Thesis for the degree of Master of Science

Photoprotective Effects of Germinated and Fermented Soybean Extract on UVB-irradiated SKH-1 Hairless Mice

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List of Abbreviations

AP-1	activator protein-1
AQP 3	aquaporin 3
CAT	catalase
COL 1α1	collagen type 1 α1
COX 2	cyclooxygenase 2
ECM	extracellular matrix
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FLG	filaggrin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFSE	germinated and fermented soybean extract
GPX	glutathione peroxidase
H&E	Hematoxylin & Eosin
HA	hyaluronic acid
HYAL 1	hyaluronidase 1
IL-1β	interleukin 1β
INOS	inducible nitric oxide synthase
INV	involucrin
JNK	c-Jun N-treminal kinase
MAPK	mitogen-activated protein kinase
MMPs	matrix metalloproteinases
NF-kB	nuclear factor kappa B
p-JNK	phosphorylated c-Jun N-treminal kinase
p-ERK	phosphorylated extracellular signal-regulated kinase
qRT-PCR	quantitative real time polymerase chain reaction
ROS	reactive oxygen species
SOD 1	superoxide dismutase 1
SPSS	statistical package for the social science
TIMPs	tissue metallopeptidase inhibitors
TEWL	transepidermal water loss
TNF-α	tumor necrosis factor-α
UV	ultraviolet
UVB	ultraviolet light B

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Abstract

Ultraviolet (UV) radiation is a primary contributor to skin aging, accounting for approximately 90% of signs of premature aging. Continuous ultraviolet irradiation rapidly induces a large accumulation of reactive oxygen species (ROS) in the body, causing various metabolic diseases and causing direct damage to skin cells due to oxidative stress. The phytochemical isoflavones present in soybeans are recognized for exerting physiological effects, including anti-tumor, anti-obesity, and especially anti-aging properties. However, there is currently limited research on phytoestrogens, including the ability of phytochemicals to improve skin aging functions. This study was to evaluate the anti-inflammatory and potent antioxidant properties of germinated and fermented soybean extract (GFSE) in improving photoaging induced by continuous UVB irradiation in hairless mouse. Photoaging was

induced in hairless mice by irradiating UVB for 8 weeks, and GFSE was orally administered daily (250 and 500 mg/kg). The skin was collected and parameters and wrinkle formation analysis and histopathological analysis were performed to indirectly assess the effects on skin conditions. Additionally, skin hyaluronic acid (HA) content, protein expression, and mRNA were evaluated for the photoprotective effects of GFSE through molecular biological approaches. GFSE significantly improved UVB-induced skin parameters and wrinkle index and reduced the increased thickness of the epidermis. In addition, the HA content reduced by UVB was increased. GFSE decreased the expression of matrix metalloproteinases, which were increased by UVB, and increased tissue metallopeptidase inhibitors and collagen type 1α1, which were decreased by UVB. It also increased antioxidant-related factors such as superoxide dismutase 1, catalase, and glutathione peroxidase, and increased factors related to the skin barrier and selective skin moisture absorption, such as involucrin, filaggrin, and aquaporin 3. GFSE administration reduced interleukin-1β, cyclooxygenase 2, and tumor necrosis factor-α which are inflammatory cytokines produced due to photoaging. Moreover, UVB reduced phosphorylated protein levels of Jun N-terminal kinase, extracellular signaling regulatory kinase, and p38, as well as cyclooxygenase 2 and inducible nitric oxide synthase protein levels.

GFSE significantly improved skin photoaging and reduced the extent of skin damage in hairless mice exposed to UVB irradiation. These findings suggest that GFSE may be a functional food ingredient for alleviating skin photoaging.

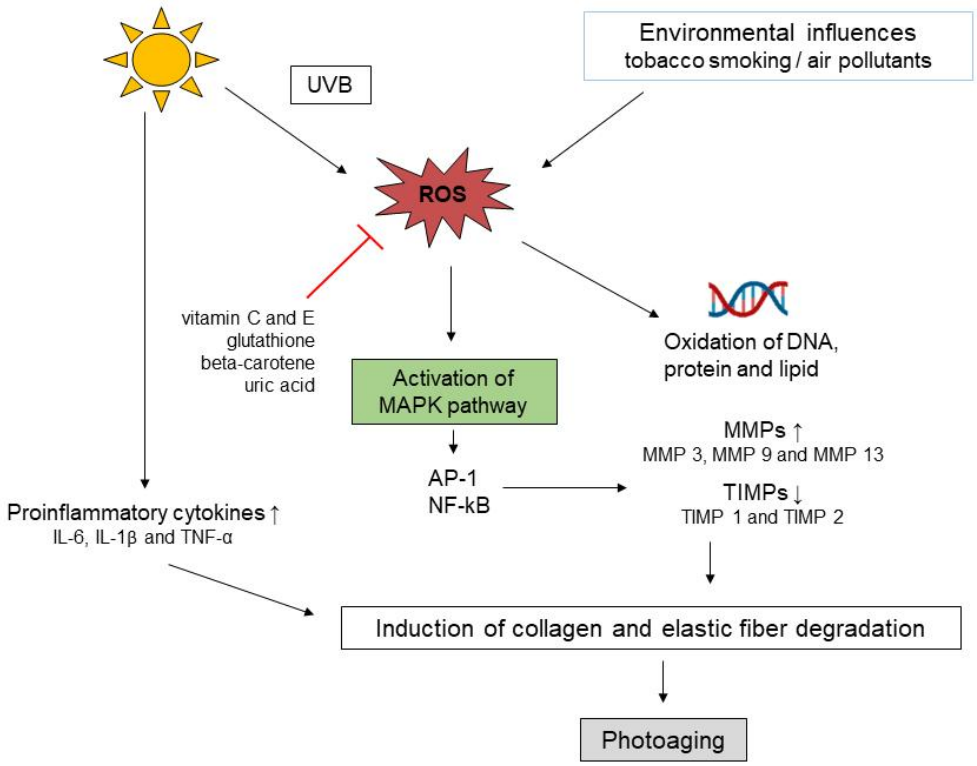
Keywords: Ultraviolet, reactive oxygen species, germinated and fermented soybean extract, phytoestrogen, antioxidants

1. Introduction

The skin is the most important and essential barrier organ that not only protects the body from dangerous substances and maintains body temperature, but also helps selective absorption of substances. Therefore, the skin that protects the body consists of two important basic layers (dermis and epidermis) that are closely connected to each other through the basal layer (Rinnerthaler, Bischof, Streubel, Trost, & Richter, 2015). It is also the core tissue of the skin, and the outermost layer, the stratum corneum, protects the body, prevents moisture evaporation, and maintains elasticity (Miri & Hyun Jeong, 2016). The stratum corneum of the epidermis is formed by combining structural proteins such as loricrin and involucrin (INV) by transglutaminase, and provides a skin barrier function. Stability and integrity are also important in the stratum corneum, which is constantly maintained by a major structural protein called filaggrin (FLG). Natural moisturizing factors such as hyaluronic acid (HA) combine with moisture in the stratum corneum to help maintain moisture and maintain skin health. (Kezic & Jakasa, 2016). If the skin lacks moisture, transepidermal water loss (TEWL) increases, causing it to dry out easily and lose elasticity. This promotes sagging skin and the formation of fine wrinkles. Skin ages naturally due to internal factors that occur due to hormonal changes and changes in the body, as well as external factors such as oxidative stress, environmental pollution, and ultraviolet (UV) irradiation (Papaccio, A, Caputo, & Bellei, 2022).

The UV are responsible for 90% of premature aging by causing the breakdown of skin structures, including the stratum corneum of the epidermis (Panich, Sittithumcharee, Rathviboon, & Jirawatnotai, 2016). Damage to skin components due to continuous and accidental sun exposure is called photoaging. Photoaging of the skin severely affects the skin layers and causes fatal, irreversible and permanent damage to the essential connective tissues of the dermis and epidermis (Scharffetter-Kochanek et al., 2000). Additionally, UV rays

promote the accumulation and production of reactive oxygen species (ROS) in the skin, causing rapid aging due to oxidative stress. (Md Jaffri, 2023). ROS includes alkoxyl ($RO^{\bullet}$), hydroxyl ($HO^{\bullet}$), superoxide radical ($O_2^{\bullet-}$), and peroxyl ($ROO^{\bullet}$), which are naturally generated due to the activities of cellular organelles such as mitochondria in the body (Juan, Pérez de la Lastra, Plou, & Pérez-Lebeña, 2021). Accumulation of ROS causes DNA damage, lipid peroxidation, immune breakdown, decomposition of connective tissue, and imbalance of cellular defense system, inhibiting normal cell function (Md Jaffri, 2023). ROS accumulated in the body by ROS accumulated in the body by UVB irradiation promote the mitogen-activated protein kinase (MAPK) signaling pathways. This promotes sustained phosphorylation of mitogen-activated protein kinase (p38), signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) thereby accelerating the degradation of the extracellular matrix (ECM) (Boo, 2020). Activated MAPK signaling pathways increase regulation of mRNA expression of matrix metalloproteinases (MMPs), a type of collagenase, promoting their degradation and inhibiting the production of collagen, a key structural protein (Rinnerthaler et al., 2015). Among them, gelatinase MMPs (MMP 2 and MMP 9) induce fragmentation of various collagens, including skin collagen types I and IV, and degrade ECM components. Additionally, collagenase MMP 13 exhibits high cleavage specificity for collagen. Therefore, suppressing MMPs expression is important for regulating collagen metabolism (Pittayapruek, Meephansan, Prapapan, Komine, & Ohtsuki, 2016). Chronic exposure to UVB changes the structural components of the ECM, leading to a decrease in important structural substances (elastin and collagen) that maintain skin elasticity, as well as the formation of wrinkles. Additionally, the moisture retained in the skin is lost, causing irritating redness due to dryness (J. W. Shin et al., 2019). If skin damage caused by UV is not recovered normally, it causes clinical symptoms such as inflammation, atopy, and skin cancer (D'Orazio, Jarrett, Amaro-Ortiz, & Scott, 2013).



(Miri et al., 2016)

Figure 1-1. Mechanisms of UVB-induced skin aging.

Demand for cosmetics and functional materials to prevent skin aging is rapidly increasing. Previously, external cosmetics that were applied topically directly to the skin were mainly used, but recently, the concept of inner beauty has emerged. 'Inner Beauty' is a compound word of 'Inner' and 'Beauty' and refers to the continuous improvement of the skin itself by managing skin health through diet and lifestyle habits. In particular, intake of antioxidant ingredients such as vitamins helps prevent and improve skin aging, so it is attracting attention as an inner beauty material (Ganceviciene, Liakou, Theodoridis, Makrantonaki, & Zouboulis, 2012).

Phytochemicals are biologically active substances extracted from natural plants and can be obtained from ingredients such as nuts, herbs, and various vegetables and fruits. According to recent research, focusing on the differentiated functions of various phytochemicals, they are actively being used as food and drugs (Kumar et al., 2023). Phytochemicals have various physiological activities that are beneficial to health, such as oxidation and antibacterial properties. In particular, phenolic compounds such as catechin, isoflavone, proanthocyanin, and resveratrol are representative phytochemicals and are known to protect against alleviate inflammation and oxidative stress by inhibiting ROS production (Pandey & Rizvi, 2009). Additionally, various physiological activities of natural products containing phytochemicals have been reported (Table 1-1).

Juglans regia L. leaves are rich in beneficial phenolic compounds that are a source of bio antioxidant-related active molecules and are consistently used in traditional medicine. This ingredient is widely used and added as a raw material for skin inflammation and diarrhea medicine, and the leaves are especially known to be rich in α-tocopherol. It is a hemolytic antioxidant found mainly in red blood cells and the stratum corneum of tissues, maintaining the structural stability of red blood cells. It also protects the body's metabolism from diseases such as cardiovascular disease by suppressing the development of inflammation and tumors

in the body (Santos et al., 2013).

Arachis hypogea L. is rich in carbohydrates, proteins, lipids, minerals and vitamins and is known to contain beneficial bioactive components polyphenols, phytosterols and triterpenes. It can reduce the incidence of coronary artery disease by participating in various physiologically active metabolism in humans. In addition, resveratrol is an effective anti-inflammatory agent that blocks inflammatory pathways, inhibiting the inflammatory response of fat cells and lowering blood sugar levels in the body (Mingrou, Guo, Ho, & Bai, 2022).

Glycine max L. is rich in phytochemical content and is used as a supplement in the food industry. Isoflavones, such as genistein, daidzein, and glycitein, are phytoestrogenic compounds. This delays skin aging by reducing collagen breakdown and preventing lipid oxidation. Recently, research has shown that genistein can increase collagen deposition, angiogenesis, and cell migration, which are important for skin improvement, and can prevent age-related skin changes caused by a decrease in estrogen in the body (Uyar, Sivrikoz, Ozdemir, Dasbasi, & Sacar, 2014).

Allium sativum L. is rich in phenolic compounds such as organosulfides and saponins, which are bioactive promoting-compounds involved in various body metabolism, and has been used in traditional medicine as it provides many health benefits. Allicin's antihypertensive activity can suppress the development of high blood pressure and lowering blood lipids and blood pressure. Additionally, phenols and saponins protect the body's metabolism by removing ROS within cells and reducing oxidative stress (Shang et al., 2019).

Camellia sinensis L. provides beneficial effects in cancer prevention as a pharmacological adjuvant. In particular, the catechin suppresses the development of various types of cancer by eliminating ROS and suppressing the formation of free radicals. It also repairs DNA damage caused by the sun's UV and inhibits cell death (Musial, Kuban-Jankowska, & Gorska-

Ponikowska, 2020).

Carica papaya L. is rich in phenolic compounds, tocopherol, and benzyl isothiocyanate phytochemicals that act as anti-obesity, anti-inflammatory and antioxidant agents. Therefore, upon ingestion, glycogen accumulation in the liver is reduced, fat accumulation is eliminated, and activation of insulin phosphorylation signaling in skeletal muscle is promoted. It can be used to improve and maintain the quality of life of people with diabetes because it reduces many of the complications associated with diabetes (Nyakundi, Wall, & Yang, 2024).

Table 1-1. Effects and mechanisms of phytochemical compounds in natural products.

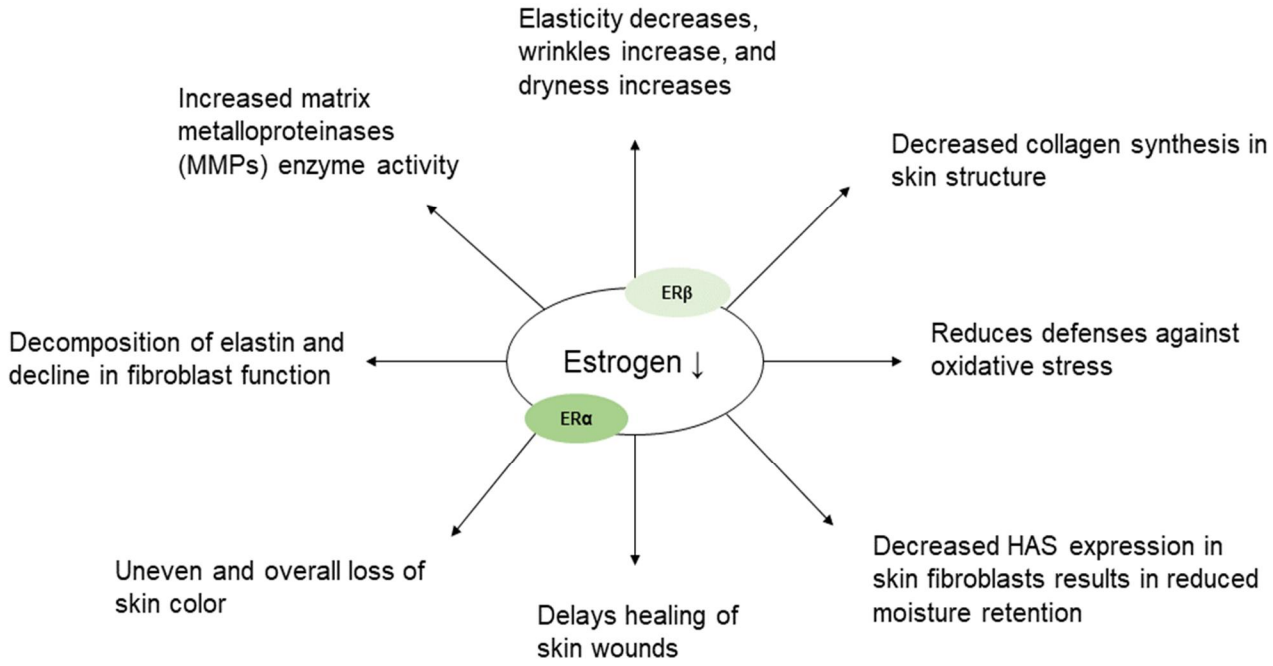
Natural product	Effects and mechanisms	Ref.
Juglans regia L. (Leaf)	It is rich in antioxidants such as tocopherol, which is a hemolytic antioxidant and can protect the safety of red blood cell structures. It protects the body's metabolism from cancer, cardiovascular disease, and various physical diseases.	(Santos et al., 2013)
Arachis hypogea L.	The antioxidant capacity of polyphenols, phytosterols and triterpenes has a beneficial effect on the body by reducing the incidence of coronary artery disease. Resveratrol inhibits the promotion of tumor and inflammation formation and lowers blood sugar levels in the body.	(Mingrou et al., 2022)
Glycine max L.	Isoflavones have beneficial effects on the skin by preventing lipid oxidation and reducing collagen breakdown. Genistein increases collagen deposition and skin angiogenesis.	(Uyar et al., 2014)
Allium sativum L.	Allicin's antihyperlipidemic activity lowers blood lipids. Phenol and saponin reduce oxidative stress by removing intracellular ROS.	(Shang et al., 2019)
Camellia sinensis L. (Leaf)	Catechin is highly effective in preventing cancer by inhibiting the production of free radicals. Repairs DNA damage caused by UV.	(Musial et al., 2020)
Carica papaya L.	It is rich in phytochemicals such as many phenolic compounds, tocopherols and benzyl isothiocyanate, which act as antioxidants, anti-obesity and anti-inflammatory agents. It improves quality of life by alleviating diabetes and obesity.	(Nyakundi et al., 2024)

Soybeans (*Glycine max* L.) are considered a plant-based complete protein source and a positive substitute for animal proteins and contain a variety of phytochemicals such as rotase inhibitors, saponins, isoflavones, phytosterols, and inositol hexaphosphatase. In particular, isoflavones and coumarins bind to the estrogen receptor (ER) in mammals and humans, acting as phytoestrogens with similar effects on the body (I. S. Kim, Yang, & Kim, 2021). It is a phytoestrogen with powerful antioxidant properties and reduces collagen degradation by inhibiting activator protein-1 (AP-1) activity and reducing MMPs transcription (Liu et al., 2020). In addition, research has shown that it has a protective effect against skin photoaging caused by UV rays, and has been shown to have a positive effect on direct skin improvement through epithelial cell proliferation (Piipponen, Li, & Landén, 2020). The physiological activity of soybeans has a positive effect on the skin in the same way as the function of estrogen in the body and has an improvement effect on aging.

Phytoestrogens are estrogen like plant compounds found in plants. It is structurally very similar to the 17-estradiol hormone and is known to exhibit the same function as estrogen by binding to ER in the body (Desmawati & Sulastri, 2019). The various biological properties of dietary phytoestrogens are similar to the actions of estrogen in the body, thereby alleviating symptoms such as breast cancer, cardiovascular disease, prostate cancer, osteoporosis, and skin aging that are prone to occur due to estrogen deficiency. (Rietjens, Louisse, & Beekmann, 2017).

Estrogen also has a particularly important effect on the skin by regulating skin physiology by acting on keratinocytes, fibroblasts, hair follicles, and sebaceous glands. Estrogen stimulates transforming growth factors and promotes the production of vascular endothelial growth factor, which promotes skin angiogenesis, increases collagen maturity and thickness, and maintains skin moisture (Stevenson & Thornton, 2007).

When estrogen in the body decreases, elastin decomposition in the skin accelerates and collagen synthesis decreases (Thornton, 2013). Estrogens can regulate MMPs activity and reduce collagen degradation by upregulating the expression of tissue metallopeptidase inhibitors (TIMPs) (Papakonstantinou, Roth, & Karakiulakis, 2012). Additionally, as antioxidant function decreases due to estrogen deficiency, sensitivity to UV increases and skin inflammation and damage is promoted.



(Liu et al., 2020)

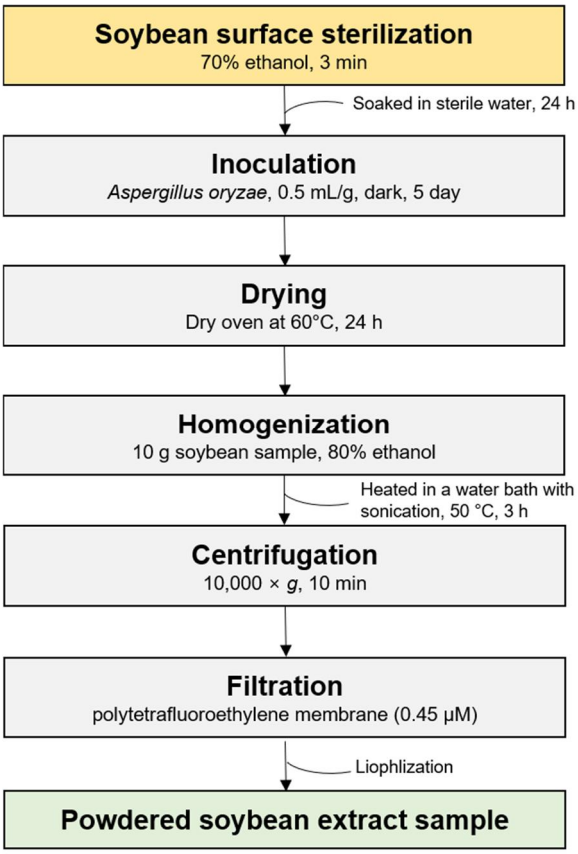
Figure 1-2. Effects of estrogens on skin aging.

Although much research has been conducted on phytochemicals, there is limited research on phytoestrogens, including their ability to improve skin aging functions. This study was to investigate the efficacy of GFSE in improving photoaging by analyzing MMP and MAPK signaling pathways along with skin parameters and histochemical staining in photoaged hairless mice.

2. Materials and methods

2.1 GFSE sample preparation

GFSE were prepared according to previous methods (E. Y. Kim, Hong, Suh, & Choi, 2015). Soybeans were immersed in 70% ethanol for 3 min to sterilize the surface, and then soaked in sterilized water for 24 h. *Aspergillus oryzae* (powder) was mixed with sterilized water and cultured at 25°C for 24 h to prepare a suspension solution of *A. oryzae* for soybean fermentation. *A. oryzae* suspension was inoculated into soybean at 0.5 mL/g and cultured inside a clean bench (25°C, dark room) for 5 days. During the inoculation period, sterilized water was applied to the soybeans five times a day, and after fermentation was completed, the soybeans inoculated with *A. oryzae* were dried at 60°C for 24 h. Dried soybean samples were homogenized by adding 80% ethanol and sonicating for 3 h at 50°C. The soybean homogenate was centrifuged for 10 min and only the supernatant was collected. The collected supernatant was filtered through a polytetrafluoroethylene filter and then lyophilized.



(Kim et al., 2015)

Figure 2-1. Schematic diagram of the germinated and fermented soybean extract manufacturing process.

2.2 Experimental animal

Female hairless mouse (SKH-1, 4 weeks old) were purchased from Orient Bio (Seongnam, Korea). Mice were housed in an environmentally controlled room with a temperature (22 ± 1°C), humidity (55 ± 5%), and a 12-h light/dark cycle. Access to drinking water and food was provided ad libitum. The experimental group was divided into four groups of five mice each.: the normal control group (NOR), UVB control group (UVB-C), low dose of GFSE-administrated group (GFSE-L, 250 mg/kg), and high dose of GFSE-administrated group (GFSE-H, 500 mg/kg). Animal experiments were approved by the Korea University Institutional Animal Care and Use Committee (KUIAUCC-2022-0038).

Table 2-1. Experimental animal treatment conditions.

Group	Treatment	UVB
NOR	Distilled water	-
UVB-C	Distilled water	+
GFSE-L	250 mg/kg of GFSE	+
GFSE-H	500 mg/kg of GFSE	+

GFSE, germinated and fermented soybean extract; NOR, normal control group; UVB-C, UVB-control group; GFSE treated group: GFSE-L, low dose group (250 mg/kg) and GFSE-H, high dose group (500 mg/kg).

2.3 UVB-irradiated photoaging model

After a one-week adaptation period, distilled water was administered to the NOR group and UVB-C group once a day for 8 weeks. The GFSE treatment groups were orally administered low-dose and high-dose (250 mg/kg and 500 mg/kg) samples. In all groups except for the NOR group, UVB exposure was induced by irradiation at an accurate dose 3 times a week for 8 weeks using a UVB irradiator (BLX-254; Vilber Lourmat, France). The doses used were 1 MED (75 mJ/cm^2) for 1 week, 2 MED for the 2 week, 3 MED for the 3 week, and 4 MED for 4-8 weeks. To avoid the effects of direct UVB damage, UVB irradiation was not applied 3 days before sacrifice.

2.4 Evaluation of skin parameters

The Multi Probe Adapter (MPA6; GmbH, Germany) was utilized to measure and analyze skin water holding capacity, TEWL, skin erythema value, and pH, parameters closely related to closely related to skin protective barrier and water retention function. Skin thickness was measured using a digital micrometer (Type 293, Mitutoyo, Kawasaki, Japan) by gently pulling the skin along the dorsal central skin line. Skin parameters were measured at 8 weeks after sample administration.

2.5 Evaluation of wrinkle formation

The back skin of the sacrificed mouse was photographed and the degree of wrinkles was visually confirmed. And then, the Visoline® Replica Kit (VL650; GmbH, Germany) was used to obtain skin replicas of each group. The following measurements were taken from the

collected replicas; mean wrinkle depth (μm), total wrinkle area (mm^2), number of wrinkles, and mean wrinkle depth (μm).

2.6 Histopathological analysis of the skin

Mice were sacrificed, dorsal skin was collected, the skin was fixed by soaking in 10% formalin solution, and then dehydrated with alcohol. Skin sections from hairless mouse were embedded in paraffin wax and stained with hematoxylin and eosin (H&E) using a kit according to the manufacturer's protocol. Histopathological changes in back skin tissue were analyzed using a light microscope (OLYMPUS BX50; Olympus, Tokyo, Japan) and Image J software (version 1.8, NIH, Bethesda, MD, USA).

2.7 Analysis of hyaluronic acid content (HA) in skin tissue

The altered HA content in mouse back skin tissue was quantified and analyzed using a mouse HA ELISA kit. (#MBS729522; MyBioSource Inc., San Diego, CA, USA). Skin tissue (100 mg) was homogenized by adding of phosphate-buffered saline, and then centrifuged to collect the supernatant. Enzyme conjugate and balance solution were added to the supernatant dispensed in a 96-well plate and incubated at 37°C for 1 h. Then, after washing and emptying the plate, substrates A and B were added, respectively, and the plate was incubated at 37°C for 20 min. The enzymatic reaction that continues after incubation is terminated by adding a stop solution, and the absorbance is measured at 450 nm using a spectrophotometer. The HA content in the tissue is then determined by comparing the measured absorbance to a hyaluronic acid standard curve.

2.8 Analysis of gene expression through qRT-PCR

Total RNA was extracted from mouse skin using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). After RNA quantification, prepared RNA samples were treated with Q1 RNase-Free DNase (Promega, Madison, WI, USA) to remove genomic DNA. Subsequently, complementary DNA (cDNA) was synthesized using SuperScript® III (Invitrogen) reverse transcriptase. The synthesized cDNA was subjected to qRT-PCR using a SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA). Results were normalized to the expression of the gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_001411843.1) and validated using the CT ($2^{-\triangle\triangle}$CT) methods (Livak & Schmittgen, 2001) and a list of primers used in the qRT-PCR is shown in Table 2-2.

Table 2-2. The primer sequences used for quantitative real-time polymerase chain reaction (qRT-PCR) in this study.

Gene	Forward (5' to 3')	Reverse (5' to 3')
MMP 2	CAAGGATGGACTCCTGGCACAT	TACTCGCCATCAGCGTTCCCAT
MMP 9	GCTGACTACGATAAGGACGGCA	TAGTGGTGCAGGCAGAGTAGGA
MMP 13	GATGACCTGTCTGAGGAAGACC	GCATTTCTCGGAGCCTGTCAAC
TIMP 1	TCCCCAGAAATCAACGAGACC	TTCCCACAGCCTTGAATCCT
TIMP 2	AGCCAAAGCAGTGAGCGAGAAG	GCCGTGTAGATAAACTCGATGTC
TNF-α	CCACGTCGTAGCAAACCAC	GTAGACAAGGTACAACCCATCG
IL-1β	CATCAGCACCTCACAAGCAG	CAGTCCAGCCCATACTTTAGG
COX 2	GCGACATACTCAAGCAGGAGCA	GTAACCGCTCAGGTGTTGCACG
COL 1a1	CCTCAGGGTATTGCTGGACAAC	CAGAAGGACCTTGTTTGCCAGG
HYAL 1	GCAAGTGGTGTGACAAGCG	GTGACTTATGGGCATGTGACTG
INV	ATGGTCCGGTATCTTCCCCA	TGACAGGCCTCCCTTTTTCC
FLG	GTGCTGGCAACCAGTCATCT	CCCATGCTGTCCAAATCCCA
SOD 1	GGTGAACCAGTTGTGTTGTCAGG	ATGAGGTCCTGCACTGGTGCAG
AQP3	CCTTGTGATGTTTGGCTGTGG	GGAAGCATTGCGAAGGTC
GPX	CGCTCTTTACCTTCCTGCGGAA	AGTTCCAGGCAATGTCGTTGCG
CAT	CATCGAGCCCAGCCCTGACA	GCGGTAGGGACAGTTCACAGG
GAPDH	CAGCAATGCATCCTGCACCAC	AGTCTTCTGGGTGGCAGTGATG

MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; COX 2, cyclooxygenase 2; COL 1α1, collagen type 1 α1; HYAL 1, hyaluronidase1; INV, involucrin; FLG, filaggrin; SOD 1, superoxide dismutase 1; AQP3, aquaporin 3; GPX, glutathione peroxidase; CAT, catalase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2.9 Western blot analysis

Mouse skin tissue (100 mg) was placed in 1 mL of RIPA lysis and extraction buffer (#89900, Thermo Fisher Scientific, Waltham, MA, USA) and homogenized using a Tissue Lyser II (Qiagen, Venlo, Netherlands). The tissue homogenate is centrifuged to collect the supernatant, and the protein concentration is quantified using a bicinchoninic acid kit (Thermo Fisher Scientific). 25 μg of protein was electrophoresed on a 10-12% SOD 1ium dodecyl sulfate-polyacrylamide gel, and transferred to polyvinylidene fluoride membranes (Mahmood & Yang, 2012). After blocking the membrane with 5% skim milk, primary antibody (Cell Signaling Technology, Danvers, MA, USA) was reacted overnight at 4°C. Subsequently, secondary antibody (anti-rabbit IgG, #7074, Cell Signaling Technology, USA) was reacted for 2 h. The primary antibodies used in the experiment are as follows: GAPDH (#5174), ERK 1/2 (#9102), JNK (#9252), p38 (#9212), p-ERK 1/2 (#9101), p-JNK (#9251), p-p38 (#9211), COX 2 (#12282), and INOS (#13120). Protein expression was confirmed using the FluorChem E System (#92-14860-00, Bio-Techne, Minneapolis, MN, USA).

2.10 Statistical analyses

The experimental results were expressed as mean ± standard error of the mean (SEM). All outcome analyzes were performed using the SPSS (Statistical Package for the Social Science, SPSS Inc., USA) version 24.0. Statistically significant differences were observed at $p<0.05$ in the data after one-way analysis of variance.

3. Results

3.1 Changes in physical condition of UVB-irradiated hairless mice

The physical condition of the experimental mice was indirectly evaluated by measuring body weight (g), food intake (g/day), and water intake (mL/day) once a week during the period of UVB irradiation and sample administration (Table 3-1). During the experiment period, it was confirmed that there was no significant difference in weight change, food intake, and water intake of all experimental groups compared to the NOR group.

Table 3-1. Effects of GFSE on body weight, food intake, and water intake in UVB-irradiated mice.

Group	Body weight (g)	Food intake (g/day)	Water intake (mL/day)
NOR	30.40 ± 0.30 ^{NS}	5.64 ± 0.16 ^{NS}	6.47 ± 0.25 ^{NS}
UVB-C	31.14 ± 0.44	5.85 ± 0.10	6.40 ± 0.29
GFSE-L	31.20 ± 0.38	5.14 ± 0.20	6.38 ± 0.15
GFSE-H	31.68 ± 0.39	5.61 ± 0.16	6.06 ± 0.22

Data are expressed means ± standard error of the mean (SEM) (n=5). GFSE, germinated and fermented soybean extract; NOR, normal control group; UVB-C, UVB-control group; GFSE administered group; GFSE-L, low dose administered group (250 mg/kg), GFSE-H, high dose administered group (500 mg/kg); NS, not significant.

3.2 Measurement of organ weights in UVB-irradiated hairless mice

After completion of the experiment, the major organs of the experimental mice were collected and weighed. There were no visible abnormalities in major organs due to administration of the sample. Additionally, no significant differences were observed in the weight of the liver, heart, kidney, and spleen in all experimental groups compared to the NOR group.

These results suggest that GFSE at the administered doses has no apparent detrimental effects on vital organs and support its potential safety.

Table 3-2. Effects of GFSE on organ weight in UVB-irradiated hairless mice.

Organ weight	Heart	Kidney	Spleen	Liver
NOR	0.62 ± 0.02^{NS}	2.26 ± 0.04^{NS}	0.36 ± 0.01^{NS}	5.68 ± 0.08^{NS}
UVB-C	0.64 ± 0.01	2.26 ± 0.04	0.36 ± 0.01	5.61 ± 0.08
GFSE-L	0.68 ± 0.04	2.33 ± 0.09	0.34 ± 0.01	5.69 ± 0.06
GFSE-H	0.61 ± 0.01	2.33 ± 0.04	0.36 ± 0.02	5.60 ± 0.06

Data are expressed means ± standard error of the mean (SEM) (n=5). GFSE, germinated and fermented soybean extract; NOR, normal control group; UVB-C, UVB-control group; GFSE administered group; GFSE-L, low dose administered group (250 mg/kg), GFSE-H, high dose administered group (500 mg/kg); NS, not significant.

3.3 Effects of GFSE on skin parameters in UVB-irradiated hairless mice

Continuous UVB irradiation has been shown to have a direct effect on the skin, worsening skin parameters related to skin hydration and barrier maintenance.

Therefore, compared to the NOR group, the UVB-C group significantly reduced skin moisture retention by 0.40-flod ($p<0.001$; Fig. 3-1A). Additionally, the UVB-C group showed significantly higher TEWL ($p<0.001$; 2.26-fold), erythema value ($p<0.001$; 1.62-fold), and skin thickness ($p<0.001$; 1.54-fold) than the NOR group (Fig. 3-1B-D). On the other hand, GFSE administration significantly improved the UVB-induced reduction in skin water holding capacity by 1.69-fold in the low dose GFSE-administered group and 2.39-fold in the high dose GFSE-administered group ($p<0.001$; Fig. 3-1A). The GFSE administration showed TEWL that was significantly lower by 1.80-fold in the low dose GFSE-administered group and 1.90-flod in the high dose GFSE-administered group compared to the UVB-C group ($p<0.001$; Fig. 3-1B). Additionally, compared to the UVB-C group, GFSE administration significantly reduced the degree of skin erythema by 1.48-flod in the low dose GFSE-administered group and 1.49-fold in the high dose GFSE-administered group ($p<0.001$; Fig. 3-1C). Skin thickness increased by continuous UVB irradiation was significantly improved by 1.44-fold in the low-dose GFSE-administrad group and 1.58-fold in the high dose GFSE-administered group 1.58-fold ($p<0.001$; Fig 3-1D). In particular, high dose of GFSE administration effectively improved changes in skin parameters caused by UVB, and this was at a similar level to that of the NOR group. Skin pH tended to increase with UVB irradiation and decrease with GFSE administration, but no significant differences between the experimental groups were observed (Fig. 3-1E).

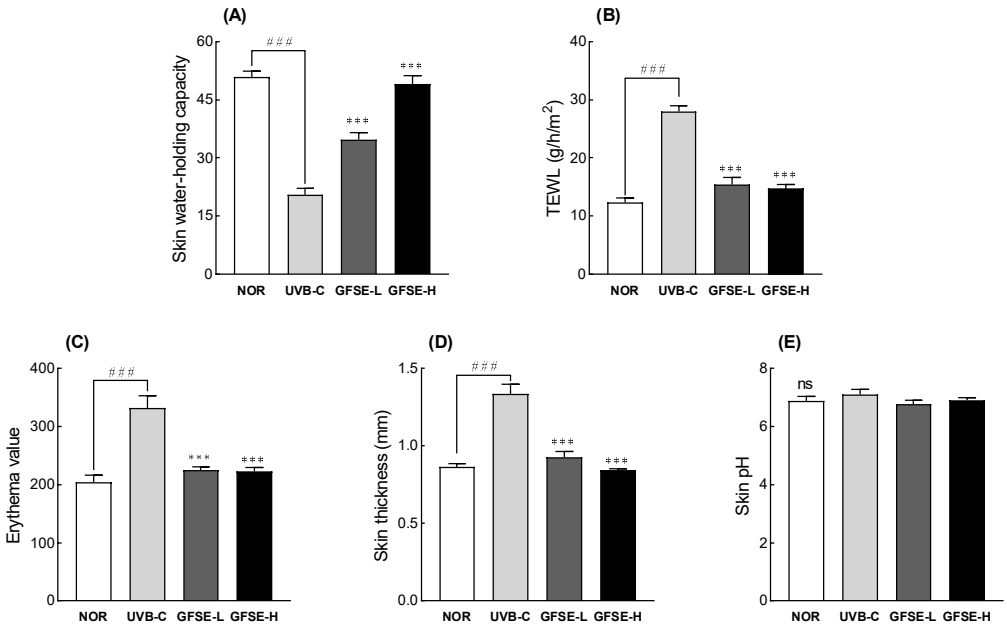


Figure 3-1. Effects of GFSE on (A) skin water holding capacity, (B) TEWL, (C) erythema value, (D) skin thickness and (E) skin pH in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). $^{###}p < 0.001$ vs. NOR group, and $^{***}p < 0.001$ vs. UVB-C group according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg).

3.4 Effects of GFSE on UVB-induced skin wrinkle formation in UVB-irradiated hairless mice

To evaluate the effect of GFSE on skin wrinkle formation, the back skin of experimental mice was visually observed and the wrinkle formation index of skin replicas was analyzed.

After 8 weeks of UVB irradiation, the back skin of the UVB-C group was thicker than the NOR group, and long and deep wrinkles were observed (Fig. 3-2A). On the other hand, the wrinkles in the GFSE-administered group were relatively shorter and thinner than those in the UVB-C group. As a result of replica analysis, the total wrinkle area ($p<0.001$; 6.96-fold), mean wrinkle length ($p<0.001$; 3.48-fold), and mean wrinkle depth ($p<0.001$; 2.37-fold) caused by UVB irradiation were significantly higher in the UVB-C group than in the NOR group. (Fig. 3-2B, D, and E). GFSE administration (250 and 500 mg/kg) dose-dependently reduced the total wrinkle area by 2.92- and 4.48-folds, respectively, compared to the UVB-C group ($p<0.001$; Fig. 3-2B). Additionally, the GFSE-administered group showed significant improvement in mean wrinkle length (GFSE-L; 3.19-fold, GFSE-H; 3.41-fold) and mean wrinkle depth (2.27-fold, and 2.37-fold) compared to the UVB-C group ($p < 0.001$; Fig. 3-2D and E). However, compared to the NOR group, the number of wrinkles in the UVB-C group was significantly reduced by 2.72-fold ($p<0.05$; Fig. 3-2C), but it recovered with GFSE administration (GFSE-L; $p<0.01$, and GFSE-H; $p<0.001$). Short and shallow fine wrinkles were observed following GFSE- administered group, while in the UVB-C group, fine wrinkles merged into thick and deep wrinkles, showing a decrease in the total number of wrinkles (Fig. 3A).

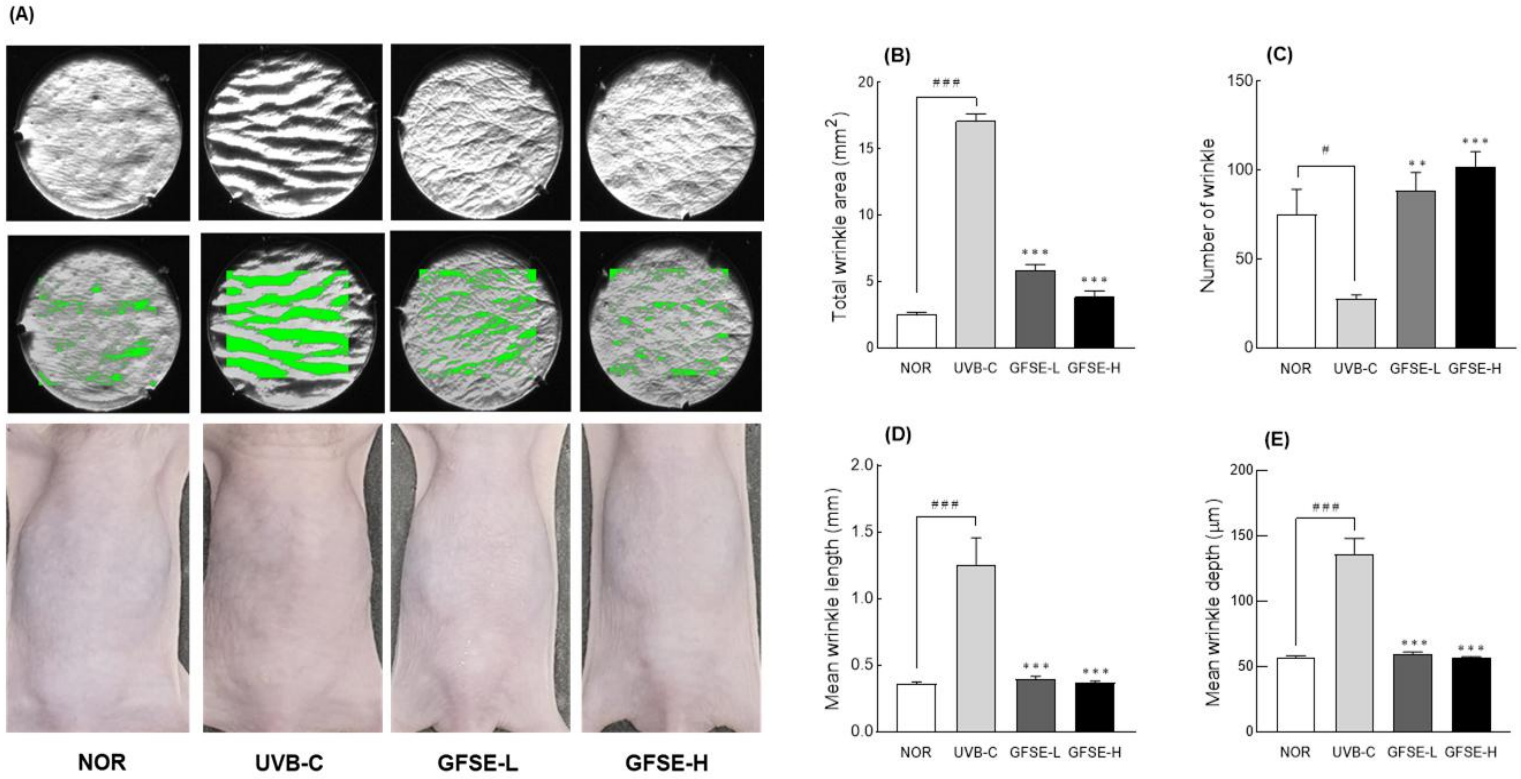


Figure 3-2. Effects of GFSE on (A) photographs and replicas of hairless mouse skin, (B) total wrinkle area, (C) number of wrinkle, (D) mean wrinkle length, (E) mean wrinkle depth. Data are expressed means ± standard error of the mean (SEM) (n=5). Different symbols indicated significant at $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. NOR group, and $^{**}p < 0.01$, $^{***}p < 0.001$ vs. UVB-C group according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg).

3.5 Effects of GFSE on epidermal thickness in UVB-irradiated hairless mice

The effect of GFSE on skin changes following UVB irradiation was quantified and analyzed by performing H&E staining on the changed epidermal thickness of hairless mouse.

Through H&E staining images, the changed epidermal thickness of the UVB-C group was significantly thicker than that of the NOR group, but the increase in epidermal thickness was improved with GFSE administration (Fig. 3-3A). As a result of measuring and quantifying the change in epidermal thickness due to UVB irradiation, the UVB-C group significantly increased 3.31-fold compared to the NOR group ($p<0.001$; Fig. 3-3B). GFSE administration alleviated the increase in epidermal thickness caused by UVB irradiation by 1.75-flod in the low dose GFSE-administered group, and 2.7-fold in the high dose GFSE-administered group ($p<0.001$; Fig. 3-3B).

These results demonstrate that the positive effect of GFSE on skin thickness may be due to its potential to stimulate collagen synthesis or inhibit UV-induced collagen degradation.

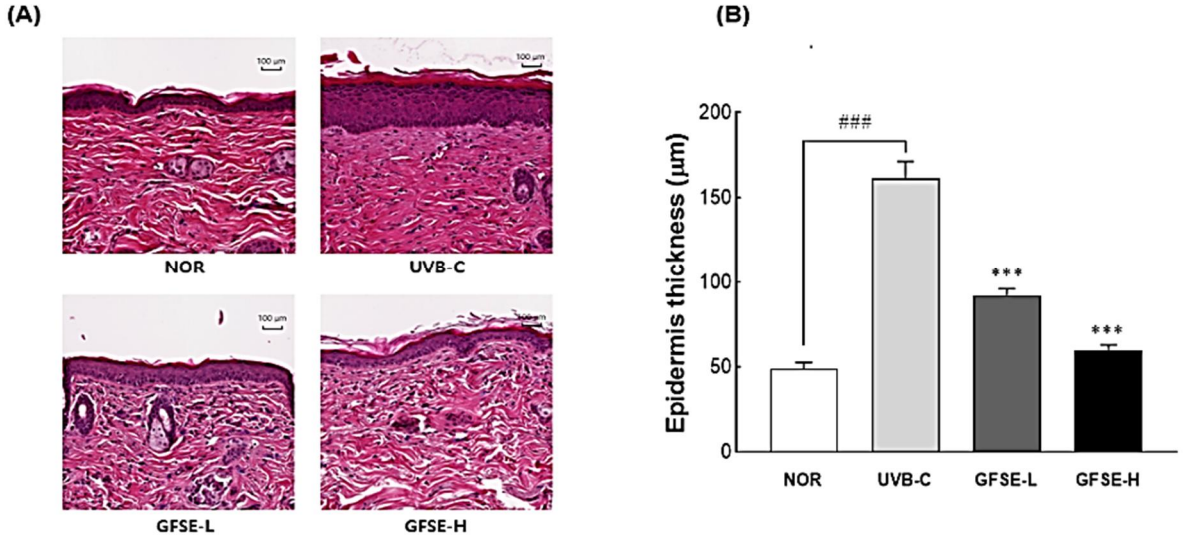


Figure 3-3. Effects of GFSE on histological alteration of epidermal thickness in UVB-irradiated hairless mouse skin. Data are expressed means ± standard error of the mean (SEM) (n=5). Different symbols indicated significant at $^{###}p < 0.001$ vs. NOR group, and $^{***}p < 0.001$ vs. UVB-C group according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg).

3.6 Effects of GFSE on skin HA content in UVB-irradiated hairless mice

Hyaluronic acid (HA), a glycosaminoglycan known for its water retention properties and role in maintaining skin elasticity, was quantified using an enzyme-linked immunosorbent assay (ELISA) kit. Notably, HA content significantly impacts overall skin health by promoting water retention and maintaining tissue flexibility.

Compared with the NOR group, the UVB-C group significantly reduced the HA content in skin tissue ($p<0.05$; 1.94-fold; Fig. 3-4A). The reduction of HA content in skin due to UVB irradiation was significantly alleviated by GFSE. In particular, the HA content in the skin of high-dose of GFSE administered group was significantly 1.9-fold higher than that of the UVB-C group (Fig. 3-4, $p < 0.05$).

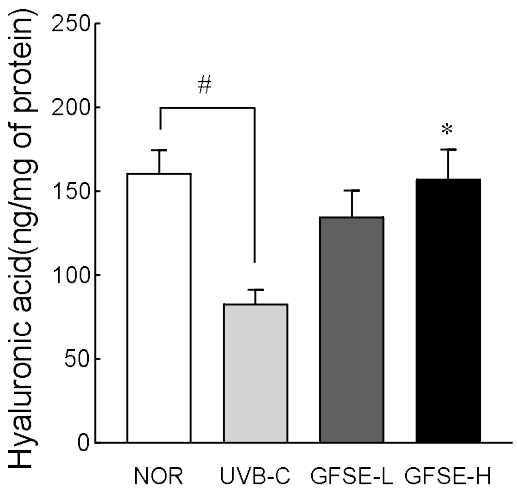


Figure 3-4. Effects of GFSE on skin HA content in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). Different symbols indicated significant at $^{\#}p < 0.05$ vs. NOR group, and $^{*}p < 0.05$ vs. UVB-C group according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg).

3.7 Effects of GFSE on collagen homeostasis-related mRNA expression in UVB-irradiated hairless mice

Collagen is important for maintaining skin structure. Continuous UVB irradiation breaks down skin collagen and inhibits its synthesis. To analyze the potential effect of GFSE on collagen homeostasis, qRT-PCR was used to evaluate collagen homeostasis-related mRNA expression in hairless mice under continuous UVB irradiation.

The mRNA expression of TIMP, which inhibits MMP activity, was significantly suppressed by UVB irradiation. In particular, the UVB-C group had significantly lower TIMP 1 expression by 1.69-fold ($p<0.001$; Fig. 3-5A) and TIMP 2 expression by 2.70-fold ($p<0.001$; Fig. 3-5B) than the NOR group. The 500 mg/kg GFSE-administered group (GFSE-H) showed significantly higher TIMP 1 expression by 2.00-fold than the UVB-C group ($p<0.001$). However, GFSE administration increased TIMP 2 expression by 3.16-fold in the low dose GFSE-administered group and 3.83-fold in the high dose GFSE-administered group compared to the UVB-C group ($p<0.001$).

Compared to the NOR group, the UVB-C group showed a significant 1.59-fold decrease in mRNA expression of COL1α1 ($p<0.01$; Fig. 3-5C). However, GFSE administration significantly improved the decrease in COL 1α1 expression level caused by UVB irradiation by 1.75-fold in the low dose GFSE-administered group and 1.95-fold in the high dose GFSE-administered group ($p<0.01$ and $p<0.001$).

Due to continuous UVB irradiation, the UVB-C group significantly increased the mRNA expression of MMP 9 ($p<0.001$; 1.56-fold) and MMP 13 ($p<0.05$; 1.70-fold), which decompose extracellular matrix, compared to the NOR group (Fig. 3-5E and F). Additionally, the mRNA expression of MMP 2 tended to increase in the UVB-C group compared to the NOR group, but there was no significant difference (Fig. 3-5D). On the other hand, GFSE

administration significantly inhibited MMP 2 mRNA expression by 1.83-fold in the low dose GFSE-administered group and 2.16-fold in the high dose GFSE-administered group ($p<0.001$). Additionally, GFSE-administered groups showed significantly lower expression of MMP 9 by 1.64-fold in the low dose GFSE-administered group and 2.00-fold in the high dose GFSE-administered group. The expression of MMP 13 was significantly lower by 1.36-flod in the low dose administered group and 1.37-fold in the high dose administered group compared to the UVB-C group ($p<0.001$).

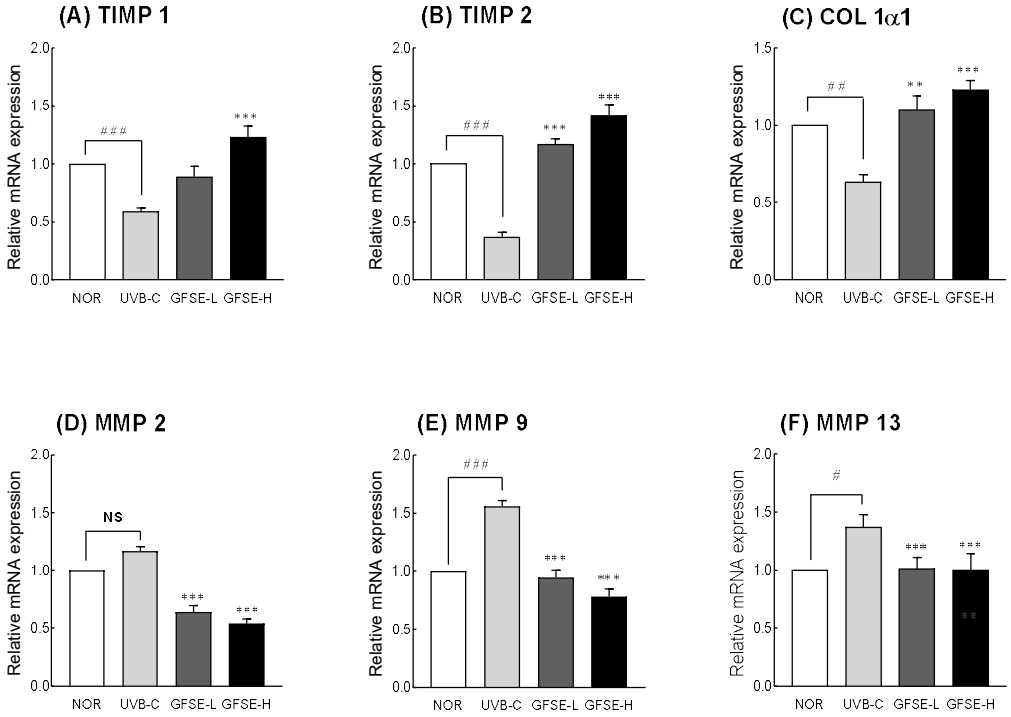


Figure 3-5. Effects of GFSE on mRNA expression of (A, B) TIMPs, (C) COL 1α1, and (D-E) MMPs in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). #p < 0.05, ##p < 0.01, ###p < 0.001 vs. NOR group, and **p < 0.01, ***p < 0.001 vs. UVB-C group according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg); TIMP, tissue inhibitor of metalloproteinase; COL 1α1, collagen type 1α1; MMP, matrix metalloproteinase, NS, not significant.

3.8 Effects of GFSE on antioxidant enzyme-related mRNA expression in UVB-irradiated hairless mice

Antioxidant enzymes play an important role in removing ROS accumulated in the body by UVB irradiation and alleviating oxidative stress. To evaluate the potential regulation of antioxidant enzyme expression by GFSE, mRNA expression was analyzed using qRT-PCR in hairless mice after UVB irradiation and are shown in Fig. 3-6.

UVB irradiation significantly suppressed the mRNA expression of antioxidant enzymes such as CAT ($p<0.001$; 1.80-fold), SOD 1 ($p<0.05$; 1.29-fold), and GPX ($p<0.01$; 1.52-fold), which are essential defense elements from ROS accumulated by UVB exposure, compared to NOR group (Fig. 3-6A-C). In particular, GFSE administration significantly increased CAT expression by 1.35-flod in the low dose GFSE-administered group and 1.54-flod in the high dose GFSE-administered group compared to the UVB-C group ($p<0.001$; Fig. 3-6A). Additionally, high-dose GFSE administration significantly restored SOD 1 expression, which was reduced by UVB irradiation, by 1.32-fold ($p<0.05$; Fig. 3-6B). The mRNA expression of GPX was significantly increased by 1.48-fold in the low dose GFSE-administered group and 1.50-fold in the high dose GFSE-administered group compared to the UVB-C group ($p<0.01$; Fig. 3-6C).

These results suggest that GFSE regulates the antioxidant response to UVB-C irradiation by upregulating antioxidant enzyme expression.

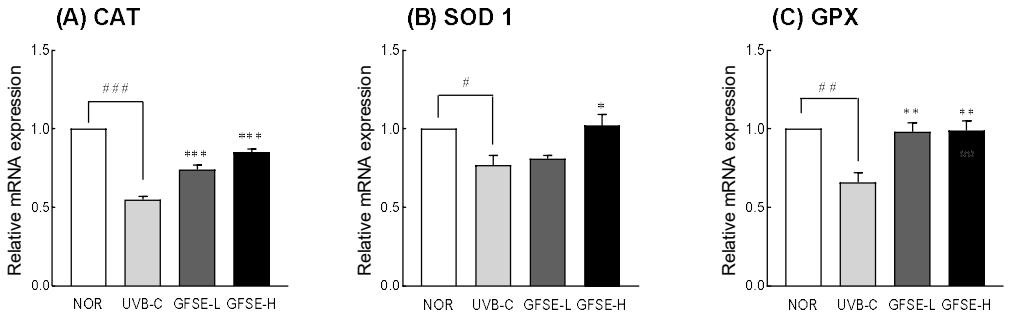


Figure 3-6. Effects of GFSE on mRNA expression of (A) CAT, (B) SOD 1 and GPX in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NOR group, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. UVB-C group according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg); CAT, catalase; SOD 1, superoxide dismutase 1; GPX, glutathione peroxidase.

3.9 Effects of GFSE on skin barrier function-related mRNA expression in UVB-irradiated hairless mice

To determine the effect of GFSE on mRNA expression essential for maintaining skin barrier function, we measured mRNA expression in the skin of hairless mouse continuously irradiated with UVB. The results obtained using qRT-PCR were analyzed and shown in Fig. 3-7.

UVB-C group significantly suppressed the expression of INV ($p<0.001$; 2.43-fold), FIG ($p<0.001$; 1.44-fold), and AQP3 ($p<0.001$; 1.40-fold) compared to the NOR group (Fig. 3-7A-C). The high dose GFSE-administered group showed significantly higher INV expression by 1.70-fold than the UVB-C group ($p<0.05$; Fig. 3-7A). GFSE administration also significantly increased the mRNA expression of FLG by 1.65-fold in the low dose GFSE-administered group and 1.78-fold in the high dose GFSE-administered group ($p<0.001$; Fig. 3-7B). Similarly, the expression of AQP 3 in the GFSE-administrated groups (GFSE-L: 1.48-fold, GFSE-H: 1.57-fold) was significantly recovered compared to the UVB-C group ($p<0.001$; Fig. 3-7C). HYAL-1 is a key enzyme responsible for the breakdown of HA, an important glycosaminoglycan that contributes to moisture retention and elasticity in the skin. In skin tissue, the expression of HYAL 1, which degrades HA, in the UVB-C group was significantly 1.94-flod higher than that in the NOR group ($p<0.001$; Fig. 3-7D). On the other hand, GFSE administration significantly suppressed HYAL 1 expression, which was increased by UVB irradiation, 2.37-flod in the low dose GFSE-administered group and 2.58-fold in the high-dose GFSE-administered group ($p<0.001$; Fig. 3-7D).

These results suggest that GFSE administration improves the essential functions of the skin barrier degraded by UVB irradiation.

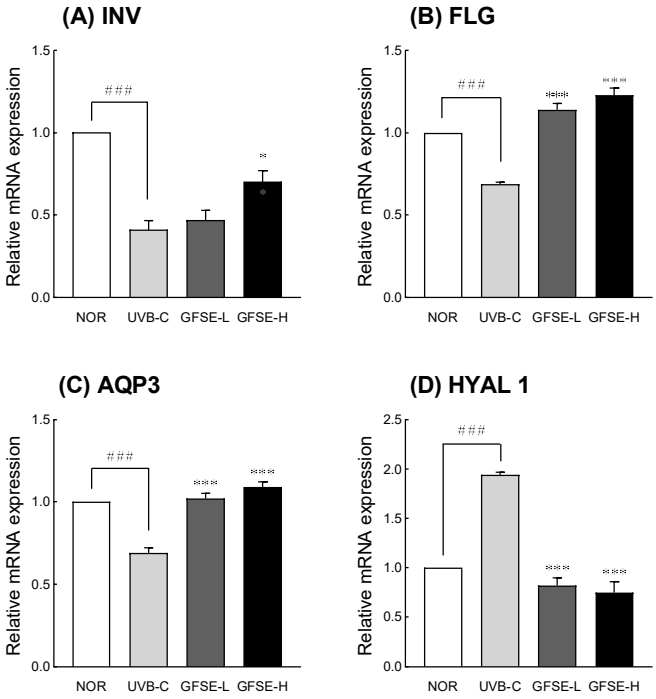


Figure 3-7. Effects of GFSE on mRNA expression of, (A) INV, (B) FLG, (C) AQP 3, and (D) HYAL 1 in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). $^{###}p < 0.001$ vs. NOR group, and $^{*}p < 0.05$, $^{***}p < 0.001$ vs. UVB-C group according to Tukey's test. INV, involucrin; FLG, filaggrin; AQP 3, aquaporin 3; HYAL 1, hyaluronidase 1; NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg).

3.10 Effects of GFSE on skin inflammation-related mRNA expression in UVB-irradiated hairless mice

To determine the anti-inflammatory potential of GFSE administration against UVB-induced skin damage, the expression of inflammatory mediator mRNA expression in hairless mouse irradiated with continuous UVB irradiation using qRT-PCR, and is shown in the Fig. 3-8.

The mRNA expression of IL-1β, a pro-inflammatory cytokine, tended to increase compared to the NOR group due to UVB irradiation, but no significant difference was observed (Fig. 3-8A). However, GFSE administration significantly inhibited the expression of IL-1β by 1.46-fold in the low dose GFSE-administered group and 1.85-fold in the high dose GFSE-administered group ($p<0.05$ and $p<0.01$; Fig. 3-8A). The UVB-C group due to UVB irradiation significantly increased the mRNA expression of TNF-α by 1.62-fold compared to the NOR group ($p<0.001$; Fig. 3-8B). GFSE-administered groups, the mRNA expression of TNF-α was significantly lower by 2.28-fold in the low dose GFSE-administered group and 2.38-fold in the high dose GFSE-administered group than the UVB-C group ($p<0.001$; Fig. 3-8B), which was at a similar level to the NOR group.

Additionally, qRT-PCR analysis revealed a significant 1.39-fold upregulation of COX-2 mRNA levels in the UVB-C group compared to the NOR group ($p<0.01$; Fig. 3-8C). However, GFSE administration (250 and 500 mg/kg) significantly inhibited UVB-induced COX 2 expression by 2.17-fold in the low dose GFSE-administered group and 2.35-fold in the high dose GFSE-administered group ($p<0.001$; Fig. 3-8C).

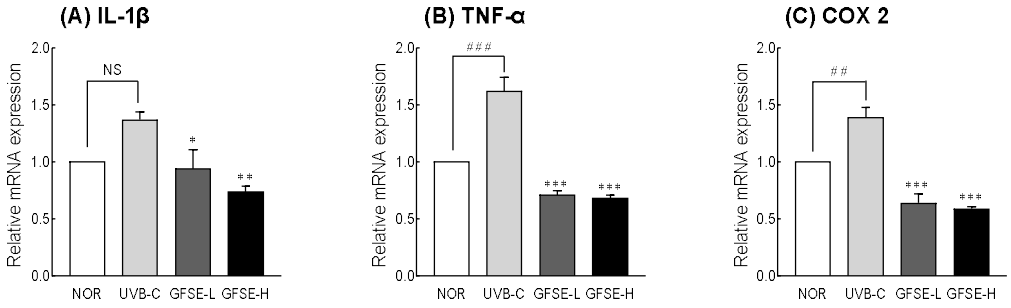


Figure 3-8. Effects of GFSE on mRNA expression of (A) IL-1β, (B) TNF-α, and (C) COX 2 in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). Different symbols indicated significant at $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NOR group, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. UVB-C group according to Tukey's test. IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; COX 2, cyclooxygenase 2. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg). NS, not significant.

3.11 Effects of GFSE on MAPK pathway-related protein levels in UVB-irradiated hairless mice

To investigate the effect of GFSE on the MAPK signaling pathways, which is known to be a major mechanism of photoaging, protein phosphorylation and quantification of factors related to MAPK signaling were performed using Western blotting in hairless mouse irradiated with UVB, as shown in the Fig 3-9.

Sustained UVB exposure accelerated phosphorylation and activation of MAPK signaling pathways (ERK, JNK, and p38; Figure 3-9), which are important drivers of cellular responses to stress and contribute to photoaging (ERK, JNK, and p38; Fig. 3-9). Phosphorylated ERK (p-ERK) protein expression increased 1.50-fold in the UVB-C group compared to the NOR group ($p<0.001$, Fig. 3-9A). GFSE administration significantly inhibited ERK phosphorylation by 1.40- and 1.45-fold, respectively, compared to the UVB-C group ($p<0.001$; Fig. 3-9A). in particular, the level of phosphorylated JNK (p-JNK) protein was significantly increased by 1.30-fold in the UVB-C group compared to the NOR group ($p<0.05$; Fig. 3-9B). On the other hand, the level of phosphorylated p-JNK in the GFSE-administered group tended to decrease compared to the UVB-C group, but no significant difference was observed (Fig. 3-9B). Continuous UVB irradiation significantly increased the protein level of phosphorylated p38 (p-p38) by 1.46-fold compared to the NOR group ($p<0.001$; Fig. 3-9C). GFSE administration significantly inhibited p38 protein phosphorylation by 0.95-flod in the low dose GFSE-administered group and 0.83-fold in the high dose GFSE-administered group ($p<0.001$; Fig. 3-9C).

These results suggest that GFSE treatment alleviates various cellular stresses and suppresses the promoted inflammatory response in the skin by the MAPK signaling pathway activated by UVB irradiation.

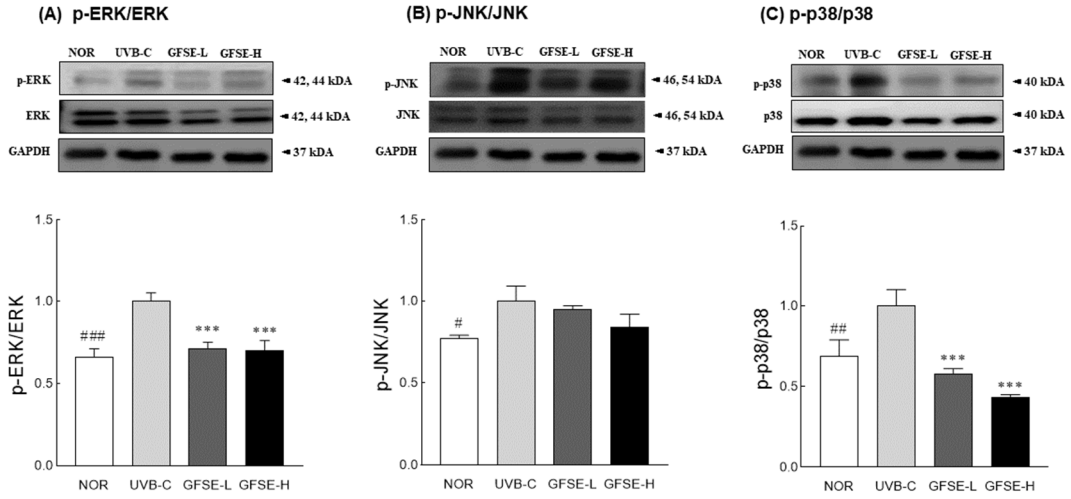


Figure 3-9. Effects of GFSE intake on the protein expression of (A) p-ERK/ERK, (B) p-JNK/JNK and (C) p-p38/p38 levels in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). Different symbols indicated significant at $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. NOR group, and $^{***}p < 0.001$ vs. UVB-C group according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GSFE administered group (500 mg/kg).

3.12 Effects of GFSE on skin inflammation-related protein levels in UVB-irradiated hairless mice

Excessive accumulation of ROS in the body can activate AP-1-related inflammatory transcription factors and MAPK pathways, causing irreversible skin damage. (Silvers, Bachelor, & Bowden, 2003). It induces the continuous and rapid production of COX 2 and INOS inflammation-promoting substances, causing the development and metastasis of skin cancer (Papaccio et al., 2022). To analyze the potential impact of GFSE on UVB irradiation-induced skin inflammation, protein levels were analyzed in hairless mice using Western blotting and shown in the Fig 3-10.

The protein levels of INOS and COX 2 in UVB-C group were significantly increased by 1.38 and 1.25-fold compared to the NOR group ($p<0.01$, $p<0.001$; Fig. 3-10). GFSE administration significantly reduced INOS protein levels by 1.50-fold in the low dose GFSE-administered group and 1.78-fold high dose GFSE-administered group compared to UVB-C ($p<0.01$, $p<0.001$; Fig. 3-10). Additionally, the protein level of COX 2 was significantly reduced by 1.42-flod in the low dose GFSE-administered group and 1.48-fold high dose GFSE-administered group ($p<0.01$ and $p<0.001$; Fig. 3-10).

These results suggest that GFSE alleviates skin damage and inflammatory responses caused by increased protein level of COX-2 and iNOS due to UVB irradiation.

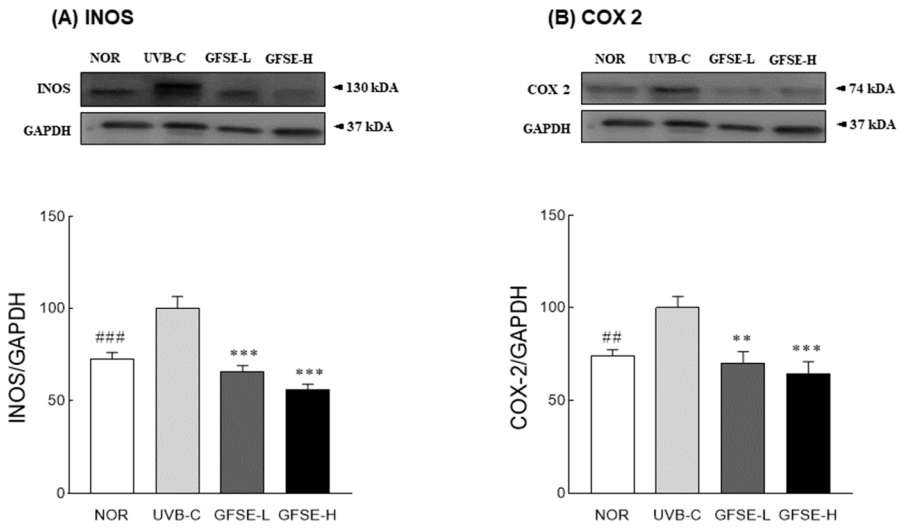


Figure 3-10. Effects of GFSE intake on protein expression of (A) INOS and (B) COX 2 levels in UVB-irradiated hairless mice. Data are expressed means ± standard error of the mean (SEM) (n=5). Different symbols indicated significant at $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NOR, and $^{**}p < 0.01$, $^{***}p < 0.001$ vs. UVB-C according to Tukey's test. NOR, normal control group; UVB-C, UVB-control group; GFSE-L, low dose of GFSE administered group (250 mg/kg); GFSE-H, high dose of GFSE administered group (500 mg/kg).

4. Discussion

Nutricosmetics, which has recently attracted attention for promoting skin health and preventing aging, is emerging as a new alternative based on traditional anti-aging approaches. Nutricosmetics is a novel concept that merges beauty and nutrition by harnessing the unique properties of natural ingredients (H. Kim, Park, & Chung, 2021). Various phytochemicals present in natural products have been used as raw materials for various functional materials for a long time. Phytochemicals help improve skin aging by strengthening the antioxidant defense system and effectively suppressing oxidative stress (Chaudhary et al., 2023). Among them, isoflavones are flavonoids found in natural plants and have a structure similar to the estrogen hormone naturally produced in the body. Genistein, glycitein, and daidzein are three important isoflavones found in high concentrations in legumes that are known to support many body biological metabolic activities and potential body health (Lee et al., 2006).

The chemical composition of soybean (Glycine max L.) is known to change during germination. Recent studies have shown that sprouted soybeans increases active ingredients, including phytoestrogenic compounds such as isoflavones, saponins, and lecithin (E. Y. Kim et al., 2015). Additionally, through germination, soy isoflavones are converted from glycosides to aglycones. Biological conversion of soybeans into aglycone increases biological activity, improves biosorption and antioxidant capacity, and increases the content of essential amino acids and vitamins, thereby improving nutritional value (Yoshiara et al., 2018). This study performed several analyzes to investigate the potential of GFSE, with its antioxidant properties and anti-inflammatory to prevent UVB-induced photoaging in hairless mouse.

The body's skin is an essential protective barrier that maintains body temperature, prevents loss of electrolytes and water, and protects the body from harmful substances. prevents loss

of electrolytes and moisture, and protects the body from harmful substances. (S. H. Shin, Lee, Rho, & Park, 2023). The skin is damaged by various internal and external factors, leading to rapid skin aging. In particular, ultraviolet rays are the main external cause of photoaging, causing pigmentation, loss of elasticity, and deep and long wrinkles (Miri et al., 2016). In fact, when the back skin of hairless mouse was irradiated with UVB for 8 weeks, water-holding capacity decreased and TEWL and erythema index increased (Fig. 3-1). Additionally, an increase in skin thickness was observed in UVB-irradiated mouse through an increase in epidermal thickness (Fig. 3-1 and 3-3). UV rays promote excessive ROS production, increasing oxidative stress in the body. Accumulation of ROS disrupts ECM homeostasis by accelerating the degradation and structure of ECM, such as elastin and collagen Accumulation of ROS activates various pathways that disrupt ECM homeostasis, accelerating the degradation and structural of ECM such as elastin and collagen (Fisher et al., 2002). Eventually, the structure and elasticity of the skin deteriorates, causing deep wrinkles, dryness, and loss of dead skin cells. Topical application of a cream containing vitamin C in healthy women has been shown to stimulate collagen synthesis and remove ROS from the skin, contributing to the reduction of skin wrinkling caused by oxidative stress (Humbert et al., 2003). In healthy adults, taking carotenoid supplements consisting of beta-carotene and lycopene reduced levels of erythema caused by UV exposure (Heinrich et al., 2003). Similarly, GFSE administration effectively improved changes in skin parameters caused by UVB irradiation and alleviated photoaging symptoms by suppressing the formation of wrinkles (Fig. 3-1 and 3-2).

HA is an important and essential natural lubricant for the ECM that is synthesized in the body by hyaluronic acid synthase (HAS). HA combines with water to keep skin elastic and moist, helping to inhibit wrinkle formation and improve skin health (Marinho, Nunes, & Reis, 2021). In addition, the skin barrier, which plays an essential role in maintaining body

moisture through selective penetration of substances and regulation of moisture and electrolytes, is closely related to the formation and function of keratinocytes (Chieosilapatham et al., 2021). UVB irradiation reduces HA content level by promoting the expression of enzymes that decompose skin HA, and suppresses the expression of skin barrier-related factors (FIG, INV and AQP3). However, GFSE administration significantly improved the impaired skin barrier function and reduced HA content caused by UVB irradiation (Fig. 3-4 and 3-7). *Aloe vera* Flower Extract restored proteins (HAS 1, AQP3, FLG, and INV) related to HaCaT cell hydration and barrier function that were reduced by UVB exposure. In particular, not only did the expression of HAS 1 increase, but the expression of HYAL 1 decreased. Atopic disease is the important cause of skin barrier failure by deforming the stratum corneum, causing damage to keratinocytes, causing them to decompose from the skin and easily dry out (Agrawal & Woodfolk, 2014).

Continuous UV irradiation on the skin induces and promotes free radical generation mechanisms and activates the MAPK pathway. Activation of the MAPK pathway promotes the expression of MMPs, causing structural disruption of the ECM and promoting the secretion of pro-inflammatory cytokines, leading to increased skin inflammation (Pittayapruek et al., 2016). Here, GFSE effectively protected against ECM degradation by inhibiting the activation of MMPs through downregulation of MAPK signaling (Fig. 3-6 and 3-9). Similarly, capsaicin from *Capsicum annuum* alleviates collagen loss in UVB-irradiated rat skin. Capsaicin inhibited MMPs-induced collagen degradation and restored ECM levels (Wu et al., 2022). In addition, gallocarbon and ellagic acids in oak reduce ERK phosphorylation and attenuate the associated protein levels of c-Jun and c-Fos in UVB-irradiated HaCaT. These compounds act as effective MMP-1 inhibitors (Hong et al., 2022).

Proinflammatory cytokines affect the proliferation and differentiation of keratinocytes that produce keratin. Chronic UVB exposure causes uncontrolled activation of inflammatory

cytokines, resulting in skin barrier dysfunction (Choi et al., 2011). In this study, UVB irradiation for 8 weeks promoted the response of inflammatory cytokines and induced oxidative stress through activation of MAPK signaling. On the other hand, GSFE administration significantly improved skin inflammation caused by UVB irradiation. Choi et al., (2011) reported that topical application of zinnia extract to mouse induced with atopic dermatitis significantly inhibited the sustained production of pro-inflammatory cytokines (IL-4, IL-5, and IL-13). Similarly, administration of genistein-rich Douchi (fermented *Glycine max* Merr.) significantly suppressed inflammatory factors such as protein kinase C and IL-4 cytokines in mouse with induced atopic dermatitis (Jung et al., 2016). Additionally, gardenia, which is rich in the carotenoid pigment crocin, protects against cellular damage caused by chronic UV exposure by reducing the expression of IL-1β and TNF-α inflammatory cytokines in HaCat cells (Park, Seok, Suh, & Boo, 2014).

The body defends against oxidative damage through enzymatic and non-enzymatic mechanisms. In the enzyme defense mechanism, important and essential antioxidant enzymes such as CAT, SOD 1, and GPX protect the body by directly eliminating free radicals in the body. Additionally, glutathione, vitamin C, and vitamin E remove toxicity by reacting with free radicals that are not affected by antioxidant enzymes (Rinnerthaler et al., 2015). Here, GFSE effectively inhibited UVB irradiation-induced oxidative stress through upregulation of antioxidant enzyme expression (Fig. 3-6). Red raspberries contain phytochemicals such as phenolic acids, tannins, lignans, and traterpenes, which have been shown to eliminate oxidative stress in mouse skin caused by UVB irradiation and to upregulate CAT and SOD 1 expression, preventing oxidative skin cell death (P.-W. Wang et al., 2019). Additionally, administration of anthocyanin-rich extract of *Vaccinium uliginosum* increases the expression of representative antioxidant related enzymes in the body, such as CAT, SOD 1, and GPX, in hairless mice, inhibiting the accumulation of ROS and effectively

suppressing oxidative damage caused by UVB (Jo, Bae, Cho, Park, Suh, & Hong, 2020). These results suggest that natural phytochemicals improve oxidative stress in the skin accumulated by UVB through antioxidant ability. Wang et al. (2013) reported that baicalin, a major component of *Scutellaria baicalensis,* alleviates UV-induced cytotoxicity, cell death, ROS generation, and DNA adduct formation in HaCaT cells. Moreover, malvidin and cyanidin, phenolic compounds isolated from the fruit of *Euterpe oleracea*, effectively suppressed with UVA-induced ROS production in BALB/3T3 cells. These compounds showed cytoprotective effects by restoring GSH levels and lipid peroxidation (Petruk et al., 2017). These results demonstrate the protective effect of phytochemicals on keratinocytes from damage caused by UV irradiation.

5. Conclusion

Based on various phytochemicals that provide abundant antioxidant capacity and health possibilities, such as isoflavone in GFSE, this study found that increased TEWL, skin thickness, and skin erythema value were reduced due to UVB irradiation, and decreased skin hydration was increased due to UVB irradiation. Mean wrinkle depth, mean wrinkle length, total wrinkle area, and number of wrinkles which directly indicate the condition of skin wrinkles, have been significantly improved. In addition, the epidermal thickness, which had been thickened by UVB irradiation, was thinned to a normal level, and the HA content reduced by UVB also increased. GFSE administration reduces MMPs expression, which increases in the body due to UVB irradiation, and helps collagen synthesis by increasing the expression of COL1α1, which synthesizes collagen, a skin component. It also increases TIMPs expression. It also increases the body's expression of important antioxidant-related factors such as CAT, GPX, and SOD 1, thereby reducing oxidative stress increased by UVB irradiation. UVB irradiation increases pro-inflammatory cytokines (COX 2, IL-1β, and TNF-α) in the body, causing inflammation in the body, and GFSE administration reduces this. Finally, continuous UVB irradiation triggered the activation of the NF-κB and MAPK signaling pathways in the skin, key regulators of cellular responses to stress and inflammation, stimulated ERK, JNK, and p38 phosphorylation and increased COX 2 and INOS protein expression. However, it was shown to inhibit phosphorylation reactions and protein expression promoted by GFSE administration, and to protect the skin from inflammatory mechanisms promoted by UV.

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국문 초록

광노화 유발된 피부에 대한 발아발효콩 추출물의 개선 효과

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요약

자외선(UV) 방사선은 피부 노화의 주요 원인으로, 눈에 보이는 조기 노화 징후의 약 90%를 차지한다. 피부 내 활성산소종 (ROS) 축적이 유도되면 산화 스트레스와 세포 손상이 발생하여 노화가 촉진된다. 콩에 존재하는 식물화학적 성분인 이소플라본은 항종양, 항비만, 특히 노화 방지를 특성으로 포함한 생리학적 효과를 발휘하는 것으로 알려져 있다. 그러나 현재 피부 노화 기능을 개선하는 식물성 화학물질의 능력을 포함하여 식물성 에스트로겐에 대한 연구는 제한적이다. 따라서 본 연구의 목적은 UVB 조사에 의해 광노화를 유발한 무모 생쥐에서 발아 및 발효 콩 추출물(GFSE)의 개선효과를 평가하는 것이다.

무모 생쥐에 8주 동안 UVB를 조사하여 광노화를 유도하였고, GFSE를 매일 경구 투여하였다 (250 및 500 mg/kg). 피부 상태에 대한 영향을 간접적으로 평가하기 위해 피부를 수집하고 매개변수와 주름 형성 분석 및 조직병리학적 분석을 수행했다. 또한, 분자생물학적 접근을 통한 피부 hyaluronic acid (HA) 함량, mRNA 및 단백질 발현을 통해 GFSE의 광보호 효과를 평가하였다. GFSE는 UVB로 인한 피부 매개변수와 주름 형성을 크게 개선하고 증가된 표피 두께를 감소시켰다. 또한, UVB에

의해 감소되는 HA 함량이 증가하였다. GFSE는 UVB에 의해 증가된 metalloproteinases (MMPs)의 발현을 감소시켰고, UVB에 의해 감소된 tissue inhibitor of metalloproteinases (TIMPs)와 collagen type 1α1 (COL 1α1)의 발현을 증가시켰다. 또한 항산화 관련 인자인 superoxide dismutase 1 (SOD1), glutathione peroxidase (GPX)를 증가시켰으며, 피부장벽 및 수분 관련 인자 involucrin (INV), filaggrin (FLG), aquaporin 3 (AQP 3)를 증가시켰다. GFSE 투여는 광노화로 인해 생성되는 염증성 사이토카인 interleukin-1β (IL-1β), tumor necrosis factor-α (TNF- α), cyclooxygenase 2 (COX 2)를 감소시켰다. 더욱이, GFSE은 extracellular signaling regulated kinase (ERK), Jun N-terminal kinase (JNK) 및 p38 인산화 단백질 수준뿐만 아니라 inducible nitric oxide synthase (INOS) 및 cyclooxygenase 2 (COX 2) 단백질 수준도 감소시켰다.

종합적으로 GFSE는 UVB 조사에 노출된 털이 없는 쥐의 피부 광노화를 크게 개선하고 피부 손상 정도를 감소시켰다. 따라서 GFSE가 피부 광노화 완화를 위한 중요한 기능성 식품 소재가 될 수 있음을 시사한다.