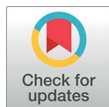


Skin Barrier-Improving and Anti-Inflammatory Effects of Exosomes Derived from the *Fructobacillus fructosus* NSH-1 Strain Isolated from the *Campsis grandiflora* Flower

Byeong-Min Choi[†], Hyehyun Hong[†], Yeon-Bo Jang, Tae-Jin Park, and Seung-Young Kim*

Department of Pharmaceutical Engineering and Biotechnology, Sunmoon University, Chungnam 31460, Republic of Korea



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*Corresponding author
E-mail: sykim01@sunmoon.ac.kr

[†]These authors equally contributed to the study.

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This study investigated the anti-inflammatory and skin barrier-improving activities of *Fructobacillus fructosus* strain NSH-1-derived exosomes in LPS-stimulated RAW 264.7 cells and HaCaT cells. Nanoparticle tracking and transmission electron microscopy analyses confirmed the concentration, purity, and morphology of NSH-1 exosomes. The exosomes showed no cytotoxicity at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml, but demonstrated concentration-dependent inhibitory activity against nitric oxide production. Furthermore, NSH-1 exosomes significantly inhibited the production of pro-inflammatory mediators, including TNF- α , IL-6, IL-1 β , and PGE₂. In HaCaT cells, treatment with NSH-1 exosomes enhanced skin barrier function by increasing the production of hyaluronan and collagen in a concentration-dependent manner and promoting wound-healing activity. Western blot analysis revealed that treatment with NSH-1 exosomes reduced the expression levels of inflammatory proteins iNOS and COX-2, while increasing the expression of skin barrier-related proteins such as filaggrin, involucrin, and loricrin. Overall, these findings indicate that NSH-1 exosomes exert strong anti-inflammatory activity by suppressing the expression of the pro-inflammatory cytokines iNOS and COX-2, while simultaneously enhancing skin barrier integrity by upregulating filaggrin, involucrin, and loricrin. Thus, exosomes derived from *F. fructosus* NSH-1, isolated from *Campsis grandiflora* flowers, demonstrate promise as natural bioactive agents for anti-inflammatory and skin barrier-improving applications.

Keywords: Anti inflammatory effect, *Campsis grandiflora* flower, *Fructobacillus fructosus*, NSH-1 exosomes, Skin-barrier

Introduction

Inflammation is an innate defense mechanism of the human body that protects against harmful external stimuli including pathogenic invasion, physical injury, and chemical irritation, and repairs damaged tissues [1]. However, chronic or excessive rather than transient activation of this immune response may exacerbate inflammation, leading to cellular damage, impaired tissue function, and even DNA injury and mutations [2, 3]. Thus, inflammation plays a crucial role in the development of chronic diseases, such as atopic dermatitis, cardiovascular disorders, metabolic syndromes, and various types of cancer [4, 5]. At the cellular level, inflammatory responses are initiated by macrophage activation. Among microbial components, lipopolysaccharide (LPS), a structural constituent of the outer membrane of gram-negative bacteria, is a potent proinflammatory agent. LPS binds to toll-like receptor 4 and activates downstream signaling cascades, including the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF- κ B) pathways [6-8]. Stimulation of these pathways enhances the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which in turn promote the production of nitric oxide (NO) and prostaglandin E₂ (PGE₂) [9]. Elevated NO and PGE₂ levels sustain the inflammatory response as well as induce the secretion of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and

interleukin-1 β (IL-1 β), further amplifying the inflammatory process [10]. These cytokines are also reported to stimulate angiogenesis, thereby contributing to cancer cell proliferation and tumor formation [11, 12]. Therefore, the development of safe and effective anti-inflammatory agents capable of suppressing or modulating the expression of these early inflammatory mediators is of great importance. In recent years, natural plant-derived resources have attracted increasing attention as promising anti-inflammatory agents due to their low toxicity compared with that of synthetic compounds and their long history of use as food and medicine, which ensures their safety. These plant-based bioactive materials provide psychological comfort to consumers as well as possess the potential to regulate inflammatory mediator expression in the human body.

More than 90% of the epidermal layer of the skin is composed of keratinocytes, which play a central role in skin regeneration and wound healing [13, 14]. When the skin is damaged by chemical or physical factors, various growth factors and inflammatory cytokines, such as epidermal growth factor, keratinocyte growth factor, transforming growth factor- β , and interleukin-1, are secreted at the wound site [15, 16]. These signaling molecules stimulate the keratinocyte migration and proliferation, thereby promoting the re-epithelialization of damaged tissue. Further, activated keratinocytes enhance the synthesis of key extracellular matrix proteins, such as collagen, contributing to improved skin elasticity, wrinkle reduction, wound repair, and skin barrier reinforcement [17].

Extracellular vesicles (EVs) are nanosized membrane-bound particles secreted by cells into the extracellular space that retain a lipid bilayer structure derived from the plasma membrane or intracellular organelles [18]. They encapsulate nucleic acids, proteins, lipids, and metabolites originating from their parental cells. The size of EVs generally ranges from approximately 40 to 1,000 nm, and their composition and function vary depending on the physiological state and microenvironment of the secreting cells. Owing to their unique biological characteristics, EVs are recognized as key mediators of intercellular communication [19, 20]. They have been reported to exhibit several advantages over synthetic nanocarriers, such as liposomes, making them highly valuable for diverse biomedical applications, including drug delivery, vaccine development, disease diagnostics, and regenerative medicine [21]. EVs are classified into several subtypes, including exosomes and microvesicles, based on their biogenesis, size, and specific protein markers [22]. Among these, exosomes have been the most extensively studied because of their structural stability and high delivery efficiency. Exosomes contain various biomolecules, including miRNAs, mRNAs, proteins, and lipids, and participate in numerous physiological processes, such as cell proliferation, differentiation, immune modulation, and inflammation regulation, through selective delivery to recipient cells [23, 24]. Furthermore, cancer cell-derived exosomes mediate metastasis, immune evasion, and tumor microenvironment formation, highlighting their potential as diagnostic and therapeutic targets [25, 26]. In contrast, exosomes derived from normal cells are reported to exhibit beneficial biological activities, including tissue regeneration, anti-inflammatory effects, and neuroprotection [27, 28]. Consequently, the utilization of exosomes as natural biodelivery systems has attracted increasing attention for therapeutic and cosmetic applications.

In this study, exosomes were isolated from *Fructobacillus fructosus* NSH-1 strain, a symbiotic microorganism isolated from the flowers of *Campsis grandiflora*, using a tangential flow filtration system. The anti-inflammatory and skin barrier-enhancing activities of purified NSH-1 exosomes were subsequently evaluated through a series of *in vitro* experiments.

Materials and Methods

Isolation of *Fructobacillus fructosus* NSH-1 Strain from *Campsis grandiflora* Flowers

Flowers of *Campsis grandiflora* were collected in 2019 from the Hongseong, Chungcheongnam-do, Republic of Korea. The collected flower samples were washed at least three times with sterile saline solution to remove surface contaminants. The washed samples were then homogenized using a sterile mortar and pestle. The resulting homogenate was serially diluted with sterile saline solution up to 10⁻⁵. Aliquots (200 μ l) of each dilution were spread onto de Man, Rogosa and Sharpe (MRS) agar plates and incubated at 30°C for 48 h under either aerobic or anaerobic conditions. After incubation, colonies that showed robust growth under aerobic conditions were selected. A total of five colonies were picked and purified by repeated streaking. Genomic DNA was extracted from the isolated strains, and the 16S rRNA gene was amplified and sequenced. Based on 16S rRNA gene sequence analysis, the isolates were identified as belonging to the *F. fructosus*.

Isolation and Purification of Exosomes

F. fructosus NSH-1 was cultured under aerobic conditions at 30°C with shaking at 150 rpm for 18 h in MRS medium to support its growth. The cultured broth was centrifuged at 4,500 $\times g$ for 15 min to remove the bacterial pellets, and the resulting supernatant was collected. The supernatant was then filtered using a 0.22 μ m pore-sized bottle-top filter (SPL, Republic of Korea) to eliminate the remaining cellular debris. Subsequently, exosomes present in the NSH-1 culture supernatant were purified using a tangential flow filtration system equipped with a hollow fiber cartridge (Cytiva, England) with internal fiber diameter 0.5 mm (Fiber ID), flow path length 110 cm, and nominal molecular weight cutoff 100 kDa.

Characterization of Exosomes

NSH-1 exosomes were diluted with 1 $\times$ phosphate-buffered saline (PBS, Biosesang, Republic of Korea) and analyzed using a ZetaView system (Particlemetrix, Inning am Ammersee, Germany) for nanoparticle tracking analysis (NTA). The analysis was conducted in accordance with the standard operating procedures recommended for NTA to ensure data reproducibility and reliability. Subsequently, for transmission electron microscopy (TEM) imaging, the exosome samples were negatively stained with 2% (w/v) uranyl acetate for 20 sec, blotted onto carbon-coated copper grids, and air-dried at room temperature. The prepared grids were examined using an Alos L120C microscope (FEI, Hillsboro, USA) to obtain high-resolution images of the exosomes.

Reagents and Cell Culture

The RAW 264.7 macrophages and HaCaT cells used in this study were obtained from the Korean Cell Line Bank (Republic of Korea). The reagents, TNF- α and IFN- γ , were purchased from MedChemExpress (Monmouth Junction, USA), and lipopolysaccharide (LPS), Griess reagent, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co. (USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM, Welgene, Republic of Korea) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After achieving stable growth through serial subculture, the cells were used for subsequent experiments.

Cell Viability Assay

The effects of NSH-1–derived exosomes on the viability of RAW 264.7 macrophage cells and HaCaT cells were evaluated using the MTT assay. RAW 264.7 cells suspended in DMEM containing 10% FBS and 100 U/ml penicillin–streptomycin were seeded at a density of 8.0×10^4 cells/well in 24-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. After pre-incubation, RAW 264.7 cells were treated with 1 µg/ml LPS and NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml for 24 h. HaCaT cells were seeded at a density of 1.0×10^4 cells/well in 96-well plates and incubated for 24 h under the same culture conditions. After incubation, the cells were stimulated with 20 ng/ml TNF-α/IFN-γ and treated with NSH-1 exosomes at the same concentrations as those used for RAW 264.7 cells. Subsequently, 200 µl of MTT solution (2 mg/ml), prepared by dissolving MTT in PBS, was added to each well and incubated for 2 h at 37°C in 5% CO₂ to allow formazan crystal formation. The resulting formazan was dissolved in dimethyl sulfoxide (DMSO), and its absorbance was measured at 570 nm using a multiwell microplate reader (ELISA reader). Cell viability was calculated as the percentage of absorbance relative to that of the untreated control group.

Measurement of Nitric Oxide Inhibitory Activity

To evaluate the effect of NSH-1 exosomes on nitric oxide (NO) production, RAW 264.7 cells suspended in DMEM containing 10% FBS and 100 units/ml penicillin–streptomycin were seeded in 24-well plates at a density of 8.0×10^4 cells/well. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. After incubation, the cells were stimulated with 1 µg/ml LPS to induce an inflammatory response and simultaneously treated with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml for 24 h. Subsequently, 100 µl of the culture supernatant was transferred to a 96-well plate, mixed with an equal volume of Griess reagent, and reacted for 10 min in the dark. Absorbance was measured at 540 nm using a microplate reader to determine NO production.

Measurement of Pro-Inflammatory Cytokine Inhibitory Activity

To evaluate the effect of NSH-1 exosomes on pro-inflammatory cytokine production, RAW 264.7 cells were seeded in 24-well plates at a density of 8.0×10^4 cells/well and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. After incubation, inflammation was induced by treating the cells with 1 µg/ml LPS, followed by co-treatment with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml for an additional 24 h. The culture medium was then centrifuged at 13,000 ×g for 3 min to remove cell debris and the resulting supernatant was collected for cytokine analysis. The concentrations of pro-inflammatory cytokines were determined using a Mouse TNF-α ELISA Kit (BMS607-3, Invitrogen, USA), a Mouse IL-6 ELISA Kit (550950, BD Biosciences, USA), and a Mouse IL-1β ELISA Kit (MLB00C, R&D Systems Inc., USA).

Measurement of Prostaglandin E2 Inhibitory Activity

To evaluate the effect of NSH-1 exosomes on PGE₂ production, RAW 264.7 cells were seeded in 24-well plates at a density of 8.0×10^4 cells/well and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. After incubation, inflammation was induced by treating the cells with 1 µg/ml LPS, followed by co-treatment with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml for 24 h. The culture medium was then centrifuged at 13,000 ×g for 3 min to remove any precipitate, and the resulting supernatant was collected. The PGE₂ concentration in the supernatant was determined using a mouse enzyme-linked immunosorbent assay (ELISA) kit (Cat. No : KGE004B, R&D Systems, Inc.).

Western Blot Analysis

RAW 264.7 cells were seeded in 6-well plates at a density of 4.0×10^4 cells/well, and HaCaT cells were seeded in 6-well plates at a density of 3×10^5 cells/well. Both cell types were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. After incubation, RAW 264.7 cells were treated with 1 µg/ml LPS along with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml. For NF-κB, IκBα and MAPK signaling analysis, cells were incubated for 2 h, whereas iNOS and COX-2 expression analyses were conducted after 24 h of treatment. Whereas HaCaT cells were treated with 20 ng/ml TNF-α/IFN-γ together with the same concentrations of NSH-1 exosomes for 24 h. The cells were then washed once with 1× PBS and harvested using 0.5× trypsin-EDTA. The collected cells were lysed in 100 µl of RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and 1% protease inhibitor cocktail for 1 h, followed by centrifugation at 13,000 ×g for 30 min at 4°C to collect the supernatant. Protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Scientific, USA). For sample preparation, the lysates were mixed with 2× Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, and 0.01% bromophenol blue) at a 1:1 ratio, supplemented with 5% 2-mercaptoethanol, and heated at 90°C for 5 min. Proteins were separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA). The PVDF membranes were blocked with 5% skim milk in 1× TBST (1× TBS, 0.1% Tween 20) at room temperature for 1 h 30 min and washed four times with 1× TBST at 10-min intervals. The membranes were then incubated overnight at 4°C with primary antibodies against iNOS (13120S, 1:500, Cell Signaling, USA), COX-2 (4842S, 1:500, Cell Signaling), phospho-ERK (9101S, 1:500, Cell Signaling), ERK (9102S, 1:500, Cell Signaling), phospho-JNK (9251S, 1:500, Cell Signaling), JNK (9252S, 1:500, Cell Signaling), phospho-p38 (9211S, 1:500, Cell Signaling), p38 (9212S, 1:500, Cell Signaling), phospho-NF-κB (#3033, 1:500, Cell Signaling), phospho-IκBα (#2859S, 1:500, Cell Signaling), filaggrin (sc-66192, 1:500, Santa Cruz Biotechnology, USA), loricrin (LS-C31863-100, 1:500, LSBio, USA), involucrin (sc-21748, 1:500, Santa Cruz Biotechnology), and β-actin (#3700, 1:5,000, Cell Signaling). After washing four times with 1× TBST at 10-min intervals, the membranes were incubated for 1 h 30 min at room temperature with horseradish peroxidase-conjugated secondary antibodies, anti-rabbit IgG, or anti-mouse IgG (1:1,000). The membranes were again washed four times with 1× TBST at 10-min intervals, and the protein bands were visualized using an enhanced chemiluminescence kit (Bio-Rad, USA). Protein expression levels were detected using an imaging densitometer (model GS-700, Bio-Rad) and quantified using ImageJ software (NIH, USA). Relative protein expression levels were expressed as graphical data.

Measurement of Wound-Healing Activity

To investigate the effect of NSH-1 exosomes on skin cell regeneration, HaCaT keratinocytes were used in a wound-healing assay. Cells were seeded in 24-well plates at a density of 1×10^5 cells/ml and incubated for 24 h. A linear scratch with a defined width of 1.0 mm was then created

across the cell monolayer using an SPLScar™ Scratcher (#201925, SPL Life Sciences, Republic of Korea), after which the cells were treated with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml to evaluate their proliferation and migration capacities.

Measurement of Hyaluronan and Collagen Production

To examine the effect of NSH-1 exosomes on the production of hyaluronan and collagen, which play crucial roles in skin barrier maintenance, HaCaT cells were seeded in 24-well plates at a density of 1×10^5 cells/ml and incubated for 24 h. The cells were then treated with TNF- α /IFN- γ (20 ng/ml) to induce inflammatory conditions, along with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml for 24 h. After incubation, the culture medium was centrifuged at $13,000 \times g$ for 3 min to remove any precipitate, and the supernatant was collected. The amounts of hyaluronan and collagen produced were determined using a Hyaluronan Quantikine ELISA Kit (DHYAL0, R&D Systems Inc.) and a Procollagen Type I C-peptide EIA Kit (MK001, Takara, Japan), respectively.

Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation. Statistical significance among the treatment groups was evaluated using a Student's *t*-test or using analysis of variance, followed by a post-hoc test for multiple comparisons. Differences were considered statistically significant at (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

Results

NTA and TEM Analysis

NTA and TEM analyses were performed to examine the concentration, particle size, and morphology of NSH-1 exosomes. The NTA results revealed that the NSH-1 exosomes had an average diameter of 151.2 nm and a concentration of 1.2×10^9 particles/ml (Fig. 1A). TEM observation further confirmed that the exosomes exhibited a spherical morphology with particle sizes of approximately 200 nm or larger (Fig. 1B).

Cell Viability

The MTT assay is a cell viability test based on the principle that yellow MTT is reduced to purple formazan by mitochondrial dehydrogenase activity in live cells [29]. The effect of NSH-1 exosomes on cell viability was evaluated in RAW 264.7 cells treated with concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml. As shown in Fig. 2A, cell viability in RAW 264.7 cells remained above 80% at all tested concentrations. In

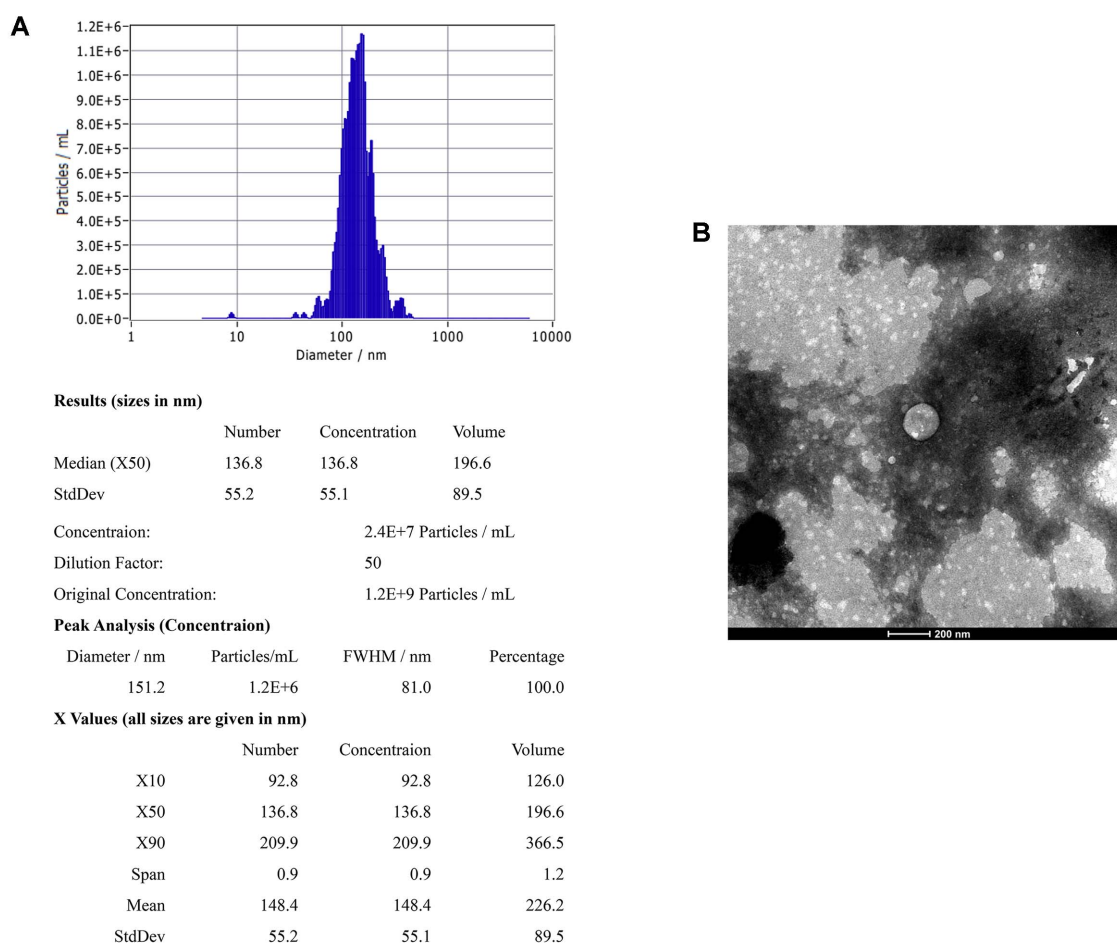


Fig. 1. Characterization of NSH-1 exosomes. (A) Nanoparticle tracking analysis of NSH-1 exosomes. (B) Transmission electron microscopy image of NSH-1 exosomes.

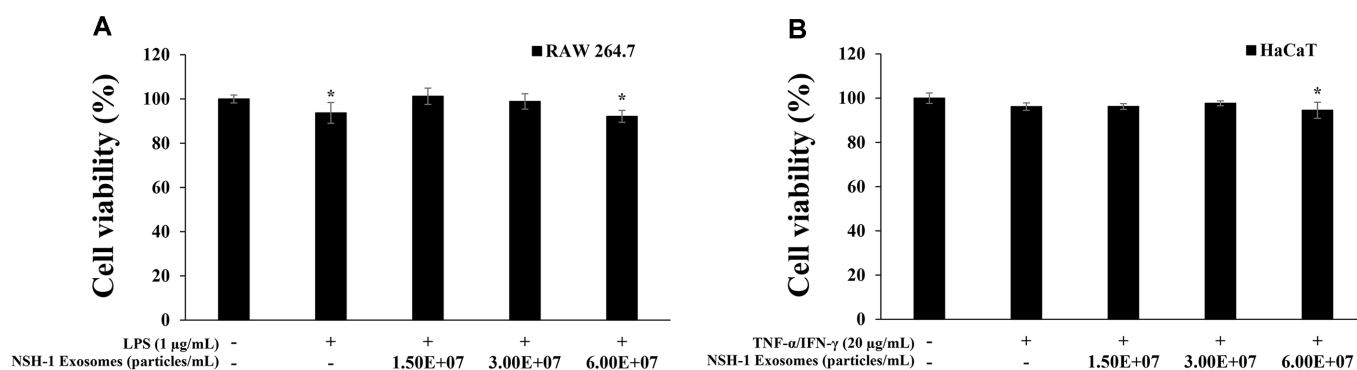


Fig. 2. Cell viability assessment of LPS-stimulated RAW 264.7 macrophages and TNF-α/IFN-γ-stimulated HaCaT keratinocytes treated with NSH-1 exosomes. (A) Cell viability of RAW 264.7 macrophages. (B) Cell viability of HaCaT keratinocytes. The results indicate that NSH-1 exosomes do not induce significant cytotoxicity in either RAW 264.7 or HaCaT cells at the tested concentrations. Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$.

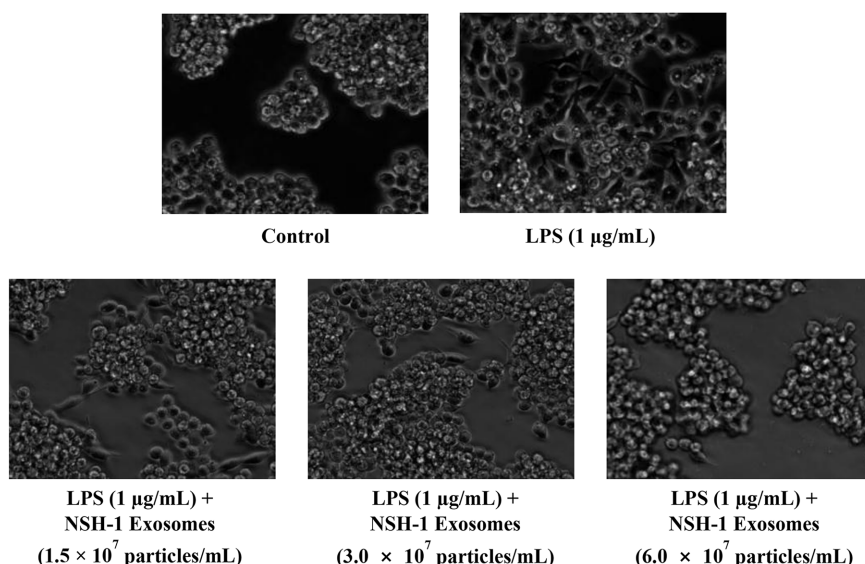


Fig. 3. Morphological changes in RAW 264.7 cells following treatment with LPS and NSH-1 exosomes. Representative phase-contrast images illustrate LPS-induced morphological alterations in RAW 264.7 cells and their attenuation upon co-treatment with NSH-1 exosomes.

addition, the effect of NSH-1 exosomes on cell viability was also examined in HaCaT cells under the same treatment conditions, and no significant cytotoxicity was observed, with cell viability remaining above 80% at all concentrations tested (Fig. 2B). Therefore, subsequent experiments were conducted using NSH-1 exosome concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml, which did not exhibit any cytotoxicity. Morphological changes in RAW 264.7 cells following treatment with LPS and NSH-1 exosomes were examined using optical microscopy. LPS stimulation induced marked morphological alterations characteristic of an activated inflammatory state. In contrast, treatment with NSH-1 exosomes attenuated these LPS-induced morphological changes, restoring cell morphology toward a state comparable to that of untreated control cells (Fig. 3).

Nitric Oxide Inhibitory Activity

Nitric oxide is a reactive nitrogen species that constitutes a final product of inflammatory responses in the body and is known, together with reactive oxygen species, to act as a factor that induces cellular damage [30, 31]. To evaluate the effect of NSH-1 exosomes on nitric oxide (NO) production, RAW 264.7 cells were treated with non-cytotoxic concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml. NSH-1 exosome treatment inhibited NO production by 16.4%, 41.3%, and 75.9% at the respective concentrations compared with that in the LPS-treated group, showing a clear concentration-dependent effect (Fig. 4). Based on these results, subsequent experiments were conducted to investigate the underlying anti-inflammatory mechanisms by analyzing pro-inflammatory cytokine expression.

Pro-Inflammatory Cytokine Inhibitory Activity

Cytokines are signaling molecules produced during immune and inflammatory responses, and they are known to modulate cellular activity and contribute to inflammatory tissue damage [32, 33]. To investigate the effect of NSH-1 exosomes on pro-inflammatory cytokine production, RAW

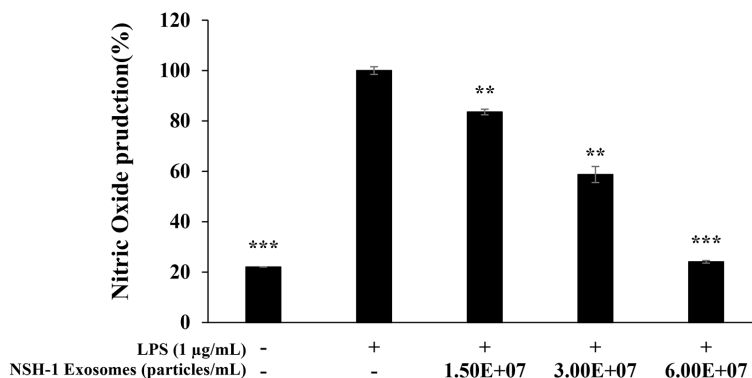


Fig. 4. Nitric oxide production assessment of LPS-activated RAW 264.7 macrophage cells treated with NSH-1 exosomes. The measurement of nitric oxide production inhibitory effect of RAW 264.7 cells was treated with LPS (1 µg/ml) for 24 h in the presence of NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml). Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

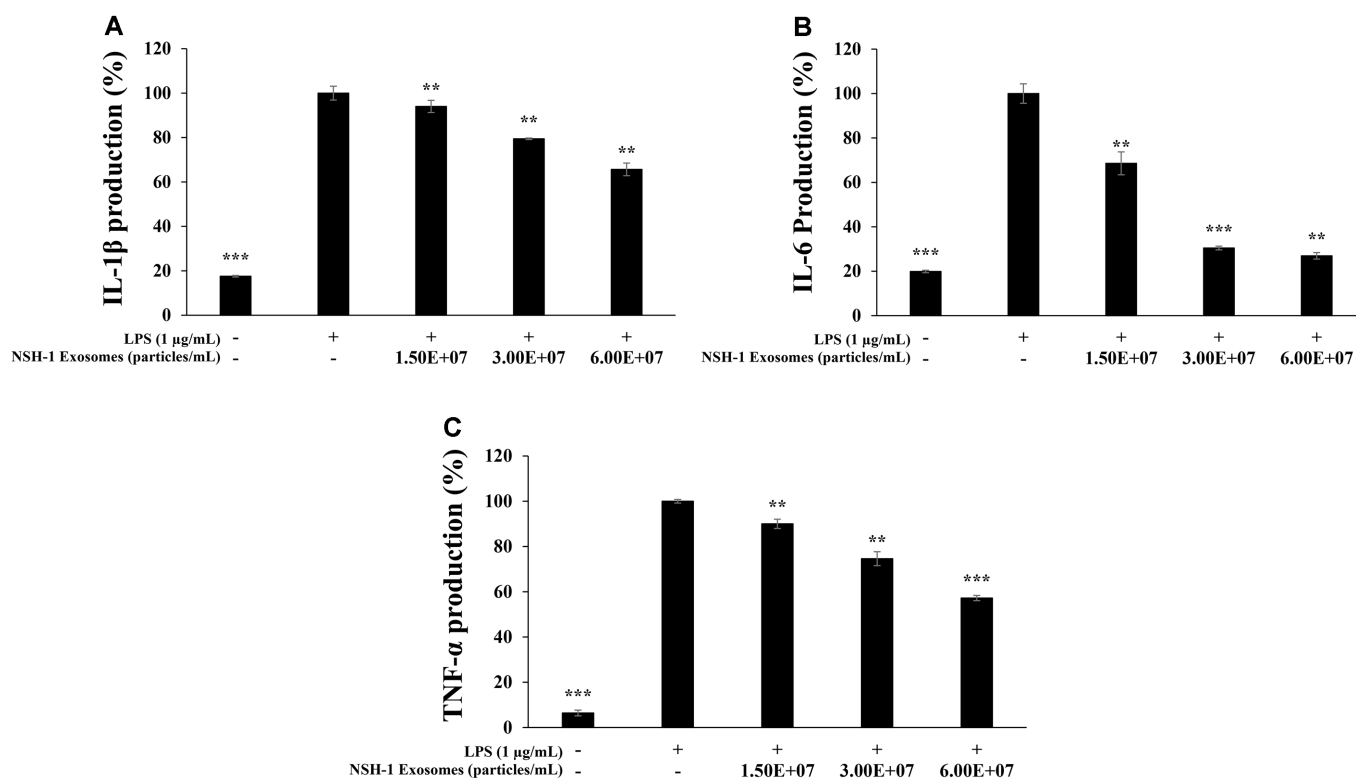


Fig. 5. Pro-inflammatory cytokines production assessment of LPS-activated RAW 264.7 macrophage cells treated with NSH-1 exosomes. The measurement of (A) interleukin (IL)-1β, (B) IL-6, (C) tumor necrosis factor alpha (TNF-α) in RAW 264.7 cells was treated with LPS (1 µg/ml) for 24 h in the presence of NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml). Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

264.7 cells were treated with non-cytotoxic concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml. The production levels of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α were then measured. The results demonstrated that treatment with NSH-1 exosomes reduced the production of IL-1β by 6.0%, 20.6%, and 34.3% (Fig. 5A), IL-6 by 31.4%, 69.5%, and 73.1% (Fig. 5B), and TNF-α by 10.0%, 25.4%, and 42.8% (Fig. 5C) at the respective concentrations, showing a clear concentration-dependent decline compared with that in the LPS-stimulated control group.

Prostaglandin E2-Inhibitory Activity

PGE₂ is an inflammatory mediator generated from arachidonic acid via COX-2-dependent catalysis, and it is known to modulate immune responses and contribute to inflammation-associated tissue damage [34, 35]. To investigate the effect of NSH-1 exosomes on PGE₂ expression, RAW 264.7 cells were treated with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml. NSH-1 exosome treatment

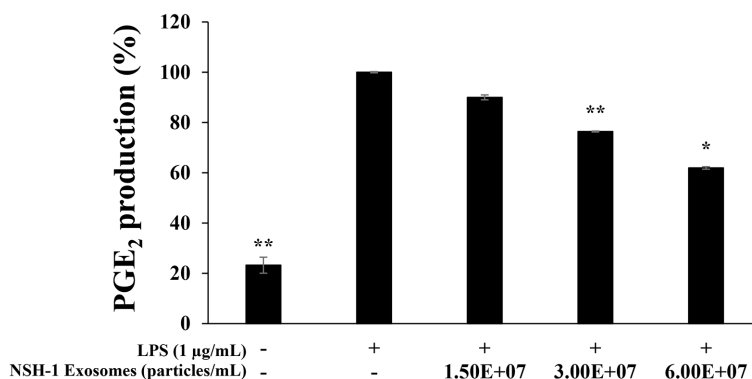


Fig. 6. Prostaglandin E₂ (PGE₂) production assessment of LPS-activated RAW 264.7 macrophage cells treated with NSH-1 exosomes. The measurement of PGE₂ production of RAW 264.7 cells was treated with LPS (1 µg/ml) for 24 h in the presence of NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml). Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$.

reduced PGE₂ expression by 10.0%, 23.6%, and 38.2% at the respective concentrations compared with that in the control group (Fig. 6). These results indicate that NSH-1 exosomes exert anti-inflammatory effects by suppressing the expression of PGE₂, a key mediator of the inflammatory response.

Measurement of iNOS and COX-2 Expression

The iNOS and COX-2 are inflammation-inducible enzymes that respectively produce nitric oxide and prostaglandins (such as PGE₂) from L-arginine and arachidonic acid, thereby serving as key mediators that amplify inflammatory responses and promote tissue damage [36, 37]. To examine the effects of NSH-1 exosomes on iNOS and COX-2 expression in RAW 264.7 macrophages, cells were treated with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml. NSH-1 exosome treatment reduced iNOS expression by 18.9%, 55.6%, and 66.6% (Fig. 7A), and that of COX-2 by 28.2%, 44.2%, and 46.6% (Fig. 7B) at the respective tested concentrations, showing a clear concentration-dependent decrease relative to β -actin.

Measurement of MAPK Phosphorylation

Phosphorylation of MAPK signaling components (ERK, JNK, and p38) activates downstream transcription factors that enhance the expression of pro-inflammatory mediators, thereby amplifying the inflammatory response [38, 39]. MAPK phosphorylation was examined using western blotting to compare the inhibitory activities of NSH-1 exosomes. Upon treatment with NSH-1 exosomes at 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml, ERK phosphorylation declined by approximately 0.1%, 8.7%, and 28.8% (Fig. 8A), while JNK phosphorylation decreased by 1.6%, 50.4%, and 61.4% (Fig. 8B), respectively. In contrast, treatment with 1.5×10^7 and 3.0×10^7 particles/ml of NSH-1 exosomes resulted in an increase in p38 phosphorylation, whereas treatment with a 6.0×10^7 particles/ml of NSH-1 exosomes effectively reduced p38 phosphorylation (Fig. 8C). This pattern suggests that NSH-1 exosomes exhibit a biphasic dose-response effect on p38 phosphorylation [40].

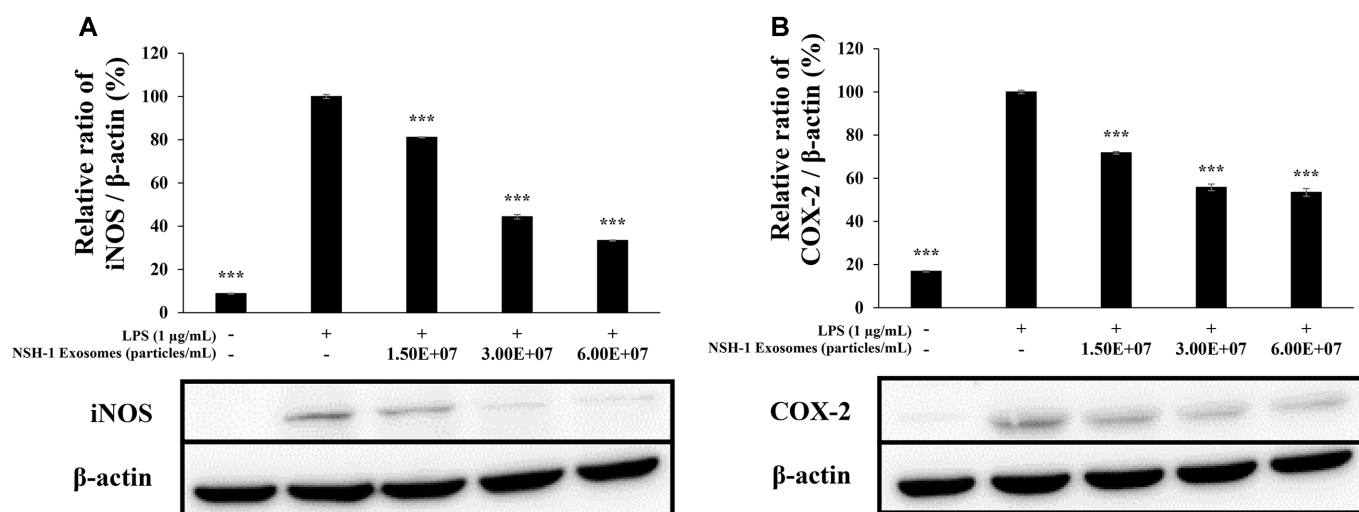


Fig. 7. Inhibitory effect of NSH-1 exosomes on the protein level of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression in LPS-activated RAW 264.7 macrophage cells. The measurement of (A) iNOS and (B) COX-2 of RAW 264.7 cells was treated with LPS (1 µg/ml) for 24 h in the presence of NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml). The protein levels of iNOS, COX-2, and β -actin were determined western blotting. Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

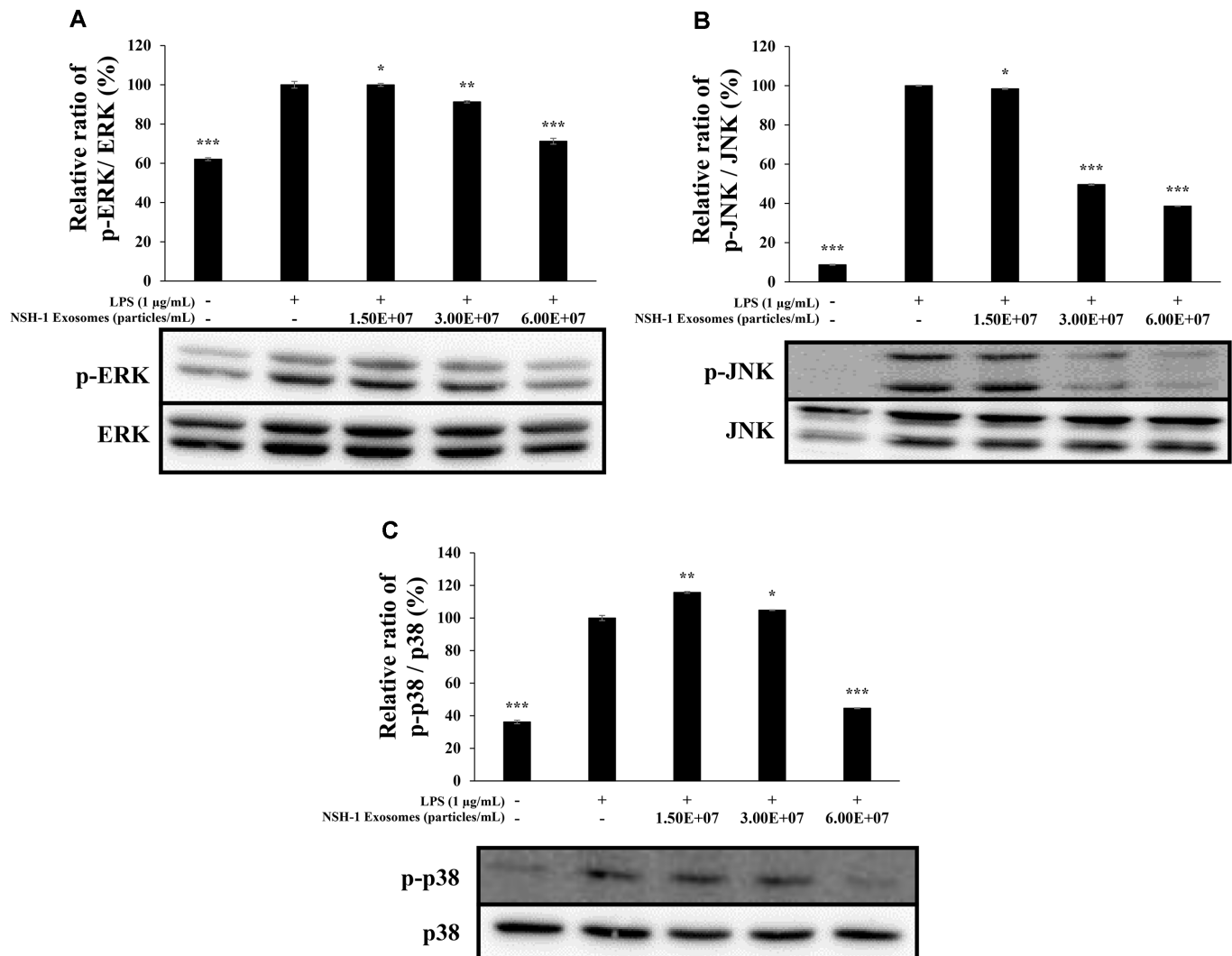


Fig. 8. Effect of NSH-1 exosomes on the phosphorylation levels of ERK, JNK, and p38. To measure the expression levels of (A) p-ERK / ERK, (B) p-JNK / JNK, and (C) p-p38 / p38, RAW 264.7 macrophage cells pre-incubated for 24 h were treated with LPS (1 µg/ml) and NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml) for an additional 2 h. The protein levels were determined western blotting. Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Measurement of NF- κ B, I κ B- α Expression

I κ B- α prevents the translocation of NF- κ B from the cytoplasm to the nucleus; however, upon receiving inflammatory signals triggered by external stimuli, I κ B- α is phosphorylated and subsequently degraded, allowing NF- κ B to translocate into the nucleus [41]. Nuclear NF- κ B then activates genes encoding pro-inflammatory mediators, such as iNOS and COX-2, thereby amplifying the inflammatory response. To examine the effects of NSH-1 exosomes on NF- κ B signaling in RAW 264.7 macrophages, cells were treated with NSH-1 exosomes at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml in the presence of LPS (1 µg/ml). As shown in Fig. 9A, NSH-1 exosome treatment significantly reduced the phosphorylation level of NF- κ B p65 by 27.0%, 91.4%, and 91.8%, respectively, compared with the LPS-treated group. In contrast, LPS stimulation markedly reduced the phosphorylation level of I κ B- α , whereas treatment with NSH-1 exosomes progressively restored p-I κ B- α levels toward those of the unstimulated control. Specifically, p-I κ B- α levels were modulated by 25.0%, 65.1%, and 77.1% at the corresponding concentrations (Fig. 9B), suggesting that NSH-1 exosomes regulate NF- κ B signaling by normalizing LPS-induced dysregulation of I κ B- α phosphorylation.

Measurement of Wound-Healing Activity

HaCaT keratinocytes-based wound-healing activity assays are commonly used as a fundamental method for evaluating skin barrier functionality [42]. In this study, a scratch was introduced into cultured HaCaT cells, after which NSH-1 exosomes were administered at concentrations of 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml to examine their wound-healing activity. As a result, the NSH-1 exosome-treated groups exhibited markedly enhanced wound-healing activity compared with the untreated control group (Fig. 10). These findings suggest that NSH-1 exosomes possess inherent skin barrier-improving activity, and subsequent experiments were conducted to assess their effects on the production of key skin structural components, including hyaluronan and collagen.

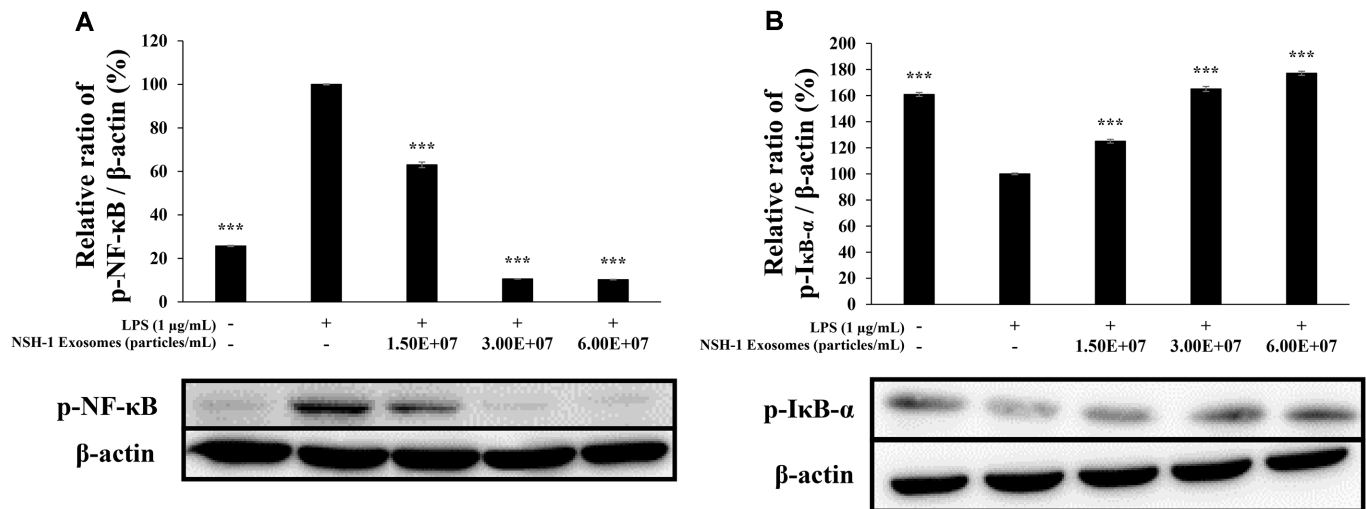


Fig. 9. Effects of NSH-1 exosomes on NF-κB signaling in RAW 264.7 macrophages. RAW 264.7 cells were pre-incubated for 24 h and then treated with LPS (1 μg/ml) in the presence of NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml) for an additional 2 h. The phosphorylation levels of (A) NF-κB p65 and (B) IκB-α were analyzed by Western blotting. β-actin was used as a loading control. Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

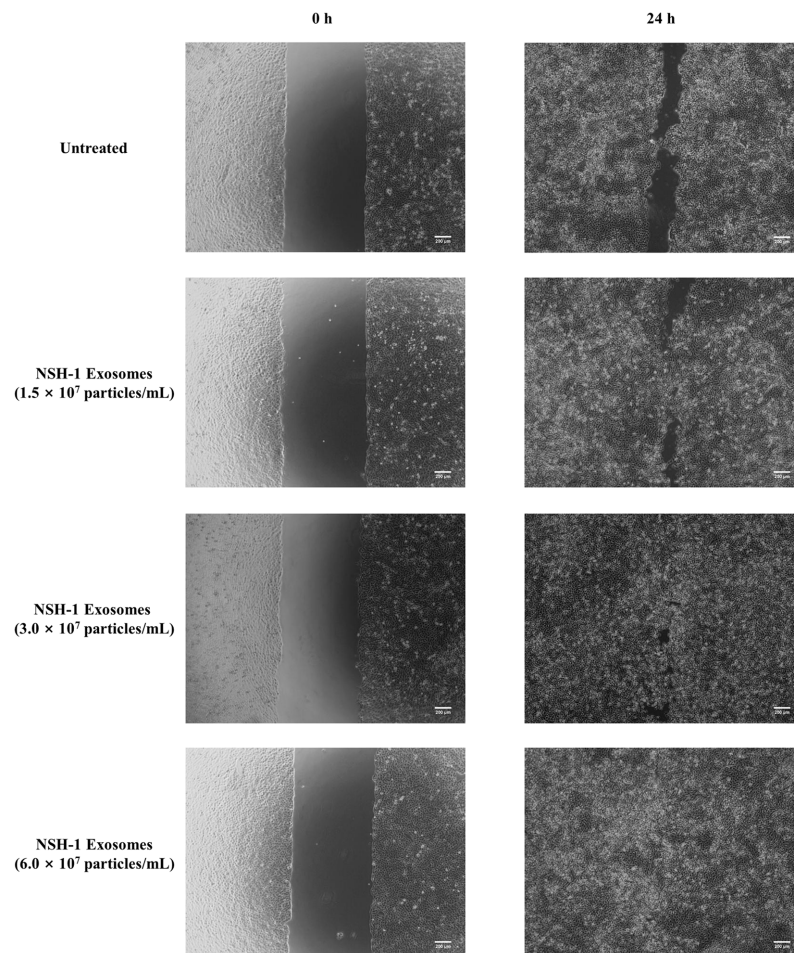


Fig. 10. Imaging of repair of scratched HaCaT cells. The HaCaT cells were seeded in 24-well plates at a density of 1×10^5 cells/ml and incubated for 24 h. After incubation, the cells were scratched and treated NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml) for an additional 24 h.

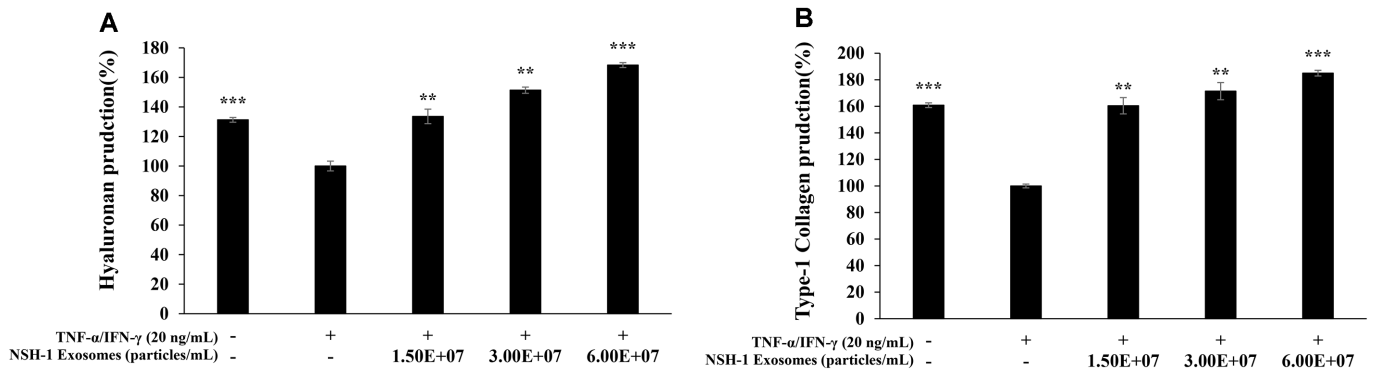


Fig. 11. Hyaluronan and type-1 collagen production assessment of TNF-α/IFN-γ-induced HaCaT cells treated with NSH-1 exosomes. The measurement of hyaluronan and type-1 collagen production of HaCaT cells was treated with TNF-α/IFN-γ (20 ng/ml) for 24 h in the presence of NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml). Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

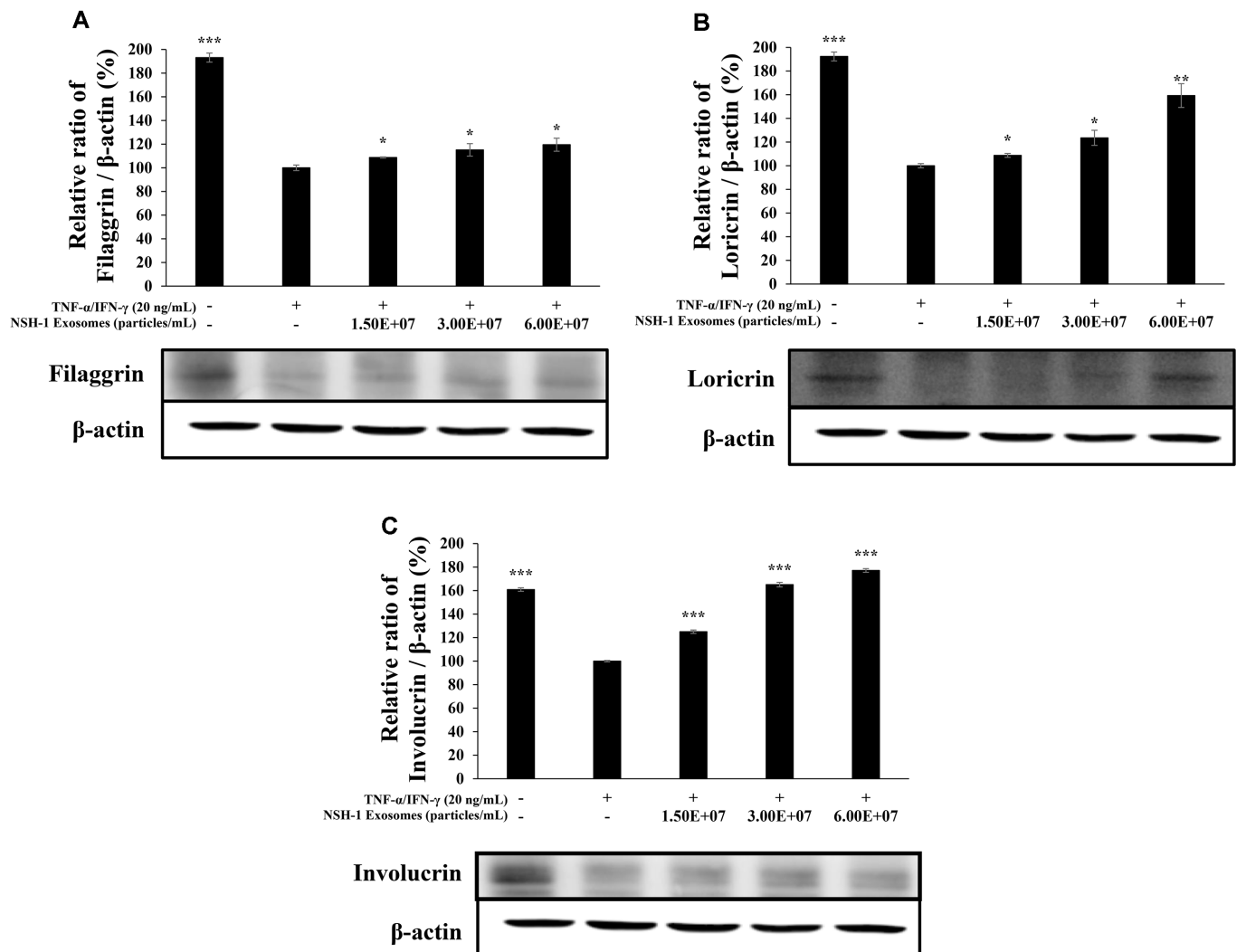


Fig. 12. Effect of NSH-1 exosomes on the expression levels of filaggrin, loricerin, and involucrin. To measure the expression levels of (A) filaggrin, (B) loricerin, and (C) involucrin, HaCaT cells pre-incubated for 24 h were treated with TNF-α/IFN-γ (20 ng/ml) and NSH-1 exosomes (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml) for an additional 24 h. The protein levels were determined western blotting. Results were expressed as percentages compared with the respective values obtained from the control. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Measurement of Hyaluronan and Collagen Production

HaCaT keratinocytes produce essential skin components such as hyaluronan and collagen, but their synthesis can be disrupted by external stimuli [43]. In this study, HaCaT cells were co-stimulated with TNF- α and IFN- γ to suppress the production of these components, followed by treatment with NSH-1 exosomes to assess whether their synthesis could be restored. NSH-1 exosomes administered at 1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml increased hyaluronan production by 33.6%, 51.4%, and 68.3% (Fig. 11A), and collagen production by 60.4%, 71.4%, and 85.0% (Fig. 11B), respectively, demonstrating clear dose-dependent enhancement. These findings indicate that NSH-1 exosomes promote wound-healing activity, as well as contribute to skin barrier restoration by stimulating the synthesis of structural components such as hyaluronan and collagen.

Measurement of Filaggrin, Loricrin, and Involucrin Expression

Filaggrin, loricrin, and involucrin are key epidermal differentiation markers that contribute to the formation and maintenance of the skin barrier by supporting corneocyte maturation and strengthening the structural integrity of the stratum corneum [44, 45]. The effect of NSH-1 exosomes on the expression of natural skin-barrier moisturizing factor-related proteins (filaggrin, loricrin, and involucrin) was evaluated in HaCaT cells using western blot analysis. NSH-1 exosome treatment increased the expression of filaggrin by 8.7%, 15.2%, and 19.5% (Fig. 12A); that of loricrin by 8.7%, 23.6%, and 59.3% (Fig. 12B); and that of involucrin by 25.0%, 65.1%, and 77.1% (Fig. 12C) at the respective tested concentrations.

Discussion

In this study, we evaluated the anti-inflammatory activity and skin barrier-enhancing effects of exosomes derived from *F. fructosus* NSH-1, a lactic acid bacterium symbiotically associated with the Korean native *Campsis grandiflora* flowers. The concentration and particle size of NSH-1 exosomes were characterized using nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM), which confirmed that they corresponded to the commonly reported size range of exosomes (30–200 nm).

NSH-1 exosomes effectively inhibited nitric oxide production in RAW 264.7 macrophages at non-cytotoxic concentrations (1.5×10^7 , 3.0×10^7 , and 6.0×10^7 particles/ml). Furthermore, they effectively suppressed the expression of pro-inflammatory cytokines, including TNF- α , IL-6, and IL-1 β , as well as prostaglandin E₂. Western blot analysis further demonstrated the significant inhibition of iNOS and COX-2, which are mediators involved in nitric oxide and PGE₂ production.

To investigate the anti-inflammatory mechanisms of NSH-1 exosomes in greater detail, we examined key phosphorylation-dependent signaling events involved in inflammatory activation. Specifically, the phosphorylation levels of NF- κ B p65 and I κ B- α , as well as MAPKs (ERK, JNK, and p38), which act at early stages of the inflammatory response and regulate its progression, were analyzed. The results showed that NSH-1 exosomes attenuated LPS-induced phosphorylation of NF- κ B p65 while modulating I κ B- α phosphorylation toward basal levels, suggesting a regulatory effect on NF- κ B signaling activation. In addition, NSH-1 exosomes suppressed the activation of MAPK pathways. Collectively, these findings suggest that NSH-1 exosomes modulate NF- κ B and MAPK signaling pathways, thereby suppressing the inflammatory response.

To evaluate the skin barrier-improving activity, HaCaT keratinocytes were wounded and subsequently treated with NSH-1 exosomes. The results demonstrated that NSH-1 exosomes promoted wound healing. Further, the presence of NSH-1 exosomes enhanced the synthesis of hyaluronan and collagen, which are key components responsible for maintaining skin structure. Furthermore, the expression of natural moisturizing factor-related proteins, including filaggrin, loricrin, and involucrin, was significantly increased.

Collectively, these findings indicate that *F. fructosus* NSH-1-derived exosomes exhibit both potent anti-inflammatory activity and skin barrier-improving effects, highlighting their potential as dual-functional agents. To further develop NSH-1 exosomes as functional agents, preclinical studies such as skin irritation tests and clinical efficacy trials are required. In addition, future studies will be necessary to achieve more comprehensive characterization of NSH-1 exosomes, including molecular-level validation of exosomal markers using complementary approaches such as PCR-based analyses, as well as detailed investigations to identify the specific exosomal components responsible for these bioactivities.

Author Contributions

S.-Y.K: Conceptualization, B.-M.C. and H.H: Data curation, B.-M.C., H.H., Y.-B.J., and T.-J.P: Methodology, B.-M.C., H.H., Y.-B.J., and T.-J.P: Validation, B.-M.C., and H.H: Writing—original draft, S.-Y.K: Writing—review and editing.

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