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RESEARCH ARTICLE

Effect of Fermented *Rhus verniciflua* Extract on DNCB Induced-Atopy like Dermatitis in BALB/c Mice

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ABSTRACT

The effect of fermented *Rhus verniciflua* extract on DNCB (1-chloro 2, 4-dinitro-benzene) induced atopic like dermatitis and associated inflammatory responses was evaluated in BALB/c mice. Controlled experiment was performed in 25 BALB/c mice randomly distributed into five groups each consisted of five mice. DNCB induced-atopy like dermatitis was formed on the back skin of mice in treatment and positive control groups. The induced-atopy like dermatitis of mice in treatment groups was topically treated with fermented *Rhus verniciflua* extract for two weeks and a day after the last treatment skin lesion was scored for severity. The mean±SD plasma histamine level of mice in *Rhus verniciflua* extract treated groups was lower than those in DNCB group. The eosinophil and basophil counts of *Rhus verniciflua* extract treated mice in FRV3 and FRV30 groups were significantly lower than values in DNCB group ($P<0.05$). The TNF-α level observed for mice in FRV30 group was significantly lower than DNCB group ($P<0.05$). The severity score of the skin lesions revealed significantly lower values in FRV30 than DNCB group ($P<0.05$). The results obtained in this study suggest the therapeutic potential of fermented *Rhus verniciflua* extract in reducing progression of atopic dermatitis.

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INTRODUCTION

Atopic dermatitis is an inflammatory condition, which is characterized by redness, edema, warmth, itching, scaling and dryness of the skin (Coenraads and Goncalo, 2007). It could occur due to activation of multiple immunological and inflammatory pathways. Although steroids are first line drugs, which have been frequently used to relief the symptoms in atopic dermatitis (Hanifin *et al*., 2004), the side effects associated with their long-term use are the prevailing problems in management of atopic dermatitis (Schäcke *et al*., 2002). Consequently, natural herbs or agents have been alternatively used in management of atopic dermatitis (Fowler *et al.,* 2010).

Rhus verniciflua is a plant species, which has been used for traditional medicine in Korea, Japan and China (Lee *et al*., 2003). In addition, *in vivo* and *in vitro* anti-inflammatory activities of *Rhus verniciflua* extract have been reported in recent studies (Jung *et al*., 2011). Although the anti-inflammatory effect of *Rhus verniciflua* extract has been studied previously, it's *in vivo* effect on blood eosinophil and basophil counts and skin TNF-α level associated with dermatitis have not been investigated. Therefore, in the current study, we evaluate the effect of fermented *Rhus verniciflua* extract on DNCB-induced atopy like dermatitis and associated inflammatory responses in BALB/c mice.

MATERIALS AND METHODS

Reagents: 1-chloro 2, 4-dinitro-benzene (DNCB), protease inhibitor and olive oil were purchased from Sigma (St. Louis MO, USA). *Acetone* (purity, 99.5%) and phosphate buffered saline and formaldehyde were

purchased from *Junsei Chemical* Co. Ltd., *Japan and* Duksan Pure Chemicals Co. Ltd. (*Ansan*, South *Korea*), respectively.

Animals: Seven-week old male BALB/c mice (n=25), weighing 23-26 g, were obtained from Orient Bio Co., Ltd (Iksan, Korea) and randomly divided into five equal groups of: control (with no treatments), DNCB (positive control; DNCB-induced atopic dermatitis with no treatments), FRV3 (T1; DNCB-induced atopic dermatitis with 3 mg/ml fermented *Rhus verniciflua* (FRV) treatments), FRV30 (T2; DNCB-induced atopic dermatitis with 30 mg/ml FRV treatments) and FRV300 (T3; DNCB-induced atopic dermatitis with 300 mg/ml FRV treatments). Animals were housed at a room temperature of 23±3°C and relative humidity of 55±10% and supplied with normal mouse chow diet and *ad libitum* filtered water. The animal experiment care was in accordance and approval of the Institute of animal uses and care committee of Kyungpook National University (Approval number: KNU 2009 -5).

Preparation of *Rhus verniciflua* stems bark extract: *Rhus verniciflua* stem bark (1 kg) was air dried, cut in to pieces and extracted in water (8 L) at 95°C for 6 h using extractor (Bio feedback cosmos-660, Korea). The extract was concentrated under vacuum at 40°C and the allergenic urushiol of *Rhus verniciflua* extract was removed according to previously described method (Sapkota *et al*., 2011).

Induction of atopic dermatitis like lesion and treatment with *Rhus verniciflua* extract: The hair on back skin of BALB/c mice in all groups was carefully shaved by electric shaver as described in previous study (Yamamoto *et al*., 2007). After one-day observation, the shaved back skin of mice in DNCB, FRV3, FRV30 and FRV300 groups was sensitized by topical application of 150 µl DNCB (2.5%) in acetone/olive oil (1:3). After five days, the skin was challenged with the same volume of 1% DNCB in acetone/olive oil (1:3) three times per week for two weeks. One day after the last DNCB application fabrics treated with *Rhus verniciflua* extract of concentrations; 3, 30 and 300 mg/ml were worn on the atopic dermatitis like lesions formed on back skin of BALB/c mice in groups; FRV3, FRV30 and FRV300, respectively. Normal saline treated fabrics were worn on back skin of mice in control and DNCB groups. The fabrics treated with the same concentrations of *Rhus verniciflua* extract were aseptically replaced every day for two weeks.

Blood histamine level, eosinophil and basophil counts: Blood samples were collected under anesthesia using K2-EDTA-coated tubes (Microvette Sarstedt, Germany) and then centrifuged at 3,000 rpm for 10 min at 4°C. Plasma histamine was analyzed using histamine ELISA kit (IBL-Hamburg, Germany). Complete differential blood count (CBC) was performed to analyze eosinophil and basophil counts.

The level of TNF-α in skin supernatant: Skin samples (1×1 cm^2) were collected at the time of sacrifice after the end of the experimentation and then homogenized at 20,000 rpm for 30 min in 5 ml phosphate buffered saline (PBS) containing protease inhibitor (Sigma, USA). After centrifugation, the supernatant of homogenized skin was collected and analyzed for the level of TNF-α using TNF-α ELISA kit (Biosource, Europe S.A).

Clinical scoring of DNCB induced atopic dermatitis: After two weeks treatment the back skin was observed for erythema/hemorrhage, scarring/dryness, edema, excoriation/ erosion, lichenification and then scored zero (none), one (mild), two (moderate) and three (severe) as described elsewhere (Dhar *et al*., 2005). Mean values of the scores were calculated for each group.

Histopathological examination: Skin samples of mice were collected immediately after sacrifice and preserved in 10% formaldehyde in PBS (PH 6.8). Histopathological changes of the skin were examined after routine tissue processing and hematoxylin-eosin staining in histo-pathology laboratory, Chungnam National University.

Statistical analysis: Data were expressed as mean±SD and the statistical analysis of the data was performed by one-way analysis of variance (ANOVA). $P<0.05$ were considered statistically significant.

RESULTS

Mean (±SD) values of plasma histamine level, blood eosinophil and basophil counts, TNF-α level of skin supernatant and clinical score for severity of skin lesions of male BALB/c mice are shown in Table 1. The values of plasma histamine level of mice in all FRV treated groups were lower than those in DNCB groups. Among FRV treated mice, those in FRV30 group showed relatively lower values in plasma histamine level than those in FRV3 and FRV300 groups. As shown in Table 1, the blood eosinophil and basophil counts of mice in FRV3 and FRV30 were significantly ($P<0.05$) lower than counts observed for mice in DNCB and FRV300 groups.

The mean TNF-α level in skin supernatant of mice in DNCB group was significantly ($P<0.05$) higher than values observed for mice in control and treatment groups. The mean value of TNF-α observed for mice in FRV30 group was significantly different from values observed in mice in other treatment groups. The mean values of clinical skin index of mice in DNCB group were significantly ($P<0.05$) higher than corresponding values in other groups. The clinical skin index values observed for mice in FRV30 were significantly ($P\leq0.05$) lower than values observed in other treatment groups (FRV3 and FRV300). The skin lesions of mice in FRV groups showed relatively fast recovery and healing than DNCB group (data not shown). Histological features of skin are displayed in (Fig. 1). Comparative epidermal thickness and infiltration of inflammatory cells of the skin for mice in FRV3 and FRV30 groups was relatively lower than DNCB and FRV300 groups.

DISCUSSION

Since dinitrochlorobenzene (DNCB) is capable to form a complex of immunogenic covalent conjugates that

Table 1: Mean values of plasma histamine level, blood eosinophil and basophil counts, TNF-α level of skin supernatant and clinical score for severity of skin lesions of male BALB/c mice in different experimental groups

Groups	Histamine (ng/ml)	Eosinophil (10^3/mm^3)	Basophil (10^3/mm^3)	TNF-α (ng/ml)	Clinical skin index
Control	0.366±0.049^a	0.002±0.002^a	0.000±0.000^a	0.900±0.400^a	0.000±0.000^a
DNCB	0.971±0.024^b	0.123±0.005^b	0.032±0.002^b	0.278±0.410^b	13.000±0.870^b
FRV 3	0.728±0.004	0.011±0.001^a	0.006±0.002^a	0.141±0.345	10.000±1.870^a
FRV 30	0.569±0.079	0.013±0.002^a	0.007±0.001^a	0.999±0.341^a	6.600±1.500
FRV 300	0.724±0.006	0.031±0.003^b	0.031±0.004^b	0.238±0.356	12.600±1.820

Values (mean±SD) sharing different superscript letters are statistically significant (P<0.05). Control (with no treatment); DNCB (positive control; DNCB-induced atopic dermatitis with no treatment); FRV3 (DNCB-induced atopic dermatitis with 3 mg/ml FRV treatment); FRV30 (DNCB-induced atopic dermatitis with 30 mg/ml FRV treatment); FRV300 (DNCB-induced atopic dermatitis with 300 mg/ml FRV treatment).

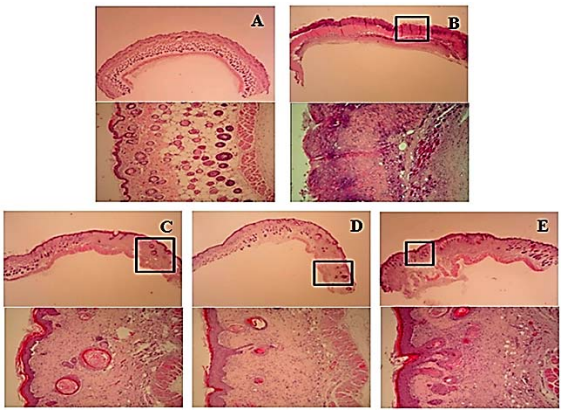

Fig. 1: Histopathological features of skin lesion of mice in control (A); DNCB (B); FRV3 (C); FRV30 (D); FRV300 (E) groups.

could be internalized, processed and then presented to T-cell (Watanabe *et al*., 2002), it has been used to induce atopy like dermatitis in animal model (Yang *et al*., 2012). In the current study, the effect of *Rhus verniciflua* extract on DNCB induced atopy like dermatitis was evaluated in BALB/c mice. DNCB modified macromolecules (immunogens) induce T-cell mediated immune response (Watanabe *et al*., 2002) that leads to synthesis of IgE from B-cells (Leung, 1997*)* and subsequent histamine release from mast or basophils cells *(*Gould *et al*., 2003). In this study*,* relatively lower plasma histamine level observed in Rhus *verniciflua* extract treated BALB/c mice than DNCB group (Table 1) could be associated with the effect of Rhus *verniciflua extract* on synthesis of IgE*,* which could sequentially induce the release of histamine from mast or basophil cells*.* In previous study DNCB induced mice had shown high level of serum IgE and application of *Chrysanthemum boreale flos* produced significant reduction in the level of serum IgE and itching behavior in atopic dermatitis mouse model (Yang *et al*., 2012). In the present study, the effect of *Rhus verniciflua* extract on intracellular calcium or cAMP was not studied. However, since de-granulation of mast cells and downstream histamine release depends on intracellular calcium and cAMP (Kim *et al*., 2006), the lower level of plasma histamine observed for mice in treatment groups (FRV3, FRV30 and FRV300) might also be attributed to the effect of *Rhus verniciflua* extract on intracellular calcium or cAMP. The observed effect of *Rhus verniciflua* extract on plasma histamine level of BALB/c mice in the present study suggests its therapeutic potential for the management of histamine induced pruritus and itching (Imaizumi *et al*., 2003).

Although there are different cells involving in patho-physiology of atopic dermatitis, the role of eosinophils in promoting tissue injury and pathogenesis of atopic dermatitis through release of cytotoxic proteins, lipid mediators, oxygen metabolites, and cytokines is also crucial (Shinagawa *et al*., 2003). Likewise, basophils are known for their role as effectors for IgE-mediated allergies (Grundström *et al*., 2012) and production and release of multiple mediators such as histamine, IL-4 and IL-13 during atopic dermatitis (Li *et al*., 1996; Khodoun *et al*., 2004; Schneider *et al*., 2010).Thus drugs or natural compounds which can reduce or tackle the role of eosinophil and basophils in pathogenesis of atopic dermatitis are very important. Although it was not investigated how high dose (300 mg/ml) topical application of *Rhus verniciflua* extract in BALB/c mice produced significant increase in both eosinophil and basophil counts than the inverse effect observed after application of low (3 mg/ml) and medium (30 mg/ml) doses (Table 1), it suggests the effect of high dose in activating immune system that leads to an increase in the production of eosinophil and basophil in bone marrow and subsequent release to circulation. This supports the previous reports that lower doses of *γ*-tocotrienol were able to prevent reduction of antioxidant enzyme (GPx) activity and apoptosis of osteoblast exposed to hydrogen peroxide (H2O2) than the opposite effect observed for high dose (Abd Manan *et al*., 2012) and anti-fibrotic effect of lower dose and reverse effect of higher dose of Chinese Herb *Rheum palmatum* L. in treating rat liver injury (Wang *et al*., 2011). The possible reasons for the controversial result in eosinophil and basophils counts in BALB/c mice observed in the current study might be due to concentration dependent positive effect of *Rhus verniciflua* extract on T-cell mediated immune responses (Th2 type cells) and Th2 driven IL-5 which is reported important for generation of eosinophils upon allergic sensitization and challenge (Simon *et al*., 2004) and negative effect on differentiation of basophils from hematopoietic pluripotent stem cell in the bone marrow (Arock *et al*., 2002).

A similar result to the significant reduction of pro-inflammatory cytokine TNFα in the current study (Table 1) were observed in previous *in vitro* study in which *Rhus verniciflua* extract and its active compound fisetin attenuated mRNA expression of TNFα in macrophage cells (Park *et al*., 2013). Although the mechanism of reduction in TNFα was not studied, the similarity of our result with previous reported values implies consistent effect of *Rhus verniciflua* extract on TNFα in both *in vitro* and *in vivo* situation. In the present study, we did not study how the level of TNFα is significantly lower in skin

samples of medium dose (30 mg/ml) *Rhus verniciflua* extract treated mice than low (3 mg/ml) and high (300 mg/ml) dose treated once. However, it might be due to dose dependent inhibitory effect of the extract on TNFα producing cells (Groves *et al*., 1995**)** or on mRNA expression of TNFα in macrophage cells (Park *et al*., 2013).

The significant lower values observed for the skin severity scores in FRV30 group when compared with DNCB treated groups (Table 1) is in accordance with previous study in which oral administration of *Rhus verniciflua* extract reduce scratching frequency and epidermal thickness on dorsal skin (Yang *et al*., 2012). Likewise, histopathological changes of the skin in DNCB induced *Rhus verniciflua* extract treated mice in the current study (Fig. 1) supports previous study (Park *et al*., 2013) in which topical treatment of *Rhus verniciflua* extract significantly reduce skin thickness and infiltration of inflammatory cells.

Conclusion: The result obtained in this study indicates the potential of the fermented *Rhus verniciflua* extract as alternative therapy for management of atopic dermatitis. However, further study, which focuses on principle active compounds and their mechanism, is recommended.

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