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Research Article

Enhancement of skin regeneration through activation of *TGF-β/SMAD* signaling pathway by *Panax ginseng* meyer non-edible callus-derived extracellular vesicles

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1. ABSTRACT

Background: This study aimed to investigate the effects of ginseng non-edible callus-derived extracellular vesicle (GNEV) on skin regeneration, particularly focusing on its impact on proliferation and migration in human dermal fibroblast (HDF).

Methods: GNEV was isolated from ginseng non-edible callus using sequential filtration and size exclusion chromatography (SEC). The extracellular vesicle was characterized using nanoparticle tracking analysis (NTA). HDF was treated with various concentrations of GNEV, and cell viability, proliferation, and migration were assessed using MTT and scratch wound healing assays. Gene expression related to collagen synthesis (*TGF-β, SMAD-2, SMAD-3, COL1A1*) was measured using RT-PCR.

Results: Treatment of HDF with GNEV resulted in a significant 2.5-fold increase in cell migration compared to the non-treated group. Furthermore, GNEV demonstrated the upregulation of collagen synthesis genes, specifically *TGF-β, SMAD-2, SMAD-3*, and *COL1A1*, by 41.7 %, 59.4 %, 60.2 %, and 21.8 %, respectively. These findings indicated that GNEV activates the *TGF-β/SMAD* signaling pathway, showcasing its potential to induce skin regeneration.

Conclusions: In conclusion, GNEV exhibits a notable ability to enhance skin regeneration through its stimulatory effects on cell migration and the upregulation of key collagen synthesis genes. The activation of the *TGF-β/SMAD* signaling pathway further suggests the potential of GNEV as a promising candidate for drug delivery systems in the fields of cosmetics and pharmaceuticals, opening avenues for further research and application in skincare and dermatology.

1. Introduction

Collagen is a major component of the extracellular matrix (ECM) and plays an important role in improving skin elasticity, building structures, and promoting skin regeneration by forming a peptide with an α-helical structure with repeating glycine, lysine, and proline [1]. When skin damage occurs, transforming growth factor-β (*TGF-β*) influences human dermal fibroblast (HDF) to enhance collagen synthesis and ECM production, crucial for wound healing and tissue regeneration [2]. *TGF-β* receptor 1 (*TβRI*) existing on the surface of HDF binds with the suppressor of mothers against decapentaplegic (*SMAD*) and initiates the process of skin regeneration through the activation of *TGF-β/SMAD* signaling pathway [3]. Accordingly, *SMAD-2* and *SMAD-3*, which are receptor-activated *SMAD*s, are phosphorylated and form a complex with *SMAD-4*, a *TGF-β* signal transduction regulator to promote the activity of collagen transcription factors such as collagen type I alpha 1 (*COL1A1*) in the HDF nucleus [4]. Therefore, the interaction of *TGF-β* and *SMAD* to promote collagen synthesis has been recognized as a critical strategy to promote skin regeneration in the field of medicinal material development, and research on new agents to use this is ongoing [5].

Extracellular vesicle (EV) is produced when the plasma membrane or intraluminal vesicle is secreted out of the cell by binding with the cell

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surface during the maturation of multivesicular endosomes [6]. In addition, EV affects various physiological effects by delivering nucleic acids, proteins, and ribonucleic acids from the originating cell to the recipient cell via endocytosis [7]. In particular, the biocompatibility and drug delivery capabilities of plant-derived EV (PDEV) have been demonstrated to exhibit efficacy and potential in various fields, including anti-inflammatory responses, tumor formation inhibition, and promotion of skin regeneration [8]. For example, ginger-derived PDEV, when absorbed by macrophages, exhibit anti-inflammatory effects through RNA delivery [9]. Furthermore, tea flower-derived PDEV exhibit the ability to selectively migrate to tumor sites, triggering apoptosis in cancer cells and impeding metastasis [10]. Additionally, wheatgrass-derived PDEV enhance the viability and migration of skin cells such as endothelium, epithelium, and dermis, suggesting that PDEV can be effectively used as functional materials and drug delivery vehicles [11]. Notably, PDEV serves as a protective barrier for encapsulated materials, offering distinct advantages in various applications by shielding them from external environmental factors such as temperature and pH fluctuations [12]. These vesicles are actively investigated as drug and skin delivery vehicles due to their exceptional attributes, which include high biological stability, minimal cytotoxicity, target specificity, and efficient tissue penetration. This combination of attributes positions PDEV as promising candidates for ensuring the safe and effective delivery of therapeutic agents to target sites.

Ginseng (*Panax ginseng* C. A. Meyer) contains high amounts of ginsenosides, plant sterols, and polysaccharides with anti-cancer and antioxidant properties, so it has been used as a medicinal plant [13]. Particularly, ginseng extract, including the ginsenoside Rb1, has been reported to have excellent skin regeneration effects by inducing the proliferation of keratinocytes and HDF and promoting collagen synthesis [14]. Nonetheless, research on how ginseng non-edible callus-derived EV (GNEV) obtained from non-edible parts of ginseng, including stem, flower, and leaves affect the proliferation of HDF and skin regeneration remains insufficient. Thus, in this study, we aimed to induce callus from ginseng leaves, effectively produce EV through cell suspension culture, and evaluate the effect on skin regeneration. To achieve this, we evaluated the effect of GNEV on HDF proliferation and migration, investigated the gene expression related to skin regeneration, and confirmed the potential applicability of GNEV as a substance with skin regeneration effects for wound healing.

2. Materials and methods

2.1. Ginseng non-edible callus culture

To isolate EV from ginseng, four-year-old Korean *P. ginseng* cultivated in Eumseong, Chungbuk, was purchased, and the roots, stems, and leaves were separated from the ginseng. The ginseng leaves, stored at 4 °C, were washed with distilled water, sterilized with 70 % v/v ethanol, and approximately 20 leaves were cut into 3–4 cm pieces. Callus was induced and cultured on solid Murashige & Skoog (MS) medium (M0222.0050, Duchefa Biochemie, Haarlem, Netherlands) supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D, D0911.0100, Duchefa Biochemie), 3 % sucrose (S0809.5000, Duchefa Biochemie), and 0.25 % GELRITE™ (G1101.1000, Duchefa Biochemie) under dark conditions at 24 °C. The ginseng non-edible part calluses were subcultured every 4 weeks and further subcultured every 2 weeks in 50 mL of MS liquid medium without GELRITE™. After the culture was completed, the culture medium was filtered through 0.45 μm membrane, and the broth containing EV was collected and stored at −20 °C for subsequent experiments.

2.2. GNEV separation

To purify ginseng exosome, fast protein liquid chromatography (FPLC) was performed using ÄKTA FPLC system (Cytiva, Little Chalfont, UK). A HiPrep 26/10 Desalting column (Cytiva) was used for size exclusion chromatography (SEC). 20 mM sodium phosphate buffer (pH 6.0) was used as a mobile phase (or eluents). Approximately 13.3 mL of culture medium was loaded, and fractions of 14 mL were collected using the buffer as the eluent. They were then filtered through a 0.45 μm syringe filter, resulting in a final volume of approximately 14 mL, and stored at −20 °C.

2.3. Characterization of GNEV

For the measurement of GNEV concentration and size distribution, 1 mL of the sample, diluted 1:10 with ultrapure water filtered through a 0.1 μm membrane filter, was injected into the Zeta View (PMX 130, Particle Metrix GmbH, Meerbusch, Germany). In the experimental procedure, the Zeta View system used to analyze a sample's size distribution. This process involved measuring the size of the sample with a 488 nm laser and dividing it into 11 sections based on the diameter. After obtaining cell concentration of diluted samples, they were meticulously adjusted by applying a dilution factor to account to compensate for variations in sample concentration. Moreover, protein was quantified using the Bradford method with BSA (Bio-Rad, Hercules, USA) as the standard.

Additionally, for the morphological analysis of GNEV, 10 μg of EV sample was adsorbed onto formvar/carbon-coated nickel grids fixed with 2.5 % glutaraldehyde for 10 min, negatively stained with 5 % uranyl acetate, and air-dried for 30 min. The samples were then observed using a transmission electron microscope (Tecnai G2 twin spirit, FEI, Eindhoven, Netherlands) for morphological analysis.

2.4. HDF cell culture

The HDF used in this study was CCD-986sk, purchased from the Korea Cell Line Bank (KCLB NO. 21947, Seoul, Korea). The cells were seeded into culture flasks at 2.5×10^4 cells/mL and subcultured at 3-day intervals. For cell culture, Dulbecco's modified Eagle's medium (Thermo Fisher, Waltham, UA) supplemented with 10.0 % v/v fetal bovine serum (FBS) and 1.0 % w/v penicillin (Thermo Fisher, Waltham, UA) was used. Cells were cultured in a CO_2 incubator (SA-MCO-18AIC, Sanyo Co., Osaka, Japan) maintained at 37 °C with 5.0 % CO_2.

2.5. Evaluation of cell proliferation

To assess the proliferation of HDF in response to GNEV treatment, cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma-Aldrich, St. Louis, USA) method. HDF was individually seeded in a 96-well plate with 2.0×10^3 cells per well. After 24 h of incubation, GNEV was added at concentrations ranging from 0.0 to 1.1×10^9 particles/mL, and the cells were cultured for an additional 48 h. Then, the medium was removed, and 0.1 mL of MTT reagent was added to each well. After incubating for 4 h, the formazan crystals formed were dissolved using dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA), and the absorbance was measured at 540 nm using a microplate reader (Infinite®200, Tecan group, Zurich, Switzerland).

$$\text{Cell proliferation}\ (\%) = (1 - A / B) \times 100 \quad (1)$$

A: Absorbance of sample group (treated with GNEV), B: Absorbance of non-treated group.

2.6. Comparison of cell migration

For cell migration assessment, the commonly applied scratch wound healing assay of tissue-culture cell monolayers was conducted in a 96-well plate. The migration ability of cells was assessed by physically removing surface-adherent cells to create a vertical scratch, where the

gap is gradually filled, allowing the evaluation of their growth and migration capabilities at different time points. The detailed experimental procedure was as follows: firstly, HDF were cultured at 1.0×10^5 cells per well in a 24-well plate for 24 h. After that, a vertical scratch was created using a sterilized 0.2 mL pipette tip with 1.0 mm width gap. Subsequently, the HDF were treated with GNEV at concentrations ranging from 6.3×10^7 to 2.5×10^8 particles/mL. Cell migration was then evaluated at 12 h intervals using an optical microscope (CX43, Olympus, Tokyo, Japan). The degree of cell migration over time was measured using Image J (National Institutes of Health, Bethesda, USA) and cell migration (%) was calculated using the formula below based on the area of the DMEM treated group.

$$\text{Cell migration rate}\ (\%) = (1 - A/B) \times 100 \quad (2)$$

A: Sample area (treated with GNEV), B: Non-treated area.

2.7. Evaluation of expression of skin regeneration gene

To evaluate the skin regeneration effect of GNEV, HDF were cultured in a 24-well plate at a initial density of 1.0×10^6 cells/mL. After culturing for 24 h, GNEV treatment was administered to the cells, and HDF were subsequently cultured for an additional 48 h, and total RNA was extracted using AccuPrep® universal RNA extraction kit (Bioneer, Daejeon, Korea). The extracted mRNA was reverse transcribed into cDNA using the AmpiRivert cDNA synthesis platinum master mix (GenDEPOT, Barker, TX, USA), and then amplified via RT-PCR using primers specific to *TGF-β, SMAD-2, SMAD-3, COL1A1, PDGF, VEGF, bFGF,* and *GAPDH* (Table 1). The conditions for reverse transcription-polymerase chain reaction (RT-PCR) for gene amplification were as follows: the amplification process consisted of an initial denaturation at 95 °C for 10 min, followed by denaturation at 95 °C for 15 s, annealing at 62 °C for 30 s, and extension at 72 °C for 7 min, repeated for a total of 35 cycles. Each RT-PCR result was visualized as bands on a 1.5 % agarose gel with GelRed® nucleic acid gel stain (Komabiotech, Seoul, Korea) and quantified using the Davinch Gel Imager (CG-550, Davinch-K, Seoul, Korea) along with CLIQS 1D analysis software (ver. 1.6.454, TotalLab, Newcastle upon tyne, UK).

2.8. Analysis of main components

For qualitative and quantitative analysis of ginsenosides encapsulated in GNEV, GNEV was lyophilized using a freeze dryer (UniFreeze FD-8, Daihan Scientific Ltd., Seoul, Korea) at a pressure of 3.00 Torr and −80 °C for over 72 h to convert it into powder. The lyophilized sample was dissolved in 70 % v/v methanol and filtered using a 0.22 μm membrane filter (Hyundai Micro Co., Ltd., Seoul, Korea) for analysis.

Table 1
Primer sequences used in reverse transcription-polymerase chain reaction of major genes related to Skin Regeneration.

Primers	Forward (5′-3′)	Reverse (3′-5′)
TGF-β[a]	CAA CCT CGT GGA ACA TGA CAA	CGT GAC AGC TTC CCC TTC TG
SMAD-2[b]	TGT GCA GAG CCC CAA CTG T	CTG AGC CAG AAG AGC AGC AA
SMAD-3[c]	CAG TAC CCC CTG CTG GAT TG	TCT GGG TGA GGA CCT TGT CAA
COL1A1[d]	TCC AGG GCT CCA ACG AGA T	CCC CAA GTT CCG GTG TGA
PDGF[e]	TAC CAG CAA AAG CGC AAA GG	CGT CCA GAT CCA GTT GTG TGA
VEGF[f]	GCT GTC TTG GGT GCA TTG G	TGC AGC CTG GGA CCA CTT
bFGF[g]	TCT GTG GTG CCC TCT GAC AA	GGC TGC GGG TCA CTG TAC A
GAPDH	GAT GGG CAT GAA GCA TGA GA	TGG CAT GGA CTG TGG TCA TT

[a] *TGF-β*: transforming growth factor-β.
[b] *SMAD-2*: suppressor of mothers against decapentaplegic-2.
[c] *SMAD-3*: suppressor of mothers against decapentaplegic-3.
[d] *COL1A1*: collagen type I alpha 1.
[e] *PDGF*: platelet derived growth factor.
[f] *VEGF*: vascular endothelial growth factor.
[g] *bFGF*: basic fibroblast growth factor.

Subsequently, a simultaneous analysis of 14 ginsenosides, including F1, F2, Rb1, Rb2, Rg1, Rg2, Rg3, Rh1, Rh2, Rc, Rd, Re, Rf, and compound K, was performed using a UPLC Vanquish Flex (Thermo Fisher, Waltham, USA) equipped with a TSQ Altis™ Plus mass spectrometer (Thermo Fisher, Waltham, USA). The mobile phases used for the analysis, including acetonitrile (JT Baker, Phillipsburg, NJ, USA), DW (Milli-Q® IQ 7000, Merck, Darmstadt, Germany), and formic acid (Alfa Aesar, Haverhill, USA), were of HPLC grade.

2.9. Statistical analysis

The data were analyzed using GraphPad Prism Software 9 (San Diego, California, USA), and the experiments were repeated three times, and the results were presented as mean ± standard deviation. The significance between each experimental group was evaluated using one-way ANOVA and Student's *t*-test, and post-hoc verification was conducted using Duncan's new multiple range test to verify the significance differences at the $p < 0.05$ level.

3. Results

3.1. Characterization of EV

EV is phospholipid bilayer structures released outside the cell, reflecting the physiological state or pathological conditions of the parent cells, and high-yield isolation is essential for utilizing EV as therapeutics or diagnostic biomarkers [15]. Ultracentrifugation (UC) is a commonly used EV isolation method, but it has low purity; therefore, various alternative isolation techniques, including differential centrifugation (DC), SEC, and polyethylene glycol precipitation, have been developed [16]. Among these, SEC is a method that minimizes alterations in the cell membrane and morphology of EV, maintaining their functional characteristics. It allows for the rapid isolation and purification of EV compared to UC and does not involve interactions between the stationary phase resin and EV, making it a suitable method for high-yield EV production [17,18].

Accordingly, to isolate EV of the desired size, minimize damage to the phospholipid bilayer structure, and improve yield, GNEV was separated into 10 fractions using SEC [19]. Based on the FPLC results, a small peak in fraction 3 and a large peak spanning fractions 4 to 6 were identified, leading to the definition of fractions 3 to 6 as GNEV, which were then separated and purified (Fig. 1-A). Also, the average diameter of GNEV was determined to be 215.5 nm, with a significant number of exosomes (15–200 nm) and microvesicles (200–1000 nm) was confirmed to be distributed within this fraction (Fig. 1-B) [20]. In addition, the total particle number in GNEV was measured to be 1.1×10^9 particles/mL. These results indicate that GNEV can separate a higher concentration of EV than the study that separated 3.6×10^7 particles/mL of EV from ginger callus using DC and UC methods [21]. The purity of the EV was determined by the ratio between particle concentration and protein, which was found to be 9.7×10^7 particles/μg protein for GNEV [22]. In addition, the morphology images of GNEV was analyzed by transmission electron microscope (TEM), and it was found a spherical membrane vesicle with a phospholipid bilayer structure, confirming that it is a typical EV structure (Fig. 1-C).

3.2. Effect of GNEV on cell proliferation

Components such as hyaluronic acid, phospholipids, free fatty acids, ceramides, filaggrin, urea, amino acids, and peptides that constitute the skin barrier are synthesized by HDF and deposited in the ECM, forming the material structure and support of the skin [23]. These components play a crucial role in skin regeneration, making the production of skin barrier substances through HDF proliferation and growth an essential process for this regeneration.

Therefore, to quantitatively measure the impact of GNEV on HDF cell

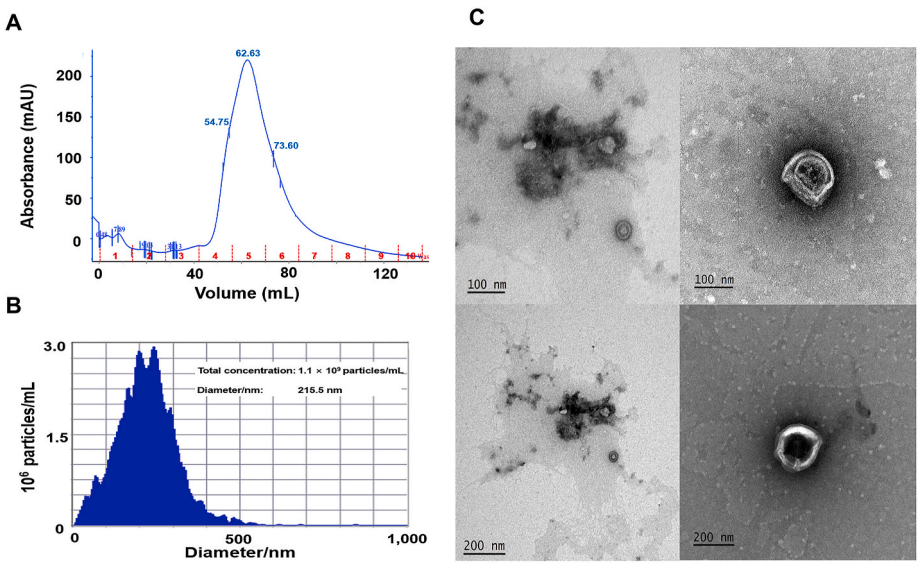

Fig. 1. GNEV characterization by transmission electron microscope (TEM), nanoparticle tracking analysis (NTA). (A) Distribution of fractions measured at 280 nm by size exclusion chromatography (SEC) using FPLC, (B) Size Distribution and Concentration of GNEV Using NTA, (C) Morphological characterization of GNEV via observation of lipid bilayer structure using TEM.

viability and proliferation, HDF were treated with GNEV at 1.1×10^9 particles/mL or lower. As a result, more than 90 % cell viability was maintained at all concentrations of GNEV, suggesting that GNEV emphasizes the safety of HDF cells. In particular, GNEV significantly increases cell viability at concentrations below 2.5×10^8 particles/mL particles/mL, suggesting its potential for enhancing cell activity ($p < 0.05$) (Fig. 2). Conversely, when GNEV was administered at concentrations of 5.0×10^8 and 1.1×10^9 particles/mL, cell viability significantly decreased to 94.8 % ($p < 0.05$) and 90.3 % ($p < 0.05$), respectively, compared to the untreated control group, indicating a substantial inhibitory effect on cell proliferation.

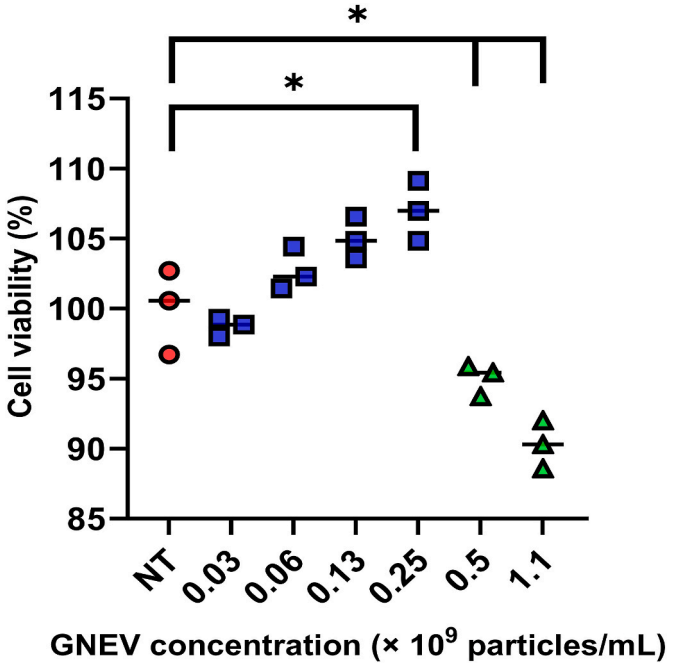

Fig. 2. Establishment of maximum non-toxic concentration through evaluation of the effect of GNEV treatment on HDF cell viability. The cell viability rate of non-treated group (N.T.) was expressed as 100 %, and bar value was calculated as percentage compared to non-treated group (*$p < 0.05$).

3.3. Effect of GNEV on cell migration

Skin regeneration depends on different stem cell compartments within the epidermis [24]. Despite variations in transcriptional and proliferative capacities, as well as anatomical locations, these stem cells play a crucial role in facilitating the proliferation and migration of skin cells for skin regeneration [25]. This process is essential for repairing damaged tissues or removing wrinkles, as it requires the movement of HDF to the damaged skin area, playing a vital role in skin regeneration [26].

To assess cell migration and skin regeneration ability, GNEV was treated in HDF at 2.5 particles/mL or lower. As the treatment concentration and duration increased, cell migration in the incision areas also increased (Fig. 3). These findings support the results of the cell proliferation evaluation, confirming that GNEV promotes the proliferation of HDF in a concentration-dependent manner. In particular, 24 h treatment with GNEV at 2.5×10^8 particles/mL increased cell migration by 2.5-fold compared with the non-treated sample group.

3.4. Effect of GNEV on angiogenesis gene expression

When skin wounds occur, proper blood flow and angiogenesis are essential for delivering nutrients and oxygen necessary for tissue regeneration. Therefore, the expression enhancement of angiogenic factors such as *PDGF, VEGF*, and *bFGF* is crucial for effective skin regeneration. *PDGF*, which increases with platelet activation, promotes cell proliferation and stimulates cell migration during skin regeneration to form a capillary-based regenerative tissue structure [27]. *VEGF* induces proliferation and migration of vascular endothelial cells in response to the nutrient and oxygen needs of newly formed capillaries, while *bFGF* not only mobilizes leukocytes to the wound site but also plays a role in HDF activation and proliferation [28,29]. Thus, for rapid skin regeneration, the interaction among these angiogenic factors is essential for ensuring a smooth supply of oxygen and nutrients, reducing inflammation, and promoting cell proliferation.

The cooperative action of growth factors regulates endothelial cell behavior and is crucial for forming vascular networks. Therefore, we evaluated the skin regeneration effects by comparing the expression of angiogenesis-related genes following GNEV treatment. GNEV (2.5×10^8 particles/mL) treatment increased the gene expression of *PDGF, VEGF,* and *bFGF* by 71.0 %, 15.9 %, and 37.0 %, respectively, confirming the

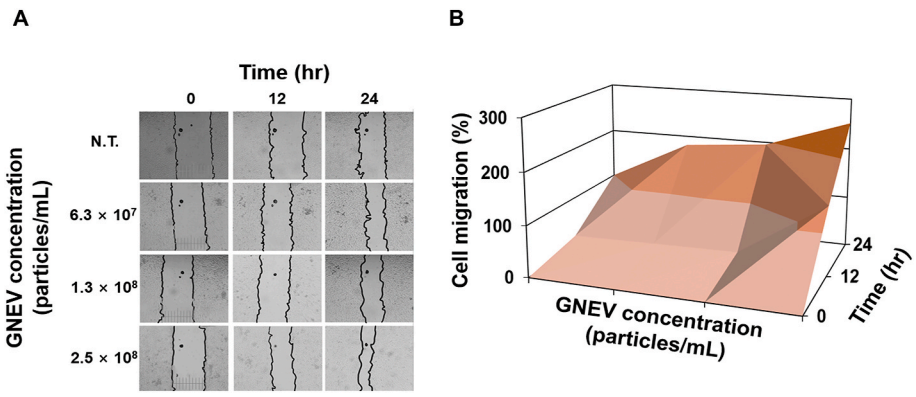

Fig. 3. Time-dependent changes in HDF cell migration following treatment with GNEV at different concentrations. (A) Images of wound-healing assay following treatment with GNEV at cell scratch area, (B) Comparison of reduction in scratch area due to GNEV treatment over time relative to non treated group (24 h).

skin regeneration effect through the promotion of angiogenesis ($p < 0.05$, Fig. 4-A).

3.5. Effect of GNEV on collagen synthesis gene expression

When sufficient blood is supplied to the wound site, oxygen transport increases, leading to the hydroxylation of proline and lysine, which promotes collagen synthesis that constitutes 90 % of the dermis [30]. Therefore, for effective skin regeneration, it is important to enhance the expression of *TGF-β, SMAD-2, SMAD-3,* and *COL1A1*, which are involved in collagen synthesis. *TGF-β*, a type of growth hormone, is reported to regulate the activation of downstream signaling pathways and ECM synthesis, as well as promote epithelial contraction and myofibroblast differentiation at the wound site [31]. *SMAD-2* and *SMAD-3* are involved in *TGF-β* signaling, regulating cell proliferation and migration, which are essential for skin regeneration, while *COL1A1* promotes re-epithelialization in the final stage of skin regeneration [32,33]. Therefore, for collagen synthesis and skin regeneration, it is essential to activate the *TGF-β/SMAD* signaling pathway by upregulating these four key genes.

Collagen fibrosis and tissue repair play important roles in skin regeneration, so the expression of key genes affecting collagen synthesis in the *TGF-β/SMAD* signaling pathway was evaluated to assess the skin regeneration effect of GNEV. Treatment with GNEV (2.5×10^8 particles/mL) increased the gene expression of *TGF-β, SMAD-2, SMAD-3*, and *COL1A1* by 41.7 %, 59.4 %, 60.2 %, and 21.8 %, respectively, confirming the skin regeneration effect through enhanced collagen synthesis ($p < 0.05$, Fig. 4-B).

3.6. Ginsenoside qualitative and quantitative analysis

Based on previous experiments evaluating the expression of genes related to angiogenesis and collagen synthesis, the aim was to identify the bioactive substances in GNEV that influence skin regeneration by analyzing the various bioactive compounds present in GNEV. Ginseng is known to have functionalities such as antioxidant, anti-inflammatory, anticancer, antidiabetic, immune-enhancing effects, and promote angiogenesis, which are reported to be due to ginsenosides, collectively referred to as saponins [34]. Therefore, considering that the ginsenoside content in ginseng leaves is reported to be approximately 2.1–5.0 times higher than in ginseng roots, qualitative and quantitative analysis of ginsenosides in GNEV, which is presumed to have similar physiological properties to ginseng leaf extract, is necessary [35–37].

To identify the bioactive substances contributing to the skin regenerative functionality of GNEV, we used UPLC-MS/MS to compare the retention times (RT) of GNEV and marker substances and conducted molecular weight (M.W.) distribution-based analysis. This confirmed that GNEV contains a total of four types of ginsenosides (Table 2, Figs. S1–S5). The RT of GNEV were 2.27, 2.29, 9.78, and 19.61 min, which showed high concordance with the RT of the standard ginsenosides Re, Rg1, Rb1, and Rg3, which were 2.28, 2.3, 9.82, and 19.62 min, respectively. Additionally, the precursor ions of GNEV at *m/z* 969.6, 799.54, 1107.7, and 783.58 matched those of the standard substances, confirming that the main substances are ginsenosides Re, Rg1, Rb1, and

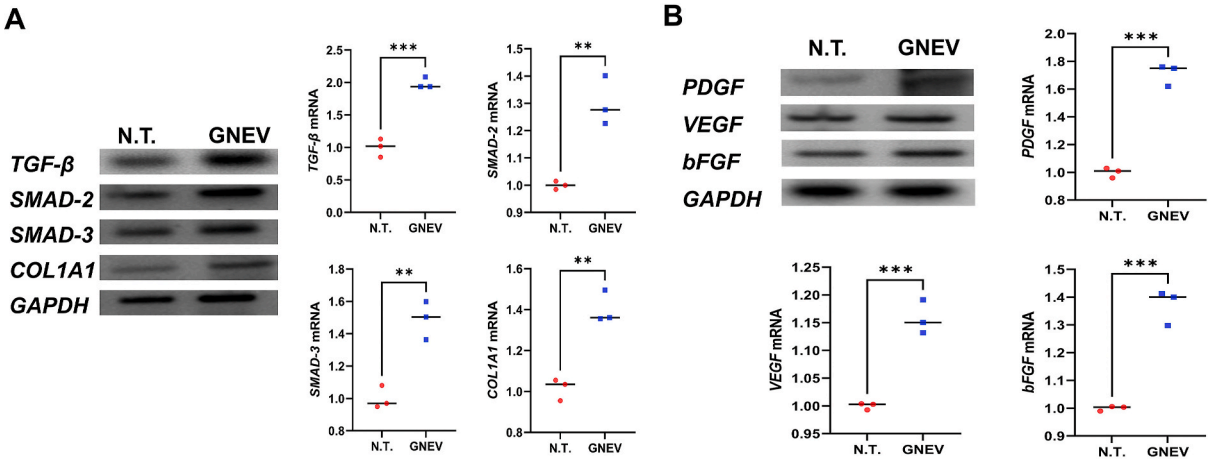

Fig. 4. Evaluation of skin regeneration effect of GNEV through comparison of (A) angiogenesis gene (*PDGF, VEGF*, and *bFGF*) and (B) collagen synthesize-related gene (*TGF-β, SMAD-2, SMAD-3*, and *COL1A1*) expression. The values are presented as mean ± standard deviation. Bars significantly different from non-treated group (N.T.) are marked with asterisk (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Table 2
Retention time and multiple reaction monitoring (MRM) results for identification of ginsenoside in GNEV.

Compound name	RT (min)	Formula	M.W.	Precursor (*m/z*)	Fragment (*m/z*)
Re	2.27	$C_{48}H_{82}O_{18}$	947.17	969.6 $[M+Na]^+$	365.20, 789.47
Rg1	2.29	$C_{42}H_{72}O_{14}$	801.02	799.54 $[M-H]^-$	637.5, 475.52
Rb1	9.78	$C_{54}H_{92}O_{23}$	1109.30	1107.7 $[M-H]^-$	179.10, 945.5
Rg3	19.61	$C_{42}H_{72}O_{13}$	785.03	783.58 $[M-H]^-$	621.46, 459.43

Rg3. For quantitative analysis, serial dilutions of the four standard ginsenosides were analyzed and calibration curves based on the peak area ratios were created to calculate the ginsenoside concentrations in GNEV, which were found to be 0.32, 0.17, 0.05, and 0.05 mg/kg for ginsenosides Re, Rg1, Rb1, and Rg3, respectively.

4. Discussion

In this study, the HDF proliferation, migration, and skin regeneration efficacy of SEC-separated GNEV were evaluated. Through electron microscopy analysis, GNEV was confirmed to exhibit the characteristics of EV, possessing an endoplasmic reticulum-like structure with a phospholipid bilayer. Furthermore, using SEC, it was determined to have an average size of 215.5 nm, and was separated successfully by SEC with a high concentration of 1.1×10^9 particles/mL, achieving a purity of 9.7×10^7 particles/μg protein. The SEC employed in this study, in contrast to DC and ultracentrifugation methods, separates EV based on the pore size within the column without direct interaction between the EV and the stationary phase, thus reducing the potential for EV damage and indicating a relatively high yield [38,39]. Therefore, the EV isolation method using SEC in this study is effective for isolating various types of EV with different biological functions. It allows for the high-yield isolation of EV from ginseng non-edible callus culture medium and can contribute to future research involving the application of EV and the use of GNEV.

Additionally, the formation of phospholipid bilayer structure is attributed to the exocytosis process, wherein the secretory endoplasmic reticulum fuses with the cell membrane, releasing EV from the cell [40]. The phospholipid bilayer forms the boundary between the hydrophilic external environment and the hydrophobic internal environment of the cell membrane, reducing substance dispersion due to the external hydrophobic environment [41]. This protects the encapsulated substances in GNEV from external environmental changes and allows them to serve as effective carriers for delivering bioactive substances between cells.

Moving to the effects of GNEV on cell proliferation, the study focused on human dermal fibroblasts (HDF), essential for synthesizing components crucial to the skin barrier. Treatment of HDF with GNEV increased cell viability at concentrations up to 2.5×10^8 particles/mL, but decreased cell viability at higher concentrations, highlighting the importance of dosage considerations. Therefore, it was concluded that applying a concentration of 2.5×10^8 particles/mL, which is the maximum non-toxic concentration with the greatest proliferation enhancement effect, is appropriate for subsequent evaluations of cell migration and the expression of skin regeneration factors in HDF treated with GNEV to assess skin regeneration.

GNEV's positive effect on HDF proliferation was extended to cell migration, a critical process in skin regeneration. The cell migration-enhancing effect of GNEV is similar to the report that ginseng root-derived EV promoted wound healing by promoting the migration of HDF, which research concluded that cell migration was enhanced by reducing the expression of apoptosis-related genes CDKN1A, CDKN2A, and P53 [42]. These results suggest that GNEV has a high similarity to the HDF cell membrane surface and can effectively penetrate the dermal layer of the skin through lipid fusion, so it is assumed that it was effectively absorbed into the HDF and that this promoted the movement of the HDF [43]. Recent studies have reported that ginseng root-derived EV not only promote the proliferation and migration of HDF, but also induce essential angiogenesis in the skin by promoting human-derived dermal keratinocyte (HaCaT) and human-derived vascular endothelial cell (HUVEC) [44]. Therefore, it is assumed that GNEV using non-edible parts of ginseng will also stimulate percutaneous regeneration and angiogenesis by inducing proliferation and migration of HaCaT and HUVEC.

In addition, the study delved into the angiogenic effects of GNEV, revealing an upregulation of key angiogenesis-related genes, including *PDGF, VEGF*, and *bFGF*. When skin damage occurs, *PDGF* induces the migration of platelets to the wound site and signals HDF to promote division and proliferation, while simultaneously increasing the expression of *VEGF* and *bFGF*, which enhances the expression of αvβ3 integrin, a type of cell adhesion molecule [45]. Activation of αvβ3 integrin induces the phosphorylation of focal adhesion kinase and activates the Ras/Mitogen-activated protein kinase signaling pathway, transmitting signals for endothelial cell differentiation, proliferation, and migration inside the cell to promote angiogenesis [46,47]. Upregulation of these growth factors supports the cell migration results that GNEV promote proliferation and migration of HDF in a concentration-dependent manner. Therefore, GNEV that increased the expression of *PDGF* to a high level was found to have angiogenic effect in this study.

Examining skin regeneration gene expression, GNEV significantly increased *TGF-β, SMAD-2, SMAD-3,* and *COL1A1* expression, indicating a role in promoting collagen synthesis. Increased expression of *TGF-β* in platelets and HDF during skin damage activates *TβRI*, which phosphorylates serine in the Ser-Ser-X-Ser sequence at the C-terminus of *SMAD-2* and *SMAD-3* and signals proliferation and migration of HDF [48]. The resulting *SMAD-2/SMAD-3/SMAD-4* complex activates the *TGF-β/SMAD* signaling pathway and translocates to the nucleus, where it regulates the transcriptional expression of collagen genes, including *COL1A1*, to promote collagen synthesis [49,50]. In particular, *SMAD-3* binds with the CAGACA sequence of *COL1A1* promoter, which is present in the transcriptional regulatory area of *TGF-β* and stimulates the transcription of *COL1A1* [51]. Hence, GNEV that increased the expression of *SMAD-3* to high levels was found to effectively promote collagen synthesis in this study.

Additionally, ginsenosides Re, Rg1, Rb1, and Rg3 were confirmed to be contained in GNEV, and were predicted to be key substances exhibiting the skin regeneration functionality of GNEV. These results are superior to recent studies detecting only 1 to 3 types of ginsenosides (Rg1, Rb1, and Rg3) in ginseng-derived EV and are consistent with the analysis of ginsenosides Re, Rg1, Rb1, and Rg3 by HPLC in studies on the antitumor effects of ginseng-derived EV [52,53]. This suggests that the EV isolation method used in this study can encapsulate more bioactive substances without destroying useful compounds. According to previous studies, ginsenoside Rg3 has been reported to increase the expression of cell proliferation genes in UV-exposed HDF, inducing ECM protein synthesis and cell proliferation, while Rg1 has been found to increase *iNOS* expression in diabetic animal models, accelerating wound healing [54,55]. Ginsenoside Re has been reported to induce proliferation and migration of endothelial cells, significantly enhancing the density of capillaries and ECM tissue hemoglobin content [56]. Ginsenoside Rb1 has been shown to greatly increase the expression of angiogenic factors in aged skin cells, demonstrating efficacy in wound healing and inducing increased expression of *COL1A1* [57,58]. Furthermore, ginsenosides have commonly been reported to increase collagen synthesis in HDF via phosphorylation of *SMAD-2*, suggesting that the physiological activities of these ginsenosides may have influenced collagen synthesis and angiogenic factor expression enhancement in GNEV [59].

In conclusion, this comprehensive study underscores GNEV's multifaceted positive impact on HDF viability, proliferation, migration,

angiogenesis, and skin regeneration gene expression. However, despite promising in vitro results, the in vivo efficacy and safety of GNEV remain unexplored, necessitating further studies in animal models and clinical trials to ensure its practical application. Hence, this study proposes the potential utilization of GNEV as a promising natural-derived therapeutic agent with skin regeneration effects, anticipating its high suitability as a material in cosmetics and pharmaceuticals for wrinkle improvement, collagen synthesis, and wound healing.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.08.002.

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