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Original Article

The Ameliorative Effect of β-sitosterol on DNCB-induced Atopic Dermatitis in Mice

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β-sitosterol, one of phytosterols, exhibited numerous pharmacological effect including anti-inflammatory, anti-cancer and immune-modulating properties. This study attempted to determine the pharmacological effects of β-sitosterol on atopic dermatitis (AD). We investigated to ascertain the pharmacological effects of β-sitosterol on 2, 4-dinitrochlrobenzene (DNCB)-induced AD symptom and histamine-induced scratching behaviors in mice. Additionally, we evaluated the effects of β-sitosterol on the interleukin (IL)-6 levels in HaCaT cells and skin tissue of AD. The findings of this study demonstrated that β-sitosterol reduced AD clinical symptoms such as eczematous, erythema and dryness and serum histamine and IgE levels in DNCB-induced AD model and histamine-induced scratching behaviors in mice. Additionally, β-sitosterol inhibited the IL-6 expression in AD-like skin lesion and HaCaT cells. Collectively, these findings provide that β-sitosterol could be a therapeutic agent for skin inflammation including AD.

Key Words: β-sitosterol; Atopic dermatitis; Interleukin-6; HaCaT cells

INTRODUCTION

Atopic dermatitis (AD) is a common skin disease characterized by a chronic and relapsing inflammatory dermatitis (Buske-Kirschbaum et al., 2001). AD is known to be the result of an immune system dysregulation, ultimately resulting in allergic inflammation (Gold and Kemp, 2005). In the past decades, AD research has enormously increased due to the rapid increase of AD related skin inflammation around the world. Generally, most therapy for AD is corticosteroids (Berke et al., 2012) but these long-term treatments cause serious side effects such as immunosuppression, and epidermal barrier dysfunction (Shiohara et al., 2004). Consequently, there is a need for anti-atopic agents that cause fewer side effects.

Keratinocytes, which are the main epidermal cells, are considered to play a critical role in AD (Eichenfield et al., 2012). Keratinocyte produces inflammatory cytokines and chemokines by various stimulations (Vestergaard et al., 2000). These mediators contribute to the infiltration of inflammatory cells to sites of inflammation in the skin. Recent studies have reported that inflammatory cytokines are involved in the initiation of AD. It was reported that cytokines were expressed at high levels in skin lesion of AD patients and it suggests that inflammatory cytokine plays an integral role in the pathogenesis of these conditions (Homey et al., 2006). Hence, there is a strong interest in the development of agents

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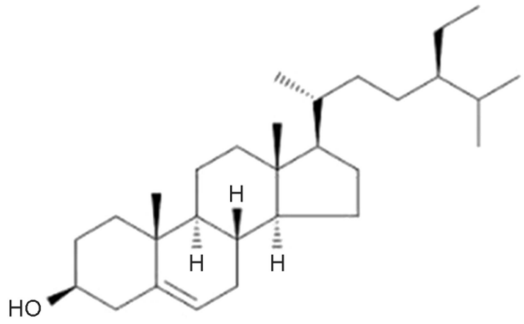

Fig. 1. Chemical structure of β-sitosterol.

that can block the generation or action of inflammatory cytokines.

As natural products are safe and devoid of toxicity, it has been the subject of increased interest for the development of new drugs against a wide range of diseases such as cancers and inflammatory diseases. β-sitosterol is a most common phytosterols and a chemical structurally related to cholesterol (Fig. 1). It presents in numerous plants such as rice, wheat and corn and exhibits anti-inflammatory, angiogenic and immune-modulating properties (Heitzman et al., 2005; Ling and Jones, 1995). In the present study, we elucidate whether β-sitosterol modulates the AD in mice. We investigated the pharmacological effects of β-sitosterol on 2, 4-dinitrochlrobenzene (DNCB)-induced AD symptom and histamine-induced scratching behaviors in mice. Additionally, we evaluated the effects of β-sitosterol on the Interleukin (IL)-6 levels in AD-like skin lesion and HaCaT cells.

MATERIALS AND METHODS

Reagents

β-sitosterol, Compound 48/80, histamine, terfenadine, avidin peroxidase (AP) and 1-chloro-2,4-dinitrochlorobenzene (DNCB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 was purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific Inc. (Somerset, NJ, USA). Anti-mouse IL-6/IgE, recombinant IL-6/IgE and biotinylated IL-6/IgE were purchased from BD Pharmingen (San Diego, CA, USA).

Animals

Male ICR mice (5 weeks, 18~20 g) and BALB/c mice (5 weeks, 19~20 g) were purchased from the Daehan biolink Co., Ltd. (Chungbuk, Korea). Animals were housed 6 heads per cage, allowed spontaneous take in food and water. Animals were kept under a 12-h light/dark cycle (light on 08:00-20:00) at room temperature (23 ± 2℃) and humidity (55 ± 10%). The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in Daegu Haany university guidelines.

Induction of AD-like skin Lesions and β-sitosterol treatment

Induction of AD-like skin lesions procedure is described in Fig. 2. DNCB (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in vehicle (3:1 acetone olive oil) and used as a sensitizer for inducing AD-like skin lesions in mice (Yoon et al., 2015). Backs of mice were shaved with a clipper and depilatory cream, washed with sterilized PBS and gauzed a day before sensitization. Mice were divided into 5 groups with 6 mice per group: vehicle, DNCB, and DNCB plus treatment of β-sitosterol (0.2 mg/kg) or β-sitosterol (2 mg/kg) or terfenadine (10 mg/kg). Exposed skin was treated with vehicle alone or with 200 μL of a 1% DNCB. On day 4 after sensitization, the back skin was challenged with 200 μL of a 0.2% DNCB solution three times per week. This procedure was repeated for 4 weeks and β-sitosterol was orally administrated 2 weeks before the end of the experiment.

Evaluation of skin dermatitis severity

The severity of dermatitis was assessed macroscopically according to the Eczema Area and Severity Index (EASI) scoring system: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. The severity of dermatitis was evaluated by the naked eye of three blind examiners. The sum of the individual scores was defined as the dermatitis score for erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness (Hanifin et al., 2001).

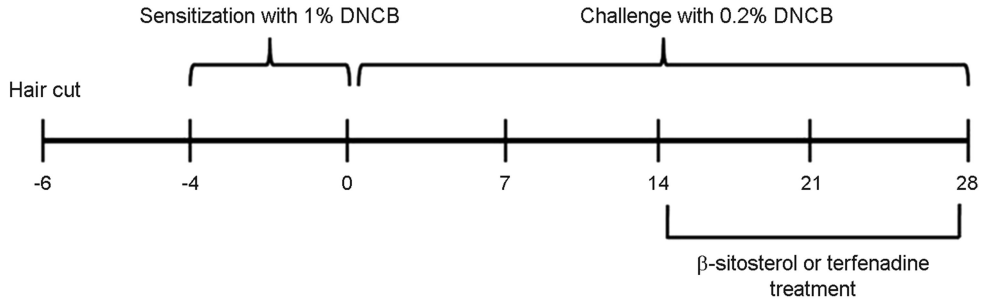

Fig. 2. Experimental protocol for induction of AD and β-sitosterol treatment.

Scratching behavioral experiment

Before the experiment, the ICR mice (n=6/each group) were put into acrylic cages (22×22×24 cm) for about 30 min for acclimation. The behavioral experiments were performed according to the method of Sugimoto et al. (2006). The rostral part of the skin on the back of mice was clipped, and histamine (100 μg/kg) for each mouse was intradermally injected. The scratching agents were dissolved in tween 80 and then used. Control mice received a Tween 80 injection in place of the scratching agent. Immediately after the intradermal injection, the mice (one animal/cage) were put back into the same cage for the observation of scratching. Scratching of the injected site by the hind paws was counted and compared with that of the other sites, such as the ears. Each mouse was used for only one experiment. The mice generally showed several scratches for 1 s, and a series of these behaviors was counted as one incident of scratching for 30 min. β-sitosterol (0.2 and 2 mg/kg) and terfenadine (10 mg/kg) was orally administered 1 h before the scratching agents.

Cell culture

Human immortalized keratinocytes (HaCaT cells) were cultured in RPMI1640 (100 unit/ml penicillin, 100 μg/ml, streptomycin, and 10% heat-inactivated FBS) at 37℃, 5% CO_2 and 95% humidity.

IL-6 and IgE assay

Skin tissue were homogenized in lysis buffer and centrifuged at 12,000 rpm for 10 min. The supernatants were used as the cytokine containing extract and protein concentrations were determined using BCA protein assay reagent (Sigma). Blood was collected and serum was separated by centrifugation at 4,000 × g for 20 min at 4℃. Levels of IL-6 in skin tissue and IgE in serum were measured using an enzyme-linked immunosorbent assay (ELISA), as previously described (Kim et al., 2010). Briefly, 96-well plates were coated with anti-mouse monoclonal antibodies and incubated overnight at 4℃. After additional washes, sample or an IL-6 or IgE standard were added and incubated at room temperature for 2 h. Plates were then washed and biotinylated anti-mouse antibody was added and incubated at room temperature for 2 h. After washing plates, avidin-peroxidase was added, and plates were incubated for 30 min at 37℃. The plates were then rewashed and ABTS substrate was added. Color development was measured at 405 nm using an automated microplate ELISA reader.

Histamine assay

Concentrations of histamine in serum isolated from sacrificed mice were measured with a specific ELISA kit according to the manufacturer's instructions (Abnova, CA, USA).

Statistical analysis

The experiments were shown a summary of the data from at least-three experiments and presented as the mean ± S.D. Statistical evaluation of the results was performed by independent *t*-test. A value of $P < 0.05$ was considered statistically significant.

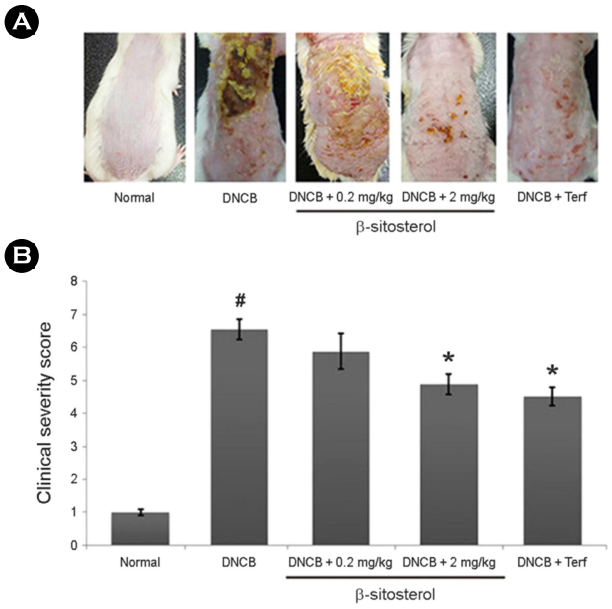

Fig. 3. Effect of β-sitosterol on DNCB-induced AD in mice. (A) The mice were sensitized with 0.1% DNCB in acetone-olive oil (3:1) or vehicle applied to the dorsal skin twice each week for a total period of 5 weeks. After 3 weeks, β-sitosterol (0.2 and 2 mg/kg) or Terfenadine (10 mg/kg) was orally administered 2 weeks prior to the end of the experiment. (B) The score of skin severity is represented. The data represents the mean ± S.D. of three independent experiments (#P<0.05 vs. control group, *P<0.05 vs. DNCB-treated group).

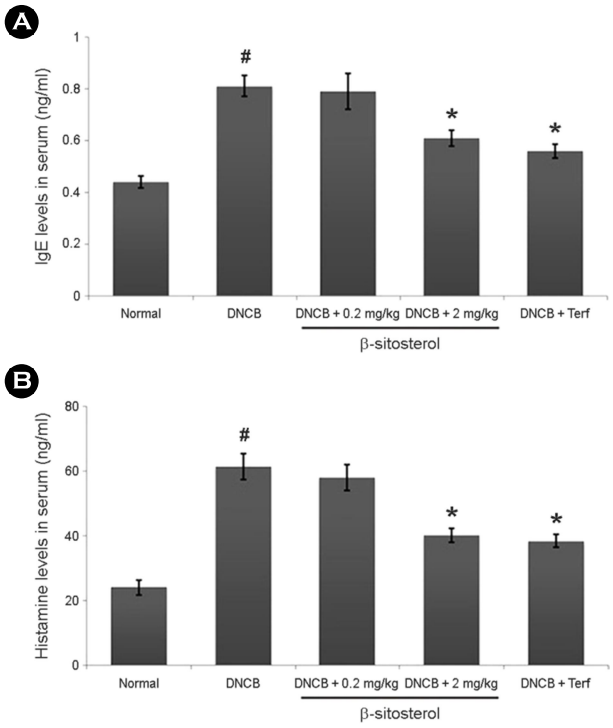

Fig. 4. Effect of β-sitosterol on the histamine and IgE serum levels. (A and B) Blood samples in DNCB-induced AD mice were collected and then levels of serum histamine and IgE were measured using ELISA method. The data represents the mean ± S.D. of three independent experiments (#P<0.05 vs. control group, *P<0.05 vs. DNCB-treated group).

RESULTS

Effect of β-sitosterol on DNCB-induced atopic dermatitis in mice

In order to evaluate the regulatory effects of β-sitosterol on AD *in vivo* model, DNCB was administered to BALB/c mice. As shown in Fig. 3A, when mice were treated for 2 weeks with β-sitosterol, DNCB-induced the AD symptoms such as eczematous, erythema and dryness were recovered to a significant extent. We observed that the skin severity scores in the β-sitosterol group and the terfenadine group were significantly lowered compared to DNCB-treated group (P<0.05). Terfenadine was used as a positive control in this study (Fig. 3B).

Effect of β-sitosterol on histamine and IgE serum levels in AD mice

An important feature of AD is the pathological secretion of histamine and IgE (Saeki et al., 2009). Thus, we evaluated the effect of β-sitosterol on histamine and IgE levels in serum using ELISA. As shown in Fig. 4A and B, the application of DNCB to mice resulted in an increased release of histamine and IgE in the serum. In contrast, the β-sitosterol-treated group showed a considerable reduction in histamine and IgE levels in the serum. The inhibition rate of histamine and IgE by β-sitosterol (2 mg/kg) was approximately 35.2% and 29.4%, respectively (P<0.05).

Effect of β-sitosterol on scratching behaviors in mice

The anti-pruritic effect of β-sitosterol was investigated on the histamine-induced scratching behavior in mice. When the β-sitosterol was orally administered 1 h before histamine injections, the scratching behaviors was reduced (Fig. 5). The inhibition rate of β-sitosterol (2 mg/kg) was approximately 39.2% (P<0.05).

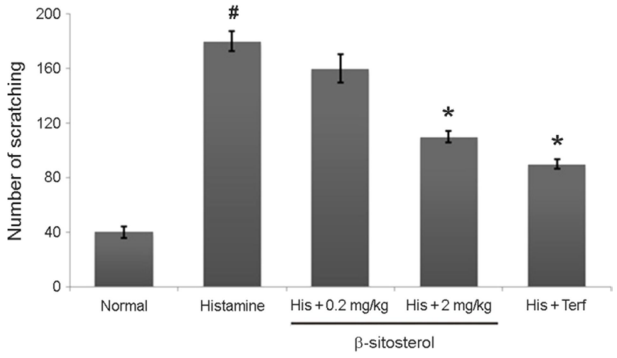

Fig. 5. The effect of β-sitosterol on scratching behavior in mice. β-sitosterol (0.2 and 2 mg/kg) was orally administered 1 h before histamine (100 μg/kg) intradermal injection. Scratching behavior was counted as one incident of scratching for 30 min. Each datum represents the means ± S.D. of three independent experiments (#P <0.05 vs. control group, *P<0.05 vs. histamine-treated group).

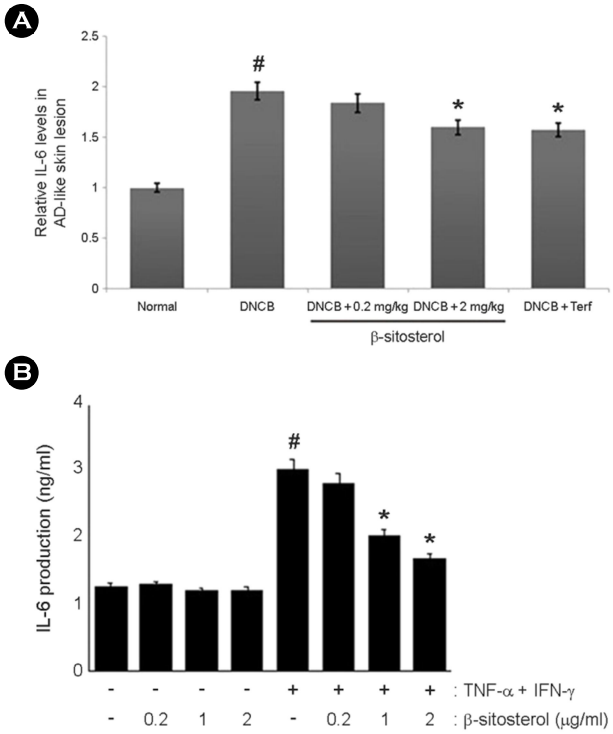

Fig. 6. The effect of β-sitosterol on the IL-6 levels in AD-like skin tissue and HaCaT cells (A) At the end of experiment, the skin tissues were cut out and homogenized. The level of IL-6 in the indicated groups was measured via ELISA. (B) Cells were pre-treated with β-sitosterol (0.2~2 μg/ml) and then stimulated with TNF-α (10 ng/ml) + IFN-γ (10 ng/ml) for 24 h. The IL-6 level in cell supernatants was measured using ELISA. All data were represented in the mean ± S.D. of triplicate determinations from triplicate separate experiments (#P<0.05 vs. control, *P<0.05 vs. DNCB-treated group, **P<0.05 vs. TNF-α+IFN-γ alone).

Effect of β-sitosterol on IL-6 levels in AD-like skin lesion and HacaT cells

Inflammatory cytokines are involved in the initiation of the inflammatory response in AD. We investigated the effect of β-sitosterol on L-6 levels in the AD-like skin lesion. At the end of experiment, the skin tissues were homogenized and ELISA was performed. The levels of IL-6 were significantly increased in the skin tissues of DNCB-treated mice compared to that of control. However, administration of β-sitosterol reduced these inductions induced by DNCB. The inhibition rate of IL-6 levels by β-sitosterol (2 mg/kg) was approximately 25.7% (Fig. 6A).

The regulatory effect of β-sitosterol on IL-6 production in HaCaT cells was also evaluated. Cells were treated with various concentrations of β-sitosterol (0.2, 1, 2 μg/ml) prior to TNF-α plus IFN-γ-stimulation. As shown in Fig. 6B, IL-6 production was increased by stimulation with TNF-α plus IFN-γ and these increases were inhibited concentration-dependently by β-sitosterol treatment. The maximal inhibition rate of IL-6 by β-sitosterol (2 mg/kg) was approximately 43.7% (P<0.05).

DISCUSSION

Generally, steroid therapy has been used for the treatment of AD because of their great anti-inflammatory and anti-allergic activities. However, it cannot be administered over the long-term, owing to its deleterious side-effects (Das and Panda, 2017). Therefore, natural product has been the subject of increased interest for its potential in the treatment of AD (Shiohara et al., 2004). β-sitosterol is a compound discovered to be present in numerous plants and various biomedical properties including immuno-modulating and anti-inflammatory activities have been reported (Heitzman et al., 2005; Ling and Jones, 1995). In the present report, we demonstrated that anti-atopic effects of β-sitosterol in AD mice model.

AD is a common chronic inflammatory skin disease inducing intense itching, edema, erythema, thickening, severe pruritus, and eczematous lesions of the skin (Leung and Bieber, 2003). It was reported that genetic, environmental

factors and immune responses are associated for the pathogenesis and progression of AD (Bieber, 2008). IgE dysregulation is implicated with the pathogenesis of AD and it was reported that serum IgE concentration is generally elevated in patients with AD (Allam and Novak, 2006; Brenninkmeijer et al., 2008). In this study, the result showed that β-sitosterol significantly reduced the AD symptoms such as eczematous, erythema and dryness. Additionally, we observed that IgE level in the DNCB group was significantly higher than that in mice in the control group and administration of β-sitosterol suppressed the increased IgE levels in serum. In pathological skin conditions, histamine is involved in the induction of itching and edema (Minami and Kamei, 2004). This study focused on the manner in which β-sitosterol regulates the scratching behaviors in mice. We showed that β-sitosterol inhibited the histamine-induced scratching behaviors in mice and attenuated the DNCB-induced histamine levels in serum. From this, β-sitosterol possibly may have a therapeutic effect that could alleviate the clinical symptoms associated with AD.

Accumulated experimental evidence shows that IL-6 is implicated with the development of AD. It was also reported that the level of IL-6 is elevated in AD patients and that it plays an integral role in their pathogenesis (Fedenko et al., 2011; Wong et al., 2001). Hence, research on new biological therapies for AD has focused on blocking components of the inflammatory cytokines. The current study confirmed that the levels of IL-6 increased in AD-like skin lesion compared with those of the control and that treatment with β-sitosterol reduced these levels. Additionally, β-sitosterol suppressed the IL-6 production in TNF-α plus IFN-γ-stimulated HaCaT cell. These results indicated that the anti-atopic effect of β-sitosterol is attributable to the regulation of inflammatory mediator. Although β-sitosterol attenuated the inflammatory cytokine, the β-sitosterol's mechanism involved in inflammatory response as not determined in present study. Therefore, further studies will be necessary in order to clarify more precisely the mechanism of β-sitosterol in AD.

In conclusion, β-sitosterol can regulate the AD response *in vivo*, including DNCB-induced atopic dermatitis and histamine-induced scratching behaviors in mice. Additionally, we demonstrated in this study that the anti-inflammatory activities of β-sitosterol could be attributed, at least in part, to the inhibition of inflammatory cytokine in AD-like skin lesion and HaCaT cell. Our current study may provide a basis for the therapeutic use of β-sitosterol in inflammatory skin diseases such as AD.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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