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Original Article

Astragalus Pretreatment but Not Posttreatment Reduces Hemorrhagic Shock-Induced Intestinal Injury in a Rat Model

Yu Gu*, Jiaxin Chen*, Nan Cheng, Dezhao Liu, Mian Ge, Chaojin Chen, and Ziqing Hei

ABSTRACT

Background: To observe the expression of inducible nitric oxide synthase (iNOS) and endothelin (ET) in small intestinal mucosa of rats which experienced hemorrhagic shock/reperfusion (HS/R) injury, and further explores the effect of astragalus in HS/R process.

Methods: Thirty-two male SD rats were randomly divided into SHAM group, HS group, HS+Pre-AS group, and HS+Post-AS group. Except for the rats in the SHAM group, the rest rats were experienced severe uncontrolled hemorrhagic shock and recovery treatment, the rats in HS+Pre-AS group were intravenous injected astragalus 15 g/kg/d for 7days before the experiment, the rats in HS+Post-AS group were intravenous injected astragalus 20 g/kg after ischemia. Then rats were killed and the small intestinal mucosa was obtained for H&E staining to observe the pathological changes of the intestine, blood was collected to detected serum MDA, SOD activity, NO levels, and the expressions of iNOS and ET in the intestinal mucosa were detected by western blot.

Results: There was no intestinal mucosa injury in SHAM group, the most serious intestinal mucosa injury appeared in HS group, astragalus had a protective effect on small intestinal mucosa in ischemia-reperfusion models. Compared with the SHAM group, the Chiu's scores in HS group were the largest, accompanied by an increase of serum MDA and decrease of serum SOD activity. Pretreatment with astragalus significantly alleviated intestinal pathological injury, decreased MDA and enhanced SOD activity, but not post-treatment. Meanwhile, the expressions of iNOS and ET-1 increased significantly in HS group and HS+Post-AS group. Compared with HS group, the expression of iNOS and ET-1 in the HS+Pre-AS group were significantly alleviated.

Conclusion: Astragalus pretreatment might protect the intestine from uncontrolled HS/R injury, which might be associated with the reduction of the iNOS and ET-1 expression. However, post-treatment with astragalus had no the same protective effect. (Funded by the National Natural Science Foundation of China, Science and Technology Project of Guangdong Province and Guangzhou Science and Technology Plan, China.)

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Hemorrhagic shock is a primary cause of death among severely injured patients, because significant blood loss may lead to multiple organ failure (1- 2). Hemorrhage shock/reperfusion (HS/R) is a whole body ischemia- reperfusion (I/R) injury, which causes the leukocyte infiltration and aggregation in ischemic tissues leads to microcirculation dysfunction and end-organ damage (3-4). Previous studies suggest HS/R has been associated with endothelial dysfunction (5-6), and iNOS and ET may regulate the function of vascular endothelium (7-8), which play a crucial role in the I/R injury. Being a traditional Chinese herb, astragalus has been reported to down-regulate adhesion molecules, stabilize macrophages and decrease activation of the inflammatory factor in endothelium, finally protect vascular endothelium and prevent the occurrence of diseases, including atherosclerosis and hypertension (9- 10). However, it is not clear whether astragalus has beneficial effects on HS/R injury and, if so, what're the mechanisms involved. Therefore, the aim of this research was to further investigate the pathogenesis of HS/R, and to verify the effect of astragalus on HS/R injury.

MATERIALS AND METHODS

Experimental Protocol

Provided by Animal Center of Sun Yat-Sen University, 32 healthy Sprague- Dawley male rats (250-300 g, 3-4 months old) were randomly allocated to four groups. The SHAM group was designed as the control group with no shock and reperfusion. Other three experimental groups were treated with hemorrhagic shock. The HS group was treated with 3 mL sterile physiological saline intravenously before reperfusion, the HS+Pre-AS group were intravenous astragalus 15 g/kg/d for 7 days before the experiment and the HS+Post-AS group were intravenous astragalus 20 g/kg before reperfusion. Astragalus was diluted in sterile physiological saline to 3 mL.

Experimental Model

Each group was fasted for 24 hours, and then they were anesthetized by intraperitoneal injection of urethane (5. 0 mL/kg, 20%). The rats were given a tracheotomy to keep ventilation and retain spontaneous respiration. The right cervical vein was catheterized for infusion of blood and drugs. The left carotid artery was catheterized for monitoring arterial pressure and the femoral artery was used to bleed for creating the experimental model.

Hemorrhagic shock model was established (11) by bleeding in 10 min after intravenous administration of heparin (250 U/kg) to make mean arterial blood pressure down to 5. 3 kPa and lasted for 60 min. Then the experimental groups were administrated with 3 mL saline or drugs intravenously. After blood doping and observation for 90 min, the specimens of small intestinal mucosa were taken.

Preparation of Specimens

After killed with deep anesthesia using carbon dioxide, the rats underwent the laparotomy. A segment of 1. 0 cm intestine was cut from 5 cm to terminal ileum and fixed in 4% paraformaldehyde solution for 24 hours. The segment of small intestine was embedded in paraffin for the section, stained with hematoxylin-eosin and observed with ZEISS optical microscope. According to the criteria of modified Chiu's scores (12), the damages of intestinal mucosa were evaluated by the changes of villus and gland.

Criteria of modified Chiu's grading system: 0, normal villus and gland; 1, changes in top of villus and initial formation of subepidermal Gruenhagen's antrum; 2, formation of subepidermal Gruenhagen's antrum and slightly injured gland; 3, enlargement of subepidermal gap and engorgement of capillary vessel; 4, epidermis moderately isolated with lamina propria and injured gland; 5, top villus shedding; 6, obvious villus shedding and capillary vessel dilating; 7, lamina propria villus shedding , and distinct injured gland; 8, initially decomposed lamina propria; 9, hemorrhage and ulcer.

ELISA Assay

MDA and NO levels in serum were measured according to the manufacturer's instructions via enzyme- linked immunosorbent (ELISA) assay (Cayman, USA). The activity of superoxide dismutase (SOD) was determined using a commercial kit (Nanjing Jiancheng Bioengineering Insti-

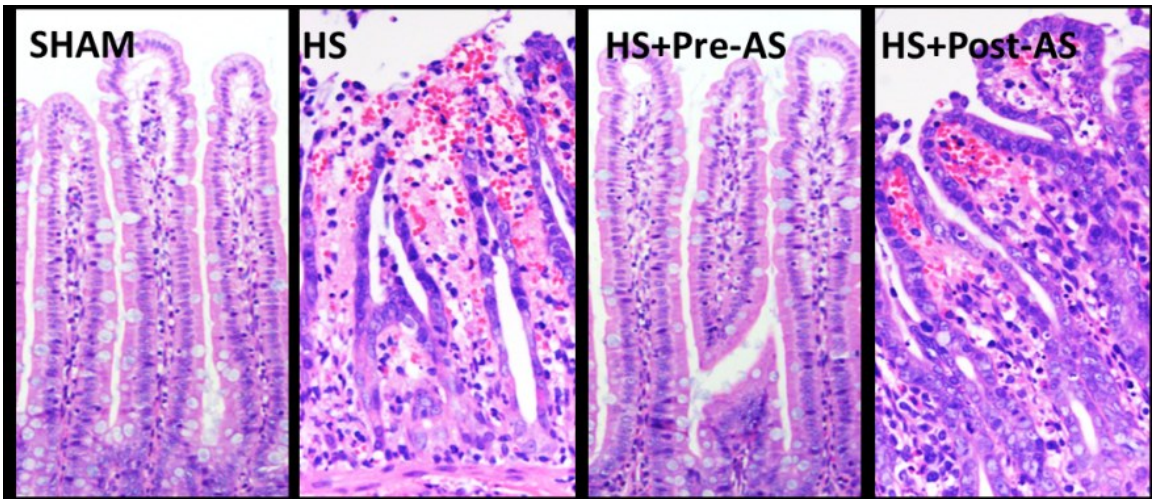

Figure 1. Photomicrograph of Small Intestine (HE staining, × 200**).**
SHAM: Normal intestine with normal villous architecture and glands in SHAM group; HS: Intestinal villi height edema, plenty of mucous epithelial cell exfoliated, villi and gland impaired obviously and some ulceration appeared in HS group; HS+Pre-AS: Nearly normal intestine with normal villous architecture and glands in HS+Pre-AS group.HS+Post-AS: Intestinal villous edema, a number of mucous epithelial cell exfoliated, villous architecture damaged, a majority of glands impaired.

tute, Nanjing, China) as we described (13).

Western Blot

Protein expression of iNOS and ET-1 in intestine tissues were detected by western blotting as described previously (14). Protein concentrations were measured using the BCA method (KeyGen BioTech, China). The primary antibodies used were rabbit anti-mouse iNOS (1:250 dilution, Santa Cruz Biotechnology, USA), anti-ET-1 (1:250 dilution, Santa Cruz Biotechnology, USA), anti- GAPDH (1:2000 dilution, Santa Cruz Biotechnology, USA). The secondary antibody used was goat anti-rabbit HRP-conjugated IgG (1:2000; Boster, China). The optical density values of the bands were normalized to those of GAPDH.

Statistical Analysis

All data were presented as mean ± SD and analyzed using SPSS10. 0 software. One way ANOVA Analysis was used for multiple mean comparisons and least significant difference test (LSD-t) was used for the independent sample mean comparison. $P<0.05$ was considered statistically significant.

RESULTS

Astragalus Alleviated Pathological Injury of Small Intestinal Mucosa

In order to explore the effects of astragalus on small intestine mucosal injury caused by severe uncontrolled HS/R, H&E staining, and Chiu's score were used to evaluate the pathological injury. As shown in Figure 1, the pathological changes of the intestine demonstrated significant villi edema, mucous epithelial cells exfoliation and some ulceration appeared in HS group, and Chiu's score increased extremely in HS group (SHAM group vs HS group, $F=72.25$, $P < 0.0001$) (Table 1). Compare with HS group, pretreatment with astragalus alleviated the pathological injury of the small intestine and lower the Chiu's score obviously (HS group vs HS + Pre-AS group, $F=10.16$, $P < 0.0001$). However, post- treatment with astragalus has little effect on small intestine injury and Chiu's score (HS group vs HS + Post- AS group, $F=1.177$, $P = 0.1998$). These results imply that astragalus pre-

Table 1. Changes of Chiu's Scores. (x±s, n=8)

Groups	SHAM	HS	HS+Pre-AS	HS+Post-AS
Chiu's score	0.52±0.12	5.68±1.02*	1.24±0.32*#	5.02±0.94*▲

Data are expressed as mean ± SD. *represents P<0.05 compared to SHAM group; #represents P<0. 05 compared to HS group. ▲represents P<0.05 compared to HS+Pre-AS group.

Table 2. Level of Serum MDA, SOD and NO. (x±s, n=8)

Groups	SHAM	HS	HS+Pre-AS	HS+Post-AS
MDA (nmol/L)	5.34±0.48	9.02±1.04*	6. 04±0.78#	8. 56±0. 62*▲
SOD (U/mL)	180.32±9. 02	102.56±10.12*	156.82±14.66*#	98.14±8.46*▲
NO	56.42±7.86	80.16±9.32*	124.64±10.86*#	88.82±9.02*▲

Data are expressed as mean ± SD. *represents P<0.05 compared to SHAM group; #represents P<0.05 compared to HS group. ▲represents P<0.05 compared to HS+Pre-AS group.

Table 3. Gray Intensity Analysis of iNOS and ET-1 Expressions. (x±s, n=8)

Groups	SHAM	HS	HS+Pre-AS	HS+Post-AS
iNOS	1.00±0.12	2.03±0.32*	1.12±0.24#	2.34±0.46*▲
ET-1	1.00±0.09	3.12±0.34*	1.42±0.28*#	3.02±0.30*▲

Data are expressed as mean ± SD. *represents P<0.05 compared to SHAM group; #represents P<0.05 compared to HS group. ▲represents P<0.05 compared to HS+Pre-AS group.

treatment, but not post-treatment, has a protective effect on intestine injury induced by severe uncontrolled HS/R.

Astragalus Pretreatment Enhanced the Level of Serum MDA, SOD, and NO

To further confirm the protective effects of astragalus against intestine injury induced by severe uncontrolled HS/R, serum MDA, SOD, and NO were detected. The levels of serum MDA were just in line with the intestinal pathological injury. When rats were subjected to severe hemorrhagic shock, serum MDA increased remarkably (SHAM group vs HS group, F=4.694, P < 0.0001), but these increase of serum MDA was almost negligible in HS + Pre- AS group (HS group vs HS + Pre- AS group, F=1.778, P < 0.0001) (Table 2). However, post- treatment with astragalus had little influence on the levels of serum MDA (HS group vs HS + Post- AS group, F=2.814, P = 0.3007). Interestingly, hemorrhagic shock caused serum SOD declined significantly (SHAM group vs HS group, F= 1.259, P < 0.0001), and pretreatment with astragalus could elevate the level of serum SOD (HS group vs HS+Pre-AS group, F=2.098, P < 0.0001). While, post- treatment with astragalus had little influence on the levels of serum SOD (HS group vs HS+Post-AS group, F=1.431, P = 0.3593). Moreover, hemorrhagic shock increased serum NO (SHAM group vs HS group, F=1.406, P < 0.0001), which could be upregulated by astragalus pretreatment (HS group vs HS+Post-AS group, F=1.358, P < 0.0001), but not post- treatment (HS group vs HS + Post- AS group, F=1.068, P = 0.0799) (Table 2). The results suggest that astragalus has a protective effect on intestinal injury induced by hemorrhagic shock and it is related to the level of serum MDA, SOD, and NO.

Astragalus Decreased the Expression of iNOS and ET-1 in Intestine

The Figure 2 and Table 3 illustrated that astragalus decreased the expression of iNOS and ET- 1 in the intestine, which suffered severe HS/R injury. Hemorrhagic shock increased the expression of iNOS (SHAM group vs HS group, F=7.111, P < 0.0001) and ET- 1 (SHAM group vs HS group, F=14.27, P < 0.0001), while pretreatment with astragalus reduced the expression of iNOS (HS group vs HS + Post- AS group, F= 1.778, P < 0.0001) and ET-1 (HS group vs HS+ Post-AS group, F=1.474, P < 0.0001) obviously. However, post-treatment with astragalus had no influence on the expression of iNOS (HS group vs HS + Post- AS group, F=2.066, P = 0.14) and ET- 1 (HS group vs HS + Post- AS group, F=1.284, P = 0.5428). The results suggest that the protective effect of astragalus on intestine injury induced by hemorrhagic shock is related to the expression of iNOS and ET-1.

DISCUSSION

Hemorrhagic shock-induced intestine I/R was reported to cause intestine barrier dysfunction and bacterial translocation, is a key contributing factor of multiple organ failure (15- 16). In this study, we confirm that hemorrhagic shock 60 min and reperfusion 90 min significantly impair small intestinal mucosa, and pretreatment but not post-treatment with astragalus provided sig-

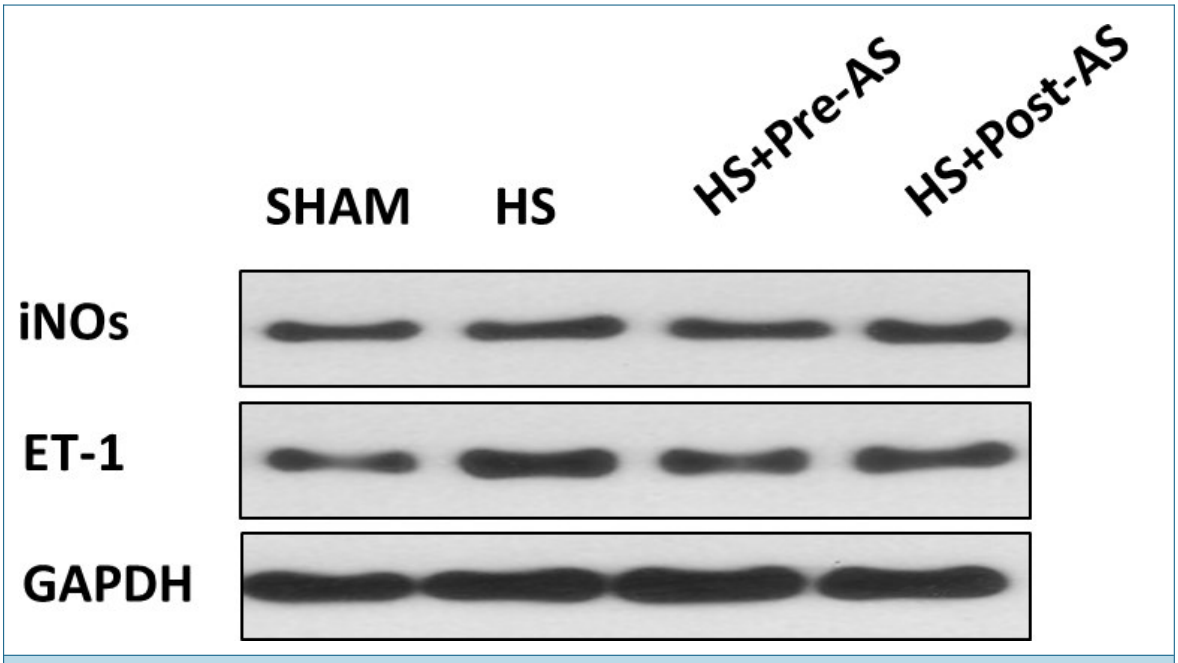

Figure 2. Western Blot Analysis of iNOS and ET Levels.
Western Blot assay was performed to detect the protein expressions of iNOS and ET-1 in intestinal mucosa tissue after hemorrhagic shock- induced intestinal injury.

nificant protection against intestine injury after hemorrhagic shock via iNOS induction.

The mechanisms of HS/R are complex, among which the Lipid peroxidation injury and inflammatory responses are thought to be the major factors causing intestinal I/R injury (17). Astragalus has been reported to decrease MDA concentration and stimulate GSH-PX and SOD activities in intestinal mucosa after ischemia-reperfusion (18). Besides the antioxidative effects, recent studies also indicated that astragalus might exert neuroprotective effects against cerebral ischemia and reperfusion through its anti-inflammatory effects by inhibiting TLR4 signaling pathway and NLRP3 inflammasome overactivation (19). Astragalus was also reported to alleviate cerebral I/R injury by improving energy metabolism and inhibiting apoptosis (20). Whether astragalus protects the intestine from HS/R injury via other potential mechanisms needs further study.

It is well known that NO is normally produced from NOS in multiple organs in animals and humans, in which iNOS is the main source of NO (21). Under pathological conditions like intestine I/R (22), iNOS is markedly induced and causes synthesis of excessive amounts of NO, which leads to oxidative injury and acts as an inflammatory mediator under proinflammatory conditions. Hassoun et. al (23) demonstrated that after intestine I/R for 90 min of hemorrhagic shock, intestinal iNOS was increased significantly and induced the expression of NO in plasma, indicating that the increased iNOS and NO play a pivotal role in the shock-induced intestinal injury. In our study, we found that 90 minutes after reperfusion, the expression of iNOS in intestinal mucosa increased significantly, which was consistent with earlier reports (23). Compared with the HS group, pretreatment with astragalus decreased the upregulation of iNOS in HS/R group, indicating that astragalus has a protective effect on intestine I/R injury, through inhibiting the enhancement of iNOS and decreased the NO production.

ET is a kind of polypeptide separated from pig aortic endothelial cells by Yanagisawa and plays various functions in animals and humans (24). Shindo et. al (25) has found that ET-1 gene expression and peptide production were marked-

ly increased by LPS in the heart, lung, kidney, liver, and aorta. In addition, our previous studies have demonstrated that intestine I/R resulted in severe injury and significant increases in ET- 1 contents (26). In the current study, we found that hemorrhagic shock upregulated ET-1 expression in intestine and pretreatment with astragalus significantly reverse this change but not post-treatment. It needs further investigation whether astragalus exerted its protective effect via ET- 1 in HS/R-induced intestinal mucosa injury.

In summary, pretreatment with astragalus might protect the intestine from uncontrolled HS/R injury, which might be associated with a reduction of the iNOS and ET- 1 expression. While, post- treatment with astragalus had no the same protective effect.

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The authors have no other potential conflicts of interest for this work.

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