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Lycium barbarum polysaccharides inhibit ischemia/reperfusion-induced myocardial injury via the Nrf2 antioxidant pathway

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ABSTRACT

Oxidative stress is considered to be one of main pathophysiological mechanisms in myocardial ischemia/reperfusion (I/R) injury. *Lycium barbarum* polysaccharides (LBP), the main ingredient of *Lycium barbarum*, have potential antioxidant activity. We aimed to investigate the effects of LBP on myocardial I/R injury and explore the underlying mechanisms. Myocardial I/R group was treated with or without LBP to evaluate oxidative stress markers and the role of Nrf2 signal pathway. Our results showed that I/R increased infarct size and the activities of creatine kinase (CK) and lactate dehydrogenase (LDH) when compared with control group. Meanwhile, the levels of reactive oxygen species (ROS), malondialdehyde (MDA), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were enhanced and the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) were decreased. These changes were associated with a significant increase in myocardial apoptosis, ultimately leading to cardiac dysfunction. LBP reduced infarct size (38.4 ± 2 % *versus* 19.4 ± 1.8 %, $p < 0.05$), CK and LDH activities and myocardial apoptotic index. Meanwhile, LBP suppressed the production of ROS and restored redox status. Additionally, LBP increased protein level of nuclear Nrf2 *in vivo* (2.1 ± 0.3 *versus* 3.8 ± 0.4, $p < 0.05$) and *in vitro* (1.9 ± 0.2 *versus* 3.8 ± 0.1, $p < 0.05$) and subsequently upregulated heme oxygenase 1 and NADPH dehydrogenase quinone 1 compared to I/R group. Interestingly, Nrf2 siRNA abolished the protective effects of LBP. LBP suppressed oxidative stress damage and attenuated cardiac dysfunction induced by I/R *via* activation of the Nrf2 antioxidant signal pathway.

1. Introduction

Myocardial ischemia induced by blood occlusion results in a variety of myocardial impairments and progressive cardiac dysfunction, which is one of the major causes of death associated with significant morbidity and disability worldwide [1]. Although the use of percutaneous coronary intervention and thrombolytic therapy have reduced the infarct area, the process of restoring blood supply to the ischemic myocardium, which is known as reperfusion, may be further deleterious to myocardial injury, ultimately leading to continuous deteriorated cardiac function [2–4]. Therefore, inhibition of myocardial ischemia/reperfusion (I/R) damage may provide treatment strategies for patients with ischemia heart disease.

The process of myocardial I/R injury is complex and may involve multiple mechanisms [5]. Previous studies have shown that oxidative stress, involved in the whole process of I/R, is considered to be one of main pathogenic mechanisms, leading to myocardial irreversible injury and cardiac dysfunction [6,7]. Liu et al. found that oxidative stress

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characterized with the overproduction of reactive oxygen species (ROS) and insufficient or ineffective supply of antioxidant system aggravated myocardial damage and resulted in cardiac dysfunction [8,9]. Therefore, inhibiting myocardial oxidative stress *via* some related signal transduction pathway may serve as an attractive strategy for treatment with cardiac dysfunction induced by I/R.

Growing evidences have shown that the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2)-signal pathway plays crucial roles in oxidative stress related cardiovascular disease by regulating heme oxygenase 1 (HO1) and NADPH dehydrogenase quinone 1 (NQO1) [10,11]. Nrf2 is inhibited by binding with Keap1 in cytoplasm under physiological state. Once intracellular ROS level increased, Nrf2 dissociated from Keap1, rapidly translocated to the nucleus. Nrf2 combined with the anti-oxidant response element to initiate the transcription of endogenous antioxidative genes and anti-oxidative enzymes subsequently, which resisted oxidative stress injury and preserved cellular homeostasis [12,13]. Xiao et al. found that activation of the Nrf2 antioxidation pathway reduced the level of ROS and attenuated I/R damage [14]. On the contrary, inhibition of the Nrf2 signal pathway by genetic modifications enhanced the production of ROS and aggravated oxidative stress injury [15]. Herein, activation of the Nrf2 antioxidation pathway by some medicines may serve as treatment options in patients with myocardial oxidative stress related I/R injury.

A number of clinical and preclinical researches have shown that *Lycium barbarum* polysaccharides (LBP), a traditional Chinese herbal medicine, extracted from the fruits of *Lycium barbarum* (goji, wolfberry, Fructus lycii), which have pleiotropic biological and pharmacological protective effects against various of diseases for thousands of years [16,17]. Previous study showed that LBP attenuated alcoholic liver injury by inhibiting apoptosis in female mice [18]. Hong et al. found that LBP improved lung function by reducing inflammation in hyperoxic lung injury animal model [19]. Po et al. found that LBP, as one of potential free radicals scavenging, played an important role in counteracting oxidative free radicals and protected against the tests from high-temperature injury [20]. Meanwhile, LBP reduced hydrogen peroxide-induced oxidative stress injury in human trophoblast HTR8/SVneo cells by improving antioxidant activity [21]. Recently studies further demonstrated that LBP also exerted anti-oxidative effect in experimental animal model *via* antioxidant pathway [22,23]. It is therefore conceivable that LBP can be used as a potential therapeutic option for preventing oxidative stress related myocardial injury induced by I/R *via* downstream signal pathway.

Therefore, the present study aimed to identify the effect of LBP on myocardial I/R injury and further investigate whether the protective effect of LBP was related to the reduction of oxidative stress *via* activation of the Nrf2 antioxidative pathway.

2. Materials and methods

2.1. Animals

Eight-week-old male Sprague-Dawley rats (weight 200 ± 20 g, n = 30) were obtained from the Experimental Animal Centre of Xi'an Jiao Tong University. The protocol of animals' experiments described below was approved by the Ethics Committee of Xi'an Jiao Tong University and performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publications No. 85-23, revised 1996)

2.2. I/R model establishment and LBP administration

The I/R injury model was constructed as previously described [24]. Briefly, rats were anesthetized using 1.5–2 % isoflurane. After opening the chest, the left anterior descending coronary artery was occlusion (8−0 silk suture) to induce myocardial ischemia for 30 min, followed by 2 h reperfusion by loosening the ligature (I/R, n = 10). The sham group represented control group, which was subjected to the same operations except ligation (Sham, n = 10). I/R rats were pretreated with LBP (100 mg/kg/day, Ningxia Qiyuan Pharmaceutical Co., Ltd, Ningxia, China) dissolved in PBS once a day for 7 days before successful establishment of the rat model with I/R (I/R + LBP, n = 10).

2.3. Hemodynamic parameters analysis

After treatment, hemodynamic parameters were recorded and analyzed using the PowerLab data acquisition system (Powerlab/4SP; AD Instruments). In brief, the right common carotid was isolated and a heparin-filled polyethylene catheter was introduced into the left ventricle to record hemodynamic parameters (heart rate—HR, mean arterial pressure—MAP, left ventricular systolic pressure—LVSP, left ventricular end-diastolic pressure—LVEDP, the maximum slope of systolic and diastolic pressure decrements—+dp/dt and −dp/dt).

2.4. Assessment of myocardial infarction

At the end of experiments, rats were euthanized by exsanguination. Serum was collected by centrifugation at 5000 *g* for 10 min. The entire heart was harvested and washed with cold PBS. Myocardial slices (2 mm) were incubated in 5 % 2, 3, 5-triphenyltetrazolium chloride (TTC) at 37℃ for 30 min and then transferred into 10 % formalin at 4℃ for 48 h. The myocardial infarction size was defined as TTC stained area (white area).

2.5. Cell culture and treatment

H9c2 embryonic rat-derived cell line (American Type Culture Collection Manassas, VA, USA) was used to further identify the possible mechanism in the present study *in vitro*. They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100U/mL) and streptomycin (100 mg/mL) at 37 °C with 95% O_2-5%CO_2. Hypoxia/reoxygenation (H/R) injury was performed as previously described [25]. Briefly, H9c2 cells were incubated with a modified ischemia-mimetic solution to induce ischemia at 37℃ for 8 h. Subsequently, H9c2 cells described above were removed from the modified ischemia-mimetic solution to a normoxic incubator with DMEM for reoxygenation (4, 8, 12, 16 h).

2.6. Nrf2 silencing by siRNA

H9c2 cells were seeded into 6-well culture plates and allowed to grow to 70 % confluence prior to transfection. According to the manufacturer's instruction (Micropoly Biotech, Nantong, China), Nrf2 siRNA and a non-silencing scrambled siRNA were used to knock down Nrf2 and as a negative control, respectively. The specific sequences of Nrf2 siRNA were: sense, 5′-GAGGAUGGGAAACCUUACUTT-3′ and antisense, 5′-AGUAAGGUUUCCCAUCCUCTT-3′. The sequences of the scramble siRNA were: sense, 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense, 5′-ACGUGACACGUUCGGAGAATT-3′. After transfection for 48 h, H9c2 cells were used for further experiments.

2.7. Cell viability assay

H9c2 cells were seed in 96-well plates and exposed to 20 μl MTT (5 mg/mL; Sigma-Aldrich) at 37 ℃ for 4 h and co-culture with 150 μl dimethyl sulfoxide. The absorbance values were measured at 570 nm using a microplate spectrophotometer (Beyotime Institute of Biotechnology, Jiangsu, China). Cell viability was represented as the percent of control.

2.8. Evaluating I/R injury enzyme

The activities of creatine kinase (CK) and lactate dehydrogenase (LDH) in serum and cell supernatant were analyzed using enzyme activity detection kits according to manufacturer's instructions (NanJing Jiancheng Biological Technology, NanJing, China). Briefly, CK and LDH activities were evaluated by means of quantitative inorganic phosphorus and dinitrophenylhydrazone *via* colorimetry, respectively. The levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were determined by enzyme-linked immunosorbent assay according to the manufacturer's protocols (R&D Systems, Inc., Minneapolis, USA). CK, LDH, TNF-α and IL-6 levels were evaluated by a microplate spectrophotometer (Beyotime Institute of Biotechnology, Jiangsu, China).

2.9. Assessment of oxidative stress

ROS level in myocardial tissue was measured and analyzed with the fluorescent dye, dihydroethidium (DHE; Beyotime Institute of Biotechnology, Nanjing, China) according to manufacturer's instructions. In brief, 5-μm fresh frozen sections from the heart were incubated in DHE at 37℃ for 30 min and observed by fluorescence microscopy (Olympus, IX71, Olympus Co., Tokyo, Japan). The level of ROS in H9c2 cells was determined by using 2, 7-dichlorodihydrofluorescein diacetate (DCHF-DA) probes (Sigma Aldrich, St. Louis, MO, USA) Briefly, H9c2 cells were incubated in DCHF-DA probes (10 μM) at 37℃ for 20 min and observed by fluorescence microscopy (Olympus). The relative fluorescence intensity of ROS was analyzed by image pro plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

The levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) in serum and cell supernatant were analyzed by a microplate spectrophotometer according to the manufacturer's protocol (Beyotime Institute of Biotechnology, Nanjing, China). Briefly, CAT, SOD and GPX activities were evaluated by measuring N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinonemonoimine, the production of WST-8 and NADPH *via* colorimetry, respectively. Meanwhile, MDA level was evaluated by testing MDA-TBA *via* colorimetry.

2.10. Assessment of apoptosis

Apoptosis in myocardial tissues and H9c2 cells was detected by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining kit according to the manufacturer's instruction (Chemicon International, Temecula, CA, USA). Nuclear in myocardial tissues and H9c2 cells were stained by DAPI and PI, respectively. The percentage of apoptosis was observed and calculated by fluorescence microscopy (Olympus) and image pro plus 6.0 (Media Cybernetics), respectively.

2.11. Western blotting

Protein samples were separated by 10 % SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocking with Tris-buffered saline containing 0.1 % Tween (TBST) and 5 % bovine serum albumin (BSA) for 1 h, and then the membrane was incubated with primary antibody overnight at 4 ℃. Primary antibodies (cytosolic Nrf2, nuclear Nrf2, NQOl, HO1, Histone 3 (H3) and GAPDH) (all from Abcam, Cambridge, UK) were used. The protein levels were quantified by image pro plus 6.0 (Media Cybernetics).

2.12. Nuclear translocation of Nrf2 by immunofluorescence analysis

Localization of Nrf2 was analyzed using immunofluorescence analysis. The cell slides were stained with PI kit (Invitrogen) and incubated with rabbit anti-Nrf2 diluted in 0.01 % PBST containing 1% BSA over night at 4℃. Then, the slides were incubated with the secondary antibody (Alexa Fluor ® 594, Molecular Probe, Eugene, OR, USA) for 1 h at room temperature and observed with fluorescence microscopy (Olympus). The relative fluorescence intensity was analyzed using image pro plus 6.0 (Media Cybernetics).

2.13. Statistical analysis

The results are expressed as means ± standard deviation. Comparisons between groups were analyzed using one-way ANVOA with Turkey's multiple as post hoc analysis. A p value < 0.05 was considered statically significant. All data analyses were performed with SPSS 19.0 (Lead Technologies, Chicago, Illinois).

3. Results

3.1. LBP protected against myocardial I/R injury

Myocardial infarct area was evaluated by TTC stain (Fig. 1A, B). There was a significant difference in infarct area between I/R group and I/R + LBP group. Interestingly, the protective effect of LBP on the myocardial infarct area almost parallels those changes of the activity of CK and LDH. Fig. 1C, D showed that the serum CK and LDH levels were increased significantly in I/R group compared with sham group, while LBP decreased the levels of serum LDH and CK.

We also monitored hemodynamic parameters and found that HR and MAP were markedly decreased in I/R group, and treatment with LBP had a significant effect on MAP and HR (Fig. 1E, F). Compared with sham group, LVSP and ± dp/dt significantly decreased, but LVEDP increased in I/R group (Fig. 1G, H). Treatment with LBP reversed these hemodynamic parameters changes. These results indicated that I/R injury led to severe cardiac dysfunction and LBP administration attenuated myocardial injury and improved cardiac function.

3.2. LBP inhibited myocardial oxidative stress damage

Oxidative stress is one of the major pathogenic mechanisms of myocardial I/R damage. The levels of oxidative stress-related markers were measured in the present study. The production of ROS and the levels of MDA, IL-6 and TNF-α were significantly increased when compared to sham group, which was associated with a marked decrease in the activities of SOD, GPX and CAT (Fig. 2A–F, I, J). Treatment with LBP decreased the levels of ROS, TNF-α and IL-6 accompanied with an increase in SOD, GPX and CAT activities, and alleviated myocardial oxidative stress damage. Moreover, we also found that I/R injury caused myocardial apoptosis in I/R group compared with sham group, while pretreatment with LBP decreased the percentage of TUNEL-positive cells in I/R + LBP group (Fig. 2G, H). These results suggested that LBP inhibited myocardial oxidative stress damage by reducing ROS levels and restoring the balance between oxidants enzymes and antioxidants.

3.3. LBP treatment activated the Nrf2 antioxidant signaling pathway

Nrf2 mediated antioxidant signal pathway against oxidative stress. The levels of cytosolic Nrf2, nuclear Nrf2, HO1 and NQO1 were evaluated by western blot. The Nrf2 antioxidant signal pathway was impaired in I/R group (Fig. 3A–E). Pretreatment with LBP increased cytosolic Nrf2, nuclear Nrf2, HO1 and NQO1 expression in myocardial tissue subjected to I/R. The abovementioned results indicated that LBP exerted protective effect by against oxidative stress *via* activation of the Nrf2 signal transduction pathway.

3.4. Pretreatment with LBP ameliorated hypoxia/reoxygenation (H/R)-induced oxidative stress damage in H9c2 cells

After 8 h hypoxia, there was a significant decreased in cell viability followed by 4 h reoxygenation in H9c2 cells (Fig. 4A). Therefore, the

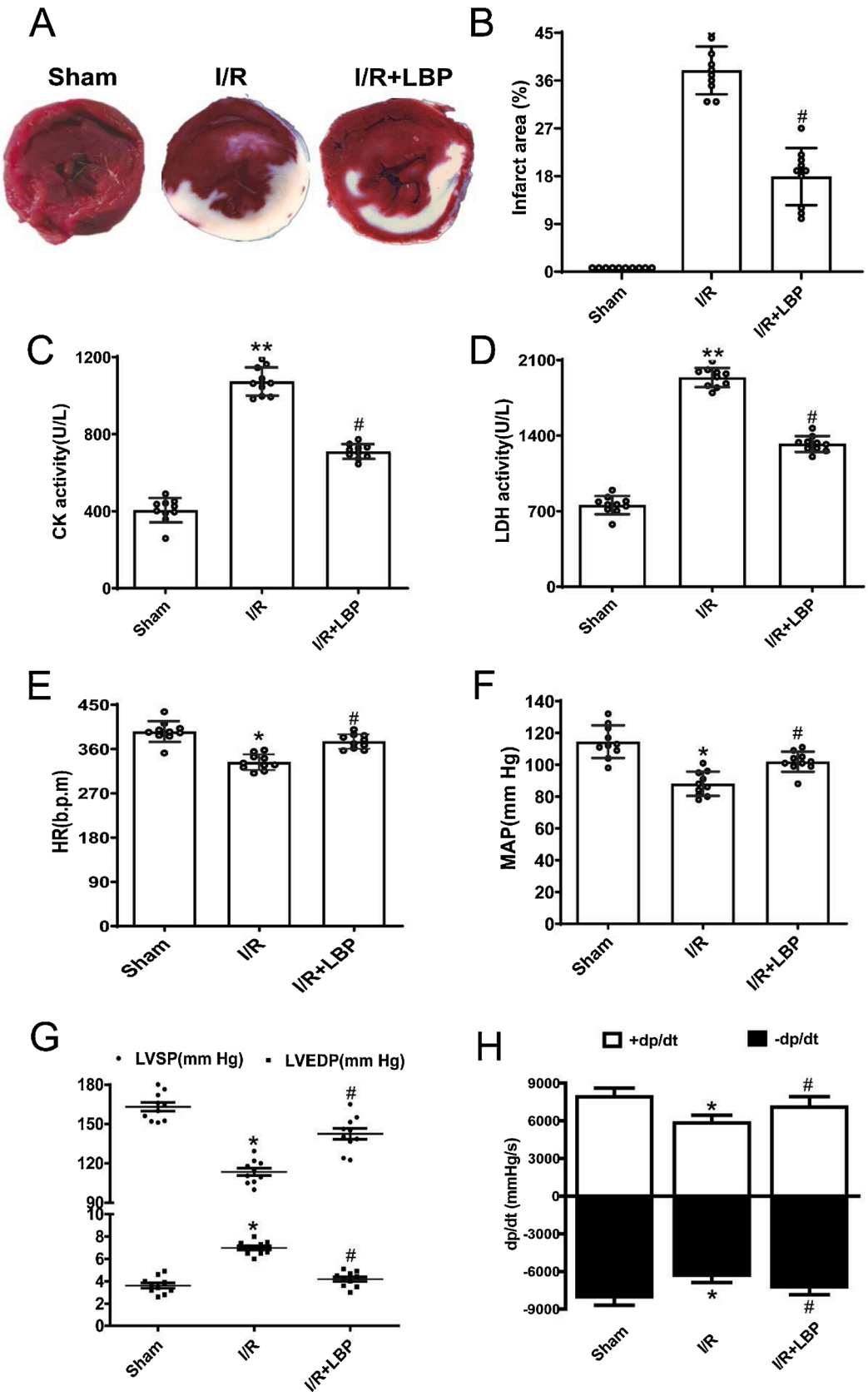

Fig. 1. Pretreatment with LBP reduced myocardial I/R injury. (A) Representative images of the infarct size in all groups. (B) Quantitative analysis of the myocardial infarct size. (C, D) Quantitative analysis of serum creatine kinase (CK) and lactate dehydrogenase (LDH) activity. (E-H) Hemodynamic parameters (HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dp/dt and −dp/dt, the maximum slope of systolic and diastolic pressure decrements) in all rats. Data are Means ± SD (n = 10). *$P < 0.05$, **$P < 0.01$ *versus* sham group, #$P < 0.05$ *versus* I/R group.

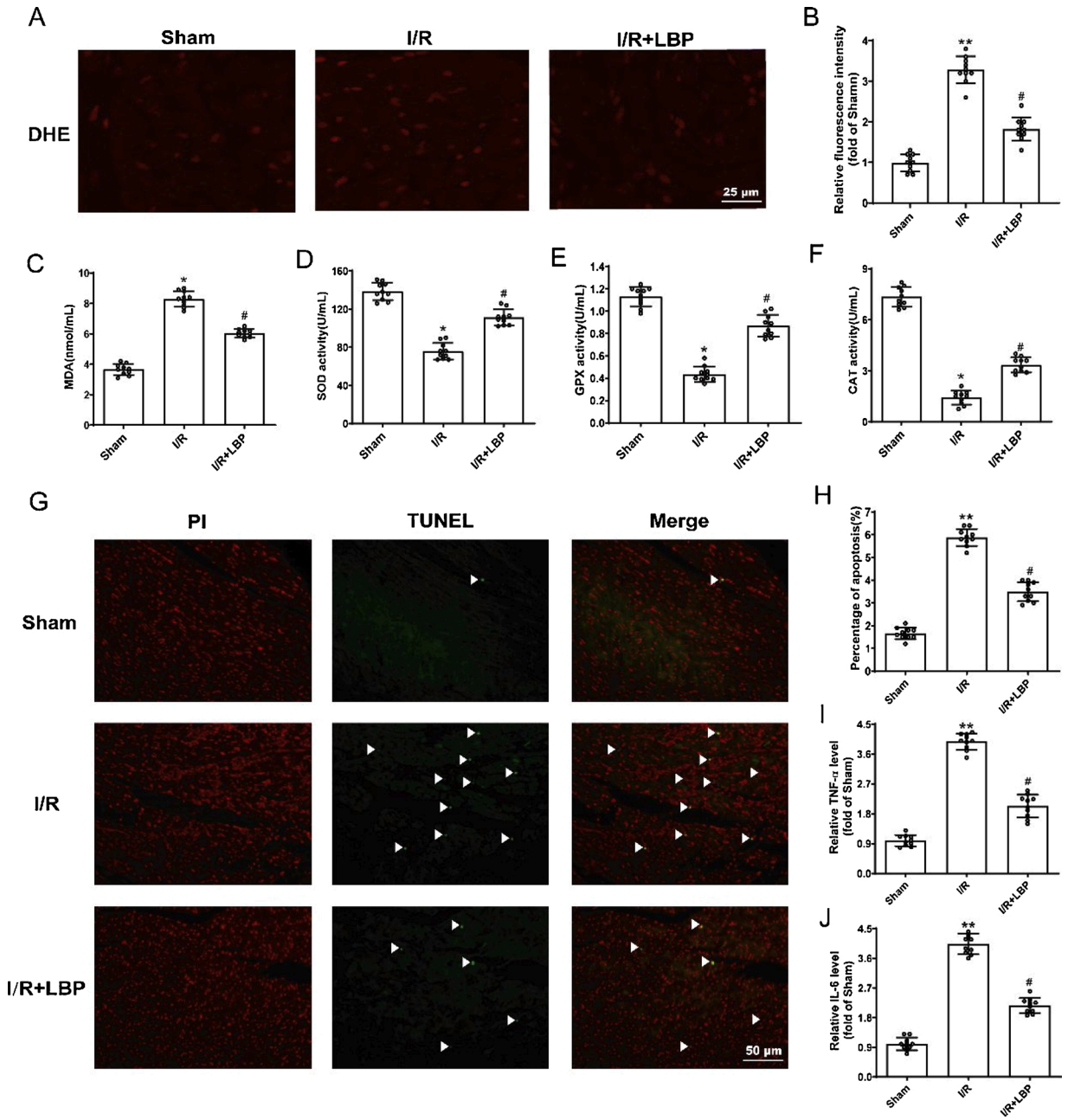

Fig. 2. LBP inhibited myocardial oxidative stress damage in I/R group. (A) Representative images using dihydroethidium (DHE) staining in myocardial tissue; scale bar =25 μm. (B) Relative fluorescence intensity of reactive oxygen species (ROS). (C-F) Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) levels in serum from all rats. (G) Representative images by propidium iodide/Annexin V staining; White triangle represents TUNEL-positive cells. (H) Quantification of the percentage of apoptosis in myocardial tissue; scale bar =50 μm. (I, J) Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels in myocardial tissue in all rats. Data are Means ± SD (n = 10). $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* sham group, $^{\#}P < 0.05$ *versus* I/R group.

model of H/R was established by 8 h hypoxia followed by 4 h reoxygenation in the next experiments. H9c2 cells were pretreated by different doses of LBP (100 μg/mL-500 μg/mL) (Fig. 4B). 100, 200 and 300 μg/mL of LBP had no effects on H9c2 cells viability, but 400 and 500 μg/mL of LBP significantly decreased H9c2 cells viability. So, 300 μg/mL of LBP was used in the following experiments. Consistent with *in vivo* study, we found that H/R reduced cell viability and increased the levels of LDH and CK in H9c2 cells (Fig. 4A–E), which was associated with a significantly enhanced oxidative stress-related markers and apoptosis as evidenced by the increased ROS, MDA, TNF-α and IL-6 levels and apoptotic index, as well as the reduced SOD, GPX and CAT activities (Fig. 5A–J). Pretreatment with LBP suppressed oxidative stress damage by reducing ROS level and restoring the balance between oxidants enzymes and antioxidants in H9c2 cells.

3.5. Activation of Nrf2 is essential in mediating the protective effect of LBP in H9c2 cells exposed to H/R

To further explore the potential mechanisms of LBP on myocardial H/R injury, the Nrf2 signal pathway related proteins were measured *in vitro* study. We also found that H/R impaired Nrf2 antioxidant signal pathway and LBP enhanced the activation of Nrf2 antioxidant signal pathway (Fig. 6A–G). To test whether or not LBP reduces oxidative stress-induced cardiomyocyte injury *via* activating Nrf2 antioxidant signal pathway, we reinvestigated the effect of LBP on H/R-mediating cardiomyocyte injury in Nrf2 deficient H9c2 cells. As shown in Fig. 7,

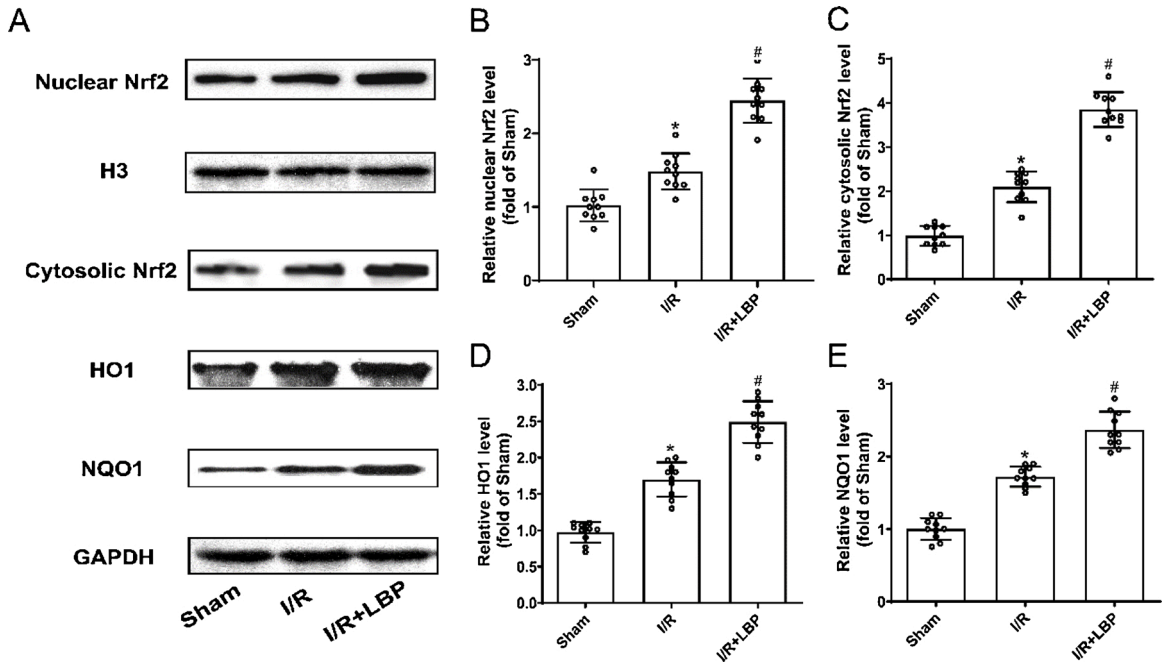

Fig. 3. LBP activated Nrf2 antioxidant signal pathway. (A)Western blots of nuclear Nrf2, histone3 (H3), cytosolic Nrf2, heme oxygenase 1(HO1), NADPH dehydrogenase quinone 1 (NQO1) and GAPDH proteins. (B-E) Western blot analysis of nuclear Nrf2, cytosolic Nrf2, HO1 and NQO1. Data are Means ± SD (n = 10). *$P < 0.05$ *versus* sham group, #$P < 0.05$ *versus* I/R group.

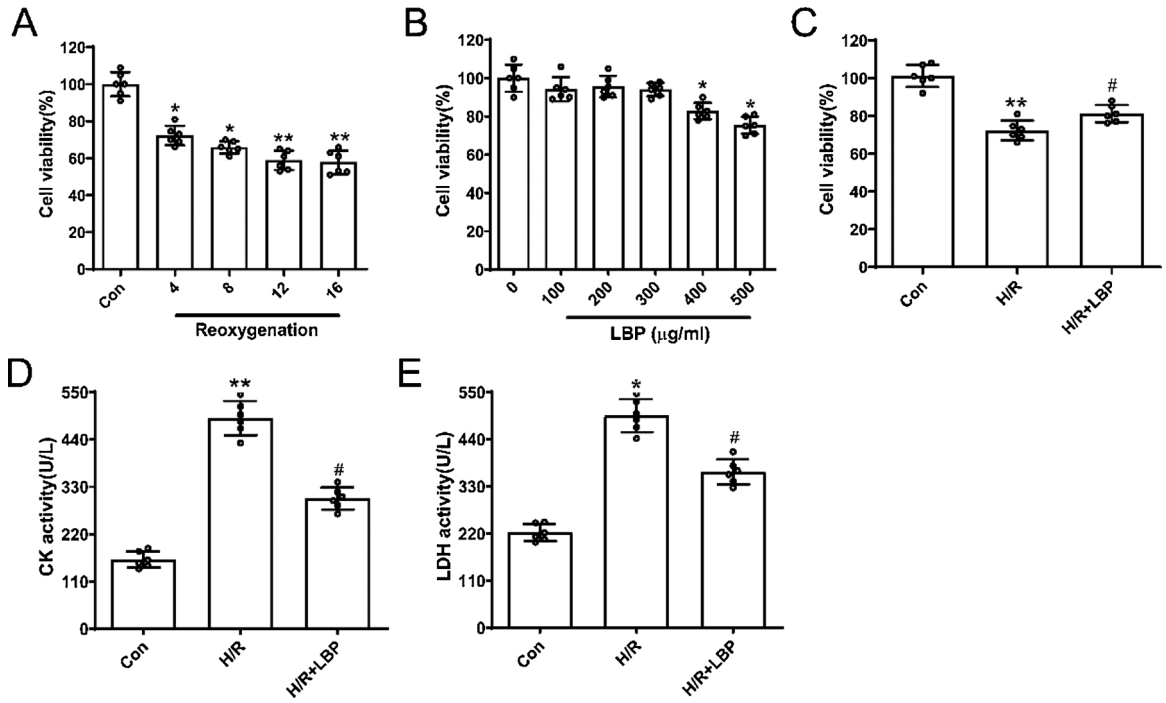

Fig. 4. LBP administration decreased hypoxia/reoxygenation (H/R) induced injury in H9c2 cells. (A-C) Viability of H9c2 cells was measured by MTT in H/R pretreated with or without LBP. (D, E) Quantitative analysis of creatine kinase (CK) and lactate dehydrogenase (LDH) activies. Data are Means ± SD (n = 6). *$P < 0.05$, **$P < 0.01$ *versus* Con group, #$P < 0.05$ *versus* H/R group.

Nrf2 siRNA abolished the protective effects of LBP on H9c2 cells evidenced by the decreased Nrf2 pathway activation and increased oxidative stress-induced cardiomyocyte injury. These results indicated that LBP exerted cardioprotective effects by reducing oxidative stress damage induced by H/R *via* Nrf2 antioxidant pathway.

4. Discussion

In the present study, we measured cardiac function, detected oxidative stress related markers and explored the activation of Nrf2 signal pathway to identify the effect of LBP treatment on myocardial I/R rats. The main findings of this study are summarized as follows: *i*) I/R triggered myocardial oxidative stress as evidenced by the

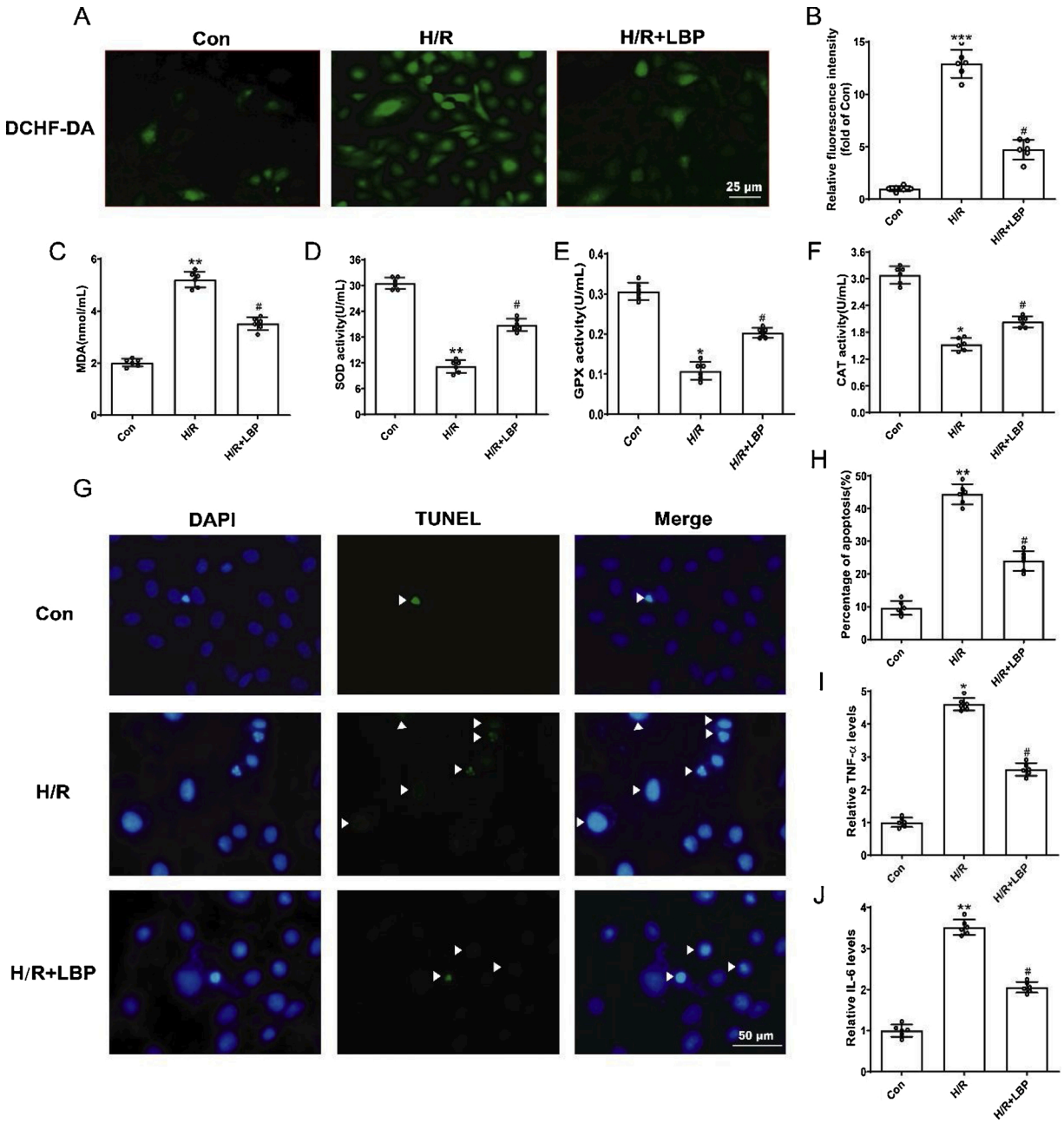

Fig. 5. LBP suppressed oxidative stress induced by hypoxia/reoxygenation (H/R) in H9c2 cells. (A) Representative images using 2, 7-dichlorodihydrofluorescein diacetate (DCHF-DA) staining; scale bar =25 μm. (B) Relative fluorescence intensity of reactive oxygen species (ROS). (C-F) Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) levels in supernatant from all groups. (G) Representative images by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining; White triangles represent TUNEL-positive cells. (H) Quantification of the percentage of apoptotic H9c2 cells; scale bar =50 μm. (I, J) Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels in cell supernatant. Data are Means ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* Con group, $^{\#}P < 0.05$ *versus* H/R group.

overproduction of ROS, the increased MDA, TNF-α and IL-6 levels and the percentage of myocardial apoptosis as well as the reduced SOD, GPX and CAT activities, which were associated with the impairment of Nrf2 signal pathway, ultimately resulted in continuous deteriorated cardiac function; *ii*) LBP, a traditional Chinese herbal medicine, attenuated the aforementioned oxidative stress related injury and improved cardiac function in I/R rats; *iii*) LBP attenuated myocardial oxidative stress *via* the activation of Nrf2 antioxidant signal pathway. Thus, our findings indicated that the therapeutic potential of LBP in preventing the development of I/R.

Myocardial I/R injury refers to insufficient myocardial blood supply in a short period of time with restored blood flow to the ischemic myocardium after a certain amount of time, which leads to additional damage to myocardium and further cardiac dysfunction [26]. Many western medicines and Chinese herbal medicines that have important cardioprotective potentials are widely used for treatment with I/R injury [27–30]. Although western medicines have prominent protective effects in cardiac function in a short period, long-term treatment with them leads to several serious side effects which cannot be ignored. Sometimes, the traditional Chinese herb with multiple pharmacological effects may become a novel approach to treatment of certain diseases. LBP, a member of the Solanaceae, is the major active ingredient extracted from *Lycium barbarum* and contains 6 monosaccharides, galactose, rhamnose, glucose, mannose, arabinose, xylose and 18 different amino acids, and

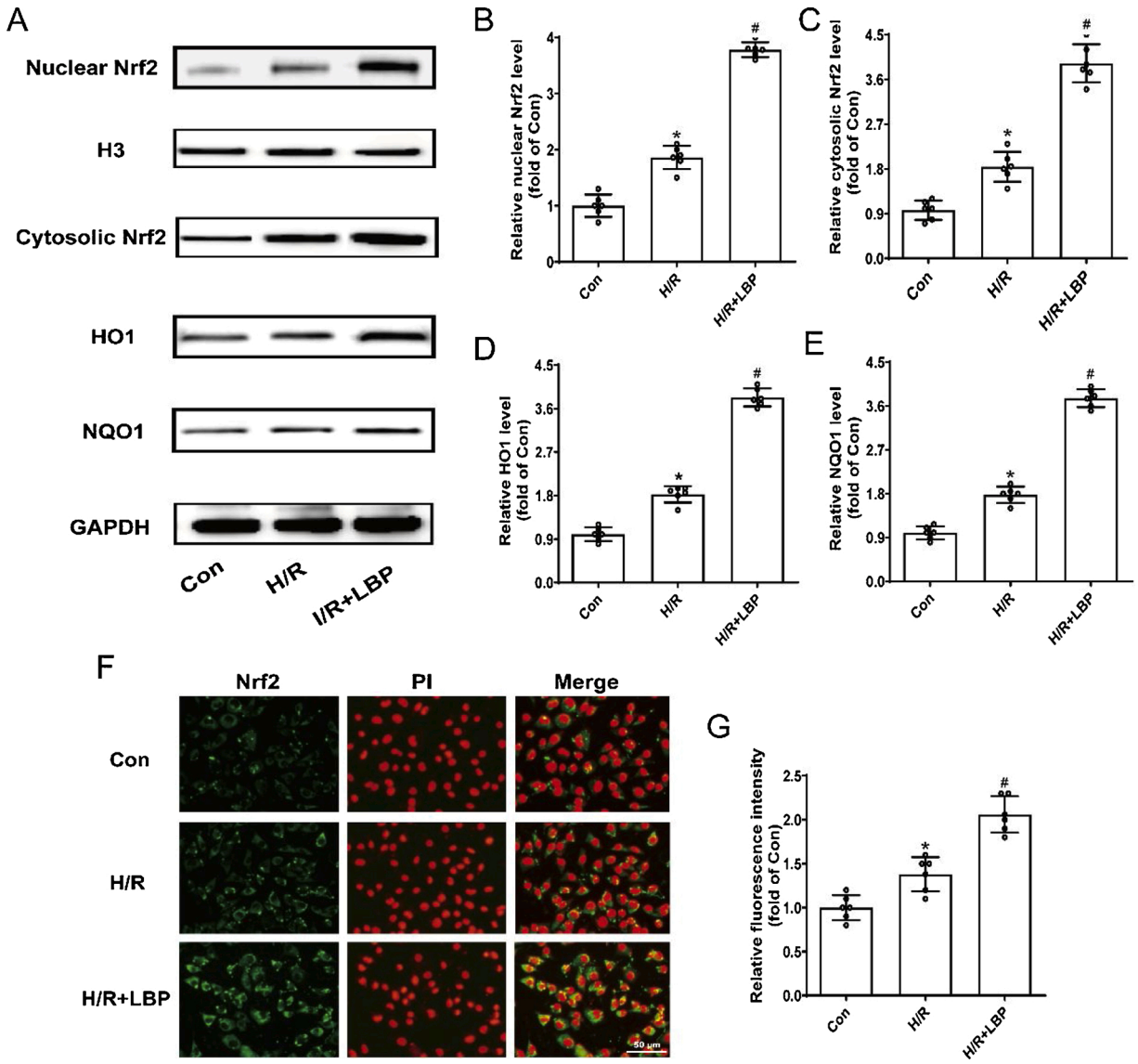

Fig. 6. LBP upregulated the expression of anti-oxidative molecular in Nrf2 signal pathway H/R in H9c2 cells. (A)Western blots of nuclear Nrf2, histone3 (H3), cytosolic Nrf2, heme oxygenase 1(HO1), NADPH dehydrogenase quinone 1 (NQO1) and GAPDH proteins. (B-E) Western blot analysis of nuclear Nrf2, cytosolic Nrf2, HO1 and NQO1. (F) Representative images of Nrf2 translocation by propidium iodide/Annexin V staining in all groups. (G) Relative fluorescence intensity of nuclear Nrf2. Data are Means ± SD (n = 6). $^{*}P < 0.05$ *versus* Con group, $^{\#}P < 0.05$ *versus* H/R group.

exerts a large variety of beneficial effects, such as immunomodulation, anti-inflammation and anti-aging [31]. Recently study further demonstrated that LBP attenuated cardiac hypertrophy and improved cardiac function in diabetic rats [32]. Accordingly, we speculated that LBP might play important cardioprotective roles in myocardial I/R rats by inhibiting oxidative stress. In the present study, we showed that treatment with LBP reduced I/R triggered severe myocardial infarct size and the leakage of serum LDH and CK from myocardial tissue, ultimately improving cardiac function and hemodynamics in I/R rats. The results of the aforementioned studies and the present study indicated that the protective effects of LBP on cardiac function might be partly associated with the inhibition of I/R-mediated myocardial injury and improved cardiac function.

Oxidative stress is involved in the pathogenesis of myocardial I/R injury [33]. The overproduction of ROS directly initiates spontaneous and self-propagating radical reactions with biomolecules that impair cardiac function. On the other hand, the excessive ROS production is also accompanied with the increased in oxidants and the decrease in endogenous antioxidative defense in I/R subjects, ultimately aggravating myocardial injury [34]. Inhibiting oxidative stress may become one of efficient treatment options for preventing myocardial I/R injury [35,36]. In the present study, we found that the overproduction of ROS, the increased MDA, TNF-α and IL-6 levels and the percentage of myocardial apoptosis as well as the reduced SOD, GPX and CAT activities were presented in I/R or H/R group. While pretreatment with LBP significantly reduced myocardial oxidative stress damage. Our results suggested that LBP reduced myocardial oxidative stress damage by restoring the balance between of redox status and suppressing oxidative stress.

There are increasing evidences from preclinical studies, suggesting that the Nrf2 antioxidant signal pathway is likely to be one of the most crucial cellular adaptive responses against oxidative stress and maintain cellular redox homeostasis in myocardial I/R injury [37,38]. Under physiological conditions, Nrf2, combination with Keap1, is in an inactivated state in the cytosol. When cells are stimulated with pathological stress, Nrf2 is released from Keap1, transferred to nucleus to bind the anti-oxidant response element, and promote the transcription and expression of HO1 and NQO1 [39,40]. Recently study showed that the

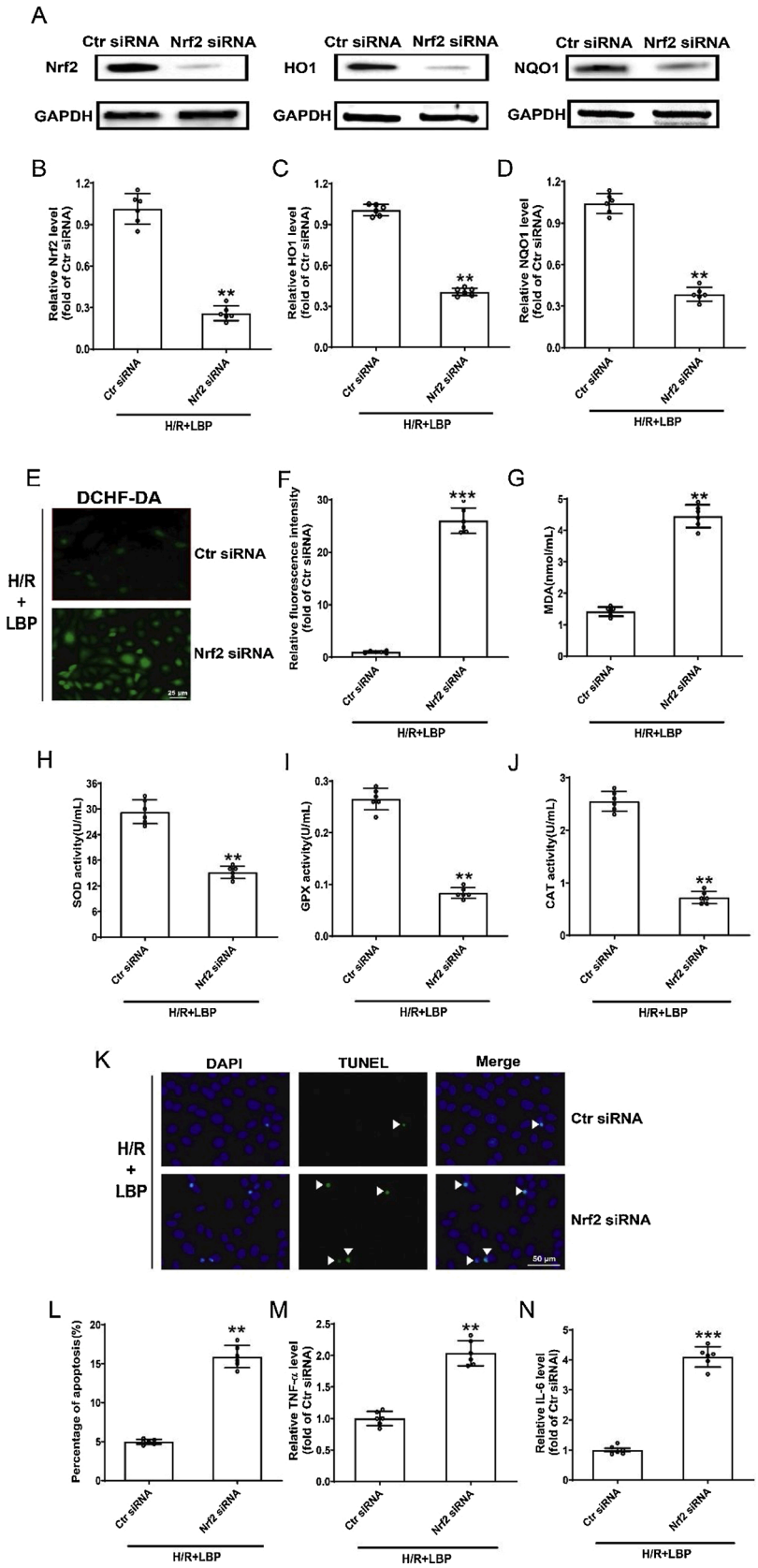

Fig. 7. The protective effects of LBP were abolished by Nrf2 knockdown. (A) Western blots of Nrf2, heme oxygenase 1(HO1), NADPH dehydrogenase quinone 1 (NQO1) and GAPDH proteins. (B-D) Western blot analysis of Nrf2, HO1 and NQO1. (E, F) The production of reactive oxygen species (ROS) in H9c2 cells; scale bar =25 μm. (G-J) Quantification of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) levels in Nrf2 knockdown cells. (K, L) Apoptotic index in Ctr siRNA and Nrf2 siRNA group; White triangle represents TUNEL-positive cells; scale bar =50 μm. (M, N) Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels in cell supernatant. Data are Means ± SD (n = 6). $^{**}P < 0.01$, $^{***}P < 0.001$, *versus* Ctr siRNA group.

highest lipoteichoic acid induced oxidative stress injury, which was associated with the increased mRNA levels of Nrf2 in H9c2 cells [41]. Li et al. further found that activation of Nrf2 signaling pathway alleviated myocardial I/R injury by reducing myocardial oxidative stress [42]. Consistent with those studies, our results showed that the Nrf2 signal pathway was impaired under the oxidative stress condition. Treatment with LBP increased Nrf2 expression, upregulated the levels of HO1 and NQO1, suppressed myocardial oxidative stress and improved cardiac function. However, Nrf2 siRNA abolished these protective benefits of LBP on myocardial I/R injury. Therefore, our results indicated that the Nrf2 antioxidant signal pathway might, at least partly, be responsible for the cardioprotective effects of LBP. Additionally, LBP provides multiple biological and pharmacological beneficial functions by inhibiting inflammation and Ca^{2+} overload and accumulating neovascularization in various disease conditions [43,44]. Based on its pleiotropic biological effects, it is conceivable that LBP may become a novel candidate for the prevention and treatment of myocardial I/R injury *via* multiple-targeting capacities and protective mechanisms.

5. Conclusions

In conclusion, we found that I/R injury mediated cardiac dysfunction by initiating oxidative stress. Treatment with LBP reduced myocardial oxidative stress and exerted protective effects in rats with myocardial I/R injury, which was partly dependent on the activation of Nrf2 antioxidant signal pathway. LBP may serve as a potential therapeutic strategy to treat oxidative stress related-myocardial I/R damage and prevent cardiovascular disease.

Authors' contributions

JJL and YL conceived the study. GXZ, ZW and LLH performed research. JNX collected the samples. BCN analyzed data. AIZ wrote the manuscript. LS revised the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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