

Original Article / 원저

Effects of Ethanol Extracts of *Anemarrhena asphodeloides* on Skin Barrier Function by Inflammation

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지모 주정 추출물이 염증으로 손상된 피부장벽 기능에 미치는 영향

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Abstract

목적 : 본 연구에서는 TNF- α 와 IFN- γ 로 자극한 인간피부각질형성세포 (HaCaT keratinocytes) 모델을 사용하여 지모가 피부장벽 기능에 미치는 영향을 알아보고자 하였다.

방법 : MTT assay를 통하여 지모 주정(70% 에탄올) 추출물 (EAA)이 HaCaT keratinocytes의 세포생존율에 미치는 영향을 확인하였으며 wound healing assay를 통해 EAA가 HaCaT 세포의 이주 능력에 영향을 주는지 관찰하였다. 또한 western blot analysis와 qRT-PCR을 통하여 EAA가 TNF- α /IFN- γ 로 자극한 HaCaT 세포에서 iNOS의 단백질 발현 및 IL-4, IL-13, IL-6의 mRNA 발현, filaggrin의 단백질과 mRNA 발현에 미치는 영향을 조사하였다.

결과 : EAA는 처리 농도 500 μ g/ml까지 HaCaT keratinocytes의 세포생존율에 영향을 미치지 않았다. EAA는 wound healing assay에서 HaCaT 세포의 이주 능력을 증가시켰으며, TNF- α /IFN- γ 로 자극한 HaCaT 세포에서 iNOS의 단백질 수준을 감소시켰다. 또한 EAA가 IL-4, IL-13, IL-6의 mRNA 발현을 억제하는 것 역시 확인할 수 있었다. 뿐만 아니라 EAA는 TNF- α /IFN- γ 자극에 의해 감소했던 filaggrin을 단백질과 mRNA 수준에서 회복시켰다.

결론 : EAA가 HaCaT 세포에서 Th2 type cytokines, pro-inflammatory cytokine의 억제와 filaggrin 회복을 통해 피부장벽 기능 손상에 대한 억제활성을 갖는 것을 확인하였으며, 이를 통해 EAA가 염증으로 인해 손상된 피부장벽 기능 개선에 효과적일 것으로 사료된다.

Key words : *Anemarrhena asphodeloides*; HaCaT; Keratinocyte; Skin barrier; Th2

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I. Introduction

As the outermost organ in the human body, skin constantly faces the external environment and serves as a primary defense system. The protective functions of skin include UV-protection, anti-oxidant and anti-microbial functions¹⁾. The defects of epidermal barrier function is considered as a key event in allergic sensitization²⁾. This study was carried out to investigate the effects of extracts of *Anemarrhena asphodeloides* (EAA) on skin barrier which has these important functions.

Intrinsic defects in skin barrier function and immune dysregulation resulting in Th2-predominant inflammation are closely associated with the pathogenesis of atopic skin inflammation³⁾. With regards to immune abnormalities, atopic skin inflammation is currently considered as a biphasic T cell-mediated disease. Atopic skin inflammation is an imbalance in the Th1/Th2 immune response and IgE-driven inflammation. Th2 signal predominates in the acute phase, whereas Th2 to Th1 switch promotes disease in chronic phase. Moreover, disturbed skin barrier function such as that caused by mutations in the filaggrin gene (FLG) significantly increases the risk of atopic skin inflammation^{4,5)}.

Recently, herbal medicine have become the source of a large proportion of drugs and active compounds. *Anemarrhena asphodeloides* Bunge (Liliaceae) is a medicinal plant widely distributed

in Korea, China, and Japan. The dried rhizomes of *A. asphodeloides* which contain steroidal saponins, xantones and polysaccharides are well-known traditional medicine used as an anti-depressant, anti-diabetics, anti-inflammatory, anti-platelet aggregation and anti-pyretic agent^{6,7)}.

Through the reports that *A. asphodeloides* water extract (AAWE) suppresses compound 48/80-induced mast cell activation by inhibition of cellular mechanisms in signaling pathways and would be beneficial for the treatment of mast cell-mediated anaphylactic response⁸⁾, mangiferin isolated from *A. asphodeloides* exerts anti-photoaging activity in UVB-irradiated hairless mice⁹⁾ and also attenuated hydrogen peroxide (H₂O₂)-induced MMP (matrix metalloproteinases)-1 activation related to oxidative stress causing skin aging, via inhibition of extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK) pathway and AP complex-1 (AP-1)¹⁰⁾, we can expect skin improvement effects of *A. asphodeloides*.

Although *A. asphodeloides* has been reported to show various biological activities, effects of ethanol (EtOH) extracts of *A. asphodeloides* (EAA) on skin barrier function remains unclear. In this study, we examined the effects of EAA on the skin barrier function in HaCaT keratinocytes.

II. Material and Methods

1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life

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● Received 2018/3/27 ● Revised 2018/4/25 ● Accepted 2018/5/2

Technologies Inc. (Grand Island, NY, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma Chemical Co. (St. Louis, MO, USA), inducible nitric oxide synthase (iNOS), filaggrin and β -actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Power SYBR® Green Master Mix was purchased from Applied Biosystems (Foster City, CA, USA), Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH), interleukin (IL)-4, IL-13, IL-6 and filaggrin oligonucleotide primers were purchased from Bioneer (Daejeon, Republic of Korea).

2. Preparation of Ethanol Extract of *Anemarrhena asphodeloides* (EAA)

The roots of *A. asphodeloides* were purchased in Nanum herb (Yeongchen, Gyeongbuk, Korea). Dried roots of *A. asphodeloides* were extracted with 2 l 70% EtOH by maceration and the extracts were filtered. The filtrate was evaporated in vacuo at 40°C, freeze-dried in vacuum. Finally, 65.72g of extract (at a concentration of 50 μ g/ml) was obtained from 200g of *A. asphodeloides* and stored at -20°C for bioassays.

3. Cell culture and sample treatment

The HaCaT keratinocytes were provided by Prof. Seung-Heon Hong (Wonkwang University, Republic of Korea). The cells were cultured in DMEM supplemented with 10% FBS, penicillin (100U/ml), and streptomycin (100 μ g/ml) in a 37°C and 5% CO₂ incubator. Cells were treated with EAA (250 and 500 μ g/ml) for 1 hr, prior to

stimulation with tumor necrosis factor- α (TNF- α)/interferon- γ (IFN- γ) (10g/ml) for the indicated time.

4. MTT assay

HaCaT keratinocytes were plated at a density of 1 \times 10⁵ cells/well in 96 well plates. To determine the appropriate concentration of EAA which has no effect on cell viability, MTT assay was performed at 24 hrs, following treatment of EAA with various concentrations in HaCaT keratinocytes. Cell viabilities were measured at a 570nm by using Epoch® autometric microplate reader (Titertek Multiskan, Huntsville, AL).

5. Quantitative real-time polymerase chain reaction analysis (qRT-PCR)

Total RNA was isolated from cells by using the Easy-Blue® Reagent according to the manufacturer's instructions Kits (Intron Biotechnology, Seongnam, South Korea). Total RNA was quantified by using an Epoch® autometric microplate reader (BioTek Instruments; Winooski, VT, USA). In addition, total RNA was converted to cDNA by using a high-capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, CA, USA) and thermocycler (Gene Amp® PCR system 9700; Applied Biosystems) with the following program: initiation for 10 min, at 25°C, followed by incubation at 50°C for 90 min, and at 85°C for 5 min. The size of the synthesized cDNAs was 200bp. qRT-PCR analysis was conducted by using a Step One Plus® Real-time PCR system (Applied Biosystems), SYBR® Green master mix and

primers were used for PCR analysis of GAPDH, IL-4, IL-13, IL-6, and filaggrin. The PCR cycling parameters were as follows: 10 min, at 95°C; 40 cycles of 5 secs, at 95°C and 45 secs, at 60°C; and a final melting curve of 15 secs, at 95°C, 1 min, at 60°C, and 15 secs, at 95°C. All primer sequences are shown in (Table 1). Gene expression was calculated according to the comparative threshold cycle (Ct) method. The results are expressed as the ratio of the optical density of the target locus to GAPDH.

6. Western blot analysis

The cells were re-suspended in protein extraction solution (PRO-PREP™, Intron Biotechnology, Seongnam, Republic of Korea) and incubated for 20 min, at 4°C. Cell debris was eliminated by micro-centrifugation, followed by immediate freezing of the supernatant. The protein supernatant concentration was measured by using the Bio-Rad protein assay reagent (Hercules, CA, USA) according to the manufacturer's instructions. Each protein sample (30 μ g) were electro-blotted transferred onto a polyvinylidene fluoride (PVDF) membrane followed by separation using 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Membranes were incubated for 30 min, with blocking solution (2.5 or 5% skim milk) at room temperature, followed by incubation overnight with a 1:1,000 dilution of primary antibody at 4°C. Membranes were washed three times with Tris-buffered saline/ Tween 20 (TBS-T) and incubated with a 1:2,500 dilution of horseradish peroxidase-conjugated secondary antibody for 1 hr, 30 min, at room temperature. Membranes

were again washed three times with TBS-T and then developed by enhanced chemiluminescence (Absignal™, Abclon, Seoul, Republic of Korea). The chemiluminescent blots were imaged on film. Densitometry analysis of the western blots was done by using ImageJ software (National Institutes of Health, Bethesda, MD). The intensity of the individual bands on western blot analysis was measured by gel analyzer tools of ImageJ and normalized either to β -actin to its total protein. The graphs represent normalized values as fold change over control condition.

7. Wound Healing Assay

HaCaT keratinocytes cells were seeded into 24-well plates (1×10^5 cells/well). After 24 hrs, of incubation, the monolayers were scratched with sterile pipette tips and treated with or without EAA (250 or 500 μ g/ml). After 72 hrs., the cells were fixed in 4% formalin for 20 min. The wound closure distance was evaluated using an Eclipse TE2000U inverted microscope with twin CCD cameras (magnification, $\times 200$; Nikon, Tokyo, Japan; n = 3).

8. Statistical analysis

All the values reported have been expressed as the mean \pm standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) with Dunnett's test, and p-values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism (version 5.00 for Windows, San Diego, CA, USA).

III. Results

1. Effects of EAA on Cell Viability in HaCaT keratinocytes

To measure the cytotoxic effects of EAA on HaCaT keratinocytes, cell viability was investigated by using MTT assay. Treatment with different concentration of EAA ranging from 7.8 to 500 $\mu\text{g/ml}$ for 24 hrs, did not have any cytotoxic effects in HaCaT keratinocytes(Fig. 1). Thus, we used EAA at a concentration of 250 and 500 $\mu\text{g/ml}$ for subsequent experiments,

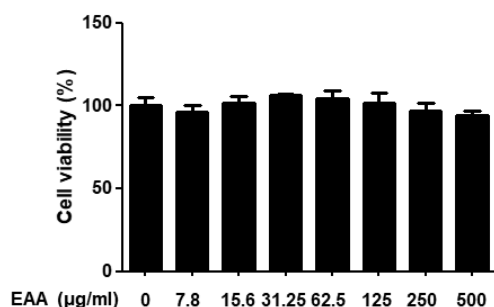


Fig. 1. Effects of EAA on Cell Viability in HaCaT keratinocytes

HaCaT keratinocytes were treated with different concentrations of EAA for 24 hrs, and their viability were determined using MTT assay. (The data shown represent mean \pm SD of three independent experiments.)

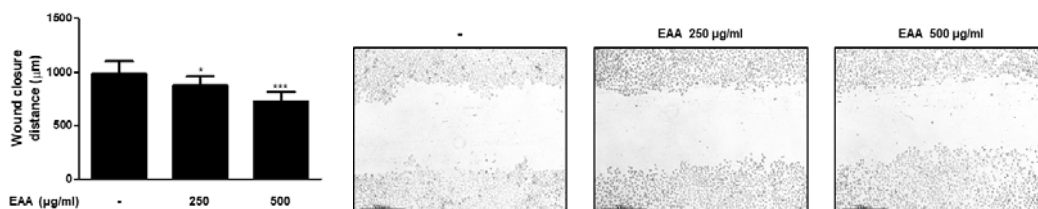


Fig. 2. Effects of EAA on Migratory Activity in HaCaT keratinocytes

After scratch of monolayers with sterile pipette tips, HaCaT keratinocytes were incubated with medium containing with or without EAA (250, or 500 $\mu\text{g/ml}$) for 24 hrs. Data were standardized to the wound closure distance (μm). (Results are expressed as the mean \pm SD of three independent experiments. * $p < 0,05$ and *** $p < 0,001$.)

2. Effects of EAA on Migratory Activity in HaCaT keratinocytes

Epidermis, the outermost layer of the skin, consists of stratified squamous epithelium which differentiates from proliferating basal keratinocytes¹¹. During wound re-epithelialisation stage, skin lesions close the wound and remodel the cytoskeleton by enhancing the proliferation and migration of keratinocytes¹². To evaluate whether EAA can influence keratinocytes migration, we assessed the effects of EAA on HaCaT keratinocytes motility using wound healing assay. The migration of HaCaT keratinocytes was evaluated by wound closure distance. Treatment of HaCaT keratinocytes with EAA for 24 hrs, increased the migration of keratinocytes(Fig. 2).

3. Effects of EAA on iNOS activation in TNF- α /IFN- γ -stimulated HaCaT keratinocytes

iNOS mediates several inflammatory responses by the overproduction of Nitric oxide (NO) and is induced in response to various pro-inflammatory stimuli such as TNF- α , IFN- γ , IL-6, and lipopolysaccharide (LPS)¹³. In addition, NO is thought to be a key disruptive factor in the

wound healing process¹²⁾. Because iNOS is highly involved in the inflammatory responses, we examined the effects of EAA on the level of iNOS protein expressions by western blot analysis. The expression levels of iNOS proteins were significantly up-regulated in response to TNF- α /IFN- γ co-stimulation, while pre-treatment with EAA inhibited the expression levels of iNOS proteins in a dose-dependent manner(Fig. 3).

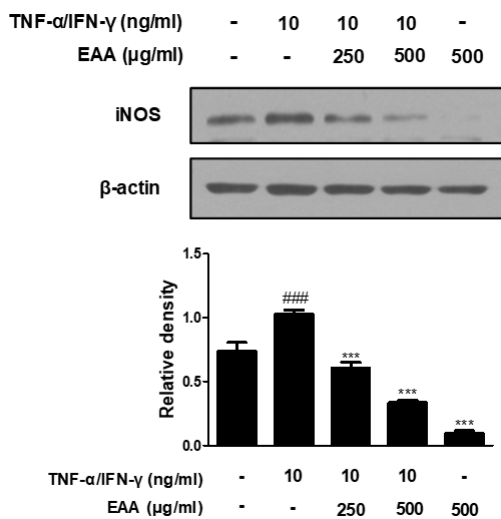


Fig. 3. Effects of EAA on iNOS activation in TNF- α /IFN- γ induced HaCaT keratinocytes

HaCaT keratinocytes were pre-treated with EAA for 1 hr, and stimulated by TNF- α /IFN- γ (10ng/ml) and the cells were further incubated for 24 hrs. Total proteins were prepared and western blotted for iNOS using specific antibodies. β -actin is used as an internal control. Densitometry analysis of the blots was done using ImageJ software. (The data shown represent mean \pm SD of three independent experiments, ###p < 0.001 vs the control group, ***p < 0.001 vs the TNF- α /IFN- γ -treated group.)

4. Effects of EAA on the mRNA Expression of IL-4 in TNF- α /IFN- γ induced HaCaT keratinocytes

Atopic skin inflammation is a kind of cutaneous inflammation reaction characterized by

Th2 cell-predominant phenotype in the initial stage with the additional acquisition of Th1 cell phenotype during the chronic phase³⁾. IL-4 has previously shown to be elevated in the skin from patients with atopic skin inflammation¹⁴⁾. IL-4 plays an important role in switching to IgE isotype by differentiating B cells and inhibits the proliferation and differentiation of Th1 cells. Also, IL-4 promotes the differentiation of naive T cells into allergic type Th2 cells¹⁵⁾. In TNF- α /IFN- γ -stimulated HaCaT keratinocytes, to elucidate the effects of EAA treatment on cytokine expression, alterations in the mRNA expression levels of various cytokines were measured using qRT-PCR. TNF- α /IFN- γ significantly increased the mRNA expression of IL-4 in HaCaT keratinocytes. However, pre-treatment with EAA significantly suppressed the mRNA expression of IL-4(Fig. 4).

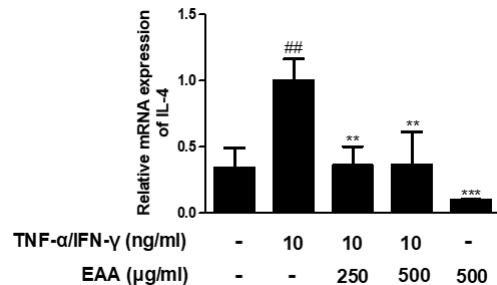


Fig. 4. Effects of EAA on the mRNA Expression of Th2 type Cytokines in TNF- α /IFN- γ induced HaCaT keratinocytes

HaCaT keratinocytes were pre-treated with EAA for 1 hr, and stimulated by TNF- α /IFN- γ (10ng/ml) and the cells were further incubated for 6 hrs. The mRNA level of IL-4 was determined by qRT-PCR. (The data shown represent mean \pm SD of three independent experiments, ##p < 0.01 vs the control group, **p < 0.01, ***p < 0.001 vs the TNF- α /IFN- γ -treated group.)

5. Effects of EAA on the mRNA Expression of IL-13 in TNF- α /IFN- γ induced HaCaT keratinocytes

It is well known that the levels of Th2 type cytokines are elevated in the skin of atopic skin inflammation patients and IL-13 levels are further increased in patients with elevated IgE levels¹⁶. IL-13 as well as IL-4, induce B cell proliferation and immunoglobulin class switching for IgE production¹⁷. EAA pre-treatment in TNF- α /IFN- γ -stimulated HaCaT keratinocytes reduced IL-13 mRNA expression, compared to TNF- α /IFN- γ -stimulation(Fig. 5).

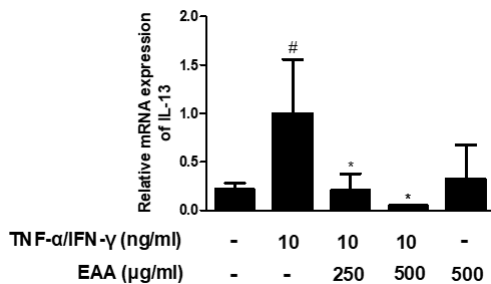


Fig. 5. Effects of EAA on the mRNA Expression of Th2 type Cytokines in TNF- α /IFN- γ induced HaCaT keratinocytes

HaCaT keratinocytes were pre-treated with EAA for 1 hr, and stimulated by TNF- α /IFN- γ (10ng/ml) and the cells were further incubated for 6 hrs. The mRNA level of IL-13 was determined by qRT-PCR. (The data shown represent mean \pm SD of three independent experiments. #p < 0,05 vs the control group, *p < 0,05 vs the TNF- α /IFN- γ -treated group.)

6. Effects of EAA on the mRNA Expression of IL-6 in TNF- α /IFN- γ induced HaCaT keratinocytes

IL-6 is one of pro-inflammatory cytokines that regulates Th2 differentiation and inhibition, as well as CD4+ T cell differentiation. Also, IL-6 is

known as negative regulator of epidermal differentiation^{18,19}. TNF- α /IFN- γ significantly increased the mRNA expression of IL-6 in HaCaT keratinocytes. However, pre-treatment with EAA significantly suppressed the mRNA expression of IL-6 in dose-dependent manner(Fig. 6). These results suggest that EAA has protective effects on skin keratinocytes by inhibiting the transcriptional level of inflammatory cytokines related to skin barrier dysfunction.

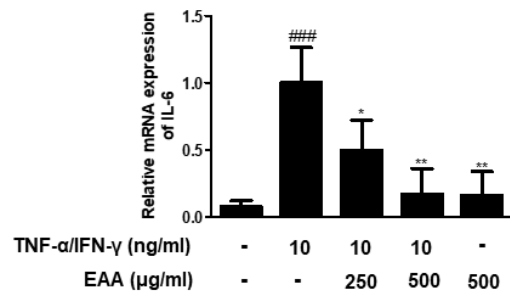


Fig. 6. Effects of EAA on the mRNA Expression of Pro-inflammatory Cytokines in TNF- α /IFN- γ induced HaCaT keratinocytes

HaCaT keratinocytes were pre-treated with EAA for 1 hr, and stimulated by TNF- α /IFN- γ (10ng/ml), and the cells were further incubated for 6 hrs. The mRNA level of IL-6 was determined by qRT-PCR. (The data shown represent mean \pm SD of three independent experiments. ###p < 0,001 vs the control group, *p < 0,05, **p < 0,01 vs the TNF- α /IFN- γ -treated group.)

7. Effects of EAA on TNF- α /IFN- γ induced Skin Barrier Dysfunction in HaCaT keratinocytes

Filaggrin is a key protein that plays an important role in formation of the cornified cell envelope which is critical for an effective skin barrier²⁰. We investigated the effects of EAA on the protein and mRNA expression levels of filaggrin by using western blot analysis and

qRT-PCR, TNF- α /IFN- γ co-stimulation decreased the protein and mRNA expression levels of filaggrin, but pre-treatment with EAA significantly increased the filaggrin protein level, although the mRNA level was increased slightly(Fig. 7). These results suggest that EAA has the effects of restoring filaggrin in TNF- α /IFN- γ -stimulated HaCaT keratinocytes.

IV. Discussion

The skin plays a major role in protection from the external environment. Stratum corneum prevents transepidermal water loss and protects the skin from external environment²¹. Among these important functions, defensive functions can be further classified as physical, thermal, immune, ultraviolet, oxidant, racial, anti-microbial and the

permeability barrier¹. In 1975, Michaels et al. suggested a schematic model to explain the permeability of the stratum corneum, called 'brick and mortar' where the corneocytes are the bricks and the lipids are the mortar²². Today this is viewed as the most appropriate model for the understanding of cellular arrangement and the winding ways of skin permeability²³.

The skin consists of epidermis, dermis and subcutaneous fat. Among the cells known to be involved in atopic dermatitis, keratinocytes constitute more than 95% of all the epidermis. Keratinocytes form keratin and produce cytokines, chemokines by various stimuli and also express various receptors on the cell surface²⁴. This barrier between the body and the environment is constantly maintained by reproduction of inner living epidermal keratinocytes which undergo a

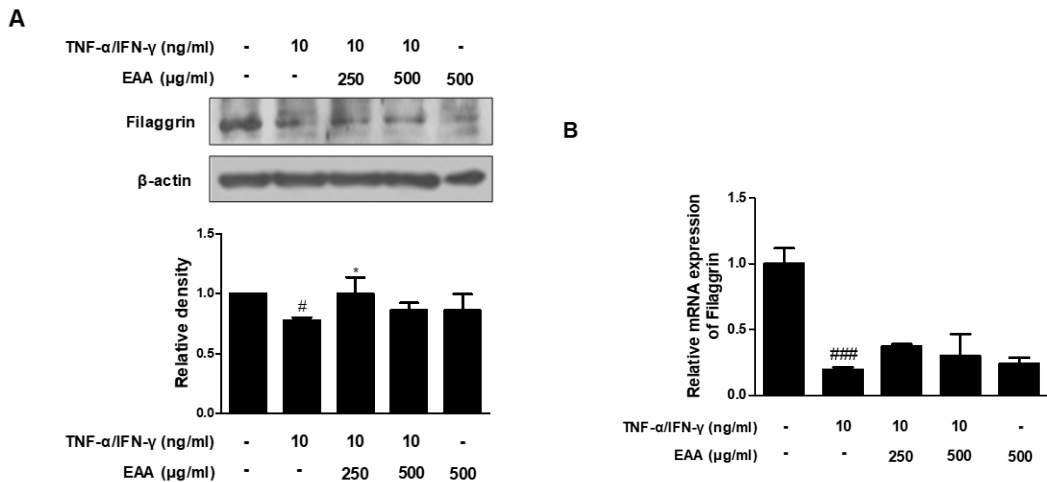


Fig. 7. Effects of EAA on TNF- α /IFN- γ induced Skin Barrier Dysfunction in HaCaT keratinocytes

A, HaCaT keratinocytes were pre-treated with EAA for 1 hr, and stimulated by TNF- α /IFN- γ (10ng/ml) and the cells were further incubated for 24 hrs. Total proteins were prepared and western blotted for Filaggrin using specific antibodies. β -actin is used as an internal control. Densitometry analysis of the blots was done using ImageJ software.

B, HaCaT keratinocytes were pre-treated with EAA for 1 hr, and stimulated by TNF- α /IFN- γ (10ng/ml) and the cells were further incubated for 6 hrs. The mRNA level of filaggrin was determined by qRT-PCR. (The data shown represent mean \pm SD of three independent experiments. #p < 0.05, ###p < 0.001 vs the control group, *p < 0.05, ***p < 0.001 vs the TNF- α /IFN- γ -treated group.)

process of terminal differentiation and then migrate to the surface as interlocking layers of dead stratum corneum cells. These cells provide the bulwark of mechanical and chemical protection and together with their intercellular lipid surroundings, confer water-impermeability²⁵⁾. Defects of the skin barrier function lead to the increased transepidermal water loss and increased penetration of harmful agent. Consequently, it allows inhaled substances such as microbes, allergens/antigens and irritants to pass more easily into the dermis to interact with immune and inflammatory cells and possibly contributes to the Th2 immune response as a common feature in many inflammatory diseases including inflammatory bowel disease, celiac disease, sinusitis, food allergy, asthma and atopic dermatitis^{21,26,27)}.

The very early event initiating atopic skin inflammation is remain unclear. However, one of the central causes of the atopic skin inflammation is the dysregulated Th1 and Th2 response that induces the characteristic Th2-dominant skin allergic inflammation and the production of primary pro-inflammatory cytokines after skin barrier disruption is a well-accepted script^{24,28)}. In atopic skin inflammation patients, genetic conditions, external stimuli or scratching episodes disrupt skin barrier, which facilitates allergen penetration and activation of keratinocytes to produce chemokines that trigger dendritic cells to induce Th2-cell mediated response. In the acute phase of disease, infiltrated CD4+ T cells in skin lesions predominantly secrete IL-4, IL-5, and IL-13²⁹⁾. Increased Th2 cytokine expression contributes to reduction in anti-microbial peptides

and reduced filaggrin expression. For instance, IL-4, a Th2 cytokine, suppresses the enhancement of ceramide synthesis and cutaneous permeability barrier function induced by TNF- α and IFN- γ in human epidermal sheets. In addition, IL-4 blocks the recovery of barrier function and enhancement of ceramide synthesis after barrier disruption by acetone treatment in living skin equivalent³⁰⁾. In this study, EAA suppressed the mRNA levels of IL-4, IL-13, and IL-6 in TNF- α /IFN- γ -stimulated HaCaT keratinocytes. These results show that EAA has inhibitory effects on skin barrier disruption by suppressing pro-inflammatory cytokines.

Cutaneous wound healing is a dynamic and complex process involving intricate interactions among a variety of inflammatory cells, extracellular matrix molecules, parenchymal cells and soluble molecules. The wound healing process consists of three steps: inflammatory, proliferative and remodelling³¹⁾. The proliferative phase of wound healing is associated with the re-epithelialisation process including collagen production and extracellular matrix molecules remodelling. Skin lesions close the wound and remodel the cytoskeleton by enhancing the proliferation and migration of keratinocytes for wound re-epithelialisation stage. During the early stage of wound healing, collagen production is promoted to increase scar formation, and then it is gradually decreased to normal levels in the final stage of wound healing¹²⁾. NOS catalyzes the synthesis of a free radical NO and l-citrulline from l-arginine. Especially, NO has many physiological functions such as vasorelaxation, neurotransmission, inflammation and wound

healing³²⁾. NO is a highly reactive radical that is generated by the activation of iNOS and contributes to various biological processes including inflammation. On the other hand, NO is thought to be a key disruptive factor in the wound healing process, although it was reported that small amounts of NO may enhance wound healing during the early stage¹²⁾. Likewise, excessive generation of Prostaglandin E2 (PGE2) by cyclo-oxygenase-2 (COX-2) is a crucial physiological factor contributing inflammation development³³⁾. In this study, it is found that treatment with EAA increased the migration of HaCaT keratinocytes and inhibited expression levels of iNOS proteins. It is possible to assume that EAA facilitates the wound healing in skin barrier through inhibition of excessive expression of NO.

Filaggrin is a filament-associated protein derived via dephosphorylation from profilaggrin which is the major component of keratohyalin granules within epidermal granular cells. The primary function of filaggrin is modulation of epidermal homeostasis via interaction with keratin filaments³⁾. Filaggrin is initially expressed as an inactive precursor protein, profilaggrin which is released from the keratohyalin F granules that are visible in the granular cell layer of the epidermis³⁴⁾. The primary role of filaggrin is to facilitate the bundling of the keratin cytoskeleton to form macrofibrils which functions as a scaffold for subsequent reinforcement stages of stratum corneum assembly. The disordered profilaggrin processing results in the complete absence of filaggrin monomers and abnormal lipid formation resulting in a lethally impaired skin barrier³⁵⁾.

The expression of barrier protein genes, such as filaggrin and loricrin is up and downregulated by various external and internal stimuli, including Th2 (IL-4 and IL-13) cytokines. The loss of mutation function of filaggrin in atopic skin inflammation, the upregulation of Th2 cytokines explain the disrupted expression of filaggrin and loricrin in the lesional skin of atopic skin inflammation patients, leading to epidermal barrier dysfunction³⁶⁾. In this study, it is observed that filaggrin protein level was restored by EAA pre-treatment in TNF- α /IFN- γ -stimulated HaCaT keratinocytes.

V. Conclusions

In this study, we determined the mechanisms of the protective effects of EAA on skin barrier function in TNF- α /IFN- γ stimulated HaCaT keratinocytes.

1. The inhibitory effects of EAA is observed in the expression of Th2 type cytokine, such as IL-4 and IL-13 and pro-inflammatory cytokine such as IL-6 at mRNA expression levels,
2. EAA increased the migration of HaCaT keratinocytes,
3. EAA inhibited expression levels of iNOS proteins,
4. EAA increased level of filaggrin at protein and mRNA expression,

These results show that EAA has inhibitory effects on skin barrier disruption due to inflammation by suppressing Th2 type cytokine,

pro-inflammatory cytokine and by filaggrin upregulation consequently. In conclusion, these data suggest that EAA is a source that can be considered as a potent candidate for the improvement of skin barrier function for external use.

※ 본 논문은 2017년도 상지대학교 석사학위논문으로 작성되었습니다.

References

1. Lee SH, Jeong SK, Ahn SK. An update of the defensive barrier function of skin. *Yonsei Med J.* 2006;47:293-306.
2. Strid J, Strobel S. Skin barrier dysfunction and systemic sensitization to allergens through the skin. *Current drug targets, Inflammation and allergy.* 2005;4(5):531-41.
3. Dang NN, Pang SG, Song HY, An LG, Ma XL. Filaggrin silencing by shRNA directly impairs the skin barrier function of normal human epidermal keratinocytes and then induces an immune response. *Brazilian J of medical and biological research.* 2015;48(1):39-45.
4. Furue M, Chiba T, Tsuji G, Ulzii D, Kido-Nakahara M, Nakahara T, et al. Atopic dermatitis: immune deviation, barrier dysfunction, IgE autoreactivity and new therapies. *Allergology international.* 2016;66(3):398-403.
5. Hönzke S, Wallmeyer L, Ostrowski A, Radbruch M, Mundhenk L, Schäfer-Korting M, et al. Influence of Th2 Cytokines on the Cornified Envelope, Tight Junction Proteins, and β -Defensins in Filaggrin-Deficient Skin Equivalents. *The J of investigative dermatology.* 2016;136(3):631-9.
6. Yang BY, Zhang J, Liu Y, Kuang HX. Steroidal Saponins from the Rhizomes of *A. asphodeloides*. *Molecules.* 2016;21(8):1075. PMID: 27548119.
7. Bak JP, Cho YM, Kim I, Park DW, Kwon JE, Jeong YJ, et al. Inhibitory effects of norlignans isolated from *A. asphodeloides* on degranulation of rat basophilic leukemia-2H3Cells. *Biomedicine & pharmacotherapy.* 2016;84:1061-6. PMID: 27780134.
8. Chai OH, Shon DH, Han EH, Kim HT, Song CH. Effects of *Anemarrhena asphodeloides* on IgE-mediated passive cutaneous anaphylaxis, compound 48/80-induced systemic anaphylaxis and mast cell activation. *Experimental and Toxicologic Pathology.* 2013;65(4):419-26.
9. Kim HS, Song JH, Youn UJ, Hyun JW, Jeong WS, Lee MY, et al. Inhibition of UVB-induced wrinkle formation and MMP-9 expression by mangiferin isolated from *Anemarrhena asphodeloides* Type. *European J of Pharmacology.* 2012;689:38-44.
10. Chae SW, Piao MJ, Kang KA, et al. Inhibition of Matrix Metalloproteinase-1 Induced by Oxidative Stress in Human Keratinocytes by Mangiferin Isolated from *Anemarrhena asphodeloides*. *Bioscience, biotechnology, and biochemistry.* 2011;75(12):2321-5.
11. Eisinger M, Sadan S, Silver IA, Flick RB. Growth regulation of skin cells by epidermal cell-derived factors: implications for wound

- healing. Proceedings of the National Academy of Sciences of the United States of America. 1988;85(6):1937-41.
12. Kim MS, Lee HJ, Randy A, Yun JH, Oh SR, Nho CW. *Stellera chamaejasme* and its constituents induce cutaneous wound healing and anti-inflammatory activities. *Scientific Reports*. 2017;7(1):42490.
 13. Chartrain NA, Geller DA, Koty PP, Sitrin NF, Nussler AK, Hoffman PE, et al. Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *J of biological chemistry*. 1994;269(9):6765.
 14. Kim BE, Leung DY, Boguniewicz M, Howell MD. Loricrin and involucrin expression is down-regulated by Th2 cytokines through STAT-6. *Clinical immunology*. 2008;126(3):332-7.
 15. Park HJ, Jang YJ, Yim JH, Lee HK, Pyo S. Ramalin Isolated from *Ramalina Terebrata* Attenuates Atopic Dermatitis-like Skin Lesions in Balb/c Mice and Cutaneous Immune Responses in Keratinocytes and Mast Cells. *Phytotherapy Research*. 2016;30(12):1978-87.
 16. Choi JK, Oh HM, Lee S, Park JW, Khang D, Lee SW, et al. Oleanolic acid acetate inhibits atopic dermatitis and allergic contact dermatitis in a murine model. *Toxicology and applied pharmacology*. 2013;269(1):72-80.
 17. Yang G, Cheon SY, Chung KS, Lee SJ, Hong CH, Lee KT, et al. *Solanum tuberosum* L. cv Jayoung Epidermis Extract Inhibits Mite Antigen-Induced Atopic Dermatitis in NC/Nga Mice by Regulating the Th1/Th2 Balance and Expression of Filaggrin. *J of medicinal food*. 2015;18(9):1013-21.
 18. Rincon M, Irvin CG. Role of IL-6 in asthma and other inflammatory pulmonary diseases. *International J of biological sciences*. 2012;8(9):1281-90. PubMed PMID: 23136556. Pubmed Central PMCID: 3491451.
 19. Son ED, Kim HJ, Kim KH, Bin BH, Bae IH, Lim KM, et al. S100A7 (psoriasin) inhibits human epidermal differentiation by enhanced IL-6 secretion through I κ B/NF- κ B signalling. *Experimental dermatology*. 2016;25(8):636-41.
 20. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, DeBenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J of Allergy and Clinical Immunology*. 2007;120(1):150-5.
 21. Souza C, de Freitas LA, Maia Campos PM. Topical Formulation Containing Beeswax-Based Nanoparticles Improved In Vivo Skin Barrier Function. *AAPS PharmSciTech*. 2017;18(7):2505-16.
 22. Michaels AS, Chandrasekaran SK, Shaw JE. Drug permeation through human skin: theory and in vitro experimental measurement. *American Institute of Chemical Engineers*. 1975;21:985-96.
 23. Addor FA1, Aoki V. Skin barrier in atopic dermatitis. *Anais brasileiros de dermatologia*. 2010;85(2):184-94.
 24. Homey B, Steinhoff M, Ruzicka T, Leung DY. Cytokines and chemokines orchestrate atopic skin inflammation. *J of Allergy and Clinical Immunology*. 2006;118(1):178-89.
 25. Nemes Z, Steinert PM. Bricks and mortar of the epidermal barrier. *Experimental &*

- molecular medicine, 1999;31(1):5-19.
26. De Benedetto A, Rafaels NM, McGirt LY, Ivanov AI, Georas SN, Cheadle C, et al. Tight junction defects in patients with atopic dermatitis. *The J of allergy and clinical immunology*. 2011;127(3):773-86.
27. Holgate ST. Epithelium dysfunction in asthma. *The J of allergy and clinical immunology*. 2007;120(6):1233-44.
28. Jin LE, Chung HJ, Pyee Y, Hong JY, Joung YU, Seo EK, et al. Suppression of inducible nitric oxide synthase expression by nyasol and broussonin A, two phenolic compounds from *Anemarrhena asphodeloides*, through NF- κ B transcriptional regulation in vitro and in vivo. *Chemistry & biodiversity*. 2014;11(5):749-59. PMID: 24827684
29. Lee HY, Stieger M, Yawalkar N, Kakeda M. Cytokines and chemokines in irritant contact dermatitis. *Mediators of inflammation*, 2013;5:1-7.
30. Kurahashi R, Hatano Y, Katagiri K. IL-4 suppresses the recovery of cutaneous permeability barrier functions in vivo. *J of Investigative Dermatology*. 2008;128(5):1329-31. Epub 2007 Oct 25.
31. Dorjsembe B, Lee HJ, Kim M, Dulamjav B, Jigjid T, Nho CW. *Achillea asiatica* extract and its active compounds induce cutaneous wound healing. *J of ethnopharmacology*. 2017;206:306-14.
32. Bruch-Gerharz D, Ruzicka T, Kolb-Bachofen V. Nitric oxide in human skin: current status and future prospects. *J of Investigative Dermatology*. 1998;110(1):1-7.
33. Emanuela R, Garret AF. Prostaglandins and Inflammation, Arteriosclerosis, thrombosis, and vascular biology. 2011;31(5):986-1000.
34. O'Regan GM, Sandilands A, McLean WH Irwin, Irvine AD. Filaggrin in atopic dermatitis. *J of Allergy and Clinical Immunology*. 2008;122(4):689-93.
35. O'Regan GM, Irvine AD. The role of filaggrin in the atopic diathesis. *Clinical and Experimental Allergy*. 2010;40(7):965-72.
36. Furue M, Chiba T, Tsuji G, Ulzii D, Kido-Nakahara M, Nakahara T, et al. Atopic dermatitis: immune deviation, barrier dysfunction, IgE autoreactivity and new therapies. *Allergology International*. 2016;66(3):398-403.