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Effects of escin on acute inflammation and the immune system in mice

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Abstract:
Escin has been used extensively to treat chronic venous insufficiency, hemorrhoids, and edema resulting from cerebral ischemic damage, trauma or operation. However, no studies have looked at the anti-inflammatory properties of escin administered by intravenous injection, and it is still not clear whether escin has an effect on the immune system. This study seeks to investigate the time-dependent anti-inflammatory properties of escin and its effect on the immune system. The anti-inflammatory effect of escin was observed in carrageenan-induced paw edema and acetic acid-induced capillary permeability in mice. The immunopharmacological effects of escin were evaluated by spleen index (SI), thymus index (TI), proliferative capacity of splenocytes (PS), lymphocyte count (LC), serum TNF-α levels, and phagocytic rate (PR) in mice. Escin treatment showed a significant anti-inflammatory effect, similar to that seen with dexamethasone treatment. However, the duration of the anti-inflammatory response was longer with escin treatment than with dexamethasone treatment. The results also demonstrated that escin had no significant effects on SI, TI, LC, PS, TNF-α levels, and PR. The findings suggest that escin is a potent anti-inflammatory drug with long-lasting anti-inflammatory effects and without any immunosuppressive effects.

Key words:
escin, dexamethasone, inflammation, immune function

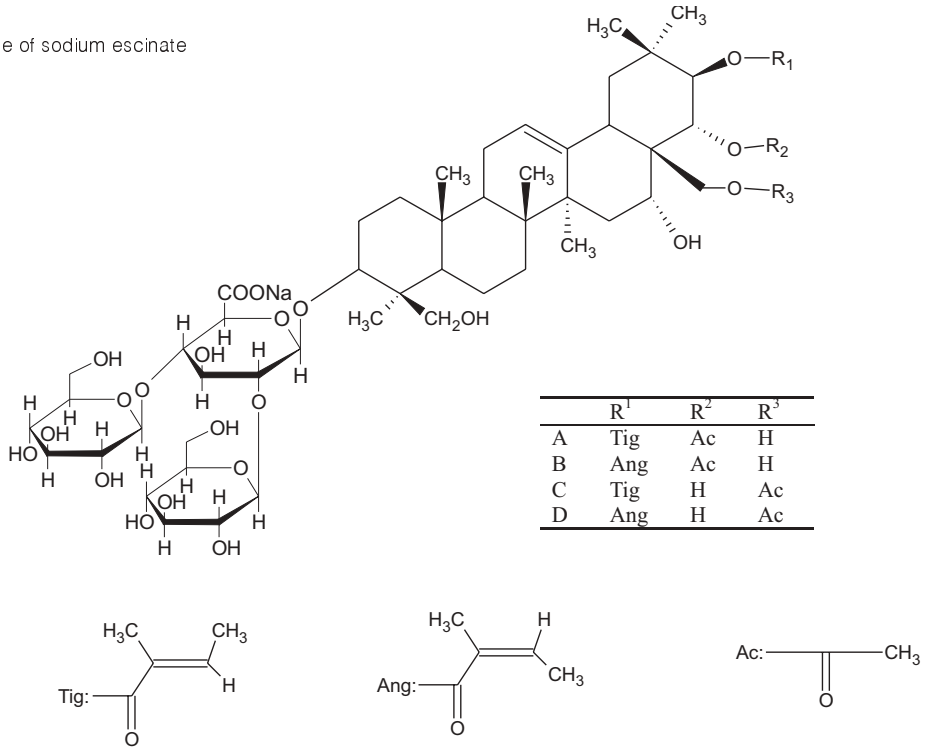
Abbrevations: CRBC – chicken red blood cells, DX – dexamethasone, LC – lymphocyte count, PR – phagocytic rate, PS – proliferative capacity of splenocytes, SA – sodium escinate, SI – spleen index, TI – thymus index

Introduction

Escin is a natural mixture of triterpene saponins, which mainly consist of A, B, C, and D escin (Fig. 1) [8]. Because escin possesses anti-inflammatory effects, it has been extensively used to treat chronic venous insufficiency, hemorrhoids, and edema resulting from trauma or operation [16]. However, up to now, there are no studies looking at the anti-inflammatory properties of escin administered by intravenous injection.

Currently, it is thought that inflammation is part of the non-specific immune response that occurs in response to any type of bodily injury. Therefore, some agents are both anti-inflammatory and immunosuppressive when administered therapeutically. Dexamethasone (DX), a steroid anti-inflammatory agent, is widely used to treat inflammatory diseases. However, DX has multiple effects that inhibit the immune

Fig. 1. The structure of sodium escinate



	R^1	R^2	R^3
A	Tig	Ac	H
B	Ang	Ac	H
C	Tig	H	Ac
D	Ang	H	Ac

system, and glucocorticoids are also associated with an increased susceptibility to infection and a risk for reactivation of latent tuberculosis [10]. Therefore, it is essential to know whether escin also suppresses immune functions to the same extent as steroids when it exerts its anti-inflammatory effect. In this paper, the duration of the anti-inflammatory effects of escin and its immunopharmacological effects were investigated in mice.

Materials and Methods

Materials and chemicals

The experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80–23, Revised 1978). Male Swiss mice (20 to 22 g body weight) were obtained from the Experimental Animal Center of Shandong Province (Certificate No. 20041225). The animals were housed under diurnal lighting conditions (12 h/12 h) and allowed free access to food and water. Sodium escinate (SA), sodium salt of escin, provided by Luye Pharmaceutical Company is a lyophilized power, which was initially made in China in 1994. Dexamethasone (DX) was purchased from Harbin Pharmaceutical Group Holding Ltd. Company (Harbin, PR China, No. 050208-2).

Carrageenan-induced paw edema

Thirty mice were randomly divided into three groups with ten mice in each of the following groups: vehicle group, SA group (2 mg/kg), and DX group (reference drug, 4 mg/kg). Mice were treated with the same volume of normal saline or drug *via* the tail vein. At the same time, inflammation was induced by a single subplantar injection of 30 μl of freshly-prepared 2% suspension of carrageenan in 0.9% normal saline solution into the right plantar aponeurosis of mice [17]. Paw volume was measured before and at 1, 2, 4, 6, 8, 12, 18, and 24 h after treatment with a plethysmometer (ZH-YLS-7A, China). For each animal, the percentage of edema was expressed as percent increase in paw volume following carrageenan injection, and the area under the curve (AUC) was calculated to evaluate the anti-inflammatory effects [18].

Acetic acid-induced capillary permeability

The effect of escin on vascular permeability induced by acetic acid in mice was determined according to

Olajide's method with minor modifications [12]. In brief, 144 mice were randomly divided into three groups with 48 mice in each of the following groups: vehicle group, SA group (2 mg/kg), and DX group (reference drug, 4 mg/kg). Mice in each group were further divided into eight subgroups. Mice were treated with the same volume of either normal saline or drug *via* the tail vein. At 1, 2, 4, 6, 8, 12, 18, and 24 h after treatment, the mice were killed by cervical dislocation. Thirty min before cervical dislocation, each mouse was injected intravenously with 0.2 ml of 1.0% Evans blue (Sigma, St. Louis, MO, USA) in saline solution *via* the tail. At 10 min after injection of the dye solution, 0.2 ml of 1% (v/v) acetic acid in saline solution was injected intraperitoneally. Then, the viscera were exposed and irrigated with distilled water, which was then poured into 10 ml volumetric flasks through the glass wool. Each flask was made up to 10 ml final volume with distilled water, and 0.1 ml of 0.1 M NaOH solution was added. The absorption of the final solution was measured at 590 nm *via* an automated ELISA reader (Synergy HT, USA).

Evaluation of the immunological functions

A total of 50 mice were randomly divided into five groups with ten mice in each of the following groups: vehicle group, mice that were given normal saline, SA (1 mg/kg) group (SA1), SA (2 mg/kg) group (SA2), and SA (4 mg/kg) group (SA4), DX group (DX), and mice that were given 4 mg/kg of DX. Mice were administered the same volume of either normal saline or drugs once a day for five days.

Neutrophil, monocyte, and lymphocyte count

Eight hours following the last administration, mice were anesthetized with sodium pentobarbital at 300 mg/kg, *ip*. Blood was obtained from the retro-orbital venous plexus using 4 mg/ml of EDTA (Sigma, St. Louis, MO, USA) as an anti-coagulant, and neutrophils, monocytes, and lymphocytes were counted using the blood cell arithmometer (ABX micros 60, France).

Spleen index and thymus index

SI and TI were measured according to the previous method [4]. Spleen and thymus of the mice were removed and weighed under sterile conditions. SI = spleen weight (mg) / body weight (g) × 100. TI = thymus weight (mg) / body weight (g) × 100.

Proliferative capacity of splenocytes

PS was determined using a previously described method [4]. Briefly, the spleens were minced into small pieces and passed through a tissue sieve (200 mesh per inch) to prepare a single-cell suspension in RPMI 1640. The cell suspension was centrifuged at 300 × g for 15 min. Then, the supernatant was discarded, and the precipitate was resuspended in RPMI 1640. The suspension was layered onto Ficoll-hypaque (density = 1.077) and separated by density-gradient centrifugation at 1,000 × g for 30 min. Splenocytes were at the top of the Ficoll and in the form of a white band. The upper liquid was gently removed. Splenocytes were transferred to a new tube and washed three times with RPMI 1640. Splenocytes were resuspended in 2 ml of RPMI 1640 complete media. The cell density was counted and adjusted to 10^7 cells/ml. 100 μl of cell suspension and 100 μl of 5 μg/ml of concanavalin A (ConA, Sigma, St. Louis, MO, USA) in RPMI 1640 or 100 l of PBS were added to a 96-well plate. The cells were incubated at 37°C with 5% CO_2. After a 72-h incubation, methylthiazolyldiphenyl-tetrazolium bromide (Sigma, St. Louis, MO, USA) was added to the cell culture to a final concentration of 5 μg/ml. The cells were incubated for another 4 h whereupon 100 μl of acidified isopropyl alcohol was added to the culture and vibrated for 10 min. The absorbance of each sample was read using an automated ELISA reader (Synergy HT, USA) at 570 nm. Splenocyte proliferation activity = A–B (A is the OD_{570} of wells with ConA, and B is the OD_{570} of wells with PBS).

Phagocytosis by peritoneal macrophages

The mice were randomly divided into five groups with ten animals in each group and treated as described the experiment of evaluation of the immunological functions. Phagocytosis by peritoneal macrophages was detected according to a previously described method [19]. Chicken blood collected under an axenic condition was put into a sterile flask containing a crystal ball and shaken to remove the fiber. The solution was rinsed three times with PBS and centrifuged at 1,000 × g for 10 min. The supernatant was discarded, and the chicken red blood cells

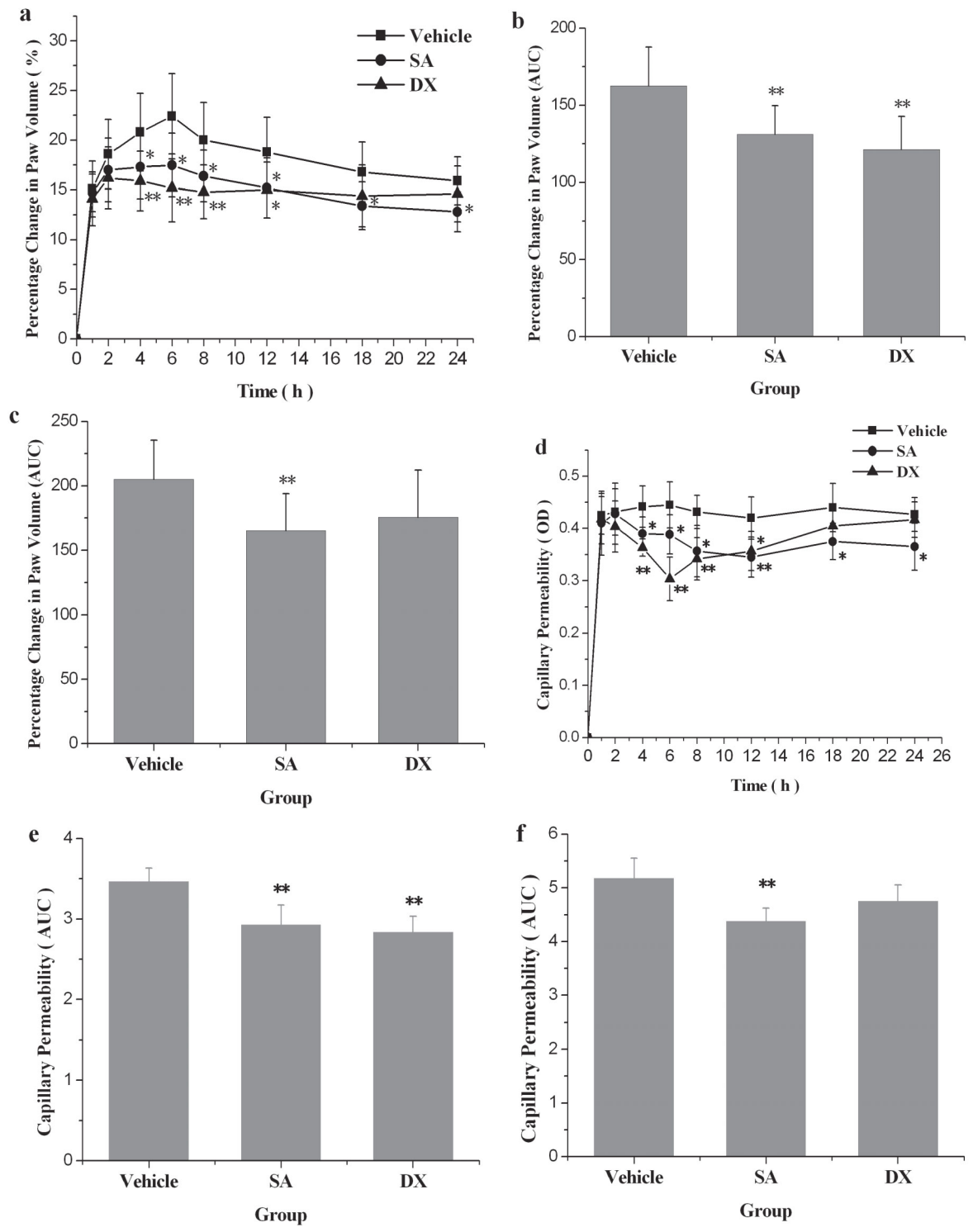


Fig. 2a, b, c. The time-dependent anti-inflammatory response of sodium escinate (SA) on carrageenan-induced paw edema. (**a**) The anti-inflammatory effect of SA is expressed as the percent increase in paw volume following carrageenan injection. (**b**) The anti-inflammatory effect of SA is expressed as the AUC during the period from 4 h to 12 h after SA injection. (**c**) The anti-inflammatory effect of SA is expressed as the AUC during the period from 12 to 24 h after SA injection. Data are represented as the means ± SEM, n = 10 mice per group. * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated group. **Fig. 2d, e, f.** The time-dependent anti-inflammatory response of SA on acetic acid-induced capillary permeability. (**d**) The anti-inflammatory effect of SA is expressed as the absorption of abdominal cavity effusion at 590 nm. (**e**) The anti-inflammatory effect of SA is expressed as the AUC during the period from 4 to 12 h after SA treatment. (**f**)The anti-inflammatory effect of SA is expressed as the AUC during the period from 12 to 24 h after SA treatment. Data are represented as the means ± SEM, n = 6 mice per group. * $p < 0.05$, ** $p < 0.01$ compared to vehicle-treated group

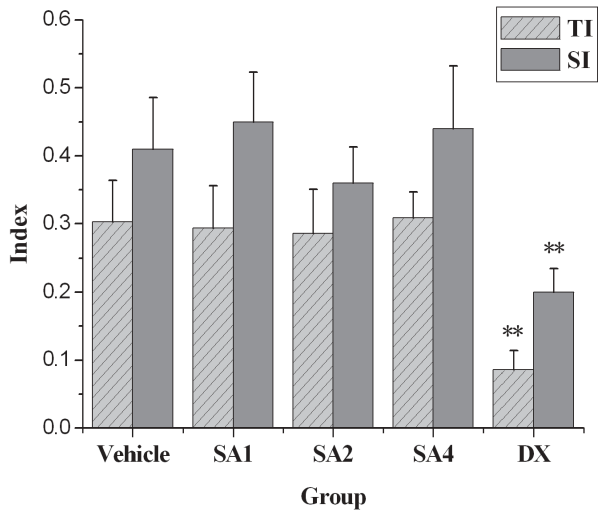


Fig. 3. The effect of sodium escinate (SA) on spleen index (SI) and thymus index (TI) in mice. SA1– SA 1 mg/kg group; SA2 – SA 2 mg/kg group; SA4 – SA 4 mg/kg group; DX – dexamethasone group. Eight hours following the last SA or dexamethasone administration, the spleen and thymus of the mice were removed and weighed under sterile conditions. Spleen index = spleen weight (mg) / body weight (g) × 100. Thymus index = thymus weight (mg) / body weight (g) × 100. Data are represented as the means ± SEM, n = 10 mice per group. ** $p < 0.01$ compared to vehicle-treated group

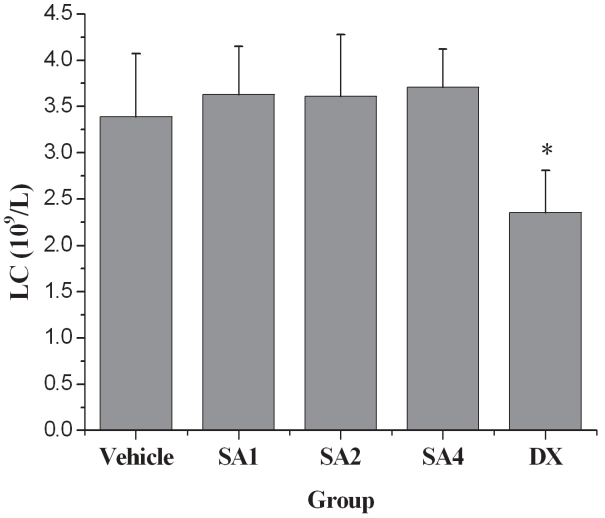


Fig. 5. The effect of sodium escinate (SA) on lymphocyte count (LC) in mice. SA1 – SA 1 mg/kg group; SA2 – SA 2 mg/kg group; SA4 – SA 4 mg/kg group; DX – dexamethasone group. Lymphocytes were counted with a blood cell arithmometer. The value 10^9/L represents the lymphocyte count in every liter of blood. Data are represented as the means ± SEM, n = 10 mice per group. ** $p < 0.01$ compared to vehicle-treated group

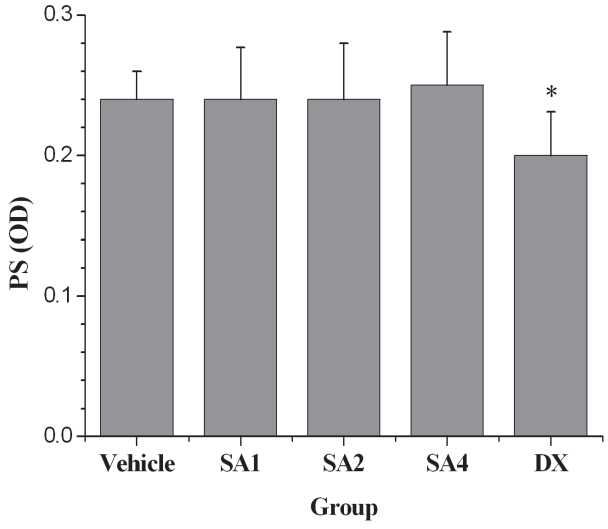


Fig. 4. The effect of sodium escinate (SA) on proliferative capacity of splenocytes (PS) in mice. SA1 – SA 1 mg/kg group; SA2 – SA 2 mg/kg group; SA4 – SA 4 mg/kg group; DX – dexamethasone group. OD represents optical density, which was assayed with an automated ELISA reader at 570 nm. Data are represented as the means ± SEM, n = 10 mice per group. * $p < 0.05$ compared to vehicle-treated group

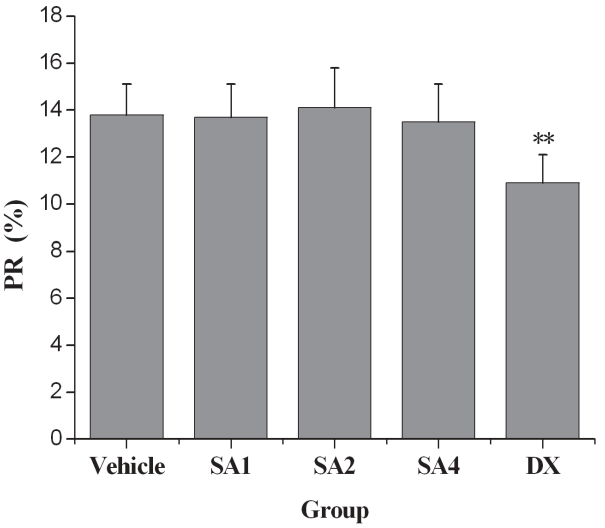


Fig. 6. The effect of sodium escinate (SA) on phagocytic rate (PR) in mice. SA1 – SA 1 mg/kg group; SA2 – SA 2 mg/kg group; SA4 – SA 4 mg/kg group; DX – dexamethasone group. The number of macrophages ingesting CRBC out of a total of at least 100 cells was calculated by direct visual enumeration using a light microscope. The PR was calculated using the following formula: PR (%) = (Number of macrophages ingesting CRBC / Number of total macrophages) ×100. Data are represented as the means ± SEM, n = 10 mice per group. ** $p < 0.01$ compared to vehicle-treated group

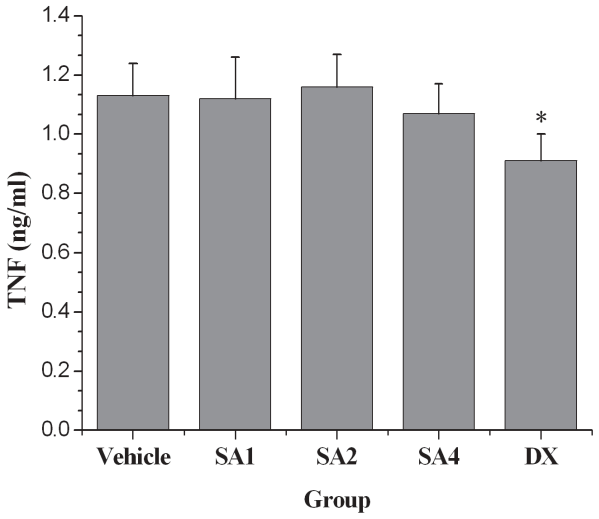


Fig. 7. The effect of sodium escinate (SA) on TNF-α in mice. SA1 – SA 1 mg/kg group; SA2 – SA 2 mg/kg group; SA4 – SA 4 mg/kg group; DX – dexamethasone group. Data are represented as the means ± SEM, n = 10 mice per group. * $p < 0.05$ compared to vehicle-treated group

(CRBC) were resuspended in PBS to a final concentration of 20% (v/v). The mice were administered the same volume of normal saline, SA or DX once a day for five days. Eight hours after the last administration, 1 ml of CRBC was injected intraperitoneally into each mouse. After 30 min, the mice were euthanized. Two milliliters of saline was injected into the abdominal cavity and 1 ml of fluid was then collected to make a smear for each mouse. The smears were incubated at 37°C for 30 min in a wet box fixed with acetone–methanol solution of a 1 to 1 (v/v) ratio and then stained with 4% (v/v) Giemsa-phosphoric acid dye. The number of macrophages ingesting CRBC out of a total of at least 100 cells was calculated by direct visual enumeration using a light microscope. The phagocytic rate (PR) was calculated using the following formula: PR (%) = (Number of macrophages ingesting CRBC / Number of total macrophages) ×100.

TNF-α assay

Fifty mice were randomly and evenly divided into five groups and were treated according to the above procedures. Eight hours after the last administration, mice were injected intraperitoneally with LPS (*Escherichia coli* serotype 0111:B4, Sigma; 10 mg/kg). After 2 h, mice were anesthetized with 300 mg/kg, *ip* of sodium pentobarbital. Blood was sampled and left at room temperature for 1 h to allow complete clotting. After centrifugation at 1,500 × g for 15 min, serum was separated from the blood cells, and the level of TNF-α in the serum was assayed by enzyme linked immunosorbent assay according to the manufacturer's instructions (Bender MedSystem, Vienna, Austria). The values for the amount of TNF-α in the serum are expressed as pg/ml. The detection limit was 0.13 pg/ml. Duplicate samples were analyzed for each sample.

Statistics analysis

Results from these experiments were analyzed using ANOVA. *Post-hoc* comparisons were carried out by Tukey-Kramer test. Data are presented as the means ± SEM. A level of $p < 0.05$ was considered statistically significant.

Results

A time-dependent inhibitory effect of escin on inflammatory responses

Compared to vehicle treatment, both SA and DX treatment significantly inhibited paw edema and capillary permeability after 4 h of treatment (Fig. 2a and 2d). When the anti-inflammatory effects of SA and DX were evaluated as the AUC during the period from 4 to 12 h after SA or DX injection, both SA and DX treatment showed significant anti-inflammatory effects (Fig. 2b and 2e, $p < 0.01$). SA also showed a significant anti-inflammatory effect during the period of 12 to 24 h after SA treatment, as evaluated by the AUC (Fig. 2c and 2f, $p < 0.01$).

The effects of escin on immune organs and immune cells

TI and SI in SA-treated mice did not change significantly ($p > 0.05$), but DX-treated mice had a significant decrease in TI and SI (Fig. 3, $p < 0.01$). SA had no effect on the PS ($p < 0.05$). The PS in the DX group was significantly lower than that of the vehicle group (Fig. 4, $p < 0.05$). Compared to the vehicle group, there was no marked change in the LC of mice treated with 1, 2, and 4 mg/kg of SA ($p < 0.05$). Treatment with DX resulted in a significant decrease in the

LC (Fig. 5, $p < 0.01$). SA had no effect on PR ($p > 0.05$). The PR of the DX group revealed a significant decrease when compared to that of the vehicle group (Fig. 6, $p < 0.01$). Neither SA nor DX had a significant effect on neutrophil and monocyte counts (data not shown).

The effect of escin on the level of TNF-α

SA treatment did not result in a significant decrease in the level of TNF-α, while the level of TNF-α in the DX group was reduced significantly (Fig. 7, $p < 0.05$).

Discussion

Carrageenan-induced paw edema and acetic acid-induced vascular permeability in animals are common models to study inflammation. Intraplantar injection of carrageenan in rats or mice leads to paw edema. The first phase of paw edema results from the concomitant release of histamine, serotonin, and kinins, and the second phase correlates with elevated production of prostaglandins, oxygen-derived free radicals, and inducible cyclooxygenase (COX-2), in addition to local neutrophil infiltration and activation [5, 6]. Although mouse paw edema elicited by carrageenan is not as well explored as rat paw edema, it has been shown to be a useful and an interesting model to study inflammation [9]. Acetic acid induces vascular permeability, which could cause an increase in prostaglandin E_2, prostaglandin F_2, serotonin, and histamine in the peritoneal fluids [7]. This leads to dilation of the arterioles and venules causing an increase in vascular permeability.

Edema or vascular permeability, which is one of the cardinal signs of acute inflammation, is an important parameter to be considered when evaluating compounds with potential anti-inflammatory activity. In the present study, the effect of escin on acute inflammation was investigated in carrageenan-induced paw edema and acetic acid-induced vascular permeability. At the same time, the anti-inflammatory effect of escin was compared to DX, which is used extensively to treat severe acute inflammation. The results indicate that both escin and DX significantly reduced carrageenan and acetic acid-induced inflammation (paw edema and vascular permeability). Compared to vehicle treatment alone, escin inhibited carrageenan-induced paw edema and acetic acid-induced vascular permeability from 4 to 24 h post-treatment. DX reduced carrageenan-induced paw edema and acetic acid-induced vascular permeability from 4 to 12 h post-treatment. These results suggest that escin has the same efficacy as DX, but with longer duration of effectiveness.

Acute inflammatory response is characterized by key elements such as an increase in the number and activity of inflammatory cells and an increase in the level of pro-inflammatory cytokines, which are considered to be fundamental components of the innate immune system. Therefore, it is essential to know whether escin affects these immune functions when it exerts its anti-inflammatory effect.

The spleen and thymus are two important immune organs. The spleen contains lymphocytes and macrophages, which phagocytose and destroy bacteria and dead tissue in order to remove them from the circulating blood. Lymphocytes are activated when microorganisms such as bacteria, fungi, and viruses circulate through the spleen [11]. Once lymphocytes are activated, they proliferate and become effector T cells or plasma cells. The main function of the thymus is to develop immature T cells into mature T cells [15]. The data from this study demonstrate that 1, 2, and 4 mg/kg of escin have no effect on the immune organs (spleen and thymus) and their function (PS).

Inflammation has very specific characteristics, and the innate immune system plays a pivotal role as the first line of response. Infiltration of innate immune cells, specifically neutrophils and macrophages, defines acute inflammation, while infiltration of T lymphocytes and monocytes are features of chronic inflammation [13]. Both monocytes and macrophages play central roles during chronic inflammation [1]. TNF-α is also one of the most prominent inflammatory mediators and is absolutely central in the stimulation of the inflammatory responses including the induction of cytokine production, activation and expression of adhesion molecules, and stimulation of growth [3, 17]. The present study demonstrates that escin did not significantly affect the LC, PR, or TNF-α levels in mice. Therefore, it suggests that escin has no effect on the immune cells and their function.

Glucocorticoids have a multitude of effects on the immune response at several sites and are both anti-inflammatory and immunosuppressive when administered therapeutically. Although glucocorticoids are ef-

fective for treatment of a wide variety of inflammatory diseases, long-term therapy with glucocorticoids causes osteoporosis, resulting in severe bone loss, which has recently become a major clinical problem [14]. However, a recent study showed that escin does not exert significant effects on bone parameters in rats, and it may reduce the unfavorable effects of prednisolone on the width of periosteal and endosteal osteoid and periosteal transverse growth in the tibia in prednisolone-treated rats [2].

In conclusion, this study demonstrates that escin is a safe and potent anti-inflammatory drug with a long-lasting anti-inflammatory effect without any immunosuppression.

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