Chinese Journal of Physiology 55(4): 229-235, 2012
DOI: 10.4077/CJP.2012.BAA023

Role of Estrogen Receptor in Astringinin–Mediated Attenuation of Intestinal Injury after Trauma-Hemorrhage

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Abstract

Astringinin protects against organ injury caused by trauma-hemorrhage though the mechanism remains unknown. We hypothesize that astringinin administration in males following trauma-hemorrhage protects against intestinal injury through an estrogen receptor-dependent pathway. To test this hypothesis, male Sprague-Dawley rats were subject to trauma-hemorrhage (mean blood pressure: 40 mmHg for 90 min) followed by fluid resuscitation. Animals were pretreated with an estrogen receptor antagonist (ICI 182,780) 30 min before vehicle or astringinin (0.3 mg/kg) administration, followed by resuscitation, and the treated rats were killed 24 h thereafter. Intestinal wet/dry weight ratio, myeloperoxidase (MPO) activity, and the levels of interleukin (IL)-6, intercellular adhesion molecule (ICAM)-1, cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-3 were measured. Trauma-hemorrhage led to an increase in intestinal wet/dry weight ratio, MPO activity, histological damage and also increases in the levels of IL-6, ICAM-1, CINC-1 and CINC-3. Administration of astringinin improved all of the above parameters. Administration of ICI 182,780 with astringinin abolished the astringinin-mediated improvement of the above parameters. These results suggest that the estrogen receptor-dependent pathway likely plays a critical role in mediating the salutary effects of astringinin on shock-induced intestinal injury.

Key Words: shock, estrogen receptor, intestine, chemokine, adhesion molecule, cytokine

Introduction

The gut is considered to be a critical organ following trauma-hemorrhage (1, 18). Estrogen receptor is reported to play an important role in limiting organ injury following trauma-hemorrhage (17, 18). An increasing body of evidence also shows that estrogen receptors lead to regulation of inflammatory response following trauma-hemorrhage (23). Estrogen-mediated attenuation of the inflammatory response to shock-induced organ injury is abolished by the presence of the estrogen receptor antagonist, ICI 182,780 (24).

Following trauma-hemorrhage, reduction of neutrophil infiltration attenuates organ injury (8, 21). Neutrophil movement and migration are mediated by multiple adhesion molecules and pro-inflammatory mediators (6, 8, 12, 21). Intercellular adhesion molecule (ICAM)-1 assumes a central role in the firm adhesion of neutrophils to the vascular endothelium and is markedly up-regulated following trauma-hemorrhage (19). The influx of neutrophils to inflammatory sites is also driven by locally-produced cytokines/chemokines (19). In this regard, interleukin

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Received: March 22, 2011; Revised: June 1, 2011; Accepted: June 28, 2011.
 ISSN : 0304-4920. http://www.cps.org.tw

(IL)-6 plays an important role in the pathophysiology of intestinal ischemia/reperfusion injury (9, 15). In addition to cytokines, chemokines such as cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-3 also activate and attract neutrophils (8, 21).

Astringinin has been shown to be protective following shock-like states in males (5). Previous studies have shown that astringinin binds to and increases the transcriptional activity of the estrogen receptor (10). Furthermore, astringinin can reduce neutrophil and cytokine production in a rodent model of lipopolysaccharide-induced septic shock (3). We have shown that astringinin treatment in male rats following trauma-hemorrhage attenuates hepatic injury under those conditions (4). Nonetheless, the role of the estrogen receptor in astringinin-mediated attenuation of intestinal injury following trauma-hemorrhage is unknown. The aim of this study was to determine whether or not the salutary effects of astringinin on the intestine following trauma-hemorrhage are mediated *via* an estrogen receptor-dependent pathway.

Materials and Methods

The current study was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. All animal experiments were performed according to the guidelines of the *Animal Welfare Act* and *The Guide for Care and Use of Laboratory Animals* from the National Institutes of Health.

Trauma-Hemorrhage Procedure

A non-heparinized rat model of trauma-hemorrhage was used in this study (22, 25). Briefly, male Sprague-Dawley rats (275-325 *g*) obtained from the National Science Council were housed in an air-conditioned room under a reverse light-dark cycle and allowed 1 week or more to adapt to the environment. Before the experiments, the rats were fasted overnight but were allowed water *ad libitum*. The rats were anesthetized using isoflurane (Attane, Minrad Inc., Bethlehem, PA, USA) inhalation prior to the induction of soft-tissue trauma *via* a 5-cm midline laparotomy. The abdomen was closed in layers, and catheters were placed in both femoral arteries and the right femoral vein (polyethylene [PE-50] tubing; Becton Dickinson and Co., Sparks, MD, USA). The wounds were bathed with 1% lidocaine (Elkins-Sinn Inc., Cherry Hill, NJ, USA) throughout the surgical procedure to reduce post-operative pain. Rats were then allowed to awake, subject to bleeding and were maintained at a mean blood pressure of 40 mmHg. This level of hypotension was continued until the mean blood pressure could not be maintained without the use of additional fluid in the form of Ringer's lactate. This duration was defined as the maximum bleed-out time, and the amount of withdrawn blood was noted. Following this, the rats were maintained at a mean blood pressure of 40 mmHg until 40% of the maximum bleed-out volume was returned in the form of Ringer's lactate. The animals were then resuscitated with four times the volume of the shed blood over 60 min with Ringer's lactate. The time required for maximum bleed out was about 45 min; the volume of maximum bleed out was about 60% of the calculated circulating blood volume, and the total hemorrhage time was about 90 min (13). Thirty minutes before the end of the resuscitation period, the rats received astringinin (0.3 mg/kg, intravenously) (4), astringinin plus an estrogen receptor antagonist (ICI 182,780, 3 mg/kg, intraperitoneally at the beginning of resuscitation) (24), or an equal volume of the vehicle (~0.2 ml, 10% DMSO). Astringinin was dissolved in 20 µl of pure DMSO and then stirred and waited for about 10 min. For administration of astringinin, rats received 20 µl of astringinin in DMSO and then received 180 µl of water to make sure that there was no residual astringinin solution in the syringe. The catheters were then removed, the vessels ligated and the skin incisions closed with sutures. Sham-operated animals underwent a surgical procedure which included a laparotomy in addition to the ligation of the femoral artery and vein, but neither hemorrhage nor resuscitation was carried out. The animals were then returned to their cages and were allowed food and water *ad libitum*. The animals were sacrificed 24 h after the end of resuscitation.

Water Content Assay

In a separate cohort, intestines were weighed and dried for 24 h at 80°C (20). Water content of the intestinal tissue was calculated as wet/dry weight ratio.

Myeloperoxidase (MPO) Activity Assay

MPO (Sigma, St. Louis, MO, USA) activity in homogenates of entire intestines was determined as described previously (18). Frozen tissue samples were thawed and suspended in phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma). Samples were sonicated on ice, centrifuged at 2,000 *g* for 15 min at 4°C, and an aliquot was transferred into phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine hydrochloride and 0.0005% hydrogen peroxide (Sigma). The change in absorbance at 460 nm was measured spectrophotometrically for 5 min. MPO activity was calculated using a standard curve generated using human MPO, and values were normalized to protein concentration.

Determination of CINC-1, CINC-3, ICAM-1 and IL-6 Levels

Intestinal tissues were homogenized in PBS (1:10 weight:volume, pH 7.4) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Boehringer Mannheim, Germany). Homogenates were centrifuged at 2,000 *g* for 20 min at 4°C and the supernatant was analyzed for the presence of CINC-1, CINC-3, ICAM-1 and IL-6 using ELISA kits (R&D, Minneapolis, MN, USA) according to manufacturer instructions and as described previously (18).

Histological Analysis of Intestines

For histological examination, intestinal tissues were fixed in 10% formalin in phosphate-buffered saline for 24 h and were sent to the histology laboratory at the Chang Gung University for further processing. Briefly, the sections were embedded in paraffin. These were then cut in 4-5 μM thickness and mounted on glass slides. Intestinal sections were stained with hematoxylin-eosin, observed under the microscope at 400X magnification for changes in intestinal morphology, and were photographed.

Statistical Analysis

Results are presented as means ± SEM (n = 11 rats/group). Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's test. Differences were considered significant at a P value of ≤ 0.05.

Results

Tissue Edema

As shown in Fig. 1, no significant differences in water content in the intestines were observed between sham groups treated with vehicle or astringinin. Trauma-hemorrhage caused a significant increase in intestinal water content which was attenuated by astringinin. To evaluate whether or not the salutary effects of astringinin are mediated by activation of the estrogen receptor, a group of astringinin-treated trauma-hemorrhage rats were administered an estrogen-receptor antagonist, ICI 182,780. Results indicated that administration of ICI 182,780 with astringinin prevented the astringinin-induced decrease in intestinal water content.

Intestinal MPO Activity

There were no differences in intestinal MPO activities between vehicle- and astringinin-treated sham groups (Fig. 2). After trauma-hemorrhage, MPO activity increased significantly in vehicle-treated rats when compared to sham-operated animals. Astringinin treatment attenuated this increase in intestinal MPO activities. Administration of ICI 182,780 with astringinin prevented the astringinin-induced attenuation of intestinal MPO activity following trauma-hemorrhage.

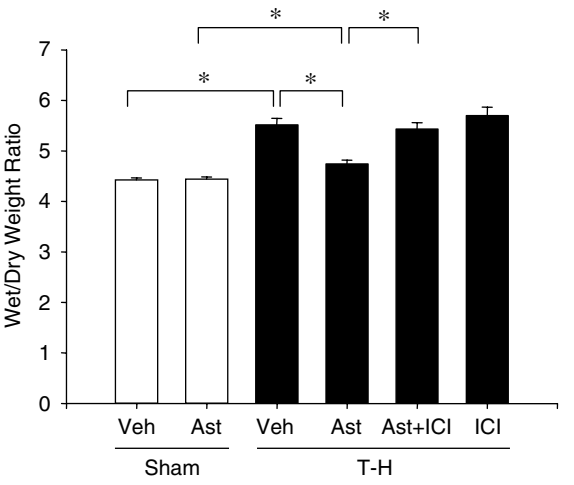

Fig. 1. Effects of astringinin treatment on tissue water content in rats after sham operation (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with vehicle (Veh), astringinin (Ast), astringinin in combination with ICI 182,780 (Ast+ICI), or ICI 182,780 only (ICI). Data are shown as means ± SEM (n = 11 rats in each group). *P < 0.05 compared to other group.

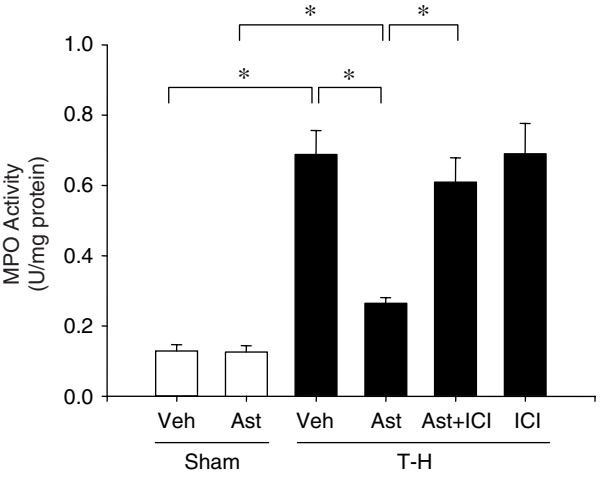

Fig. 2. Effects of astringinin treatment on intestinal MPO activities in rats after sham operation (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with vehicle (Veh), astringinin (Ast), astringinin in combination with ICI 182,780 (Ast+ICI), or ICI 182,780 (ICI). Data are shown as means ± SEM (n = 11 rats in each group). *P < 0.05 compared to other group.

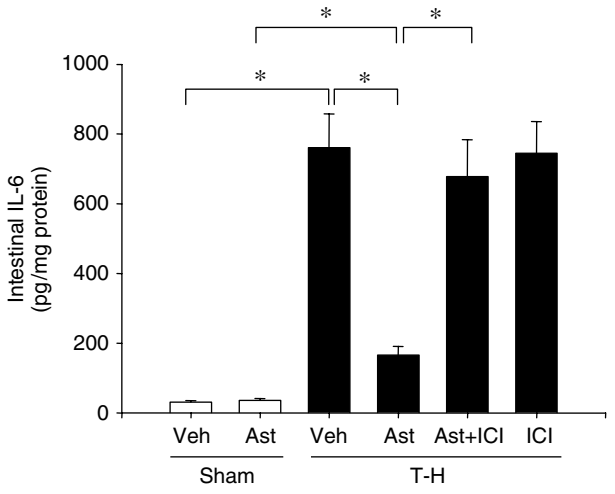

Fig. 3. Effects of astringinin treatment on intestinal IL-6 levels in rats after sham operation (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with vehicle (Veh), astringinin (Ast), astringinin in combination with ICI 182,780 (Ast+ICI), or ICI 182,780 (ICI). Data are shown as means ± SEM (n = 11 rats in each group). *$P < 0.05$ compared to other group.

Intestinal IL-6 Levels

Intestinal IL-6 levels for vehicle- and astringinin-treated sham animals were similar (Fig. 3). Trauma-hemorrhage significantly increased intestinal IL-6 levels in vehicle-treated rats when compared with sham animals; this was attenuated by astringinin treatment. Astringinin-mediated reductions of intestinal IL-6 levels were abolished by ICI 182,780 co-administration.

Intestinal Expression of CINC-1, CINC-3 and ICAM-1

Trauma-hemorrhage significantly increased CINC-1, CINC-3 and ICAM-1 expression in the intestine (Figs. 4A, 4B and 5). Treatment with astringinin attenuated this increase in CINC-1, CINC-3 and ICAM-1 expression. Co-administration of ICI 182,780 with astringinin prevented the astringinin-induced reductions in CINC-1, CINC-3 and ICAM-1 levels.

Histological Analysis of the Intestine

Representative photomicrographs of the intestine are presented for sham animals treated with vehicle (Fig. 6A), sham animals treated with astringinin (Fig. 6B), trauma-hemorrhage animals treated with vehicle (Fig. 6C), trauma-hemorrhage animals treated with astringinin (Fig. 6D), trauma-hemorrhage animals treated with astringinin and ICI 182,780 (Fig. 6E), and trauma-hemorrhage animals treated with ICI

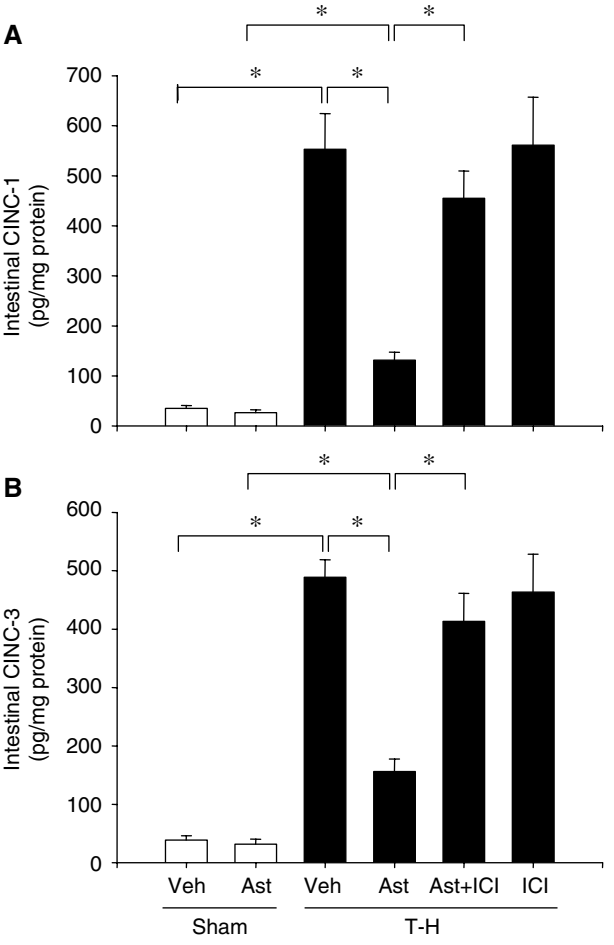

Fig. 4. Effects of astringinin treatment on intestinal CINC-1 (A) and CINC-3 (B) levels in rats after sham operation (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with vehicle (Veh), astringinin (Ast), astringinin in combination with ICI 182,780 (Ast+ICI), or ICI 182,780 (ICI). Data are shown as means ± SEM (n = 11 rats in each group). *$P < 0.05$ compared to other group.

182,780 (Fig. 6F). Similar results were obtained from four or more animals in each group. Together, these results, as presented in Fig. 6, suggest that astringinin ameliorated the trauma-hemorrhage-induced damage in the intestine; however, the damage remained higher than in the shams.

Discussion

Our present results indicated that at 24 h after trauma-hemorrhage, intestinal water content, MPO activity and the levels of IL-6, CINC-1, CINC-3 and ICAM-1 were markedly increased in the male rats tested. Administration of a single dose of astringinin during resuscitation attenuated the increase in these inflammatory markers. Administration of an estrogen-receptor antagonist ICI 182,780 with astringinin after

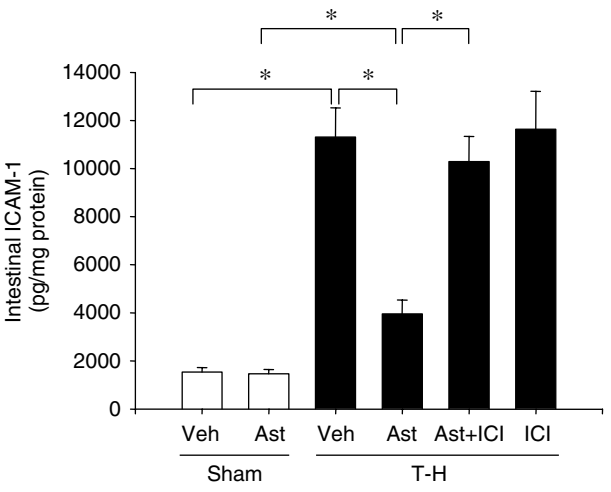

Fig. 5. Effects of astringinin treatment on intestinal ICAM-1 levels in rats after sham operation (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with vehicle (Veh), astringinin (Ast), astringinin in combination with ICI 182,780 (Ast+ICI), or ICI 182,780 (ICI). Data are shown as means ± SEM (n = 11 rats in each group). *$P < 0.05$ compared to other group.

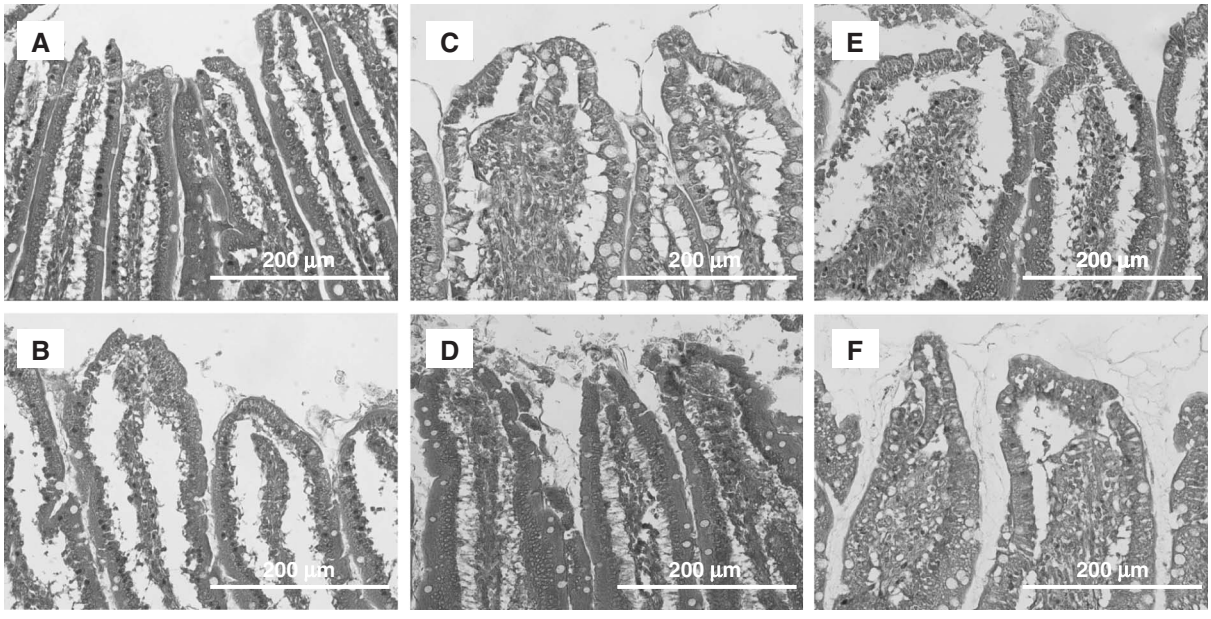

Fig. 6. Representative photomicrographs of the intestine from sham-operated rats receiving vehicle (A), sham-operated rats receiving astringinin (B), trauma-hemorrhage rats receiving vehicle (C), trauma-hemorrhage rats receiving astringinin (D), trauma-hemorrhage rats receiving astringinin and ICI 182,780 (E) and trauma-hemorrhage rats receiving ICI 182,780 (F).

trauma-hemorrhage prevented the above-mentioned astringinin-induced effects. These studies collectively suggest that the salutary effects of astringinin in the intestine appear to be mediated by estrogen receptors.

IL-6 is an important mediator in intestinal inflammation (9, 15) and is required for the expression of adhesion molecules and production of chemokines (8). CINC-1 and CINC-3 levels also correlated with activities of tissue MPO, a marker of neutrophil infiltration, following trauma-hemorrhage (21). Our results, thus, suggest that astringinin-mediated attenuation of intestinal injury after trauma-hemorrhage occurs through down-regulation of production of intestinal pro-inflammatory mediators and attenuation of neutrophil infiltration, which is consistent with the findings that astringinin attenuates ICAM-1 expression and chemokine productions in shock status (4).

Our hypothesis was that astringinin mediated its beneficial effects *via* the estrogen receptor. Our results showed that administration of astringinin in

conjunction with an estrogen receptor antagonist (ICI 182,780) blocked the astringinin-mediated attenuation of intestinal injury. Previous studies demonstrated the importance of sex steroids on the maintenance of organ function in shock status (2, 7, 11, 14, 17). Investigation has revealed the critical roles of estrogen receptors and their receptor agonists/antagonists under injurious conditions (2, 11, 14). The present study shows that estrogen receptor pathways are critical to astringinin-induced post-resuscitation intestinal-protection, consistent with the view that estrogen receptor pathways might be critical to the utility of potential therapies in the treatment of trauma patients.

Previous studies have shown that expressions of the estrogen receptor is down-regulated in the intestine 2 h after trauma-hemorrhage (18). However, it is not known whether the levels of the estrogen receptor are changed 24 h after trauma-hemorrhage or whether astringinin has any effect on the levels of estrogen receptor. The precise mechanism still needs to be determined.

Our previous results have shown that administration of astringinin following trauma-hemorrhage improves hepatic function under those conditions (4). The present results indicate that astringinin administration after trauma-hemorrhage also attenuates intestinal injury and down-regulates pro-inflammatory mediators. The data also suggest that the salutary effects of astringinin are mediated in part through the estrogen receptor-dependent pathway. Support for this suggestion comes from the fact that administration of an estrogen receptor antagonist following trauma-hemorrhage abolished the salutary effects of astringinin on the intestine. However, since astringinin can mediate its effects in multiple ways, we do not propose that activation of estrogen receptors is the exclusive action of astringinin following trauma-hemorrhage. Astringinin produces various beneficial effects through multiple pathways, *e.g.*, it is recently shown that astringinin suppresses colotis *via* the inhibition of inducible nitric oxide synthase which is considered to contribute beneficial effects (16). It is important to further characterize the molecular mechanism by which this agent improves and maintains various cell and organ functions following low-flow conditions. Future studies may reveal the precise mechanism by which astringinin produces the above-mentioned salutary effects following low-flow conditions. A better understanding of the relationship between astringinin and other signaling pathways should enable us to develop new therapeutic models for the treatment of hemorrhagic shock.

Acknowledgments

This work was supported, in part, by grants from the National Science Council (NSC 98-2314-B-182A-034-MY3) and Chang Gung Memorial Hospital (CMRPG381072), Taiwan, ROC.

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