

• BASIC RESEARCH •

Protective effect of *Astragalus membranaceus* on intestinal mucosa reperfusion injury after hemorrhagic shock in rats

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Abstract

AIM: To study the protective effect of *Astragalus membranaceus* on intestinal mucosa reperfusion injury and its mechanism after hemorrhagic shock in rats.

METHODS: A total of 32 SD rats were randomly divided into four groups ($n = 8$, each group): normal group, model group, low dosage group (treated with 10 g/kg *Astragalus membranaceus*) and high dosage group (treated with 20 g/kg *Astragalus membranaceus*). The model of hemorrhagic shock for 60 min and reperfusion for 90 min was established. Therapeutic solution (3 mL) was administered before reperfusion. At the end of the study, the observed intestinal pathology was analyzed. The blood concentrations of lactic acid (LD), nitric oxide (NO), endothelin-1 (ET-1), malondialdehyde (MDA) and the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) in intestinal mucosa were determined.

RESULTS: The intestinal mucosa pathology showed severe damage in model group and low dosage group, slight damage in high dosage group and no obvious damage in normal group. The Chiu's score in low dose group and high dose group was significantly lower than that in model group. The content of MDA in model group was higher than that in low and high dose groups, while that in high dose group was almost the same as in normal group. The activity of SOD and GSH-PX was the lowest in model group and significantly higher in high dose group than in normal and low dose groups. The concentrations of LD and ET-1 in model group were the highest. The concentrations of NO in model group and low dose group were significantly lower than those in high dose group and normal group.

CONCLUSION: High dose *Astragalus membranaceus* has

much better protective effect on hemorrhagic shock-reperfusion injury of intestinal mucosa than low dose *Astragalus membranaceus*. The mechanism may be that *Astragalus membranaceus* can improve antioxidative effect and regulate NO/ET level during hemorrhagic reperfusion.

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Key words: Hemorrhage shock; Intestinal reperfusion injury; *Astragalus membranaceus*

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INTRODUCTION

Under normal conditions, the integrity of intestinal mucosa as a barrier, can prevent bacterial translocation^[1,2]. Bacterial and endotoxin can enter into blood across the barrier when the intestinal mucosal barrier is demolished due to anoxia, ischemic and reperfusion injury. It is possible to induce systemic inflammatory response syndrome (SIRS) or multiple organ dysfunction syndrome (MODS), leading to the hemorrhagic shock^[3-6].

Free oxygen radical is one of the major activating factors in ischemia/reperfusion injury of intestinal mucosa^[7]. The maladjustment of NO/ET can not only aggravate oxidative damage, but also lead to dysfunction of the microcirculation of intestinal mucosa^[8-11]. Many drugs have been tried to palliate the ischemia-reperfusion injury of intestinal mucosa after hemorrhagic shock^[8,12,13]. However the results are still controversial and unsatisfactory.

Astragalus membranaceus is a traditional Chinese medicine which can improve microcirculation and has good curative effect. The main purpose of our study was to investigate whether *Astragalus membranaceus* could protect intestinal mucosa against ischemia-reperfusion injury after hemorrhagic shock, and to observe the effect of *Astragalus membranaceus* on intestinal oxidative damage, NO and ET levels.

MATERIALS AND METHODS

Animal experimental protocol

Approved by the University Animal Study Committee, 32 healthy male Sprague-Dawley rats (200-300 g, provided by

Animal Center of Sun Yat-Sen University) were randomly divided into four groups: normal group ($n = 8$, sham-operation, no shock and reperfusion), model group ($n = 8$, with hemorrhagic shock and treated only with 3 mL normal saline intravenously prior to reperfusion), low dose group ($n = 8$, with hemorrhagic shock and treated with 10 mg/kg *Astragalus membranaceus* which was five times of the human clinical dose) and high dose group ($n = 8$, with hemorrhagic shock and treated with 20 mg/kg of *Astragalus membranaceus* which was 10 times of the human clinical dose). The latter three groups were experimental groups.

Astragalus membranaceus (provided by Dioujiuhong Pharmaceutical Co., Ltd, Chengdu, China) was diluted in 3 mL normal saline and infused intravenously for 3 min prior to reperfusion.

Experimental model of hemorrhagic shock and resuscitation

Laboratory temperature was kept at 25–27 °C. The rats were anesthetized by intraperitoneal injection of urethane (5.0 mL, 20%) after they were fasted for 24 h. Tracheotomy was performed for ventilation. The right cervical vein was cannulated for monitoring central venous pressure and fluid infusion and drugs. The left carotid artery and femoral artery were catheterized for monitoring arterial pressure. The femoral artery was used to withdraw blood samples and to create hemorrhagic shock model.

Preparation of hemorrhagic shock model

Hemorrhagic shock model was established by withdrawing blood by femoral artery until mean arterial blood pressure (MABP) reached about 5.3 kPa (40 mmHg) and maintained for 60 min.

Rat resuscitation

Rats were administrated with 3 mL solution/drugs. Then their blood was reinfused for approximately 5 min and observed for 90 min. The segment samples of small intestinal mucosa were taken. The rats were killed at the end of the experiment.

The parameters included room temperature, rat weight and blood loss.

Mean arterial pressure and pulse rate were recorded every 10 min during the hemorrhagic shock and resuscitation period.

Preparation of specimens and measurements

After successful establishment of experimental model, the rats were killed and paunched rapidly. A segment of 0.5–1.0 cm intestine was cut from 5 cm to terminal ileum, fixed in 4% formaldehyde polymerisatum and embedded in paraffin for section. Another segment of small intestine was washed with frozen saline. The intestinal mucosa was scraped off, dried with suction paper and preserved at -70 °C.

The segment of small intestine was stained with hematoxylin-eosin. The damages of intestinal mucosa were evaluated by criteria of modified Chiu method. Criteria of modified Chiu grading system were divided into 10 subdivisions according to the changes of villus and gland of intestinal mucosa: 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.

Detection of lactic acid content in intestinal mucosa

Intestinal mucosal tissues were weighed and made into 10% homogenate. The lactic acid content in tissues was determined by the method of minim quick measurement (Jiancheng Bioengineering Ltd, Nanjing, China) and the concentration of protein was determined by Coomassie brilliant blue. The results were expressed as mmol/gpro.

Detection of content of MDA (malondisdehyde) in intestinal mucosa

Intestinal mucosal tissues (100 mg) were homogenized with normal saline. MDA content was determined by TBA method (Jiancheng Bioengineering Ltd, Nanjing, China). Homogenate (0.1 mL) was taken to detect MDA content. Briefly, 0.1 mL 8.1% SDS, 0.8 mL acetic acid buffer, 0.8 mL 0.8% TBA and 0.2 mL distilled water were added into the sample tubes and one standard tube (containing 0.1 mL tetraethoxypropane). Then all the tubes were incubated at 100 °C for 1 h. After cooled at -20 °C for 5 min, 2 mL n-butyl alcohol was added into the sample, which was then vibrated for 1 min and centrifuged for 10 min at 3 000 r/min. The supernatant of the samples was taken to detect absorbance at 533 nm with spectrophotometer (content of MDA (nmol/100 mg) = absorbance of each sample/absorbance of standard \times dilution times).

Detection of activity of superoxide dismutase (SOD) in intestinal mucosa

Intestinal mucosal tissues (100 mg) were weighed and made into 10% homogenate with 0.9 mL normal saline, frozen in refrigerator at -20 °C for 5 min and centrifuged for 15 min at 4 000 r/min. Supernatants were transferred into fresh tubes for the evaluation of SOD activity. SOD activity was evaluated with SOD detection kit according to the manufacturer's instructions (Kits were provided by Jiancheng Bioengineering Ltd, Nanjing, China). SOD activity (μ /mL) = $(A_0 - A_1) / A_0 \times 360 \times V$ (A_0 : absorbance of self-oxidation rate per minute; A_1 : absorbance of each sample per minute; V : volume of extracted tissues).

Detection of activity of glutathione peroxidase (GSH-PX) in intestinal mucoa

Intestinal mucosal tissues (100 mg) were weighed and made into 10% homogenate with 0.9 mL normal saline. The activity of GSH-PX was detected according to the manufacturer's instructions (reagents were purchased from Jiancheng Bioengineering Ltd, Nanjing, China). Protein of homogenates was detected with Coomassie brilliant blue. The calculated results were expressed by U/100 mg protein.

Detection of content of NO in intestinal mucosa

Intestinal mucosal tissues (100 mg) were weighed and made into 10% homogenate with 0.9 mL normal saline. After being centrifuged for 10 min at 10 000 *g*, the supernatants were placed in boiling water for 3 min and then centrifuged for 5 min at 10 000 *g*. Supernatant (0.1 mL) was taken for detection, 0.2 mL 35% sulfosalicylic acid was added into the sample to make protein deposits. The sample was homogenized and centrifuged at 10 000 *g* for 10 min. The supernatants were taken again and preserved at -20 °C in refrigerator. One hundred μ L supernatant was detected by indirect nitric acid deoxidized enzyme method (Kits were provided by Jingmei Bioengineering Ltd). After 100 μ L nitrate reductase was homogenized gently, the sample was placed in water at 37 °C for 1 h with KNO₂ standard succi homogenized. After being placed in ambient temperature for 10 min and with zero setting with blank tube at 530 nm wavelength and 0.5 cm colorimetric cylinder, *A* value of detection tube and standard tube was read respectively.

Detection of ET-1 concentration in intestinal mucosa

Intestinal mucosal tissues (100 mg) were weighed and made into 10% homogenate with 0.9 mL normal saline. Homogenate ET-1 levels were measured by radioimmunoassay (Kits were obtained from Beijing East Asian Radioimmunoassay Technology Institute, Beijing, China).

Statistical analysis

Data were expressed as mean \pm SD and analysis of variance was performed using SPSS10.0 software. One-way analysis of variance was used for multiple comparisons and least significant difference test (LSD-*t*) was used for intra-group comparison. *P*<0.05 was considered statistically significant.

RESULTS

Basic status of each group

There was no difference in the four groups (*P*>0.05). The amount of withdrawn blood in three groups was similar, except in normal group (Table 1).

Table 1 Status of animals in each group (mean \pm SD)

Group	<i>n</i>	Weight (g)	Ambient temperature (°C)	Bloodletting volume (mL)
Normal	8	265 \pm 18	26.38 \pm 1.06	-----
Model	8	281 \pm 20	26.67 \pm 0.52	5.10 \pm 1.80
Low dose	8	269 \pm 14	25.88 \pm 1.13	5.20 \pm 1.60
High dose	8	272 \pm 16	26.20 \pm 1.10	4.80 \pm 1.50

Table 2 Changes of MBP (kPa) and HR (bpm) during shock and reperfusion period (mean \pm SD)

Groups/ <i>n</i>		Pre-shock	30 min of shock	10 min after reperfusion	30 min after reperfusion	60 min after reperfusion	90 min after reperfusion
Normal (<i>n</i> = 8)	MABP	12.66 \pm 1.28					
	HR	316.50 \pm 22.12	-----	-----			
Model (<i>n</i> = 8)	MABP	12.68 \pm 0.82	5.33 \pm 0.25 ^d	12.66 \pm 1.35	12.83 \pm 1.60	11.65 \pm 0.68	11.60 \pm 1.82
	HR	322.50 \pm 28.12	239.75 \pm 35.50 ^d	256.40 \pm 35.63 ^b	292.55 \pm 41.96	325.36 \pm 36.81	309.25 \pm 38.68
Low dose (<i>n</i> = 8)	MABP	13.07 \pm 1.27	5.32 \pm 0.20 ^d	13.21 \pm 1.17	12.46 \pm 1.46	12.21 \pm 0.82	12.05 \pm 1.58
	HR	320.87 \pm 38.83	221.75 \pm 49.89 ^d	250.00 \pm 45.63 ^b	282.76 \pm 45.63	319.50 \pm 42.28	300.62 \pm 32.28
High dose (<i>n</i> = 8)	MABP	13.00 \pm 1.36	5.30 \pm 0.20 ^d	12.16 \pm 1.35	12.90 \pm 1.60	12.05 \pm 0.82	11.82 \pm 1.42
	HR	310.83 \pm 25.63	236.16 \pm 51.67 ^b	266.00 \pm 38.10 ^b	316.83 \pm 30.91	306.66 \pm 22.11	298.50 \pm 22.11

^b*P*<0.01 vs pre-shock, ^d*P*<0.01 vs pre-shock.

Changes of animals' vital signs

There was no difference in MAP and HR in four groups before the experiment (*P*>0.05). The MAP in experimental groups was maintained at 5.33 kPa (40 mmHg), but the HR decreased during hemorrhage shock period. There was no significant difference after resuscitation in experimental group (*P*>0.05, Table 2).

Changes of intestinal mucosa under light microscope

The villus and glands were normal and no inflammatory cell infiltration was observed in mucosal epithelial layer in normal group. Severe edema of mucosa villus and infiltration of necrotic epithelial and inflammatory cells were found, indicating that damage was severe in model group. Light edema of mucosa villus and infiltration of few necrotic epithelial inflammatory cells were found in mucosa epithelial layer in low dosage group. No significant edema and necrotic mucosa villus were observed, but infiltration of a few inflammatory cells in mucosal epithelial layer was found in high dosage group, which was also the same as those in normal intestinal mucosa (Figures 1-4).

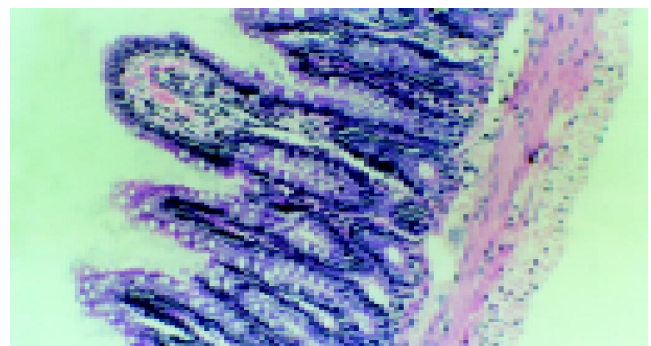


Figure 1 Normal villus and glands in normal group.

Chiu's score of small intestinal structure

The Chiu's score was the lowest in normal group and the highest in model group. Compared to model group, the Chiu's score was significantly lower in high dose group (*P*<0.05, Table 3).

Changes of MDA in small intestinal mucosa

The content of MDA in intestinal mucosa was the highest in model group, compared to that in normal, high and low dose groups. However there was no difference in Chiu's

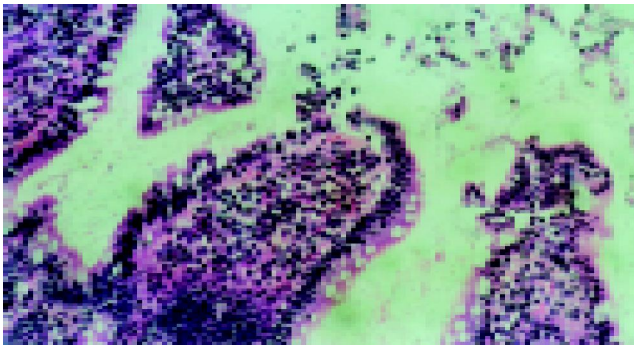


Figure 2 Severe edema of mucosa villus and infiltration of necrotic epithelial cells and inflammatory cells in model group.

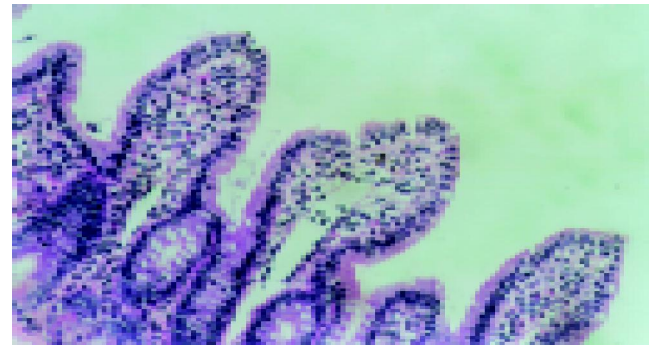


Figure 4 No significant edema and necrotic mucosa villus and infiltration of a few inflammatory cells in high dose group.

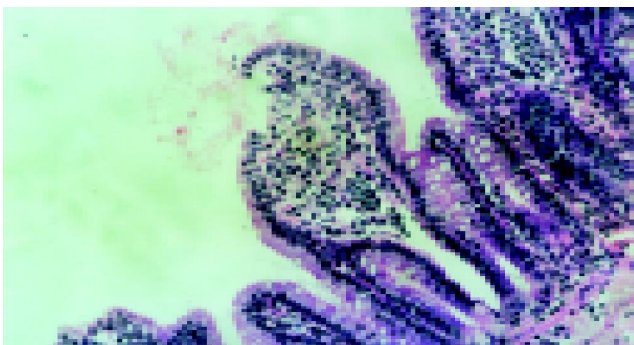


Figure 3 Light edema of mucosa villus and few necrotic epithelial cells and infiltration of few inflammatory cells in low dose group.

score among normal, low and high dose groups ($P>0.05$, Table 3).

Changes of activity of SOD

The activity of SOD in intestinal mucosa was the highest in high dose group ($P<0.05$ or 0.01), and the lowest in model group ($P<0.05$, Table 3).

Change of activity of GSH-PX in intestinal mucosa

The activity of GSH-PX was the lowest in model group ($P<0.05$) compared to that in the other three groups. There was no difference between normal group and low dose group ($P>0.05$, Table 3).

Lactic acid content in intestinal mucosa

The content of lactic acid in model group and low dose group was significantly higher than that in normal and high dose groups ($P<0.05$). There was no significant difference

among model group, low and high dose groups ($P>0.05$, Table 3).

Change of NO content in small intestinal mucosa

The content of NO in small intestinal mucosa in model group and low dosage group was significantly lower than that in normal group and high dosage group ($P<0.05$ or 0.01). There was no significant difference between model group and low dosage group ($P>0.05$) and between normal group and high dosage group ($P>0.05$, Table 3).

Changes of ET-1 content in intestinal mucosa

The content of ET-1 in intestinal mucosa was significantly higher in three experimental groups ($P<0.01$). The content of ET-1 in model group was higher than that in low and high dose groups ($P<0.05$ or 0.01 , Table 3).

DISCUSSION

Blood supply of small intestine from celiac branch of superior mesenteric artery accounts for 20% of total body blood volume. The blood flow in mucous layer is about 70-80% of total intestine blood flow, while the blood flow in muscular layer and serosa is about 15-25% of intestine blood flow. The blood flow in submucous layer is less than 5% of intestine blood flow. But 60% of blood flow in mucous layer is concentrated on the top of mucosa which provides enough hemoperfusion of endotheliocytes and villus^[14]. When shock occurs, the blood flow in intestinal mucosa decreases sharply. That is why the small intestinal mucosa is most easily subjected to ischemia and reperfusion injury^[15,16].

Studies have proved that ischemia reperfusion injury of intestinal mucosa plays an important role in inducing

Table 3 Effects of *Astragalus membranaceus* on LD, NO, MDA and SOD GSH-PX during hemorrhagic shock-resuscitation (mean \pm SD)

Group	<i>n</i>	Chiu's score	Lactic acid nmol/gpro	NO nmol/100 mg	ET Pg/100 mg	MDA content nmol/100 mg	SOD activity (U/100 mg)	GSH-PX activity (U/100 mg)
Normal	8	0.90 \pm 0.86	2.62 \pm 0.45	41.27 \pm 8.60	274.62 \pm 44.2	28.89 \pm 4.90	42.42 \pm 4.37	58.04 \pm 7.18
Model	8	6.25 \pm 2.75 ^b	3.11 \pm 0.53 ^a	20.21 \pm 4.14 ^b	481.50 \pm 109.98 ^b	62.70 \pm 15.37 ^b	27.85 \pm 10.75 ^b	42.92 \pm 10.62 ^a
Low dosage	8	4.05 \pm 1.96 ^{b,c}	3.00 \pm 0.36 ^a	26.86 \pm 5.33 ^{a,c}	414.53 \pm 26.15 ^{b,c,e}	36.80 \pm 9.35 ^{b,d}	39.13 \pm 7.62 ^{a,c,e}	55.91 \pm 11.27 ^{a,e}
High dosage	8	3.27 \pm 1.82 ^{a,c}	2.69 \pm 0.19 ^a	42.41 \pm 9.89 ^d	353.51 \pm 31.90 ^{a,d}	31.31 \pm 11.45 ^d	54.38 \pm 6.84 ^{a,d}	77.78 \pm 13.56 ^{b,d}

^a $P<0.05$, ^b $P<0.01$ vs normal group, ^c $P<0.05$, ^d $P<0.01$ vs low dose group and high dose group, ^e $P<0.05$ vs low dose group.

multiple organ dysfunction syndrome (MODS)^[3-6]. Therefore prevention of intestinal mucosal barrier from hemorrhagic shock is very important. The results of our study showed that small intestinal mucosa was severely injured after simple hemorrhagic shock. However, the injury of small intestinal villus was alleviated after *Astragalus membranaceus* was administrated. The best result was obtained with high dose *Astragalus membranaceus* treatment, indicating that *Astragalus membranaceus* can protect intestinal mucosa against reperfusion injury, after hemorrhagic shock in a dose-dependant manner.

NO and ET-1 are active substances released by vascular endothelial cells (especially in lung) and the most important endogenous regulatory factors. It is most important to keep the dynamic balance between NO and ET in order to maintain tissue and organ hemoperfusion. Some studies^[9,17,18] reported that NO and ET-1 increase in plasma sharply during hemorrhagic shock and after resuscitation. At the same time, the intestinal barrier is also damaged.

NO/ET imbalance and endothelial disorder can induce intestinal mucosal hypoperfusion or ischemic injury, when hemorrhagic shock occurs.

Our study showed lactic acid content in intestinal mucosa in model group increased markedly, suggesting that anaerobic metabolism of intestinal mucosa occurs. NO level decreased and ET level increased sharply, suggesting that microcirculation of the intestinal mucosa is destroyed during and after hemorrhagic shock / reperfusion injury.

Bauer *et al.*^[19,20], found that intestinal damage can be relieved by drugs, when endogenous NO release is evoked. Oktar *et al.*^[21], and Anadol *et al.*^[22], used ET receptor antagonists to suppress endogenous ET activity, and found that they can relieve the damage due to intestine ischemic reperfusion, demonstrating that ET receptor antagonists can relieve ischemia reperfusion injury of intestine by modulating NO/ET level.

We found that *Astragalus membranaceus* could increase endogenous NO level and decrease endogenous ET level in intestinal mucosa, suggesting that *Astragalus membranaceus* can relieve endothelial dysfunction and ameliorate microcirculation via regulating NO/ET level during hemorrhagic-reperfusion.

Oxygen free radical is another major factor in inducing ischemia reperfusion injury. It could damage the structure of cell membrane and mitochondrial membrane through lipid peroxidation and result in cellular structure destroy and cell dysfunction^[7,8]. MDA is the direct products of lipid peroxidation^[7,8]. The extent of lipid peroxidation could be accessed by measuring MDA level in tissues. Our study also showed that the content of MDA in intestinal mucosa in model group was significantly higher than that in other groups, suggesting that significant lipid peroxidation occurs in small intestinal mucosa during hemorrhagic shock and reperfusion period. Some previous studies found^[23,24] that peroxide dismutase significantly relieves small intestine mucosal injury after 3 h ischemia, demonstrating that oxygen free radical plays an important role in ischemic injury of small intestinal mucosa.

SOD and GSH-PX are the major enzymes for scavenging oxygen free radical, whose activity could reflect its functional

status^[25-27]. The activity of SOD and GSH-PX in *Astragalus membranaceus*-treated groups was markedly higher than that in normal group and model group, which would be beneficial to scavenging oxygen free radical.

The content of MDA in intestinal mucosa in *Astragalus membranaceus*-treated groups was obviously lower than that in model group, demonstrating that *Astragalus membranaceus* has powerful antioxidative effects and protects small intestinal mucosa against hemorrhagic- reperfusion injury.

In conclusion, *Astragalus membranaceus* protects intestinal mucosa against hemorrhagic-reperfusion injury in a dose-dependant manner by regulating NO/ET level of intestinal mucosa after ischemia-reperfusion.

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