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Diosgenin Protects Rats from Myocardial Inflammatory Injury Induced by Ischemia-Reperfusion

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Diosgenin, a phytosteroid sapogenin, has anti-inflammatory properties shown to reduce myocardial ischemia-reperfusion injury (MIRI). However, the specific mechanism by which this is achieved is not clear. This study investigated the protective effects of diosgenin on myocardial ischemia/reperfusion (I/R) and the potential anti-inflammatory mechanisms.

Material/Methods: Healthy adult male SD rats, body weight (b.w.) 250–280 g, were used to model ischemia-reperfusion injury (IRI) and were administered diosgenin (50 mg/kg and 100 mg/kg b.w.) intragastrically for 4 consecutive weeks before surgery. The left anterior descending artery (LAD) was ligated to induce myocardial ischemia for 30 min and reperfusion for 30 min, 60 min, and 120 min while relevant indicators were detected.

Results: Both 50 mg and 100 mg diosgenin oral administration increased left ventricular developed pressure (LVDP) and maximum changing rate of ventricular pressure ($\pm dp/dt_{max}$), decreased left ventricular end-diastolic pressure (LVEDP), and myocardial enzyme markers. TTC staining suggested that diosgenin reduced myocardial infarct size in the rat model. Pathological results showed that myocardial ischemia and inflammation were alleviated by diosgenin. In addition, the increased expression of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) in serum, and myeloperoxidase (MPO) in myocardium were significantly suppressed by diosgenin administration. Diosgenin further inhibited the phosphorylation of transcription factor NF-κB and modulated the expression of downstream inflammatory cytokines by regulating the activation of p38-MAPK and JNK pathways.

Conclusions: Results demonstrate diosgenin plays an anti-inflammatory role in the protection of MIRI through regulation of p38-MAPK and JNK pathways and phosphorylation of NF-κB.

MeSH Keywords: Anti-Inflammatory Agents • Diosgenin • MAP Kinase Signaling System • Myocardial Reperfusion Injury

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Background

Coronary atherosclerotic heart disease (CHD) is a common cause of death and disability worldwide, with myocardial infarction being the most serious complication. Although timely reperfusion therapy can reduce the infarct size and improve survival rates, occurrence of injury (e.g., arrhythmia, cardiac insufficiency, and death) can increase tissue damage after blood flow recovery. Myocardial ischemia triggers a cascade of tissue injuries that can be exacerbated by reperfusion, metabolic disorders, and dysfunction of cardiomyocytes. This can cause myocardial stunning and hibernation, leading to functional and structural damage of the heart. Given the serious adverse effects, preventing myocardial ischemia-reperfusion injury (MIRI) is important for the treatment of CHD [1].

Several mechanisms are involved in the impairment of ischemia-reperfusion (I/R). Resent research suggests reperfusion injury is a result of an increased expression of inflammatory cytokines [2]. The release of cytokines and inflammatory mediators activates neutrophils and endothelial cells, which both mediate the inflammatory response of I/R. MIRI promotes the production of inflammatory cytokines and facilitates the infiltration of inflammatory cells. Interleukin-1β (IL-1β) directly up-regulates cellular adhesion molecule-1 (ICAM-1) and endothelial-leukocyte adhesion molecule-1 (ELAM-1) at the molecular level and promotes the inflammatory response. Tumor necrosis factor α (TNF-α), a cell signaling protein, promotes the expression of inflammatory mediators, infiltration of neutrophils, production of oxygen free radicals, and apoptosis, which further damages ischemic tissue. NF-κB, a protein complex, plays an important role in activation and expression of downstream inflammatory factors in MIRI [3,4], and inhibition of NF-κB nuclear translocation reduces the adhesion and infiltration of leukocytes and alleviates ischemia-reperfusion injury (IRI) [5]. Ischemic preconditioning (IPC) has been shown to attenuate IRI through the inhibition of NF-κB [6]; however, IPC is difficult to implement in clinical practice.

Diosgenin is a natural steroidal sapogenin extracted from *Solanum* and *Dioscorea* plant species, and is important for the synthesis of steroid hormones. It has many pharmacological effects, including anti-tumor [7,8], anti-inflammation [9,10], and anti-oxidation [11] effects, and has been shown to successfully treat cardiovascular disease, type 2 diabetes, and neurodegenerative diseases [12]. Research has shown diosgenin inhibits TNF-α and regulates the process of atherosclerosis inflammation and immune response [13]. In the human umbilical vein endothelial cells (HUVECs) model, diosgenin regulates the NF-κB pathway and down-regulates the expression of adhesion molecules to inhibit leukocytes migration and adhesion [14]. Diosgenin also attenuates macrophage-mediated inflammatory mediators by inhibiting the activation of NF-κB and c-JUN N-terminal kinase (JNK) [15]. In myocardial I/R models, diosgenin alleviates acute MIRI by activating mitochondria K_{ATP} (mitoK_{ATP}) channels and stabilizing lysosomal membrane potential [16,17]. It appears there is no recent research examining the anti-inflammatory effects of diosgenin in MIRI. Therefore, the present study explored the role of diosgenin in reducing myocardial injury after I/R, and assessed whether p38-MAPK, JNK, and NF-κB signaling pathways are involved in the underlying molecular mechanism.

Material and Methods

Animals and chemicals

Approval for these experiments was obtained from the Ethics Committee of Nanjing Medical University. Healthy adult male SD rats (250–280 g) were provided by the Animal Center of Nanjing Medical University. All animals were fed a standard diet and were subjected to 1 week of adaptive feeding. During this period, the animals were allowed free access to drinking water and were housed at a temperature of 23–25°C and humidity of 55–70%. Animals were fasted for 12 h except for water before the experiment. Diosgenin was purchased from Sigma (St Louis, MO). All other chemicals used were of analytical grade.

Experimental design

Rats were randomly divided into 4 groups (n=10): the sham operation group (sham group) (n=10), I/R group (n=10), the diosgenin (50 mg/kg/d)+I/R group (n=10), and the diosgenin (100 mg/kg/d)+I/R group (n=10). Diosgenin (freshly prepared daily in different doses in salt water) groups were administered intragastrically once every day for 4 weeks. Rats in the sham group and I/R group were given an equal volume of saline for 4 weeks. The dose of diosgenin was determined based on previous protective studies [18,19].

Acute I/R model in rats

The acute cardiac I/R model in rats was conducted according to the methods described in a previous report [20]. Rats were weighed and 60% urethan was injected intraperitoneally (0.5 ml/100 mg). Rats were fixed in supine position on the operating table and connected a standard lead II electrocardiogram. The neck was cut in the middle and a small animal ventilator was connected for mechanical ventilation (respiratory rate 60 beats/min, tidal volume 8.0 ml, frequency 5: 4). After endotracheal intubation, the left common carotid artery was detached and catheterized and connected to the biological information collection system (BL-410, Nihon Kohden, Japan) via the pressure sensor. Left thoracotomy and pericardotomy were performed to expose the heart. The LAD artery was ligated (4/0

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silk) 2 mm away from the left auricle, and a small vinyl tube was passed through the ligature to form a snare. Myocardial ischemia was induced by LAD ligation for 30 min followed by 120 min of reperfusion. If the typical ST-segment elevation was observed in EKG, the operation was regarded as a success and a reduction of ST-segment greater than 1/3 was considered the qualified model. The sham group underwent the same operation and the LAD was threaded but not ligated.

Determination of cardiac function parameters

Cardiac hemodynamic parameters were recorded in each group for different time periods (including ischemia, ischemia 30 min, reperfusion 30 min, 60 min, and 90 min). Heart rate (HR), left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), and $\pm dp/dt_{max}$ were recorded and analyzed.

Detection of myocardial enzymes

After reperfusion for 120 min, blood was taken from the left common carotid artery and centrifuged at 3500 rpm for 10 min. The supernatant was collected and the concentrations of CK-MB and cTNI were detected using the corresponding kits (KHB, Shanghai, China).

Measurement of myocardial infarct size

LAD was re-ligated and 1% Evans Blue 1 ml was injected into the left common carotid artery to distinguish ischemic and non-ischemic areas. The heart was removed and washed repeatedly in pre-chilled saline and the left ventricle was cut into thin slices of about 1–2 mm in thickness and placed in 1% TTC solution and incubated for 15–30 min in a 37°C water bath, then fixed with 10% formaldehyde for 10 min. Photos were then taken. The percentage of infarct size for the left ventricular area was calculated using Image-pro plus 6.0 (Media Cybernetics, MD, USA).

Histopathologic examination

The heart was removed and placed in pre-cooled PBS to remove the connective tissue and adipose tissue, and the myocardium was cut transversely about 5 mm. HE-stained sections were fixed, dehydrated, embedded, stained, and sealed. Finally, the pathological changes of myocardium were observed under a light microscope.

TNF-α and IL-β activity

After centrifugation, serum was collected and the expressions of TNF-α and IL-1β were detected using an ELISA kit (R&D Systems, Emeryville, CA).

Myeloperoxidase (MPO) activity

Approximately 200 mg of ventricular tissue was weighed and cut into pieces in 2 mL of ice-cold lysis buffer. The homogenate was centrifuged at 15 000 g for 30 min at 4°C. The supernatant was collected and MPO activity was detected using a kit (Jiancheng Biology, Nanjing, China).

Western blotting

Proteins were extracted from myocardial tissue and separated by SDS-PAGE, transferred to the polyvinylidene fluoride membrane (Solarbio, Shanghai, China), and blocked in 5% skim milk for 1 h. They were then washed 3 times in TBST and incubated with primary antibody overnight at 4°C. Proteins were incubated by secondary antibody for 1 h. ECL chemiluminescent reagent (Thermo Fisher Scientific, Rockford, AL) was added before exposure and the grayscale values were scanned and analyzed using a gel imaging system (FlourChem HD2, ProteinSimple, CA). Each protein was repeated 3 times and normalized analysis was performed to obtain the mean and standard deviation. Antibodies against NF-κB p65 (4764S), p-NF-κB p65 (3033S), IκBa (9242S) p38 (8690S), p-p38 (4092S), JNK (2305S), p-JNK (4668S), anti-rabbit IgG (14708S), and β-actin (12620S) were acquired from Cell Signaling Technology (Danvers, MA).

Statistics analysis

Data are expressed as mean ±SD, and normality and variance homogeneity were tested. The between-group parameters were analyzed using one-way ANOVA followed by the Tukey post hoc test. Differences were considered statistically significant when $p<0.05$.

Results

Diosgenin improves cardiac function parameters of I/R rats

Cardiac function parameters (HR, LVDP, LVEDP, and $\pm dp/dt_{max}$) were measured at different times. Results indicated diosgenin (50 mg and 100 mg) increased LVDP and $-dp/dt_{max}$ and decreased LVEDP at 60 min and 120 min of reperfusion compared with the I/R group. Diosgenin slowed the decline of $+dp/dt_{max}$, but the effect on heart rate was not statistically significant (Figure 1). These results suggest that diosgenin improves left ventricular function after I/R.

Diosgenin decreases the expression of myocardial enzymes

CK-MB and cTNI in the I/R group were significantly higher compared to the sham group, while diosgenin pretreatment

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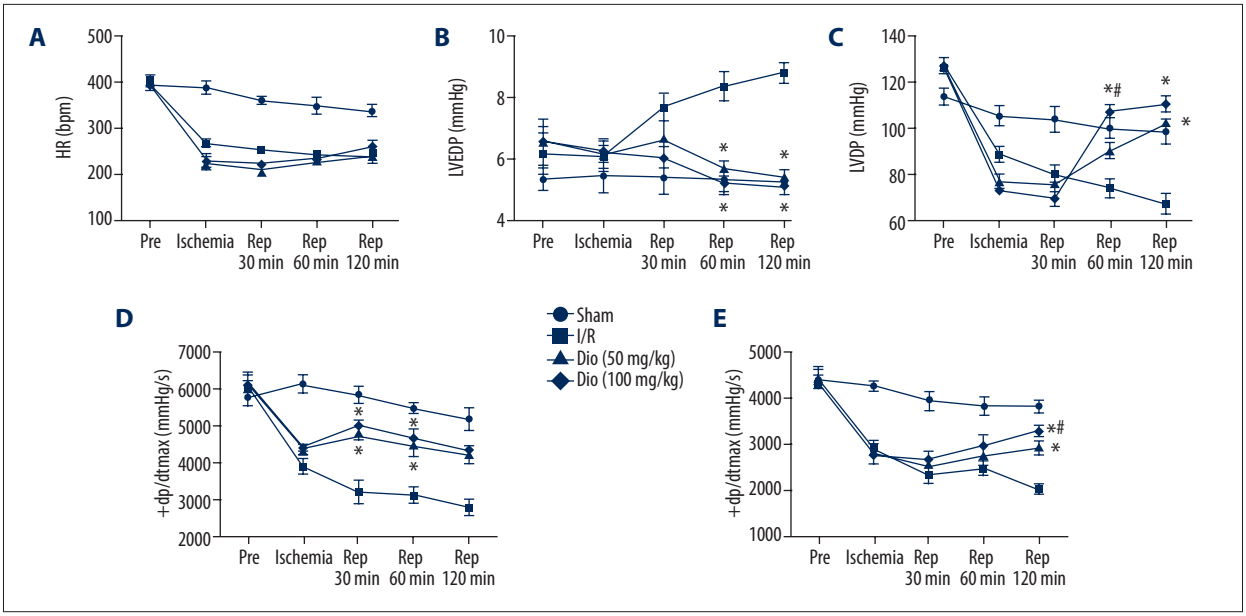

Figure 1. Effects of diosgenin on cardiac function parameters of I/R rats. (**A**) Changes of HR within 2 h. (**B**) Changes of LVEDP within 2 h. (**C**) LVDP is calculated by LVSP (left ventricular systolic pressure) minus LVEDP. (**D, E**) Changes of $\pm dp/dt_{max}$ within 2 h. Data represent means ±SD. * $p<0.05$ compared with I/R Group. # $p<0.05$ compared with Dio (50 mg/kg) group.

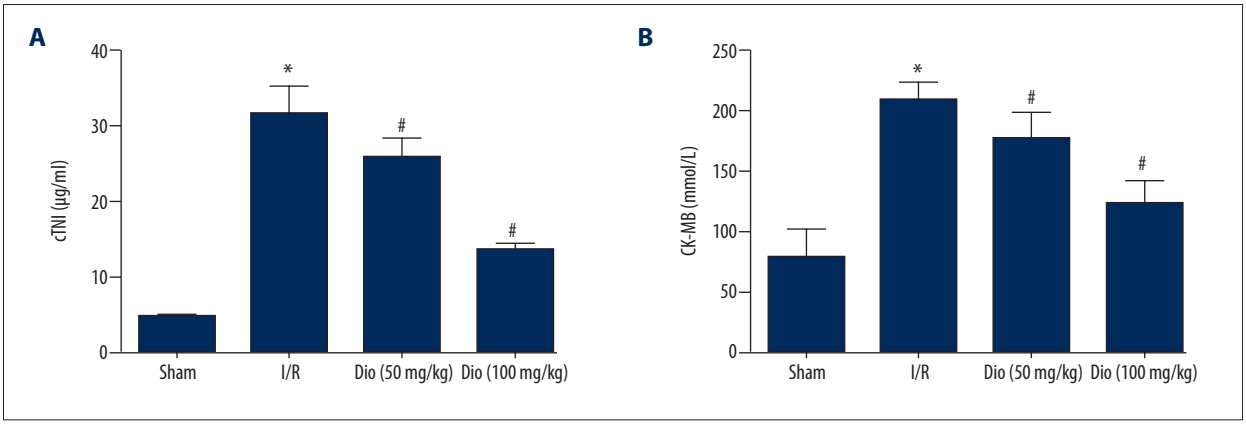

Figure 2. Diosgenin decreases the expression of myocardial enzymes. (**A**) CK-MB. (**B**) cTNI. Data represent means ±SD. * $p<0.05$ compared to sham operation (sham) group. # $p<0.05$ compared to I/R group.

(especially 100 mg diosgenin administration) decreased myocardial enzyme activity (Figure 2). Our results suggest that diosgenin moderately alleviated MIRI.

Diosgenin reduces myocardial injury and myocardial infarct size

After conventional HE staining, myocardium of the I/R group showed regional degeneration and necrosis, muscle fiber disorder and rupture dissolution, and interstitial hyperemia and edema, accompanied by inflammatory cell infiltration. Diosgenin pretreatment markedly reduced myocardial injury, and muscle fibers were arranged neatly. Part of the muscle bundle gap widened, there was mild interstitial swelling, and there was little inflammatory cell infiltration (Figure 3A). The percentage of infarcted myocardium in the total ventricular area can represent the extent of myocardial infarction. Compared with the sham group, infarct size of the I/R group was notably increased, and the infarct size of the diosgenin group was significantly lower than in the I/R group (Figure 3B). These results further demonstrate the protective effects of diosgenin on ischemic myocardium.

Diosgenin decreases serum levels of TNF-α and IL-1β and myocardial MPO in I/R rats

The levels of TNF-α and IL-1β were significantly increased after reperfusion for 2 h. Oral administration of diosgenin (50 mg and

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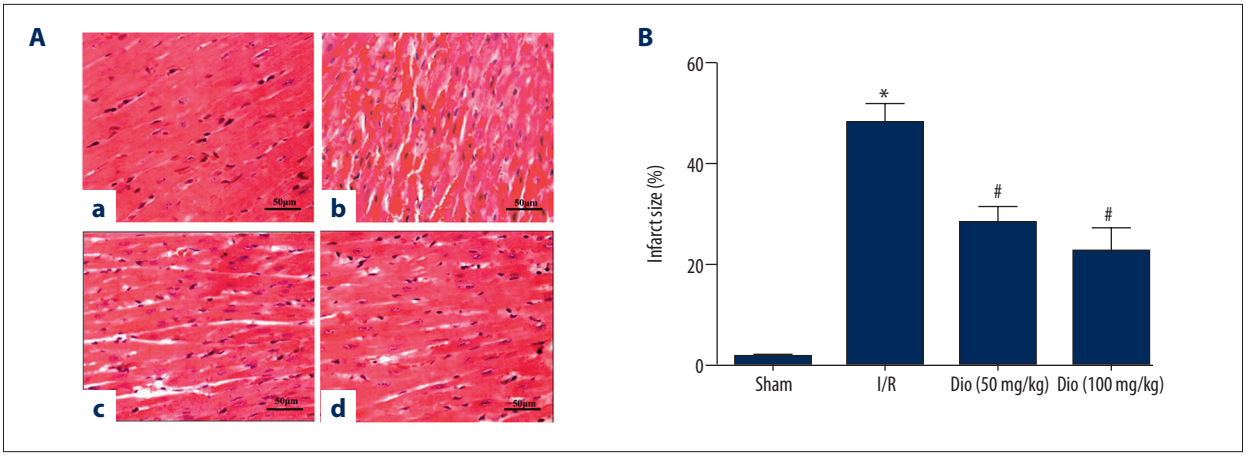

Figure 3. Diosgenin reduced myocardial injury and myocardial infarct size after 2-h reperfusion. (**A**) A representative micrograph (400× magnification) of left ventricular myocardial is presented and (**a**), (**b**), (**c**), and (**d**) represent the sham group, I/R group, Dio (50 mg/kg) group, and Dio (100 mg/kg) group, respectively. The infarction area was characterized by regional degeneration and necrosis, cardiac muscle fiber swelling, disorder, and fiber destruction in the control group, but minimal histopathological changes were observed in the Dio (50 and 100 mg/kg)-treated groups. (**B**) Effect of diosgenin on I/R-induced myocardial infarct size. Data represent means ±SD. * $p<0.05$ compared to sham group. # $p<0.05$ compared to I/R group.

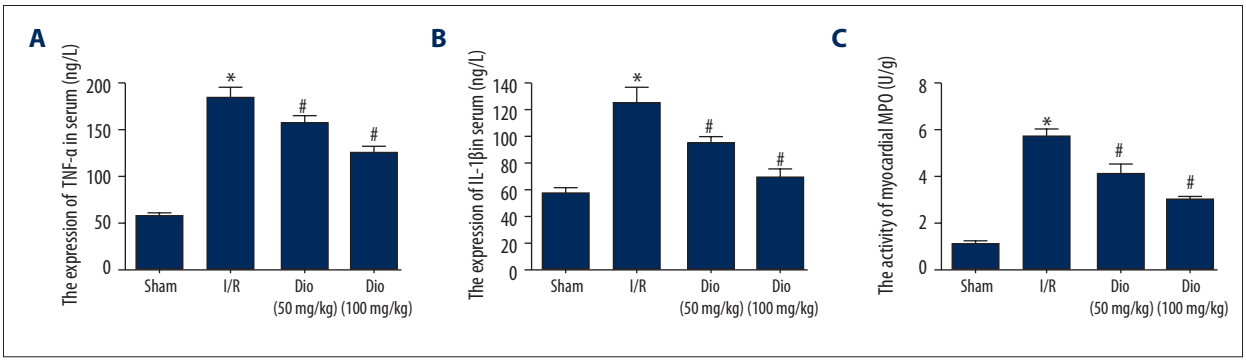

Figure 4. Diosgenin decreases serum levels of TNF-α and IL-1β and myocardial MPO in I/R rats. (**A**) The expression of TNF-α in serum. (**B**) The expression of IL-1β in serum. (**C**) The expression of MPO in myocardium. Data represent means ±SD. * $p<0.05$ compared to sham group. # $p<0.05$ compared to I/R group.

100 mg) decreased the expression of TNF-α and IL-1β, as show in Figure 4A, 4B. The degree of neutrophil infiltration is closely related to expression of myocardial MPO [21]. Figure 4C shows the relationship between the dose of diosgenin and the MPO value.

Diosgenin inhibits phosphorylation of the NF-κB signaling pathway

The expression of IκBa and p65 protein was detected by Western blotting after reperfusion for 2 h. As shown in Figure 5, I/R stimulation increased the degradation of IκBa and up-regulated the ratio of p-p65/p65. However, 50 mg and 100 mg diosgenin dramatically reversed the down-regulation of IκBa and phosphorylation of NF-κB p65 induced by I/R. Our data suggest that diosgenin suppressed the activation of the NF-κB pathway, which can block the production of I/R-induced inflammatory molecules by diosgenin.

Diosgenin regulates the activation of p38-MAPK and JNK signaling pathways

To assess the role of p38-MAPK and JNK signaling pathways in MIRI and explore the protective mechanism of diosgenin, p38-MAPK and JNK phosphorylation were detected by immunoblotting. As shown in Figure 6, phosphorylation of p38-MAPK and JNK pathways were evidently increased in I/R group, while diosgenin pretreatment significantly inhibited their activation.

Discussion

In the present acute MIRI model, changes in hemodynamics and myocardial enzymes suggested diosgenin markedly improved systolic and diastolic function of the left ventricle *in vivo*, similar to previous *in vitro* results [16]. LVEDP and LVDP

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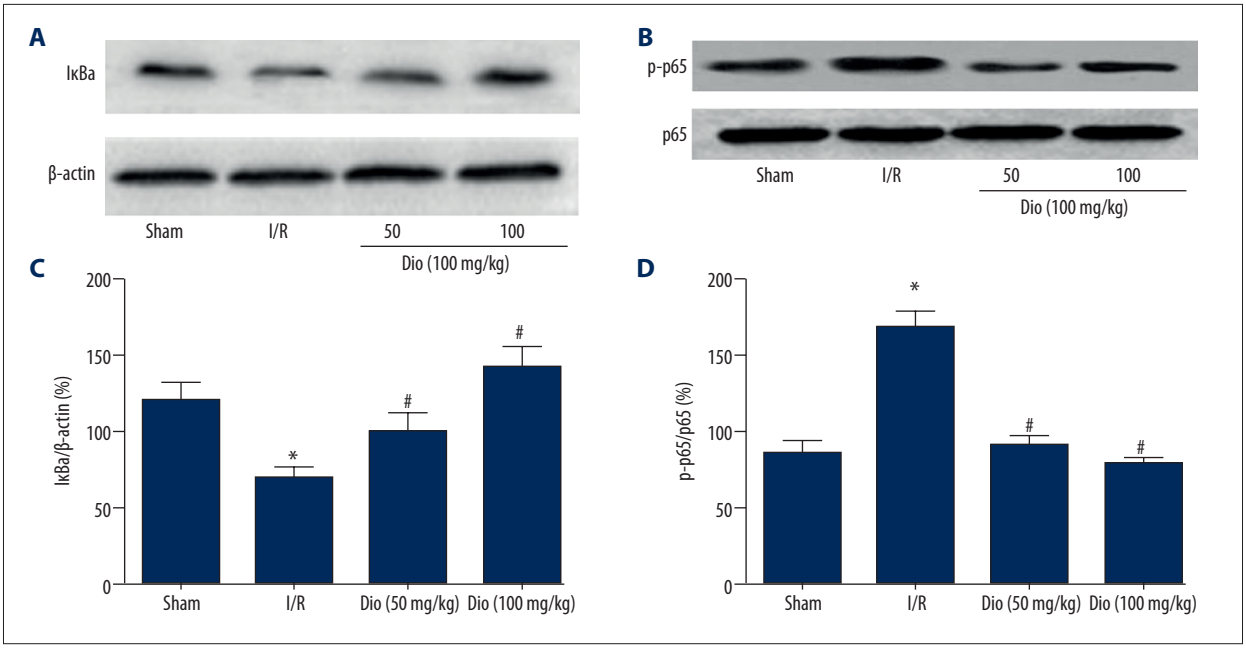

Figure 5. Diosgenin inhibits phosphorylation of NF-κB signaling pathway in I/R rats. (**A**) Effect of diosgenin on I/R-induced decreased of IκBa. (**B**) Effect of diosgenin on I/R-induced up-regulated ratio of p-p65/p65. (**C, D**) Quantitative analysis of IκBa and p-p65 were performed after 3 replicates. Data represent means ±SD. * $p<0.05$ compared to sham group. # $p<0.05$ compared to I/R group.

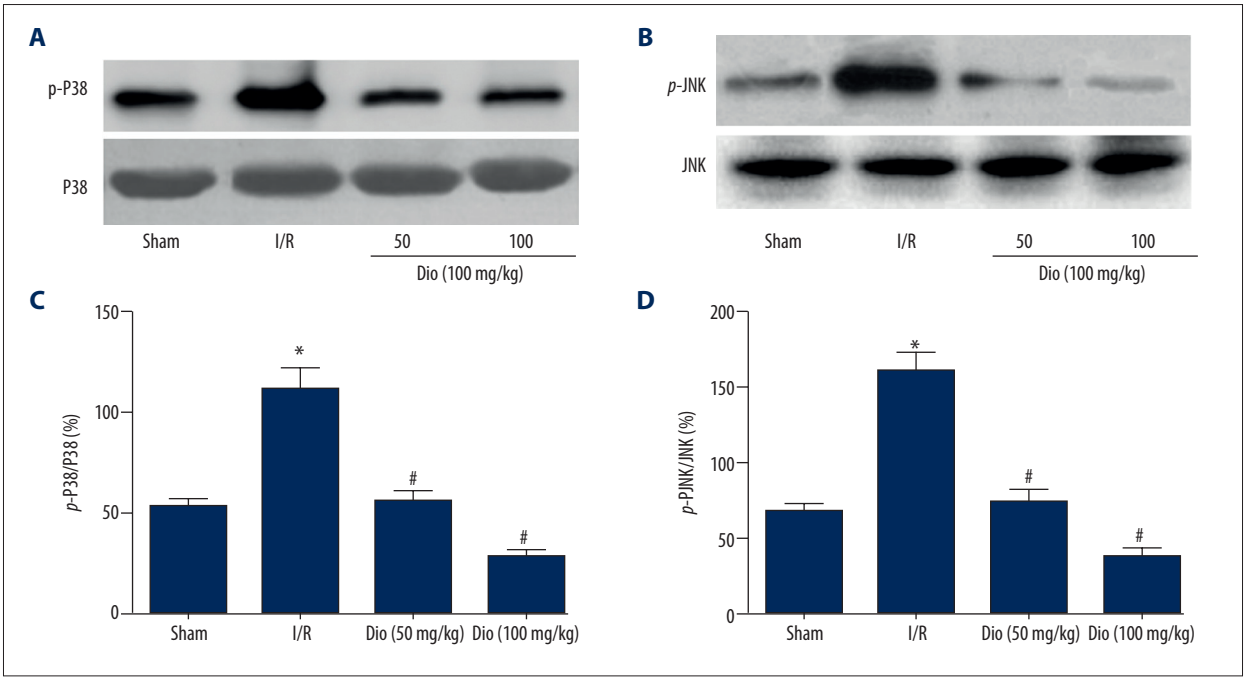

Figure 6. Diosgenin regulates the activation of p38-MAPK and JNK signaling pathways. (**A**) Effect of different dosages of diosgenin on phosphorylation of p38-MAPK. (**B**) Effect of different dosages of diosgenin on phosphorylation of JNK. (**C, D**) The phosphorylation of p38 protein and JNK protein relative quantification, each protein repeated 3 times. Data represent means ±SD. * $p<0.05$ compared to sham group. # $p<0.05$ compared to I/R group.

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are important indicators of left ventricular diastolic function, and $\pm dp/dt_{max}$ reflects left ventricular systolic and diastolic velocities, which is influenced by the efficiency of left ventricular cardiomyocytes. Elevated LVEDP can cause a decrease in left ventricular filling pressure, which in turn affects the left ventricular systolic preload. The present study shows that diosgenin improves stunning myocardial and pumping efficiency of left ventricle by decreasing LVEDP and increasing $\pm dp/dt_{max}$ during reperfusion, particularly in 120-min reperfusion.

Serum proinflammatory factors were significantly elevated after reperfusion and diosgenin demonstrated anti-inflammatory properties [22], resulting in a decrease in the level of serum inflammatory markers TNF-α and IL-1β (Figure 4A, 4B). Lysosomal enzyme MPO is involved in diseases such as inflammation, vasculitis, and atherosclerosis [23]. Studies have shown that MPO reflects neutrophil polymorphonuclear leukocyte (PMN) function and activity status. Accumulation of PMN not only damages vascular endothelial cells and tissue morphology, but also induces respiratory burst, leading to lipid peroxidation [24]. In this study, diosgenin decreased the value of MPO after reperfusion, and reduced inflammation by inhibiting the infiltration of PMN to improve cardiac function. The improved pathological morphology and reduced myocardial infarct size demonstrated the protective effect of diosgenin on injured myocardium. This suggests the anti-inflammatory effects of diosgenin act through the suppression of proinflammatory mediators and PMN infiltration during myocardial I/R.

NF-κB plays an essential role in the gene expression of cytokines, such as TNF-α and IL-1β [25], and is activated in myocardial I/R models [16,17]. IκBa protein and phosphorylated p65 were detected by Western blotting, and the down-regulated expression of IκBa in the I/R group suggests increased degradation of IκBa and dissociation of p65 with IκBa. When pretreated with diosgenin, the separation of IκBa with NF-κB and the phosphorylation of p65 were significantly inhibited. These data demonstrate that diosgenin alleviates MIRI by inhibiting NF-κB signaling pathways and downstream inflammatory cytokines.

Mitogen-activated protein kinase p38 is important in regulating cell signaling pathways. It participates in IRI by exacerbating inflammation and metabolic disorders and promoting apoptosis [26]. Sun et al. found that the activation of p38-MAPK and JNK pathways is closely related to inflammatory injury and apoptosis in a cardiomyocytes hypoxia/reoxygenation model [27]. Gao et al. confirmed that diosgenin can attenuate LPS-induced acute lung injury through p38-MAPK and NF-κB signaling pathways [28]. In the present study, the expression of p38-MAPK and JNK pathways in each group by immunoblotting was examined and we found similar results. Similarly, diosgenin oral administration inhibited the phosphorylation of both in I/R injury. Although the present results support the activation of the p38-MAPK pathway to exacerbate I/R injury, it has been shown that p38-MAPK activation in different environments has a protective effect on the heart. This may be explained by the different roles isomers play [29], but more research is needed.

Conclusions

In the present study, administration of *in vivo* diosgenin improved left ventricular function and reduced myocardial infarct size after I/R. Diosgenin suppressed the inflammatory response and infiltration of PMN by decreasing proinflammatory factor and MPO expression. Diosgenin inhibited the phosphorylation of NF-κB induced by activation of p38-MAPK and JNK, thereby alleviating the cascade of inflammation in I/R. These results suggest that diosgenin reduces inflammatory damage by regulating p38-MAPK and JNK signaling pathways and could play a role in the treatment of MIRI.

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