SUPPLEMENTARY MATERIAL

Topical application of *Scutellariae baicalensis* suppresses 2,4-dinitrochlorobenzene-induced contact dermatitis

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Allergic contact dermatitis is a prototypic T-cell-mediated cutaneous inflammatory response. In the present study we describe the anti-allergic effect of topically applied *Scutellaria baicalensis* aqueous extract (WSBE) in suppressing 2,4-dinitrochlorobenzene (DNCB)-induced allergic contact dermatitis in BALB/c mice. Topically applied WSBE attenuated the epidermal thickness and mast cell infiltration into the skin in DNCB-induced contact dermatitis. Furthermore, WSBE suppressed DNCB-induced production of serum IgE as well as IL-4, IFN- γ , and TNF- α in the skin. Topical application of WSBE also ameliorated the significant decrease in dermal glutathione and superoxide dismutase levels. Moreover, present results demonstrated that the baicalin, bioactive compound of WSBE, was able to penetrate into the skin following topical application, which was confirmed by the HPLC analysis using rat model. Taken together, topical application of WSBE exerts beneficial effects in contact dermatitis model, suggesting that WSBE might be a candidate for the treatment of contact dermatitis.

Keywords

2,4-dinitrochlorobenzene; contact dermatitis; mouse; *Scutellariae baicalensis*

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Experimental

Preparation of standardized S. baicalensis aqueous extract

The roots of *S. baicalensis* was obtained from local herbal market in Jangheung, Korea and authenticated by Dr. Kim of Jeollanamdo Development Institute for Traditional Korean Medicine. A voucher specimen (No.2011-SB-07) was deposited at the herbarium of Jeollanamdo Development Institute for Traditional Korean Medicine. The WSBE was prepared following the previous method (Kim et al., 2013). In brief, the roots of *S. baicalensis* had extracted by refluxing with boiling water for 1 h, and the resultant extract was spray-dried. The dried powder was stored at 4°C before use. The quantitative analysis of WSBE was performed using high-performance liquid chromatography (HPLC) according to the previous method and the contents of baicalin, wogonoside, baicalein, and wogonin in WSBE were 6.45%, 3.37%, 2.07%, and 0.48%, respectively (Kim et al., 2013).

Animals and treatments

For dermatitis model study, female BALB/c mice weighing 16-18 g (7 weeks old) were obtained from Orient Bio (Seungnam, Korea). They were housed in the animal facilities of Chungnam national University Animal Research Resources Center on a $23 \pm 2^\circ\text{C}$ of room temperature, $55 \pm 3\%$ of relative humidity and 12 h of light/dark cycle. Animals were fed food and water ad libitum. After 1 week of acclimation, the backs of mice were shaved with an electric clipper and depilatory cream, and washed with sterilized PBS-gauzed a day before sensitization. The mice were randomly divided into six groups ($n=5/\text{group}$) as follows: negative control group (cream base alone), positive group (DNCB + cream base), dexamethasone group (DNCB + 0.1% dexamethasone cream), 0.1% WSBE group (DNCB + 0.1% WSBE cream), and 0.5% WSBE group (DNCB + 0.5% WSBE cream). Each gram of creams contains (w/w) 1 mg of dexamethasone (Sigma Aldrich, MO, USA) and WSBE (1 and 5 mg) in an emollient cream base (containing propylene glycol, stearyl alcohol, acetyl alcohol, sorbitan monostearate, polysorbate 60, mineral oil and purified water; Kyonghee Skin biotechnology Center, Suwon, Korea) respectively. Mice were topically given with approximately 20 mg of the freshly prepared dexamethasone cream, WSBE creams or emollient cream base alone on the back skin of each mouse every day from day 1 to 14. Allergic contact dermatitis was induced following the methods from the Manetz et al. (2001) with minor modification. For the sensitization process, 1 cm² gauze attached patch (3M Health Care, MN, USA), applied with 0.1 mL of 1% DNCB in acetone/olive oil (3:1), was attached to the shaved backs of the mice for four hours on day one and four. At four days later, mice were challenged on dorsal back by 0.2% DNCB patch on day 8 and 11. Three days after last challenge

of 0.2% DNCB (day 14), mice were killed by cardiac puncture after being anesthetized lightly with carbon dioxide. Collected blood samples were separated by centrifugation at 800 g for 15 min, and the serum samples were subjected to biochemical investigations. Back skin samples of each mice were taken for histopathological and biochemical examination. For skin absorption study, male Sprague-Dawley rats weighing 210-230 g (Orient Bio) were used. They were acclimated and housed under same condition with mice. For the topical application, the backs of rats were shaved with an electric clipper and the rats (n=3) were given topically 500 mg kg⁻¹ of WSBE cream formulation once daily for 3 consecutive days. Occlusive dressing was placed on the applied region for 24 h to withhold licking or scratching the area. Blood samples were collected from the tail vein into heparinized tubes at designated times after last topical application. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Chungnam National University (Deajeon, Korea).

Serum IgE antibody measurements

Total IgE level in serum were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA; Komabiotec, Seoul, Korea) according to the manufacturer's protocols.

Cytokine production in skin

Cytokines levels were determined by ELISA. Skin samples from back of each animals were weighed (100 mg) and homogenized in 1 ml of T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail. Homogenates were then centrifuged at 12,000 g for 20 min at 4°C to obtain the supernatant. IL-4, TNF- α , and IFN- γ levels were analyzed using ELISA kit (Komabiotec, Seoul, Korea) according to the manufacturer's instructions. Cytokine levels were normalized with a protein contents and expressed as pg mg⁻¹.

Lipid peroxidation, nitric oxide, superoxide dismutase, and glutathione content in skin

The content of malondialdehyde (MDA), a terminal product of lipid peroxidation, was measured by the TBARS reduction based on Ohkawa et al. (1979) method using a commercially available kit (BioAssay systems, CA, USA). The skin GSH levels were determined using the improved DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] method first described by Tietze (1969) (BioAssay Systems). NO was determined by colorimetric assay based on Griess reaction (BioAssay Systems). Total SOD activity in

skin homogenates was assessed by the disappearance of superoxide detected by a tetrazolium salt (Cayman Chemical, MI, USA). All the assays were performed according to the manufacturer's guidelines.

Histopathological examination

Skin were fixed immediately in a 10% buffered formalin phosphate solution, embedded in paraffin, and cut into 5 μ m sections. Skin samples were stained with hematoxylin and eosin to evaluate epidermal hyperplasia and infiltration of immune cells in the dermis, as well as toluidine blue to count the number of mast cells in five random fields ($\times 200$) using a biological image analysis package (ImageJ, MD, USA).

Skin absorption following topical administration of WSBE

After blood collection at each time point, the samples were centrifuged immediately, and the plasma was collected and stored at -70°C until analysis. Plasma concentration of the baicalin was determined using HPLC as described in Lu et al.'s study (Lu et al., 2007).

Statistical analysis

Results were expressed as means $\pm$ standard error (SEM). The significance of differences among experimental groups was determined using one-way analysis of variance (ANOVA) or the corresponding nonparametric (Kruskal-Wallis) test, as required. Where significant effects were found, post hoc analysis using the Tukey's multiple comparison test or Mann-Whitney U-test was performed, and $p < 0.05$ was considered to be statistically significant.

References

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Figure S1. Histopathological features of 2,4-dinitrochlorobenzene-induced skin lesions in the *S. bacalensis* aqueous extract (WSBE) treated BALB/c mice. The skin sections were stained with hematoxylin and eosin (A) and toluidine blue (B). Skin thickness (C) and the number of mast cell per high power field (D) were determined in five random sites. Results are presented as mean $\pm$ standard error of the mean. ^a $p < 0.05$, a significant difference in comparison with the negative control group. ^b $p < 0.05$, a significant difference in comparison with the positive group.

Figure S2. The effect of topically applied *S. bacalensis* aqueous extract (WSBE) on serum immunoglobulin E (IgE) level in 2,4-dinitrochlorobenzene-induced skin lesion. Values are means $\pm$ standard error of the mean. ^a $p < 0.05$, a significant difference in comparison with the negative control group. ^b $p < 0.05$, a significant difference in comparison with the positive group.

Figure S3. The effect of topically applied *S. bacalensis* aqueous extract (WSBE) on TNF- α (tumor necrosis factor- α ; A), IL-4 (interleukin-4; B), and IFN- γ (Interferon- γ ; C) production in 2,4-dinitrochlorobenzene-induced skin lesion. Values are means $\pm$ standard error of the mean. ^a p <0.05, a significant difference in comparison with the negative control group. ^b p <0.05, a significant difference in comparison with the positive group.

Figure S4. The effect of topically applied *S. bacalensis* aqueous extract (WSBE) on GSH (glutathione; A), NO (nitric oxide; B), SOD (superoxide dismutase; C), and TBARS (thiobarbituric acid; D) content in 2,4-dinitrochlorobenzene-induced skin lesion. Values are means $\pm$ standard error of the mean. ^a $p < 0.05$, a significant difference in comparison with the negative control group. ^b $p < 0.05$, a significant difference in comparison with the positive group.

Figure S5. Mean plasma concentration–time curve of baicalin following topical application of *S. bacalensis* aqueous extract (WSBE) in SD rats. Three rats were given topically WSBE (500 mg kg⁻¹) once daily for 3 days with WSBE cream. Results are presented as mean $\pm$ standard error of the mean.