Inhibitory effect of membrane-free stem cell components derived from adipose tissues on skin inflammation in keratinocytes

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Abstract. Inflammatory disorders of the skin are major public health concerns due to constant exposure to external stimuli. Skin cells are associated with prominent immune mechanisms to defend against adverse reactions. In the present study, the anti-inflammatory properties of membrane-free stem cell components (MFSCC) from adipose tissue-derived stem cells (ADSCs) and their basic preventive effects on skin wrinkle formation using human keratinocytes (HaCaT) and fibroblast (Detroit 551) cells, were investigated. Initially, a human inflammation antibody array was used on tumor necrosis factor-α (TNF-α)/interferon-γ (IFN-γ)-induced and MFSCC-treated HaCaT cells. Array spots revealed three differential proteins, interleukin (IL)-1 F1 (IL-1α), IL-6, and TIMP2. Of these three proteins, IL-6 was significantly downregulated by MFSCC treatment. Western blot analysis revealed that IL-6 and its key downstream proteins JAK2 and STAT3 were suppressed in MFSCC-treated HaCaT cells. Further analysis revealed that MFSCC decreased the expression of TNF-α/IFN-γ-induced phosphorylated (p)-IκB-α, p-p65, p-JNK, p-ERK, and p-p38 by inhibiting the activation of MAPK and NF-κB pathways. Treatment of Detroit 551 cells with MFSCC increased COL1A1 and elastin but suppressed matrix metalloproteinase (MMP)-1 and MMP-8 protein expression levels. Collectively, these data indicated that MFSCC exhibited a primary inhibitory effect on inflammation and wrinkle formation in skin. These results provide a basis for further extensive studies and application of MFSCC in treating skin inflammatory disorders.

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Introduction

The skin is the first line of defense against infection. Skin is a mechanical barrier between the body and the surrounding environment (1). The skin protects an organism from external hazards, toxins, and pathogen infections by forming a barrier between the host and the environment (2). However, frequent, prolonged, and permanent contact with exogenous stimuli will eventually activate immune responses. Hence, it is not surprising that chronic immune-mediated skin diseases are among the most common disorders of humans (3,4).

Keratinocytes are the major cell type of the epidermis, the outermost layer of the skin that is involved in the pathogenesis of inflammatory skin diseases. Keratinocytes are involved in epidermal inflammatory responses upon exposure to infectious agents and infiltration of immune cells (5). Inflammatory responses in keratinocytes are initiated and maintained by pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (6). Stimulation of keratinocytes eventually leads to the production of cytokines, chemokines, and adhesion molecules (7).

Interleukin (IL)-6 is a cytokine with a wide range of effects on the immune system and the host. It is actively researched as a promising target for clinical investigation (8). IL-6 has been demonstrated to activate Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) in signaling cascades that control inflammation (9). Nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) are two major signaling pathways in the pathogenesis of inflammation. NF-κB and MAPK activation mediates the response to critical pro-inflammatory cytokines (such as TNF-α, IL-1β, IL-6, and IL-8) and chemokines (10).

NF-κB is a transcription factor that plays an important role in the inflammatory mechanism by regulating genes, particularly inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), cytokines, and growth factors (11).

The activation of NF-κB occurs through association with an endogenous inhibitor protein of the IκB (inhibitor of NF-κB) family (12). Once NF-κB is phosphorylated, it translocate to the nucleus and binds to inflammation-related genes, causing the production of pro-inflammatory mediators (13).

Mitogen-activated protein (MAP) kinases are a family of serine/threonine kinase proteins regulated by phosphorylated cascades of three kinases that serially phosphorylate one another: p38 isoforms (p38s), c-Jun NH2-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs) (14). The activation of MAPKs through any of the downstream signaling cascades (p38, JNK, or ERK) mediates the regulation of the inflammatory mechanism (15). Thus, anti-inflammatory drug modulators with phosphorylation activity on any of these cascades can be an attractive strategy to inhibit chronic inflammation (16).

The cosmetic and pharmaceutical industries have been widely screening for products with favorable properties such as anti-inflammatory, antiaging, and anti-melanogenic effects (17). Adipose tissue-derived stem cells (ADSCs) have shown potency in dermatological treatment through topical application due to their improved ability to treat inflammation (18). Recent research has introduced improved and fascinating approaches to modify ADSCs or component derivatives to be useful in treatment options (19). Membrane-free stem cell components (MFSCC) from ADSCs are considered as an alternative source of therapeutics because they have better regenerative effects than stem cells. In a previous study, our research group reported the effects of MFSCC on lipopolysaccharide (LPS)-stimulated Raw 264.7 cells through its preliminary anti-inflammatory action (20). Another study reported that MFSCC could regulate the NF-κB/MAPK pathway in IL-1α-induced rat primary chondrocytes (21). In the present study, for the first time, to the best of our knowledge, the inhibitory effect of MFSCC on skin keratinocytes and fibroblast cells, was reported. The anti-inflammatory mechanism of MFSCC through various inflammatory pathways in human keratinocytes was also investigated using HaCaT cells.

Materials and methods

Cell culture and reagents. HaCaT human keratinocytes cells and Detroit 551 human fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM) containing 10% fetal bovine serum (FBS; all Gibco; Thermo Fisher Scientific, Inc.), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (both Thermo Fisher Scientific, Inc.). HaCaT human keratinocytes cells were provided by Professor Hae Young Chung, Department of Pharmacy, Longevity Science and Technology Institutes, Research Institute for Drug Development, Pusan National University, Korea (cat. no. 300493; CLS GmbH). Detroit 551 human fibroblast cells were purchased from Korean Cell Line Bank (cat. no. 10110; KCLB). The cells were incubated at 37˚C and 5% CO_2. Recombinant human TNF-α and IFN-γ were obtained from Enzynomics, Inc. and R&D systems, Inc. TNF-α and IFN-γ were re-suspended in PBS (0 ng/ml). Antibodies IL-6 (product no. 12153S), JAK2 (product no. 3230S), STAT3 (product no. 4904S), phosphorylated (p)-p65 (product no. 3033S), p65 (product no. 8242S), p-IκB-α (product no. 2859S), IκB-α (product no. 4812S), p-JNK (product no. 4671S), JNK (product no. 9258S), p-p38 (product no. 9216S), p38 (product no. 8690S), p-ERK1/2 (product no. 4370S), ERK1/2 (product no. 4695S), collagen type I α1 chain (COL1A1) (product no. 39952S), and matrix metalloproteinase (MMP)-1 (product no. 54376S) were purchased from Cell Signaling Technology, Inc. Antibodies p-JAK2 (product code ab195055), p-STAT3 (product code ab30647), elastin (product code ab217356), and MMP-8 (product code ab154507) were obtained from Abcam. Horseradish peroxidase (HRP)-conjugated secondary antibodies to anti-rabbit (cat. no. A120-101P) and anti-mouse (cat. no. A90-116P) were obtained from Bethyl Laboratories, Inc.

Preparation of membrane-free stem cell components (MFSCC). MFSCC used in this study were prepared using patented technology by T-Stem Co., Ltd. The adipose tissues used for the preparation of MFSCC were obtained from Tiara Clinic (Changwon, Korea) upon agreement from the respective donors. The donors provided written informed consent and the Regional Ethics Committee on Biomedical Research approved the clinical protocol. The fat tissues were provided by females in their twenties with a BMI of 25 to 29.9 (Overweight) considered appropriate based on blood tests and physician diagnosis. The blood was tested for viruses including hepatitis B virus (HVB), hepatitis C virus (HCV), human immunodeficiency virus (HIV), human T-cell lymphocytic virus (HTVL), parvovirus B19, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and *Treponema pallidum*. The cells were cultured at 37˚C and 5% CO_2, in a standard incubator using serum-free cell culture medium. After the cell growth reached 70-80% confluence, the cells were sub-cultured until 6 to 8 passages. ADSCs were characterized using specific markers (positive markers, CD105 and CD29; negative marker, CD34) by an immunofluorescence assay (data not shown). A certain amount of stem cells ($1x10^6$ cells/ml) was collected; the cell membranes were removed by ultra-sonication, and the debris of the membranes was eliminated by centrifugation at 800-1,500 x g (Room temperature, 3 min.), following successive filtration. The final product of MFSCC was obtained upon separating the stem cell membranes, and performing nine non-toxic-based safety tests conducted by accreditation authority certified under Good Laboratory Practice (GLPs). The overall preparation of MFSCC in the present study was based on the protocol by Venkatarame Gowda Saralamma *et al* (20).

Cell viability assay. Cell viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). HaCaT cells were seeded at a density of $5x10^4$ cells/well and Detroit 551 cells were seeded at a density of $3x10^4$ cells/well in 48-well plates. After treatment with the indicated concentrations of MFSCC (0, 1, 5, 10, 15, 20, and 25 μg/ml) with or without TNF-α/INF-λ (10 ng/ml), cells were incubated for 24 h. MTT solution was added to each well, and the cells were incubated for 2 h at 37˚C. The formazan was dissolved in DMSO, and then the absorbance was measured at 540 nm by microplate reader (BioTek Instruments, Inc.).

Human inflammation antibody array. Antibody array was analyzed using RayBio Human Inflammation Antibody Array C1 kit (cat. no. AAH-INF-1-2). HaCaT cells were seeded at a density of $4x10^5$ cells/well in a 100-mm plate, and treated with

TNF-α/IFN-γ (10 ng/ml) with or without MFSCC (10 μg/ml) for 24 h. The cell lysates were then collected and processed according to manufacturer's instructions. Arrays membranes were directly detected using a chemiluminescence detection system (Bio-Rad Laboratories, Inc.) to obtain production levels of the following cytokines/proteins: Eotaxin-1 (CCL11), Eotaxin-2 (MPIF-2/CCL24), GCSF, GM-CSF, IFN-γ, IL-1α (IL-1 F1), IL-1β (IL-1 F2), IL-2, IL-3, IL-4, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-11, IL-12 p40, IL-12 p70, IL-13, I-309 (TCA-3/CCL1), and TIMP metallopeptidase inhibitor 2 (TIMP2). Data were analyzed with the Image Studio Lite software (version 5.2; LI-COR Biosciences). Data are expressed as the relative signal intensity (RSI) between the MFSCC co-treated test group and the only TNF-α/IFN-γ-treated control group [RSI=(test group/control group)].

Western blot analysis. HaCaT cells were treated with the indicated concentration of MFSCC with TNF-α/IFN-γ (10 ng/ml) for 24 h. Detroit 551 cells were treated with the indicated concentration of MFSCC for 24 h. Then the incubated cells were lysed using RIPA buffer (iNtRON Biotechnology, Inc.) containing a protease inhibitor cocktail and a phosphatase inhibitor (Thermo Fisher Scientific, Inc.). The protein quantification of each cell lysate sample was measured using BCA assay (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Equal amounts of protein (10 μg) were separated on 8-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene fluoride (PVDF) membrane (Immunobilon-P, 0.45 mm; EMD Millipore), using the semi-dry transfer system (Atto Corp). The membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline containing 1% Tween-20 (TBS-T, pH 7.4) at room temperature for 1 h, followed by incubation overnight at 4˚C with a 1:1,000 dilution of the respective primary antibody. The membranes were washed five times with TBS-T for 10 min each at room temperature, and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at room temperature. The membranes were then rewashed 5 times using TBS-T, detected by chemiluminescence detection system and analyzed using Image Lab 4.1 program (both Bio-Rad Laboratories, Inc.). The densitometry analysis using ImageJ software (version 1.50i) (National Institutes of Health) of each of the protein bands was normalized by comparing with the expression of β-actin.

Protein-protein interactions using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis. The protein-protein interaction network was constructed using STRING (https://string-db.org/). The STRING database provides the interactions of different proteins based on a confidence level score. The interactive network with the most connected neighborhood proteins of IL-6 were identified. The confidence score for the interactive network was set up with a medium score of 0.4 to 0.9 respectively.

Statistical analysis. All experimental results are expressed as the mean ± standard deviation (SD) of at least triplicate samples using GraphPad Prism software (version 8.02; GraphPad Software, Inc., San Diego,). Significant differences were calculated by one-way factorial analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A value of $P<0.05$ was considered statistically significant.

Results

Cytotoxicity of MFSCC to human keratinocytes. To evaluate the cytotoxic effect of MFSCC, human keratinocyte HaCaT cells were treated with MFSCC at indicated concentrations (0, 1, 5, 10, 15, 20 and 25 μg/ml) for 24 h. Cell viability assessment results indicated that MFSCC at concentrations of up to 25 μg/ml did not cause 50% inhibition of HaCaT cells as revealed in Fig. 1A. Co-treatment with TNF-α/IFN-γ did not show significant cytotoxicity to HaCaT cells either (Fig. 1B). Based on these results, concentrations of 1, 5 and 10 μg/ml MFSCC were selected for subsequent experiments.

Detection and identification of proteins in TNF-α/IFN-γ-induced HaCaT cells associated with inflammation using an antibody array. An antibody array was used for HaCaT cells treated with only TNF-α/IFN-γ (10 ng/ml) considered the control, and those co-treated with MFSCC (0, 1, 5, and 10 μg/ml) and TNF-α/IFN-γ (10 ng/ml) in order to identify proteins involved in the anti-inflammatory action of MFSCC. Proteins were extracted from cell lysates and subjected to Human Inflammation array using a kit (RayBio® C-series Human Inflammation Array C1). RayBio® C1 array contains 20 duplicate spots of inflammatory-related proteins. A plot of all protein spots with their expression is depicted graphically based on their signal intensities (Fig. 2A). Among the expressed protein spots, the protein expression levels with clear signal intensities were selected to pivot and the significant proteins related to anti-inflammatory roles were selected as shown in Fig. 2B. These three protein spots were found to be IL-1 F1 (IL-1α), IL-6, and TIMP2. IL-1 F1 (IL-1α) and IL-6 are involved in the activation of the acute phase of inflammatory responses and cytokine-mediated pathways (22). TIMP2 is involved in regulation of MMP proteins (23). Proteins along with their biological functions are displayed in Table I. Among these three proteins, IL-6 was specifically focused on, which exhibited significant downregulation in the MFSCC treatment group compared with that in the TNF-α/IFN-γ-treated group. These results indicated the anti-inflammatory action of MFSCC by inhibiting the inflammation marker IL-6 upon its treatment. Thus, IL-6 was subjected to further analysis.

The STRING database search tool was used to analyze protein-protein interactions and to retrieve genes/proteins interacting with significant protein IL-6. An interactive protein-protein network of IL-6 was constructed, with a score of 0.900 as the highest degree of confidence (Fig. S1). In the interactive network, IL-6 was identified to be closely associated with nodes JAK2 and STAT3. Both nodes, JAK2 and STAT3 were observed to have strong associations with IL-6 through the JAK/STAT signaling pathway in the network with a strength parameter of 1.96. Closely-associated node partners and their functional parameters related to inflammation are presented in Table SI.

Western blot analysis of the expression levels of IL-6, p-JAK2, and p-STAT3 proteins. To validate antibody array results, western blot analysis was conducted using HaCaT cells treated with only TNF-α/IFN-γ (10 ng/ml) or co-treated with MFSCC

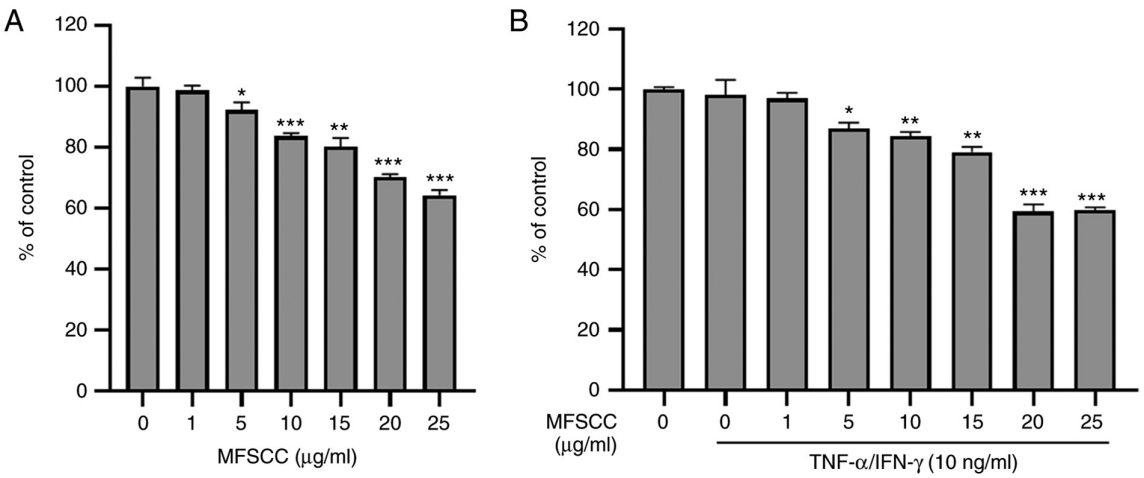

Figure 1. Cytotoxic assessment of MFSCC in HaCaT cells. (A) Effect of MFSCC on cell viability in HaCaT cells treated with indicated concentrations (0, 1, 5, 10, 15, 20 and 25 μg/ml) of MFSCC for 24 h. (B) Effect of MFSCC (0, 1, 5, 10, 15, 20 and 25 μg/ml) on the cell viability of TNF-α/INF-γ-induced (10 ng/ml) HaCaT cells. Data are presented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01 and ***P<0.001 vs. the untreated group. MFSCC, membrane-free stem cell components; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

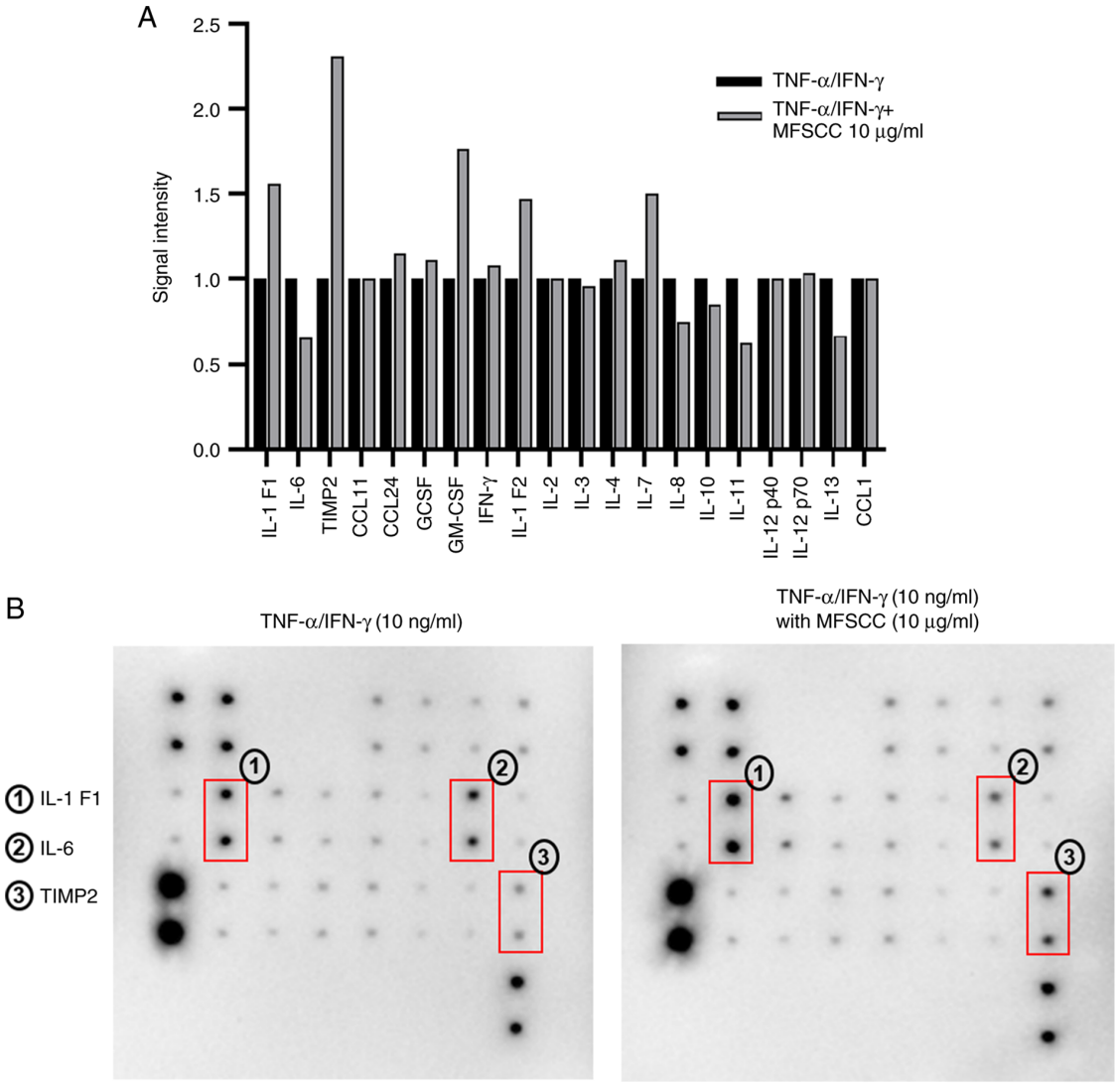

Figure 2. Antibody array analysis of HaCaT cells. (A) The relative expression graph of the protein spots are represented graphically based on the signal intensities between the TNF-α/IFN-γ-treated only group (10 ng/ml) as the control and the TNF-α/IFN-γ (10 ng/ml) co-treated with MFSCC (10 μg/ml) group. (B) Three significantly identified protein spots related to inflammation in TNF-α/IFN-γ-treated cells and TNF-α/IFN-γ (10 ng/ml) co-treated with MFSCC (10 μg/ml) cells. Protein spots: ① IL-1 F1 (IL-1α), ② IL-6, and ③ TIMP2. TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MFSCC, membrane-free stem cell components; IL, interleukin; TIMP2, TIMP metallopeptidase inhibitor 2.

Table I. List of proteins identified in varied expression in HaCaT cells treated only with TNF-α/IFN-γ (10 ng/ml) and co-treated with MFSCC (10 μg/ml) detected by antibody array analysis.

UniProt ID	Symbol	Protein name	Up/Down regulation	Biological function
P01583	IL-1 F1 (IL-1α)	Interleukin-1α	↑	Inflammatory response, cytokine-mediated signaling pathway
P05231	IL-6	Interleukin-6	↓	Acute-phase response, interleukin-6-mediated signaling pathway
P16035	TIMP-2	Metalloproteinase inhibitor 2	↑	Activation of matrix metalloproteinases

The biological functions of the proteins are presented according to UniProt protein database (https://www.uniprot.org/).

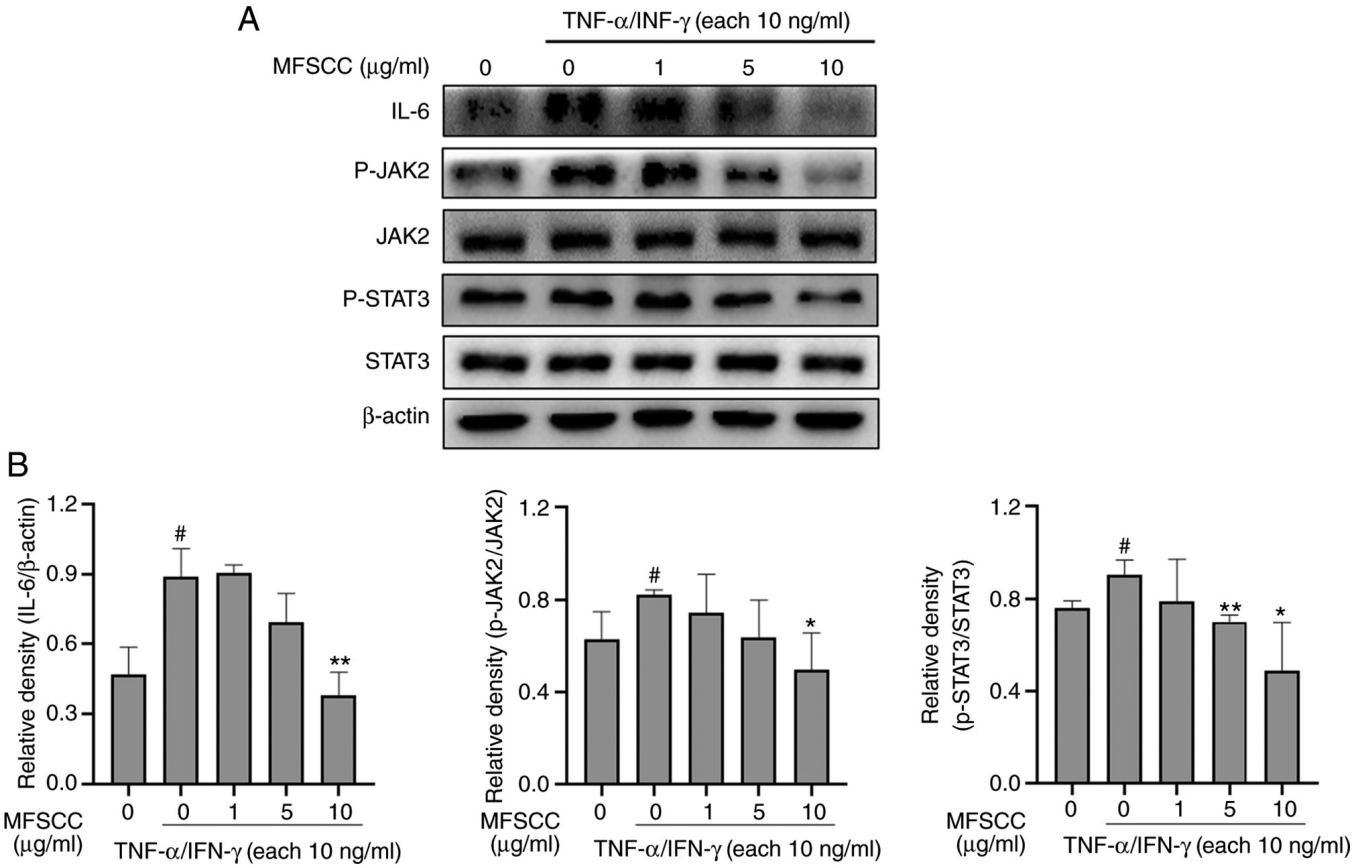

Figure 3. Western blot analysis of the expression of IL-6, p-JAK2, and p-STAT3 proteins in HaCaT cells. (A) Protein levels of IL-6, JAK2, p-JAK2, STAT3, and p-STAT3 in the TNF-α/IFN-γ-treated only group (10 ng/ml) and the co-treated MFSCC (0, 1, 5 and 10 μg/ml) group. (B) The expression of the proteins are shown graphically based on their relative density normalized against β-actin which served as the acting and internal control. #P<0.05 vs. the untreated group; *P<0.05 and **P<0.01 vs. the TNF-α/IFN-γ-treated only group. IL, interleukin; p-, phosphorylated; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MFSCC, membrane-free stem cell components.

(0, 1, 5 and 10 μg/ml) and TNF-α/IFN-γ (10 ng/ml). Blots obtained from the western blotting of protein IL-6 along with phosphorylated forms of JAK2 and STAT3 are shown in Fig. 3. The expression levels of IL-6, p-JAK2, and p-STAT3 proteins were decreased in the MFSCC-treated group compared with the group treated only with TNF-α/IFN-γ. These western blot results were consistent with the antibody array results which demonstrated IL-6 reduction and inhibition of inflammatory responses through JAK2 and STAT3 phosphorylation in HaCaT cells.

Effect of MFSCC on NF-κB signaling in TNF-α/IFN-γ-stimulated HaCaT cells. Inflammatory responses through small regulator molecules involve crucial mechanisms mediated by NF-κB signaling. The activator of signal transcription factors through JAK/STAT signaling also plays a pivotal role in the pathogenies of inflammatory disorders (24). In this aspect, the inhibitory potential of MFSCC on the NF-κB signaling pathway was investigated by assessing protein markers using western blotting. The results revealed that stimulation by TNF-α/IFN-γ (10 ng/ml) increased the phosphorylation levels of IκB-α and

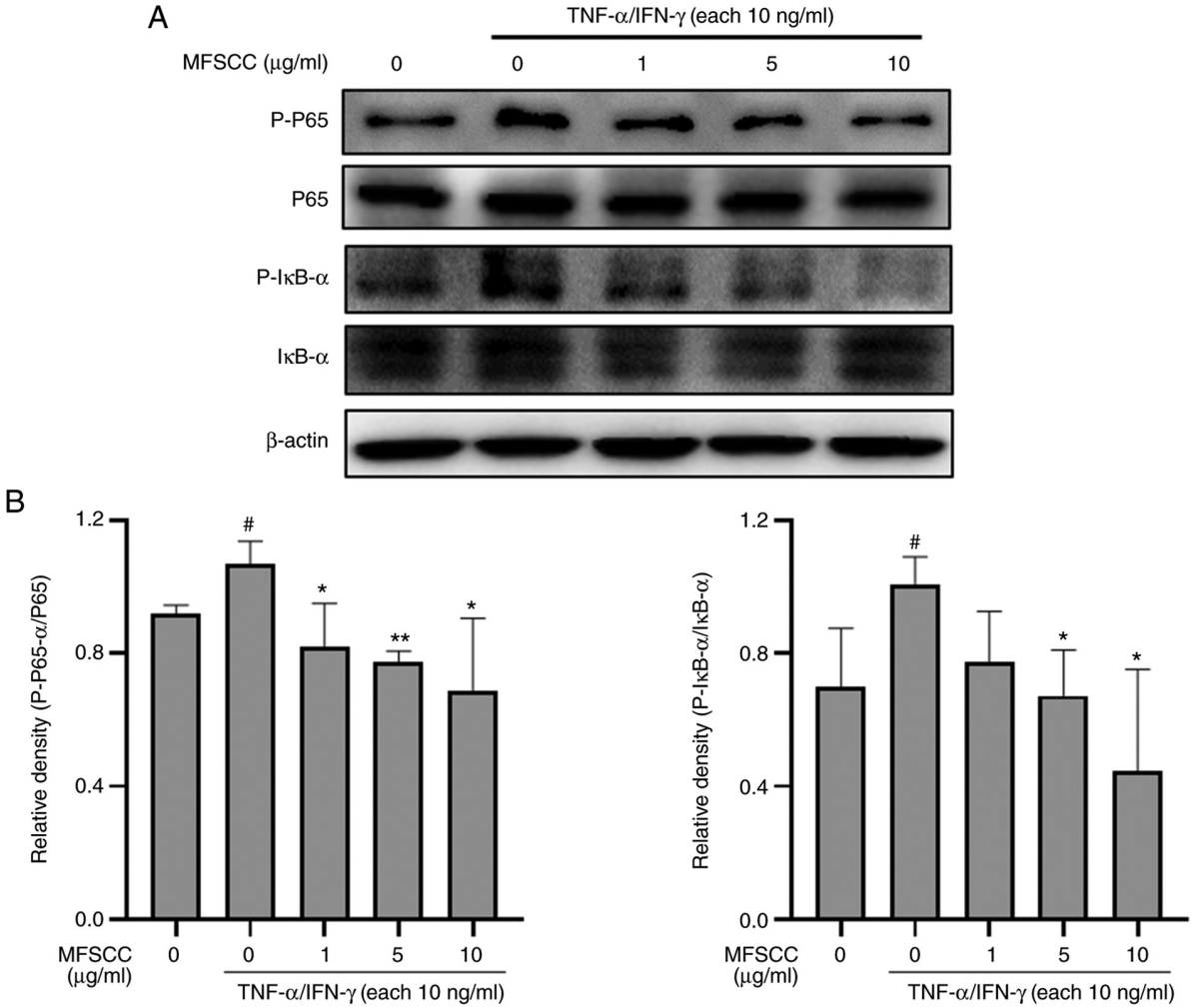

Figure 4. Western blot analysis of the NF-κB signaling pathway in HaCaT cells. (A) Protein levels of p65, p-p65, IκB-α, and p-IκB-α in the TNF-α/IFN-γ-treated only group (10 ng/ml) and the co-treated MFSCC (0, 1, 5, and 10 μg/ml) group. (B) The expression of the proteins are shown graphically based on their relative density normalized against the internal control. $^{\#}$P<0.05 vs. the untreated group; *P<0.05 and **P<0.01 vs. the TNF-α/IFN-γ-treated only group. NF-κB, nuclear factor-κB; p-, phosphorylated; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MFSCC, membrane-free stem cell components.

p65, which were decreased by co-treatment with MFSCC (0, 1, 5 and 10 μg/ml) (Fig. 4). The reduction in the phosphorylation of NF-κB proteins by co-treatment with MFSCC indicated that MFSCC could decrease inflammatory responses by inhibiting the respective signaling pathway in HaCaT cells.

Effect of MFSCC on MAPK phosphorylation in TNF-α/IFN-γ-stimulated HaCaT cells. Fundamental inflammatory responses mediated by any stimuli are highly associated with MAPKs regulation in anti-inflammatory mechanisms (25). To identify the association of the MAPK pathway in HaCaT cells with MFSCC treatment, the phosphorylation levels of JNK, p38, and ERK as downstream proteins were examined. TNF-α/IFN-γ (10 ng/ml) treatment increased the phosphorylation levels of MAPK proteins. However, co-treatment with MFSCC (0, 1, 5 and 10 μg/ml) significantly reduced their phosphorylation levels as shown in Fig. 5. These results indicated that MFSCC could induce anti-inflammatory responses through regulation of the MAPK pathway in HaCaT cells.

Expression of collagen and elastin in MFSCC-treated Detroit 551 cells. The skin can be damaged by inflammatory reactions, resulting in aging symptoms such as wrinkles (26). Thus, wrinkle-related proteins were investigated using MFSCC-treated human fibroblast Detroit 551 cells. Cell viability assay results (Fig. 6A) demonstrated that MFSCC at concentrations of 1, 5 and 10 μg/ml had no cytotoxicity. Therefore, these concentrations were used in subsequent experiments. Wrinkle formation is closely associated to the reduction of collagens and the extracellular matrix (ECM) in dermal skin (27). According to previous studies, the induction of collagen and ECM can lead to potent treatment for anti-wrinkle conditions (24,28). Thus, the expression levels of wrinkle-related proteins, including COL1A1, elastin, MMP-1, and MMP-8 were determined. As revealed in Fig. 6B, MFSCC significantly downregulated the protein levels of MMP-1 and MMP-8 but upregulated the protein levels of COL1A1 and elastin. These findings indicated that MFSCC could induce anti-wrinkle effects in Detroit 551 cells by reducing collagen and ECM degradation.

Discussion

Skin forms the essential interface between the intrinsic body and the extrinsic insults from the environment. It can attenuate

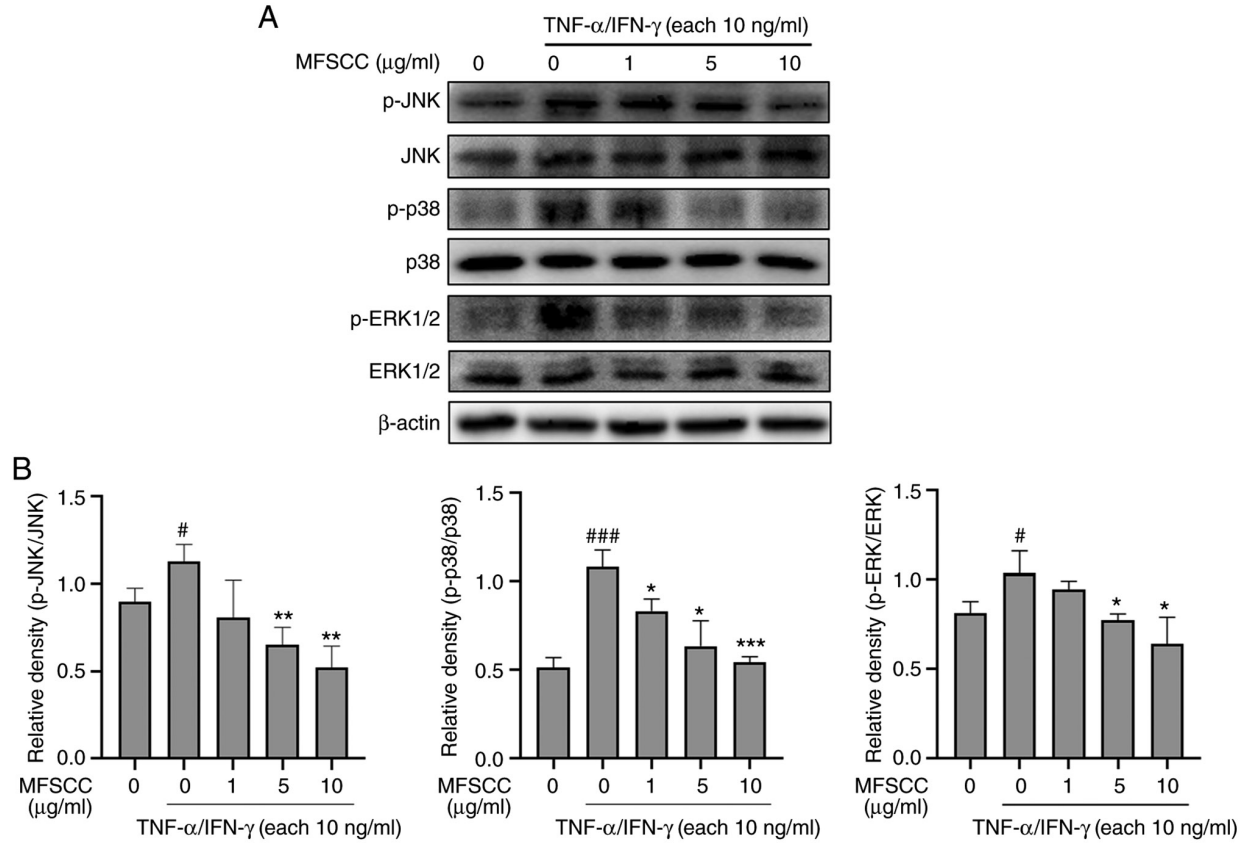

Figure 5. Western blot analysis of the MAPK signaling pathway in HaCaT cells. (A) Protein levels of JNK, p-JNK, p38, p-p38, ERK1/2, and p-ERK1/2 in the TNF-α/IFN-γ-treated group (10 ng/ml) and co-treated MFSCC (0, 1, 5 and 10 μg/ml) group. (B) The expression of the proteins are shown graphically based on their relative density normalized against the internal control. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. the untreated group; *P<0.05, **P<0.01 and ***P<0.001 vs. the TNF-α/IFN-γ-treated only group. MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; p-, phosphorylated; ERK, extracellular signal-regulated kinase; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MFSCC, membrane-free stem cell components.

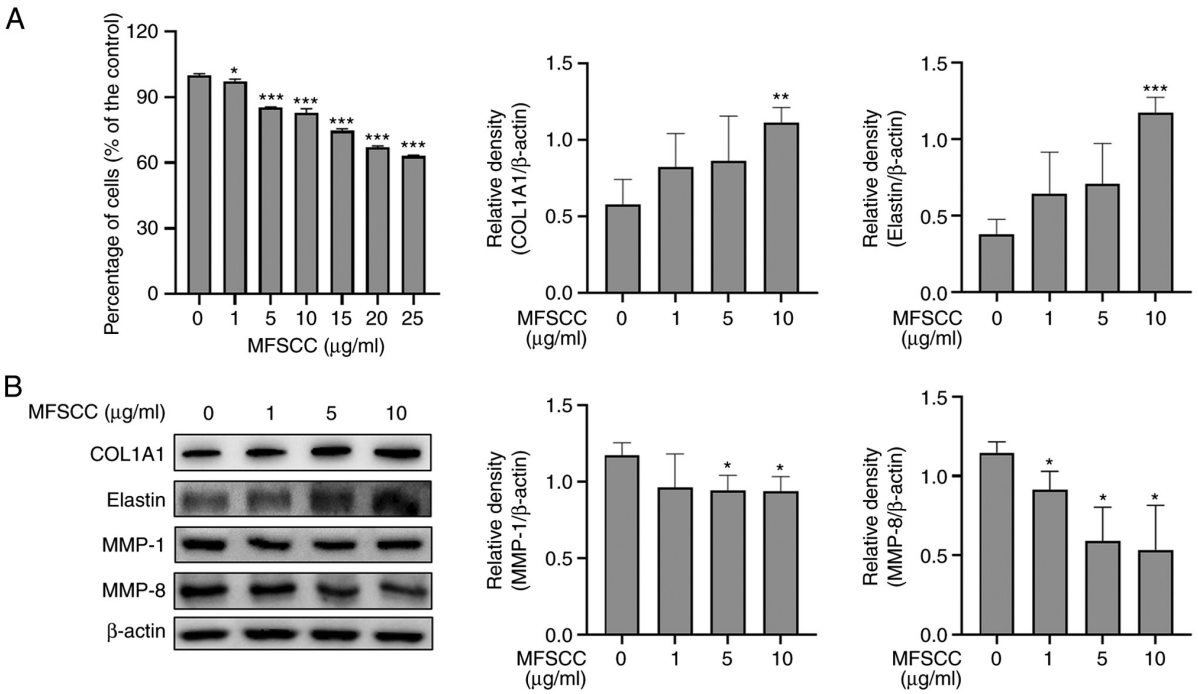

Figure 6. Expression of collagen and elastin in MFSCC-treated Detroit 551 cells. (A) Effect of MFSCC on the cell viability of Detroit 551 cells treated with various concentrations (0, 1, 5, 10, 15, 20 and 25 μg/ml). (B) Western blot analysis of the expression levels of protein markers COL1A1, elastin, MMP-1, and MMP-8 in MFSCC-treated Detroit 551 cells (0, 1, 5 and 10 μg/ml). The protein expression levels are shown graphically based on their relative density normalized against β-actin as the acting control. *P<0.05, **P<0.01 and ***P<0.001 vs. the untreated group. MFSCC, membrane-free stem cell components; COL1A1, collagen type I α1 chain; MMP, matrix metalloproteinase.

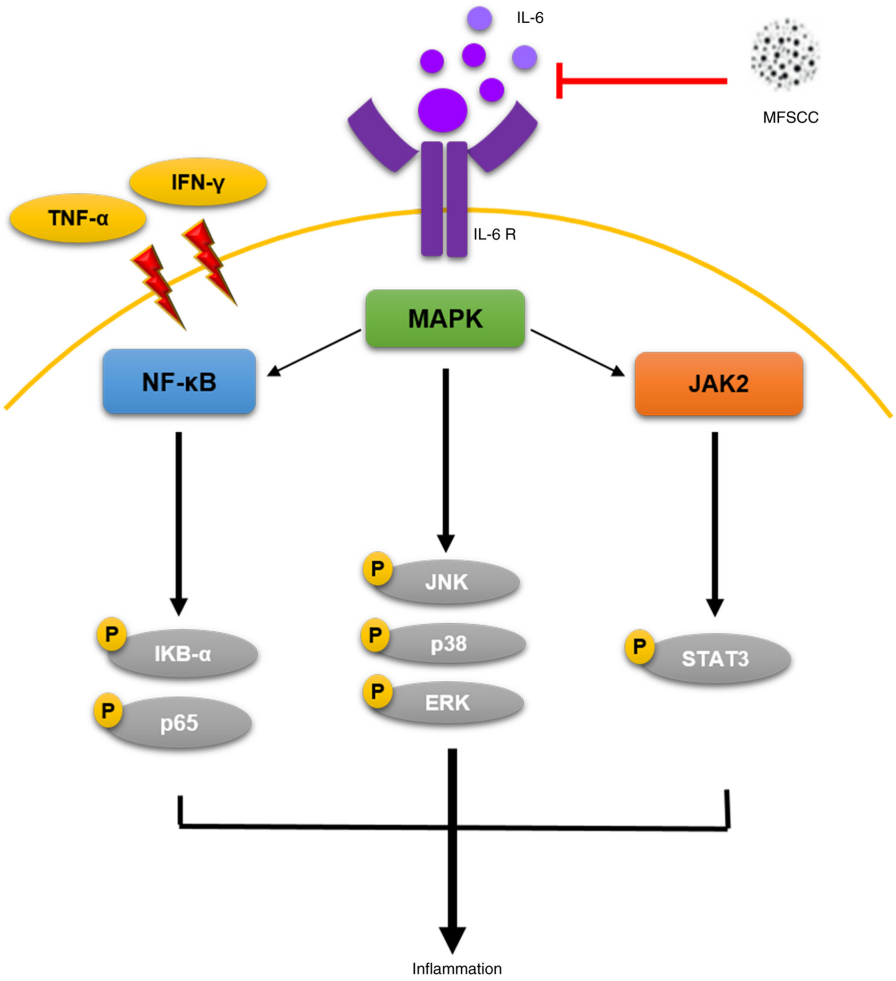

Figure 7. Schematic representation of the mechanism of MFSCC inhibiting the inflammatory responses through JAK2/STAT3, NF-κB, and MAPK signaling pathways in HaCaT cells. MFSCC, membrane-free stem cell components; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3; NF-κB, nuclear factor-κB; MAPK, mitogen-activated protein kinase.

infections caused by microbes and radiation produced by ultraviolet emission by activating innate immune responses (1). The activation of the inflammatory system can aid skin homeostasis and control inflammatory disorders, allergic reactions, and skin cancer (29).

Keratinocytes are highly active immunological cells that execute responses to external stimuli in skin. They can activate a variety of molecules involving different forms of cytokines, pro-inflammatory chemokines, and other proteins (30). Production levels of chemokines and cytokines are elevated upon stimulation of keratinocytes with inflammatory cytokines such as TNF-α and IFN-γ (31). Previous research findings on HaCaT cells have used TNF-α/IFN-γ activation to stimulate various intracellular signaling pathways, including MAPKs, NF-κB, and STAT-1/JAK-2 (32,33). Thus, keratinocyte-mediated inflammatory molecules activated via immune responses are highly associated with the pathology of skin inflammatory disorders (34). In the present study, the anti-inflammatory action of MFSCC was investigated through antibody array-mediated analysis using TNF-α/IFN-γ-stimulated human keratinocytes HaCaT cells.

Regarding the cytotoxic effect of MFSCC, cell viability was moderately inhibited by MFSCC compared with the control group of TNF-α/IFN-γ-stimulated HaCaT cells. Human inflammatory antibody array analysis using TNF-α/IFN-γ-stimulated HaCaT cells identified three differential proteins: IL-1 F1 (IL-1α), IL-6, and TIMP2. All these three identified proteins are known to contribute to the inflammatory response pathway (35). IL-6 was found to be significantly reduced in its expression upon treatment with MFSCC. IL-6 is one of the well-characterized pro-inflammatory cytokines that exerts a vital role in performing multiple functions of the immune system (36). With the observed downregulated expression in the array, it was hypothesized that MFSCC could mediate the anti-inflammatory action through IL-6 (37). The host defense mechanism involves the activation of several intracellular signaling pathways, including JAK2/STAT3, mammalian MAPK, and NF-κB pathways (38,39).

IL-6 is a prominent inflammatory mediator that can activate JAK2 and STAT3 pathways with extracellular signals regulated by the ERK pathway (40). The JAK2/STAT3 signaling pathway is known to effectively contribute to inflammatory responses. Considerable attention has been directed towards its inhibition for the treatment of inflammation (41). The protein-protein interaction network in the present study revealed that JAK2 and STAT3 are closely associated interactors. Protein expression of IL-6, p-JAK2, and p-STAT3 in HaCaT cells was determined to be downregulated by MFSCC treatment. The present data indicated that MFSCC

could significantly inhibit the activation of the IL6-mediated JAK2/STAT3 signaling pathway.

Accumulating evidence has revealed that a variety of signaling networks are involved in the preventive effect against inflammation, such as NF-κB and MAPKs (42). Interestingly, in the present study, MFSCC appeared to control the activation of NF-κB and MAPK signaling pathways. The constitutive activation of NF-κB is driven by major inflammatory factors such as TNF-α, IL-6, IL-1, and IL-8. The most commonly triggered stimulant has been reported to be TNF-α that can lead to complex activation (43). Furthermore, it has been suggested that the interactive nature of STAT3 activation with NF-κB signaling can lead to the induction of inflammation (44).

Inflammatory mediator production is primarily managed by NF-κB signaling that is highly associated with MAPK signaling (45). In terms of MAPK activation, phosphorylation of ERK, p38, and JNK occurs in both the nucleus and cytosolic layer to stimulate the expression of relevant inflammatory factors (46). MAPKs are also involved in the activation of JAK/STAT, a critical signaling transduction pathway for the biological function of many cytokines (47). Our experiments also analyzed the impact of MAPKs on the expression of JNK, p38, and ERK1/2. The results showed that their phosphorylated forms were suppressed in MFSCC-treated HaCaT cells. Divergent active signals from MAPK pathways are prominent mediators of inflammation. They play a vital role in degenerative disorders (48). Conversely, there is evidence that MAPK inhibitors are involved in treating systemic inflammation caused by stimulation with TNF-α (49). Based on our findings, stimulation with TNF-α/IFN-γ induced phosphorylation of p65, IκB-α, ERK, p38, and JNK in HaCaT cells, although MFSCC treatment downregulated the activation of MAPKs mediated by the NF-κB signaling pathway.

Inflammation of the skin is analogous with the degeneration of elastic fibers and loss in elasticity, resulting in wrinkle formation (50). Changes associated with the microenvironment can impact the skin and lead to aging and drying with loss of important ECM components (51). Inflammation in skin mediated by cytokines such as IL-6 has been subsequently linked to the formation of wrinkles, leading to poor integrity of skin structure (50). The inflammatory mechanism triggers responses of rapid collagen degradation by the family of MMPs (52). Collagen degradation in the phase of a wound response mechanism is followed by fibroblast inducing new collagen synthesis to replace lost collagen fibers in the ECM (53). The present results also revealed that the expression levels of pro-collagen and elastin were increased upon MFSCC treatment in fibroblast cells. In addition, decreased expression levels of MMP-1 and MMP-8 were also observed in human fibroblast Detroit 551 cells treated with MFSCC. This indicated that MFSCC could also trigger anti-wrinkle effects by decreasing MMPs via IL-6 alteration. Thus, the use MFSCC on skin inflammation has an added therapeutic significance in that it also has a cosmetic function. In consideration of overall outcomes, MFSCC provides a primary defense against skin inflammation. It also tends to possess an anti-wrinkle effect, indicating that it could be a potential treatment option for inflammatory skin disorders in the future.

In conclusion, the results of the present study suggest that MFSCC can inhibit primary inflammatory response stimulated by TNF-α/IFN-γ through regulation of IL-6 and JAK2/STAT3 expression (Fig. 7). The present study also revealed preliminary inhibitory effects on NF-κB and MAPK signaling pathways in human keratinocyte HaCaT cells. Furthermore, MFSCC treatment could improve collagen and elastin expression, eventually leading to decreased expression of MMP proteins in human fibroblast Detroit 551 cells. Collectively, these findings form an initial study on the anti-inflammatory properties of MFSCC and provide support for future studies about its therapeutic effect on skin inflammation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GSK and YSK conceived the study and designed the experiments. SEH performed the experiments and collected and analyzed data. PV performed the experiments. SMK, PBB and HHK analyzed data. JEP and JDH interpreted the results. GSK, YSK, SEH, PV, SMK, PBB, HHK, JEP and JDH confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

All donors provided written informed consent and the Regional Ethics Committee on Biomedical Research approved the clinical protocol (Ministry of Health and Welfare, Sejong).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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