The Effect of Extract from Sea Buckthorn on DNCB-induced Atopic Dermatitis in NC/Nga Mice

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Abstract - Sea Buckthorn (Hippophae rhamnoides L.) has been used in traditional medicine for the treatment of cough, indigestion, circulatory problems and pain. The associated anti-inflammatory effect of this agent is achieved via the inhibition of Nf-κB signaling, a property that has been demonstrated to effectively control the symptoms of various skin disorders, including atopic dermatitis. Accordingly, the purpose of this study was to assess the efficacy of Sea Buckthorn in reducing the production of lipopolysaccharide (LPS) activated nitric oxide (NO) by inhibiting the Nf-κB pathway, as measured by the symptoms of atopic dermatitis (AD) occurring secondarily to inflammation and immune dysregulation. Our data demonstrate that Sea Buckthorn significantly decreased the LPS-induced production of NO (p<0.001). Atopic dermatitis was induced by repeated application of 2,4-dinitrochlorobenzene to the dorsal skin of mice. Topical application of 5% Sea Buckthorn extract improved the symptoms of AD, specifically reducing disease severity scores, scratching behaviors and epidermal thickness. When compared to the control group, animals treated with Sea Buckthorn exhibited increased serum IL-12 levels and decreased serum TNF-α, IL-4 and IL-5 levels. Such a modulation of biphasic T-helper (Th)1/Th2 cytokines may result in a reduction in serum IgE levels. Our findings suggest that mechanism of action of Sea Buckthorn in the treatment of AD is associated with a marked anti-inflammatory effect as well as an inhibition of Th2-mediated IgE overproduction via the modulation of biphasic Th1/Th2 cytokines. Such results suggest that topical Sea Buckthorn extract may prove to be a novel therapy for AD symptoms with few side effects.

Key words - Atopic Dermatitis, Cytokine, IgE, Sea Buckthorn (Hippophae rhamnoides L.).

Introduction

Atopic dermatitis is a chronic inflammatory skin disease affecting all age groups, which often presents as dry skin and pruritus (Novak, 2009). Although the exact underlying cause of atopic dermatitis remains unknown, it has been associated with immunologic dysregulation, specifically imbalances of Th1 and Th2 cytokines (Pate et al., 2010).

Physiologically, Th1 cells are believed to regulate cell-mediated immunity via interleukin 2 (IL-2) and interferon γ (IFN-γ), while Th2 cells are thought to induce humoral immunity and promote antibody production in B cells via IL-4, -5, and -10 (Reinhold et al., 1988). In general, the balance of the Th1 and Th2 axes skews more toward Th2 in the setting of atopic dermatitis, as manifested by accelerated IgE production, increases in mast cells, and permeation of eosinophils (Coffman and Carty, 1986; Namkoong et al., 2012). In turn, IgE binds to specific cell surface receptors on mast cells, basophils and eosinophils, thus stimulating the activation of these cell populations after antigen bridging to various discharge substances (Barnes, 2000).

In addition to immunologic dysregulation, defective skin barrier function is also believed to play an important role in the underlying pathophysiology of atopic dermatitis. Under normal conditions, the skin barrier is comprised of a sequential bed structure of lipids that exists between keratinocytes (Chen et al., 2008). These keratinocytes, which...
contain keratin bundles, contribute to skin elasticity and stability. Furthermore, the lipids between the keratinocytes are composed of ceramides, cholesterol and free fatty acids, and act to inhibit water and electrolytes losses (Choi, 2000; Lee et al., 1999; Park et al., 2005; Park et al., 2011). The skin barrier defects associated with atopic dermatitis cases classically result from decreased ceramide levels, which often manifests clinically as xerosis, a near ubiquitous complaint in affected individuals (Park and Lee, 2000). Moreover, atopic dermatitis and psoriasis are the two main skin diseases associated with skin barrier defects resulting from decreased ceramide levels (Lee et al., 1999; Park and Lee, 2000). Such decreases in ceramide act to further reduce sphingosine levels (formed via ceramide decomposition) and thus induce protein kinase C (PKC) activation, which is normally suppressed by sphingosine (Hunnum and Bell, 1989). This activation of PKC then prompts nuclear factor (NF)-κB activation, thereby inducing the expression of inflammation-related and apoptosis-suppressing genes (Baueuerle and Baltimore, 1996) and up-regulating nitric oxide synthase (iNOS) expression to produce increased amounts of nitric oxide (NO) (Baueuerle, 1998; Kim et al., 2012; Li et al., 2000; Morise et al., 1998).

As the secondary signal transducer within cells, NO has been suggested to exert a potent pro-inflammatory effect, as high-concentrations of NO in vivo have been shown to disrupt host cells, induce an increase in eosinophil production, expand blood vessels, and generate tissue damage by triggering inflammation (Anggard, 1994).

While dexamethasone, cyclosporine A and other various steroids are often regarded as the standard of care in the treatment of atopic dermatitis, the use of natural therapeutic agents is garnering significant attention due to the lower risk of side-effects. Sea Buckthorn (Hippophae rhamnoides), commonly known as the vitamin tree, is a deciduous shrub in the Elaeagnaceae family (which includes Sandthorn, Swallowthorn and Sea-berry). Though Sea Buckthorn grows naturally throughout central Asia to Europe, it is also extensively cultivated in Russia, Europe, Canada, China, Mongolia and other nations surrounding the Himalayas (Merja et al., 2006; Richard and Paul, 2008; Rousi, 1971). The leaves of the Sea Buckthorn have been shown to be rich in flavonoids, tannin, vitamin C, protein, fat, fiber, amino acids and minerals. Not surprisingly, the 70% EtOH extract of Sea Buckthorn leaves has been suggested to exert potent antioxidative, immune regulatory (Geetha et al., 2003) and anti-inflammatory effects (Ganju et al., 2005). Moreover, Sea Buckthorn fruit is abundant in vitamins A, C, E and K, carotenoids, and organic acids, and has also been demonstrated to have powerful anti-oxidative (Eccleston et al., 2002; Suleyman et al., 2002) and photo-protective effects (Goel et al., 2002), as well as the ability to suppress ulcer formation (Suleyman et al., 2001). However, to date no research has been conducted in relation to the effect of Sea Buckthorn on atopic dermatitis.

NC/Nga mice – the atopic dermatitis animal model used in this experiment – lose their earflaps at week 7 as a result of erythema, hair loss, bleeding, and crusting secondary to dermatitis-related behaviors (scratching/itching). As these findings mirror the clinical symptoms associated with atopic dermatitis, these animals are widely used as the experimental model for spontaneous atopic dermatitis (Horiuchi et al., 2004).

The present study attempts to assess the effect of Sea Buckthorn in the treatment of atopic dermatitis. After triggering atopic dermatitis in NC/Nga mice, visual and histologic assessments of the effects of the Sea Buckthorn in atopic dermatitis were conducted, with the anti-inflammatory effects verified via NO assay. Secondarily, we attempted to demonstrate the effect of this agent on serum immunoglobulin cytokine concentrations in splenocytes.

Materials and Methods

Materials

Seven-week-old NC/Nga male mice were purchased from Central Laboratory Animals Inc. (Seoul, Korea) and maintained under conventional conditions. All aspects of this study were performed in accordance with the Institutional Animal Care and Use Committee guidelines of Kyung Hee University (Yongin, Republic of Korea).

All reagents used for cell culture, including penicillin/streptomycin, fetal bovine serum, trypsin, trypan blue and Dulbecco’s modified Eagle’s medium (DMEM), were purchased from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS), Griess reagent, 2,4-dinitrochlorobenzene (DNCB),
dexamethasone, paraformaldehyde, phosphate buffered saline (PBS), hematoxylin, eosin and toluidine blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ketamine was purchased from Yuhan Corp. (Seoul, Korea). Rompun was purchased from Bayer (Leverkusen, Germany).

**Sea Buckthorn leaf extract preparation**

Over the course of 3 hours, 20 kg of Sea Buckthorn leaves were extracted with 30 L of 70% aqueous ethanol via a sonicator. Next, the Vita 70% extract was evaporated using a rotary vacuum evaporator (NE-2001 & AC 1112A, Eyela Co., Tokyo, Japan) (1.4 L, 30 brix). Additionally, 250 kg of Sea Buckthorn leaves were extracted with 3,600 L of distilled water at temperatures between 70 and 80 °C for 3 hours. This extract was then evaporated to obtain the Vita water extract (50 kg, 20% yield).

**Cell culture and nitric oxide (NO) measurement**

Raw 264.7 cells, a mouse leukemic monocyte macrophage cell line from ATCC (Manassas, VA, USA), were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. All experiments were performed in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were first plated in 24-well plates at a density of 5×10³/well and allowed to stabilize for 24 hours. Following this period, the cells were stimulated for 24 hours with 0.5 μg/mL LPS, dexamethasone and Sea Buckthorn extract. The production of nitric oxide (NO) was then assessed by measuring the accumulation of nitrite in the culture supernatants via colorimetric reaction with Griess reagent per the manufacturer's instructions.

**Degranulation assay**

Rat basophilic leukemia (RBL-2H3) cells were obtained from the Korean Cell Line Bank (Seoul, Korea, KCLB 22256) and cultured in DMEM supplemented with 10% FBS. The RBL-2H3 cells were then plated in 96-well plates at a density of 5×10³/well and incubated overnight. The cells were then sensitized with IgE against 0.1 μg/mL DNP-BSA (Sigma) for 1 hr in DMEM without FBS and subsequently washed three times with Tyrode’s assay buffer containing 10 M HEPES and 0.1% BSA, pH 7.4 in order to remove the unbound IgE. Next, 50 μl aliquots of the solution were treated with Tyrode’s assay buffer for 1 hour, after which 50 μl of 2 μg/mL DNP-BSA was added. After 30 minutes of additional stimulation, the supernatants were collected and the unsensitized cells were lysed using 0.1% Triton X-100. The β-hexosaminidase activity in the supernatant and lysate was measured using a spectrophotometer as previously described (Suzuki et al., 2003). The activity in the unsensitized cell lysate was defined as the total activity for the calculation of the release percentage.

**Induction of AD-like skin lesions in NC/Nga mice and topical Sea Buckthorn extract application**

Fifty 7-week-old NC/Nga male mice were randomly divided into five study groups and acclimated under standard conditions for 1 week prior to the experiments. DNCB-induced AD-like dermatitis was induced using the following protocol: The mice’s abdomens and ears were sensitized with 200 μl applications of 1% DNCB dissolved in a mixture of acetone and olive (4:1, v/v) twice with a three-day interval between applications. Five days after sensitization, the mice’s dorsal skin was challenged with 200 μl of 0.2% DNCB three times per week for 4 weeks. After this period, 150 μl of 70% EtOH or water Sea Buckthorn extract (5% in 1,2-propanediol:ethyl alcohol:distilled water = 5:3:2) or 0.1% dexamethasone was applied topically every day for 4 weeks.

**Scratching behavior**

Scratching behavior was assessed for 3 hours with MicroAct (Neuroscience, Japan), a system that automatically and objectively detects and evaluates murine scratching behavior (Inagaki et al., 2002; Inagaki et al., 2003). For these assessments, a small magnet (1 mm in diameter, 3 mm in length) was implanted subcutaneously into both hind paws of each mouse under ether anesthesia one week prior. The MicroAct analysis parameters for detecting waves were a threshold of 0.1 V, event gap of 0.2 s, minimum duration of 1.5 s, maximum frequency of 20 Hz, and minimum frequency of 2 Hz.

**Histologic change analysis**

Mice were sacrificed 4 weeks after the first topical
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Fig. 1. Inhibition of lipopolysaccharide (LPS) induced production of nitric oxide (NO) by Sea Buckthorn. As indicated, macrophage cell line, RAW 264.7 was treated with LPS and Sea Buckthorn. After 24 hours incubation, the amounts of NO in the media were measured as nitrite by Griess reagent (Normal, No LPS treatment; Negative Control (NC), LPS 0.5 μg/mL treatment; Positive Control (PC), LPS+Dexamethasone 20 μM treatment; Vita 70%, LPS+Sea Buckthorn leaf 70% EtOH extract 10 μg/mL treatment; Vita water, LPS+Sea Buckthorn leaf water extract 10 μg/mL treatment; *, P<0.05; **, P<0.01; ***, P<0.001).

Serum IgE and cytokine measurements

Blood was collected from the retro orbital plexus using heparinized glass capillaries at the time of sacrifice. Sera were obtained by centrifuging the whole blood for 10 minutes at 16,000 g; sera were stored at -80 °C until analysis. Serum levels of IgA, IgE, IgG1, IgG2a and IgG2b were quantified by ELISA (BD Biosciences, Franklin Lakes, NJ, USA). Serum levels of TNF-α, IFN-γ, IL-4, IL-5, IL-12 and MCP-1 were quantified by ELISA (BD Biosciences, Franklin Lakes, NJ, USA).

Statistics

All data are expressed as the mean ± standard deviation, with differences compared to the control group assessed by Student's t-test.

Results

Inhibition of LPS-induced production of NO by Sea Buckthorn extract

Murine macrophage Raw 264.7 cells were used to assess whether Sea Buckthorn extract produces an anti-inflammatory effect on LPS-activated NO production. Enhanced production of NO in response to stimulation has been reported to cause inflammatory diseases (Ko et al., 2010). As demonstrated in Figure 1, the Sea Buckthorn water extract suppressed NO formation by 67% (p ≤ 0.001). Furthermore, the 70% EtOH extract suppressed the NO formation by 73%. These results indicate that the water and 70% EtOH extracts of Sea Buckthorn have a strong anti-inflammatory effect. These extracts were subsequently used in experiments designed to determine whether they could alleviate the symptoms of atopic dermatitis induced by DNCB in an animal model.

Mast cell degranulation inhibition

The release of β-hexosaminidase, a biomarker of degranulation, was observed in IgE-sensitized RBL-2H3 cells after antigen exposure. Treatment with Sea Buckthorn extract at concentrations of 1 mg/mL for 1 hr prior to antigen stimulation significantly reduced this release (p<0.01; Fig. 2A). Cell survival rates were above 80% at all concentrations (Fig. 2B).

Skin lesion scratching frequency

Scratching frequencies were measured immediately after triggering atopic dermatitis via DNCB (0 weeks). These measurements were used to allocate the animals into experimental groups, among which the differences were insignificant. The number of scratching events was measured 2 and 4 weeks after application of the substances. The number of scratching events was significantly higher at 0 weeks in the atopic dermatitis group when compared to the controls. The number of scratching events then spontaneously decreased after the application, and the upper dorsal skin was removed for histological examination. Skin sections were fixed in 10% phosphate-buffered formalin (pH 7.2) for 24 hours, embedded in paraffin, sectioned at a thickness of 4 μm and stained with hematoxylin and eosin and toluidine blue.
cessation of DNCB application. However, the topical application of the 70% EtOH extract or water extract of Sea Buckthorn leaf significantly decreased the number of scratching events at 2 and 4 weeks when compared to that in the negative control group (Fig. 3). Dexamethasone also significantly reduced the number of scratching events at 2 weeks, but not at 4 weeks. These results were in good agreement with the corresponding
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macroscopic observations (Fig. 4). At 0 weeks, the mice exhibited skin lesions associated with symptoms mirroring atopic dermatitis in humans. In addition to erythema and edema affecting the entire back, crusting and pyogenic erythema were observed in severe cases. Ear flap bleeding and edema was also observed in severe cases. At week 2, the lesions in animals with moderate atopic dermatitis spontaneously improved, with only erythema and slight edema noted. At week 4, skin lesion improvement was observed in the groups treated with the 70% EtOH extract of Sea Buckthorn leaf and the water extract of Sea Buckthorn leaf when compared with the negative control group.

**Histological observation**

H&E staining revealed that the epidermis was significantly thicker in the negative control group (NC) when compared to the atopic dermatitis group (Fig. 5A). Specifically, the mean epidermal thickness was 10.70±1.09 μm in the normal group and 29.94±10.43 μm in the negative control group (NC). The application of dexamethasone (PC) decreased the thickness of the epidermis (24.81±12.74 μm), however not significantly. In contrast, animals treated with the 70% EtOH extract of Sea Buckthorn and the water extract of Sea Buckthorn exhibited the significantly decreased epidermal thicknesses of 17.01±6.59 μm and 17.87±3.38 μm (p ≤ 0.001), respectively (Fig. 5C).

Generally, skin tissue of atopic dermatitis patients is...
infiltrated by degranulated mast cells from the epidermis to the upper layer of the subcutaneous tissue (Kenichiro et al., 2009). Therefore, this study compared the number of mast cells in each group through toluidine blue staining. Toluidine blue staining showed that DNCB treatments recruited mast cells to the epidermis (Fig. 5B), with a mean of 42.19 ± 18.58 mast cells in the negative control group. In comparison, a noticeable increase of 7.58±5.72 was observed in the normal group (Normal). Furthermore, the mean number of mast cells was significantly decreased in the positive control group (PC) (29.41±12.55) when compared with the negative control group (p ≤ 0.01). Finally, the mean number of mast cells in the groups treated with the 70% EtOH extract and the water extract was 18.33±6.74 (p ≤ 0.001) and 21.48±6.94 (p ≤ 0.001), respectively (Fig. 5D).

Immunoglobulin measurements in NC/Nga mouse serum

The immunoglobulin within the serum is used as an indicator for evaluating allergy diseases such as atopic dermatitis (Robert et al., 2009). Therefore, this study measured the quantities of IgA, IgE, IgG1, IgG2a, and IgG2b 4 weeks after beginning treatments (Fig. 6A-E). While DNCB treatment resulted in significant differences among groups in terms of serum levels of IgE, IgG1, and IgG2b (p<0.01), no such differences were observed in serum IgA and IgG2a levels. IgE, an effector molecule of atopic dermatitis, is known to be elevated in atopic dermatitis. Here, treatment with DNCB induced IgE elevation, although the differences never reached significance. However, treatment with the water extract of Sea Buckthorn significantly lowered serum IgE levels (p<0.05) when compared to the negative control. Similar, although insignificant, trends were also observed in the
animals treated with the 70% EtOH extract of Sea Buckthorn and dexamethasone. Interestingly, while Serum IgG1 levels were elevated by DNCB, none of the treatment modalities affected this finding. Moreover, treatment with the water extract of Sea Buckthorn significantly elevated the serum IgG2a ($P<0.05$) level when compared to the normal and negative controls.

**Serum cytokine measurements**

As atopic dermatitis is also related to the inflammatory response, inflammatory cytokines also play an important role in improving atopic diseases (Ciebiada *et al.*, 2011). Here, the serum levels of several additional cytokines and chemokines in addition to TNF-$\alpha$ were quantified, including IFN-$\gamma$, IL-4, IL-5, and IL-12 and MCP-1 (Fig. 7A-F). There was no difference between treatments for any of the analyzed factors.

On average, the serum levels of TNF-$\alpha$ were elevated, and were reduced by treatment with both dexamethasone and the Sea Buckthorn extracts. Serum IL-4 was similarly elevated by DNCB sensitization and subsequently reduced by treatment with both dexamethasone and the Sea Buckthorn extracts. Reciprocally, serum IL-12 levels were lower after DNCB sensitization, and later increased after treatment with both dexamethasone and the Sea Buckthorn extracts. Lastly, no general trend was observed in serum IFN-$\gamma$, IL-5, and MCP-1 levels.

**Discussion**

Our data suggest that the water extract of Sea Buckthorn leaf may improve symptoms of atopic dermatitis, as demonstrated by the changes in appearance, scratching behavior and epidermal
thickness in the NC/Nga atopic dermatitis model induced with DNCB. These results coincided with a decrease in mast cells infiltrating the epidermis. The water extract of Sea Buckthorn leaf may inhibit the release of β-hexosaminidase, an indicator of antigen-induced mast cell degranulation anchored by IgE. As such, we contend that the water extract of Sea Buckthorn leaf suppressed scratching behavior by inhibiting the release of inflammatory mediators such as histamine. Moreover, the water extract of Sea Buckthorn leaf reduced the serum levels of IgE and IL-4, while simultaneously raising serum IL-12 levels.

Sea Buckthorn leaves are believed to exert a potent antioxidant effect via several phenolic compounds such as quercetin, kaempferol and the associated glycosides (Maheshwari et al., 2011). In the setting of rat basophilic leukemia (RBL-2H3), these compounds have been suggested to suppress antigen and immunoglobulin E-induced allergic inflammation, such as β-hexosaminidase secretion (Lee et al., 2010). Similarly, mast cells are equipped with the high affinity receptor FcεRI for IgE, the antigen of which is known to induce mast cell degranulation and initiate hypersensitivity, both of which are seen in atopic dermatitis (Kawakami et al., 2009). Histamine, a preformed inflammatory mediator, induces scratching and thus aggravates the symptoms of atopic dermatitis. In addition to emollients, preventing the antigen-induced degranulation of mast cells has been suggested as a prophylactic target in identifying new therapies for atopic dermatitis (Darsow et al., 2010).

Data now suggest that Nf-κB activity is increased in children affected by atopic and non-atopic eczema (Angelini et al., 2007). This transcriptional factor controls the expression of many downstream genes that encode immune receptors, cytokines, chemokines and chemokine receptors, and adhesion molecules critical in the pathogenesis of atopic dermatitis. Moreover, the induction of iNOS by LPS through Nf-κB results in the formation of NO (D’Acquisto et al., 1997), which may act to subsequently effect immunoglobulin class switching (Pate et al., 2010). The data presented here indirectly show that the extract of Sea Buckthorn effectively inhibits Nf-κB signaling, as evidenced by the suppression of LPS-mediated NO formation. Such a blockade of Nf-κB signaling has been suggested to have potential therapeutic value in the treatment of atopic dermatitis (Nakagami et al., 2006).

Atopic dermatitis is characterized by inflammation and immune dysfunction, with Th2 axis predominance. One of the representative Th2 cytokines, IL-4, is elevated in atopic dermatitis and is involved in IgE class switching (Pate et al., 2010). Conversely, IL-12 is associated with the Th1 axis, and together both cytokines are believed to reciprocally control the expression of each other. In addition to antibody therapy versus IgE (Vichyanond, 2011), cytokine and anti-cytokine therapies (Numerof and Asadullah, 2006) have been suggested as effective treatments for atopic dermatitis. In this way, the modulatory effect of Sea Buckthorn on the Th1/Th2 balance may represent a possible therapeutic mechanism of action, as treatment with this agent reduced serum levels of IL-4 while raising serum IL-12.

The findings of the present study suggest that Sea Buckthorn extract may improve atopic dermatitis associated with scratching by stabilizing mast cells and inhibiting IgE expression via modulation of the Th1/2 balance. However, the exact bioactive compounds in Sea Buckthorn extract and the underlying signaling pathways remain to be identified.

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