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Anti-inflammatory Effect of Shea Butter Extracts in Canine Keratinocytes

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Abstract : Shea butter (*Vitellaria paradoxa*) is a fat extracted from shea tree nuts and contains relatively high levels of non-glycerides. Triterpenes, the main non-glyceride component, exhibit a variety of biological activities such as antitumor, antibacterial, and anti-inflammatory. Shea butter extract (SBE) has been used to treat various skin problems such as burns, eczema, and rash in human medicine, but little is known about the activity of SBE on canine skin. This study evaluated the cytotoxicity and anti-inflammatory effect of SBE in canine keratinocytes. Cytotoxicity of lipopolysaccharide (LPS, 5-50 ng/mL) and SBE (50-200 μg/mL) was evaluated using the CCK-8 assay. Non-cytotoxic concentrations of LPS and SBE were administered to canine cell cultures to evaluate anti-inflammatory effects. To evaluate the anti-inflammatory activity of SBE, the levels of IL-1β, IL-8, IL-12, and TNF-α were measured using ELISA kits. The concentration of each cytokine was quantified in control, LPS-treated, LPS + SBE-treated groups. Increased levels of IL-1β, IL-8, and IL-12 were found in LPS-treated groups relative to control groups. LPS + SBE-treated groups showed a lower level of IL-1β, IL-8, and IL-12 than LPS-treated groups. These results suggest that SBE may have application as a topical agent for canine inflammatory skin diseases. However, further *in vivo* study is needed to evaluate the safety and efficacy of SBE in dogs.

Key words **:** Shea butter extracts (SBE), anti-inflammatory, cytokine, keratinocyte, dog.

Introduction

Shea butter is a fat extracted from Shea trees (*Vitellaria paradoxa*) which grows predominantly in Africa (11,18). Shea butter contains higher levels of non-glyceride constituents compared to typical vegetable oils (6,12). The primary non-glyceride constituents are triterpenes such as lupeol, butyrospermol, α-amyrin, and β-amyrin (22). Triterpenes are bioactive compounds, and the composition of the trierpenes in shea butter have no striking regional differences (2). Triterpenes are reported to exhibit a variety of biological activities such as antitumor, antibacterial, and anti-inflammatory activities (3,8,9,30).

Canine Skin is the largest organ of the body and is exposed to numerous biological or environmental stresses; the body responds to changes caused by stress factors to restore tissue and maintain tissue hemostasis (6,27). The epidermis is the outermost layer of the skin and acts as a physical barrier against various agents that are physically, chemically, or biochemically harmful (7,15,27). Exogenous stimuli induce the biosynthesis of inflammatory mediators such as leukotrienes and prostaglandins, causing an immune response (7,14,17,28).

Keratinocytes are the predominant cell type of the epidermis and synthesize a variety of pro-inflammatory and anti-inflammatory cytokines, including TNF-α, IL-1, IL-6, IL-8, and I-10 (14,17). In several skin diseases, abnormal production of cytokines occurs, and this production is belived to contribute to the pathogenesis of inflammatory dermatosis (20,24).

In veterinary dermatology, the use of topical therapy to manage of skin diseases has been increasing (21,23). Topical agents are applied directly to the skin and have been used to treat numerous skin diseases, including allergic and infectious disorders (23). In allergic skin diseases, topical therapy reduce inflammation and pruritus by desensitizing or improving the barrier function of skin (21). Numerous commercial topical agents have been developed and used in veterinary dermatology (10).

Several studies have shown the anti-inflammatory effect of shea butter extract (SBE), indicating potential for reducing skin stress induced by environmental factors (6,13,15,25). Shea butter has been used to treat various skin problems such as eczema, dry skin, burns, rashes, and wrinkles in humans (1).

Although shea butter is reported to have beneficial effect on skin, little is known about the activity of SBE on canine skin. In this study, we evaluated the cytotoxicity and anti-inflammatory effects of SBE in lipopolysaccharide (LPS)-induced inflammation in canine keratinocytes, using the CCK-8 assay and ELISA.

Materials and Methods

Shea butter extracts (SBEs)

Refined shea butter extracts (ULAB SWITZERLAND, Co; Korea) were dissolved in dimethyl sulfoxide (Sigma-Aldrich; Korea) to make a stock solution (100 mg/mL) and stored at −80°C until required (30). Lipopolysaccharide (LPS) was dissolved in sterile phosphate-buffered saline (PBS) and prepared as a 1 μg/mL stock solution.

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Cell culture

Canine epidermal keratinocyte progenitors (CPEK) were purchased from CELLnTEC Advanced Cell Systems (Switzerland). The CPEK cells were cultured in T75 cell culture flasks in CnT-09 medium (CELLnTEC; Switzerland) at 37°C under 5% CO_2 in a humidified incubator until 70-80% confluence was reached. The culture medium was removed, and the cells were trypsinized by the addition of 5 mL of trypsin. The cell suspension was incubated for 5 min at 37°C, treated with 5 mL of fetal bovine serum (FBS), and centrifuged at 800 rpm for 10 min at 25°C. The supernatant was removed, and cell pellets were resuspended in serum-free media (DMEM/F12 1:1 mixture, 1% gentamicin). Cell counts were obtained using a hemocytometer. Fifth-passage cells were used for all experiments.

Cell counting kit (CCK)-8 assay

CCK-8 assays were performed to determine the cytotoxic effects of LPS and SBE. Suspensions of CPEK cells were seeded into a 96-well plates at 10^3 cells/well and cultured for 24 h in a humidified incubator at 37°C under 5% CO_2. Cells were treated with various concentrations of LPS (5-50 ng/mL) and SBE (50-150 μg/mL) for 24 h. SBE and LPS stock solutions were diluted with serum-free medium, and the concentration of DMSO in each solution was less than 1%. The cell culture medium served as negative control. After 24 h, 10 μg/well of CCK-8 solution (Abbkine Scientific Co.; China) was added, and mixtures were incubated for 1 h. The absorbance of solutions was measured at 450 nm to assess cell viability using a spectrophotometric plate reader (Epoch, BioTek; USA).

The cell viability was calculated as follows: cell viability (%) = $(A_{treatment} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$, where A is the absorbance at 450 nm of the indicated sample.

Measurement of IL-1β, IL-8, IL-12, and TNF-α

CPEK cells were seeded into 6-well plates at 0.4×10^6 cells/well and grown to approximately 100% confluency. The culture medium was removed, and the cells were washed three times with sterile PBS. Cells were treated with LPS ± SBE. A non-treatment group served as a negative control. The culture supernatants were collected after 24 h. The levels of IL-1β, IL-8, IL-12, and TNF-α were determined by ELISA kits (MyBioSource; USA) according to the manufacturer's instructions. The measured concentration calculated from the standard curve was multiplied by dilution factor (x2). Each group was assayed in triplicate, and the optical density was measured at 450 nm using a spectrophotometric plate reader.

Statistical analysis

Data are presented as the mean ± standard deviation (S.D.). Data were analyzed using SigmaPlot for Windows, Version 12.0 (Systat Software; USA). Differences between groups were assessed with one-way ANOVA. *P*-values < 0.05 were considered statistically significant.

Results

Cytotoxicity of LPS and SBE

Cytotoxicity of LPS in CPEK cells

The cytotoxicity of LPS in CPEK cells was evaluated using the CCK-8 assay (Table 1). Table 1. show the cell viability of CPEK cells exposed to various concentrations of LPS (5-50 ng/mL). The cell viability of the negative control was set as 100%. Relative cell viabilities are 94.8%, 94.9%, and 62.6% when cells were treated with 5, 10, and 50 ng/mL LPS, respectively. LPS shows no cytotoxic effect at 5 and 10 ng/ml. However, cell viability is significantly decreased when treated with 50 ng/ml LPS ($P < 0.01$).

Cytotoxicity of SBE in CPEK cells

Table 2 show the optical density and cell viability of CPEK cells, respectively, exposed to SBE (50-200 μg/mL). The relative cell viabilities are 92.6%, 99.4%, 73.6%, and 57.7% when treated with 50, 100, 150, and 200 μg/mL SBE. SBE at 50 and 100 μg/mL shows no cytotoxic effect in CPEK

Table 1. Cytotoxicity of LPS in CPEK cells measured by CCK-8 assay (mean ± S.D.)

	Control	LPS-treated groups		
		5 ng/mL	10 ng/mL	50 ng/mL
OD_{450}	1.88 ± 0.06	1.79 ± 0.07	1.79 ± 0.06	1.20 ± 0.06
Cell viability (%)	100	94.81 ± 3.94	94.90 ± 3.17	62.63 ± 3.26**

OD_{450}: optical density at 450 nm.
**$P < 0.01$ relative to the control group.

Table 2. Cytotoxicity of SBE in CPEK cells measured by CCK-8 assay (mean ± S.D.)

	Control	SBE treated groups			
		50 μg/mL	100 μg/mL	150 μg/mL	200 μg/mL
OD_{450}	1.88 ± 0.06	1.75 ± 0.13	1.88 ± 0.07	1.40 ± 0.03	1.11 ± 0.05
Cell viability (%)	100	92.55 ± 2.99	99.42 ± 3.90	73.58 ± 1.88	57.68 ± 2.48

OD_{450}: optical density at 450 nm.
**$P < 0.01$ relative to the control group.

cells. The cell viability is significantly decreased when treated with 150 and 200 μg/mL of SBE ($P < 0.01$). The cytotoxicity of SBE increased in a dose-dependent manner.

The cytotoxicity of a mixture of 10 ng/mL LPS and 100 μg/mL SBE was evaluated. The cell viability of this mixture shows no significant difference compared to the negative control.

The anti-inflammatory effect of SBE

The levels of IL-1β, IL-8, IL-12, and TNF-α were measured to evaluate the anti-inflammatory effect of SBE in LPS-induced inflammation of CPEK cells using ELISA assays. The CPEK cells were divided into Control (no treatment), LPS-treated, and LPS + SBE-treated groups. Fig 1 show the levels of IL-1β, IL-8, IL-12, and TNF-α, respectively. Cytokine concentrations were calculated by comparing the optical density of the sample to the standard curve.

Levels of IL-1β

The levels of IL-1β are 83.7 ± 5.7, 98.1 ± 6.1, and 82.8 ± 2.1 pg/mL for control, LPS-treated, and LPS+SBE-treated groups, respectively (Fig 1A). The LPS-treated group exhibits an increased level of cytokine compared to the control group ($P < 0.05$). The concentration of IL-1β in the LPS + SBE-treated group is lower than that of the LPS-treated group ($P < 0.05$).

Levels of IL-8

The levels of IL-8 are 44.8 ± 3.3, 63.2 ± 2.9, and 46.5 ± 4.4 pg/mL for control, LPS-treated, and LPS + SBE-treated groups, respectively (Fig 1B). An increased level of IL-8 is observed in the LPS-treated group ($P < 0.05$). In the LPS + SBE-treated group, a decreased level of IL-8 is observed compared to the LPS-treated group ($P < 0.05$).

Levels of IL-12

The levels of IL-12 are 39.6 ± 7.5, 62.8 ± 3.2, and 41.6 ± 2.3 pg/mL for control, LPS-treated, and LPS+SBE-treated groups, respectively (Fig 1C). The level of IL-12 is increased

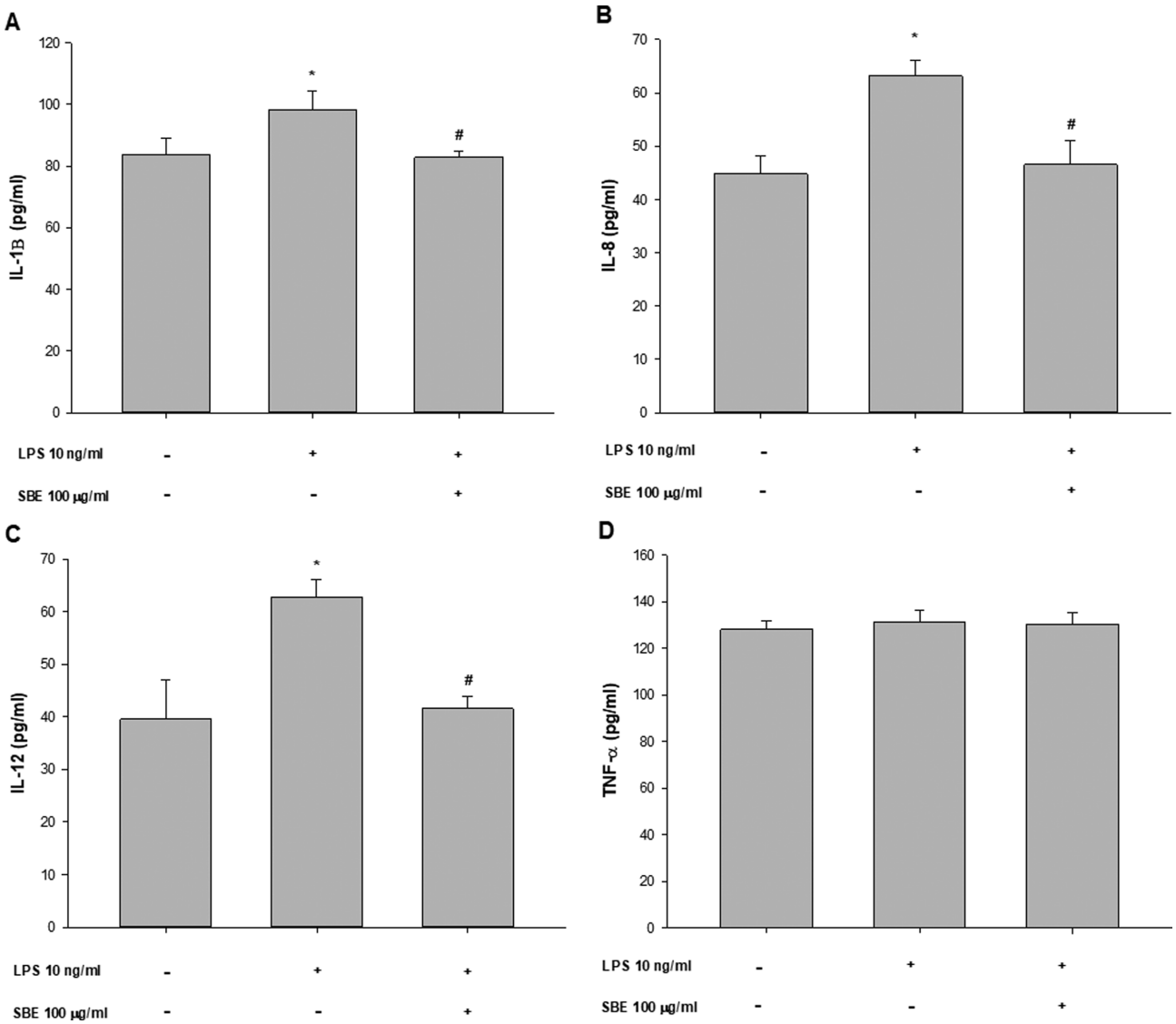

Fig 1. Concentrations of IL-1β, IL-8, IL-12, and TNF-α (pg/mL) in control, LPS-treated, and LPS + SBE-treated groups. The CPEK cells were treated with LPS ± SBE and optical density was measured at 450 nm using a spectrophotometric plate reader. *$P < 0.05$ relative to the control group. #$P < 0.05$ relative to the LPS-treated group.

in the LPS-treated group compared to the control group ($P < 0.05$). LPS + SBE-treated group exhibits a decreased level of IL-12 ($P < 0.05$), and no significant difference in IL-12 level is observed between the control and LPS + SBE-treated groups.

Levels of TNF-α

The levels of TNF-α are 128 ± 3.9, 131.1 ± 5.2, and 130.5 ± 5.0 pg/mL for control, LPS-treated, and LPS + SBE-treated groups, respectively (Fig 1D). No significant differences are observed between the three groups.

Discussion

Shea butter is reported to exhibit numerous biological activities, including anti-inflammatory, antiarthritic, chemopreventive, and sun-screening (3,13,25). Several studies have revealed that shea butter has skin-moisturizing and anti-aging activities (4,17). Shea butter contains high percentage of bioactive compounds including triterpenes, tocopherols and phenols, and has become a popular component of cosmetic formulations (15,19). In human medicine, shea butter has been used as a topical agent in skincare and to treat various skins disorders. In this study, we evaluated the cytotoxicity and anti-inflammatory effects of SBE in canine epidermal progenitor keratinocytes (CPEK).

The cytotoxic effects of LPS and SBE in CPEK cells were assessed using the CCK-8 assay. Based on the results of the CCK-8 assays, the concentrations of LPS and SBE used to treat cells before ELISA assays were selected. Cell viability tests showed that a mixture of 10 ng/mL LPS and 100 μg/mL SBE has no cytotoxic effect in CPEK cells.

ELISA assays revealed increased levels of inflammatory cytokines in LPS-treated groups. LPS induced the production of IL-1β, IL-8, and IL-12, and statistically significant differences were observed between control and LPS-treated groups. The levels of IL-1β, IL-8, and IL-12 were significantly decreased in LPS + SBE-treated groups relative to LPS-treated groups. The levels of TNF-α exhibited no significant differences between the three groups. Although skin inflammation results from the effects of several cytokines rather than the dysregulation of a single cytokine, these results suggest that SBE may act on inflammation related to keratinocytes.

The exact mechanism of anti-inflammatory effect of SBE is not confirmed in this study, but previous study showed that shea butter suppressed the production of TNF-α, IL-1β, and IL-12 in LPS-activated macrophage cells (30). This study revealed that triterpenes, the main non-glyceride component in SBE, suppressed the NF-κB nuclear translocation induced by LPS (30). LPS is a component of Gram-negative bacteria and triggers an inflammatory response (30). LPS binds with Toll-like receptors and induces the activation of NF-κB.

IL-1β is a cytokine that produced by numerous cells, including macrophages, keratinocytes, neutrophils, NK cells, and T cells (17). IL-1 is a primary mediator of inflammatory skin diseases and is constitutively produced by keratinocytes (26). Trauma, bacterial products, and UV-irradiation stimulate keratinocytes to produce IL-1. TNF-α has overlapping functions with IL-1. IL-8 has chemotactic effects on neutrophils and T lymphocytes. IL-1 and TNF-α augment the production of IL-8 and induce neutrophil accumulation in the dermis (17). IL-12 increases Th-1 responses and is important in maintaining a balance between Th-1 and Th-2 cells. Cytokines produced by Th-1 and Th-2 cells are thought to contribute to various allergic conditions. In ELISA assays, non-cytotoxic concentration of SBE significantly suppressed the production of IL-1β, IL-8, and IL-12 in LPS-induced inflammation of canine keratinocytes. Previous studies showed increased level of TNF-α in LPS-treated group and decreased level in SBE-treated group. However, this study showed no significant difference between groups. This result may be due to unskilled technician or low concentration of LPS to induce the sufficient production of TNF-α in canine keratinocytes.

A variety of environmental stimuli affect the immune response and biosynthesis of cytokines (14). Abnormal production of cytokines induces numerous skin diseases, including atopic dermatitis, allergic contact dermatitis, and psoriasis (5). Systemic or topical treatment is required for the management of skin disorders. Topical therapy is extremely important in allergic, infectious, and seborrheic disorders and topical anti-inflammatory agents provide relief from inflammation in skin (10,23). Numerous plant oils have been used for skin care because they many physiological benefits such as moisturizing, skin barrier repairing, and antioxidant activities (15, 29). The topical application of plant oils may act synergistically with conventional therapeutic regimens (15). Although commercial topical agents containing various plant oils have been used in human medicine, registration is required for their application in dogs as canine skin is more sensitive than human skin (10).

Topical agents that contain shea butter extract for canine skincare are available, but the effects of shea butter in canine keratinocytes are not well established (13). SBE caused decreased production of several cytokines in LPS-induced inflammation in canine keratinocytes. Although numerous mechanisms are involved in the pathogenesis of inflammation, these results suggest that SBE may become a valuable component of topical agents for canine inflammatory skin diseases. However, a comparison between conventional anti-inflammatory agent and SBE is not performed in this study. To evaluate the relative anti-inflammatory effect of SBE, further study is needed. Furthermore, this study evaluated the anti-inflammatory effect of SBE against several cytokines and was performed *in vitro*. Further study to evaluate the safety and clinical effectiveness of SBE in canines is needed.

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