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Cultivated ginseng inhibits 2,4-dinitrochlorobenzene-induced atopic dermatitis-like skin lesions in NC/Nga mice and TNF- α /IFN- γ -induced TARC activation in HaCaT cells

Jae Ho Choi^{a,1}, Sun Woo Jin^{a,1}, Bong Hwan Park^a, Hyung Gyun Kim^a, Tilak Khanal^a, Hwa Jeong Han^a, Yong Pil Hwang^{a,b}, Jun Min Choi^c, Young Chul Chung^c, Sang Kyu Hwang^d, Tae Cheon Jeong^{e,*}, Hye Gwang Jeong^{a,*}

^aDepartment of Toxicology, College of Pharmacy, Chungnam National University, Daejeon 305-764, Republic of Korea

^bDepartment of Pharmaceutical Engineering, International University of Korea, Jinju, Republic of Korea

^cDepartment of Food Science, International University of Korea, Jinju, Republic of Korea

^dDepartment of Administration, International University of Korea, Jinju, Republic of Korea

^eCollege of Pharmacy, Yeungnam University, Gyeongsan 712-749, Republic of Korea

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ABSTRACT

Ginseng contains many bioactive constituents, including various ginsenosides that are believed to have anti-allergic, anti-oxidant, and immunostimulatory activities; however, its effects on atopic dermatitis (AD) remain unclear. In the current study, we hypothesized that cultivated ginseng (CG) would inhibit 2,4-dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in NC/Nga mice by regulating the T helper (Th)1/Th2 balance. Also, CG inhibits TNF- α /IFN- γ -induced thymus- and activation-regulated chemokine (TARC) expression through nuclear factor-kappa B (NF- κ B)-dependent signaling in HaCaT cells. CG ameliorated DNCB-induced dermatitis severity, serum levels of IgE and TARC, and mRNA expression of TARC, TNF- α , IFN- γ , IL-4, IL-5, and IL-13 in mice. Histopathological examination showed reduced thickness of the epidermis/dermis and dermal infiltration of inflammatory cells in the ears. Furthermore, CG suppressed the TNF- α /IFN- γ -induced mRNA expression of TARC in HaCaT cells. CG inhibited TNF- α /IFN- γ -induced NF- κ B activation. These results suggest that CG inhibited the development of the AD-like skin symptoms by modulating Th1 and Th2 responses in the skin lesions in mice and TARC expression by suppressing TNF- α /IFN- γ -induced NF- κ B activation in keratinocytes, and so may be a useful tool in the therapy of AD-like skin symptoms.

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1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease with a clinical presentation of erythematous skin, lichenification with cutaneous hypersensitivity, and intense pruritus (Leung, 2000). AD is a multifactorial skin disease, with complex interactions of innate and adaptive immune responses based on a strong genetic, environmental, pharmacological, and psychological predisposition and triggered by environmental factors (Udompatikul and Limpa-o-vart, 2012). Various factors, including immunological abnormalities contribute to the pathogenesis and development of AD. The early onset of AD in infancy results in it being the most

prevalent chronic skin disorder in childhood and can affect individuals throughout their lifetimes (Choi et al., 2012a,b).

The NC/Nga strain originated from Japanese mice and is the most extensively studied animal model of AD. Skin changes develop spontaneously in NC/Nga mice exposed to various allergens and closely mimic human AD. NC/Nga mice have been reported to develop AD-like eczematous skin lesions when kept in conventional conditions but not when maintained under specific pathogen-free (SPF) conditions (Hiramoto et al., 2010). To reduce experimental error and to induce a stable clinical AD-like skin disease, many researchers have used the chemical haptens, DNCB, 2,4-dinitrofluorobenzene (DNFB), and 2,4,6-trinitro-1-chlorobenzene (TNCB). Application of the haptens DNCB, DNFB, or TNCB produces an AD-like skin disease in NC/Nga mice (Park et al., 2012; Kim et al., 2011). DNCB was used on the basis of evidence of its efficacy in creating AD-like skin lesions and IgE hyperproduction in NC/Nga mice (Jung et al., 2010; Yang et al., 2011; Fujii et al., 2009). The pathogenesis of DNCB-induced contact hypersensitivity is predominantly the result of a T cell mediated immune response (Zhang

* Corresponding authors. Tel.: +82 53 810 2819 (T.C. Jeong), tel.: +82 42 821 5936 (H.G. Jeong).

E-mail addresses: hgjeong@cnu.ac.kr (T.C. Jeong), taecheon@ynu.ac.kr (H.G. Jeong).

¹ These authors are contributed equally to this work.

et al., 2009). The scratching behavior is the first symptom of the AD-like skin changes and is followed by rapidly developing hemorrhages, edema, scarring, and erosion of the face, ears, neck, and back. Histological examination shows dermal infiltration with mast cells, mononuclear cells, and eosinophils prior to the appearance of clinical skin symptoms.

The pathogenesis of AD is not fully understood, but specific immune and inflammatory mechanisms culminate in a complex series of cellular interactions, leading to the signs and symptoms of AD (Liu et al., 2011). Along with the AD-like skin changes, NC/Nga mice show preferential Th differentiation toward Th2 cells in the spleen, accumulation of eosinophils and mast cells in the skin lesions, and increased IgE levels (Kohara et al., 2001; Vestergaard et al., 1999). Acute AD skin lesions reveal the important role of the Th2 type immune response, characterized by the infiltration of CD4⁺ T cells and the secretion of interleukin (IL)-4, IL-5, and IL-13, whereas in chronic AD skin lesions, Th1 type immune responses, characterized by the production of tumor necrosis factor (TNF)- α and interferon (IFN)- γ by Th1 cells, and delayed-type hypersensitivity reactions are responsible (Jin et al., 2009; Leung and Bieber, 2003; Werfel et al., 1996). Recent reports have shown that typical AD symptoms involve increased levels of Th2-mediated cytokines and a deficiency in Th1-mediated cytokines (Sawada et al., 2012). Immunoglobulin E (IgE) production is also closely related to AD; indeed, elevated IgE levels are a hallmark of AD and the expression of IL-4 contributes to this elevation. IL-4 stimulates IgE production in B cells. IgE is released from B cells and then binds to mast cells. Mast cells degranulate and release various biological mediators in IgE-mediated AD (Amin, 2012; Kishimoto and Hirano, 1988; Poulsen and Hummelshøj, 2007). AD is dependent on the secretion of the cytokines IL-4, IL-5, and IL-13 by Th2 cells that are generated from precursors. Furthermore, recent studies have shown that T cells constitutively expressing CD25, T regulatory cells (Tregs), regulate AD (Stassen et al., 2004; Szegedi et al., 2009). It has been suggested that IL-10 production by Tregs suppresses both Th1- and Th2-mediated immune responses (Wilson et al., 2007). Thus, it is important to consider the balance among Th1, Th2, and Treg cells in AD patients.

Cultivated ginseng (CG, *Panax ginseng* C.A. Meyer) is one of the most widely used medicinal herbs in traditional and oriental medicine (de Andrade et al., 2007). It has many biological and pharmacological effects, including anti-inflammatory, anti-oxidative, anti-tumor, and anti-aging activities (Jung et al., 2012; Kim et al., 2012; Wong et al., 2010; Ho et al., 2010). We previously reported that CG suppresses ultraviolet B-induced collagenase activation via mitogen-activated protein kinases and nuclear factor-kappa B (NF- κ B)/activator protein-1 (AP-1)-dependent signaling in human dermal fibroblasts (Hwang et al., 2012). Although CG has been reported to have activity in various assays, the effects of CG as an anti-atopic agent for AD remain unknown. In this study, we examined the inhibitory effects of CG on the development of AD in *in vivo* and *in vitro*.

2. Materials and methods

2.1. Chemicals

DNCB (2,4-dinitrochlorobenzene) was obtained from Sigma-Aldrich (Milwaukee, WI, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IgE and TARC were obtained from BD Biosciences (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA). Polymerase chain reaction (PCR) oligonucleotide primers were custom synthesized by Bioneer Co. (Daejeon, Korea). All chemicals and solvents were of the highest commercially available grade.

2.2. Preparation of cultivated ginseng (CG)

Mountain CG (*Panax ginseng* C.A. Meyer; Jangnoisam), naturally grown in forested areas, was collected and identified by the Ham Yang Agricultural Association (Ham Yang, Korea). Cultivated ginseng roots used in this experiment were 5 years

old. The CG roots (585 g) were cut into small pieces and extracted twice at 90 °C in distilled water (DW) for 5 h. The solutions were combined, filtered through Whatman No. 1 filter paper, concentrated using a rotary vacuum evaporator (EYELA, Tokyo Rikakikai Co., Tokyo, Japan) under reduced pressure, refrigerated in a recirculating chiller (EYELA CCA-1110) to obtain concentrated extracts, and then lyophilized (EYELA FD-800). The concentration of ginsenosides, the major active ingredients, was measured by high-performance liquid chromatography (HPLC) (Lee et al., 2007). The composition of CG was described previously (Choi et al., 2007; Lee et al., 2007). CG was dissolved with distilled water and then mixed with emollient cream at 5 or 20 mg/kg concentration.

2.3. Cell culture

The spontaneously immortalized human keratinocyte cell line HaCaT (gift from Fusenig, German Cancer Research, Germany) (Fusenig and Boukamp, 1998) was cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. Cells (2.5 $\times$ 10⁵) were plated in 60 mm plates and cultured until confluent. Cells were treated with CG for 1 h, and then stimulated with TNF- α and IFN- γ (each 20 ng/mL) for 24 h in serum-free culture medium. Control cells were treated with DW alone.

2.4. Measurement of cytotoxicity

Cytotoxicity was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay and a lactate dehydrogenase (LDH) release assay kit (Roche, Indianapolis, IN, USA), both according to the manufacturer's instructions. Briefly, the cells (5 $\times$ 10⁴/500 μ L) were seeded in 48-well plates in 10% FBS-containing medium. After cells had been subjected to the MTT reduction assay, the supernatants were used in the LDH release assay. After incubation, the cells were treated with MTT solution (final concentration, 1 mg/mL) for 1 h. The dark blue formazan crystals that formed in intact cells were solubilized with dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was measured with a spectrophotometer (Varioskan, Thermo Electron Co., Finland). LDH levels in the supernatants were quantified using a colorimetric test based on the NADH concentration. CG did not interfere with the absorbance measurements at the wavelengths used in these assays.

2.5. Animals and treatment

Specific pathogen free male 6-week-old NC/Nga mice were obtained from SLC, Inc. (Shizuoka, Japan). Animals were acclimatized to the temperature (22 $\pm$ 2 °C) and humidity (55 $\pm$ 5%) in controlled rooms with a 12/12-h light/dark cycle for at least 2 weeks prior to experiments. Animals were allowed free access to Purina rodent chow (Seoul, Korea) and tap water. All experimental protocols for animal care were performed according to the rules and regulations of the Animal Ethics Committee, Chungnam National University. The mice were divided into four groups (n = 5 per group). To induce AD-like immunological and skin lesions, DNCB was applied to the dorsal skin and ears. After complete removal of dorsal hairs within an area of approximately 8 cm², 200 μ L of 0.2% DNCB solution (dissolved in a 3:1 mixture of acetone and olive oil) was challenged for three times per week for 9 weeks. After inducing AD, emulsion containing CG was applied topically to the dorsal skin and ears of the mice six times per week for 4 weeks. In the CG-treated mice, emollient cream containing CG (5 or 20 mg/kg mouse body weight), 100 μ L, was applied topically 1 h before DNCB application each time. Control and DNCB-treated mice received topically applied 100 μ L emulsions without CG on the dorsal skin and ears at the same time. Animals were sacrificed 64 days after the first application of DNCB (Fig. 1). Blood was collected from the vena cava, and the right ear was removed and subjected to histopathological examination.

2.6. Measurement of ear thickness

Ear thickness was measured with a micrometer (Mitutoyo, Kawasaki, Japan) on the day of sacrifice. The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges, and the thickness was recorded in micrometers. To minimize technique variation, a single investigator performed the measurements throughout each experiment.

2.7. Histopathological studies

The right ear lesions were sliced and tissue slices were fixed in 10% buffered-neutral formalin for 24 h. The fixed ear tissue slices were embedded in paraffin wax, sectioned, deparaffinized, and rehydrated using standard techniques. Sections 5 μ m thick were subjected to hematoxylin and eosin (H&E) staining for the detection of various inflammatory cells. Histopathological changes were examined by light microscopy. An arbitrary scope was given to each microscopic field viewed at a magnification of 100 $\times$.

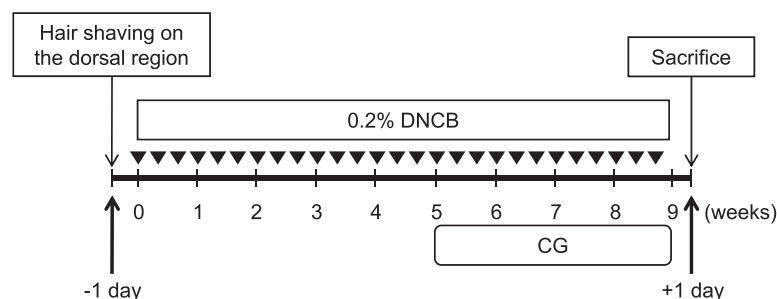


Fig. 1. Schematic diagram of the experimental protocol. Mice were divided into four groups ($n = 5$ per group). To induce AD-like immunological and skin lesions, DNCB was applied to the dorsal skin and ears. After complete removal of dorsal hairs within an area of approximately 8 cm^2 , $200\text{ }\mu\text{L}$ of 0.2% DNCB solution was applied three times per week for 9 weeks. After inducing AD, an emulsion containing CG was applied topically to the dorsal skin and ears of the mice six times per week for 4 weeks. In CG-treated mice, CG was applied topically 1 h before each application of DNCB. Control and DNCB-treated mice received topical emulsion without CG on the dorsal skin and ears. Animals were sacrificed 64 days after the first application of DNCB.

2.8. Evaluation of skin dermatitis severity

The severity of dermatitis in the dorsal skin and ear lesions was evaluated twice per week. The development of (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, and (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores was defined as the dermatitis score (Matsuda et al., 1997).

2.9. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from left ear lesions or HaCaT cells using the RNAiso reagent (Takara, Kyoto, Japan), according to the manufacturer's protocol, and stored at $-80\text{ }^{\circ}\text{C}$ until use. Then, $0.5\text{ }\mu\text{g}$ RNA was used for reverse transcription and amplified by PCR using the access RT-PCR system Takara thermal cycler (Takara, Seoul, Korea). PCR was performed using primers for mouse TARC, TNF- α , IFN- γ , IL-4, IL-5, IL-13, and GAPDH or human TARC and β -actin. The sequences of the primers are indicated in Table 1. The PCR products were separated on a 2% agarose gel containing ethidium bromide, visualized, and photographed using a gel documentation system (UVP, Cambridge, UK). Band intensity was quantified by densitometry using the ImageJ software (NIH, USA). The relative expression levels of target genes were normalized using GAPDH as the internal control.

2.10. ELISA assay

Levels of cytokines in serum were measured by a sandwich ELISA using the Opt-EIA Set mouse IgE kit from BD Biosciences and the DuoSet mouse CCL17/TARC kit from R&D Systems, according to the manufacturers' instructions. Cytokine concentrations were calculated using a linear regression equation obtained from standard absorbance values.

2.11. Transient transfection and luciferase assays

Cells were transfected with a NF- κB reporter vector and *Renilla* luciferase reporter vector using serum- and antibiotic-free LipofectAmine 2000 reagent. At 4 h after transfection, the transfection medium was replaced with growth medium. The cells were then pretreated with CG ($5\text{--}20\text{ }\mu\text{g/mL}$) for 1 h, treated with TNF- α and IFN- γ (each 20 ng/mL) for 24 h, and lysed. After incubation, cells were lysed and luciferase activity was measured using a luminometer (Luminoscan Ascent, Thermo Electron). For each sample, the luciferase signal was normalized to *Renilla* luciferase activity and expressed relative to the control value.

2.12. Preparation of the nuclear fraction

Cells were washed with ice-cold phosphate-buffered saline (PBS) and resuspended in $100\text{ }\mu\text{L}$ of lysis buffer containing 10 mM HEPES (pH 7.9), 0.5% Nonidet P-40, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After cell membranes had been disrupted by vortexing, the lysates were incubated for 5 min on ice and centrifuged ($7500g$, 5 min). The pellets containing crude nuclei were resuspended in $50\text{ }\mu\text{L}$ of extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, and then incubated for 30 min on ice. The samples were centrifuged ($15,800g$, 30 min) to obtain supernatant fractions containing the nuclear extracts.

2.13. Western blot analysis

HaCaT cells were pretreated with CG ($5\text{--}20\text{ }\mu\text{g/mL}$) for 1 h and then treated with TNF- α and IFN- γ (each 20 ng/mL) for 30 min. To evaluate NF- κB p65 translocation, cellular nucleus ($50\text{ }\mu\text{g}$) proteins were normalized by the Bradford method (Bradford, 1976), resolved on 10% polyacrylamide gels, transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and probed with the appropriate primary and secondary antibodies. NF- κB p65 and lamin B1 antibodies were obtained from Santa Cruz Biotechnology (Santa

Table 1
Primer sequences and the reaction conditions for semi-quantitative RT-PCR.

Gene	Primer sequences (5' $\rightarrow$ 3')	Annealing temperature ($^{\circ}\text{C}$)	Gene Bank No.
TARC	(F) TGA GGT CAC TTC AGA TGC TGC (R) ACC AAT CTG ATG GCC TTC TTC	60	NM_011332.3
TNF- α	(F) CCT GTA GCC CAC GTC GTA GC (R) TTG ACC TCA GCG CTG AGT TG	56	NM_013693.2
IFN- γ	(F) CTC AAG TGG CAT AGA TGT (R) GAG ATA ATC TGG CTC TGC AGG ATT	60	NM_008337.3
IL-4	(F) GAA TGT ACC AGG AGC CAT ATC (R) CTC AGT ACT ACG AGT AAT CCA	58	NM_021283.2
IL-5	(F) TCA CCG AGC TCT GTT GAC AA (R) CCA CAC TTC TCT TTT TGG CG	62	NM_010558.1
IL-13	(F) GAC CCA GAG GAT ATT GCA TG (R) CCA GCA AAG TCT GAT GTG AG	62	NM_008355.3
GAPDH	(F) CAT CTT CCA GGA GCG AGA CC (R) TCC ACC ACC CTG TTG CTG TA	56	NM_008084.2
TARC	(F) ACT GCT CCA GGG ATG CCA TCG TTT TT (R) ACA AGG GGA TGG GAT CTC CCT CAC TG	60	NM_002987.2
β -Actin	(F) ATG GAT GAT GAT ATC GCC GCG (R) TCT CCA TGT CGT CCC AGT TG	55	NM_001101.3

Cruz, CA, USA). The secondary antibody was a horseradish peroxidase-coupled anti-rabbit IgG (Beverly, MA, USA). Membranes were visualized using an enhanced chemiluminescence Western blotting detection kit (iNtRON Biotechnology Co., Ltd., Korea). The intensities of Western blot bands were measured using the ImageJ software.

2.14. Statistical analyses

All experiments were repeated at least three times. Results reported are means $\pm$ standard error of the mean (SEM). Statistical significance was determined using a one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test, setting $p < 0.05$ as the level of significance.

3. Results

3.1. CG suppressed DNCB-induced AD-like skin severity in NC/Nga mice

To assess the effectiveness of CG against AD-like skin lesions, mice were treated with 5 or 20 mg/kg CG per day after induction of AD-like skin lesions by DNCB application. The dermatitis severity was assessed twice per week for 64 days. As shown in Fig. 2A and C, repeated topical application of DNCB significantly increased dermatitis scores and ear thickness in the DNCB-treated group compared with the control group. Also, repeated topical application of DNCB significantly increased AD symptoms of hemorrhage, edema, scarring, dryness, and erosion in NC/Nga mice (Fig. 2B). Furthermore, ear histopathological results showed thickening of the dermis and epidermis and excessive lymphocyte infiltration into the dermis was observed in the DNCB-treated group versus the control group (Fig. 2D). CG significantly reduced DNCB-induced AD severity, ear thickness, and lymphocyte infiltration, in a dose-dependent manner. These results demonstrate that CG decreased AD symptoms in NC/Nga mice.

3.2. CG suppressed DNCB-induced serum IgE and TARC levels in NC/Nga mice

The elevated serum levels of IgE and TARC are related to disease severity in AD patients (Kabashima-Kubo et al., 2012). An important characteristic of AD is the secretion of IgE and TARC. To assess the effectiveness of CG in terms of influencing serum IgE and TARC levels, mice were treated with 5 or 20 mg/kg CG per day after induction of AD-like skin lesions by DNCB application. As shown in Fig. 3A and B, repeated topical application of DNCB significantly increased secretion of IgE and TARC in the DNCB-treated group versus the control group. CG significantly reduced DNCB-increased serum levels of IgE and TARC, in a dose-dependent manner.

3.3. CG suppressed DNCB-increased mRNA expression of TARC, TNF- α , IFN- γ , IL-4, IL-5, and IL-13 in NC/Nga mice

Th2 cytokines are dominant in the acute phase; Th1 cytokines are highly expressed and contribute to AD pathogenesis in the chronic phase (Leung and Bieber, 2003). TARC is a functional ligand for CCR4, a chemokine receptor expressed by most skin-homing Th cells in the circulation by skin-infiltrating lymphocytes (Campbell et al., 1999). To gain a better understanding of the molecular mechanism(s) involved in the development of AD reactions, we assessed the mRNA expression of TARC chemokine and Th1 and Th2 cytokines in the ear lesions by semi-quantitative RT-PCR. As shown in Fig. 3C, repeated topical application of DNCB significantly increased mRNA expression of TARC, TNF- α , IFN- γ , IL-4, IL-5, and IL-13 in the DNCB-treated group versus the control group. CG treatment significantly inhibited these chemokine and cytokines' mRNA expression, dose-dependently. These results demonstrate that CG decreased DNCB-induced inflammatory cytokines and chemokine

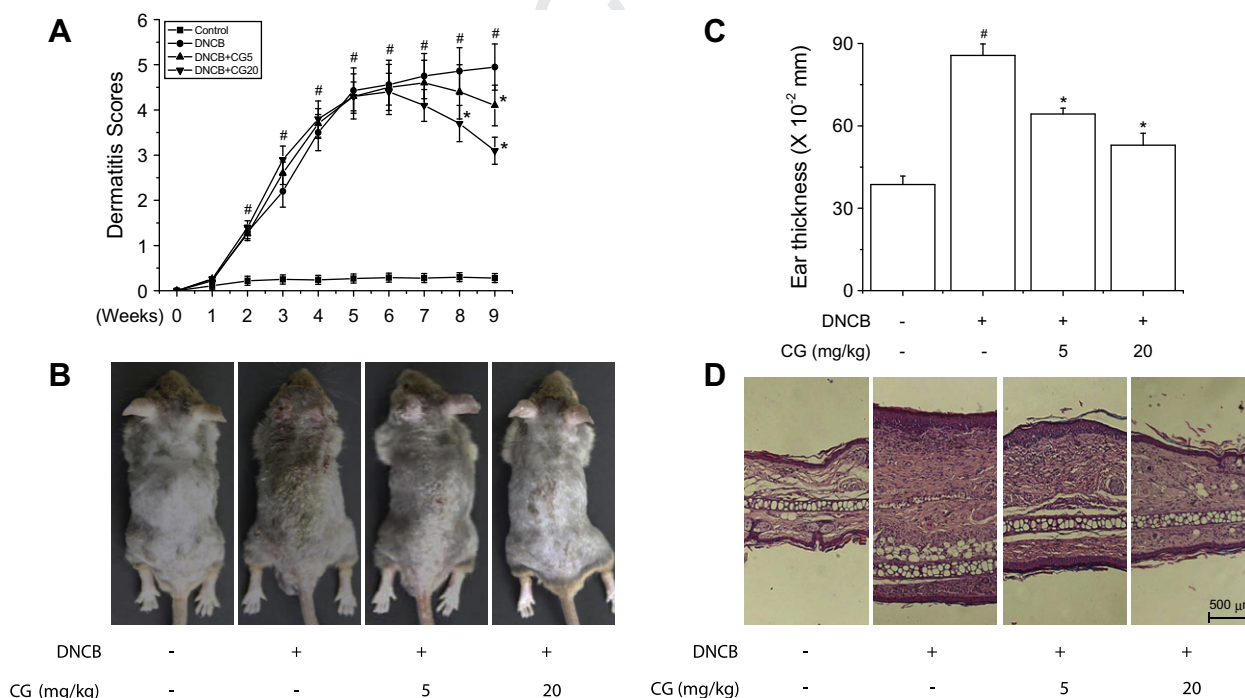


Fig. 2. Inhibitory effects of CG on DNCB-induced AD skin symptoms in NC/Nga mice. (A) The dermatitis score was defined as the sum of scores for seven clinical criteria: erythema, hemorrhage, edema, excoriation, erosion, scarring, and dryness. (B) Example of the clinical severity of AD-like skin lesions. (C) Ear thickness was measured with a micrometer. (D) Ear lesions were removed and fixed in 10% formaldehyde solution. Thin sections were cut and stained with hematoxylin and eosin. The photographs were taken on day 64 after the sacrifice of the mice. Magnification, 100 $\times$. Results shown are representative of five observations. Results are presented as means $\pm$ SEM ($n = 5$). $^{\#}p < 0.05$, significantly different from the control group; $^{*}p < 0.05$, significantly different from the DNCB-treated group.

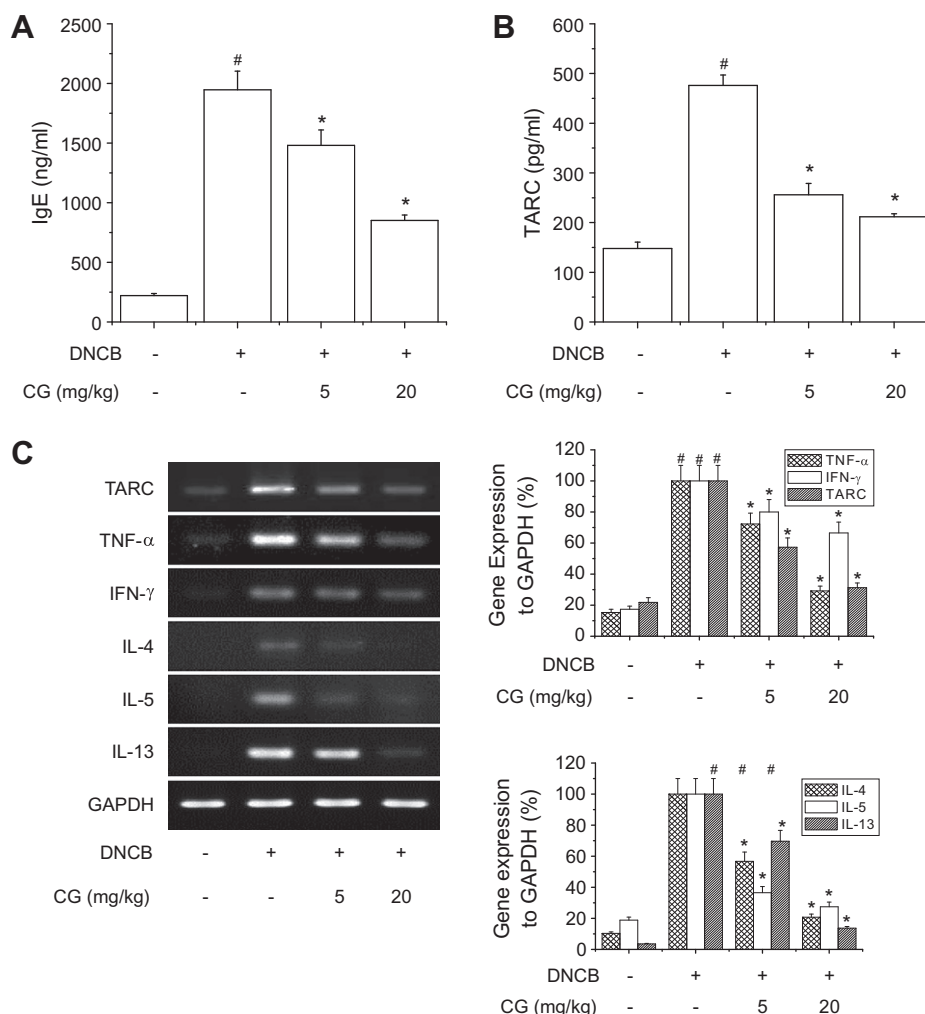


Fig. 3. Inhibitory effects of CG on DNCB-induced serum levels of IgE and TARC and mRNA expression of Th1 and Th2 cytokines in NC/Nga mice. Serum was collected 24 h after the last CG and/or DNCB treatment. Serum levels of (A) IgE and (B) TARC were determined by ELISA. (C) Total RNA was extracted from ear tissue and mRNA levels of TARC, TNF- α , IFN- γ , IL-4, IL-5, IL-13, and GAPDH were determined by semi-quantitative RT-PCR. Results shown are representative of five observations. The intensities of PCR bands were measured using the ImageJ software. Results are presented as means $\pm$ SEM ($n = 5$). # $p < 0.05$, significantly different from the control group; * $p < 0.05$, significantly different from the DNCB-treated group.

expression, leading to inhibition of the skin inflammation caused by the infiltration of inflammatory cells.

3.4. CG suppressed TNF- α /IFN- γ -increased Th2 chemokine mRNA expression in HaCaT cells

To obtain a suitable concentration range to investigate the effects of CG on cell viability in HaCaT cells, we treated cells with CG concentrations ranging from 5 to 50 μ g/mL for 24 h. We observed no significant alteration in cell viability following CG treatment at these concentrations. Thus, 5–20 μ g/mL concentrations of CG were used in subsequent experiments. We further examined the inhibitory effects of CG on TNF- α and IFN- γ -activated Th2 chemokine expression in HaCaT cells. Keratinocytes are known to express TNF- α and IFN- γ -activated chemokines (Vestergaard et al., 1999; Xiao et al., 2003). Chemokines are known to be important in immune responses and inflammatory reactions by recruiting selected subsets of leukocytes (Ben-Baruch et al., 1995). To assess the inhibitory effects of CG, HaCaT cells were stimulated with TNF- α and IFN- γ in the presence of CG for 24 h, and the mRNA expression of TARC was then evaluated by semi-quantitative RT-PCR. CG treat-

ments inhibited significantly and dose-dependently TNF- α and IFN- γ -increased mRNA expression of TARC in the cells (Fig. 4C).

3.5. CG suppressed TNF- α /IFN- γ -increased TARC mRNA expression by inhibiting NF- κ B activation

NF- κ B activation is known to be involved in the regulatory mechanism of TARC mRNA expression (Ju et al., 2009). To assess the inhibitory effects of CG on NF- κ B promoter activity, HaCaT cells were transfected with reporter plasmids containing NF- κ B responsive elements and then treated with CG in the presence of TNF- α and IFN- γ for 24 h. CG treatment inhibited significantly and dose-dependently TNF- α and IFN- γ -increased NF- κ B luciferase activity (Fig. 5A). NF- κ B is composed of p65 and p50 subunits, and inactive NF- κ B dimers are sequestered in the cytosol in association with various inhibitory molecules of the I κ B family. Stimulation of cells with TNF- α and IFN- γ causes phosphorylation of I κ B α , leading to its polyubiquitination and proteasome-mediated degradation and the release of active NF- κ B (Kwon et al., 2012). We examined the inhibitory effects of CG on the NF- κ B signaling pathway in TNF- α and IFN- γ -stimulated HaCaT cells. Nuclear and cytosolic extracts from cells were evaluated in terms of NF- κ B acti-

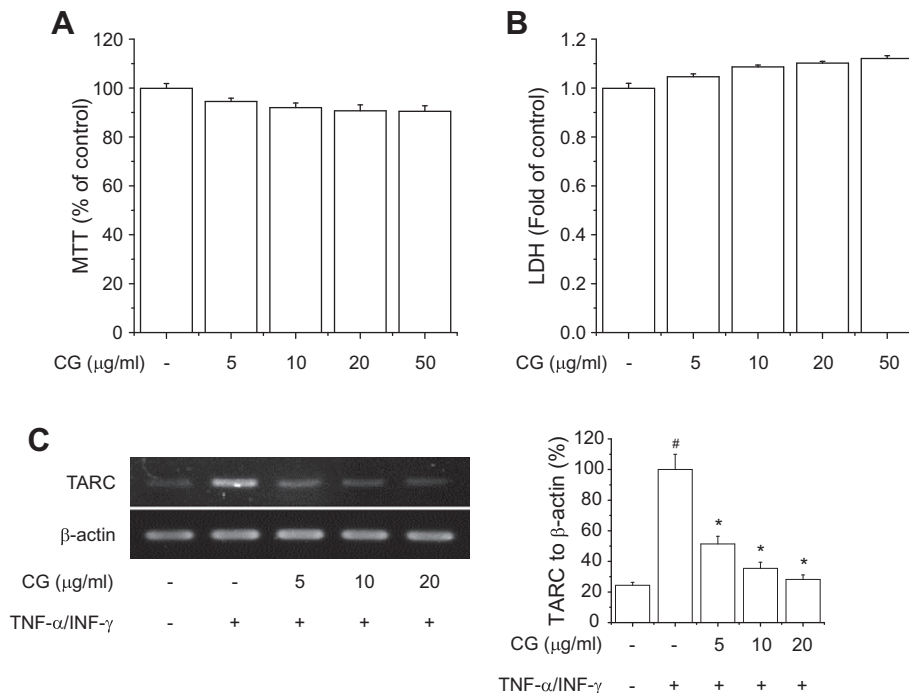


Fig. 4. Inhibitory effects of CG on TNF- α - and IFN- γ -induced mRNA expression of Th2 chemokine in HaCaT cells. (A and B) Cells were cultured in 48-well plates and treated with CG (0, 5, 10, 20, or 50 μ g/mL). After 24 h, cell viability was measured using the MTT reduction assay and LDH release assay. (C) Cells were treated with CG (0, 5, 10, or 20 μ g/mL) in the presence of TNF- α and IFN- γ (each 20 ng/mL). After 24 h, total RNA was extracted from cells and mRNA levels of TARC and β -actin were determined by semi-quantitative RT-PCR. The intensities of PCR bands were measured using the ImageJ software. Results are presented as means $\pm$ SEM of three independent experiments. [#] $P < 0.05$, compared with the control group; ^{*} $P < 0.05$, compared with the TNF- α - and IFN- γ -treated group.

vation by Western blotting. CG inhibited TNF- α and IFN- γ -induced nuclear translocation of NF- κ B p65, in a dose-dependent manner (Fig. 5B). Furthermore, the NF- κ B activation inhibitor JSH-23 inhibited TNF- α and IFN- γ -increased TARC mRNA expression (Fig. 5C). These results demonstrate that CG inhibited TNF- α and IFN- γ -increased activation of TARC by suppressing NF- κ B activation in HaCaT cells.

4. Discussion

Treatment of inflammatory disease by traditional herbal extracts is widely used as a folk remedy in Asia (Choi et al., 2011). Many *in vitro* and *in vivo* studies of herbal medicines have been conducted to identify natural products effective for treatment of AD. Ginseng is one of the most widely used herbal medicine plants. CG is an important traditional herbal extract that has diverse functions, such as anti-inflammatory, anti-allergic, and anti-cancer effects, with less side effects (Ye et al., 2011). Ginseng root extracts have long been used to treat various diseases, including respiratory and hepatic dysfunction, hypertension, inflammation, and diabetes in China, Korea, and Japan (Seida et al., 2011; Huu Tung et al., 2012; Pan et al., 2012; Fan et al., 2012; Sawiress et al., 2011). Nevertheless, the mechanism(s) of action(s) of ginseng as an anti-AD agent remain unclear. In the present study, we demonstrated the inhibitory effect of CG on DNCB-induced AD-like skin lesions in NC/Nga mice and TNF- α - and IFN- γ -induced TARC activation in HaCaT cells.

AD is the most common chronic skin disease worldwide, and onset occurs typically during early infancy or childhood, but can also occur in adulthood (Leung and Bieber, 2003; Yang et al., 2009). To identify better clinical parameters for AD, NC/Nga mice are commonly used as an experimental model. NC/Nga mice manifest clinical characteristics of human AD-like skin symptoms, including increased serum IgE levels (Pokharel et al., 2008). IgE is

one of the most important targets in AD because high serum IgE levels mediate the critical clinical characteristics of AD. Specifically, IgE binds to mast cells, causing the release of inflammatory mediators; this increase correlates with AD severity (Leung, 2000). Naive CD4⁺ T helper cells differentiate into Th2 cells and release cytokines, such as IL-4, IL-5, and IL-13. These cytokines, in turn, induce class switching, making IgE producing plasma cell (Fujita et al., 2012). We found that IgE production was elevated in the DNCB-treated NC/Nga mice. This IgE production was reduced significantly by CG treatment.

DNCB-induced AD-like skin disease is a condition predominantly mediated by cellular immune responses (Tuckermann et al., 2007; Kehren et al., 1999; Xu et al., 1997). In support of this, the severity of AD-like skin was lessened significantly in mice lacking CD4⁺ or CD8⁺ T cells, and these results demonstrated the involvement of T cells in the development of AD-like skin (Wang et al., 2000). AD is thus a T cell-mediated skin inflammatory condition. Various cells, inflammatory mediators and cytokines, are involved in the regulation of immunological and inflammatory processes in AD. Consistent with a previous study, we found that mRNA expression of TNF- α , IFN- γ , IL-4, IL-5, and IL-13 were elevated in the DNCB-treated NC/Nga mice (Choi et al., 2012a,b). This expression of cytokines was significantly reduced by CG treatment, in a dose-dependent manner. We also evaluated the effects of CG on skin severity and histopathological changes in DNCB-treated NC/Nga mice. CG treatment significantly suppressed topical application of DNCB-induced AD-like skin severity, including hemorrhage, excoriation, and dryness, and histopathological changes, including infiltration of leukocytes into the ear lesions. These results suggest that the therapeutic effects of CG in NC/Nga mouse models are associated with both down-regulations of inflammatory cytokines and AD-like skin symptoms.

AD is also mediated by chemotactic factors, such as TARC, MDC, and CTACK, which accelerate the inflammatory cell infiltration into

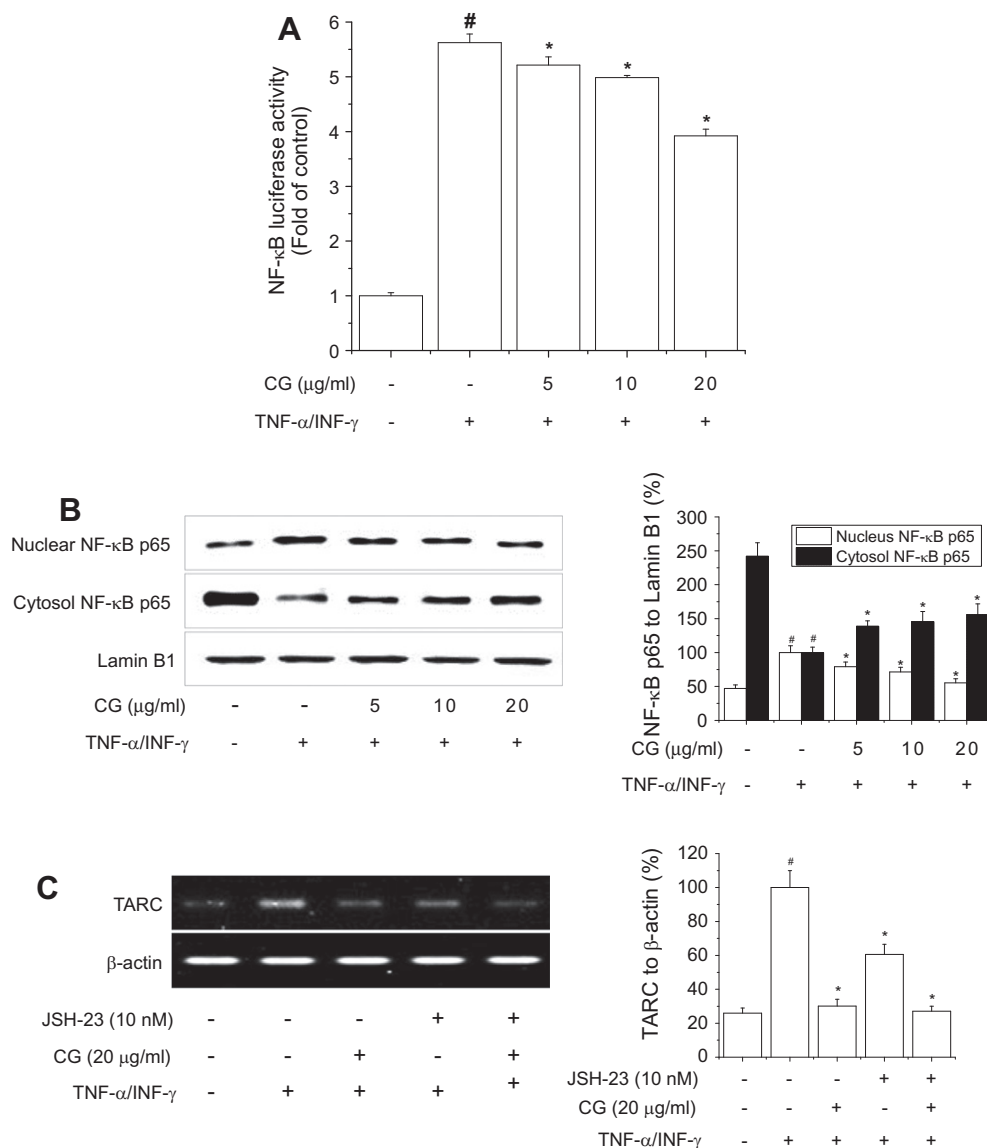


Fig. 5. Inhibitory effects of CG on TNF-α- and IFN-γ-induced NF-κB activation in HaCaT cells. (A) Cells were transiently transfected with reporter plasmids containing tandem elements of NF-κB binding sites. After 5 h, the transfected cells were treated with CG (0, 5, 10, or 20 μg/mL) in the presence of TNF-α and IFN-γ (each 20 ng/mL), and luciferase activity was determined. Luciferase activities were normalized to *Renilla* activity and expressed as fold increases over the control. (B) Cells were pretreated with CG (0, 5, 10, or 20 μg/mL) for 1 h and then treated with TNF-α and IFN-γ (each 20 ng/mL) for 30 min. Cell lysates were prepared from HaCaT cells treated with TNF-α and IFN-γ and the levels of signaling molecules leading to NF-κB activation were determined by Western blotting. (C) Cells were treated with CG in the presence of TNF-α and IFN-γ (each 20 ng/mL) and/or NF-κB inhibitor JSH-23. After 24 h, total RNA was extracted from cells and mRNA levels of TARC and β-actin were determined by semi-quantitative RT-PCR. The intensity of PCR bands was measured using the ImageJ software. Results are presented as means ± SEM of three independent experiments. [#]*p* < 0.05, compared with the control group; ^{*}*p* < 0.05, compared with the TNF-α- and IFN-γ-treated group.

the pathological lesions. The infiltration of inflammatory cells into sites of inflammation is dependent on the local production of various members of the chemokine family that exhibit leukocyte chemoattractant activity (Oshio et al., 2009). TARC is a selective chemoattractant and assists in the recruitment and migration of Th2 cells that express the CC chemokine receptor 4 (Sandoval-López and Teran, 2001). TARC is an important mediator that exacerbates AD. TARC is overexpressed in AD mice and human patient keratinocytes (Kawasaki et al., 2001). Consistent with a previous study, we found that TARC production was elevated in DNCB-treated NC/Nga mice (Choi et al., 2012a,b). This TARC production was significantly reduced by CG treatment. We have examined that serum TARC level reflect the AD, which is thought to be a Th2-dominant inflammatory skin disease in the acute phase. TARC may be a useful laboratory marker for the diagnosis of AD, which are moder-

ate to severe, and for the evaluation of AD. TARC and its receptor CCR4 are believed to play important roles in the pathogenesis of the AD, TARC and CCR4 may be possible future targets for therapy of these diseases.

In the skin, stimulation of keratinocytes with various stimuli leads to abnormal induction of TARC, which selectively increases the infiltration of CCR4⁺ lymphocytes into the site of inflamed tissues (Saeki and Tamaki, 2006). This is a characteristic feature of development of Th2-mediated inflammatory responses. We conducted experiments to explore the effects of CG on the TNF-α and IFN-γ-induced expression of TARC in HaCaT cells. Pretreatment with CG significantly inhibited mRNA expression of TARC in TNF-α- and IFN-γ-stimulated HaCaT cells.

NF-κB is known to be part of the regulatory mechanism for TARC activation (Ju et al., 2009). Aggarwal reported that TNF-α

and IFN- γ can activate NF- κ B, which is responsible for expression of pro-inflammatory cytokines (Aggarwal, 2003). Upon this cytokine stimulus, the I κ B proteins are phosphorylated and degraded, which allows NF- κ B to translocate into the nucleus where it can bind specific promoter regions of target genes and activate expression of inflammatory cytokine genes. Thus, inhibition of NF- κ B activation has been suggested as an anti-inflammatory strategy in AD. We evaluated whether the anti-inflammatory effects of CG were mediated through the regulation of NF- κ B activation. TNF- α and IFN- γ -induced phosphorylation of NF- κ B p65 and I κ B α and degradation of I κ B α was inhibited significantly in HaCaT cells treated with CG, indicating that CG can affect the NF- κ B signaling pathway (data not shown). CG inhibited TARC expression by repressing transcriptional activation of the NF- κ B promoter site. We further confirmed NF- κ B transcriptional activation by semi-quantitative RT-PCR in HaCaT cells. Treatment with JSH-23, a potent inhibitor of NF- κ B transcriptional activation, reduced the TNF- α - and IFN- γ -induced mRNA expression of TARC. These findings collectively suggest that CG significantly inhibited expression of TARC via blockade of NF- κ B activation in TNF- α and IFN- γ -treated HaCaT cells.

In conclusion, our results suggest that topical application of CG attenuated the Th1 and Th2 responses, and that leukocyte infiltration may be a major mechanism in the development of AD-like skin lesions in DNCB-treated NC/Nga mice. Also, we have provided evidence that CG exerts inhibitory effects on TARC expression by suppressing the signaling pathway that lead to the activation of NF- κ B in TNF- α - and IFN- γ -stimulated HaCaT cells. These results suggest topical application of CG to be an effective alternative therapy for the management of AD. Further studies are needed to determine the molecular mechanism and the therapeutic potential of CG in the prevention and treatment of AD.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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