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Astringinin–Mediated Attenuation of the Hepatic Injury following Trauma-Hemorrhage

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Abstract

Although astringinin administration under adverse circulatory conditions is known to be protective, the mechanism by which astringinin produces the salutary effects remains unknown. We hypothesize that astringinin administration in males following trauma-hemorrhage decreases cytokine production and protects against hepatic injury. Male Sprague-Dawley rats underwent trauma-hemorrhage (mean blood pressure: 40 mmHg for 90 min, then resuscitation). Different doses of astringinin (0.01, 0.03, 0.1, 0.3 mg/kg of body weight) or vehicle were administered intravenously during resuscitation. Concentrations of plasma aspartate aminotransferase (AST) with alanine aminotransferase (ALT) and various hepatic parameters were measured (n = 8 rats/group) at 24 h after resuscitation. One-way ANOVA and Tukey testing were used for statistical analysis. Trauma-hemorrhage significantly increased plasma AST and ALT levels at 24 h postresuscitation; there was a dose-related benefit when astringinin was administered at doses of 0.01 to 0.3 mg/kg. In astringinin-treated (0.3 mg/kg) rats subjected to trauma-hemorrhage, there were significant improvements in liver myeloperoxidase (MPO) activity (237.80 ± 45.89 *vs*. 495.95 ± 70.64 U/mg protein, $P < 0.05$), interleukin-6 (IL-6) levels (218.54 ± 34.52 *vs*. 478.60 ± 76.21 pg/mg protein, $P < 0.05$), cytokine-induced neutrophil chemoattractant (CINC)-1 (88.32 ± 20.33 *vs*. 200.70 ± 32.68 pg/mg protein, $P < 0.05$), CINC-3 (110.83 ± 26.63 *vs*. 290.14 ± 76.82 pg/mg protein, $P < 0.05$) and intercellular adhesion molecule (ICAM)-1 concentrations (1,868.5 ± 211.5 *vs*. 3,645.0 ± 709.2 pg/mg protein, $P < 0.05$), as well as in histology. Results show that astringinin significantly attenuates proinflammatory responses and hepatic injury after trauma-hemorrhage. In conclusion, the salutary effects of astringinin administration on attenuation of hepatic injury following trauma-hemorrhage are likely due to reduction of pro-inflammatory mediator levels.

Key Words: astringinin, hemorrhagic shock, chemokine, adhesion molecule, cytokine

Introduction

A large number of studies have demonstrated that the enhanced secretion of pro-inflammatory cytokines by mast cells, dendritic cells and macrophages is an important factor in initiation and perpetuation of inflammation in different tissues (16). These cytokines recruit other immune cells such as neutrophils thereby increasing leukocyte trafficking and hepatic injury (21). Neutrophils can release superoxide anions and proteolytic enzymes, which diffuse across the endothelium and injure parenchymal cells, or alternatively, neutrophils can leave the microcirculation and migrate and adhere to matrix proteins or other cells (3, 26). Intercellular adhesion molecule (ICAM)-1 is known to play a major role in the firm adhesion of neutrophils to the vascular endothelium. ICAM-1 is constitutively present on the surface of

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endothelial cells, and is markedly upregulated following trauma-hemorrhagic shock (4). In addition to adhesion molecules, chemokines such as cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-3 are also potent chemotactic factors for neutrophils (10).

Hemorrhagic shock results in excessive production of pro-inflammatory mediators such as cytokines and chemokines, which play a significant role in the development of multiple organ dysfunctions under those conditions. Studies have shown that neutrophils are activated following hemorrhagic shock and that hepatic injury is associated with an increased neutrophil accumulation in the liver after hemorrhagic shock (21, 29, 30). The activated neutrophils appear to infiltrate the injured liver, with increased expression of adhesion molecules on endothelial cells and elevated local chemokine/cytokine levels following hemorrhagic shock (30). Furthermore, trauma-hemorrhagic shock increases endothelial cell P-selectin and ICAM-1 levels in the liver (27). Moreover, levels of CINC-1 and CINC-3 are elevated in the liver after trauma-hemorrhage (21, 28). Interleukin (IL)-6 also appears to be an essential component of the inflammatory cascade associated with hepatic injury in hemorrhagic shock (21). Moreover, IL-6-deficient mice show less neutrophil infiltration and organ damage when compared with wild-type mice under these conditions (17).

Astringinin (3, 39, 49, 5-tetrahydroxystilbene), a resveratrol analogue reported to have higher antioxidant activities and a greater radical scavenging capacity than resveratrol, has been shown to possess antiarrhythmic, anti-tumorigenic and apoptosis-inducing effects (2, 7, 11, 12, 25). Previous studies have shown that astringinin can reduce cytokine production and demonstrates cardioprotective activities after shock-like states in ischaemic-reperfused rat hearts (6). Since the liver is the critical organ for inflammation following trauma-hemorrhagic shock, we hypothesized that astringinin administration following trauma-hemorrhage attenuates hepatic injury and cytokine production (21). To test the hypothesis, we examined the effects of astringinin treatment on hepatic injury by measuring hepatic chemokine and cytokine production following trauma-hemorrhage.

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 of the National Institutes of Health, USA.

Trauma-Hemorrhage Procedure

A non-heparinized rat model of trauma-hemorrhage was used in this study (31). Briefly, male Sprague-Dawley rats (275-325 g) obtained from the National Science Council were housed in an air-conditioned room under a reversed light-dark cycle and allowed 1 week or more to adapt to the environment. Before the experiment, they 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* 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 & Co., Sparks, MD, USA). The wounds were bathed with 1% lidocaine (Elkins-Sinn Inc., Cherry Hill, NJ, USA) throughout the surgical procedure to reduce postoperative pain. Rats were then allowed to awaken, subjected to bleeding, and maintained at a mean blood pressure of 40 mmHg. This level of hypotension was continued until the mean blood pressure could no longer 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 for 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 (23). Thirty minutes before the end of the resuscitation period, the rats received astringinin (0.3 mg/kg, intravenously) or an equal volume of the vehicle (~0.2 ml, 10% DMSO, Sigma) (6). 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.

Measurement of Hepatic Injury

Twenty hours after resuscitation or sham surgery, blood samples with heparin were obtained and plasma was separated by centrifugation, immediately frozen and stored at -80°C until assayed. Hepatic injury was determined by measuring plasma levels of AST and ALT using a colorimetric analyzer (Dri-Chem 3000, Fuji Photo Film Co., Tokyo, Japan).

Measurement of Myeloperoxidase (MPO) Activity

MPO activity in homogenates of the entire liver was determined as described in previous studies (28, 30). All reagents were purchased from Sigma (St. Louis, MO, USA). Briefly, equal masses (100 mg wet weight) of liver from various groups were suspended in 1 ml buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) and sonicated twice at 30 cycles for 30 sec on ice. Homogenates were cleared by centrifuging at 2,000 g at 4°C and the supernatants were stored at -80°C. Sample protein content was determined using the Bio-Rad (Hecules, CA, USA) assay kit. Samples were incubated with a substrate of o-dianisidine hydrochloride. This reaction was done in a 96-well plate by adding 290 µl 50 mM phosphate buffer, 3 µl substrate solution (containing 20 mg/ml o-dianisidine hydrochloride), and 3 µl H_2O_2 (20 mM). A sample (10 µl) was added to each well to start the reaction. Standard MPO (Sigma) was used in parallel to determine MPO activity in the sample. The reaction was stopped by adding 3 µl sodium azide (30%). Light absorbance at 460 nm was read and MPO activity was determined using the curve obtained from the standard MPO.

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

CINC-1, CINC-3, ICAM-1 and IL-6 levels in the liver were determined using ELISA kits (R&D, Minneapolis, MN, USA) according to manufacturer instructions and as described in previous studies (8, 10). Briefly, the samples 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 2000 × g for 20 min at 4°C and the supernatant was assayed for CINC-1, CINC-3 and ICAM-1 levels. An aliquot of the supernatant was used to determine protein concentration (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA, USA).

Histological Examination of the Liver

For histological examination, three pieces of the middle lobe were fixed in 10% formalin in phosphate-buffered saline for 24 h and were sent to the histology laboratory at Chang Gung University for further processing. Briefly, sections were embedded in paraffin and then cut (4-5 µM) and mounted on glass slides. Liver sections were stained with hematoxylin-eosin, observed under the microscope (Nikon Eclipse TS100) at 400X magnification for changes in liver morphology, and photographed (SPOT, RTcolor, Diagnostic Instrument, Inc., Iowa City, Iowa, USA) using a microscope-attached camera.

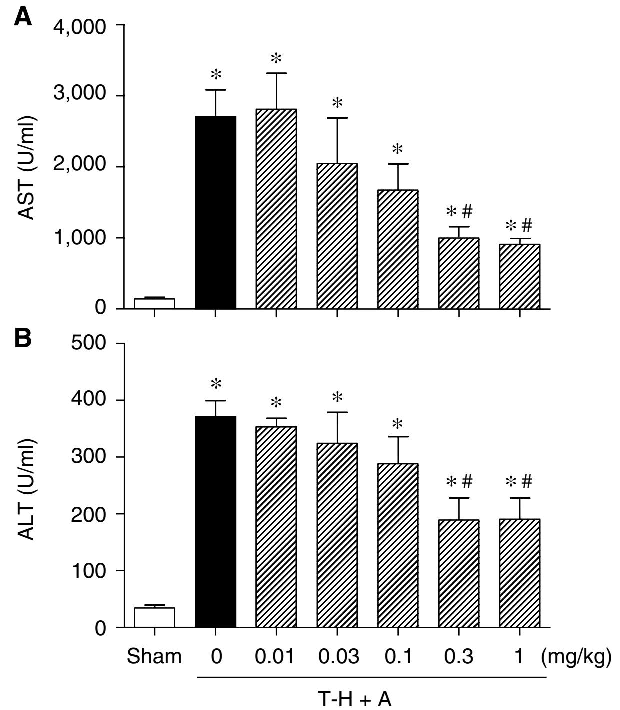

Fig. 1. Dose-dependent responses to astringinin treatment of plasma AST (A) and ALT (B) in rats at 24 h after sham surgery (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with astringinin at doses of 0, 0.01, 0.03, 0.1, 0.3 or 1 mg/kg. Data are shown as means ± SEM for 6 rats in each group. $^*P < 0.05$ compared to sham; $^{\#}P < 0.05$ compared to T-H + A (0 mg/kg); A indicates astringinin.

Statistical Analysis

Results are presented as means ± SEM (n = 8 rats/group). Data was analyzed using one-way analysis of variance (ANOVA) and Tukey's test. Differences were considered significant at a P value of ≤ 0.05.

Results

Dose-Response Effects for Astringinin on Plasma AST and ALT Levels

As shown in Fig. 1, trauma-hemorrhage significantly increased plasma AST and ALT levels at 24 h postresuscitation. Administration of astringinin at doses of 0.01, 0.03, 0.1, 0.3 or 1 mg/kg was used to evaluate its effects on the attenuation of hepatic injury after trauma-hemorrhage. There was a dose-related benefit when astringinin was administered at doses of 0.01, 0.03 or 0.1 mg/kg (Fig. 1). Effects were equivalent when doses were 0.3 or 1 mg/kg (Fig. 1).

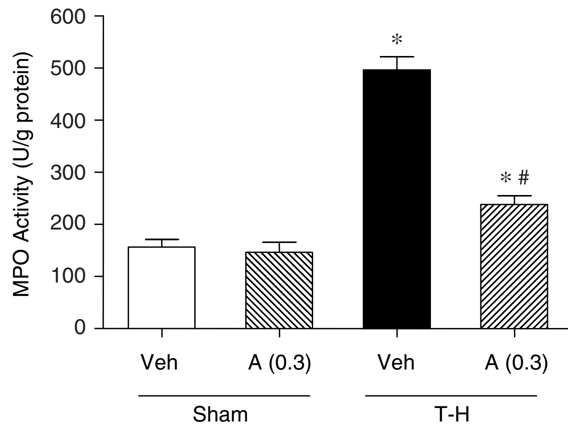

Fig. 2. Effects of astringinin treatment on hepatic MPO activity in rats at 24 h after sham surgery (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with either vehicle (Veh) or astringinin (A: 0.3 mg/kg). Data are shown as means ± SEM for 8 rats in each group; $*P < 0.05$ compared to sham; $^{\#}P < 0.05$ compared to T-H + Veh.

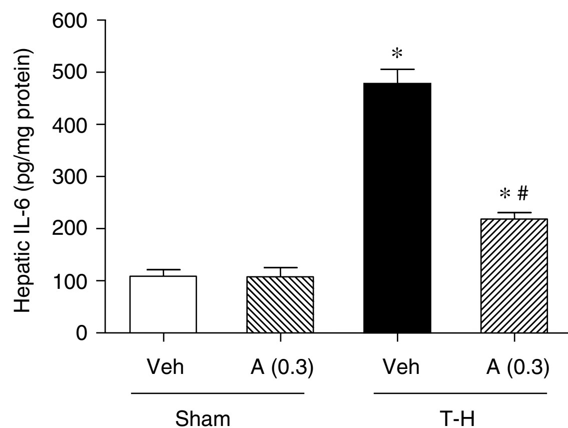

Fig. 3. Effects of astringinin treatment on hepatic IL-6 levels in rats at 24 h after sham surgery (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with either vehicle (Veh) or astringinin (A: 0.3 mg/kg). Data are shown as means ± SEM for 8 rats in each group; $*P < 0.05$ compared to sham; $^{\#}P < 0.05$ compared to T-H + Veh.

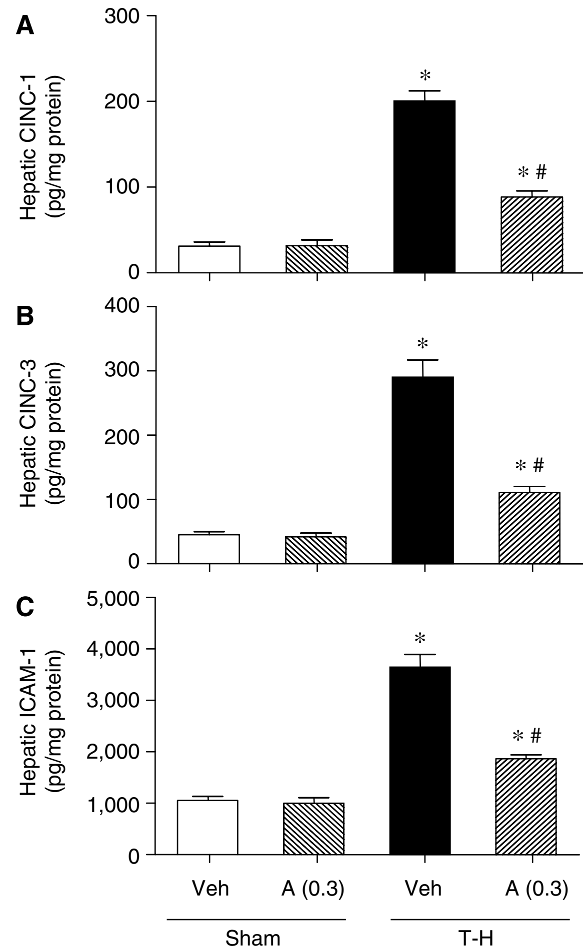

Fig. 4. CINC-1 (A), CINC-3 (B) and ICAM-1 (C) levels in the liver in rats after sham surgery (Sham) or trauma-hemorrhage and resuscitation (T-H). Animals were treated with vehicle (Veh) or astringinin (A: 0.3 mg/kg). Data are shown as means ± SEM for 8 rats in each group; $*P < 0.05$ compared to sham; $^{\#}P < 0.05$ compared to T-H + Veh.

Alteration in Hepatic MPO Activity

Hepatic MPO activity in sham surgery or trauma-hemorrhaged animals, with and without astringinin treatment, is shown in Fig. 2. In sham-operated rats, astringinin did not alter hepatic MPO activity. Trauma-hemorrhage resulted in a significant increase in hepatic MPO activity in vehicle-treated animals. Furthermore, astringinin treatment attenuated the increase in hepatic MPO activity.

Alteration in Hepatic IL-6 Levels

Hepatic IL-6 levels were not influenced by astringinin administration in sham animals compared with shams that received vehicle (Fig. 3). Trauma-hemorrhage significantly increased hepatic IL-6 levels when compared with sham animals. Astringinin administration after trauma-hemorrhage, however, significantly reduced the elevated hepatic IL-6 levels.

Alteration in Hepatic CINC-1, CINC-3, and ICAM-1 Expression

Trauma-hemorrhage significantly increased CINC-1 and CINC-3 expression in the liver (Fig. 4, A and B). However, treatment with astringinin prevented the said increases. In addition, hepatic ICAM-1 levels increased significantly in vehicle-treated rats following trauma-hemorrhage (Fig. 4C). Astringinin

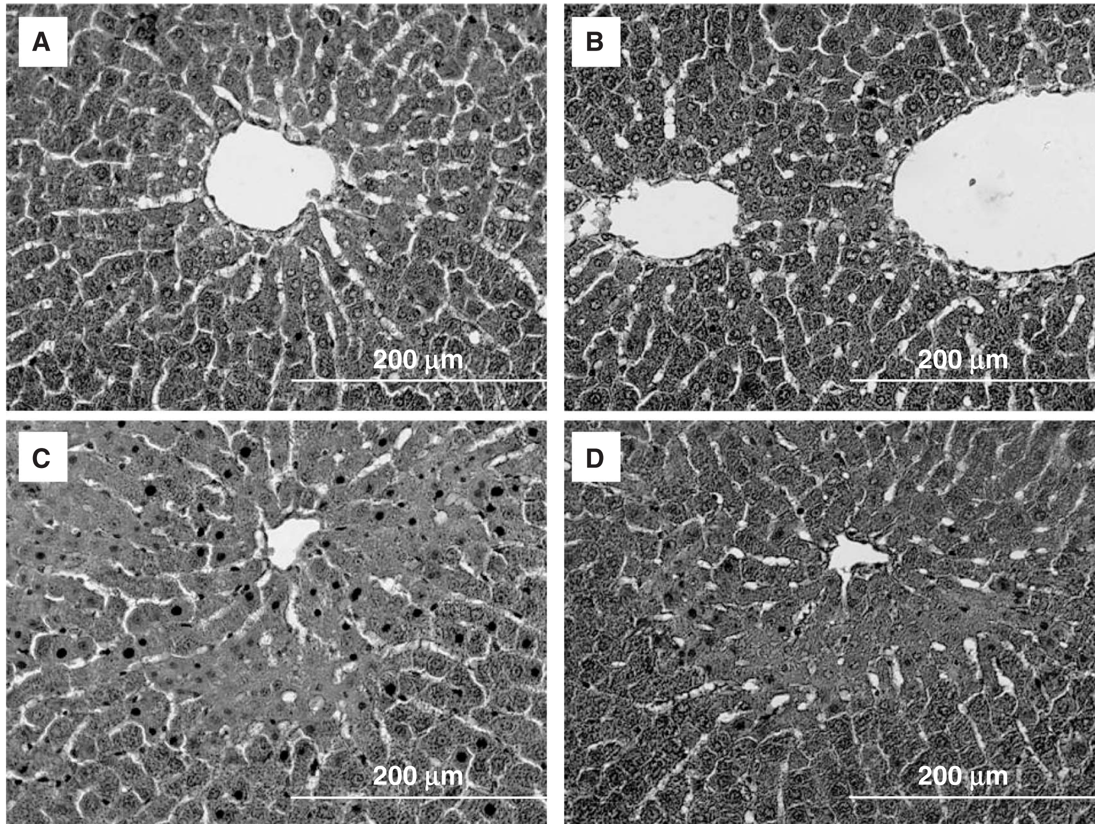

Fig. 5. Representative photomicrographs of livers of sham animals that received vehicle (A), sham animals that received astringinin (0.3 mg/kg) (B), trauma-hemorrhage animals that received vehicle (C) and trauma-hemorrhage animals that received astringinin (D). Astringinin decreased histological liver necrosis at 24 h after trauma-hemorrhage with resuscitation. Liver sections were stained with hematoxylin-eosin, examined at an original magnification ×400 and photographed.

administration following trauma-hemorrhage prevented the increase in hepatic ICAM-1 levels.

Histological Analysis of the Liver

Representative photomicrographs of liver are presented for sham animals treated with vehicle (Fig. 5A), sham animals treated with astringinin (Fig. 5B), trauma-hemorrhage animals treated with vehicle (Fig. 5C) and trauma-hemorrhage animals treated with astringinin (Fig. 5D). Similar results were obtained from four or more animals in each group. Together, these results, as presented in Fig. 5, suggest that astringinin ameliorates trauma-hemorrhage-induced damage in the liver; however, the damage remained higher than in shams.

Discussion

The liver is considered a critical organ in the development of delayed organ dysfunction in patients suffering from traumatic injuries and severe blood loss (21). Multiple organ failure or dysfunction secondary to systemic inflammatory response remains the major cause of mortality and morbidity (24). Neutrophils are the principal cells involved in host defenses against acute bacterial and fungal infections and thus are protective (15). However, under conditions such as those described in this study, infiltration of these cells may cause tissue damage (28, 30). Neutrophil movement and migration are mediated by multiple adhesion molecules on the neutrophil and endothelial cell surfaces and chemotactic factors. Initially, neutrophils interact with endothelial selectins resulting in neutrophils rolling along the endothelial surface. This rolling process appears to allow the neutrophils to become activated ("primed") by chemokines and other mediators secreted by the endothelium, resulting in their firm adhesion to endothelial adhesion molecules *via* the β1-integrins and β2-integrins (5, 22). Among adhesion molecules, ICAM-1 is an important mediator in the firm adhesion of neutrophils to the vascular endothelium and is strongly upregulated following trauma-hemorrhagic shock (4). Regarding chemokines, rat CINC-1 and CINC-3 are both members of the IL-8 family and are potent chemotactic factors for neutrophils (10). Chemotaxis of neutrophls is an important functional

response to chemokines and is a key event in the recruitment of neutrophils in inflammation. Using antibodies to CINCs, it has been demonstrated that CINC-1 and CINC-3 contribute significantly to the influx of neutrophils in rat inflammation models, including lung injury and lipopolysaccharide-induced inflammation (8, 20). Our previous studies have also indicated that CINC-1 and CINC-3 levels are correlated with tissue MPO activity, a marker of neutrophil content, following trauma-hemorrhage (21, 30). Astringinin, a natural polyphenolic compound found in nuts, red grapes and red wine, has been shown to have antioxidant activity and greater radical scavenging activity than resveratrol. Astringinin also plays a critical role in the regulation of immune and inflammatory responses of hematopoietic cells (18). Some studies have shown that astringinin possesses cardioprotection effects associated with antiarrhythmia and attenuates ischemia-reperfusion injury in rat hearts. Since free radicals generated in neutrophils are responsible for ischemic injury in myocardial tissues, it is possible that antioxidative and free radical scavenging activities of astringinin play a role not only in inhibition of reperfusion injury but also in suppression of ischemic damage by infiltrating neutrophils in the heart (22).

There is now considerable evidence for a role for astringinin in mediating the production of proinflammatory cytokines (19). A common link between the inhibitory effects of astringinin mentioned above could be its ability to inhibit factors involved in inflammatory proteins such as JNK and NF-κB (1, 9, 13). The cytokines IL-1, IL-6 and TNF-α are important early mediators in the liver and are required for expression of adhesion molecules and chemokines (14, 21). The ability of astringinin to mediate expression of inflammatory cytokines as well as adhesion molecules and chemokines suggests a role for astringinin in the regulation of hepatic inflammation. The present study is the first to examine the protective effects of astringinin in the liver following trauma-hemorrhage and to indicate that astringinin administration following trauma-hemorrhage decreases CINC-1, CINC-3 and ICAM-1 levels.

In conclusion, our study indicates that astringinin administration ameliorates hepatic injury and IL-6 production following trauma-hemorrhage. The improvement in hepatic injury following astringinin administration is likely due to a reduction of hepatic neutrophil accumulation associated with downregulation of CINC-1, CINC-3 and ICAM-1 following trauma-hemorrhage. Furthermore, the suppression of hepatic cytokine production caused by astringinin appears to contribute to the decrease in hepatic expression of chemokines and adhesion molecules. Since astringinin administration following trauma-hemorrhage decreases hepatic injury and cytokine production, this agent appears to be a novel adjunct for improving depressed hepatic functions in male animals under adverse circulatory conditions.

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