Abstract. Background/Aim: The skin plays an important role in protecting the body from mechanical damage, microbial infection, ultraviolet radiation, and extreme temperatures. Many products as well as ongoing studies have focused on skin injury and repair; however, unlimited challenges are still being faced. Furthermore, the drugs that are currently on the market are not adequate to meet the increasing medical needs. This study aimed to discover whether our new product can efficiently promote wound repair and skin restoration. Materials and Methods: In this study, we applied a new AIMP1-derived peptide (AdP), NeoPep S, administered in two dose types (1 ppm and 3 ppm), and determined their effect on skin wound repair in rat models. Cell proliferation and inflammatory responses were assessed using immunofluorescence (IF) staining and ELISA assay. Results: As expected, our results showed more rapid and satisfactory progress in wound closure upon treatment with NeoPep S 3 ppm than with NeoPep S 1 ppm. The 3 ppm peptide derived from AIMP1 protein, harmoniously interacted with the wound to promote re-epithelialization and collagen regeneration, as well as the down-regulation of several types of cytokines and chemokines, such as TNF-α, IL-6, IL-8, IL-1β, MCP-1, and F4/80. Moreover, it was demonstrated to promote fibroblast proliferation, migration, and differentiation by TGF-β1 and TGF-β3 modulation, as well as nitrite and reactive oxygen species scavenging. Conclusion: The novel peptide NeoPep S 3 ppm showed high effectiveness and safety in wound healing.

The skin is not only the largest organ of the human body and the most important protective barrier against external environmental agents, but it also has many other essential roles, such as temperature regulation, metabolism, absorption, reception, and secretion, as well as immunity. Therefore, the consistency of the skin structure in ensuring the complete performance of all the functions is crucial. However, skin can be affected by irregular external factors, such as mechanical, thermal, chemical, and radiative factors, leading to damage and imbalance, or serious structural effects on function (1, 2). Following these injuries, the human body generally initiates a multi-stage wound repair and healing process that includes: hemostasis, inflammation, proliferation, and remodeling. Many variables, including cell types such as keratinocytes, fibroblasts, macrophages, platelets, and endothelial cells, as well as cell-derived mediators, such as vascular endothelial growth factor (VEGF), transforming growth factor-beta, interleukins, and platelet-derived growth factors, tightly control and coordinate each stage (3, 4).

Despite the fact that skin regeneration is a natural process, a variety of conditions may interfere with the phases of healing, producing extensive healing or chronic wounds. Pathogens can more easily infiltrate and harm chronic wounds, resulting in inflammation, septicemia, electrolyte and water...
imbalances, multi-organ damage, and secondary diseases, as well as increasing the psychological burden and financial economy of patients (5). The formation of scar tissue and keloids are common side effects of extensive wound healing that can influence the aspect, function, and esthetics of the skin (6). Both chronic wounds and extensive healing have an impact on the biological mechanisms of the skin, resulting in the creation of more significant health problems, as well as increased clinical treatment charges (7-9).

Many efforts have been made to identify effective procedures for wound healing treatments. Although there are many new technologies that have been applied that are attractive, including stem cells and tissue engineering, other therapies are still urgently being researched (4, 10-12). Nowadays, the most common medical drugs consist of small molecules and grown factors. However, these single-type drugs have many disadvantages, including: being unstable carriers and being difficult to synthesize; carrying out unsatisfactory activities; needing to be stored under strict situations; being easily rendered inactive through storage or transport; and, most importantly, their use may result in intemperate wound repair, giving rise to hypertrophic scars (13-15). Nevertheless, wound repair and skin restoration still face unlimited challenges, and the current drugs on the market are inadequate to meet the increasing medical needs. Therefore, finding and developing new medications with high activity and low production costs is important.

In the last five years, 15 peptide molecules and peptide derivatives drugs have been approved by the U.S. Food Drug Administration (FDA) with many great successes, such as Afamelanotide Scenese®, Semaglutide Ozempic®, Bremelanotide Vyleesi®, and Abaloparatide Tymlos®, accounting for 7% of the total number of drugs (208 new drugs) and 25.8% of the biological drugs (16, 17). These peptide drugs indicate many advantages worth considering, including straightforward compound discoveries, high affinity and specificity for receptors in vivo, ideally high reliability, finite poisonous metabolism, rapid approval rate, low levels of accumulation, and fewer medication interchanges (18, 19). However, among these achievements, very few non-growth factor peptides have emerged to have the capability to improve the skin wound repair process (20). Thus, wound healing peptides with increased activity, high stability, and low cost are still needed in the market.

Aminocyl tRNA synthetase complex interacting 1 (AIMP1) is a prominent protein with many applications in medications. The drugs developed from AIMP1 protein and its derivatives have been demonstrated for use in therapies for systemic lupus erythematosus, alopecia, and rheumatoid arthritis (21-23). Moreover, AIMP1 has been modified under peptide variants, i.e., so-called AIMP1-derived peptides (AdP). In addition, the impacts of the derivatives of the protein on skin cells have opened up many possibilities for cutaneous studies. For example, the potential of AdP (amino acid region 6-46) in skin repair was shown through ERK activation, resulting in fibroblast proliferation and collagen generation that improved wound healing (24, 25). In the cosmeceutical field, AdP was applied as an ingredient in an anti-aging and whitening cosmetic product (26, 27). Nevertheless, the potential of AdP in skin wound repair has not yet been discovered. In this study, we applied a new generation of AdP, NeoPep S cream, containing a short peptide chain with a length of 15 amino acids, and carried out in vivo experiments to examine the contribution as well as effect of NeoPep S on the wound healing process. The experiments were performed using H&E staining, MT staining, immunofluorescence staining, and ELISA assay. The results from NeoPep S showed great efficacy, including promotion of the wound healing process and inhibition of inflammatory cytokines, indicating that it could be a promising drug for the treatment of wounds.

Materials and Methods

Materials and reagents. Fetal bovine serum (#16000044) and Dulbecco’s Modified Eagle Medium (DMEM) (#11965118) were purchased from Gibco (Grand Island, NY, USA). Nitrite and Griess reagent (modified) were ordered from Sigma Aldrich (St. Louis, MO, USA). PRO-PREP Protein Extraction Solution (#17081) was from iNtRON (Seongnam, Gyeonggi, Republic of Korea). The Pierce™ BCA Protein Assay Kit (#23225) was from Thermo Fisher Scientific (Waltham, MA, USA). Tissue Extraction Reagent I was ordered from Invitrogen (Carlsbad, CA, USA). Mouse monoclonal IgG1 k vimentin antibody (sc-6260), smooth muscle actin antibody (sc-53015), and F4/80 antibody (sc-377009) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Purified hamster anti-mouse/rat TNF (cat: 557516), purified mouse anti-rat MCP-1 (cat: 555072), and purified mouse anti-rat IL6 (cat: 550644) were obtained from BD Pharmingen™ (San Diego, CA, USA). Rat IL-1 beta (cat: MAB501) and rat IL-8 antibody were purchased from R&D Systems (MN, Canada). Rabbit anti-mouse IgG (H+L) Cross-Adsorbed Secondary Antibody Alexa Fluor 488 (#A-11059) was from Invitrogen (Carlsbad, CA, USA). ELISA kits (TNF-α, IL8, and IL-1β) were obtained from MyBioSource (San Diego, CA, USA). The nitrocellulose membrane was a product of Bio-Rad (Hercules, CA, USA). Anti-TGF beta 1 antibody was from Abcam (1:100, #ab92486; Cambridge, MA, USA).

Peptide preparation and examination. The NeoPep S peptide with high purity (>95%) used in this research was synthesized and prepared by CG Bio Co., Ltd (Seoul, Republic of Korea). A new variant of AIMP1-derived peptide composed of 15 amino acids, 1,700 Da, was generated. The original substance was formed under white powder, then creamed in distilled water with neither color nor fragrance. The creams, at a mass of 1 ppm and 3 ppm, were tested and then used to treat wound areas after surgery.

In vivo wound healing experiment. For the in vivo wound experiment, we used 8-week-old Sprague Dawley rats (BioOrient Company, Republic of Korea) weighing 250-300 g that were grown for 12/12 h in a light/dark cycle and maintained in pathogen-free...
conditions with free access to food and drinking water. The animal experiments were authorized by the Institutional Animal Care and Use Committee of Bundang Seoul National University Hospital (approval number: BA-1802-241-014-08), and all the methods followed the NIH Guidelines for the Care and Use of Laboratory Animals. Ten rats from each group were wounded and then treated with NeoPep S creams at 1 ppm and 3 ppm doses separately. The animals were first anesthetized with isoflurane (Hana Pharm, Republic of Korea), the dorsum was shaved, and their skin was then cleaned with 70% alcohol and betadine. A 10-mm-diameter round wound was made by a puncture on the skin in the mid-dorsal region. The wounds were treated with the creams and covered with a light dressing. The cream treatments were repeated once a day for 10 days. We monitored the wound healing progress daily and calculated the wound sizes. Moreover, five rats per group were chosen randomly and biopsied on days 7 and 10, respectively. The 12-mm-diameter circle area of the wound tissue for biopsy consisted of the epidermis and dermis.

Figure 1. NeoPep S promoted the wound healing process, re-epithelialization, and collagen deposition. (A) Wound healing was analyzed postsurgery. Wound closure photos were captured from the control, NeoPep S 1 ppm, and NeoPep S 3 ppm on days 0, 3, 7, and 10 post-surgery by macroscopic photography. (B) Schematic diagram of the healing process of all groups by wound sizes. The circle represents the entire wound, while the sections in skin color and white color indicate the healing and unhealing areas, respectively. (C) Quantification analysis of wound size at days 0, 3, 7, and 10 show that NeoPep S promoted the wound healing process in vivo. (D) H&E staining images of the wound groups on day 7 and day 10. (E) Re-epithelialization thickness of each group on day 7 and day 10. (F) MT staining images of all groups on day 7 and day 10. (G) Collagen density of various groups on day 7 and day 10. Statistical differences were performed using an unpaired two-tailed t-test. ****p<0.0001, ***p<0.001, **p<0.01, and *p<0.05 compared with the control group.

Wound size measurement. The kinetics of the wound closures from all samples was captured using digital photography on days 0, 3, 7, and 10 after removing all wound dressings. The wound sizes were then measured and calculated using ImageJ software (version 1.53c, National Institute of Health, Bethesda, MD, USA). The following equation was used to calculate the percentage of wound healing:

\[
\text{Percentage of wound healing} = \left(\frac{A_0 - A_n}{A_0}\right) \times 100\%
\]

where \(A_0\) represents the original wound size immediately after surgery, and \(A_n\) indicates the wound size on day \(n\) after surgery. The rats in each group were analyzed on the same day to evaluate the approximate levels of wound closure.
**Hematoxylin and eosin staining.** The tissue sections underwent deparaffinization in xylene for 30 min before being rehydrated in different declining concentrations of ethanol (100%, 95%, and 80%), and were then washed with distilled water every 5 min. To reduce background staining, after 5 min in hematoxylin, the samples were rinsed for 3 min in PBS. The samples were then stained for 2 min with eosin, rinsed for 5 min with distilled water, and then dehydrated every 5 min in 80%, 90%, and 100% ethanol, followed by 15 min in xylene. Finally, the slides were mounted with neutral resin and coverslipped. A light microscope (Zeiss Axioscope.A1, Carl Zeiss, Göttingen, Germany) was used to examine the stained sections and to capture photographs. The thickness of the epidermis was measured using ImageJ software, and statistical analysis was performed.

**Masson’s trichrome staining.** Following the manufacturer’s indicated techniques, the tissue sections were performed through deparaffinization and rehydration, and then stained using a Masson’s trichrome staining kit. Briefly, the cell nuclei were stained for 5 min with A1:A2 (1:1), and then thoroughly rinsed with water and soaked in acid alcohol for 3 s to differentiate. The fibrous tissues were stained for 5 min with a Ponceau acid fuchsin solution, and then followed with a series of 1 min with 2% acetic solution, 30 s with phosphomolybdic for differentiation, and 20 s with aniline blue before being dehydrated, mounted, and coverslipped, as described above. Masson’s trichrome stained tissues were used to assess collagen fiber synthesis and intensity by using ImageJ software.

**Immunofluorescent staining.** The tissue sections underwent deparaffinization and hydration, followed by the blocking of the non-specific binding sites by dropping 4% BSA in PBS at RT for 1 h. The blocked sections were then incubated with a mouse monoclonal IgG1 k vimentin antibody (1:100, sc-6260, Santa Cruz Biotechnology) diluted in 4% BSA in PBS solution overnight at 4˚C. After washing with PBS, a rabbit anti-mouse IgG Alexa Flour 488®-conjugated secondary antibody (1:1,000, #A-11059, Invitrogen) was prepared and incubated for the sections in the dark for 1 h at RT, followed by nuclear labeling with DAPI and mounting with antifade mounting medium. Imaging of the fluorescent signals was then performed using a confocal microscope (Zeiss LSM 710 or LSM 800, ZEN software, Germany). The images were then analyzed for vimentin intensity using ImageJ software.

**ELISA assay.** The tissue sections from the wound area were obtained from the rats on days 7 and 10 post-surgery, and then immersed in liquid nitrogen to snap freeze. The samples were next homogenized in PRO-PREP™ Protein Extraction Solution (C/T) (#17081, iNtRON, Republic of Korea) following the manufacturer’s suggested protocol. After the last step of centrifugation at 14,000 rpm for 15 min at 4˚C, the supernatants were collected and the expression levels of TNF-α, IL-1β, IL-6, MCP-1, and IL-8 cytokines in the wound tissues were determined using ELISA kits (MyBioSource, CA, USA) following the manufacturer’s instruction accordingly.

**Nitric oxide (NO) assay.** A nitric oxide assay was used for the quantitative determination of nitrite and nitrate in the wound tissue lysates using the Griess modified reagent. The wound sections were collected on day 7 and day 10, and protein was extracted as described above. The extracts were cleared by centrifugation at 14,000 rpm for 15 min at 4˚C, and then the supernatants were collected and diluted 1:1 with distilled water. A mix of 100 μl of 1X Griess modified reagent and 100 μl of diluted protein extract was incubated at room temperature for 15 min. The absorbance was measured at the wavelength 540 nm. The nitrite standard solution was prepared for dilution with ranges from 0 to 50 μM.

**DCF-DA assay.** The wound sections were collected on days 7 and 10, and protein was extracted as described above. The extracts were cleared by centrifugation at 14,000 rpm for 15 min at 4˚C, and then the supernatants were collected. Next, 20 μM of DCF-DA solution was added and incubation occurred at 37˚C, 5% CO₂ for 45 min in the dark. The fluorescent signals were read at 485 and 535 nm excitation and emission, respectively.

**Evaluation of NeoPep S with LPS.** Raw 264.7 cells (2.5×10⁵ cells/well) in 24-well plates were cultured at 37˚C, 5% CO₂ overnight. Next, concentrations of LPS at 500 ng/ml were co-incubated with or without NeoPep S at 0.5, 5, and 50 μg/ml at 37˚C, 5% CO₂ for 3 h. The supernatants were collected and stored at –80˚C for further experiments such as ELISA assay and nitric oxide assay.

**Evaluation of intracellular ROS level.** Raw 264.7 cells (12×10⁴ cells/well) in 96-well plates were grown and incubated at 37˚C, 5% CO₂ overnight. Subsequently, concentrations of LPS at 500 ng/ml were co-incubated with or without NeoPep S at 0.5, 5, and 50 μg/ml at 37˚C, 5% CO₂ for 3 h. All media were removed and washed with 1× PBS buffer. The cells were washed with 1× PBS buffer, 20 μM of DCF-DA solution was added, and incubation occurred at 37˚C, 5% CO₂ for 45 min in the dark. The fluorescence was measured using a microplate reader at 485 and 535 nm excitation and emission, respectively.

**Evaluation of nitric oxide production.** Inflammation of raw 264.7 cells was conducted using 500 ng/ml LPS with and without NeoPep S (0.5, 5, and 50 μg/ml) co-incubated for 3 h at 37˚C, 5% CO₂. Next, 100 μl of the inflamed media supernatant was mixed with 100 μl of 1X Griess modified reagent. The mixed solution was incubated at room temperature for 15 min. Subsequently, measurements of nitric oxide were determined by carrying out a microplate reader of the absorbance at 540 nm.

**Cell viability.** NIH-3T3 cells (4×10⁴ well/cell) were grown in 96-well plates overnight. The cells were then treated and incubated with 0.5, 5, and 50 μg/ml of NeoPep S for 24, 48, and 72 h. The viability of the cells was analyzed using the MTT assay. In brief, the cells were incubated with media containing 0.5 mg/ml MTT at 37˚C, 5% CO₂ for 4 h. Subsequently, all media were removed and rinsed with 1phosphate buffered saline (PBS). The formazan product was dissolved in 100 μl of dimethyl sulfoxide. The absorbance at 570 nm was then measured. The viability of the non-treated cells and the cells treated with NeoPep S were evaluated.

**Statistical analysis.** All information was gathered through measurements and provided as mean±standard error of the mean (SEM). Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA), and the differences between the groups were established using an unpaired two-tailed t-test. To be considered statistically significant, we required a p-Value <0.05 (95% confidence interval) for three to five independent experiments.
Results

Effect of NeoPep S on wound healing in vivo. The NeoPep S 1 ppm and 3 ppm were applied to full thickness wounds every day to investigate *in vivo* wound healing. All the rats survived and in time showed gradual wound healing without infection. The wound healing process was photographed at days 0, 3, 7, and 10, and the percentage of wound healing was then measured. In the NeoPep S 1 ppm and 3 ppm groups, the wound healing size showed faster wound closure than for the control group each time. As shown in Figure 1A-C, the wound healing size was 67.88% in the control group after 3 days, whereas the wound healing sizes in the NeoPep S 1 ppm and 3 ppm groups were 48.75% and 42.97%, respectively, for the same period. On day 7 post-wounding, application of the NeoPep S 1 ppm and 3 ppm resulted in 16.39% and 12.20% wound closure, respectively, whereas only 25.33% wound closure was observed in the control group. The wounds treated with NeoPep S 1 ppm and 3 ppm were completely healed with 3.62% and 2.76% wound closure, respectively, by day 10, while 10.56% of the wound closure remained unhealed in the control group. The quantification of the wound healing size revealed that the NeoPep S 3 ppm group had the highest percentage of wound closure, which was considerably different from that in the control group.

In order to further evaluate the wound healing, a histological analysis was conducted using H&E staining to determine the restoration level of the epidermis layer. Figure 1D presents the H&E staining pictures of all the groups on day 7 and day 10. It is clear from the results that 7 days after surgery, there was already epidermis formation in all groups. The thickness of the re-epidermis tissue in each group was measured based on H&E staining. On day 7, the average thickness of the re-epithelialized tissue in the NeoPep S 3 ppm group was the highest, followed by that in the NeoPep S 1 ppm and control groups, respectively. The results of day 10 showed a similar trend after a certain increase. The thickness of the re-epithelialized tissue in the NeoPep S 3 ppm and NeoPep S 1 ppm groups were both higher than that of the control group (61.80±3.92 μm), at 73.01±3.59 μm and 80.5±5.33 μm, respectively. A histological evaluation of the wound sections observed a decrease in wound width for the NeoPep S groups compared with the control group at days 7 and 10 of wound healing (Figure 1E). In addition, at days 7 and 10, a histological examination of the H&E-stained wound sections demonstrated a reduction in wound width for the NeoPep S 1 ppm and 3 ppm treatment groups, compared with the control group. The wound width and thickness of the re-epithelialized tissue in the NeoPep S 3 ppm group was consistently the highest among all groups during the whole wound healing process.

Collagen production and deposition are also important for wound healing. As shown in Figure 1F, Masson’s trichrome staining revealed that the collagen fibers were relatively rare and disordered in the untreated group, while those in the NeoPep S 1 ppm and 3 ppm groups were bundled on day 7. On day 10, the intensity of the collagen fibers in the NeoPep S 1 ppm (37.04%) and NeoPep S 3 ppm (39.55%) groups was significantly denser compared with those in the control group (28.68%) (Figure 1G). We can conclude from this result that the treated wounds in the NeoPep S groups (especially the NeoPep S 3 ppm group) had a higher collagen content than that in the control group. Taken together, these results show that NeoPep S 3 ppm accelerates wound healing by promoting inflammation in the early stages as well as collagen synthesis.

NeoPep S promoted fibroblasts. To evaluate the effect of NeoPep S 1 ppm and NeoPep S 3 ppm on wound healing, the collected tissues from days 7 and 10 were stained with vimentin, which acts as an integrator of the wound healing processes, controlling fibroblast proliferation and the EMT-like transdifferentiation of keratinocytes, both of which are critical for successful wound repair using the immunofluorescence (IF) staining method. As Figure 2A-B shows, the intensity of vimentin significantly increased in both NeoPep S groups compared with the control group over time. On day 7, the vimentin intensity was highest in the NeoPep S 3 ppm group (68.71±2.12), while it was 64.97±1.92 and 45.44±2.4 in the NeoPep S 1 ppm group and the control group, respectively. The same trend was observed on day 10. The NeoPep S 3 ppm group still released the highest vimentin intensity at 78.86±7.17, followed by the NeoPep S 1 ppm group at 70.21±2.24, while the vimentin intensity in the control group was the lowest at 51.71±2.70 (Figure 2C).

NeoPep S supported the down-regulation of pro-inflammatory cytokines in the wound. To quantify the remaining inflammatory cytokines during the wound healing processes, tissues from days 7 and 10 were stained with inflammatory cytokine biomarkers, such as IL-1β and IL-6, using the IF staining method. Regarding the IL-1β biomarker, a key mediator of the inflammatory response, a significant reduction in intensity was revealed in the NeoPep S 3 ppm group-treated wound sites on day 7 at 55.05±3.59, whereas they were at 65.37±3.29 and 97.05±4.64 for the NeoPep S 1 ppm and control groups, respectively (Figure 3A-C) for the same period. On day 10 post-wounding, the intensity of IL-1β in the NeoPep S 3 ppm group was the lowest at 44.39±2.16, followed by the NeoPep S 1 ppm group at 53.54±2.89, and only 80.62±5.99 IL-1β intensity was observed in the control group (Figure 3). In addition, IL-6, a key modulator marker of inflammatory cytokine, was also examined using the IF staining method. The results showed there was a significant down-regulation of the cytokine profile during the examination time, indicating a
sharp reduction in inflammation. The IL-6 intensity was relatively high in the control group (71.27±4.35) but was significantly decreased in the NeoPep S 1 ppm group (42.38±2.25) and NeoPep S 3 ppm group (38.58±2.16). Similarly, on day 10, the NeoPep S 3 ppm group showed the lowest IL-6 intensity at 32.50±1.52, whereas 38.76±2.25 and 57.21±3.97 were observed in the NeoPep S 1 ppm and control groups, respectively (Figure 4A-C).

**Effect of NeoPep S on cytokine expression, nitric oxide, and ROS level in vivo.** To measure the expression of cytokines in the wound healing tissues, proteins were purified from collected skin tissues from day 7 and day 10 using a protein extraction kit. Cytokine expression was quantified by ELISA assay. The concentration of IL-1β in the NeoPep S groups was observed as lower than that in the control group over time. The concentrations of IL-1β in the NeoPep S 1 ppm (26.01±2.82 pg/mg, p=0.037) and NeoPep S 3 ppm (21.05±1.94 pg/mg, p=0.034) groups at day 7 were lower compared with those in the control group (41.50±5.78 pg/mg). In addition, at day 10, the concentration of IL-1β in the NeoPep S 3 ppm group was the lowest (13.62±1.07 pg/mg, p=0.034) compared with those in the NeoPep S 1 ppm (14.78±0.75 pg/mg, p=0.028) and control groups (18.8±1.3 pg/mg), respectively (Figure 5A). Furthermore, Figure 5B-E shows a similar trend for IL-6, MCP-1, TNF alpha, and IL-8, and they decreased over time. These findings suggest that NeoPep S 3 ppm has a substantial anti-inflammatory impact and aids in the wound healing process.

Similarly, we determined the level of reactive oxygen species (ROS) at day 7 and day 10, and the inflammation in wound was observed with a high expression level of ROS. Interestingly, the ROS levels in theNeoPep S 1 ppm and 3
ppm groups were reduced compared with those in the control group over time. The level of ROS in the NeoPep S 3 ppm group at day 10 showed the strongest reduction (Figure 5F-G). Based on these results, we believe that NeoPep S 3 ppm could be introduced as a wound healing treatment to remove excess pro-inflammatory mediators, such as nitric oxide and ROS.

Moreover, to determine the function of NO during wound healing and in the regeneration of new tissue in the skin of rat models in vivo, purified proteins from the collected tissues at day 7 and day 10 were analyzed to measure the concentration of nitrate (i.e., an end product of nitric oxide generation). High levels of NO cause inflammation, later initiating the synthesis of pro-inflammatory mediators and the degradation of extracellular matrix components. As shown in Figure 5H, it was observed that the concentration of NO in NeoPep S decreased over time compared with that in the control group. In particular, the concentration of NO in the NeoPep S 3 ppm group on day 10 was measured at 17.63±1.41 μM/mg (p=0.016), while it was measured at 18.07±1.62 μM/mg (p=0.03) in the NeoPep S 1 ppm group, and 20.21±0.81 μM/mg in the control group (Figure 5H).

**Effect of NeoPep S on inflamed raw 264.7 cells.** The effect of NeoPep S on LPS-induced inflammatory cytokine production was quantified by using raw 264.7 cells. Raw 264.7 cells were co-treated with 0.5, 5, and 50 μg/ml of NeoPep S, and 500 ng/ml of LPS for 3 h. The level of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and MCP-1, in the supernatant of the media was determined using the ELISA method. As shown in Figure 6A, the concentration of TNF-α in the LPS-stimulated raw 264.7 cells showed a more than ten-fold increase compared with that in the control group. However, the level of TNF-α production reduced with the NeoPep S treatment. In particular, 50μ/ml of NeoPep S significantly
inhibited TNF-α production in the LPS-stimulated raw 264.7 cells. The concentration of TNF-α decreased by over 70% compared with that in the LPS group.

Similar results were exhibited for other inflammatory cytokines, such as IL-6, IL-1β, and MCP-1 (Figure 6B-D). The concentration of inflammatory cytokines surged by a large margin in the LPS group compared with the control group, showing that LPS can give rise to the secretion of inflammatory cytokines. However, the concentration of inflammatory cytokines declined to relatively low degrees with an increasing concentration of NeoPep S treatment. In short, this finding revealed that NeoPep S can inhibit the secretion of inflammatory cytokines and play a significant role in relieving inflammation. This function contributes to wounds recovering quickly during the wound healing process.

Moreover, the effect of NeoPep S on nitric oxide production in the culture supernatant of the inflamed raw 264.7 cells was examined using an NO assay method. As shown in Figure 6E, NO production was significantly increased in the LPS-treated raw 264.7 cell group compared with the control group. The amount of NO production in the inflamed raw 264.7 cells treated with different concentrations of NeoPep S showed meaningful declines, ranging between 72.79% and 87.56%.

Furthermore, the effect of NeoPep S was examined by looking at the ROS level in the raw 264.7 cells. As demonstrated in Figure 6F, the LPS-induced 264.7 cells produced a ROS level that was two-fold higher than that in the control group. Meanwhile, the levels of ROS production in the NeoPep S treatment groups were significantly decreased compared with those in the LPS treatment group. At a high concentration of NeoPep S, there was a considerably reduced ROS level in the LPS-stimulated raw 264.7 cells (Figure 6F).
**Discussion**

The multi-aminoacyl-tRNA synthetase complex is linked to AIMP1 and was identified as a cofactor protein, providing an efficient aminoacyl-tRNA synthesis trafficking pathway for translation (28). Furthermore, AIMP1 levels are higher in damaged skin, and the roles of AIMP1 in the wound healing process (e.g., stimulating re-epithelialization and revascularization, neutralizing LPS, and performing as immunomodulators) are worth noting (29-35).

The process of wound healing is impacted by a variety of factors. In the present research, we investigated the effects of NeoPep S on the process of wound healing, confirmed as: faster re-epithelialization, granulation tissue thickness, increased fibroblasts, elevated collagen deposition, and decreased inflammation. Moreover, NeoPep S can inhibit the inflammatory production of cytokines such as TNF-α, IL-1β, MCP-1, IL-6, and IL-8 in LPS-stimulated raw 264.7 cells, suggesting that NeoPep S may contribute to the wound healing process via the promotion of fibroblast migration in the wound site.

Wound healing is a complicated process consisting of a series of steps, including hemostasis, inflammation, proliferation, migration, extracellular matrix formation, and remodeling to...
reconstruct the natural barrier between the body and the external environment (36). A variety of wound-related signaling networks contribute to re-epithelialization, such as nitric oxide, which is mostly produced by macrophages (37), cytokines, and growth factors, including heparin-binding epidermal growth factor (HB-EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), nerve growth factor (NGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), secreted from many cell types in the wound (38-43). In our in vivo study, the formation of re-epithelialization occurred more quickly compared with the control group on days 7 and 10. The addition of NeoPep S promoted the fast formation of the epidermis layer, as well as wound closure. These results may be explained by the enhancing rate of proliferation, migration, and differentiation of keratinocytes in response to NeoPep S.

Macrophages and inflammation are known to play an important role in wound healing through inflammation, proliferation, and remodeling. Inflammation and chronic inflammation are both important stages in wound healing. Shortly after an injury, the innate immune system is activated, triggering a local inflammatory response that initiates the complement cascade and leads to neutrophil infiltration surrounding the wound site, the primary role of which is to prevent infection. Neutrophils phagocytose foreign substances and bacteria in the wound environment, killing them using proteolytic enzymes and oxygen-derived free radical species. However, the long-time gathering of leukocytes will influence the wound healing process. In the current investigation, the wounds treated with NeoPep S demonstrated a reduced level of inflammatory cytokines, such as IL-1β, TNF-α, MCP-1, IL-6, and IL-8 (via ELISA assay and the immunofluorescence staining method) compared with those in the control group, as evidenced by the considerably lower neutrophil and macrophage production in the wound bed. In wound healing, the local macrophage population shifts from the pro-inflammatory (M1 phenotype) to anti-inflammatory (M2 phenotype). Macrophages in M1 have the ability to kill off pathogens in wounded tissues that are virally infected, whereas macrophages in M2 play an important role in the formation of granulation tissue, cell proliferation, and tissue repair (44-46). The polarization of macrophages from M1 to M2 has been reported to help in the process of wound healing and regeneration (47, 48). In our research, treatment of the wounds with NeoPep S promoted the polarization of the macrophages forward to the M2 phenotype. The levels of pro-inflammatory cytokines in our in vivo research were decreased in the treated wounds in the NeoPep S groups. Moreover, the results of this in vivo study demonstrated that NeoPep S suppresses the production of inflammatory cytokine...
biomarkers, including IL-1β, TNF-α, MCP-1, and IL-6, secreted from inflamed raw 264.7 cells. Therefore, the decrease in inflammation cytokines in the treated wounds in the NeoPep S groups and the inflamed raw 264.7 cells suggests that NeoPep S contributed to inhibiting the inflammation and improving the wound-healing process observed in this study.

Fibroblasts are crucial in processes such as breaking down the fibrin clot and establishing new extracellular matrix and collagen structures to assist other cells that are needed for efficient wound healing, as well as wound closure (49). Vimentin is expressed in activated fibroblasts and play an important role in extracellular matrix production, TGF-1β–slug signaling, collagen accumulation, and the remodeling that occurs during wound healing (50, 51). Recent studies have shown that vimentin knockout mice present systemic defects that are related to development and wound repair (52, 53). Therefore, the results showed the intensity of vimentin observed at days 7 and 10 using an immunofluorescence assay (Figure 2A-C). The expression of vimentin in the wounds treated with NeoPep S was higher compared with that in the control group over time. The NeoPep S 3 ppm group measured the highest expression during the wound healing process. In our in vivo experiment, the results showed high expression of vimentin similar to that found in previous studies, in which the role of vimentin in wound healing was discovered (50, 54). Our findings showed that NeoPep S may contribute to the process of wound healing through the expression of vimentin by regulating fibroblast proliferation, collagen accumulation, and epithelial–mesenchymal transition (EMT), all of which influence keratinocyte activation. Furthermore, the process of collagen synthesis plays an important role in each phase of wound healing. Collagen encourages the formation of new collagen in the wound bed by the stimulation of fibroblasts. It contributes to the physical strength and flexibility of the tissue (55). Delayed or extended collagen deposition, on the other hand, can lead to the creation of chronic inflammation of wounds and scars. Therefore, the support of balanced collagen deposition may be beneficial to a successful cell therapy (56). As shown in Figure 1F and G, in the in vivo experiment, the synthesis of collagen in the wounds treated with NeoPep S was displayed at a higher expression level than that in the control group. These results show that NeoPep S plays an important part in promoting wound repair.

Recent research from both animal and human studies have recognized that nitric oxide (NO) plays an important role in biological functions. NO has been observed in variety of wound healing processes, such as inflammation response, cell proliferation, collagen synthesis in wound healing, vasodilatation, angiogenesis, tissue fibroblasts, antimicrobial activity, and immunological responses (57, 58). The key role of NO in wound healing has drawn a lot of attention and led to NO-based wound healing (59). In our research, the level of NO in the wounds treated with NeoPep S was significantly decreased, lower than the level in the control group in both in vitro and in vivo experiments. This indicates that NeoPep S has an important function in inhibiting the inflammation phase of the wound healing process.

Moreover, ROS also play a significant part in the orchestration of the normal wound healing process. Low ROS levels have a beneficial impact on wound healing, whereas high ROS production causes oxidative stress, which can be harmful to wound healing (60). In our study, the level of ROS generation significantly decreased in the groups treated with NeoPep S compared with that in the control group. This finding suggests that NeoPep S contributes to reducing the level of ROS to accelerate the pro-inflammatory process.

**Conclusion**

To conclude, the results of in vivo and in vitro experiments have confirmed that AIMP1-derived peptides have the ability to promote the skin wound healing process. We applied a very small amount of the peptide (15 amino acids) as a new generation NeoPep S—a new material that promotes wound healing. Through many careful examinations and strict verifications, NeoPep S showed various activities on inflammatory cells, fibroblasts, and myofibroblasts, which all play important roles in the wound healing process. For raw264.7 cells stimulated by LPS, Adp significantly inhibited TNF-αexpression. The NeoPep S 3 ppm dose was the most efficient treatment for rat wound healing in comparison with the 1ppm dose. The findings contribute strong evidence of the safety and effectiveness of using a natural protein derivative in the ingredients of clinical products.

**Conflicts of Interest**

The Authors declare no conflicts of interest regarding this study.

**Authors’ Contributions**

Xin Rui Zhang: Investigation, Data curation, Writing original draft. Pham Ngoc Chien: Investigation, Data curation, Methodology, Writing original draft and editing. Nguyen Thi Thanh Ho: Writing original draft, Conceptualization. Van Anh Le Thi: Investigation. Jun Kyu Park: Development of NeoPep S cream. Sun Yeong Nam: Conceptualization, Methodology, Supervision, Investigation. Heo Chan Yeong: Conceptualization, Methodology, Supervision, Investigation, Validation.

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