Protective effects of escin against water immersion and restraint stress-induced gastric ulcer in mice

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ABSTRACT

Background: Escin was a natural mixture of triterpenoid saponin that was isolated from the seed of the horse chestnut. The previous study from our laboratory demonstrated that escin had a protective effect against indomethacin-induced gastric ulcers in mice. The aim of this study was to investigate the effect of escin on gastric mucosal lesions induced by water immersion and restraint stress (WRS).

Materials and methods: Gastric lesion was estimated morphometrically and histopathologically 6 h after the escin administration. The activities of superoxide dismutase (SOD), catalase and the level of malondialdehyde (MDA) in the gastric mucosa were measured. Moreover, the contents of TNF-α, IL-1β, and IL-6 in gastric tissues were determined.

Results: The exposure of mice to WRS produced acute gastric lesions accompanied by a significant rise in the contents of MDA, TNF-α, IL-1β, IL-6, and a significant fall in the activities of SOD and catalase. Escin protected gastric tissues against WRS-induced gastropathy as demonstrated by a reduction in the ulcer index and an attenuation of histopathological changes. Escin significantly decreased the contents of MDA, TNF-α, IL-1β, and IL-6. The altered activities of SOD and catalase in the stomach tissues were also ameliorated by escin treatment.

Conclusion: Escin protects against a WRS-induced gastric ulcer by virtue of its antioxidant potential and anti-inflammatory effect.

Keywords: Escin, Stress, Gastric ulcer, Antioxidant, Anti-inflammatory.

INTRODUCTION

Gastric stress ulceration and bleeding were common occurrences in critically ill patients. Patients with stress ulcers had a longer hospitalization stay and a higher mortality rate than those who do not have stress ulcers (Janicki et al., 2007). Moreover, the mortality rate from stress-related mucosal bleeding was nearly 50% despite aggressive acid suppression treatment (Cook et al., 1994). Stress ulceration was diffuse lesions of the mucosal layer of the stomach and sometimes the esophagus and intestine, and frequently occurs as a result of major stressful events such as burns, shock, sepsis, surgery, and trauma. Antacids such as histamine H₂ receptor antagonist and proton pump inhibitor had been used to treat patients with stress ulcers. However, they had been suggested to be associated with the incidence of nosocomial pneumonia and the inhibition of immune function (Spirt, 2004).

Among various animal models of stress, water immersion and restraint stress (WRS) was a commonly used and clinically relevant experimental model for stress ulceration (Silen, 1988). Under WRS conditions, reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals were rapidly and continuously produced, and the resulting oxidative stress is proven crucially responsible for the development
and progression of gastric epithelial necrosis and mucosal ulceration (Shian et al., 2000). In addition, WRS was also accompanied by an acute inflammatory response of the gastric mucosa characterized by the accumulation of inflammatory cells and a multitude of inflammatory mediators (Liu et al., 1998). Recruitment and activation of neutrophils contributed greatly to the pathophysiological processes occurring in gastric mucosa at the later stage of stress (Hamaguchi et al., 2001). Synthesis and release of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have recently emerged as important determinants of mucosal inflammation and gastric injury following WRS (Okajima et al., 2000).

Escin, the major active component of Aesculus hippocastanum, was a natural mixture of triterpene saponins. Accumulating experimental evidence suggested that escin had potent antioxidative and anti-inflammatory properties (Wang et al., 2009). Aesculus hippocastanum was traditionally used to treat stomach ache in China. It was also reported that escin exerted a gastroprotective effect against ethanol-induced gastric mucosal lesions (Matsuda et al., 1999). The previous study from our laboratory demonstrated that escin had a protective effect against indomethacin-induced gastric ulcers in mice (Wang et al., 2014). However, the protective effect of escin in stress-associated ulcers has never been investigated. The aim of this study was to examine the protective effect of escin on stress-induced gastric mucosal ulceration in mice.

MATERIALS AND METHODS

Drug and chemicals

Escin, a lyophilized powder used in this experiment, was obtained from Luye Pharmaceutical Company. Nitroblue tetrazolium (NBT), trichloroacetic acid, horseradish peroxidase, H₂O₂ were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The enzyme-linked immunosorbent assay (ELISA) kits of TNF-α, IL-1β, and IL-6 were obtained from Abcam Company (Cambridge, England).

Animals

Swiss albino mice weighing 19-23 g were provided by the Experimental Animal Center of Shandong Luye Pharmaceutical Company (Yantai, China). The animals were housed in a climate-controlled room, maintained on a 12 h/12 h light/dark cycle, and had ad libitum access to food and water. The experiments were performed according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (publication 86-23, revised in 1986), and were approved by the Animal Ethics Committee of Yantai University. All efforts were made to minimize the number of animals used and their suffering.

Experimental design

A WRS-induced ulcer was produced as described by Senay et al with minor modification (Senay et al., 1967). After fasting for 18 h, the animals were randomly divided into the five groups each consisting of 16 mice: the control group, WRS group, escin (0.45 mg/kg) group, escin (0.9 mg/kg) group, and escin (1.8 mg/kg) group. The mice in the escin groups were treated orally with escin at a dose of 0.45, 0.9, or 1.8 mg/kg. The doses were selected on the basis of previous results published by our laboratory (Jiang et al. 2011 etc.). Two hours later, the mice were immobilized in a supine position on a wooden board and then were immersed up to the depth of the xiphoid level in a 25°C water bath for 4 h.

Histopathological analysis of gastric mucosal lesions of mice

Four hours after water immersion and restraint, the animals were sacrificed by means of an overdose of chloral hydrate (600 mg/kg, i.p.). The stomachs of eight mice in each group were removed and opened along the greater curvature and were then washed with ice-cold saline and examined for macroscopical mucosal lesions. The ulcer index was assessed as the method previously described (Aboubakr et al., 2013). Two persons blinded to the groups of animals performed the histological experiments. In order to eliminate an observer bias, histological sections were coded. The ulcerated portions of the stomach were fixed in 10% formaldehyde for 24 hours, embedded in a paraffin block, and cut into 4-μm sections and deparaffinized in xylene and rehydrated through a series of decreasing concentrations of ethanol. The sections were stained with hematoxylin and eosin. The pathological observation of the tissues was performed under a light microscope.

Preparation of the homogenate of gastric tissues of mice

The eight gastric tissues of each group were harvested and weighed. The gastric tissues were then homogenized in 4 volumes of 0.1 mol/L ice phosphate buffer (pH 7.4) and centrifuged at 5000 g for 30 min at 4°C. The supernatants were immediately stored at -80°C and used for the determination of the biochemical analysis and ELISA assay. The total protein was assayed by the previous method (Lowry et al., 1951).

Assays of superoxide dismutase, catalase, and malondialdehyde in the gastric tissues of mice

Superoxide dismutase (SOD) activity was determined by the NBT reduction method (McCord et al., 1969). It was
based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which reacts with NBT to form formazan dye. SOD activity was measured at 560 nm by the degree of inhibition of this reaction. Catalase (CAT) activity in the gastric tissues was measured at 37°C by following the rate of disappearance of H₂O₂ at 240 nm (Aebi et al., 1984). One unit of CAT activity was defined as the amount of enzyme catalyzing the degradation of 1 μmol of H₂O₂ per min at 37°C. The determination of malondialdehyde (MDA) content was performed according to the previous method (Ohkawa et al., 1979). The homogenate (200 μL) was added to a solution containing 8% sodium lauryl sulfate (100 μL), 20% acetic acid (800 μL), 8% 2-thiobarbiturate (800 μL) and distilled water (150 μL). The mixture was incubated at 98°C for 1 h. Upon cooling, 5 mL of n-butanol: pyridine (15:1) was then added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 g. The supernatant absorbance was measured at 532 nm.

**Assays of TNF-α, IL-1β, IL-6 in gastric tissues of mice**

The levels of TNF-α, IL-1β, and IL-6 in the gastric tissues of mice were estimated with an ELISA plate reader, using commercially available ELISA kits and following the manufacturer’s protocols. Duplicate samples were analyzed for each sample.

**Statistical analysis**

Data were expressed as means ± SD. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test. The p<0.05 was considered as statistically significant.

**RESULTS**

**Effect of escin on WRS-induced ulcer index of mice**

WRS induced a significantly high ulcer index when compared to the control group. Treatment of mice with escin at a dose of 0.45, 0.9, or 1.8 mg/kg reduced the intensity of WRS-induced ulcers and decreased the ulcer index (Figure 1).

**Effect of escin on gastric mucosal lesion formation in WRS-challenged mice**

Gross pathological studies showed that WRS caused multiple hemorrhagic erosions and bleeding in the stomach. In contrast, treatment of escin inhibited the gastric lesions induced by WRS. The histology of mice in the control group showed intact mucosal epithelium, submucosa, and an absence of any inflammatory cells infiltration. In the WRS group, superficial erosion in the mucosal epithelium with a presence of neutrophil

![Figure 1. Effect of escin on WRS-induced ulcer index of mice.](image-url)

WRS: Immersion and restraint stress.

Data were expressed as means ± SD (n=8). Statistical significances were determined using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

*“p<0.01 compared with control group; ’p<0.05, “p<0.01 compared with WRS group.*
infiltration in the serosal layer, and disruption of the mucosal layer were observed. On the other hand, stomach tissues of mice pretreated with escin showed mild neutrophil infiltration in the serosal layer and slight disruption of the mucosal layer (Figure 2).

**Figure 2.** Effect of escin on gastric mucosal lesion formation in WRS-challenged mice.

Gross pathological studies of effect of escin on gastric mucosal lesion formation. A-Control group, B-WRS group, C-Escin (0.45 mg/kg) group, D-Escin (0.9 mg/kg) group, E-Escin (1.8 mg/kg) group. Photomicrographs of escin on gastric mucosal lesion formation. (H&E). F-Control group, G-WRS group, H-Escin (0.45 mg/kg) group, I-Escin (0.9 mg/kg) group, J-Escin (1.8 mg/kg) group. Bar = 40 μm. WRS : Immersion and restraint stress.

**Effect of escin on the level of MDA in gastric tissues of mice**

The lipid peroxidation end product MDA was quantitated in the gastric tissues of mice. Compared with the control group, the level of MDA was significantly increased in the WRS group. Treatment with escin at a dose of 0.45, 0.9, or 1.8 mg/kg inhibited the increase of MDA content in gastric tissues of mice (Figure 3).

**Figure 3.** Effect of escin on the level of MDA in gastric tissues of mice.

WRS : Immersion and restraint stress.

Data were expressed as means ± SD (n=8). Statistical significances were determined using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

##p<0.01 compared with control group; *p<0.05, **p<0.01 compared with WRS group.
Effects of escin on the activities of SOD and CAT in gastric tissues of mice

A significant reduction in the activities of SOD and CAT in the WRS-challenged mice was observed as compared with that of the control group. In contrast, administration of escin at a dose of 0.45, 0.9, or 1.8 mg/kg to WRS-challenged animals partially prevented the reduction in the activities of SOD and CAT in gastric tissues (Figures 4 and 5).

Figure 4. Effect of escin on the activity of SOD in gastric tissues of mice.
WRS: Immersion and restraint stress.
Data were expressed as means ± SD (n=8). Statistical significances were determined using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.
##p<0.01 compared with control group; *p<0.05, **p<0.01 compared with WRS group.

Figure 5. Effect of escin on the activity of CAT in gastric tissues of mice.
WRS: Immersion and restraint stress.
Data were expressed as means ± SD (n=8). Statistical significances were determined using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.
##p<0.01 compared with control group; *p<0.05, **p<0.01 compared with WRS group.
Effects of escin on the levels of TNF-α, IL-1β, IL-6 in gastric tissues of mice

In this study, WRS caused a significant increase in the levels of TNF-α, IL-1β, and IL-6 in gastric tissues. However, treatment with escin at a dose of 0.45, 0.9, or 1.8 mg/kg inhibited the increase of the levels of TNF-α, IL-1β, IL-6 significantly (Figures 6, 7 and 8).

Figure 6. Effect of escin on the level of TNF-α in gastric tissues of mice. WRS: Immersion and restraint stress. Data were expressed as means ± SD (n=8). Statistical significances were determined using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. ##p<0.01 compared with control group; *p<0.05, **p<0.01 compared with WRS group.

Figure 7. Effect of escin on the level of IL-1β in gastric tissues of mice. Data were expressed as means ± SD (n=8). WRS: Immersion and restraint stress. Statistical significances were determined using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. ##p<0.01 compared with control group; **p<0.01 compared with WRS group.
DISCUSSION

Escin attenuated gastric mucosal injury induced by WRS in mice. Escin decreased stress-induced gastric mucosal hemorrhage and ulceration. Escin inhibited neutrophil infiltration into gastric mucosa in WRS-challenged mice. Furthermore, escin modulated the antioxidative parameters and the proinflammatory mediator production in gastric mucosa. The present findings suggested that escin protected against WRS-induced gastric ulcers by virtue of its antioxidant potential and anti-inflammatory effect.

The pathogenesis of the experimental mucosal damage induced by WRS, including the generation of ROS, seemed to play an important role, accompanied by impairment of the antioxidative enzyme activity of cells (Kwiecien et al., 2002). Overproduction of ROS resulted in oxidative damage, including lipid peroxidation, protein oxidation, and DNA damage, which could lead to cell death. Tissues contained various endogenous antioxidant enzymes like SOD and CAT, which scavenged ROS and therefore prevented lipid peroxidation formation. In the present study, escin increased the activities of SOD and CAT and decreased the content of MDA in the stomach tissues of mice. These findings indicated that escin possessed a protective effect against the WRS-caused oxidative damage in the gastric tissues of mice.

ROS were known to act as second messengers to regulate the expression of numerous proinflammatory genes, hence leading to tissue and cell to inflammatory injuries (Droge et al., 2002). Given that the pathogenesis of WRS-induced gastric mucosal lesions was typically associated with ROS overproduction and inflammatory molecules overexpression, and that the local generation of ROS was an initial event in the early phase of stress (Yasukawa et al., 2004), it was therefore plausible to speculate that ROS generated during stress in gastric mucosa might contribute to the development of mucosal inflammation and gastric injury. It was also reported that overproduction of inflammatory cytokines, such as TNF-α and IL-1β, was responsible for the stress-induced gastric mucosal injury. In addition, pretreatment of anti-TNF-α antibody prevented stress-induced gastric injury by decreasing TNF-α expression in rat gastric mucosa in the WRS model (Hamaguchi et al., 2001). Reducing the recruitment of inflammatory cells in the gastric mucosa by escin decreased cytokine production. It was likely that sesamol attenuated stress-related gastric mucosal ulceration by inhibiting the inflammation in the stomach.
CONCLUSION

In summary, escin protected against WRS-induced gastric ulcers by virtue of its antioxidant potential and anti-inflammatory effect. However, the other mechanism that might involve the escin-related gastric mucosal protection (e.g. the inhibition of H⁺/K⁺ ATPase) required further studies. Furthermore, although escin showed promising gastric protective effects against WRS-induced mucosal injury in the animal study, to increase its potential for clinical use required additional clinically oriented investigations.

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CONFLICT OF INTEREST

None declared.

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