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Original Article

Garlicin Post-conditioning Suppresses Adhesion Molecules in Porcine Model of Myocardial Ischemia-Reperfusion Injury*

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ABSTRACT **Objective**: To evaluate whether garlicin post-conditioning can attenuate myocardial ischemia-reperfusion injury in a catheter-based porcine model of acute myocardial infarction (AMI) by affecting adhesion molecules integrin β1/CD29 and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31). **Methods**: Twenty-two swine were devided into 3 groups: 6 in a sham-operation group, and 8 each in the model and garlicin groups. AMI porcine model was established in the model and garlicin groups. The distal parts of the left anterior descending coronary artery in the animals of the model and garlicin groups were occluded by dilated balloon for 2 h, followed by reperfusion for 3 h. Garlicin (1.88 mg/kg) was injected over a period of 1 h, beginning just before reperfusion, in the garlicin group. Real-time polymerase chain reaction, immunohistochemistry and Western blot were carried out to detect mRNA and protein expressions of CD29 and CD31 3 h after reperfusion. **Results**: Hematoxylin-eosin staining showed a better myocardial structure in the garlicin group after reperfusion. Compared to the model group, garlicin inhibited both the mRNA and protein expression of CD29 and CD31 in reperfusion area and no-reflow area (both $P<0.05$). **Conclusions**: Garlicin post-conditioning induced cardio-protection against myocardial ischemia-reperfusion injury in this catheter-based porcine model of AMI. The cardio-protective effect of garlicin is possibly owing to suppression of production of CD29 and CD31, by inhibition of the mRNA expression of CD29 and CD31.

KEYWORDS garlicin, reperfusion injury, myocardial infarction, CD29, CD31

In clinical practice it is quite well-established that early and effective myocardial reperfusion, using either thrombolysis or primary percutaneous coronary intervention (PCI), helps in limiting myocardial infarct size, preventing left ventricular remodelling, preserving left ventricular systolic function and improving clinical outcomes in patients presenting with acute myocardial infarction (AMI). However, there is possibility that reperfusion, in itself, can lead to myocardial cell death through a process known as reperfusion injury, which comprises of apoptosis, necrosis, and autophagy. Reperfusion injury can be manifested by arrhythmias, transient mechanical dysfunction of the heart (myocardial stunning), micro-vascular injury and "no-reflow", as well as various inflammatory responses. In order to improve clinical outcomes in patients with AMI, novel therapeutic strategies are required to protect the myocardium from acute ischemia-reperfusion (I/R) injury and preserve cardiac function. Pharmacological post-conditioning may provide novel approaches for protecting the heart in acute myocardial I/R injury.[1]

Garlicin is a chemical compound extracted from garlic (*Allium sativum* Linn.) in which diallyltrisulfide is a principal constituent. Studies have shown that garlic is beneficial for persons suffering from cardiovascular diseases. It has several cardio-protective and pro-circulatory properties, such as hypo-lipidemic, anti-oxidation, antiplatelet, fibrinolytic, antihypertensive, hypoglycemic, antithrombotic, and anti-atheromatic effects.[2-7] In a previous study, we observed that garlicin could attenuate reperfusion no-reflow in our catheter-based porcine model possibly through lowering of serum pro-inflammatory cytokines and endothelin-1.[8,9] Although various mechanisms have been postulated to describe the cardio-protective effect of garlicin, there is still a need for further research to identify the specific molecular pathway of

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cardio-protection in cardiac diseases.

The objective of the present study is to evaluate whether garlicin post-conditioning has an effect on myocardial adhesion molecules integrin β1/CD29 and platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) in our catheter-based porcine model of AMI.

METHODS

AMI Model and Animal Grouping

The animal experiments undertaken in the current study were approved by the Animal Care and Use Committee of China-Japan Friendship Hospital, Beijing, China and were in compliance with the regulations of "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (Publication No. 85–23, revised in 1996). Twenty-two male Chinese mini swine, weighing 22±3 kg, procured from China Agricultural University were randomized into 3 groups: 6 in the sham-operation group, and 8 each in the model and garlicin groups. Our porcine model of AMI was modified on the basis of a previous study by Suzuki, et al.[10] Detailed steps referred to our previously published article.[8] Garlicin (Shanghai Harvest Pharmaceutical) infusion at a dosage of 1.88 mg/kg body weight was started just prior to beginning of reperfusion, and continued for 1 h, in the garlicin group. Saline was used instead of garlicin in the model group.

Pathological Staining

Double-staining with Evans blue dye and thioflavin-S was performed to delineate reperfusion area (RA) and no-reflow area (NRA). Three hours after reperfusion, 1 mL/kg thioflavin-S was injected into the left ventricle. The reperfusion region was stained but not the no-reflow region. After another complete occlusion of left anterior descending (LAD) by dilated balloon, Evans blue was injected into the left ventricle and normal myocardium was stained. Animals were then sacrificed with an intravenous bolus injection of potassium chloride. The hearts of the slain animals were then excised and the left ventricles were sectioned into 10 mm-thick cross-sectional myocardial slices. Among the myocardial slices, the cross-section at the papillary muscle level was chosen for analysis. Normal area, RA and NRA were then fixed using 10% formalin and embedded in paraffin for histopathological examination by hematoxylin-eosin (HE) staining.

Real-Time Polymerase Chain Reaction for mRNA Expression of CD29 and CD31

Three hours after reperfusion, swine hearts were excised and the left ventricles were sectioned into 10-mm-thick cross-sectional myocardial slices. Among the myocardial slices, the cross-section at the left ventricular papillary muscle level was chosen. Normal area, RA and NRA were then frozen and crushed in liquid nitrogen. Total RNA was then extracted with RNA extraction kit (TaKaRa, Japan) as per the manufacturer's guidelines and quantified by absorbance at 260 nm. The reaction volume of polymerase chain reaction (PCR) was set at 10 μL, while the reaction temperature, for a total of 40 cycles, was fixed at 95 ℃ for 5 s and 60 ℃ for 30 s. Primer sequences used for this procedure are depicted in Table 1. β-Actin was used as the internal standard. PCR amplification was performed in a volume of 10 μL containing 2 μL of the cDNA template, 2×SYBR® Premix Ex TaqTm (TaKaRa, Japan) 5 μL, primers (5 μmol/L, forward and reverse each, Invitrogen, USA) and RNase free dH_2O 1 μL.

Immunohistochemical Staining for CD29 and CD31

Three hours after reperfusion, swine hearts were excised as before, and the left ventricles were sectioned into 10-mm-thick cross-sectional myocardial slices. For this procedure, cross-sectional myocardial slices at the level of left ventricular papillary muscles

Table 1. Primer Sequences Used for Real-Time PCR

Gene	Direction	Sequence	Length (bp)	Genebank No.
β-Actin	Forward primer	TTCCAGCAGATGTGGATCAGC	70	DQ-845171
	Reverse primer	AGCATTTGCGGTGGACGAT		
CD29	Forward primer	CTGGTGTGGTTGCTGGAATTG	53	NM-213968
	Reverse primer	CCAAATGAGCAGCAGTGCAAG		
CD31	Forward primer	CGCGGTATTCAAAGATAACCCA	67	NM-213907
	Reverse primer	GGAATGGCAATTATCGGCG		

were chosen. Normal area, RA and NRA were then fixed using 10% formalin and embedded in paraffin. Tissue sections (5 μm) were deparaffinized by xylene, followed by re-hydration with graded ethanol series. Samples were then subjected to 0.1% Triton X-100 for permeability. The endogenous peroxidase activity was subdued by treating with 3% hydrogen peroxide for 10 min. Antigen was retrieved in tris-ethylene diamine tetraacetic acid buffer (20×, pH 9.0), heated in a microwave oven at 92–98 ℃ for 8 min. After being rinsed thrice in phosphate-buffered saline (PBS), the sections were incubated overnight at 4 ℃ with specified primary antibodies: mouse antibody CD29 (1:500, BD Bioscience, USA) or CD31 (1:500, Abcam, UK). The slides were again rinsed 3 times in PBS and incubated with the peroxidase-conjugated anti-mouse antibody (Duko, Denmark) for 2 h. Bound antibody was detected by adding 3-3'-diaminobenzidine (Duko, Denmark). Positive areas were selected and 6 clockwisely selected fields were photographed at a magnification of ×200. The sum of integrated optical density (IOD) and the total positive areas of each group were measured using an image processing software IPP 6.0. IOD to area ratios were calculated as the sum of IOD divided by the total positive area. These measurements were performed thrice and were analyzed by two independent observers who were blinded to the treatment allocation.

Western Blotting Analysis for CD29 and CD31

To identify the protein expression of CD29 and CD31 in the swine myocardium 3 h after reperfusion, Western blot was performed. Swine hearts were excised and the left ventricles were sectioned into 10-mm-thick cross-sectional myocardial slices. Among the myocardial slices, the cross-section at the left ventricular papillary muscle level was chosen. Normal area, RA and NRA (n=5) were then frozen and crushed in liquid nitrogen and protein were extracted using a protein extraction kit (Keygen Biotech, China). After sonication, the lysates were centrifuged, and the proteins were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidenedifluoride membrane (Abcam). After being blocked with 5% skim milk in tris-buffered saline at room temperature for 1 h, the membrane was incubated with primary antibody against integrin-β1/CD29 (CD29: 1:1000; Abcam), platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31: 1:1000; Abcam), or β-actin (1:3000; Abcam) overnight at 4 ℃. Then, the membranes were rinsed with PBS 3 times and incubated with horseradish peroxidase-conjugated IgG antibody (Abcam) for 1 h at 37 ℃. The labeled proteins were visualized with enhanced chemiluminescence reagents and exposed to film with a UVP Bio-Imaging System (Alpha Innotech, USA).

Statistical Analysis

Numerical data were presented as mean ± standard deviation ($\bar{x} \pm s$). Comparisons of parameters among three groups were performed by a one-way analysis of variance (ANOVA). Comparisons of parameters between two groups were performed with unpaired Student's t-test (SPSS, Chicagom IL, USA). Probabilities of P<0.05 were considered statistically significant.

RESULTS

Procedure Success and Mortality

Twenty-six male Chinese mini swine were used for the current study, and among them 4 died during the study. Barring the 6 swine of the sham-operation group, complete occlusion of LAD by dilated balloon was confirmed by coronary angiography (CAG, Figure 1). CAG was performed again to confirm if blood flow at thrombolysis in myocardial infarction (TIMI) 3 level was recovered in LAD after removing the balloons in other 16 swine belonging to other two groups, to ensure successful simulation of reperfusion after AMI.

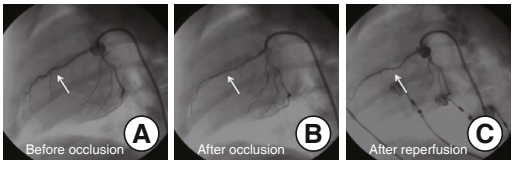

Figure 1. Procedure of AMI and Reperfusion Swine Model

Notes: A: normal LAD of swine model before occlusion. B: LAD was occluded by a dilated balloon. C: LAD was reperfused without balloon and TIMI 3 blood flow was recovered.

Effect of Garlicin Postconditioning on Pathological Changes

By HE staining, myocardium was normal with clear striations in the sham-operation group. No necrosis or neutrophil infiltration was observed (Figure 2A). In contrast, the model group showed local swelling, myocardial necrosis, disorganized myocardial fibers, and ruptured cells with large number of inflammatory cells in the cytoplasm (Figures 2B and 2C). In the garlicin group, myocardial

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cells with normal structure and shape can be seen, though the myocardial fibers were mildly swollen or partially ruptured and slight edema can be seen in the interstitial tissues with small amount of inflammatory cells (Figures 2D and 2E).

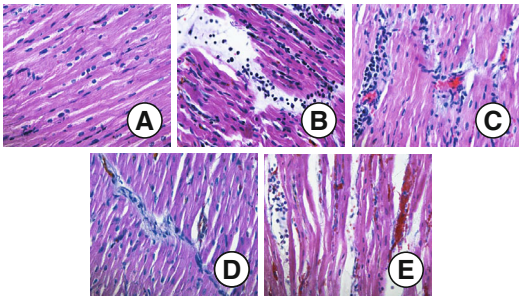

Figure 2. Histological Study by HE Staining of Myocarium from Different Groups (× 400)

Notes: A represents normal area: normal size cardiomyocyte, no hemorrhage, or neutrophil granulocyte infiltration. B and C represent RA and NRA in model group: cardiomyocyte degeneration, hemorrhage, edema, and significant interstitial neutrophil granulocyte infiltration. D and E represent RA and NRA in garlicin group: no significant cardiomyocyte degeneration, slight edema between the myocardial fibers, and mild neutrophil granulocyte infiltration.

Garlicin Decreased mRNA Levels of CD29 and CD31

The mRNA level of CD29 was similar in normal myocardial tissues of the sham-operation and model groups, but increased significantly in RA and NRA in the model group (number of fold increase *vs*. normal myocardium: RA 1.47; NRA 1.97; *P*<0.05 in both, Figure 3). However, after garlicin post-conditioning, CD29 mRNA level of RA and NRA both decreased significantly (number of fold increase *vs*. normal myocardium: RA 1.06; NRA 1.07; *P*<0.05 in both) compared to the model group. Similar findings were also observed for mRNA expression of CD31. Garlicin post-conditioning had an inhibitory effect on mRNA expression of CD31 in RA and NRA (*P*<0.05) compared to the model group.

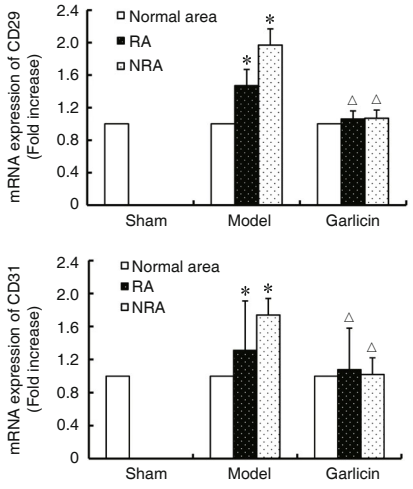

Figure 3. mRNA Levels of CD29 and CD31 in Each Group by Real-Time PCR

Notes: $^{*}P$<0.05 *vs*. sham group; $^{\triangle}P$<0.05 *vs*. model group

Garlicin Decreased Protein Expression of CD29 and CD31

IOD to area ratio of each group was shown by immunohistochemical staining (Figure 4), while the

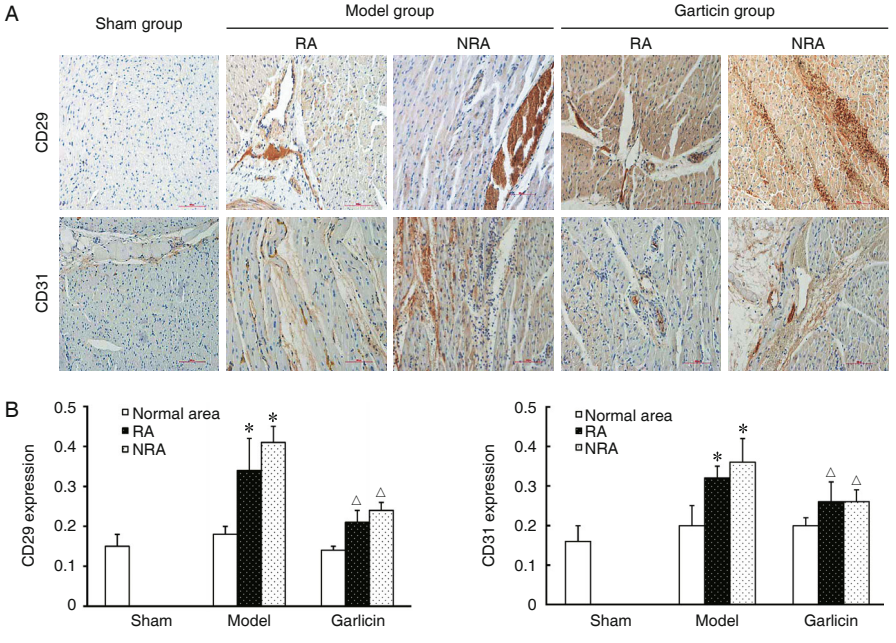

Figure 4. Immunohistochemical Staining for CD29 and CD31

Notes: A: Magnification: × 200; B: $^{*}P$<0.05 *vs*. sham group; $^{\triangle}P$<0.05 *vs*. model group

protein expression of CD29 and CD31 was shown by Western blot (Figure 5). Both of the two methods showed the similar results: compared to the normal myocardium, the protein expression of CD29 and CD31 in RA and NRA in the model group increased significantly (both $P<0.05$), while garlicin reduced the protein expression of CD29 and CD31 in RA and NRA compared to the model group (both $P<0.05$).

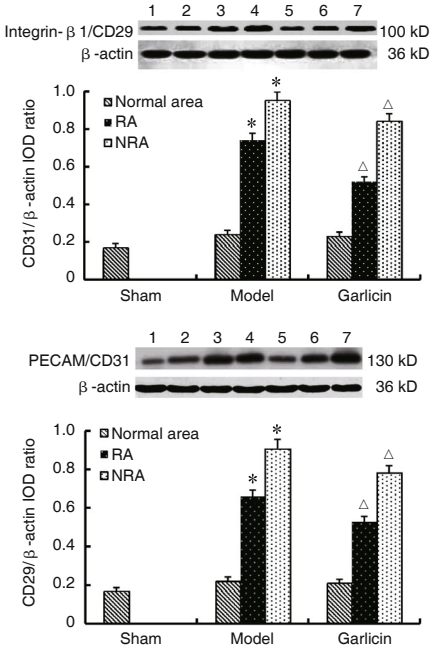

Figure 5. Western Blot Analysis of CD29 and CD31

Notes: 1: CD 29 and CD31 protein expression in sham-operation group. 2–4: CD29 and CD31 protein expression in normal area, RA and NRA of model group respectively. 5–7: CD29 and CD31 protein expression in normal area, RA and NRA of garlicin group respectively. $^{*}P<0.05$ *vs*. normal area, $^{\triangle}P<0.05$ *vs*. model group

DISCUSSION

All of the studies pointed to the fact that garlic reduces cholesterol, inhibits platelet aggregation, reduces blood pressure, and increases antioxidant status.[5] Thus, as the most important one of compounds abstracted from the garlic, garlicin appears to hold promise in reducing parameters associated with cardiovascular disease. In our porcine model of AMI, we observed significant amelioration in myocardial reperfusion injury after garlicin therapy using intravenous of injection, which not only improved left ventricular systolic pressure and left ventricular end diastolic pressure, but also reduced NRA after reperfusion in AMI.[8] Our findings provided evidences on potential benefits of garlicin reducing NRA in microcirculation level during reperfusion.[8] Then ajoene in garlicin has been shown to inhibit *in vitro* platelet aggregation indifferent species of animals.[5] Adhesion molecules are considered to be indicators of activation of leukocytes, platelets and endothelial cells. In patients with acute coronary syndrome, these molecules would increase in ischemic and reperfusion area.[11] It has been shown that patients with high levels of these adhesion molecules have poor prognosis in future. Integrin ligation and signaling are involved in the cardiac hypertrophic response pathway. Overexpression of CD29 has been shown to augment the myocardial hypertrophic response. CD31, an immunoglobulin gene superfamily member, is expressed constitutively on neutrophils and endothelium, which mediates the adhesive events immediately following I/R injury. Blocking CD31 mediated pathway can exert significant protective effect on myocardial I/R injury via blockade of neutrophil accumulation in the myocardium. Inhibition of CD31 interactions via agents that bind to or modulate the function of CD31 may represent a novel adjunctive therapeutic approach in the current treatment protocol for AMI.

The principal findings of this study were that (i) garlicin inhibited both the mRNA and protein expression levels of CD29 and CD31 in RA and NRA after reperfusion and (ii) garlicin post-conditioning played an important role in ameliorating the I/R injury. Therefore garlicin was inferred to suppress production of CD29 and CD31 after myocardial reperfusion, possibly via inhibition of the mRNA expression of CD29 and CD31. Such adhesion molecules as CD29 and CD31 were decreased not to activate the aggregation of leukocytes and platelets in microcirculation. Thus micro-spasm or micro-embolism could not happen in capillaries and the successful reperfusion was protected in microcirculation, which could possibly salvage the risk or stunned myocardium. Experiments involving adhesion molecule receptor blockade and CD29/CD31 knockout animals will, it is anticipated, also yield information concerning the mechanisms by which garlicin produces its cardio-protective actions.

In conclusion, this study was aimed at obtaining additional evidence for the proposition that garlicin post-conditioning could prove valuable in limiting myocardial damage and reducing myocardial I/R injury by affecting adhesion molecules CD 29 and CD31 when administered at pharmacological doses

just before or during reperfusion. However, studies involving human tissue (rather than animal tissues) will provide information which goes further to consolidating our results. In future we will investigate our hypothesis that garlicin may induce cardio-protective mechanism by mediating the expression of CD29/CD31, given its influence on those kinases implicated in reperfusion injury salvage kinases (RISK) or survivor activating factor enhancement (SAFE) pathway. This will be postulated to be an important cardioprotective mechanism of garlicin.

Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions

Li XL designed research; Yang P, Li AL and Li J performed experiments; Yang P and Li JH analyzed data and prepared figures; Li JH drafted manuscript; Li JH, Yang P, Wang Y and Ren SY revised manuscript; Li XL approved final version of manuscript.

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