, ischemia, and endoplasmic reticulum stress [10, 11, 13-16]. NOX4 is the main NOX isoforms expressed in heart, and its activity is the highest in vascular endothelial cells [17]. Recent studies demonstrated that the overactivation or abnormal expression of NOX4 can lead to cardiac damage. Kuroda et al. reported that NOX4 is the main source of oxidative stress in heart failure [5]. It was also reported that inhibition of NOX4/ROS could suppress neuronal and blood-brain barrier injury in intracerebral hemorrhage [18]. Moreover, hypertrophic stimuli triggered the expression of NOX4 in cardiac myocytes, which can promote apoptosis and mitochondrial dysfunction [19]. In the paraventricular nucleus, inhibition of NOX4 improves MI-induced cardiac dysfunction by suppressing the apoptosis of periinfarct and sympathoexcitation [20]. In addition, Xie et al. reported that NOX4 inhibition can improve the blood-brain barrier damage and attenuate oxidative stress caused by intracerebral hemorrhage [18]. NOX4 is closely related to oxidative stress in cardiovascular and cerebrovascular diseases, however, the specific quantitative relationship between NOX4 and oxidative stress in MI model is still unknown. In addition, although NOX4 knockout in mice exhibited protective effects on MI [5], the applicability of this method is limited in therapeutic administration. In this study, the role of NOX4 and its relationship with oxidative stress in MI were explored.

MATERIALS AND METHODS

Animal and treatment

Ten to twelve-week-old male C57BL/6J mice obtained from GemPharmatech (Nanjing,

China) were used in this study. Mice were housed under standard conditions (12/12 light/dark cycles) with free access to water and food. MI surgery was carried out by the ligation of left anterior descending coronary artery as described in the protocols published previously [21, 22]. After the MI modeling, the left ventricle samples were collected at 6, 12, 24, 48, 72 h, 7-day (d), and 14 d after the surgery for the following biochemical analyses. For sham group, surgical procedures were the same as those in the MI groups, except for ligation. Then the left ventricle samples were collected at 24 h after the surgy. For all the MI treatments, samples were collected at different times after MI without additional setting of the corresponding Sham group (n=6 each group). This research was approved by the Institutional Animal Care and Use Committee in Nanjing Medical University Affiliated Wuxi No.2 People's Hospital.

NOX4 knockdown

In this study, siRNA was used to knockdown NOX4 *in vivo*. Both NOX4 siRNA (sc-41587) and control scramble siRNA (sc-37007) were ordered from GeneWiz (Suzhou, China), and sub-cloned into pGB vector, respectively. The efficiency of Nox siRNA was verified by qRT-PCR. For the *in vivo* transfection, control or NOX4 siRNA was mixed with Entranster^{TM}-in vivo (Engreen Biosystem, Beijing, China). Thirty minutes after the ligation, the anesthetized mice were intramyocardially injected with transfection reagents at five points around the MI region. There were four groups in total: MI, MI+si-NC, MI+si-NOX4, and Sham. The treatment of the Sham group was same as those in the MI groups, except for ligation. Tissue acquisition time was consistent with the experimental group (n=6 each group). Myocardial infarct size was analyzed to evaluate the status of myocardial infarction of treated groups, there were three groups: MI, MI+si-NOX4, and Sham. The treatment of

the Sham group was same as those in the MI groups, except for ligation. The 2,3,5-triphenyltetrazolium chloride (TTC) staining was performed to measure the infarct size as described previously [23, 24] (n=6 each group). In brief, 2 mm thick heart sections were incubated in 2% TTC solution for 25 min at 37℃. After washing and mounting, sections were photographed, and infarct size was measured using Image J.

qRT-PCR

Myocardial tissues of mice in Sham and MI groups were harvested at 6 h, 12 h, 24 h, 48 h, 72 h, 7 d, and 14 d after the surgery. Tissue samples were homogenized, and the total RNA was extracted by using the Trizol Reagent (Invitrogen, Waltham, USA), following the protocol provided by the manufacture. One μg RNA was used as template to synthesize the cDNA using the cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA). The qPCR analysis was performed by using the Applied Biosystems™ 7500 Real-Time PCR System (Thermo Fisher Scientific). Gene expression level was calculated using the $2^{\wedge(-\text{delta delta CT})}$ method [21]. Primers used in this study were listed below:

NOX4 sense, 5'-GGT CAC AGA AGG TCC CTA GCA G-3';

NOX4 anti-sense, 5'-GCA GCA CAT GCA CAC CTG AGA A-3';

β-actin sense, 5’-CAT CCC TTC CTC CCT GGA GAA GA-3’;

β-actin anti-sense 5’-ACG GAT TCA TAC CCA AGA AGG AAG G-3’.

Western blot

Myocardial tissues of mice from the indicated groups were lysed using the radioimmunoprecipitation assay buffer lysis buffer (Beyotime, Shanghai, China), and protein concentration was determined by BCA protein assay (Sigma-Aldrich, St. Louis, USA). Protein level of NOX4 was analyzed using Western blot as described previously

[22]. NOX4 primary antibody (ab133303, 1:1000 dilution) was obtained from Abcam (Shanghai, China). GAPDH primary antibody (#2118, 1:2000 dilution) was purchased from Cell Signaling Technology, Inc. (Danvers, USA).

Echocardiographic evaluation

Visual Sonics animal ultrasonic instrument (Toronto, Canada) was used to detect the cardiac cycle M-mode echocardiography as described previously [22]. Both left ventricular end-systolic dimension (LVESD) and left ventricular end diastolic diameter (LVEDD) were measured. The percentage of left ventricular fractional shortening (LVFS) and left ventricular ejection fractions (LVEF) were calculated as described previously [25].

Enzyme-linked immunosorbent assay (ELISA)

Multiple oxidative stress indicators, hydrogen peroxide (H_2O_2), oxidative stress indicators malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD), in left ventricle tissues of Sham and MI mice were determined using the commercial ELISA kits obtained from Beyotime (Shanghai, China), following the instructions provided by the manufacturer.

Statistical analysis

GraphPad Prism 8.0 was used to perform all the statistical analyses in this study. One-way analysis of variance (ANOVA) method followed Dunn's multiple comparisons test were used to analyze the differences between groups. Pearson’s correlation analysis was carried out to measure the correlations between NOX4 protein expressions and oxidative stress indicators. The data were shown as mean ± standard deviation (SD).

RESULTS

NOX4 is upregulated in the myocardial tissues of MI mice

To investigate the pathological role of NOX4 in myocardial infarction, we established the MI mouse model and evaluated NOX4 expression level at different time points after surgeryNOX4. As shown in Figure 1A, in comparison with Sham mice, NOX4 mRNA level gradually increased with the postoperative time (from 6 h to 14-day) in left ventricle tissues, which showed a time-dependent pattern and peaked at 7-day after MI surgery. Similar pattern was observed on the protein level of NOX4 in myocardial tissues of MI mice, NOX4 protein significantly elevated after MI surgery (6 h) and reached the platform at day 7 (Figure 1B and 1C). These data indicated that NOX4 is upregulated in the myocardial tissues of MI mice.

Oxidative stress is upregulated in the myocardial tissues of MI mice

Since oxidative stress is one of the major contributors of the progression of MI, we examined multiple oxidative stress indicators in the myocardial tissues of Sham and MI mice by ELISA assay. The levels of H_2O_2 and MDA in the left ventricle tissues of MI mice increased significantly compared to those in Sham mice (Figure 2A and 2B), which showed a time-dependent pattern and peaked at 7-day after MI surgery. In contrast, the levels of GPx and SOD, two antioxidants, in the MI mice decreased gradually with the postoperative time (Figure 2C and 2D). The above results suggested that oxidative stresses are upregulated in the myocardial tissues of MI mice.

The expression of NOX4 positively corelate with the oxidative stress level in MI mice

Next, we performed Pearson's correlation analysis between the expression level of NOX4 and oxidative stress indicators. As shown in Figure 3A and 3B, NOX4 level positively correlated with the oxidative stress factors, H_2O_2 (r=0.59, p<0.001) and MDA (r=0.48,

p<0.001), in left ventricle tissues of MI mice. Interestingly, nox4 protein level negatively correlated with the antioxidants, GPx (r=-0.52, p<0.001) and SOD (r=-0.36, p=0.013), in left ventricle tissues of MI mice (Figure 3C and 3D).

NOX4 inhibition promotes cardiac function *in vivo*

To further dissect the role of NOX4 in MI progression, we silenced NOX4 in myocardial tissues through intramyocardial injection of siRNA 30 min after the ligation. Fourteen days after myocardial infarction, left ventricle tissues were harvested to verify the knockdown efficiency of NOX4 siRNA by qRT-PCR and Western blot. Upon NOX4 siRNA injection, the MI-induced NOX4 upregulation in myocardial tissues was significantly reduced comparing that in MI and MI+siNC groups on both mRNA and protein levels (Figure 4A-4C), which indicated that the local siRNA injection could knockdown NOX4 efficiently. Next, we performed echocardiography using Visual Sonics animal ultrasonic instrument to evaluate the cardiac function of mice in Sham, MI, MI+si-NC, and MI+si-NOX4 groups 14 days post-myocardial infarction. The results showed that both LVEDD and LVESD were reduced after the NOX4 inhibition (Figure 5A and 5B). In contrast, the percentage of LVEF and LVFS were significantly decreased in MI mice compared to that in Sham mice, however, NOX4 inhibition could rescue the MI-induced reduction of LVEF and LVFS (Figure 5C and 5D). These echocardiographic results suggested that NOX4 inhibition promotes cardiac function in myocardial infarction mice.

NOX4 inhibition attenuates MI-induced oxidative stress in myocardial tissues

The above results showed NOX4 expression positively correlated with oxidative stress in myocardial tissues (Figure 3). Next, we examined the oxidative stress levels in myocardial tissues of NOX4 normal and silent MI mice. The MI-induced elevation of oxidative stress

factors, H_2O_2 and MDA, were significantly reduced in left ventricle tissues after NOX4 inhibition (Figure 6A and 6B). Moreover, NOX4 silence restored the MI-induced reduction of antioxidants (GPx and SOD) in myocardial tissues significantly (Figure 6C and 6D). Furthermore, TTC staining showed that NOX4 inhibition significantly attenuated the myocardial infarct size in MI mice compared to that MI group (Figure S1). All these data indicated that inhibition of NOX4 improves oxidative stress after myocardial infarction *in vivo*.

DISCUSSION

Oxidative stress is considered as a critical risk factor involving in the progression of myocardial infarction [26]. ROS are the major molecules that result in elevation of oxidative stress, and then significantly affect the progression and severity of heart damage [27]. NOX enzyme system is the main driver of ROS production in mammals [28]. In particular, NOX4 is the most abundant subtype of NOX that expresses in cardiovascular system [29]. In this study, we demonstrated the increased expression levels of both NOX4 and oxidative stress indicators in MI mouse models at different times after modeling, revealed the positive correlation between NOX4 expression and ROS production in myocardial tissues of MI mice. Interestingly, with the heart specific NOX4 knockdown, both cardiac damage and oxidative stress were significantly improved in MI mice.

Recent studies demonstrated that acute injury led to the upregulation of NOX4 in heart failure and cardiac ischemia [5, 30]. Kuroda et al. reported that NOX4 is the main source of ROS and oxidative stress in heart failure, NOX4 knockout mice exhibited protective effects on MI [5]. However, the germline gene knockout strategy is limited for therapeutic

administration. Here, we extended this field to include the myocardial infarction, which is the first time to show the expression level of NOX4 was increased abnormally after MI and developed an applicable strategy for MI administration. Several groups revealed that the NOX4 expression is closely related to the ROS production and oxidative stress levels in multiple tissues [5, 31, 32]. Indeed, we observed that both NOX4 expression and oxidative stress indicators production were significantly elevated in the myocardial tissues of MI mice. Noticeably, NOX4 expression positively correlated with the oxidative stress factors (H_2O_2 and MDA), and negatively correlated with the antioxidants (GPx and SOD). Interestingly, similar pattern was observed in brain injury, Casas et al. reported that the expression level of NOX4 was elevated after brain injury, and Xie et al. found that both NOX4 and oxidative stress levels were increased in the brain with intracerebral hemorrhage [18, 33]. Ours and other groups' studies suggest that NOX4 plays an important role in the oxidative stress imbalance that happens in the injury tissues.

MI results in multiple progressive deterioration of the cardiac function, including cardiac arrhythmias, apoptosis, and ventricular hypertrophy [34-36]. Patients with heart failure had obvious left ventricular apoptosis which correlates with reduced cardiac function [37]. Our findings of a significant increase of NOX4 expression accompanying the evaluations of LVEDD and LVESD, two key physiological parameters of cardiac function. Furthermore, we performed heart specific NOX4 knockdown using siRNA and observed remarkably improvement in the cardiac function and reduced size of myocardial infarct of MI heart, which are in line with studies that demonstrated the correlation between oxidative stress inhibition and cardiac enhancement in heart failure models [38]. Upon NOX4 silence, the oxidative stress levels were significantly reduced in myocardial tissues, which might

interpret why inhibition of NOX4 can improve cardiac function of MI mice, however, the underlying molecular mechanism need to be addressed in the future studies. Our findings suggest that inhibition of NOX4/ROS axis in heart provide a potential treatment for MI-induced cardiac dysfunction.

CONCLUSION

NOX4 is upregulated in myocardial tissues of MI mice, which mediates the elevation of oxidative stress and cardiac dysfunction. Selective knockdown of NOX4 in heart attenuates MI-induced oxidative stress and improve cardiac function, suggesting inhibition of NOX4/ROS axis in heart using siRNA is a potential therapeutic treatment for MI.

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Conflict of interest

None declared.

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FIGURE

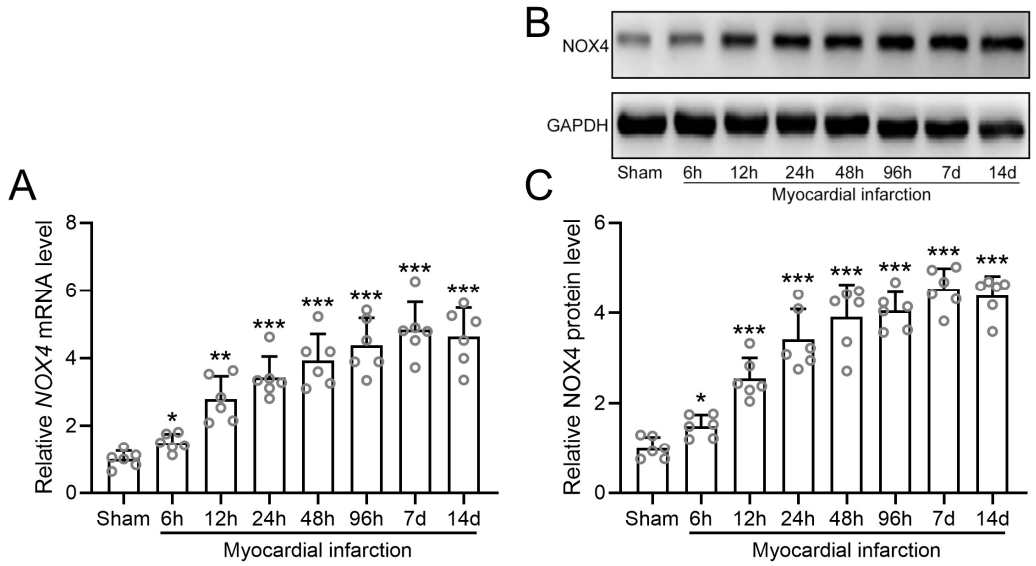

Figure 1. NOX4 was upregulated in the myocardial tissues following myocardial infarction in mice. RT-qPCR and western blotting were used to analyze the mRNA (A) and proteins (B, C) levels of NOX4 in left ventricle tissues at different time after myocardial infarction. The β-actin and GAPDH were used as control, respectively. NOX4 expression levels were normalized to sham group. Data were shown as mean ± SD. (n=6 each group). *p < 0.05, **p < 0.01, ***p < 0.001 compared to Sham group.

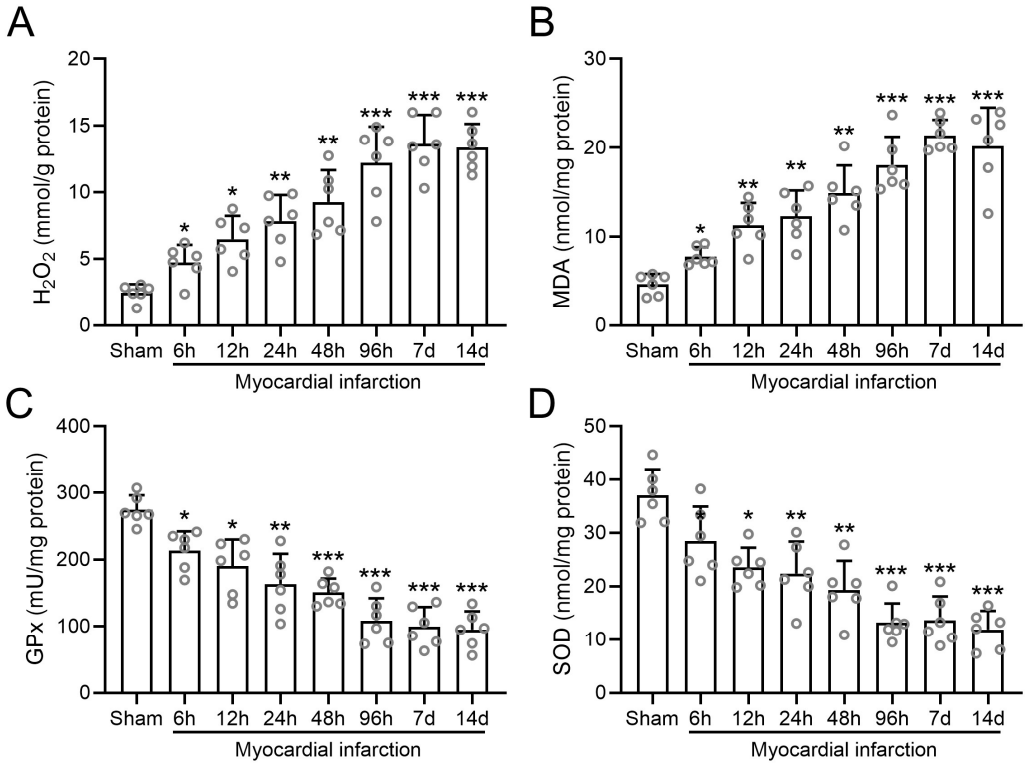

Figure 2. Oxidative stresses were upregulated in the myocardial tissues following myocardial infarction in mice. The levels of hydrogen peroxide (H_2O_2) (A), oxidative stress indicators malondialdehyde (MDA) (B), glutathione peroxidase (GPx) (C) and superoxide dismutase (SOD) (D) in left ventricle tissues were examined at different time points after myocardial infarction. Data were shown as mean ± SD. (n=6 in each group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to Sham group.

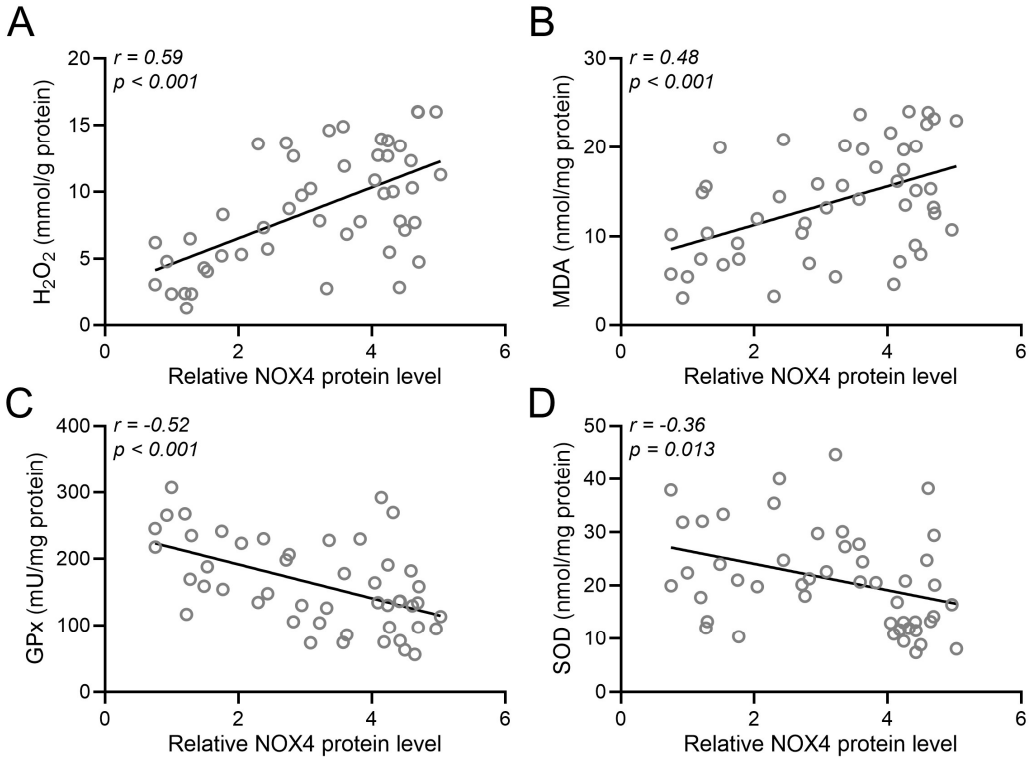

Figure 3. Pearson's correlation analysis between NOX4 expression and oxidative status. Pearson's correlation analysis was carried out to measure the correlations between NOX4 protein expressions and oxidative stress indicators hydrogen peroxide (H_2O_2) (A), oxidative stress indicators malondialdehyde (MDA) (B), glutathione peroxidase (GPx) (C) and superoxide dismutase (SOD) (D) in left ventricle tissues (n = 48 from 8 groups).

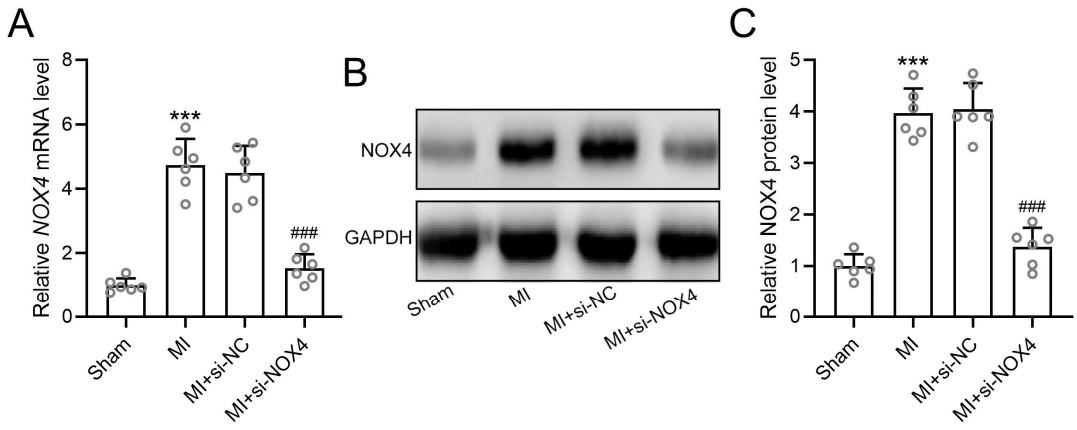

Figure 4. NOX4 was inhibited after NOX4 siRNA treatment in myocardial infarction mice. RT-qPCR and western blotting were used to analyze the mRNA (A) and protein (B, C) levels of NOX4 in left ventricle tissues 14 days after myocardial infarction. The β-actin and GAPDH were used as control, respectively. NOX4 expression was normalized to sham group. Data were shown as mean ± SD (n=6 in each group). ***$p < 0.001$ compared to Sham group. ###$p < 0.001$ compared to MI group.

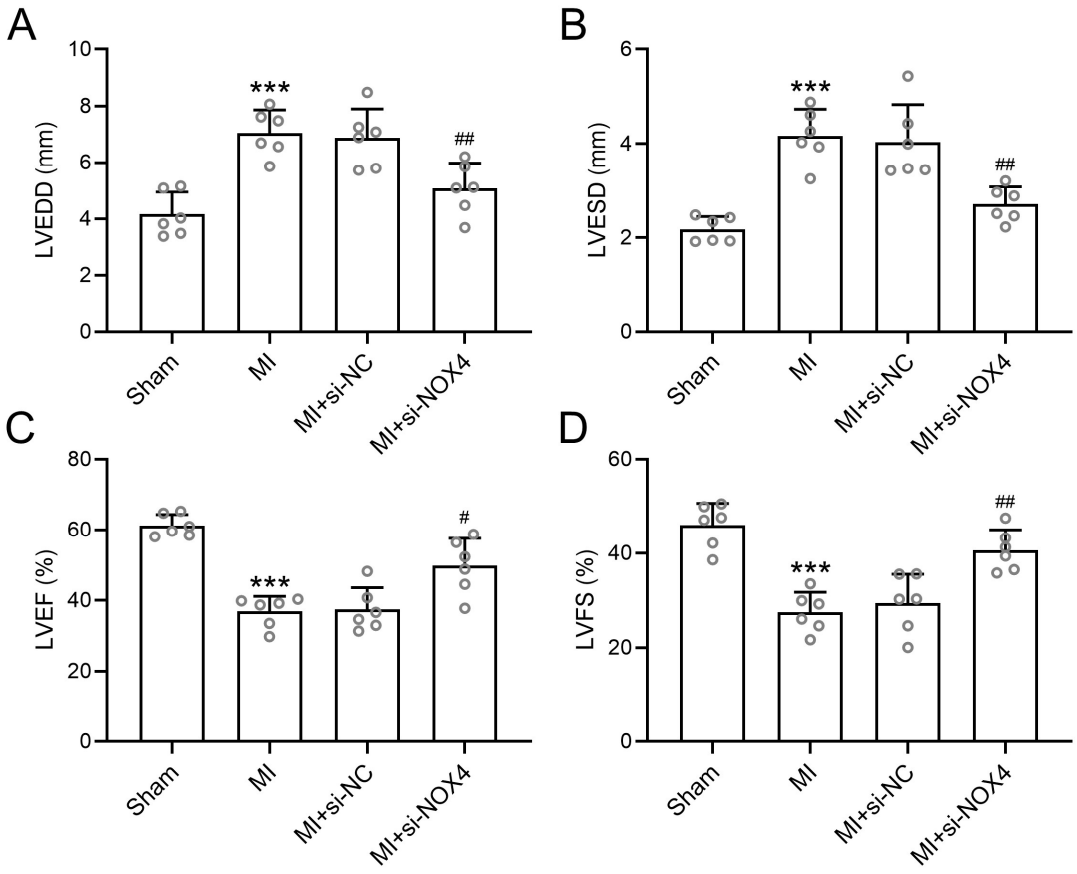

Figure 5. NOX4 inhibition promoted cardiac function in myocardial infarction mice. Evaluations of left ventricular end diastolic dimension (LVEDD) (A), left ventricular end systolic diameter (LVESD) (B), left ventricular ejection fraction (LVEF) (C) and left ventricular fractional shortening (D) 14 days post-myocardial infarction from echocardiography. Data were shown as mean ± SD (n=6 in each group). ***$p < 0.001$ compared to Sham group. #$p < 0.05$, ##$p < 0.01$ compared to MI group.

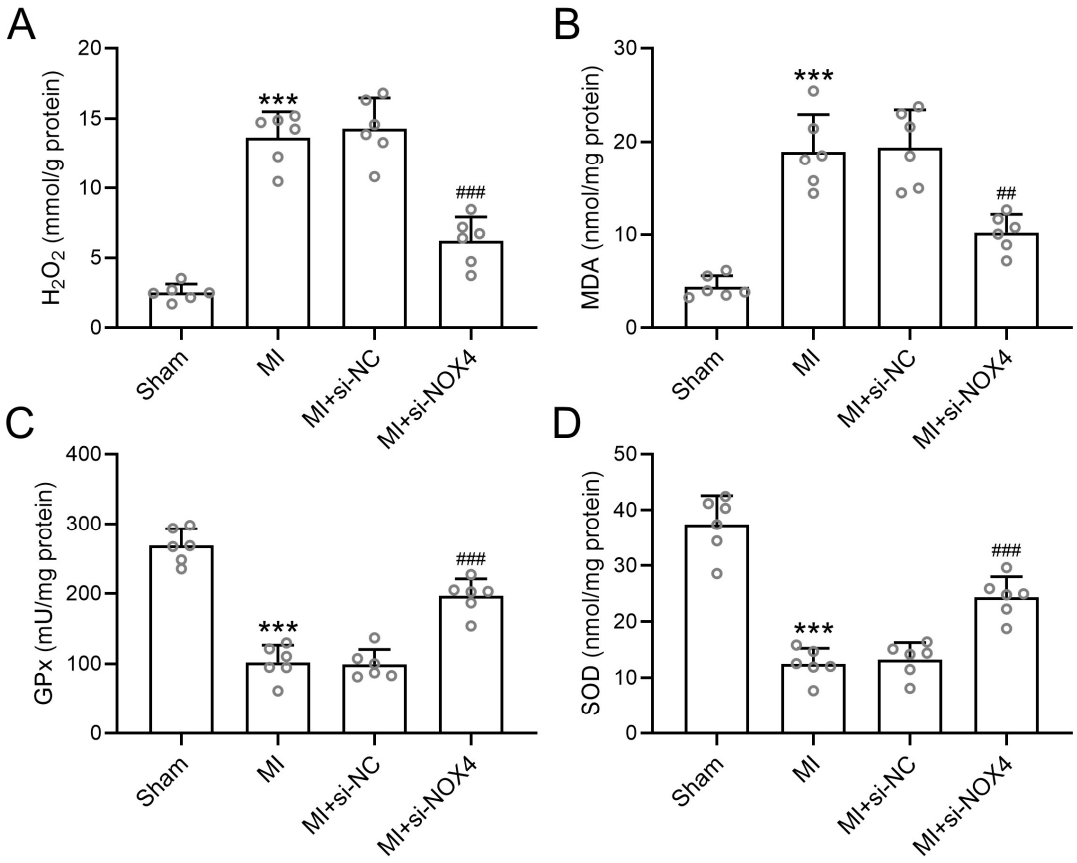

Figure 6. NOX4 inhibition attenuated myocardial infarction induced oxidative stress in the myocardial tissues of mice. The levels of hydrogen peroxide (H_2O_2) (A), oxidative stress indicators malondialdehyde (MDA) (B), glutathione peroxidase (GPx) (C) and superoxide dismutase (SOD) (D) in left ventricle tissues were examined 14 days post-myocardial infarction. Data were shown as mean ± SD (n=6 in each group). ***$p < 0.001$ compared to Sham group. ##$p < 0.01$, ###$p < 0.001$ compared to MI group.