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Article

Salidroside induced repair of myocardial infarction through Nrf2/HO-1

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Salidroside (SAL) has been confirmed to have some protective effects against inflammatory injury. However, little information was established as to the mechanism of these protective effects. To this effect, we designed this study to explore the protective effects and mechanisms of SAL against myocardial infarction (MI). A rat MI model was established and divided into five groups (n = 6): sham, MI, MI+SAL, MI+ LY294002 (PI3K inhibitor), and MI+SAL+ LY294002. The cardiac function and histological pathology were analyzed with a color Doppler ultrasonic diagnostic instrument. Anti-oxidative enzyme activities and the production of inflammatory media were assayed by biochemical kits and ELISA. MI size and fibrosis were assayed by Masson's trichrome staining while Bax/Bcl-2 and PI3K/Akt/Nrf2/HO-1 were assayed by Western blotting and immunofluorescence. The results showed that SAL significantly improved the left ventricle ejection fraction and fractional shortening, decreased the MI size and fibrosis, inhibited apoptosis and promoted blood vessel formation. SAL promoted anti-oxidative and anti-inflammatory abilities. Moreover, SAL enhanced PI3K/ Akt/Nrf2/HO-1 expression. To this effect, we designed this study suggested that SAL induced repair of MI via PI3K/A kt/ Nrf2/HO-1.

Keywords: Fibrosis. HO-1. Myocardial Infarction. Salidroside. PI3K/Akt.

INTRODUCTION

Coronary atherosclerotic disease (CAD), such as myocardial infarction (MI), is a disease characterized by myocardial cell necrosis due to the interruption of cardiac blood flow. This interruption is induced by coronary artery occlusion. The myocardial cell is a terminal differentiation cell. To this effect, myocardial injury cannot be repaired through cell regeneration. It is instead repaired through the formation of a fibrotic scar. Thus, ventricular remodeling is the main cause of cardiac insufficiency and cardiogenic death in patients with infarct. The treatment methods of current clinical applications such as drug therapy (Thrombolysis), percutaneous coronary intervention and coronary artery bypass graft can make recanalization and myocardial revascularization progress. These, to a certain extent, reduce ventricular remodeling and improves the patient's symptoms, thereby reducing the mortality rate (Montalescot *et al.* 2013; Varenne *et al.,* 2018). However, these treatments cannot promote the regeneration of infarcted myocardium. Furthermore, the long-term prognosis of patients with MI cannot be significantly improved.

Salidroside (SAL), molecular formula C14H2007 (relative molecular mass 300.31), is a colorless transparent needle-like crystal at room temperature, which is soluble in water, ethanol, butyl alcohol and other solvents. The pharmacokinetic experiments established that SAL was mainly metabolized by the liver, excreted by the kidneys, and confers no genotoxicity to animals. Studies have

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shown that SAL has a variety of pharmacological effects that enhance immunity, tolerance of hypoxia, and anti-free radical damage (Zhao *et al*., 2013; Sun *et al*., 2018). With the in-depth study of the anti-damage protection effect of SAL, it has been found that SAL can play an anti-oxidation, anti-inflammatory and anti-apoptosis role in various ways. These can inhibit the release of cytokines such as TNF, IL-6, IL-1, NO, iNOS, PGE2, and COX-2. In addition reduction in the intracellular calcium ion concentration, and increased level of cAMP and cGMP. Also, it has effects on p-IκB, p-ERK, and p38 (Wang *et al*., 2019a; Lu *et al*., 2017).

Nrf2/HO -1 is an important regulatory signal pathway for cell antioxidant stress response.It is an indispensable part of cell resistance to various environmental stress and endogenous stress defense mechanisms (Lin *et al*., 2019). Under oxidative stress conditions, the Nrf2 protein in the plasma is dissociated from the Keel protein and transferred to the nucleus of the cell. Then binds to the specific site on the antioxidant reaction element (ARE) to initiate the expression of an ARE-regulated series of oxidase enzymes. These enzymes include catalase (CAT), superoxide dismutases (SOD), HO-1, etc. ARE-regulation increases the ability of cell antioxidant stress (Yu *et al*., 2019).

A large number of studies have established a significant negative correlation between HO-1 and the incidence of CAD (Fiorelli *et al*., 2019; Kishimoto *et al*., 2018). In this study, we studied the protective effect of SAL on the myocardial ischemia-reperfusion model and observed its inhibitory effect of it on the P13K/Akt/Nrf2 signal pathway *in vivo.*

MATERIAL AND METHODS

Ethics statement

Male wild-type SD rats were purchased from the Hubei University of Chinese Medicine. They were maintained under specific pathogen-free conditions. All experiments referring to the use of animals were approved by the Committee of Animal Care and Use of Hubei University of Chinese Medicine (HUCM-001765).

Establishment of rat models

Male SD rats weighing (220 – 250 g) were divided into five groups (n = 6): sham, MI, MI+SAL, MI+ LY294002, and MI+SAL+ LY294002. After Sodium Pentobarbital of 35 mg/kg was injected intraperitoneally into rats for anesthesia and fixation. The limbs of rats were connected to SIEMENS Sequoia (512 Color Doppler Ultrasonic Diagnostic Instrument, probe frequency 8-13 MHZ). The chest was opened and the left anterior descending coronary artery was ligated for about 30 min. Then the ligation was loosened for reperfusion for about 120 min. Intravenous injection of SAL (50 mg/kg) or saline. The SAL used in this experiment was provided by the School of Pharmacy of Fujian University of Traditional Chinese Medicine (purity >99 %). The drug was dissolved with 0.9 % saline. LY294002 (purity > 98%), a PI3K inhibitor, was purchased from Keygen, Jiangsu, China. LY294002 was dissolved in DMSO and injected intravenously with a concentration of 0.25 mg/kg according to experiments in advance. The limbs of rats were connected to SIEMENS Sequoia (512 Color Doppler Ultrasonic Diagnostic Instrument, probe frequency 8-13 MHZ) and the Heart function including ejection fraction (EF), fractional shortening (FS), and left ventricle inner diameter at systole (LVIDs) was measured at 24 h after MI for each group.

Determination of serum and heart CAT, SOD, LDH and MDA

After 24h of reperfusion, the femoral artery blood was extracted from various groups, left for 30 min, and centrifuged at 300 g/min for 15 min to obtain serum. The total protein from myocardial tissues of the infarct area was extracted according to the instructions. The protein concentrations were determined by BCA methods. The CAT, LDH, SOD activities and MDA contents were evaluated by kits according to the instructions (Jiancheng, Nanjing, China). Thiobarbituric acid (TBA)methods were used for MDA assay. It can condense with TBA to form a red product with the largest absorption peak at 532 nm. WST-1 methods were used for the SOD assay. WST-1

is 4-Iodophenyl-3-4-nitrophenyl-5-2,4-disulfophenyl-2h-tetrazolium, monosodium salt, which reacts with a superoxide anion catalyzed by Xanthine oxidase to produce a water-soluble formazan dye. This dye can be inhibited by SOD. The absorbance at 450 nm was measured. H_2O_2, which remains after the reaction, forms a stable yellow complex with ammonium molybdate in serum or plasma under the optimal enzymatic reaction conditions. This yellow color is inversely proportional to the enzyme activity. The absorbance at 405 nm was measured for the CAT assay. According to the instructions, 20 µl of samples, 25 µl of Matrix buffer and 5 µl of Coenzyme I were mixed and incubated at 37 ºC for 15 minutes. Then 25 µl of 2, 4-dinitrophenylhydrazine was added and incubated for another 15 minutes at 37 ºC. Finally, 0.4 mol/L of NaOH was added for 5 minutes at room temperature and the absorbance at 450 nm was measured for LDH assay.

ELISA for TNF-α, IL-1A and IL-6

The total protein from myocardial tissues of the infarct area was extracted according to the instructions. The protein concentrations were determined by BCA methods. According to the kit instructions, the sample was added into the wells (100 µl/ well) and incubated at 37ºC for 90 min. After washing the plate 5 times, biotinylated antibody (100 µl/ well) was added and incubated for 60 min at 37ºC. Then after washing the plate 5 times, the enzyme conjugate working solution (100 µl/ well) was added and incubate at 37ºC for 30 min in the dark. After washing the plate 5 times, 100 µl/ well of the chromogenic substrate was added and incubate at 37ºC in dark for 15 min. After adding 100 µl/ well of stopping solution, the OD450 value was measured immediately after mixing evenly.

Masson's trichrome staining and immunostaining

Hearts were harvested and fixed in 4% Paraformaldehyde for 24 h, cut into five transverse slices through the infarcted area. The slices were embedded in Paraffin and 5 µM histological sections were stained with Masson's Trichrome. Infarct Size and Fibrosis were quantified using the Image J software. The vessel density was identified by immunohistochemistry using an anti-VWF antibody. Nrf2 activation was assayed by immunofluorescence using an anti-Nrf2 polyclonal antibody and FITC-labeled secondary antibody.

Western Blotting

Myocardial tissues of the infarct area were lysed with RIPA buffer containing Protease inhibitors (Dingguo, Beijing, China). The primary antibody, Rabbit anti-Bax, Bcl-2, Nrf2, HO-1, p-PI3K and p-Akt polyclonal antibodies (1:250) were provided by Santa Cruz Biotechnology, Santa Cruz, CA. A Horse Radish Peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5,000, Boster, Wuhan, China) was used as a secondary antibody (Li *et al*., 2016).

Statistical Analysis

All the statistical analyses were performed with SPSS 15.0 to assess differences among the groups. The measurement data were expressed as mean ± standard deviation. A comparison among multiple groups was performed by one-way ANOVA. The LSD *t*-test was used for comparison between the two groups. A '*p*' value less than 0.05 was considered to be statistically significant.

RESULTS:

SAL reduced cardiac injury

We tested the effects of SAL on cardiac function in the rat MI model. Echocardiography was performed to evaluate the therapeutic efficacy of different treatments in the rat MI-induced heart. Heart function including ejection fraction (EF), fractional shortening (FS), and left ventricle inner diameter at systole (LVIDs) was measured 24h after reperfusion. The EF and FS in the SAL group were much higher than those in the MI group. Similarly, LVIDd in the SAL group was significantly lower compared to that of the MI group. The P13K inhibitor LY294002 could effectively reverse the effects of SAL (Figure 1A, $p < 0.05$).

Next, we conducted a histological analysis to better understand the improvement of cardiac function by Masson's Trichrome Staining of the rat hearts at 24h after reperfusion. Fibrosis due to MI appeared blue while the preserved myocardium appeared red in Masson's Trichrome Staining. The MI size was greatly reduced in the SAL group compared to those in the MI group (Figure 1B). Moreover, the ventricular fibrosis rate was significantly reduced in the SAL group compared with that of the MI group (Figure 1C). The P13K inhibitor LY294002 could effectively reverse the effects of SAL.

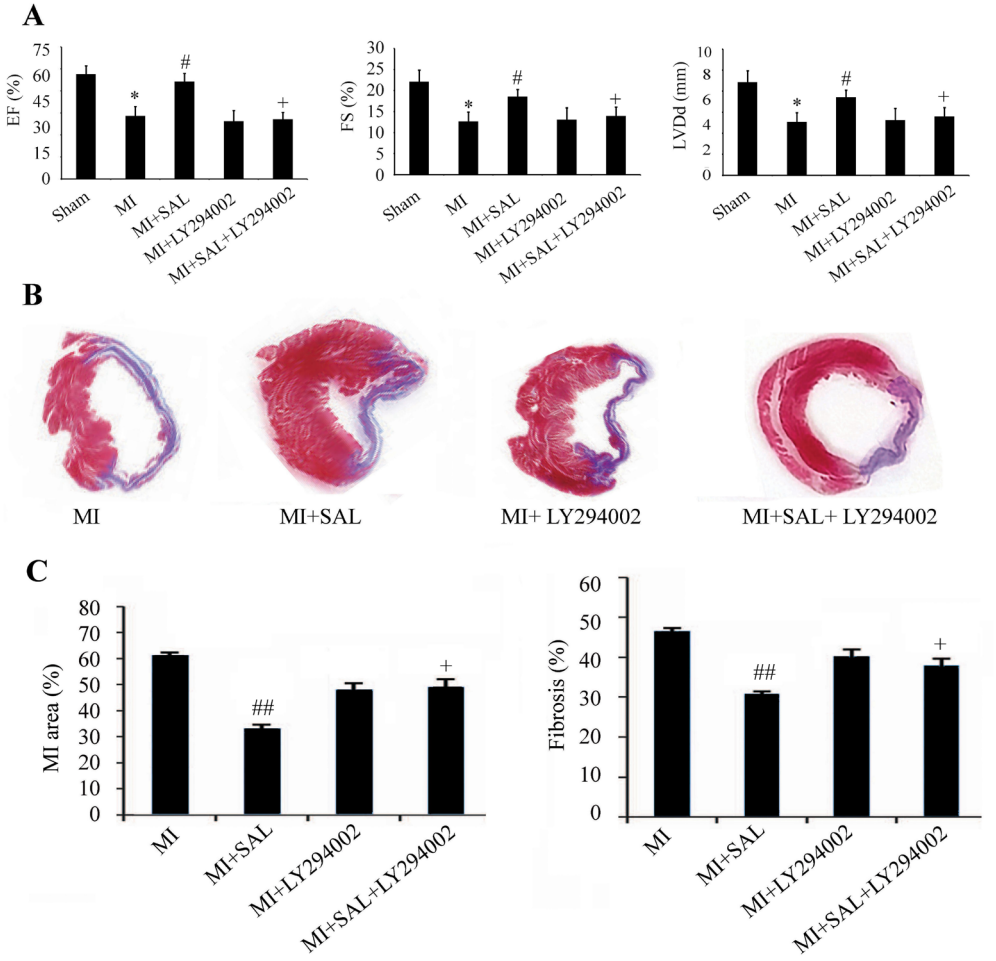

FIGURE 1 - SAL reduced cardiac injury. (A) Recovery of cardiac function after SAL treatment was assayed using echocardiography measurements at 24 h after MI induction. (B) Representative images of heart sections stained with Masson's Trichrome. (C) MI size and fibrosis were compared among different groups. * $p < 0.05$ vs. Sham; # $p < 0.05$ and ## $p < 0.01$ vs. MI; + $p < 0.05$ vs. MI+SAL.

SAL promoted anti-oxidative and anti-inflammatory activities

By determining serum and heart CAT, SOD, LDH and MDA, we found that the SAL group showed a significant increase in the release of SOD, CAD and a reduction in the release of LDH and MDA (Figure 2A, B). Furthermore, the results of ELISA showed that the SAL group had significantly inhibited the production of TNF-α,IL-1β and IL-6 compared to the MI group (Figure 2C). The P13K inhibitor LY294002 could effectively reverse the effects of SAL, indicating that the SAL exhibit strong anti-oxidative and inflammatory effects.

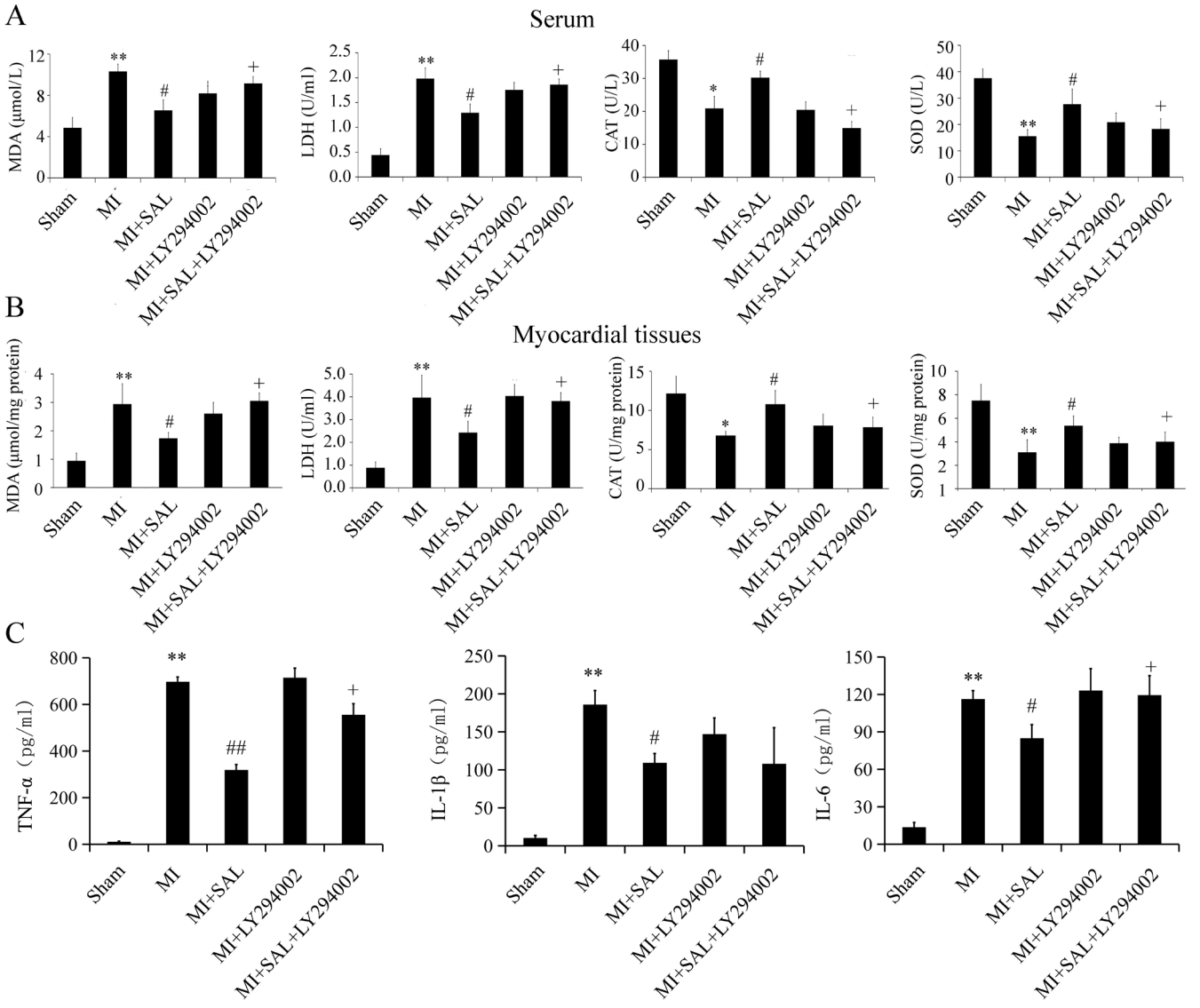

FIGURE 2 - Effects of SAL on the anti-oxidative enzyme activities in serum (A) and myocardial tissues (B) and inflammatory media (TNF-α,IL-1ects of SAL (C). * p <0.05 and ** p < 0.01 vs. Sham. # p < 0.05 and ## p < 0.01vs. MI; + p < 0.05 vs. MI+SAL.

SAL inhibited apoptosis and promoted vascular regeneration

MI can increase the expression of pro-apoptotic protein Bax while reducing the expression of anti-apoptotic protein Bcl-2. The expression of Bax decreased gradually while the expression of Bcl-2 increased gradually with the administration of SAL. Furthermore, anti-VWF staining showed that the SAL group had significantly increased vessel density in the infarcted areas compared to the MI group (Figure 3B). The P13K inhibitor, LY294002 could effectively reverse the effects of SAL. This indicates that the SAL inhibited apoptosis and promoted vascular regeneration.

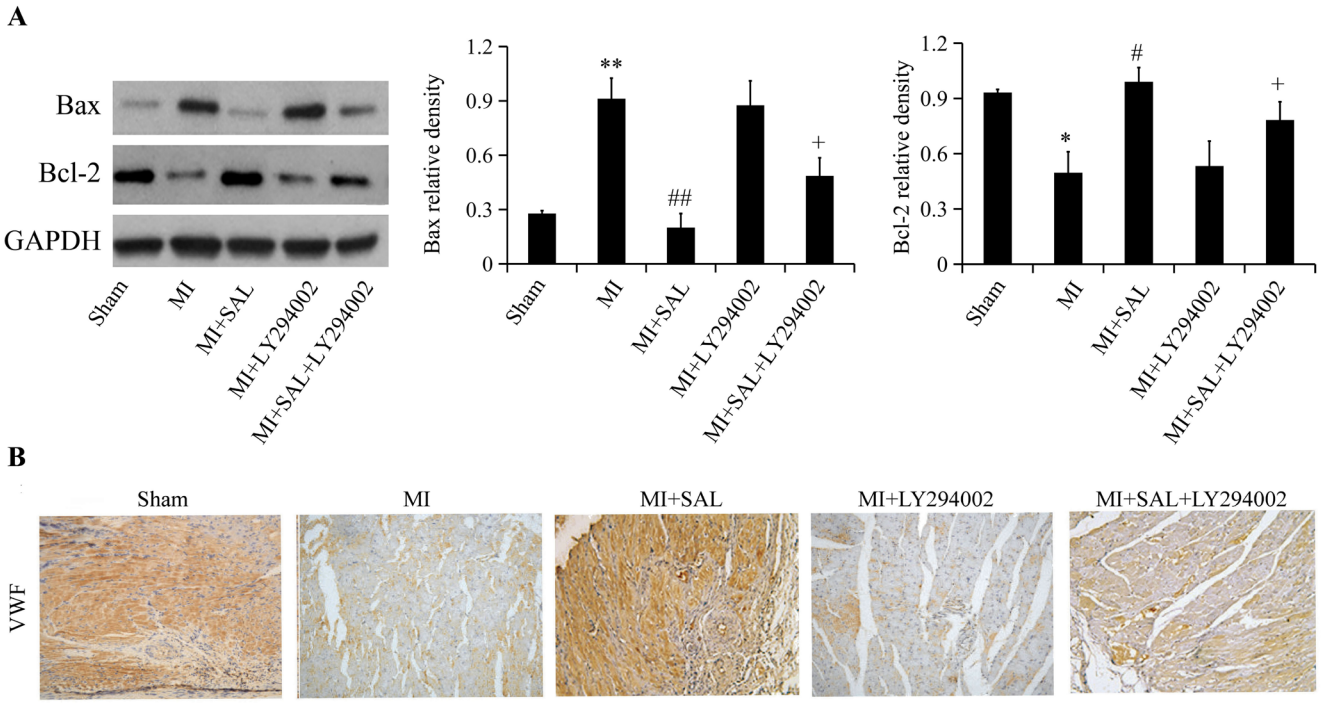

FIGURE 3 - SAL inhibited apoptosis (A) and promoted vascular regeneration (B).* $p < 0.05$ and **$p < 0.01$ vs. Sham. # $p < 0.05$ and ## $p < 0.01$ vs. MI; + $p < 0.05$ vs. MI+SAL.

SAL acted through PI3K/Akt/Nfr2/HO-1

Western blotting was performed to investigate p-PI3K, p-Akt, Nfr2, HO-1 expression in the infarcted areas following various treatments. The infarcted area in the SAL group showed a high expression of p-PI3K, p-Akt, nuclear-Nfr2 (n-Nfr2) and HO-1, which was weak in the MI control (Figure 4A, B). Next, we determined the expression of Nrf2 in heart tissues using immunostaining. Although a small number of Nrf2 was detected in the myocardium of the MI group, more Nrf2 activation and expression were detected in the myocardium of the SAL group (Figure 4C). The P13K inhibitor LY294002 could effectively reverse the effects of SAL.

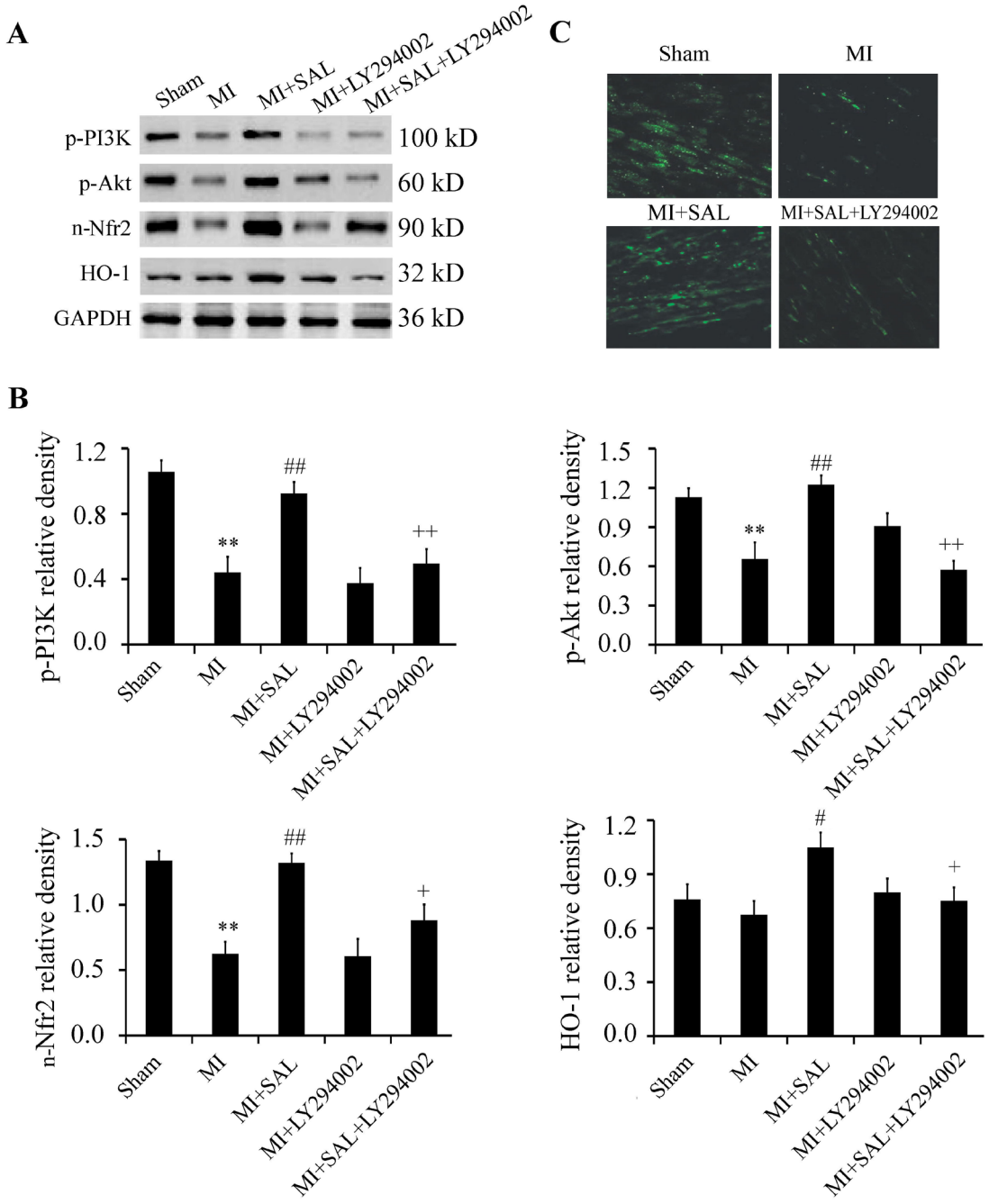

FIGURE 4 - p-PI3K/p-Akt/n-Nrf2/HO-1 were assayed using Western blotting (A), and Nrf2 was assayed by immunofluorence (B). n-Nrf2 refers to nuclear Nrf2. ** $p < 0.01$ vs. Sham; # $p < 0.05$ and ## $p < 0.01$ vs. MI; + $p < 0.05$ and ++ $p < 0.01$ vs. MI+SAL.

DISCUSSION

SAL has been confirmed to possess protective effects of inflammatory injury. These include cardiovascular diseases, neurodegenerative diseases, diabetes, sepsis, and cancer (Pu *et al.*, 2020). A profound number of studies reported that SAL exhibits neuroprotective activities through the regulation of oxidative stress response, inflammation, apoptosis, and neural regeneration (Zhong *et al.*, 2018). In this study, we verified that SAL has

protective effects against MI through improving cardiac function, the MI size and ventricular fibrosis.

Some studies have suggested that the excessive generation of ROS and inflammation play a central role in myocardial injury. MDA and LDH as the biological products of ROS and lipid peroxidation. SOD and CAT are the main antioxidant enzymes responsible for mobilizing free radicals (Bajinka *et al.,* 2020). This study revealed that SAL increased SOD and CAT activities and decreased MDA and LDH contents in serum and myocardial tissues of the infarct area.

Moreover, the SAL group had significantly inhibited the production of TNF-α,IL-1β and IL-6 induced by MI. This confirms the myocardial protection of SAL partly due to the inhibiting oxidative and inflammatory effects. Bax and Bcl-2 proteins play an important role in regulating proliferation, differentiation and apoptosis (Loginov *et al,* 2017). The expression of Bax decreased gradually and the expression of Bcl-2 increased gradually with the administration of SAL. Furthermore, anti-VWF staining showed that the SAL group had significantly increased vessel density in the infarcted areas compared to the MI group. This indicates that the SAL inhibited apoptosis and promoted vascular regeneration.

Nrf2 is an important transcription factor regulating the oxidative stress response. In the condition of oxidative stress, Nrf2 is translocated into the nucleus and binds with ARE. This signals the onset of the transcription of detoxifying enzymes and antioxidant enzyme gene expression such as HO-1. Of note, HO-1 protects the body from ROS (Wang *et al*., 2019b). Continuous expression of HO-1 in endothelial cells can reduce the inflammatory damage and apoptosis of cells caused by ischemia (Liu *et al*., 2006). The present results showed that SAL can significantly promote p-PI3k, p-AKT, Nrf2 and HO-1 expression of rats with MI.

CONCLUSION

We concluded that SAL significantly improved the MI remodeling process and improved the cardiac injury through PI3K/AKT/Nrf2 /HO-1 pathway, which provides an effective treatment for MI.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE:

All animal experiments and procedures were approved by the Institutional Animal Care and Use Committees of Hubei University of Chinese Medicine (HUCM-001765).

FUNDING

None.

CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

YF, WW and LT performed the experiments; YF carried out data analysis; JY designed the study and wrote the manuscript. All authors approved the submission.

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