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Preliminary report

Effects of topically applied Korean red ginseng and its genuine constituents on atopic dermatitis-like skin lesions in NC/Nga mice☆

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ABSTRACT

Ginseng (the root of *Panax ginseng* C.A. Meyer, family Araliaceae) possesses various biological activities, including anti-inflammatory and anti-tumor actions. However, their topical effect on atopic dermatitis (AD) has not been studied yet. The aim of this study was to examine the therapeutic effects of topical Korean red ginseng saponin fraction (KRGS) and its genuine constituents on AD-like skin lesions in an AD mouse model. The therapeutic effect of topical KRGS and ginsenosides in 2-chloro-1,3,5-trinitrobenzene (TNCB)-treated NC/Nga mice was assessed by measuring the skin severity scores, ear thickness, histological changes of lesional skin including mast cell count, tissue tumor necrosis factor (TNF)-α, interleukin (IL)-4, and interferon (IFN)-γ mRNA expression, and total serum IgE. Topical administration of 0.1% KRGS, 0.1% Rh2 and 0.1% Rh2 + 0.1% Rg3 significantly reduced the clinical skin severity scores, ear thickness and mast cell infiltration in the TNCB-induced AD-like skin lesions. Topical application of KRGS and its constituents significantly reduced TNCB-induced increase in ear TNF-α and IL-4 mRNA expression, but not IFN-γ mRNA expression. There was little change of serum total IgE level by topical KRGS and its constituents. In this study, topical KRGS and ginsenosides Rh2 and Rg3 were found to exert an anti-inflammatory effect *in vivo* and proved to be beneficial in an animal model of AD. Our results suggest that KRGS and its ginsenosides Rh2 and Rg3 have potential as a topical agent for the treatment of AD.

1. Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin condition which has steadily increased over the past 20–30 years [1]. The main treatment goals include the elimination of inflammation and infection, preserving and restoring the barrier function and controlling exacerbation factors. Most people with mild to moderate AD are treated with topical agents alone. Although topical glucocorticosteroids and topical calcineurin inhibitors have strong anti-inflammatory properties, there are safety concerns with their long-term usage. There are many known long-lasting and possible irreversible adverse effects associated topical steroids including skin atrophy, striae, and telangiectasia. Overall local side effects commonly noted with topical calcineurin inhibitors include local cutaneous effects (e.g., erythema, pruritus and irritation), but preclinical animal studies with topical calcineurin inhibitors have demonstrated an increased risk of cutaneous malignancy [2]. It is therefore imperative that alternative topical agents be developed to manage this condition.

To date, several natural substance based anti-atopic agents have been topically tested as potential therapeutics for AD [3–5]. Korean red ginseng (KRG, the steamed root of *Panax ginseng* C.A. Meyer, family Araliaceae) is frequently used as a crude substance taken orally in Asian countries as a traditional medicine. The major components of raw ginseng are ginsenosides, which contain an aglycone with a dammarane skeleton [6]. Ginsenosides have been reported to exhibit various biological activities, including anti-inflammatory and anti-tumor effects [7,8]. In addition, the anti-allergic properties of ginsenosides have recently been noted [9–13]. Tachikawa et al. reported that ginsenoside Rg3 suppress histamine release from mast cells after stimulation with compound 48/80 *in vitro* [12], and Ro et al. reported that ginsenosides Rb1 and Rc partly inhibit the release of histamine and leukotrienes during the activation of guinea pig lung mast cells *in vitro* [10]. The anti-allergic and anti-inflammatory effects of ginsenosides Rh1, anti-allergic effect of ginsenosides Rh2 and anti-allergic and anti-contact dermatitis activity of KRGS and ginsenosides Rg3, Rf and Rh2 have also been identified [9,11,13]. The anti-allergic

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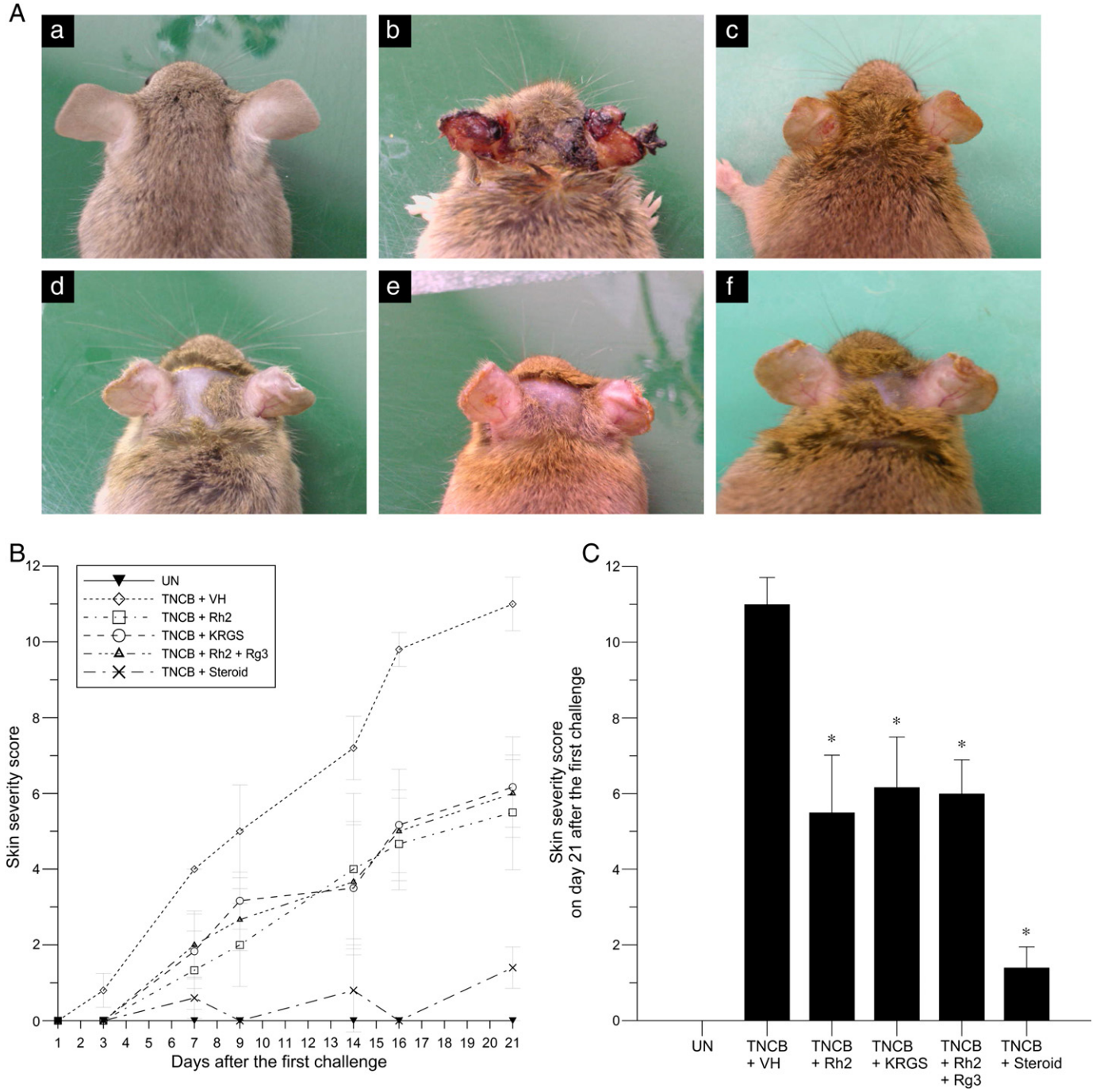
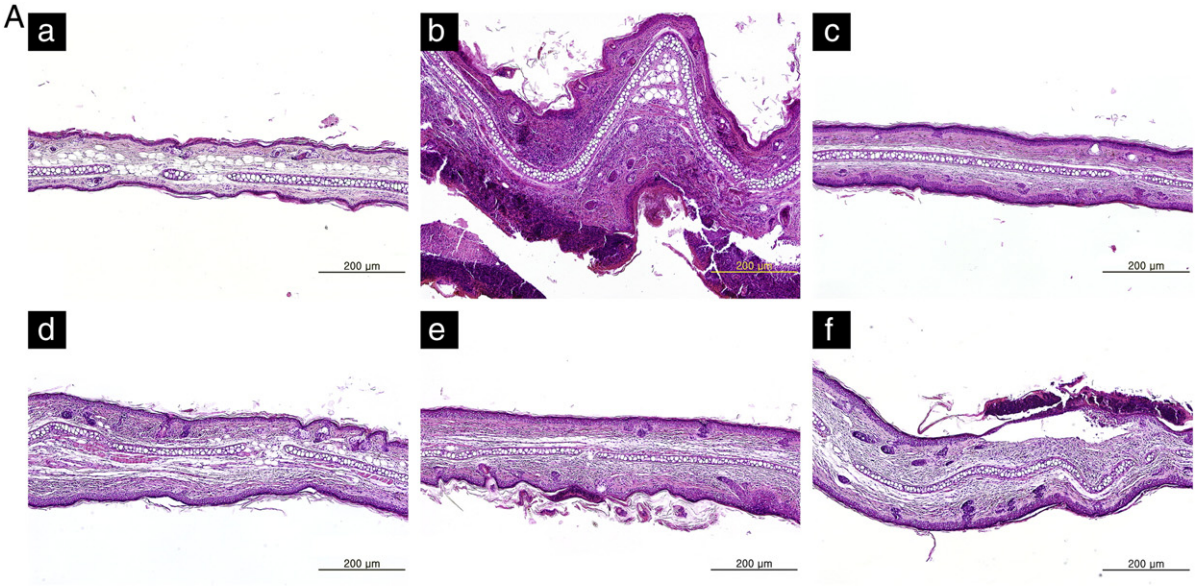


Fig. 1. (A) Photographs of the clinical features of NC/Nga mice (a) in the untreated (UN), (b) TNCB plus vehicle (TNCB + VH), (c) TNCB + 0.1% Rh2, (d) TNCB + 0.1% KRGS, (e) TNCB + 0.1% Rh2 + 0.1% Rg3, and (f) TNCB plus 0.05% betamethasone (TNCB + steroid), on day 21 following the first challenge. (B) Clinical skin severity scores. (C) Clinical skin severity scores on day 21 following the first challenge. Data are presented as mean ± SEM ($n = 7$). *$p < 0.05$ vs. TNCB + VH.

properties of KRGS and ginsenosides have strongly suggested their possibility as potential anti-atopic agents. However, the effect of topical KRGS and ginsenosides in AD has not been studied yet.

NC/Nga mice spontaneously develop AD-like skin lesions when maintained under conventional condition, and has been considered as one of the most valuable mouse models representing human AD [14]. However, the low incidence of AD-like lesions, late onset of the disease and poor reproducibility are disadvantages of this model [15]. To overcome this problem, contact sensitizers have been adopted in NC/Nga mice [16,17]. Repeated application of TNCB to the same skin site is known to result in a shift in the time course of antigen-specific hypersensitivity response from a typical delayed type hypersensitivity to an immediate-type response followed by a late reaction. Development of this hypersensitivity response is antigen specific, and the shift is associated with epidermal hyperplasia, accumulation of large numbers of mast cells and CD4+ T cell beneath the epidermis, and elevated serum levels of antigen-specific IgE [16]. Such TNCB-induced features are representative of AD.

In this study, we examined the therapeutic effect of topical Korean red ginseng saponin fraction (KRGS) and ginsenosides Rh2, Rg3 on the AD-like skin lesions in NC/Nga mice. Assessment was made by measuring the skin severity scores, ear thickness, histological changes of skin including mast cell count, skin TNF-α, IL-4, and IFN-γ mRNA expression and total serum IgE levels in each of the study groups.


A
a
b
c
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e
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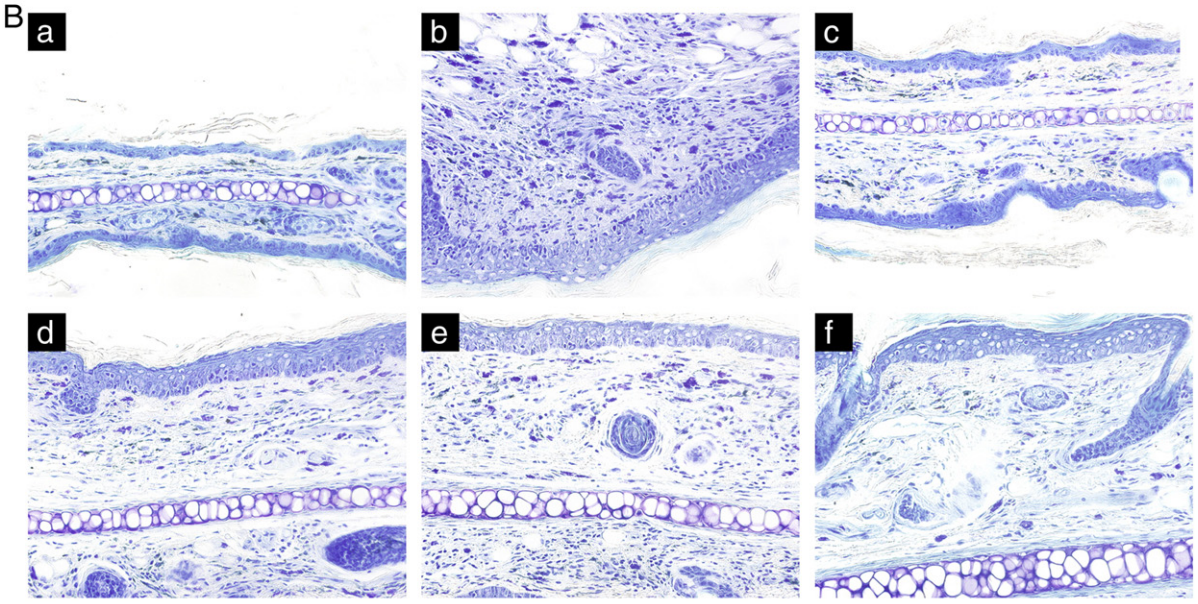
200 μm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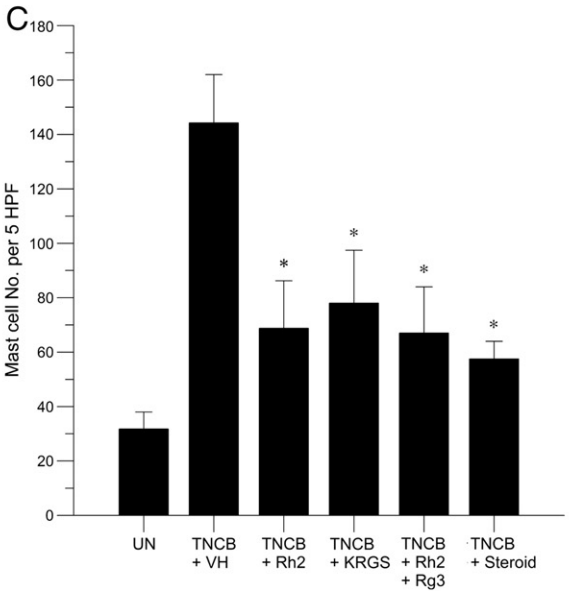
C
Mast cell No. per 5 HPF
0
20
40
60
80
100
120
140
160
180
*
UN
TNCB + VH
TNCB + Rh2
TNCB + KRGS
TNCB + Rh2 + Rg3
TNCB + Steroid

2. Materials and methods

2.1. Chemicals and animals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Forty-two 4-week-old female NC/Nga mice were purchased from Japan SLC (Shizuoka, Japan) and maintained under semi-SPF conditions at 24 ± 1 °C and a humidity of 50 ± 10%, under a 12-hour/12-hour light/dark cycle. Food and water was freely available. The animal care, handling, and experimental procedures were performed in accordance with a protocol approved by the Animal Care and Use Committee of The Catholic University of Korea and all procedures were conducted in accordance with the U.S. National Institutes of Health guidelines. The KRGS and ginsenosides Rh2 and Rg3 in this study were kindly provided by Prof. Dong Hyun Kim (College of Pharmacy, Kyung Hee University, Seoul, Korea). The KRGS in our study contained ginsenosides Rb1 (4.5%), Rg1 (2.8%), Rg3 (6.7%), and Rh2 (0.4%). The characteristics of the ginsenosides are as follows: ginsenoside Rh2 (purity, >95%). White needle (MeOH); m.p. 219–221 °C; (+)-FAB-MS *m/z*: 623 $[M+H]^+$, ginsenoside Rg3 (purity, >95%). White needle (MeOH); m.p. 248–250 °C; (+)-FAB-MS *m/z*: 785.5 $[M+H]^+$. The KRGS and ginsenosides Rh2 and Rg3 were all free of endotoxin.

2.2. Induction of AD-like skin lesions and topical application of KRGS and ginsenosides in NC/Nga mice

The NC/Nga mice were equally allocated into 6 groups (the untreated (UN), TNCB plus vehicle (TNCB + VH), TNCB + 0.1% Rh2 (TNCB + Rh2), TNCB + 0.1% KRGS (TNCB + KRGS), TNCB + 0.1% Rh2 + 0.1% Rg3 (TNCB + Rh2 + Rg3) and TNCB plus 0.05% betamethasone (TNCB + steroid)). 2-Chloro-1,3,5-trinitrobenzene (TNCB)-induced AD-like skin lesions in NC/Nga mice was produced as previously described with slight modifications [17]. Briefly, the abdomens and ears were sensitized epicutaneously by applying 150 μl of 5% TNCB dissolved in ethanol/acetone mixture (4:1 vol/vol) once. Five days after sensitization, the dorsal surface of the ears was challenged, each with 20 μl of 1% TNCB dissolved in acetone/olive oil (4:1 vol/vol). 1% TNCB was repeatedly applied to the ears 3 times a week for 21 days following the first challenge.

Rh2 (0.1%), KRGS (0.1%), and Rh2 (0.1%) + Rg3 (0.1%) each dissolved in dimethyl sulfoxide (DMSO)/ethanol (1:20 vol/vol) were topically applied to the dorsal side of the ears (20 μl per ear) of each group 30 min before and 3 h after TNCB painting starting from the first challenge (TNCB + Rh2, TNCB + KRGS, TNCB + Rh2 + Rg3 groups). DMSO/ethanol (1:20 vol/vol) and 0.05% betamethasone were applied in the same manner to the TNCB + VH and TNCB + steroid groups, respectively.

2.3. Measurement of skin severity scores and ear thickness

The severity of dermatitis was assessed macroscopically and photographed in a blinded fashion using the following scoring procedure twice a week starting from the first challenge. The total skin severity score was defined as the sum of individual scores for each of the following 4 signs and symptoms: erythema (hemorrhage), edema (swelling), excoriation (erosion) and dryness. Each of these items were scored as 0 (none), 1 (mild), 2 (moderate) and 3 (severe), as previously described [7]. Ear thickness was measured using a dial caliper (Kori Seiki MFG, Japan) twice weekly. Assessment was performed by 2 separate investigators who were blind with the grouping of the animals.

2.4. Histopathology

The mice were sacrificed on day 21 after the first challenge. Ear samples were fixed in 10% vol. phosphate-buffered formalin solution, embedded in paraffin and sectioned at 4 μl. The tissue sections were stained with hematoxylin and eosin (H&E) for microscopic examination. For identification of the mast cells, skin sections were stained with toluidine blue. The mast cells were counted independently by 2 researchers in 5 randomly chosen visual fields at ×400 magnification.

2.5. Reverse transcription–polymerase chain reaction (RT-PCR)

The expression of mRNA transcripts of TNF-α (forward: 5′-CATCTTCTCTCAAAATTCGAGTGACAA-3′, reverse: 5′-TGGGAGTAGACAAGGTACAACCC-3′), IL-4 (forward: 5′-TCTCGAATGTACCAGGAGCCATATC-3′, reverse: 5′-AGCACCTTGGAAGCCCTACAGA-3′) and IFN-γ (forward: 5′-CGGCACAGTCATTGAAAGCCTA-3′, reverse: 5′-GTTGCTGATGGCCTGATTGTC-3′) were determined by real-time RT-PCR. Total RNA was isolated using TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the protocol provided by the manufacturer. Equal amounts of RNA were reverse transcribed into cDNA using oligo$(dT)_{15}$ primers. iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) and iCycler iQ™ Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) were used for real-time PCR analysis. For amplification, samples were heated to 94 °C for 8 min and cycled 30 times at 94 °C for 30 s, and 60 °C for 30 s, and 72 °C for 40 s. Using standards, the amount of TNF-α, IL-4, and IFN-γ cDNA was determined and normalized by the amount of β-actin cDNA.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Blood was collected from the retroorbital plexus using heparinized glass capillary tubes at the end of the experiment (day 21 following the first challenge). Serum samples obtained by centrifugation (3000*g* for 5 min at 4 °C) were stored at −80 °C until use. Concentration of total IgE in serum was determined using the mouse IgE ELISA kit (Shibayagi Co. Ltd., Gunma, Japan) according to the manufacturer's instruction.

2.7. Statistical analysis

All data are expressed as the mean ± standard error of the means (S.E.M). One-way analysis of variance followed by Tukey's multiple comparison test was used for statistical analysis. In all cases, p values <0.05 were considered significant.

3. Results

3.1. Effect of topical KRGS and its ginsenosides on TNCB-induced AD-like skin lesions

Repeated topical application of TNCB on the ears of NC/Nga mice induced AD-like skin lesions such as erythema, edema, excoriation and scaling (Fig. 1A). The skin severity scores of the TNCB + VH group progressively increased with time, reaching a score of more than 11 points on day 21 after the first challenge. The skin severity scores of the groups TNCB + Rh2, TNCB + KRGS, and TNCB + Rh2 + Rg3 were significantly lower compared to that of TNCB + VH group from day 7 ($p<0.05$) (Fig. 1B). On day 21 after the first challenge, the skin severity scores were reduced by 50%, 44%, and 45% in the 0.1% Rh2, 0.1% KRGS, and 0.1%

Fig. 2. (A) Histological features of ear skin in the untreated (UN) (H & E; original magnification, × 100). (B) Effect of KRGS and ginsenosides on TNCB-induced infiltration of mast cells in the ears of NC/Nga mice (toluidine blue; original magnification, × 400). (C) The number of mast cells in 5 randomly chosen visual fields at ×400 magnification. HPF indicates high-power fields. (a) The untreated (UN), (b) TNCB plus vehicle (TNCB + VH), (c) TNCB + 0.1% Rh2, (d) TNCB + 0.1% KRGS, (e) TNCB + 0.1% Rh2 + 0.1% Rg3, and (f) TNCB plus 0.05% betamethasone (TNCB + steroid) at day 21 following the first challenge. Data are presented as mean ± SEM ($n=7$). *$p<0.05$ vs. TNCB + VH.

Rh2 + 0.1% Rg3 applied groups, respectively, compared to the TNCB + VH group (Fig. 1C). TNCB also significantly increased the ear thickness in NC/Nga mice. Treatment with 0.1% Rh2, 0.1% KRGS, and 0.1% Rh2 + 0.1% Rg3 significantly reduced TNCB-induced ear thickening on day 21 by 61%, 47%, and 44%, respectively ($p<0.05$) (data not shown). However, there was no significant difference in the skin severity scores and ear thickness among the KRGS and ginsenoside groups.

3.2. Effect of topical KRGS and its ginsenosides on TNCB-induced epidermal thickening and mast cell infiltration in the ear skin

As shown in Fig. 2A, marked epidermal thickening and excessive leukocyte infiltration in the dermis were observed in the ears of NC/Nga mice following TNCB application (TNCB + VH group). In contrast, ear sections from the TNCB + Rh2, TNCB + KRGS and TNCB + Rh2 + Rg3 groups all showed marked decrease in epidermal hyperplasia and inflammatory cell infiltration in the dermis.

We further investigated the effect of topical KRGS and its constituents in the infiltration of mast cells, one of the most important effector cells involved in AD. As shown in Fig. 2B, treatment with TNCB increased mast cell infiltration in the dermis ($p<0.05$). The mast cell number was significantly reduced in groups treated with 0.1% Rh2, 0.1% KRGS, and 0.1% Rh2 + 0.1% Rg3 as well as 0.05% betamethasone, compared to the TNCB + VH group ($p<0.05$) (Fig. 2C). We found no significant difference in mast cell number among groups treated with 0.1% KRGS, 0.1% Rh2, 0.1% Rh2 + 0.1% Rg3 and 0.05% betamethasone.

3.3. Effect of topical KRGS and its ginsenosides on TNCB-induced mRNA expression of TNF-α, IL-4, and IFN-γ in the ear skin

The mRNA expression of IL-4, IFN-γ and TNF-α was minimal in the UN group. However, there was a significant increase in their expression following TNCB application (Fig. 3). KRGS and its constituents as well as 0.05% betamethasone significantly suppressed TNCB-induced mRNA expression of TNF-α and IL-4 compared to the vehicle ($p<0.05$) (Fig. 3A and B). TNCB-induced IFN-γ mRNA expression was significantly reduced by 0.05% betamethasone, but not by KRGS and its constituents (Fig. 3C).

3.4. Effect of topical KRGS and its ginsenosides on TNCB-induced total serum IgE levels

The total serum IgE levels on day 21 following the first challenge were markedly higher in the TNCB + VH group compared to the UN group ($p<0.05$) (Fig. 4). KRGS and ginsenosides as well as 0.05% betamethasone failed to reduce total serum IgE levels following TNCB application.

4. Discussion

We demonstrate for the first time that topical KRGS and ginsenosides Rh2 and Rg3 markedly improves AD in NC/Nga mice, an animal model of AD. Macroscopic analysis revealed severe erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness in the TNCB plus vehicle group, whereas topical KRGS and its constituents alleviated the severity of these skin changes. In addition, topical KRGS and ginsenosides suppressed TNCB-induced increase in ear thickness. Histologically, topical KRGS and its constituents led to a decrease in hypertrophy, hyperkeratosis and infiltration of inflammatory cells in the skin. Mast cells are key effector cells in the development of various allergic diseases including AD [18], and in the TNCB-treated mice model, the number of mast cells was found to be directly proportional

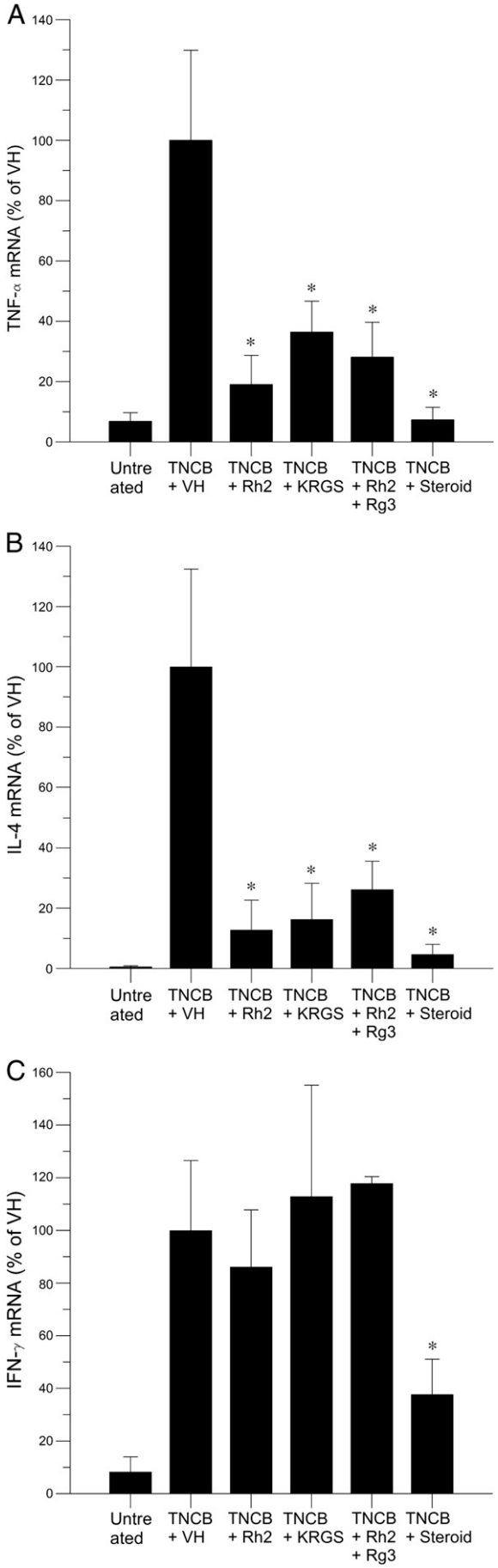

Fig. 3. Effects of KRGS and ginsenosides on mRNA expression of (A) TNF-α, (B) IL-4 and (C) IFN-γ in the ears of NC/Nga mice. Data are presented as mean ± SEM ($n=7$). *$p<0.05$ vs. TNCB + VH.

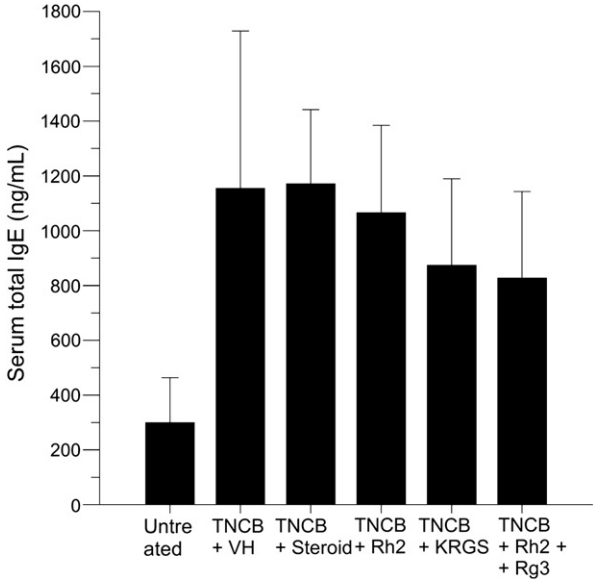

Fig. 4. Serum levels of IgE on day 21 following the first challenge. Data are presented as mean ± SEM ($n = 7$).

to the ability of the mice to mount an immediate-type response to the contact sensitizer [16]. In our study, repeated topical application of TNCB on the ears of NC/Nga mice resulted in heavy mast cell infiltration in the dermis, fulfilling the criteria of an AD animal model [14]. The extensive mast cell infiltration in the TNCB plus vehicle group was largely suppressed by topical KRGS and ginsenosides. Together with the information from Bae and coworkers [11], who previously demonstrated KRGS and ginsenosides to block the degranulation of mast cells, we are able to speculate that topical KRGS and ginsenosides have significant suppressive effects on local mast cells.

The production of proinflammatory cytokines, such as TNF-α, by epidermal cells is known as one of the main events mediating the initiation of AD [19]. TNF-α and other proinflammatory cytokines produced at the initiation stage of AD induce the expression of a variety of chemokines and adhesion molecules which direct the recruitment, proliferation and survival of leukocytes within the skin [19]. In our study, KRGS and ginsenosides significantly suppressed TNCB-induced elevation of TNF-α mRNA expression in the ears of NC/Nga mice which likely have affected leukocyte recruitment at the site. Kitagaki et al. [20] reported that Th2-type cytokines, especially IL-4, are predominantly produced by T cells and mast cells in the chronic contact hypersensitivity model. Therefore, the production of these cytokines in our own chronic contact hypersensitivity model was measured. The quiescent nature of the normal skin was striking with minimal background of IL-4 and IFN-γ observed in the untreated group. Repeated exposure to TNCB caused in an increase in mRNA levels of IL-4 and IFN-γ, where topical KRGS and ginsenosides were found to significantly alter tissue IL-4, but not IFN-γ mRNA expression. The results suggest that the suppressive effects of topical KRGS and ginsenosides on AD-like skin lesions in NC/Nga mice are possibly mediated by local tissue Th2 response modification.

The development of an immediate-type response to TNCB is linked to the capacity of the mice to develop antigen-specific IgE antibodies [16]. We were not able to measure the TNCB-specific IgE level, but only the serum total IgE in our study, to find a definite increase following TNCB application. Rh2 (0.1%), KRGS (0.1%), Rh2 (0.1%) + Rg3 (0.1%) as well as betamethasone (0.05%) failed to modify the TNCB-induced elevation of serum total IgE. Contrasting reports of oral and intraperitoneal administration of KRGS and ginsenosides Rh2 and Rg3 inhibiting the passive cutaneous anaphylaxis (PCA) reaction induced by IgE [11] suggest that their effect is largely dependent on the administration route.

In conclusion, our results demonstrate that topical KRGS and its ginsenosides suppress TNCB-induced AD-like skin lesions in NC/Nga mice. This may have been mediated, at least in part, by suppressing the local Th2 response and TNF-α mRNA expression. Further studies are required to elucidate the mechanism of action of topical KRGS and ginsenosides on AD, but based on our findings, KRGS and ginsenosides may be considered a potential topical therapeutic agent in the management of AD. Investigations to identify the major components of KRGS that are responsible for its therapeutic effect on AD are currently in progress.

In this preliminary study, we were not able to measure the skin permeability of the individual substances that we have tested. Although we did not find significant difference in the anti-atopic effect between the KRGS and ginsenosides Rh2 and Rg3, it is possible that agents with higher permeation would result in better outcomes when topically applied. With the positive results of topical KRGS and ginsenosides on an atopic dermatitis mouse model, we hope to perform the skin permeation test in our next study and identify ginsenosides with the best bioavailability.

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