

Original Article

# Danshensu protects vascular endothelia in a rat model of hyperhomocysteinemia

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**Aim:** To examine whether danshensu could protect vascular endothelia in a rat model of hyperhomocysteinemia.

**Methods:** The model was established by feeding rats with a methionine-rich diet (1 g·kg<sup>-1</sup>·d<sup>-1</sup>) for 3 months. Immediately following the discontinuation of methionine-rich diet, rats were treated with danshensu (67.5 mg·kg<sup>-1</sup>·d<sup>-1</sup>, po) or saline for 3 additional months. One group of rats receiving vitamin mixture (folic acid, vitamin B12 and vitamin B6) was included as a positive control. One group of rats not exposed to methionine-rich diet was also included as a blank control. The expression of tumor necrosis factor-alpha (TNF-alpha) and intercellular adhesion molecule-1 (ICAM-1) protein in the descending aorta was examined using immunohistochemistry and Western blot. Homocysteine and blood concentration of endothelin and nitric oxide (NO) was also examined.

**Results:** Methionine-rich diet resulted in accumulation of “foam cells”, up-regulated expression of TNF-alpha and ICAM-1 in the descending aorta, and significantly increased serum homocysteine. Plasma endothelin concentration was significantly increased; NO was decreased. Danshensu treatment, either simultaneous to methionine-rich diet or afterwards, attenuated the above mentioned changes.

**Conclusion:** Chronic treatment with danshensu could prevent/attenuate the formation of atherosclerosis. Potential mechanisms include inhibited expression of representative proinflammatory cytokines and adhesion molecules in arterial endothelia. Changes in homocysteine and circulating molecules that control vascular contraction/relaxation via endothelial cells (eg, endothelin and NO) were also implicated.

**Keywords:** hyperhomocysteinemia; danshensu; endothelin; nitric oxide; tumor necrosis factor-alpha; intercellular adhesion molecule-1

Acta Pharmacologica Sinica (2010) 31: 1395–1400; doi: 10.1038/aps.2010.167; published online 27 Sep 2010

## Introduction

Hyperhomocysteinemia (elevated circulating level of homocysteine) often occurs in patients with one or more defective genes (eg, methylenetetrahydrofolate reductase) that control the breakdown of homocysteine<sup>[1]</sup>, and is an independent risk factor for atherosclerosis<sup>[2]</sup>. Deficiency of folate, vitamin B6 and B12 could also result in hyperhomocysteinemia<sup>[3]</sup>. High level of homocysteine damages endothelial cells in vasculature via increased formation of reactive oxygen species (ROS) and inflammation, and therefore increases the likelihood to develop heart attack and stroke<sup>[4–7]</sup>. Hyperhomocysteinemia also promotes LDL oxidation and internalization by macrophages, which in turn is the initial step of atherosclerosis<sup>[8]</sup>.

Supplementation of folate, vitamin B6 and B12 could reduce the level of homocysteine in patients with hyperhomocysteinemia<sup>[9]</sup>, but do not affect the process of atherosclerosis directly<sup>[10]</sup>. As a result, the benefit is limited<sup>[11–13]</sup>.

Radix salviae miltiorrhizae (commonly known as “Danshen”) has been used by practitioners of the Traditional Chinese Medicine for decades in the treatment of a variety of cardiovascular diseases. Danshensu [3-(3,4-dihydroxyphenyl)-2-hydroxy-propionic acid], a major active component of Danshen, could improve microcirculation, suppress the formation of ROS, inhibit platelet adhesion and aggregation, and protect myocardium against ischemia<sup>[14–16]</sup>. Danshensu could also protect endothelial cells against injury induced by hyperhomocysteinemia<sup>[17]</sup> or inflammation<sup>[18]</sup>.

Effects of danshensu on homocysteine, TNF-alpha, ICAM-1, endothelin and NO have been reported by many labs. For example, Cao and colleagues<sup>[19]</sup> reported decreased homocysteine level in rats in response to danshensu treatment.

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Received 2010-05-08 Accepted 2010-08-19

Ding *et al*<sup>[20]</sup> showed that danshensu inhibits the expression of ICAM-1 induced by TNF-alpha in endothelial cells. In addition, danshensu also inhibits proliferation of vascular smooth muscle cells induced by elevated NO level and decreased ET-1<sup>[21]</sup>. In the current study, we examined potential effects of danshensu on the formation of atherosclerosis in a rat model of hyperhomocysteinemia. Signaling molecules that control vascular contraction/relaxation, and representative proinflammatory cytokines and ICAM-1 were also investigated.

## Materials and methods

### Animal treatment

Animal experiments were conducted in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (Document #55, 2001) and were approved by the Animal Care and Use Committee of Shandong University. Wistar rats were purchased from Shandong Academy of Medical Sciences, and housed individually in stainless cages at 24 °C with a 12-h light-dark cycle. Rats (200 g) received a methionine-rich diet (provided by Dr Weidong ZHANG, Shandong Academy of Medical Sciences) for 3 months. Intake of methionine-rich diet was monitored daily and adjusted to a level of 1 g methionine per day per kg body weight. Access to water was unrestricted. At the end of the 3-month methionine-rich diet, rats received daily treatment of danshensu (67.5 mg·kg<sup>-1</sup>·d<sup>-1</sup>, *po*; (Ang Sheng Shaanxi Biomedical Technology, Xi'an, China), a vitamin mixture (folic acid 0.45 mg·kg<sup>-1</sup>·d<sup>-1</sup>, vitamin B12 6.75 µg·kg<sup>-1</sup>·d<sup>-1</sup>, vitamin B6 2.7 mg·kg<sup>-1</sup>·d<sup>-1</sup>; *po*), or saline for another 3 months before sacrifice. Each group consisted of 5 male and 5 female rats. An additional "prevention" group received danshensu (67.5 mg·kg<sup>-1</sup>·d<sup>-1</sup>, *po*) during the 3-month period of methionine-rich diet. Data collected from this group were compared to a "model" group that was sacrificed immediately after the 3-month methionine-rich diet. A group of rats not exposed to methionine-rich diet was also included as a blank control.

At the end of the experiments, rats were sacrificed with chloral hydrate (0.36 g/kg). Trunk blood was collected; blood samples were stored at -80 °C. Segments of descending aorta were collected for Western blot analysis, or fixed with 4% paraformaldehyde and processed for immunohistochemical analysis or routine H&E staining.

### ICAM-1 and TNF-alpha expression

The expression of ICAM-1 and TNF-alpha was examined with a 3-step streptavidin-biotin immunoperoxidase method<sup>[22]</sup>. Tissue sections (5 µm) were deparaffinized and rehydrated, and then heated in a microwave oven for 10 min to enhance antigen retrieval. Slides were incubated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min to quench endogenous peroxidase, and then blocked with 5% normal goat serum for 20 min. Tissue sections were incubated with a rabbit anti-rat ICAM-1 or rabbit anti-rat TNF-alpha antibody (1:200 dilution; Bosider, Wuhan, China) for 2 h in a moisture chamber at 37 °C. After extensive washing, sections were incubated with biotinylated goat anti-rabbit IgG (1:200 dilution) and avidin for 30 min at 37 °C, and

developed using a diaminobenzidine method. Sections were counterstained with hematoxylin for 15 s. For blank controls, primary antibody was omitted. Sections were examined under a microscope for positively stained cells (brown or yellow). Data were analyzed with CMIAS series of true color multi-functional pathological image analysis system (Beijing University of Aeronautics and Astronautics, Beijing, China). The mean optical density of positively stained cells from 10 randomly selected fields (×400) was calculated, and used to estimate the expression of ICAM-1 and TNF-alpha.

### Western blot analysis

The descending aorta was homogenized and then digested with a lysis buffer, and centrifuged at 13000×g for 15 min at 4 °C. Protein concentration in the supernatant was measured with a BCA method (Pierce Corporation, Tempe, AZ, US). Samples (20 µg protein) were separated with 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with a rabbit anti-rat TNF-alpha or rabbit anti-rat ICAM-1 (both 1:500) for 1 h. After extensive washing, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (1:1000; Bosider, Wuhan, China) prior to ECL visualization (Pierce Corporation). Optical density of the band at appropriate molecular weight was used to estimate the amount of protein. Experiments were repeated 3 times independently.

### Blood biochemical analysis

Serum homocysteine concentration was measured using a HPLC method as described previously<sup>[23]</sup>. Blood endothelin concentration was measured using a radioimmunoassay (RIA Institute of People's Liberation Army General Hospital Science and Technology Development Center)<sup>[24]</sup>. Serum NO concentration was measured using a kit from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China)<sup>[24]</sup>.

### Statistical analysis

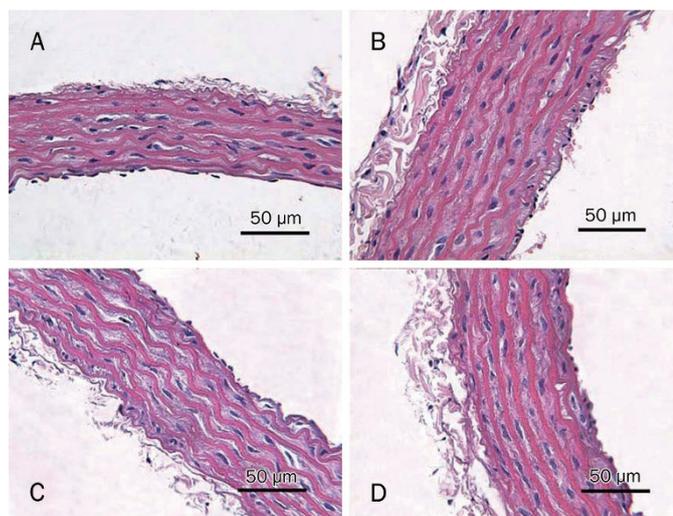
Data are expressed as mean±SD. Statistical analysis was performed with one-way ANOVA followed by least-significant difference *t*-test for *post hoc* comparison. Statistical significance was set at *P*<0.05.

## Results

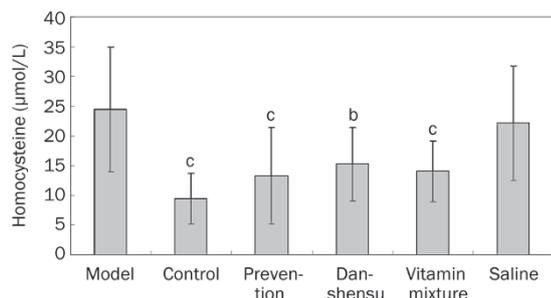
In contrast to the normal appearances in the blank control rats, the intima of descending aorta in rats receiving methionine-rich diet was thickened, and contained scattered foam cells (Figure 1). Danshensu treatment, either simultaneous with methionine-rich diet or afterwards, significantly decreased the number of foam cells.

### Effect of danshensu on homocysteine level

Methionine-rich diet significantly increased serum homocysteine concentration (*P*<0.01 *vs* blank controls; Figure 2). Such an increased homocysteine was significantly attenuated by treatment with folate-VitB6-VitB12 vitamin and danshensu after the 3-month methionine-rich diet (*P*<0.01 and <0.05



**Figure 1.** Morphology of aortic wall was observed by light microscopy. (A) blank controls; (B) model group; (C) "preventive" group; (D) danshensu group.

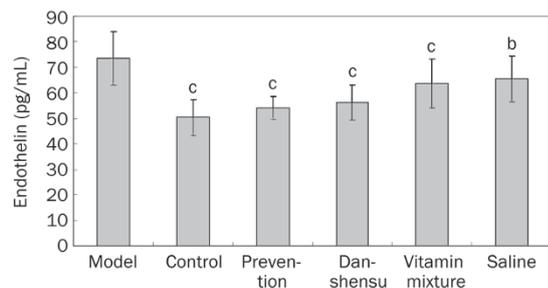


**Figure 2.** Serum homocysteine concentration in different groups. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs model group.

respectively). Preventive treatment (daily danshensu during the 3-month methionine-rich diet) also significantly decreased serum homocysteine level ( $P < 0.01$  vs the "model" group).

#### Effect of danshensu on blood endothelin and NO

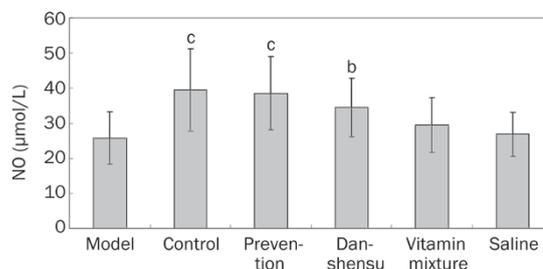
Methionine-rich diet significantly increased plasma endothelin concentration ( $P < 0.01$  vs blank controls; Figure 3). Such



**Figure 3.** Plasma endothelin levels in different groups. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs model group.

a response was significantly attenuated by treatment with vitamin mixture and danshensu ( $P < 0.01$  for both). Preventive danshensu treatment also significantly decreased endothelin concentration. Saline treatment also produced a statistically significant reduction in endothelin concentration in rats exposed to methionine-rich diet ( $P < 0.05$ ).

Serum NO level was significantly decreased by methionine-rich diet ( $P < 0.01$  vs blank controls; Figure 4). Treatment with danshensu, either after or simultaneously with methionine-rich diet, significantly increased serum NO level ( $P < 0.05$  and  $< 0.01$ , respectively). Treatment with vitamin mixture produced a trend of increasing serum NO, but the difference was not statistically significant.



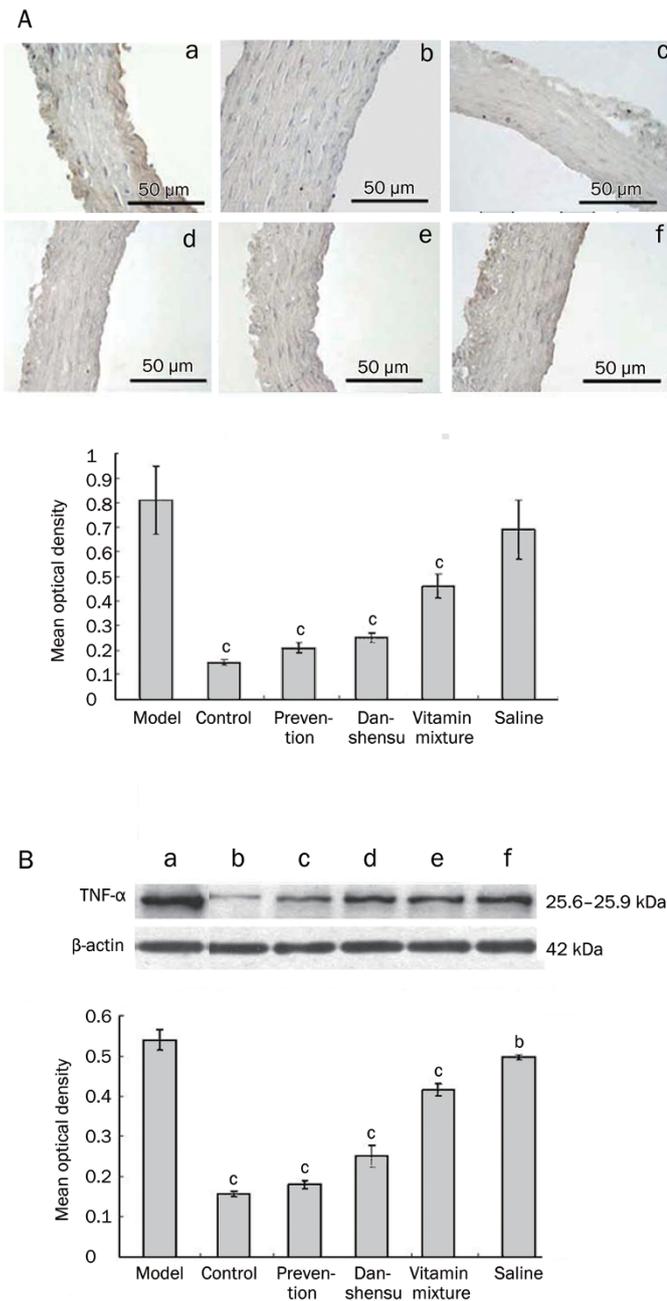
**Figure 4.** Serum NO concentration in different groups. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs model group.

#### Effect of danshensu on TNF-alpha and ICAM-1

Immunohistochemical analysis revealed dramatically increased expression of TNF-alpha (Figure 5A) and ICAM-1 (Figure 6A) in endothelial cells in response to methionine-rich diet. Increased expression of TNF-alpha and ICAM-1 was significantly attenuated by vitamin mixture and danshensu treatment, regardless of the treatment timing ( $P < 0.01$  for all). Results obtained from Western blot analysis (Figure 5B and 6B) were largely consistent with the immunohistochemical findings.

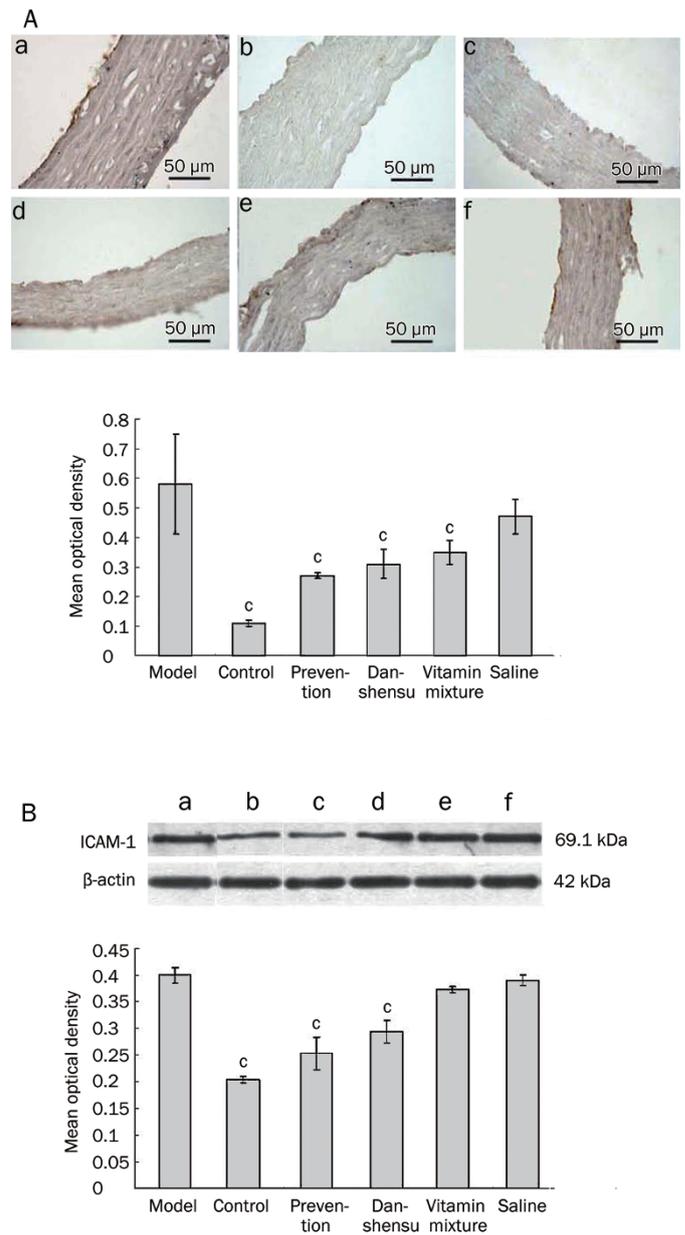
#### Discussion

Folate, vitamin B6 and vitamin B12 are key cofactors to the enzymes that metabolize homocysteine, and have been used to manage hyperhomocysteinemia<sup>[25]</sup>. Hyperhomocysteinemia is associated with increased risk of thrombotic and atherosclerotic vascular diseases<sup>[25]</sup>. Elevated level of homocysteine causes lipid peroxidation, inflammatory reactions, and vascular endothelial injury<sup>[26]</sup>. In the current study, danshensu treatment significantly lowered the serum homocysteine concentration in rats receiving a methionine-rich diet. The magnitude of such an action was similar to that of a vitamin mixture containing folate, VitB6 and VitB12. Effects of danshensu were more robust when given simultaneous to methionine-rich diet, indicating the superiority of preventive treatment. We speculate that contributing factors to this phenomenon is the ability of danshensu to promote the catabolism<sup>[19]</sup> and excretion of homocysteine during the period of methionine intake.



**Figure 5.** The effect of Danshensu on TNF- $\alpha$  expression in endothelium in a rat model of hyperhomocysteinemia. (A) TNF- $\alpha$  expression determined by immunohistochemistry. Upper: Representative photographs. Bottom: a statistical analysis of immunohistochemical results. <sup>c</sup> $P < 0.01$  vs model group. (B) TNF- $\alpha$  expression determined by Western blot. Upper: Representative photographs. Bottom: a statistical analysis of Western blot results. (a) model group; (b) blank controls; (c) prevention group; (d) danshensu group; (e) vitamin mixture group; (f) saline group. <sup>c</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs model group.

A major function of NO in the cardiovascular system is to relax both large arteries and small resistance vessels<sup>[26]</sup>. In contrast, endothelin induces vasoconstriction, participates in inflammation, cellular injury, and vascular events<sup>[27]</sup>. Con-



**Figure 6.** The effect of Danshensu on ICAM-1 expression in endothelium in a rat model of hyperhomocysteinemia. (A) ICAM-1 expression determined by immunohistochemistry. Upper: Representative photographs. Bottom: a statistical analysis of immunohistochemical results. <sup>c</sup> $P < 0.01$  vs model group. (B) ICAM-1 expression determined by Western blot. Upper: Representative photographs. Bottom: a statistical analysis of Western blot results. (a) model group; (b) blank controls; (c) prevention group; (d) danshensu group; (e) vitamin mixture group; (f) saline group. <sup>c</sup> $P < 0.01$  vs model group.

sistent with previous studies<sup>[8, 28–32]</sup>, our results indicated that NO is decreased upon hyperhomocysteinemia. In contrast, endothelin was significantly increased. These changes would result in increased vascular contraction, and might have contributed to the eventual development of atherosclerosis. Treatment with danshensu clearly attenuated the changes of NO

and endothelin in response to methionine-rich diet, suggesting that the preventive/therapeutic effects of danshensu on atherosclerosis are at least partially mediated by vasodilation secondary to decreased level of serum homocysteine. In addition to indirect effect via decreased homocysteine level, danshensu has direct action on vascular endothelium. For example, a previous report<sup>[33]</sup> indicated that danshensu could result in significant vasodilation through promoting the opening of non-selective potassium channels and small-conductance calcium-sensitive potassium channels in vascular smooth muscle cells. In our experiments, the level of endothelin but not NO was significantly lower in rats receiving 3-month saline treatment after methionine-rich diet (saline group) in comparison to immediately after the methionine-rich diet ("model" group), indicating differential spontaneous recovery of various pathological changes after discontinuation of the methionine-rich diet.

A previous study demonstrated that hyperhomocysteinemia could increase inflammatory responses mediated by NF- $\kappa$ B and TNF- $\alpha$  via activating the ERK(1/2)/p38MAPK pathway<sup>[10]</sup>. TNF- $\alpha$  is mainly secreted by monocytes and macrophages<sup>[34]</sup>. But under certain circumstances, TNF- $\alpha$  can also be secreted by endothelial cells, vascular smooth muscle cells, and cardiac fibroblast cells<sup>[35]</sup>. TNF- $\alpha$  could damage endothelial cells and vessel wall, and thus contribute to the formation of plaques<sup>[36]</sup>, possibly through stimulating the synthesis of matrix metalloproteinases-9 (MMP-9) by macrophage<sup>[37,38]</sup>.

ICAM-1 is expressed constitutively at low levels by vascular endothelial cells under normal conditions, but expressed at large quantity upon stimulation by inflammatory cytokines such as TNF- $\alpha$ <sup>[39]</sup>. Increased ICAM-1 allows the attachment of leukocytes to the endothelium and subsequent migration into peripheral tissue<sup>[39]</sup>. Antioxidants can decrease TNF- $\alpha$  synthesis/release by a variety of cell types and ICAM-1 expression by vascular endothelial cells<sup>[39]</sup>. Danshensu is a known antioxidant. Therefore, decreased TNF- $\alpha$  and ICAM-1 expression upon danshensu treatment could partially be attributed to its antioxidant property.

In our experiment, high level of TNF- $\alpha$  and ICAM-1 expression was observed in rats exposed to methionine-rich diet. Danshensu treatment, either simultaneous to methionine-rich diet or afterwards, significantly decreased TNF- $\alpha$  and ICAM-1 expression. It remains to be investigated whether such effects are mediated by the ERK1/2/p38MAPK pathway as suggested by Bai *et al*<sup>[10]</sup>.

In summary, our findings demonstrated that danshensu could lower homocysteine level and attenuate atherosclerosis in rats receiving a methionine-rich diet. Signaling molecules that favor vasoconstriction, such as endothelin, were decreased by danshensu. Molecules that relax arteries, such as NO, were increased. Danshensu treatment also attenuated the overexpression of TNF- $\alpha$  and ICAM-1. Considering its excellent safety profile, we propose that danshensu could be used in people at high risk to develop atherosclerotic diseases.

## Acknowledgements

This study was supported by the National Basic Research Program of China (national "973" Project, 2005CB523301) and grants from the National Natural Science Foundation of China (No. 30801503, 30772810, 30873325). Assistance was provided by the key laboratory of cardiovascular remodeling and function research and the Chinese Ministry of Education.

## Author contribution

Yu-xia ZHAO and Yun-fang LIU designed this research project; Rui-xue YANG carried out the experiments, analyzed the data, and wrote the manuscript; Shan-ying HUANG, Fang-fang YAN, Xiao-ting LU, Yi-fan XING, and Yan LIU contributed analytical tools and reagents; Yu-xia ZHAO revised the manuscript.

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