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Protective effect of notoginsenoside R1 in a rat model of myocardial ischemia reperfusion injury by regulation of Vitamin D3 upregulated protein 1/NF-κB pathway

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The aim of this study was to investigate the protective effects of notoginsenoside R1 (R1) in the rat model ofmyocardial ischemia reperfusion injury and the possible mechanisms. Myocardial ischemia/reperfusion injury (MIRI) was induced by ischemia for 30 min and reperfusion for 60 min. Fifty male SD rats (250-300 g), were randomly divided into 5 groups: sham, model, R1 (20 mg/kg, 40 mg/kg, 60 mg/kg). The activities of serum lactate dehydrogenase (LDH), creatine kinase (CK), myeloperoxidase (MPO), total superoxide dismutase (T-SOD), and malondialdehyde (MDA)were determined after 60 min of reperfusion. Interleukin-1β (IL-1β), interleukin-8 (IL-8) and tumor necrosis factor (TNF)-α were evaluated by enzyme-linked immunosorbent assay (ELISA), VitaminD3 Upregulated Protein 1 (VDUP1), IκB α, P-IκB α, NF-κBP65, pNF-κBP65 were measured by western blotting. Our study demonstrated that R1 can ameliorate the impaired mitochondrial morphology and oxidation system; IL-1β, IL-8 and TNF-α were recovered. Western blotting studies demonstrated that R1 substantially inhibited p-IκBα, NF-κBP65, p- NF-κBP65 protein levels and increased VDUP1 protein level. These findings suggest that R1 may effectively ameliorate the progression of 1/R injury and could be used as a therapy for patients with myocardial ischemia/reperfusion injury.

1. Introduction

Myocardial ischemia caused by coronary heart disease is a major cause of death. Currently, reperfusion therapy is the most effective way of treating acute myocardial infarction (Rodríguez-Sinovaset al. 2007). However, myocardial ischemia/reperfusion injury (MIRI) is an important factor affecting reperfusion treatment.Myocardial ischemia/reperfusion (MI/R) injury is an injury that is induced when blood returns to transiently ischemic myocardial tissues. It is associated with microvascular dysfunction including impaired endothelium-dependent dilation in arterioles, enhanced fluid filtration and leukocyte plugging in capillaries (Armstrong 2004). MI/R is a complicated pathological process, the mechanism of which remains unclear. MI/R injury poses great harm to patients. It may lead to a decline in cardiac function and necrosis of myocardial tissue in ischemic areas, particularly in hypertensive patients.
VDUP1 is a multifunctional 46-kDa protein that was initially isolated in B16 melanoma cells and HL-60 leukemia cells, and its expression is up-regulated by vitamin D3 administration (Chung et al. 2006). VDUP1 directly interacts with the catalytic active center of thioredoxin (Trx). The ubiquitous location of TRX implies its multiple roles as a biological regulator. These studies have shown that it is considered to increase the vulnerability of cells to oxidative stress (Junn 2000). In addition, it plays an important role in lipid metabolism. Moreover, VDUP1 has also been related to up-regulation by various stresses, including H_2O_2, ROS production, irradiation, heat shock, and serum starvation (Pan and Dong 2015). Although deficits in antioxidants and increased oxidative stress accompanying myocardial infarction have been directly implicated in the pathogenesis of post-infarct heart failure, a possible role for VDUP1 in the adverse outcomes accompanying myocardial ischemia has not yet been investigated.
Panax notoginseng, also known as sanchi ginseng, is famous in China and other countries for its obvious the rapeutic effects on the cardiovascular system (Ng 2010). Notoginsenoside R1 (R1), the unique saponin solely contained in *Panax notoginseng*, is known to possess anti-inflammatory, anti-oxidative and anti-ischemia-reperfusion injury of the kidney and intestine as well as LPS-induced septic shock (Sun et al.2012; Zhang 1997). Hence, we hypothesized that R1, which has protective effects in myocardial ischemia/reperfusion injury *in vivo*, effects might also be associated with the inhibition of inflammation and anti-oxidative effects. The present study was under taken to evaluate the protective effects of R1 and to elucidate the mechanism underlying these protective effects in rats.

2. Investigations and results

2.1. Effect of R1 on the myocardial infarction size of hearts

To evaluate the direct effect of R1 onmyocardial I/R injury, TTC staining was used to analysis the infarct size (Fig. 1). In the I/R group, the infarct size was increased to 7.5 fold compared with

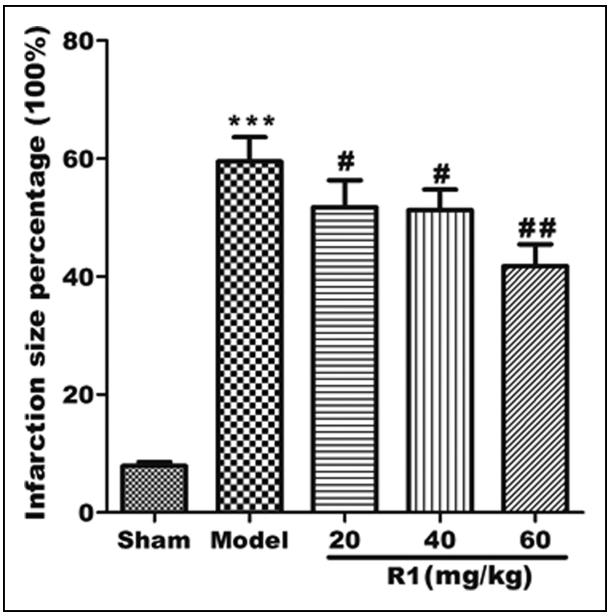

Fig. 1: Effects of R1 on myocardial infarct size. TTC staining to assess the extent of myocardial necrosis. Bars represent the percent of ischemic area at risk in hearts. Values are means ± SEM, n = 6 per group. ***P < 0.001vs. sham; #P < 0.05, ##P < 0.01 vs. model group.

the sham group. In contrast, this effect was abolished by R1 treatment, particularly at the high dose.

2.2. Myocardial ultra structure

The ultrastructure of cardiac muscle cells in each condition were examined, the representative electron micrograms are presented in Fig. 2. In the sham group, cardiac myofibrils stood regularly arranged with well-preserved myofilaments, and mitochondria occupied the cytoplasm between myofibrils with densely packed cristae. The I/R challenge provoked dramatic injury in cardiac muscle cells, as indicated by disrupted myofibrils and ruptured mitochondria. Pretreatment withR1 at each of the three doses attenuated the ultrastructural alterations induced by I/R injury.

2.3. R1 reduces LDH, CK, MPO, T-SOD, MDA release after I/R injury in rats

The measurements of LDH, CK, MPO, T-SOD and MDA are shown in the Table. After 60 min of reperfusion, treatment with R1 at dosages of 20–60 mg/kg resulted in a dose-dependent decrease of LDH, and CK release as compared with the I/R group.

In MDA and T-SOD activity assay, to exclude the possibility that pro-treatment with R1 may affect the lipid peroxidation and oxidative stress in I/R injury, the absorbance was measured MDA level was decreased and T-SOD activity was increased in adose-dependent manner by R1 treatment in I/R. At 60 mg/kg ofR1, T-SOD activity increased and the reductions in CK, LDH, MPO and MDA release were consistent with the decrease.

2.4. Expression of IL-1β, IL-8 and TNF-α following I/R injury

Figure 3 shows the change of serum IL-1β, IL-8 and TNF-α concentrations of all the tested rats. The concentrations of IL-1β, IL-8 and TNF-α were significantly increased (P < 0.001) in the I/R group compared to the sham group. However, pretreatment with R1 effectively antagonized the cytokines release. As expected, the treatment of R1 dose-dependently protected the rats from I/R injury. At the dose of 60 mg/kg, the levels of inflammatory cytokines were mostly close to the sham rats. Serum IL-1β, IL-8 and TNF-α concentrations decreased to 2.4, 1.5, 2.5 folds, respectively.

2.5. Effect of R1 on VDUP1/NF-κB pathway

The expression of VDUP1/NF-κB pathway proteins changed in myocardial tissue. As shown in Fig. 4, the protein expression of p-IκBα, NF-κBP65, p- NF-κBP65 protein levels in model group were significantly increased (P < 0.05) compared with the sham group. After treatment with R1(40 and 60 mg/kg), the phosphorylation levels of IκBα, NF-κBP65 were significantly decreased compared to the model group. Compared with the sham group, VDUP1 protein expression in the model group was significantly decreased (P < 0.01). InR1, the VDUP1protein level was significantly increased compared to the model group (P < 0.05).

3. Discussion

Ischemic heart disease remains the most common cause of death in the industrialized world, (Barski et al. 2007). Further, reperfusion therapy, which promotes the rapid recovery of blood flow to the myocardial ischemic zone, may result in further complications such as diminished cardiac contractile function and irreversible tissue necrosis, which are collectively known as I/R injury (Sajkowska et al. 1999). Therefore, reducing reperfusion injury has become one of the focuses of clinical research. In the present study, we demonstrated the protective effect of R1 in the rat model of myocardial ischemia/reperfusion injury, whereas R1 affected the pathway ofVDUP1/NF-κB.

Along with MIRI, inflammatory response and oxidative stress aggravate myocardial injury. Lipid peroxidation refers to oxidative reduction of lipids (Berg et al. 2005). In this process, free radicals steal electrons from the lipids in cell membranes resulting result cell damage. In the I/R group, significantly increased levels of MDA indicated severe oxidative damage caused by ischemia/reperfusion. Following reperfusion, CK shows an early and rapid rise to a high maximum value with rapid normalization. LDH is a late release following myocardial infarction. LDH and CK are serum biomarkers reflecting myocardial damage (Hou et al. 2015). In our study, R1 dose-dependently lowered the increased levels of MDA, LDH and CK, especially at 60 mg/kg dose. The effects were dose related and higher doses contributed to oxidative stress. Moreover, R1 enhanced the activity of the antioxidase level of T-SOD, compared with the I/R group. Taken these results together with the experimental data, it was suggested the protective ability of R1 against ischemia/reperfusion injury *in vivo* by mediating reactive oxygen species. Moreover, the inflammatory response during I/R injury is exacerbated by production of inflammatory mediators, such as TNF-α and IL-1β. Here, we determined the levels of IL-1β, IL-8 and TNF-α in serum. However, R1-treated rats showed significantly lower levels of cytokines compared to the model group. R1 has been widely studied for its strong anti-inflammatory properties in rats. The present study was carried out at three different doses of R1 (20, 40 and 60 mg/kg body weight). All doses have shown potential protective effects. However, the 60 mg/kg dose level showed better response on anti-oxidant and anti-inflammatory parameters.

To further characterize the mechanism of R1 protective effect on MIRI rats, we examined the effects of R1 on the activation of the VDUP1/NF-κB signaling pathways. NF-κB is the critical transcription factor needed to express genes associated with a proinflammatory response. As can be seen, the level of

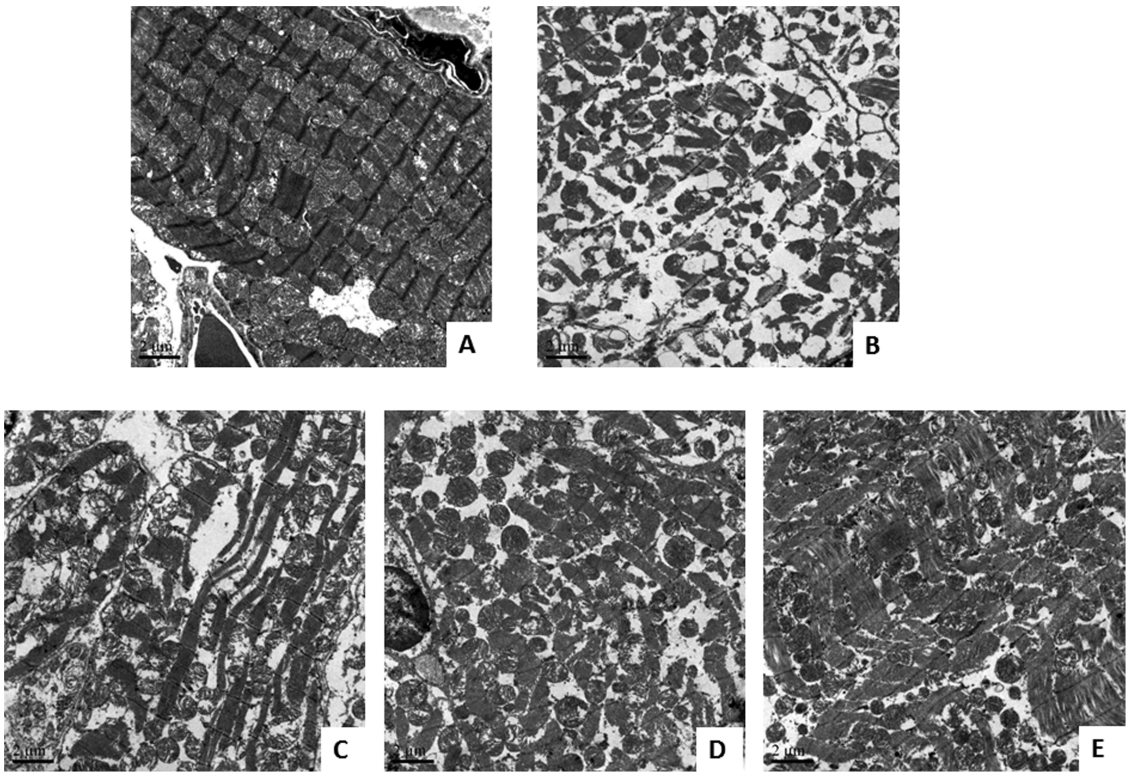

Fig. 2: Myocardial ischemia was induced by LAD ligation. Tissues of the surrounding infarction areas of the left ventricle were stained and observed under a transmission electronic microscope. (A) Sham-operated group: cardiac myofibrils stood regularly arranged with well-preserved myofilaments, and mitochondria occupied the cytoplasm between myofibrils with densely packed cristae. (B) Model group: the myocardial fiber was disordered, even dissolved. The mitochondria were enlarged, even exhibiting vacuolar degeneration. (C 20 mg/kg, D 40 mg/kg, E 60 mg/kg) R1-treated group: the ultrastructural changes of myocytes were extensively attenuated. Mitochondria and myocardial fiber showed slight edema. A–E was 5000 ×, bar = 2 μm.

Table: Effect of R1 on the biochemical parameters in serum of rats with ischemia/reperfusion myocardial injury

Group	Dose(mg/kg)	n	CK(U/ml)	LDH(U/ml)	T-SOD(U/ml)	MDA(μM/ml)
Sham	—	6	1.01 ± 0.18	0.11 ± 0.02	50.78 ± 3.65	5.54 ± 2.01
Model	—	6	$1.98 \pm 0.35^{**}$	$0.15 \pm 0.03^{**}$	$46 \pm 2.24^{***}$	$11.21 \pm 3.68^{**}$
R1	20	6	1.61 ± 0.21	0.14 ± 0.02	$48.79 \pm 3.22^{\#\#}$	$6.88 \pm 3.45^{\#}$
	40	6	$1.45 \pm 0.23^{\#}$	$0.12 \pm 0.04^{\#}$	$50.12 \pm 2.25^{\#\#}$	$5.77 \pm 1.78^{\#\#}$
	60	6	$1.34 \pm 0.17^{\#\#}$	$0.12 \pm 0.03^{\#}$	$51.09 \pm 3.10^{\#\#\#}$	$5.52 \pm 2.62^{\#\#}$

Rats in R1 treated groups were pretreated with intragastric administration of R1 for 5 days prior to the myocardial ischemia/reperfusion. LDH, CK, and T-SOD activities and MDA levels in serum werespectrophotometrically determined. Data were expressed as the mean ± SEM (n = 6). **P < 0.01, ***P < 0.001 vs. sham; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. model group.

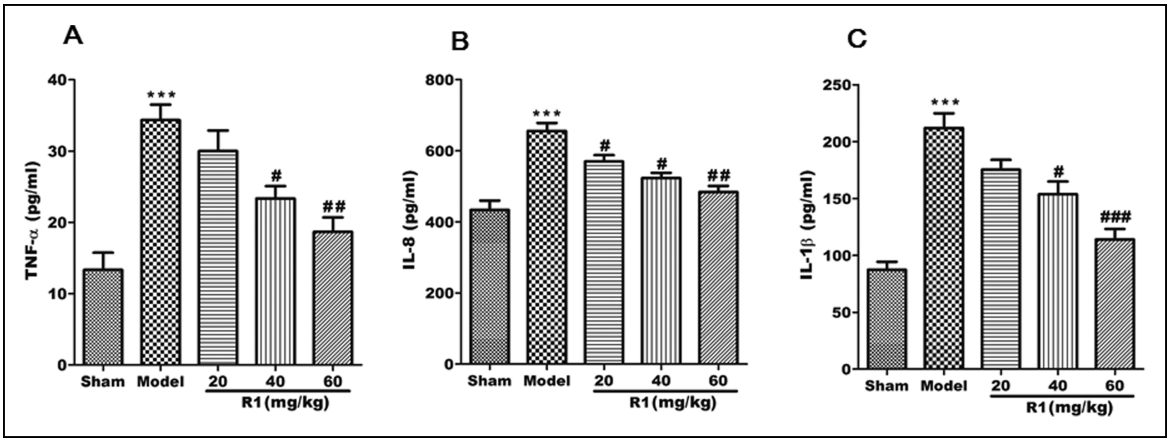

Fig. 3: Effect of R1 on inflammatory cytokine production in the serum. The levels of TNF-α, IL-1β and IL-8 were measured by ELISA kits. Data represent the means ± SEM in each group (n = 6), **p < 0.01 compared with the sham group; #p < 0.05, ##p < 0.01 compared with the model group.

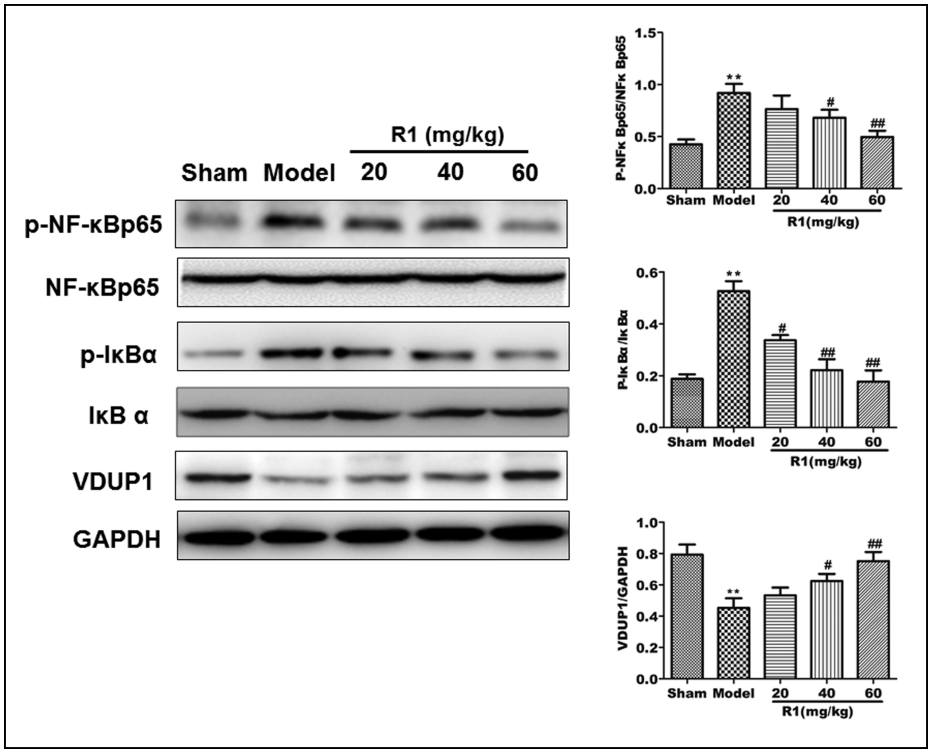

Fig. 4: The protein levels of p-IκBα, IκBα, NF-κB, p-NF-κB and VDUP1 in rat myocardial tissue after 5 days treated were detected by western blot. Data represent the means ± SEM in each group (n = 3), **p < 0.01 compared with the control group; #p < 0.05, ##p < 0.01 compared with the model group.

VDUP1was basically recovered to normal values after R1 treatment in the 60 dose group. The levels of phosphorylation of NF-κB P65 and IκBα were markly increased in the I/R group, and administration of R1 impairs phosphorylation of these molecules in a dose-dependent manner. The presented results clearly demonstrate that R1 significantly regulated VDUP1/NF-κB pathway. Our findings support the possible use of R1 as a therapeutic drug for patients with I/R injury.

4. Experimental

4.1. Materials

R1 (pure: 95%) was purchased from National Institutes for Food and Drug Control (Beijing, China). ELISA kits were purchase d from ((R&D, Minneapolis, MN, USA). Superoxide dismutase (SOD), malondialdehyde (MDA) and lactate dehydrogenase (LDH) kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). IL-1β, IL-8 and TNF-α Elisa kits were from R&D Systems Inc. (Minneapolis, MN, USA).

4.2. Animals

Fifty male Sprague-Dawley rats weighing 250-300 g were acquired from Shanghai Slac Laboratory Animal Ltd (Shanghai, China). The animals were maintained under standard laboratory conditions at 22 ± 2 °C and a relative humidity of 55 ± 5 % and had free access to food and water under a natural day/night cycle. Rats were acclimated for 7 days before any experimental procedures. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of China.

4.3. Experimental design

The I/R injury animal model was established by occlusion of the left anterior descending coronary artery (LAD) for 30 min followed by 1 h reperfusion (Yu et al. 2015). Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg), ventilated with a positive pressure respirator at a stroke volume of 12 ml/kg and a rate of 60 strokes per minute with 95% O_2 and 5% CO_2. A left parasternal incision was made through the third and fourth ribs, and the pericardium was then gently opened to expose the heart. A 6–0 silk suture slipknot was placed at the distal 1/3 of the left anterior descending artery. In addition, a small glass tube was placed between the ligature and myocardial tissues. For the ischemia injury, the coronary artery was occluded by tightening the tension. After 30 min of ischemia, the slipknot was released, and the myocardium was reperfused for 1 h. Sham-operated animals underwent the same surgical procedures except that the suture around the LAD was not ligatured. In the R1 + I/R group, R1dissolved in normal saline was given through intra gastric for 5 days before operation, with the concentration of 20, 40, and 60 mg/kg respectively for the three R1 pretreatment groups. 50 animals were randomly divided into the sham, I/R, R1 (20 mg/kg) + I/R, R1 (40 mg/kg) + I/R, and R1 (60 mg/kg) + I/R, with 10 animals in each group.

4.4. Measurement of myocardial infarct size

Myocardial infarct sizes were evaluated by tetrazolium chloride (TTC) staining. After reperfusion, the hearts were immediately removed and sectioned into 5 mm thick short-axis slices from the apex towards the base of the heart. The slices were incubated in 1% TTC (pH 7.4) at 37 °C in PBS for 15 min and then photographed with a digital camera (Canon, Japan). Red parts in the heart, which were stained by TTC, represented ischemic but viable tissue. White part areas were defined as the infarcted myocardium. Areas of infarct size were measured digitally using Image Pro Plus software.

4.5. Myocardial ultrastructure

Fresh myocardial tissues of rats were excised from the surrounding infarction areas of the left ventricle after reperfusion. Tissues were fixed in 2.5% glutaric dialdehyde and post-fixed with 1% osmium tetroxide. The specimens were processed for ultrathin sections. The sections were stained with uranium acetate and lead citrate, and the ultrastructural changes of myocardial tissue were then examined in a Hitachi H-7560 (Tokyo, Japan) transmission electron microscope. In each group, specimens were taken from six hearts and for each heart six tissue blocks were obtained.

4.6. Measurement of creatine kinase (CK) and lactate dehydrogenase (LDH) release

To test for CK and LDH activity, 2 mL of femoral veinblood was drawn and centrifuged at 3,000 rpm for 15 min. The samples were analyzed by colorimetry according to the manufacturer's instructions. The activities of these enzymes were expressed in U/L.

4.7. Determination of serum total superoxide dismutase (T-SOD), malondialdehyde (MDA)

Detection of the T-SOD activity at 550 nm and MDA content was quantified by thiobarbituric acid assay with 1,1,3,3-tetramethoxypropane as an external standard at 532 nm according to the manufacturer's instructions. Each measurement was performed in duplicate.

4.8. Serum concentrations of IL-1β, IL-8 and TNF-α

Blood was collected 60 min after I/R and the serum was extracted. The concentrations of IL-1β, IL-8 and TNF-α in the serum were determined using the respective ELISA kits according to the manufacturers' instructions.

4.9. Western blotting ofVDUP1/NF-κB pathway

Myocardial tissue sample were processed for Western blot analysis. An equal amount (50 μg) of protein was subjected to 10% SDS-PAGE gel, and transferred onto a PVDF membrane in a semi-dry system (Bio-Rad, USA). The blots were incubated with the appropriate concentration of specific anti body. After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated second antibody. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Millipore Co., Bedford, USA). The band intensities were quantified using the ChemiDoc^{TM} MP System (Bio-Rad, Hercules, USA).

4.10. Statistical analysis

The results were presented as mean ± SEM. All data were statistically analyzed with SPSS 11.0 statistical package for Windows version. The means among groups were compared using one-way ANOVA, followed by Student–Newman–Keuls's post hoc test. Statistical significance was set at $P < 0.05$.

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References

Armstrong SC (2004) Protein kinase activation and myocardial ischemia/reperfusion injury. Cardiovasc Res 61: 427–436.

Barski L, Porath A, Novack V, Jotkowitz A (2007) Prevalence, recognition and treatment of cardiovascular risk factors in outpatients with atherothrombosis in Israel. Israel Med Assoc J 9: 376–379.

Berg K, Jynge P, Bjerve K, Skarra S, Basu S, Wiseth R (2005) Oxidative stress and inflammatory response during and following coronary interventions for acute myocardial infarction. Free Radical Res 39: 629–636.

Chung J W, Jeon J, Yoon S, Choi I (2006) Vitamin D 3 upregulated protein 1 (VDUP1) is a regulator for redox signaling and stress-mediated diseases. J Dermatol 33: 662–669.

Hou X, Han J, Yuan C, Ren H, Zhang Y, Zhang T, Xu L, Zheng Q, Chen W (2015) Cardioprotective effects of total flavonoids extracted from xinjiang sprig Rosa rugosa against acute ischemia/reperfusion-induced myocardial injury in isolated rat heart. Cardiovasc Toxicol Doi:10.1007/s12012-015-9308-0.

Junn E (2000) Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function. J Immunol 164: 6287–6295.

Ng TB (2010) Pharmacological activity of Sanchi Ginseng (Panaxnotoginseng). J Pharm Pharmacol 58: 1007–1019.

Pan CL, Dong ZW (2015) Antiasthmatic effects of eugenol in a mouse model of allergic asthma by regulation of Vitamin D3 Upregulated Protein 1/NF-κB Pathway. Inflammation 38: 1385–1393.

Rodríguez-Sinovas A, Abdallah Y, Piper HM, Garcia-Dorado D (2007) Reperfusion injury as a therapeutic challenge in patients with acute myocardial infarction. Heart Failure Rev 12:207–216.

Sun B, Xiao J, Sun XB, Wu Y (2013) Notoginsenoside R1 attenuates cardiac dysfunction in endotoxemic mice: an insight into estrogen receptor activation and PI3K/Akt signaling. Brit J Pharmacol 168: 1758–1770.

Sajkowska A, Wykretowicz A, Szczepanik A, Kempa M, Minczykowski A, Wysocki H (1999) Fibrinolytic therapy and n-acetylocysteine in the treatment of patients with acute myocardial infarction: its influence on authentic plasma hydroperoxide levels and polymorphonuclear neutrophil oxygen metabolism. Cardiology 91: 60–65.

Yu D, Li M, Tian Y, Liu J, Shang J (2015) Luteolin inhibits ROS-activated MAPK pathway in myocardial ischemia/reperfusion injury. Life Sci1 22:15–25.

Zhang WJ (1997) Notoginsenoside R1 counteracts endotoxin-induced activation of endothelial cells in vitro and endotoxin-induced lethality in mice in vivo. ArteriosclerThrombVasc Biol 17: 465–474.