Sea Buckthorn (*Hippophaë rhamnoides* L.) Oil Improves Atopic Dermatitis-Like Skin Lesions via Inhibition of NF- κ B and STAT1 Activation

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Keywords

Sea buckthorn oil · Atopic dermatitis · DNCB · *Hippophaë rhamnoides* L. · NF- κ B · STAT1

Abstract

Background and Objectives: The objective of this study was to evaluate the topical effects of sea buckthorn (SBT) oil on atopic dermatitis (AD)-like lesions in a mouse model generated by repeated topical administration of DNCB in BALB/c mice. **Methods:** DNCB was applied repeatedly on the dorsal skin of mice to induce AD-like lesions. Following AD induction, SBT oil was applied daily on the dorsal skin for 4 weeks. The severity of skin lesions was examined macroscopically and histologically. We further measured the production of MDC/CCL22 and TARC/CCL17 in IFN- γ /TNF- α activated HaCaT cells. **Results:** Topically applied SBT oil in DNCB-treated mice ameliorated the severity score of dermatitis, decreased epidermal thickness, reduced spleen and lymph node weights, and prevented mast cell infiltration. In addition, SBT oil suppressed the Th2 chemokines TARC and MDC

via dose-dependent inhibition of NF- κ B, JAK2/STAT1, and p38-MAPK signaling pathways in IFN- γ /TNF- α -activated HaCaT cells. **Conclusion:** These results suggest that SBT oil had a beneficial effect on AD-like skin lesions, partially via inhibition of the Th2 chemokines TARC and MDC in inflamed skin.

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease accompanied by an itchy and scaly rash. As a serious global public health problem [1], the morbidity of AD has increased in recent years [2]. AD is a disease arising from complex interaction of genetic factors with skin barrier dysfunction and environmental factors [3, 4]. AD is associated with Th2 cell-mediated allergic reactions characterized by increased production of Th2 cytokines in skin lesions, mast cell activation, and aberrant IgE production [5, 6].

Table 1. Major fatty acids and contents of sitosterol and β -carotene in sea buckthorn oil

Fatty acids, %							Sitosterol, mg/g	β -Carotene, mg/g
myristic acid (C14:0)	palmitic acid (C16:0)	palmitoleic acid (C16:1)	stearic acid (C18:0)	oleic acid (C18:1)	linoleic acid (C18:2)	linolenic acid (C18:3)		
0.83	26.87	27.52	1.2	33.1	4.3	1.5	15.0	6.9

Therapies for AD mainly include systemic anti-histamines, topical steroids, and immunosuppressive agents [7]. As most of these treatment methods are associated with the rebound phenomenon, side effects, or intermittent recurrences [8, 9], many patients are still worried about adverse effects of long-term treatment with these agents [10]. Presently, there is global interest in seeking new anti-inflammatory agents from natural materials [11, 12].

Sea buckthorn (SBT) (*Hippophaë rhamnoides* L.) is a wild shrub belonging to the Elaeagnaceae family. Berries of SBT have been used in the Chinese, Mongolian, and Tibetan traditional medicine for the treatment of various diseases for more than 1,000 years [13]. Many researchers have paid much attention to SBT for its various medicinal values, such as anti-inflammatory and immunoregulatory effects [14–16]. All parts of SBT are rich sources of bioactive agents [9]. SBT berry oil is a kind of multifunctional natural oil and contains abundant bioactive substances such as ω -3 and ω -6 fatty acids, tocopherols, phytosterols, and carotenoids [17].

This study estimated the topical effects of SBT oil on AD mouse models induced by 2,4-dinitrochlorobenzene (DNCB). It was observed that topical application of SBT oil reduced the severity of dermatitis, infiltration of mast cells, epidermal thickness, and spleen and lymph node weights. Furthermore, there was an inhibitory effect of SBT oil on TARC and MDC expression in IFN- γ /TNF- α -treated HaCaT keratinocytes.

Materials and Methods

SBT Oil

SBT oil was purchased from Dongning Pharmaceutical Co., Ltd. The oil was extracted from dried SBT residues (including seeds, berry flesh, and peel), using an aseptic supercritical carbon dioxide process. An HP-5MS capillary column (30 m $\times$ 0.25 mm, 0.25 μ m; Agilent Technologies Inc., Santa Clara, CA, USA) in a GC/MS (5975C, Agilent Technologies Inc.) was adopted for the analysis of samples. Samples were injected into the column and run using the split mode (split ratio = 10:1). The helium carrier gas was programmed to maintain a constant flow rate of 1 mL/min. Oven

heating was initially 80°C for 3 min and then finally raised to 300°C at 4°C/min. Fatty acids were identified by a reference standard mixture FAME (Supelco, Bellefonte, PA, USA) analyzed under the same operating conditions as those employed for FAME of the samples. The SBT oil components are exhibited in Table 1.

Animals

Female BALB/c mice, aged 6 weeks, were adopted in this study. The mice were provided by Liaoning Changsheng Biotechnology Co., Ltd (Benxi, China). All mice were housed under pathogen-free conditions and allowed free access to water and food. All experimental procedures were in accordance with the guidelines approved by the Ethical Committee of Experimental Animal Care at the China Medical University (Shenyang, China).

Animal Treatment

Mice were divided into 5 groups: the normal control group (a), DNCB group (b), and groups treated with DNCB plus topical dexamethasone acetate (DEX) (c), DNCB plus topical olive oil (d), and DNCB plus topical SBT oil (e). Each group comprised 6 mice.

To induce AD-like skin lesions, DNCB (Sigma-Aldrich, St. Louis, MO, USA) was applied onto the dorsal skin [18]. An area of approximately 6 cm² was shaved on each mouse back using an electric clipper 1 day before DNCB sensitization. As shown in Figure 1, for sensitization, 200 μ L of 1% DNCB solution (dissolved in acetone) was applied for 4 times from day 1 to day 11. To induce AD-like lesions, 200 μ L of 0.5% DNCB solution was applied on the dorsal skin once every 3 days from day 15 to day 42. The SBT oil (40 μ L) was prepared in cream form dosed at 200 μ L for each treatment. The cream was applied to the dorsal skin once a day from day 15 to day 42. Olive oil was provided by Sinopharm Chemical Reagent Co., Ltd, Shanghai, China. DEX (1 mg/kg) cream was purchased from Hubei Ketian Pharmaceutical Co., Ltd, Hubei, China. Mice were sacrificed by CO₂ inhalation, and samples were collected. Spleen and axillary lymph nodes were removed and weighed.

Evaluation of AD Severity

Dermatitis severity was assessed according to 4 specific parameters once a week: (1) hemorrhage/erythema, (2) dryness/crusting, (3) erosion/excoriation, and (4) edema [19]. Each component was scored on a scale from 0 (none), 1 (mild), 2 (moderate), to 3 (severe); the sum of the individual scores was defined as the overall dermatitis score.

Histopathological Studies

The dorsal skin of mice was collected on the final day of the schedule, fixed in 10% formalin, and embedded in paraffin. Skin sections (5 μ m thick) were cut and stained with either H&E stain-

Fig. 1. Schematic diagram of the experimental protocol. SBT, sea buckthorn.

Fig. 2. Effects of sea buckthorn (SBT) oil on DNCB-induced atopic dermatitis (AD)-like skin lesions and body weight of mice. **A** Example of the clinical severity of AD-like skin lesions in the normal control group (a); DNCB group (b); DNCB plus olive oil treatment group (c); DNCB plus dexamethasone (DEX) acetate

treatment group (d); and DNCB plus SBT oil treatment group (e). **B** The dermatitis scores were defined as the sum of scores based on the various AD symptoms. **C** The body weight of the mice. ## $p < 0.01$ vs. the control group; *** $p < 0.001$ vs. the control group; ** $p < 0.01$, * $p < 0.05$ vs. the DNCB group; ΔΔ $p < 0.01$, Δ $p < 0.05$ vs. the DNCB + DEX group.

ing for counting of inflammatory cell numbers or with toluidine blue staining for counting mast cells.

Cell Culture

Human keratinocyte HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose), which was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified 5% CO₂ incubator. FBS, DMEM, PBS (pH 7.4), penicillin, and streptomycin were all provided by Hyclone Co. (USA).

TARC/CCL17 and MDC/CCL22 Production

HaCaT cells (1.0×10^6 cells/well) were seeded in 6-well plates. After 24 h, the cells were washed and treated with SBT oil in 1 mL serum-free media containing IFN-γ (10 ng/mL)/TNF-α (10 ng/mL) (R&D Systems, Minneapolis, MN, USA) for 24 h. The culture medium of the cells was harvested, and the production of TARC and MDC were determined using an ELISA kit (Cusabio, Wuhan, China).

Western Blots

Total protein was extracted from HaCaT cells using RIPA lysis buffer (Beyotime Biotechnology, Nantong, China) according to the manufacturer's protocol. The protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins were electrophoresed on 10% SDS polyacryl-

amide gel and transferred to a nitrocellulose membrane by electroblotting. The membrane was incubated with primary antibodies (p-IkBα, IkBα, p-NF-κB p65, NF-κB p65, p-p38, p38, p-STAT1, STAT1, p-JAK2, JAK2, and β-actin) at 4°C overnight followed by incubation with the appropriate HRP-conjugated secondary antibodies (Proteintech, Wuhan, China) for 1 h at room temperature. All primary antibodies were provided by Ruiying Biological Technology Co., Ltd, Suzhou, China. The membranes were visualized by the ECL detection system (Bio-Rad Laboratories).

Statistical Analysis

Results are presented as means ± SD. The significance of differences of all results was analyzed by one-way analysis of variance (ANOVA) followed by the Dunnett t test. $p < 0.05$ was considered significant.

Results

SBT Oil Suppressed the Development of DNCB-Induced AD-Like Mouse Skin Lesions

To evaluate the effect of SBT oil against AD-like skin lesions, 1 mL/kg SBT oil was applied on mice once a day along with induction of AD-like skin lesions by DNCB

Fig. 3. Effects of sea buckthorn (SBT) oil on histological dorsal skin lesions in the normal control group (a); DNCB group (b); DNCB plus olive oil treatment group (c); DNCB plus dexamethasone (DEX) acetate treatment group (d); and DNCB plus SBT oil treatment group (e). **A** H&E. $\times 200$. **B** Epidermal thickness was ana-

lyzed in H&E-stained tissue. **C** Toluidine blue. $\times 200$. **D** The number of mast cells was analyzed in the toluidine blue-stained sections. ## $p < 0.01$ vs. the control group; ** $p < 0.01$ vs. the DNCB group; Δ $p < 0.05$, $\Delta\Delta$ $p < 0.01$ vs. the DNCB + DEX group.

Fig. 4. Effect of sea buckthorn (SBT) oil on spleen and lymph node weights in DNCB-induced atopic dermatitis-like skin. Spleen (**A**) and lymph node weights (**B**) in the normal control group (a); DNCB group (b); DNCB plus olive oil treatment group (c); DNCB

plus dexamethasone (DEX) acetate treatment group (d); and DNCB plus SBT oil treatment group (e). ^{##} $p < 0.01$ vs. the control group; ^{**} $p < 0.01$, ^{*} $p < 0.05$ vs. the DNCB group; ^{ΔΔ} $p < 0.01$ vs. the DNCB + DEX group.

administration. The severity of dermatitis was evaluated once a week. The DNCB-induced AD-like skin lesions resulted in severe itching, mild erythema, and hemorrhage, which was followed by abnormal keratinization, dryness, excoriation, and edema of the skin. After SBT oil or DEX application for 4 weeks, SBT oil significantly decreased DNCB-induced AD severity; the dermatitis scores of the DEX group decreased more markedly than that of SBT oil group (Fig. 2A, B). However, more body weight reduction was observed in the DEX group than in the SBT oil group (Fig. 2C). Compared to the DNCB group, olive oil had no protective effect against AD-like skin lesions in mice.

Histologically, SBT Oil Improved the Skin and Decreased Mast Cell Infiltration in Mice

To evaluate whether SBT oil reduces infiltration of inflammatory cells into AD skin lesions, H&E staining was performed on the skin sections. Repetitive DNCB treatments induced epidermal hyperplasia and increased epidermal thickness. In contrast, SBT oil application suppressed epidermal hyperplasia and epidermal thickness induced by DNCB (Fig. 3A, B). Toluidine blue staining was also performed to trace mast cells. Repetitive DNCB treatments increased mast cell numbers in the skin sec-

tions. However, this feature was inhibited by SBT oil compared with the DNCB group (Fig. 3C, D). The effects of SBT oil on DNCB-induced epidermal hyperplasia and mast cell infiltration were also studied.

SBT Oil Decreased the Spleen and Lymph Node Weights in Mice with AD-Like Skin Lesions Induced by DNCB

To determine whether SBT oil decreases spleen and lymph node weights, we weighed the spleen and axillary lymph nodes. Results demonstrated an increase in both spleen and lymph node weights in DNCB-treated mice, which were both decreased by SBT oil application (Fig. 4).

SBT Oil Inhibited IFN- γ /TNF- α -Induced Production of TARC and MDC in HaCaT Cells

In order to determine whether SBT oil suppresses TARC and MDC production, HaCaT cells were co-treated with IFN- γ and TNF- α in the presence or absence of SBT oil for 24 h, and culture supernatants were harvested for chemokine detection with ELISA. TARC and MDC production in HaCaT cells increased significantly following stimulation with IFN- γ /TNF- α . Increased TARC and MDC production was suppressed by SBT oil in a dose-dependent manner (Fig. 5). The results suggest that SBT

Fig. 5. Effects of sea buckthorn (SBT) oil on IFN- γ /TNF- α -induced TARC (**A**) and MDC levels (**B**) in HaCaT cells. ## $p < 0.01$ vs. the control group; ** $p < 0.01$ vs. the TNF- α /IFN- γ -treated group.

oil can inhibit the expression of Th2 chemokines such as TARC and MDC in IFN- γ /TNF- α -stimulated HaCaT cells.

SBT Oil Inhibited IFN- γ /TNF- α -Induced NF- κ B, JAK/STAT1, p38-MAPK Activation in HaCaT Cells

In order to determine whether SBT oil has an influence on the activation of the NF- κ B, JAK2/STAT1, and p38-MAPK signaling pathways in IFN- γ /TNF- α -stimulated HaCaT cells, HaCaT cells were co-treated with IFN- γ and TNF- α in the presence or absence of SBT oil for 24 h. Total proteins extracted from cells were assessed in terms of NF- κ B, JAK2-STAT1, and p38-MAPK activation by immunoblotting. As shown in Figure 6, SBT oil inhibited IFN- γ /TNF- α -induced phosphorylation of I κ B α , NF- κ B p65, JAK2, STAT1, and p38 expression in a dose-dependent manner. These results suggest that suppression of IFN- γ /TNF- α -induced TARC and MDC production by SBT oil is probably mediated by the inhibition of NF- κ B, JAK/STAT1, and p38-MAPK activation.

Discussion

In this study, we demonstrated the inhibitory effects and molecular mechanisms of SBT oil on AD-like skin symptoms both in vivo and in vitro.

AD is associated with immune system dysregulation, genetic and psychological factors, epidermal barrier defects, and environmental factors [20]. The current standard treatment for severe AD has long consisted of topical steroid (corticosteroids) application or administration of immunosuppressive agents, but these treatments

may be associated with potentially serious adverse effects [21]. It is necessary to develop new and effective treatments for patients suffering from AD. Traditional herbal extracts have been widely used in the treatment of inflammatory diseases as a folk remedy in Asia [8].

SBT has been ascribed a wide variety of pharmacological effects such as cytoprotective, anti-inflammatory, and immunomodulatory activity [22]. Topical application of SBT oil improved burns, healing of wounds, and irradiation dermatitis [23]. Based on the anti-inflammatory effects and its application in skin diseases, we examined the effects of SBT oil in AD-like skin lesions in vivo and in vitro.

In mice, skin and immunological alterations in DNCB-induced dermatitis were similar to those observed in human AD [24–26], including clinical features such as skin erosion, hemorrhage, edema, and increased serum IgE levels [27]. Therefore, the DNCB-induced BALB/c mice model was adopted to evaluate the effects of SBT oil on AD in vivo.

The symptoms observed in the mice studied here are consistent with those observed in the DNCB-induced model of AD-like lesions, such as edema, erythema/hemorrhage, scaling/dryness, and excoriation/erosion. In this model, SBT oil remarkably suppressed the development of AD-like skin lesions in mice after treatment for 4 weeks. The effect of SBT oil on AD-like lesions was lower than with DEX, but a higher body weight reduction was observed in the DEX group than the SBT oil group, which indicated that DEX had more side effects than SBT oil.

The thickness of the epidermis was significantly reduced following application of SBT oil compared with the

Fig. 6. Effects of sea buckthorn (SBT) oil on TNF- α /IFN- γ -induced NF- κ B, JAK2/STAT1, p38-MAPK activation in HaCaT cells. Total protein extracted from cells treated with IFN- γ /TNF- α were analyzed for the expression of signaling molecules NF- κ B (**A**), JAK2/

STAT1 (**B**), and p38-MAPK by Western blot analysis (**C**). β -Actin was adopted to confirm an equal protein loading. ^{##} $p < 0.01$, [#] $p < 0.01$ vs. the control group; ^{**} $p < 0.01$ vs. the TNF- α /IFN- γ -treated group.

DNCB group, demonstrating that SBT oil could inhibit lichenification and protect the skin barrier function [28, 29].

Infiltration of mast cells is one of the main features of AD lesions. Mast cells can release inflammatory mediators to mediate inflammatory responses such as allergic reactions [30, 31]. Histologically, there was a high degree of mast cell and inflammatory cell infiltration in the skin tissue of DNCB-sensitized mice, and SBT oil decreased

the infiltration of mast cells and inflammatory cells in the skin.

The weights of the spleen and local lymph nodes increased after topical application of allergen [32, 33]. Local lymph node and spleen weights were both markedly increased in DNCB-induced mice, which were significantly reduced following application of SBT oil.

Compared to the DNCB group, olive oil had no protective effect against AD-like skin lesions in mice. There

was a report that topical treatment with olive oil significantly damaged the skin barrier, and researchers considered that olive oil has the potential to promote the development of, and exacerbate existing, AD. The use of olive oil for the treatment of dry skin and infant massage should, therefore, be discouraged [34]. There are great differences in ingredients between olive oil and SBT oil. Besides oleic acid, SBT pulp oil has an obviously higher palmitoleic acid content than olive oil [35]. Palmitoleic acid shows anti-inflammatory ability via reducing pro-inflammatory cytokines [35, 36]. The SBT fruit is a rich source of flavonoids (0.2% in juice and 0.6% in dry fruits). SBT flavonoids also have anti-inflammatory and immunomodulatory activity [37].

This study confirmed the inhibitory effects of SBT oil on the expression of TARC and MDC in IFN- γ /TNF- α -treated HaCaT cells, an in vitro model of AD [38]. CCR4 is a predominant chemokine receptor on Th2 cells [39]. TARC and MDC are both ligands of CCR4, which can attract inflammatory lymphocytes to the skin [12]. Thus, TARC and MDC are both critical in the pathogenesis of AD, and inhibiting TARC and MDC expression in skin keratinocytes may improve AD symptoms [40]. The results suggested that SBT oil might improve AD-like symptoms via reduced TARC and MDC production by keratinocytes.

Studies have indicated that the regulation of TARC and MDC expression in HaCaT cells is related to the NF- κ B and JAK/STAT signaling pathways [38, 41]. Our results showed that IFN- γ and TNF- α can activate NF- κ B and STAT1 in keratinocytes; however, this feature was significantly suppressed by SBT oil.

STAT1 is activated by ERK, JNK, p38 MAP, and JAK kinases in response to cytokines such as TNF- α and IFN- γ [42]. Previous studies showed that p38 MAPK and JAK2, but not ERK and JNK kinases, are involved in the regulation of IFN- γ /TNF- α -induced TARC and MDC expression in HaCaT cells [12, 38, 43]. The results indicated that SBT oil suppressed IFN- γ /TNF- α -induced phosphorylation of p38 and JAK2 in HaCaT cells, which suggested

that SBT oil inhibited the production of TARC and MDC in IFN- γ /TNF- α -stimulated HaCaT cells by suppression of JAK2 pathways and p38 MAPK activated by IFN- γ and TNF- α .

Taken together, our results indicated that SBT oil suppressed TARC and MDC production in IFN- γ /TNF- α -stimulated HaCaT cells by inhibiting activation of the NF- κ B, JAK2/STAT1, and p38-MAPK signaling pathways.

Conclusion

In summary, the study indicated that SBT oil had anti-inflammatory effects against DNCB-induced atopic symptoms in mice and suppressed the production of Th2-type chemokines TARC and MDC in IFN- γ /TNF- α -stimulated HaCaT cells via blockade of NF- κ B/STAT1 signal pathway activation. It is expected that SBT oil might be a useful therapeutic agent for the treatment of patients with AD.

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Statement of Ethics

All experimental procedures were conducted according to the guidelines provided by the ethical committee of experimental animal care at China Medical University (Shenyang, China).

Disclosure Statement

The authors state no conflict of interest.

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