Effect of adipose-derived stem cell sheets on wound healing

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Effect of adipose-derived stem cell sheets on wound healing

Directed by Professor Dae Hyun Lew

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Chae-Eun Yang

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

Effect of adipose-derived stem cell sheets on wound healing

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Human adipose-derived stem cells (*h*ASCs), an attractive mesenchymal stem cell source, can accelerate cutaneous wound healing due to their plasticity and paracrine activities. To maximize therapeutic efficacy, highly-concentrated cells should be properly delivered to the wound. Traditionally, cultured stem cells are separated using proteolytic enzymes before re-suspension, which can deteriorate cell functions. Here, *h*ASCs were harvested as sheets through a non-invasive photothermal technique, and their effect on wound healing was compared with that of conventional cell delivery.

In vitro, mono-layer sheets maintained cellular contacts and exhibited properties consistent with respective ASCs. Cell viability decreased over time; however, secretion of growth factors including bFGF, TGF-ß1, EGF, HGF, PDGF, and VEGF was more active in these cells than enzyme-treated cells.

In vivo, dorsal full-thickness wounds were created on SKH-1 hairless mice. Compared to conventional methods, *h*ASC sheets application resulted in earlier and faster re-epithelialization based on the gross photography and H&E staining. Immunohistochemistry

showed higher CD31 and VEGF expression in sheet-transplanted wounds.

To verify the effect of *h*ASC sheets on chronic wound healing, the dorsum of SKH-1 hairless mice was irradiated and wounds were created after 6 weeks. Faster re-epithelialization was observed in the sheet-transplanted group than in the control group.

Unlike conventional methods, since enzymes are not used, cell surface proteins and cellular contacts are preserved, and thus, stem cells secrete more growth factors and effectively engraft to the wound. In conclusion, improved wound healing might be expected by applying *h*ASC sheets obtained via non-invasive photothermal cell harvesting.

Key words: adipose tissue-derived stem cells, photothermal cell sheet harvest, wound healing,

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I. INTRODUCTION

The cutaneous wound healing process consists of a complex, but well-orchestrated, cascade of numerous feedback and feed-forward regulatory loops governed by several factors. Normally, the wound is subjected to the following phases which overlap each other; hemostasis, inflammation, proliferation and remodeling[1]. However, disruption of any of these phases, which occurs in conditions such as diabetes, infection or radiation, can lead to transformation towards a chronic non-healing wound.

A chronic wound generally refers to a wound that has not healed after 4 weeks, or a wound that fails to achieve 20-40% reduction in area after 2-4 weeks of optimal therapy[1]. This results in the suffering of the individual, as well as great economic and social losses[2]. In total, 6.5 million patients have been reported to be suffering from chronic wounds and the annual cost was estimated to be approximately US$25 billion in the United States in 2009. Moreover, the associated burden is rapidly increasing because of an increasing proportion of elderly individuals and the prevalence of life-style diseases such as diabetes and obesity. Thus, interest in wound healing, in the

context of healthcare, continues to increase[3,4].

Various treatment methods for wound healing have constantly been evolving from ancient times to the present. In addition to changes in the approach to wound dressing, new therapeutic modalities have been introduced such as negative pressure wound therapy (NPWT), electrical stimulation, hyperbaric oxygen therapy (HBOT), lasers, and phototherapy, whereas advanced wound healing therapies have also evolved. Since the 1980s, growth factor therapy has emerged and has been followed by tissue engineering, and by the early 2000s, stem cell therapy had become popular and various studies related to this approach have been conducted[5]. The exogenous transplantation of mesenchymal stem cells during wound healing has shown potential therapeutic efficacy in animal models. Transplanted stem cells secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities, resulting trophic effects[6].

Mesenchymal stem cells (MSCs) are non-hematopoietic cells that can differentiate into, and contribute to the regeneration of mesenchymal tissues such as bones, cartilages, muscles, ligaments, tendons, fats, dermis and other connective tissues[7,8]. These cells have been isolated from various sources, such as bone marrow, adipose tissue and amniotic fluid. Human adipose-derived stem cells (*h*ASCs) are an attractive source of mesenchymal stem cells because, relatively, they can be harvested with ease using minimally invasive procedures. These cells exhibit high cellular density

(approximately 1×10^6/10ml of adipose tissue)[9] and have extensive proliferative capacity *ex vivo*.

To maximize their therapeutic effect for wound healing, it is essential to properly deliver these cells to the target area to ensure that a highly-concentrated population of cells is integrated into the wound surface. Traditionally, cultured stem cells are delivered into or around the wound through topical application or intradermal injection[10]. For transplantation, they can also be seeded into scaffolds such as collagen sponge[11], fibrin glue[12], calcium phosphate cement[13], or hydrogels[14]. For these, cultured cells are detached from the culture dish and separated using proteolytic enzymes before re-suspension. Trypsin is frequently used to release adherent cells from the surface or to disaggregate tissues into single cell suspensions. This proteolytic enzyme cleaves adhesion proteins involved in cell-cell and cell-matrix interactions. However, it can also damage cells by striping cell surface proteins, which might alter cell functions[15]. Although previous studies have shown enhanced wound healing after stem cell treatment with conventional delivery methods[16-20], it is expected that the therapeutic effect could be increased by minimizing cellular damage during cell harvesting. In addition, injection is accompanied by pain, and a large amount of the suspension may be lost by flowing outward from the wound through spraying.

To promote stem cell engraftment and encourage the secretion of tissue-trophic

mediators after implantation, a cell sheet harvesting technique has recently been introduced. (Figure 1) Unlike the enzymatic treatment which separates cells individually, this technique utilizes a substrate-coated culture dish, which responds to various stimuli. Cultured cells rise up into sheet-form when a stimulus such as temperature, electricity, light, pH and magnetic field is applied to the substrate[21]. As this enables cells to maintain cell-cell contacts and adherence proteins, the secretory ability of cells might be improved, which could facilitate better adherence to the wound surface, thereby preventing the loss of *h*ASCs[22]. In this study, we harvested cultured *h*ASC sheets, using poly(3,4-ethylenedioxythiophene; PEDOT)[23], a conductive polymer with a photothermal effect as a substrate, and investigated *in vitro* characteristics and effects on *in vivo* cutaneous wound healing.

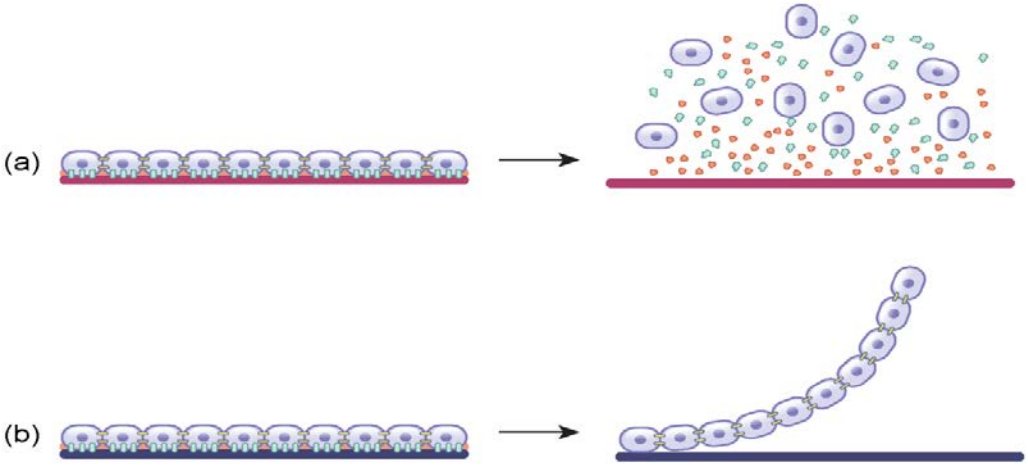


Figure 1. Concept of cell harvesting technique (a) Conventional method using proteolytic enzyme. Samples are separated into individual cells. (b) Cell sheet

harvesting technique. Cultured cells are harvested as a monolayer while maintaining their cell-to-cell contacts and cell-to-extracellular matrix environment.

II. MATERIAL AND METHODS

1. *h*ASC sheet preparation

For cell harvesting, a noninvasive photothermal detachment method, which uses cell culture surfaces coated with near-infrared (NIR)-sensitive conductive PEDOT polymers was used. A photothermal polymer film (PEDOT) was directly coated onto polystyrene petri dishes, and collagen type I (0.3 wt%) was dropped onto the surface to form a thin collagen layer. Cultured *h*ASCs were seeded on collagen-coated PEDOT petri dishes and cultured for 1 day. The adipose tissue collection procedure was approved by the Yonsei University Institutional Review Board (4-2015-1085). After a 5-minute irradiation with NIR light (808 nm, 270 mW), adsorbed collagens on the PEDOT surface dissociated, and the cell sheets (8-mm in diameter) were detached from the substrate (Figure 2).

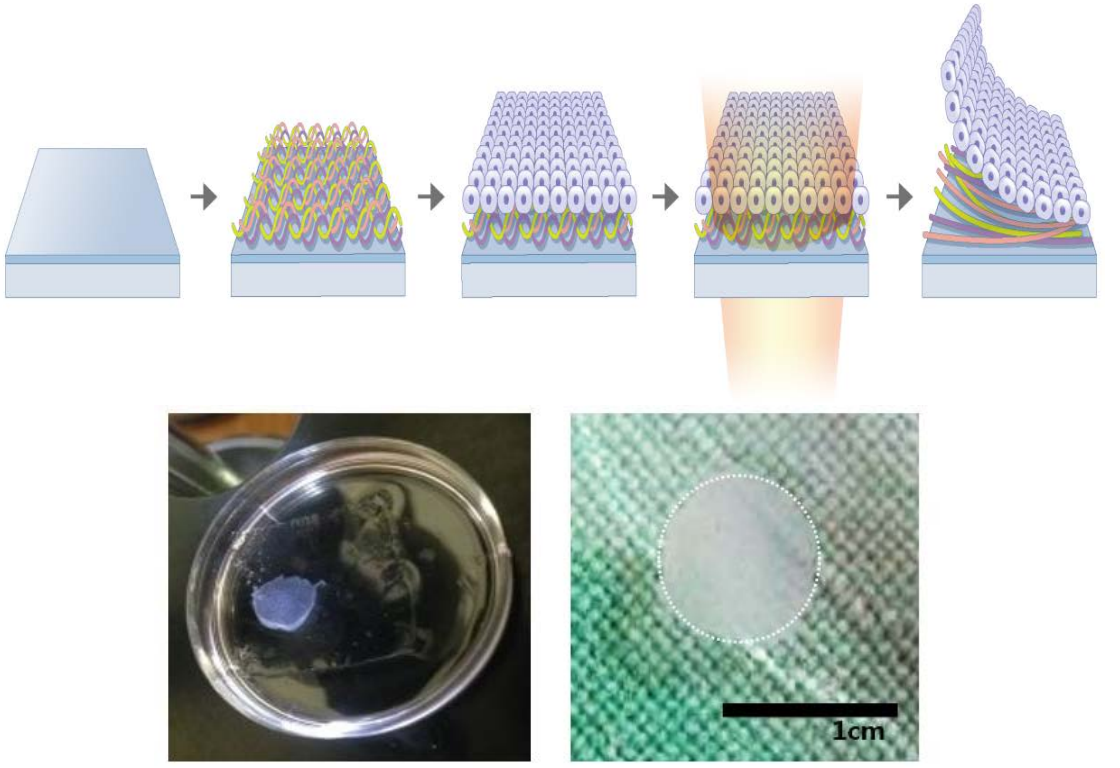


Figure 2. Photothermal cell harvesting technique. A biocompatible conductive polymer (PEDOT) and collagen were used to coat cell culture dishes and human adipose-derived stem cells were cultured on the coated surface. NIR light was used to irradiate an 8 mm diameter circle. As the collagen structure dissociated, a gap was created between the cell layer and the media, and, cell sheets were then floated on the medium.

2. *In vitro* studies

A. Morphology of *h*ASC sheets

The cellular morphology of the harvested *h*ASC sheets was assessed by electron microscopy. Treatment medium from the dishes was decanted and cells were fixed with freshly prepared 2.5% glutaraldehyde. Subsequently, cells were dehydrated with increasing concentrations of ethanol, and critical-point dried. The samples were then coated with a thin layer of platinum-palladium and visualized using a field emission-scanning electron microscope (SEM, HITACHI S-800, Japan).

B. Identification of ASC surface markers after harvesting sheets (flow cytometry)

Cell surface markers in the harvested stem cell sheets were analyzed using the Cytomics FC500 (Beckmann Coulter, Krefeld, Germany). ASCs were stained with the following fluorescein isothiocyanate conjugated monoclonal antibodies, CD14-FITC, CD29-FITC, CD31-PE, CD34-FITC, CD44-PE, CD45-PE, CD73-PE, CD90-FITC, CD105-PE, and CD106-FITC (BD Pharmingen, San Diego, CA, USA). Briefly, cells were stained with antibodies for 30 minutes at room temperature. Stained cells were washed with PBS and fixed with 1% paraformaldehyde. Data was evaluated using WinMDI, version 2.9.

C. Viability of cells in the sheet

The trypan blue dye exclusion test was used to determine the number of viable cells present in a cell suspension. Suspension from PEDOT-coated dishes before and immediately after NIR irradiation for 2, 4, 6, 24 and 48 hours, were mixed with trypan blue and then examined for dye uptake or exclusion.

D. Comparison of growth factor release after trypsinization and NIR irradiation

The media from the trypsin-treated cell suspensions and cells harvested from sheets were collected. The supernatant of cells subjected to normal conditions was used as a control. Briefly, the supernatant was analyzed after combining with coated beads from a human premixed multi-analyte kit (R&D systems) for VEGF, PDGF, HGF, and EGF. TGF-ß1 and bFGF were quantified using a human ELISA kit (KOMA Biotech Inc., Seoul, Korea).

3. *In vivo* acute wound model

A. Experimental animals and groups to assess acute wound healing

SKH-1 hairless mice (7-week-old females) were selected as the wound healing model. All animal experimental procedures conformed to the guidelines of the Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Yonsei University College of Medicine (IACUC No. 2015-0306). Forty mice were randomly divided into experimental and control groups, and the experimental group was subdivided into three groups according to the method of stem cell transplantation as follows: group I, sheets; group II, injection; group III, spray.

B. Anesthesia and creation of an excisional wound

Mice were anesthetized through the intraperitoneal injection of sodium pentobarbital. Isoflurane gas inhalation was used to maintain the anesthesia. The dorsal surface was disinfected with povidone-iodine followed by one rinse with ethanol. After stretching the dorsal skin of the anesthetized mice manually, an 8-mm full-thickness skin wound was created using an 8-mm-biopsy punch. The skin flap was removed from the wound bed. Next, 0.020-inch-thick donut-like silicone splints (Medical Grade Silicone Sheeting, Bio-Plexus, Vernon, Connecticut, USA), with a 20-mm outer diameter and an 8-mm inner diameter, was placed around the wound. The wound was centered

within the splint to prevent wound closure by skin contraction, and thus, closure was only achieved through re-epithelialization[24]. The splint was fixed with an instant bonding adhesive (Krazy glue, Columbus, cat. no. 744-3514, USA) and fixation was secured to the skin with 6-0 nylon sutures (Figure 3). Photographs of individual wounds were taken with a digital camera.

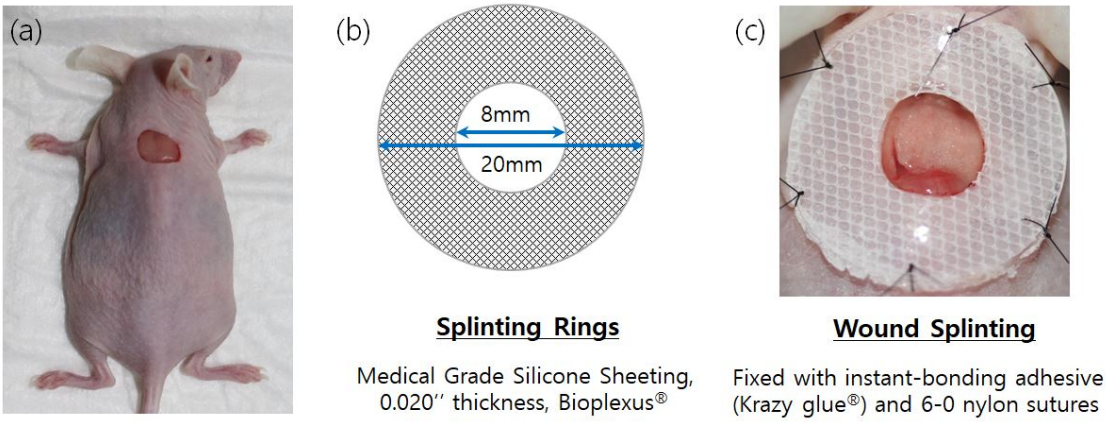


Figure 3. Excisional wound splinting model. (a) After anesthesia, an 8-mm full-thickness cutaneous wound was created on the dorsal skin of a 7-week-old SKH-1 hairless mouse. (b) To prevent wound closure by skin co ntraction, splinting rings were prepared with a 20-mm outer diameter and an 8-mm inner diameter to ensure that the size of the inner hole matched the wound size. (c) The splint was placed with glue on the underside to ensure that the wound was centered, and the splint was secured with simple interrupted sutures.

C. *h*ASC transplantation

For experimental group I (n = 10), *h*ASC sheets, 8-mm in diameter and containing 1.2×10^6 cells, were transferred onto the wounds. For experimental group II (n = 10), we injected 0.2 ml of PBS containing 1.2×10^6 cells into the dermis at four sites surrounding the wound with a 31-gage, 1-ml syringe. For experimental group III (n = 10), the same number of cells in 0.1 ml PBS was sprayed onto the wound bed using a syringe. For the control group (n = 10), 0.1 ml of PBS was sprayed onto each wound bed to facilitate a hydrated environment similar to that in the experimental group. Subsequently, all wounds were covered with Tegaderm3M sterile transparent dressing and dressed with self-adhering elastic bandages (Coban, 3M, MN, USA).

D. Measurement of wound sizes

Wound dressings were uncovered, and photographs were taken of individual wounds every day from the 5th day. Digital photographs of the injury sites were taken using a Canon 60D digital camera. Using the Image J software (National Institutes of Health, Bethesda, MD), the pixel data from gross wound areas were determined by tracing the wound margin on the photograph. Since the inside edge of the splint matched the margin of the original wound, the splinted hole was used as a reference for the original wound size. Setting the first wound size to 100%, we calculated the residual wound area (area of the actual wound at each day/area of original wound).

E. Histologic analysis

Wounded skin and surrounding normal skin were collected after mice were sacrificed at the indicated time points (day 2 and 8 post-wounding). Samples were fixed in 4% paraformaldehyde, embedded, and sectioned at 8 μm. The sections were stained with hematoxylin and eosin (H&E). Images were captured using a microscope (DM5000, Leica Microsystems Gmblt, Wetzlar, Germany) to evaluate re-epithelialization.

F. Evaluation of CD31 and vascular endothelial growth factor (VEGF) by immunohistochemistry

Some histological sections at day 8 were used for immunohistochemistry to probe for CD31 and VEGF. For CD31, sections were incubated with an anti-CD31 mouse monoclonal antibody (ab119339, Abcam, Cambridge, UK) diluted at 1:100 in 3% BSA-PBS overnight at 4 °C. For VEGF, sections were incubated with an anti-VEGF rabbit polyclonal antibody (ab46154, Abcam, Cambridge, UK) diluted at 1:100 in 1% BSA-PBS for 45 minutes. Images were captured using a microscope (DM5000, Leica Microsystems Gmblt, Wetzlar, Germany). The tissue area with the highest vessel density was identified at low magnification (100×); five adjacent pictures of this identified area were then taken at a high magnification (400×). Microvessel density was then assessed by enumerating the number of CD31-positive vessels. Vessels characterized by thick muscular walls or with a lumen greater than 20 μm in diameter

were excluded. Anti-VEGF-stained sections were also exported to the Metamorph® (Molecular Devices, Sunnyvale, CA) microscopy automation and image analysis software for analysis of VEGF expression.

4. *In vivo* model of chronic wound healing

A. Experimental Animals and Groups for chronic wound healing

Twenty-four 7-week-old female SKH-1 hairless mice were irradiated and randomly divided into two groups as follows: control (no treatment) and experimental (*h*ASC sheet transplantation). Anesthetized mice were irradiated with 20 Gy using a biological irradiator (XRAD-320, PRECISION X-RAY, Softex, Korea) with a single exposure to X-rays at a dose rate of 2 Gy/min on a dorsal area of 2×2 cm^2 in skin depth[25]. After 6 weeks[26], wound creation and splinting were performed as previously described.

B. Measurement of wound size

Wound dressings were removed and photographs were taken of individual wounds every third day from the 5th day as previously described and wound size was calculated in the same manner.

5. Statistics

Residual wound area (%) was evaluated using a linear mixed model as implemented in the MIXED procedures of SAS (version 9.3, SAS Inc., Cary, NC, USA). Data are expressed as the mean ± SD. We analyzed the interaction between treatment groups by time interaction depending on treatment. Additionally, we performed post hoc analyses to estimate the time points at which treatment effects differed among the four groups. P-values were obtained by performing a Bonferroni correction for multiple comparisons, and $P < 0.05$ was considered statistically significant.

III. RESULTS

1. In vitro study

A. Electron microscopy analysis of harvested hASC sheets

Based on SEM, the cell sheet shape matched the pattern of the circular laser source, and cells in the harvested sheets were closely attached to each other (Figure 4).

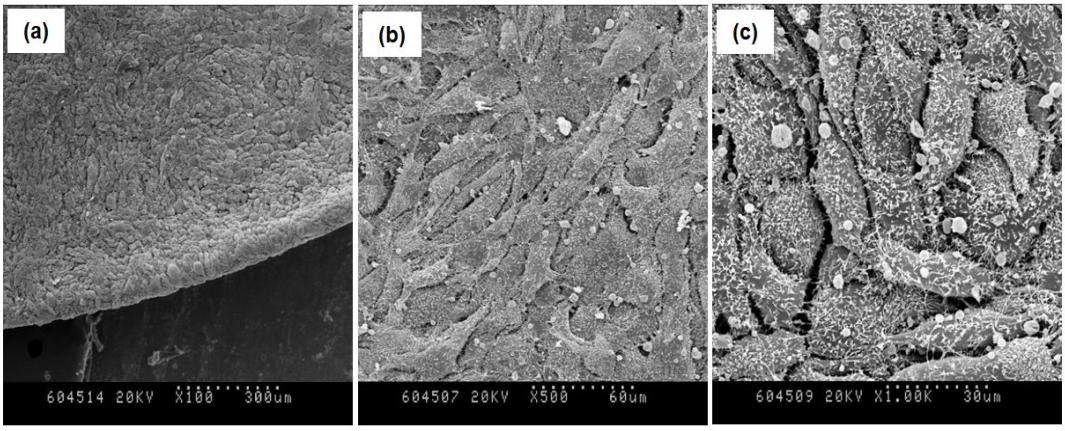


Figure 4. Scanning electron microscopy (SEM) of harvested human adipose-derived stem cells sheet. The shape of the cell sheet matches the pattern of the circular laser source. Cells maintained cell-to-cell contacts, and abundant microvesicles are shown on cell surfaces. (a) 100×, (b) 500×, (c) 1,000×

B. *h*ASCs in sheets show intrinsic properties consistent with respective ASCs after harvesting.

The *h*ASCs in the sheets expressed high levels of CD90, CD44, CD29, CD105, and CD73. These cells were also negative for endothelial- and hematopoietic-specific markers such as CD31, CD106, CD34, and CD45, indicating that cell surface marker expression in cultured ASCs was maintained after sheet formation[27-29]. (Figure 5)

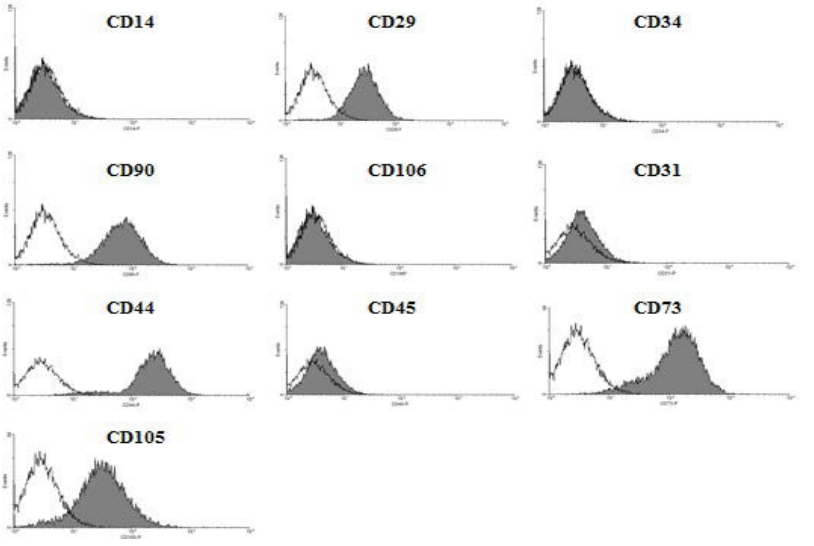


Figure 5. Flow cytometric analysis of cultured human adipose-derived stem cells (*h*ASCs) in photothermally-harvested sheets. Cells were positive for the adipose tissue-derived MSC-specific markers CD29, CD44, CD73, CD90, and CD105, but were negative for the endothelial-specific marker CD31 and the hematopoietic-specific markers CD14, CD34, and CD45. Thus, the intrinsic properties of cultured *h*ASCs were maintained after harvesting the sheets.

C. Viability of cells in sheets

Before NIR irradiation, viability was 84%, as cells were trypsin-treated to obtain single cells and to measure viability. Immediately after the harvesting of the sheets, viability was decreased to 80% and gradually decreased over time. At 6 hours after harvesting, more than 50% of the cells survived (Figure 6).

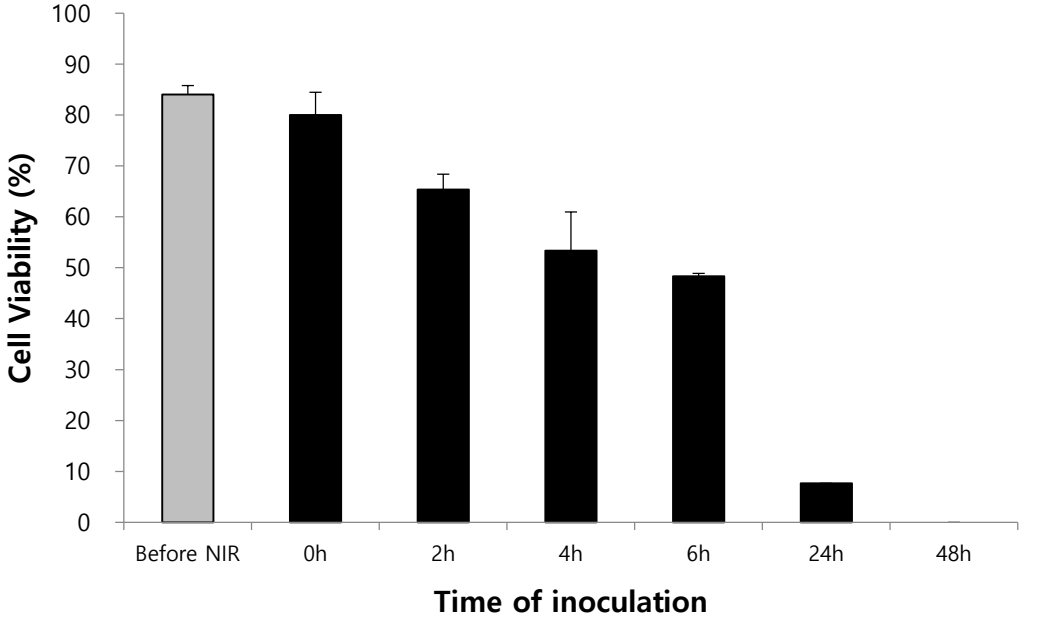


Figure 6. Viability of cells in human adipose-derived stem cell sheets over time. Cells were harvested using a non-invasive photothermal technique and viability, as assessed by trypan blue dye exclusion assays, was found to gradually decrease over time after harvesting. Error bars represent mean±SD.

D. Sheet-harvested *h*ASCs secrete higher levels of bFGF, TGF-ß1, HGF, and PDGF

Analysis of growth factor secretion revealed that bFGF, TGF-ß1, EGF, HGF, and PDGF, which have positive effects on wound healing, were secreted at higher levels in cell sheets than in other conditions. VEGF secretion level in cell sheets was higher than that in trypsin-treated cells, but was lower than that in controls (Figure 7)

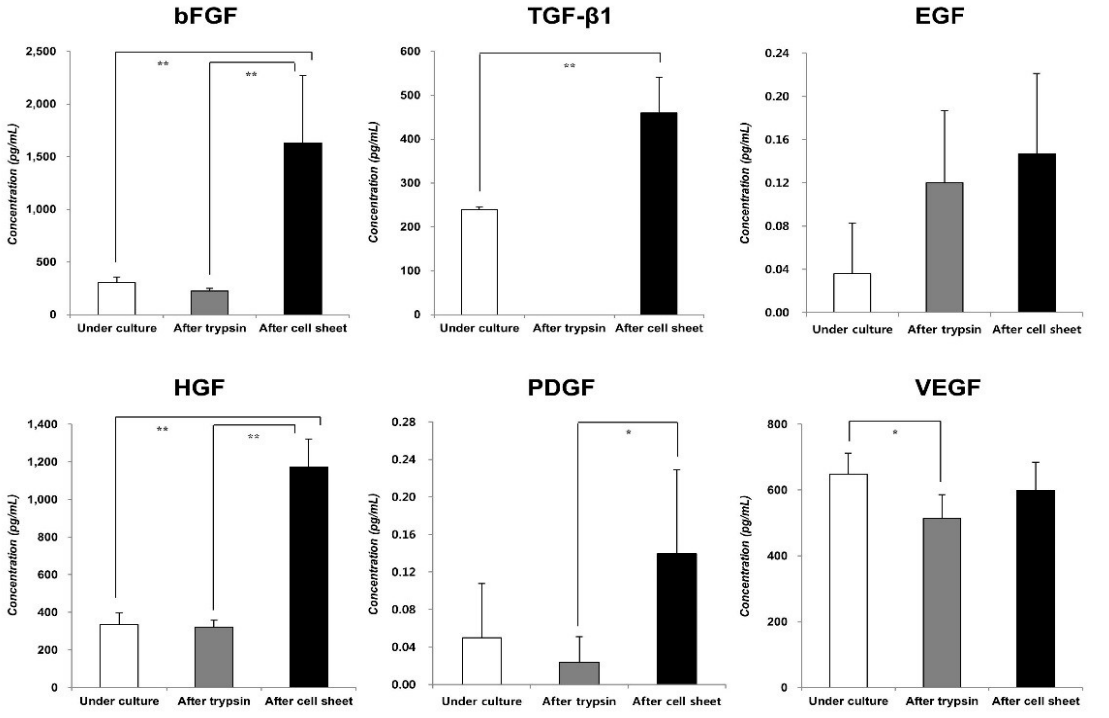


Figure 7. Comparisons of growth factor release from human adipose-derived stem cells in each condition. Growth factor secretion was assessed by ELISA-based methods. Methods of cell harvesting, specifically after trypsinization and after sheet

harvesting using a non-invasive photothermal technique, were compared. For all growth factors, sheet-harvested cells secreted higher levels of growth factors than trypsin-treated individualized cells (*P < 0.05, **P < 0.01). Error bars represent mean ±SD.

2. *In vivo* study using acute wound model

A. Analysis of wound re-epithelialization

Photographs taken every 3 days showed that healing in the experimental groups was faster during the early period (Figure 8). In vivo, wound healing was faster in the experimental groups (I, II, and III) than in the control group. A post hoc analysis to determine the time point at which the treatment effects differed among the four groups, revealed significantly faster healing at day 5 in the sheet group, and this trend continued until day 14. At day 8, the residual wound areas were significantly smaller in all experimental groups than in the control group. At day 11, the treatment effects of cell injection and cell sheets were significantly enhanced compared to those in control and spray groups; the effects observed in the spray group were not significantly different compared to those in the control group. After day 14, no statistically significant difference was noted among all groups. Representative images of the wound healing process are displayed in Figure 9.

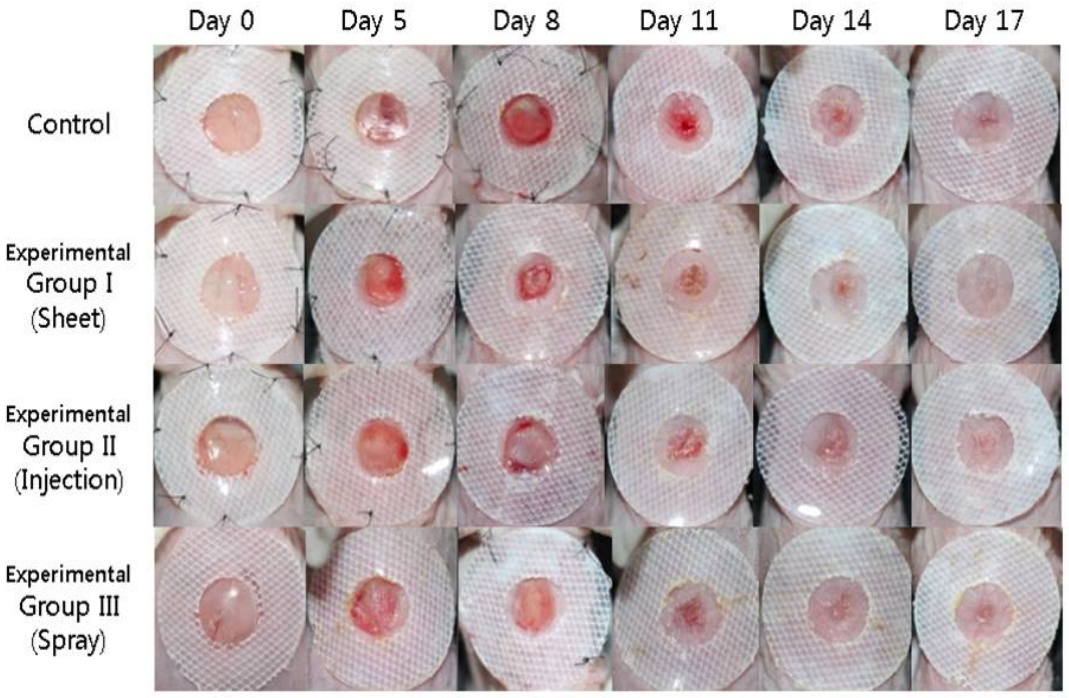
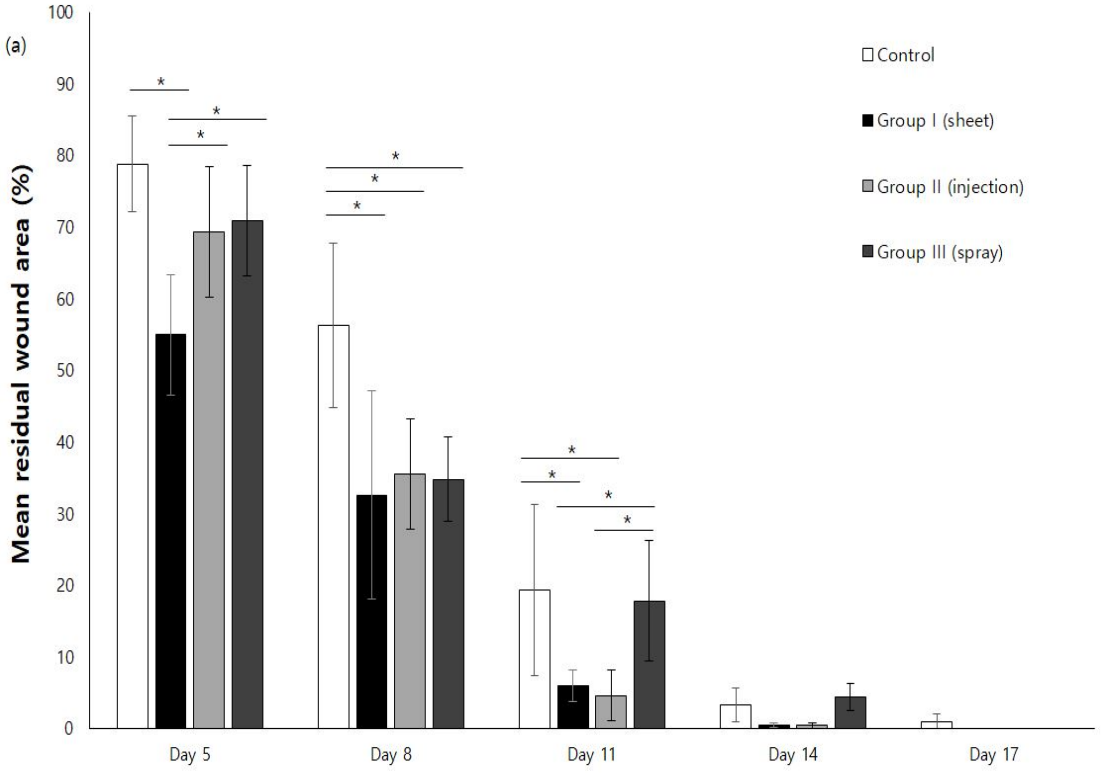


Figure 8. Photographic images of dorsal full-thickness wounds after treatment with different human adipose-derived stem cell preparations. Experimental groups included preparations of 1.2×10^6 cells as follows. Group I, cell sheets harvested by a non-invasive photothermal technique; Group II, individual cells injected into the wound site, Group III, individual cells sprayed on to the wound site. Experimental groups showed faster healing in early period than the control (untreated) group.



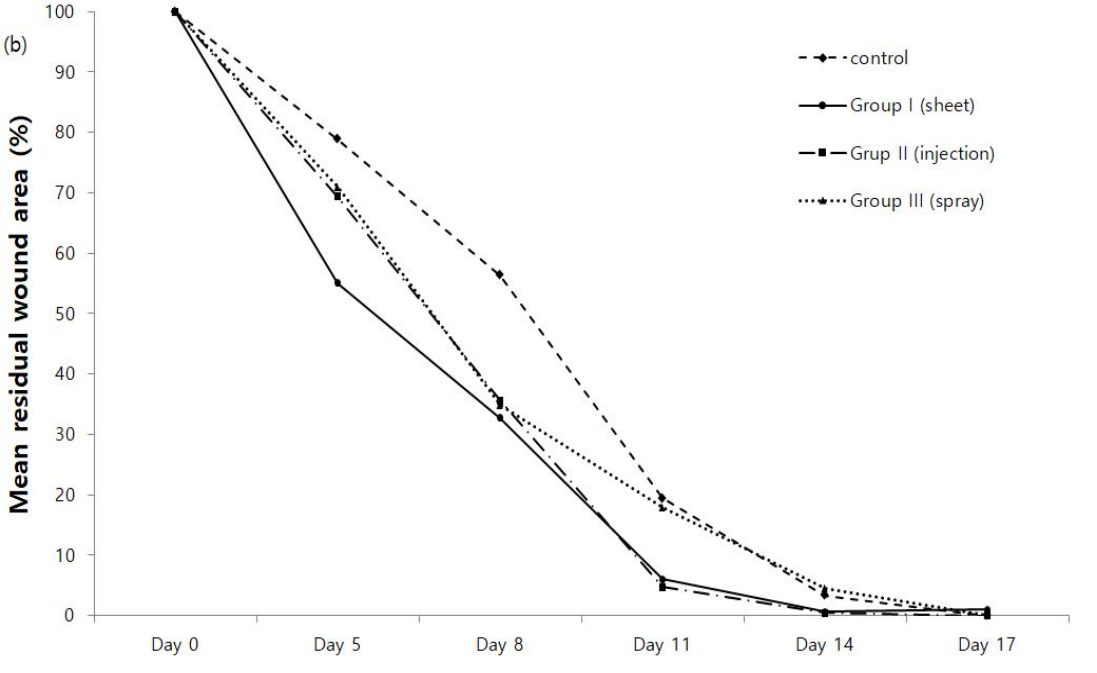


Figure 9. Comparison of wound healing with different human adipose-derived stem cell preparations. Mean residual wound area (area of the actual wound at each day/area of original wound, %) was indicated by a bar graph, and the tendency of wound decreasing was represented by a linear graph by day. Error bars represent mean ± SD. Overall, the experimental groups showed faster healing rates than the control group. The difference between the groups was significant on days 5, 8, and 11. (*p < 0.05) On day 5, the sheet group showed a significantly faster re-epithelialization than the other groups. On day 8, the control group showed a significantly slower healing than all experimental groups, but the difference among experimental groups was not significant. On day 11, there was no significant difference between the control and the spray groups, whereas the sheet and injection groups showed significantly better treatment effect than the control group. Likewise, the treatment effects of injection and sheet group were significantly faster than those of spray group. However, there was no significant difference between sheet and injection. There was no significant difference among the groups on day 14 and 17.

B. Histological evaluation of treatment groups

At day 8, the epithelial layer was observed in all groups. However, there was a difference in the degree of wound covering. In the experimental group, re-epithelialization proceeded faster than in the control group, and in the experimental group, it was in the order of sheet, injection, and spray. (Figure 10)

C. Expression of CD31 and VEGF in treatment groups

Revascularization (measurement of CD31-positive blood vessels) was observed at 20.4(12-26)/HPF in the experimental group I, 12(2-19)/HPF in experimental group III, 7.8(2-11)/HPF in experimental group III, and 5.2(3-7)/HPF in the control group on average. Compared to that in the control group, higher microvessel density was observed for all experimental groups (Figure 11). Metamorph analysis also revealed higher VEGF expression in the experimental groups than in the control group (Figure 12).

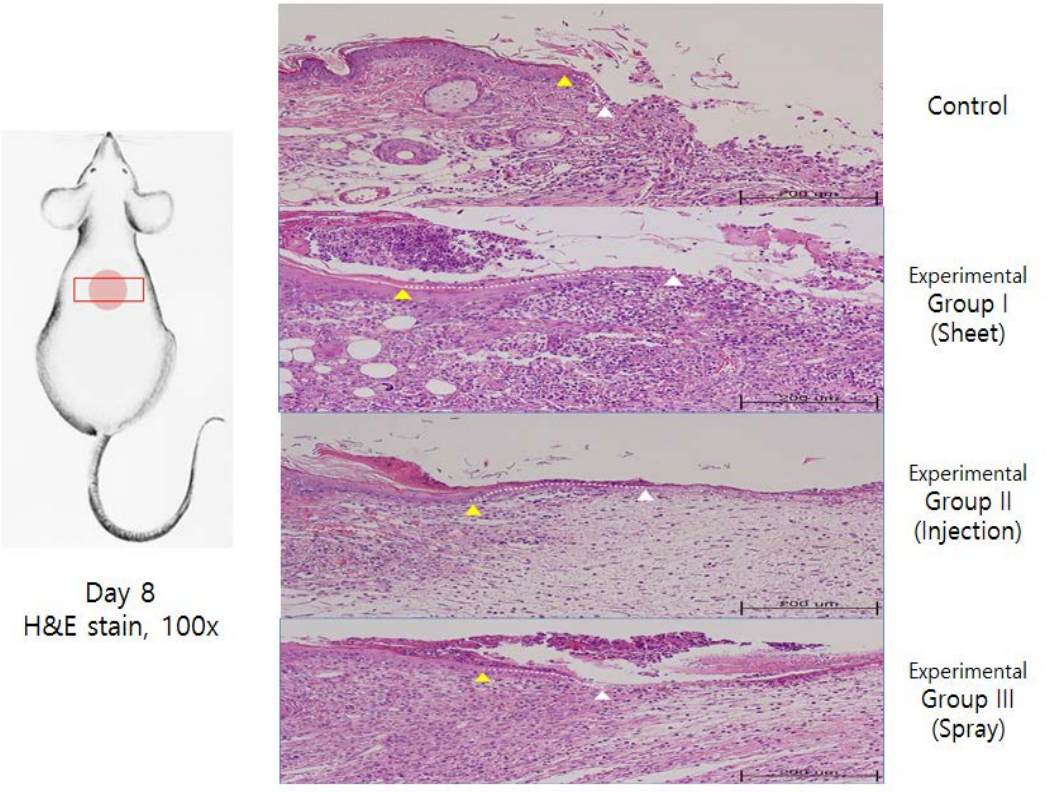


Figure 10. Histological evaluation of re-epithelialization among human adipose-derived stem cell treatment groups. H&E staining was performed at day 8 (100× magnification). Wounded skin and surrounding normal skin were collected after mice were sacrificed at day 8 (as depicted, left). Yellow arrows indicate the edge of the wound and white arrows indicate the edge of re-epithelialization. By day 8, the degree of re-epithelialization was highest in group I and lowest in the control group.

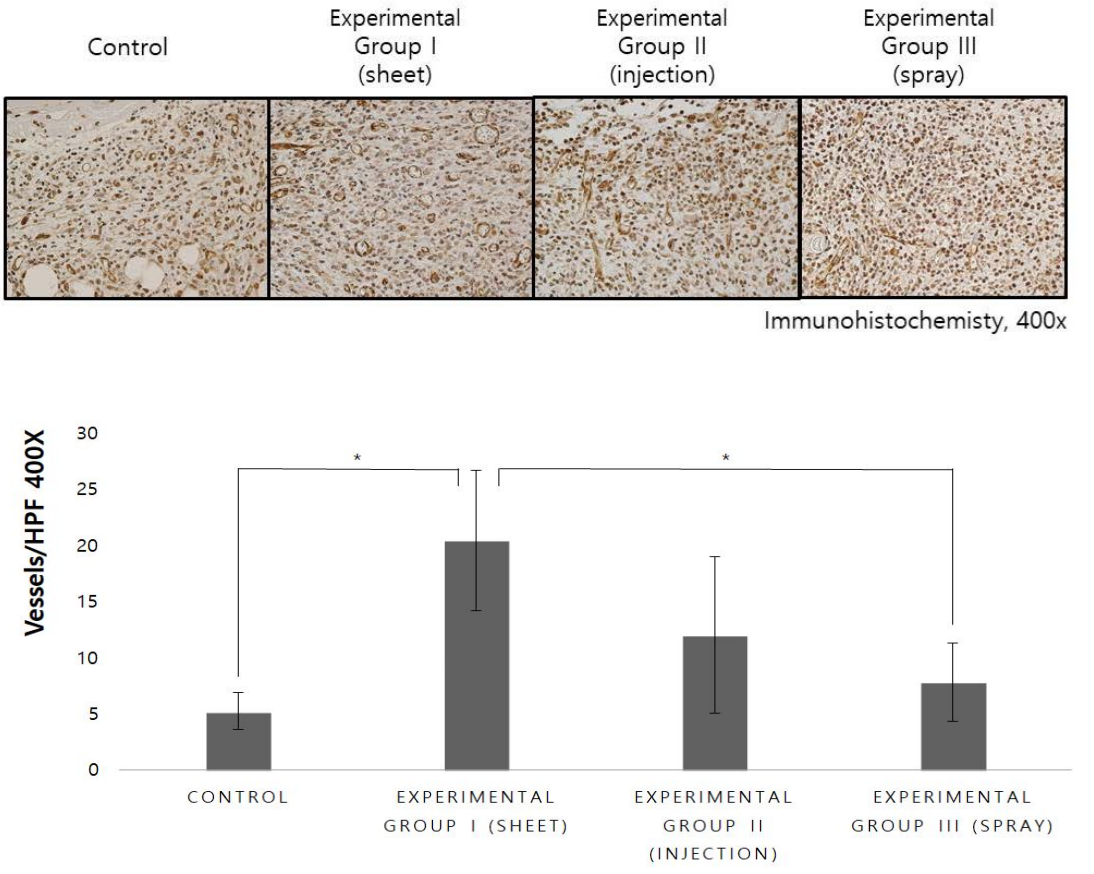


Figure 11. Comparison of microvessel density among wounds treated with different preparations of human adipose-derived stem cell sheets. At day 8, wound samples were obtained and after immunohistochemical staining, blood vessels were counted (400×). Blood vessels were quantitated, and the mean values were obtained from 10 samples per group. Compared to that in the control group, higher vessel density was observed for experimental groups, and especially group I. (*p < 0.05)

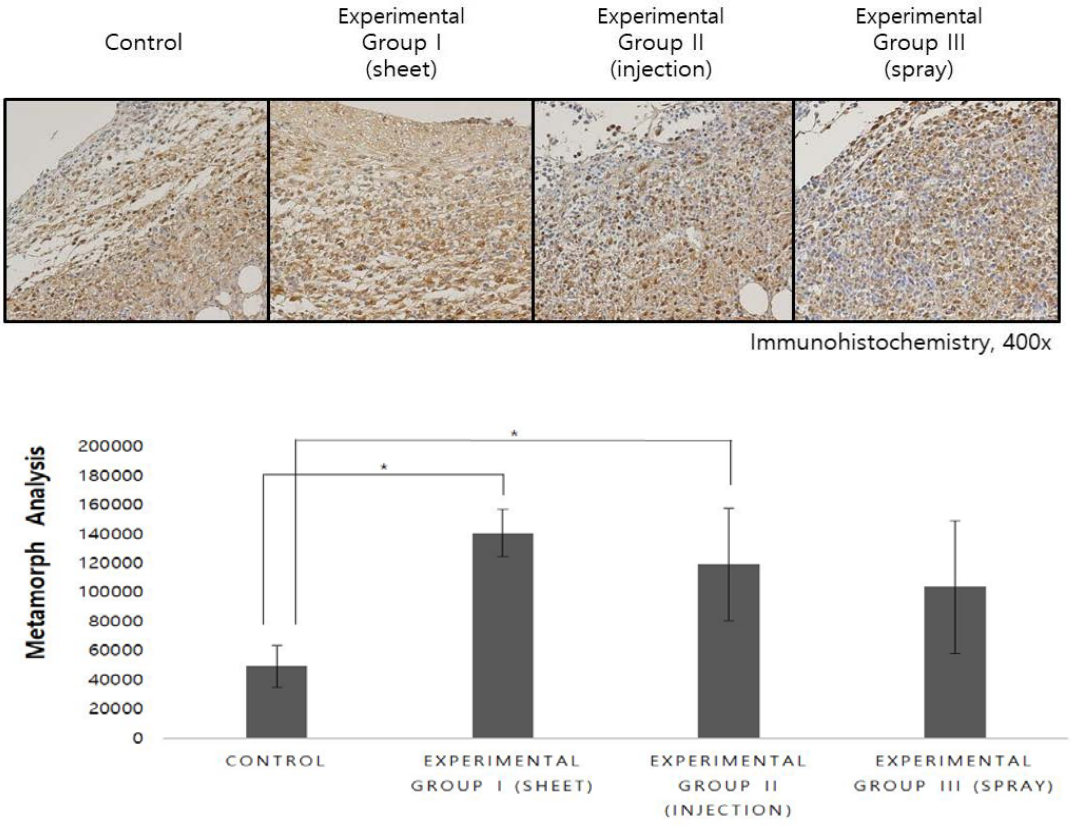


Figure 12. Metamorph quantitative analysis of VEGF expression after treating acute wounds with human adipose-derived stem cells (*h*ASCs). After immunohistochemical staining (for VEGF) of tissue obtained on day 8, the mean expression value was quantitated by performing Metamorph analysis using 10 samples per group (400×). VEGF expression was higher in the experimental groups than in the control group. Among the experimental groups, the highest expression was observed in the *h*ASC sheet group (I), followed by the injection (II) and spray groups (III). Group I and II exhibited higher VEGF expression than the control group and this difference was statistically significant. (*$p < 0.05$).

3. *In vivo* chronic wound model

Using an irradiated wound model, complete wound healing required more time, compared to that in the acute wound model (Figure 13). Compared to that in the control (untreated) group, healing of irradiated wounds was faster in the experimental (*h*ASC sheet transplantation) group, and this was statistically significant on day 5, 8, and 11 (Figure 14).

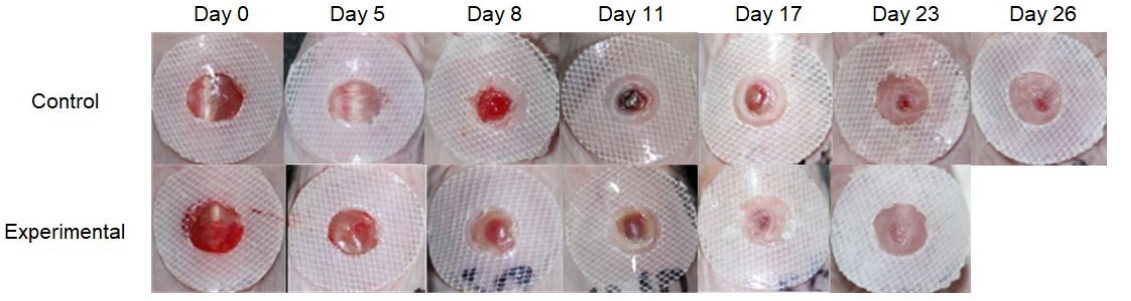
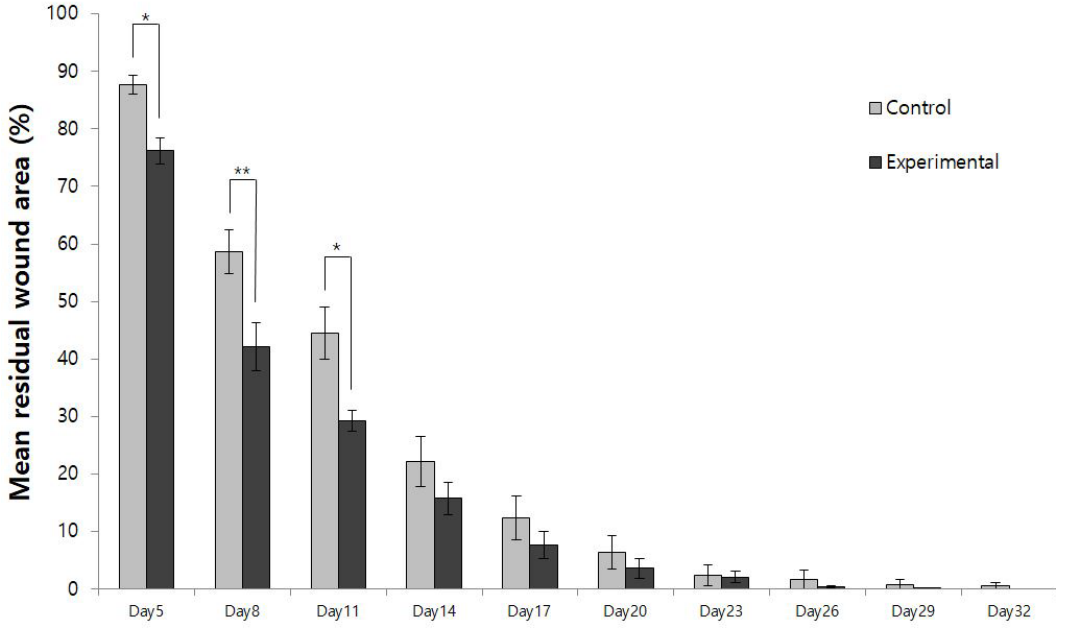


Figure 13. Photographic images of dorsal full-thickness wounds of the irradiated model. Experimental group was treated with human adipose-derived stem cell sheet containing 1.2×10^6 cells after wound formation. Experimental groups showed faster healing in early period than the control (untreated) group.



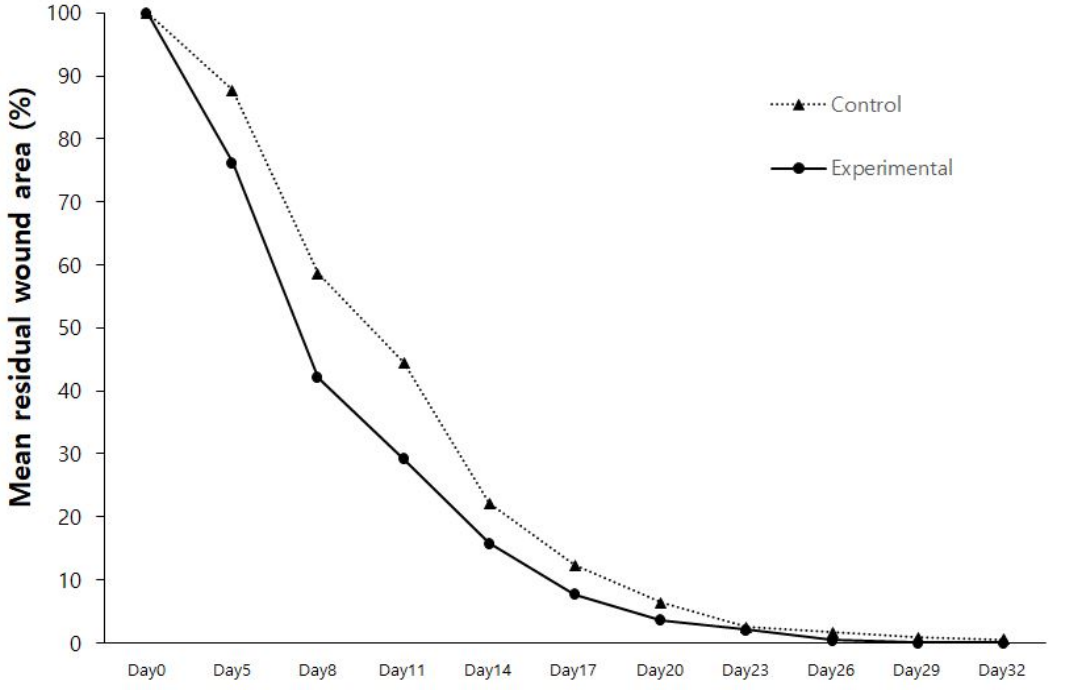


Figure 14. Comparison of wound healing of irradiated model. Mean residual wound area (area of the actual wound at each day/area of the original wound, %) was indicated by a bar graph, and the tendency of wound decreasing was represented by a linear graph by day. Error bars represent mean±SD. Irradiated wound healing was faster in the human adipose-derived stem cell sheet-transplanted group than in the control group. The difference in healing rate was apparent during the early period of wound healing (*$p < 0.05$, **$p < 0.01$).

IV. DISCUSSION

The positive effect of transplanted MSCs on tissue repair has previously been reported in therapeutic settings for various medical conditions such as renal[30-32], myocardial[33-35], neurological disorders[8,36,37]. The exogenous application of MSCs on the cutaneous wound is known to facilitate wound healing. *In vivo*, these cells respond to chemotactic signals associated with inflammation, wound repair, and tissue regeneration[38]. Moreover, they secrete various cytokines and growth factors that have both autocrine and paracrine activities, resulting in the stimulation of angiogenesis, anti-inflammatory properties, immunomodulation, inhibition of fibrosis, and extracellular matrix production[8,17,39,40]. They can also directly reconstitute epidermal and dermal components by differentiating into epidermal keratinocytes, endothelial cells, and pericytes[40].

Although previous studies have shown that wound healing is enhanced after stem cell treatment with conventional delivery methods such as injection or topical application, both of which use proteolytic enzyme [16-20], it is expected that the therapeutic effect could be increased if cellular damage is decreased. The emergence of cell sheet harvesting technology thus represents an alternative to minimize cell damage and maintain the surrounding environment, as proteolytic enzymes are not used. Without the requirement for such enzymes, cultured cells are released in a non-invasive manner, as a confluent monolayer, and essential cell surface proteins are

preserved[23,41].

Currently, the majority of cell sheet harvesting techniques use thermo-responsive systems for cell detachment[21]. Cells are grown on thermo-responsive polymer-coated surfaces, and the cell sheet is harvested as an intact monolayer through temperature reduction (20°C ~ 4°C)[42-44]. However, the preparation of this polymer is complicated, and as the cells are exposed to a low temperature for greater than 1 hour, there is a risk of hindering cellular metabolism and inducing cell necrosis. To overcome these limitations, we used a photothermal cell harvesting technique. Unlike a thermo-responsive dish, living cell sheets are harvested by dissociating the collagen layer on a conductive polymer (PEDOT) surface. The triple helix collagen structure, which provides an interface between the PEDOT and cell layer can be unfolded into single strands within a few minutes after NIR light irradiation. As this layer is dissolved into solution, the upper cellular layer can be floated and harvested noninvasively. NIR light, which is required for cell sheet harvesting, does not noticeably change the morphology or viability of cells[23]. The PEDOT and collagen layer, located between cells and PEDOT, also prevent direct cell exposure to NIR. Although the temperature at the PEDOT layer increases (up to 43 ℃) for collagen dissociation, the temperature of the cell medium is maintained at 37~38℃, which was shown to have no damaging effects on cells within a short irradiation time[45]. The harvested stem cells were found to be viable and proliferated well despite the temperature increase induced by NIR.

The viability of cells in harvested sheets was reported to be greater than 90%, compared to 86% after trypsinization[45]. Furthermore, by modifying thermal patterns and the optical system, sheets can be harvested as multiple shapes and sizes using this technique. More importantly, using this technique, we observed that the intrinsic characteristics and multi-lineage differentiation capacities of harvested *h*ASCs were preserved.

In this study, we observed a 4% reduction in cell viability immediately after NIR irradiation. The survival rate was gradually decreased and only fewer than 10% of cells survived after 24 hours. When the amounts of secreted growth factors were compared, it was observed that cells harvested in sheet form secreted much more growth factors than cells processed with trypsin. This seems to be related to the rapid re-epithelialization observed in the experimental group, especially in the sheet group, at the initial phase of wound healing.

Complete healing in the acute wound was accomplished at approximately 17 days, and the healing was much faster in the experimental group than in the control group during the early phase. In particular, histologic examination showed significantly faster re-epithelialization and angiogenesis in the sheet group, on day 8 during the proliferation phase of cutaneous wound healing. The secretion of various growth factors that facilitate wound healing has been shown to be more active in photothermally harvested *h*ASCs than in trypsinized *h*ASCs. In addition, the cells in

sheet form make direct contact with the wound surface to exhibit treatment effects, and only a few cells are lost to the outside of the wound. The injection method localizes cells around the wound, which results in slower effect than the sheet because the therapeutic effect of the cells should be exerted at the wound site. When the cells are sprayed, a large amount flows down around the wound and the efficiency decreases. Since stem cell treatment was performed only once at the beginning, it is believed that the survival of cells and growth factor secretion diminished over time, making differences according to different cell delivery method less likely to occur.

Immunohistochemistry also showed that secretion of angiogenic factors and angiogenesis were active in the stem cell treated groups. The distinct superiority in the sheet group suggests that stem cells stimulate the formation of blood vessels and angiogenesis to contribute to wound healing, and these effects may be maximized when applied in sheet form.

Wounds in the irradiated tissue tend to heal more slowly than those in normal tissues. Radiation affects not only the target tissue but also the surrounding normal tissues. Radiation-induced cutaneous changes can cause radiation dermatitis such as ulcerations, necrosis, and fibrosis[46-48]. These changes make the skin vulnerable to injury and it is more likely to progress to a chronic wound when injured. Two hypotheses dominate current knowledge regarding the mechanism of chronic radiation injury: one is depletion of parenchymal and stromal elements, and the other is hypoxia

and ischemia in the tissue[49,50]. In addition, excessive fibrosis and direct cellular damage may prevent normal cell replication in radiated wounds[48,50]. Applying stem cells on irradiated wounds may accelerate tissue regeneration; stem cells secrete various growth factors and promote angiogenesis, thereby rescuing tissues from stem cell niche depletions and ischemic conditions. Furthermore, stem cell themselves have anti-inflammatory and anti-fibrotic effects. There have already been reports of positive results for the application of stem cells to radiation-induced injuries of brain[51], heart[52], and lung[53,54]. In this study, we found that the application of stem cell sheets also induces rapid re-epithelialization of irradiated wounds.

There are two limitations to the clinical use of these sheets. First, as these sheets consist of cell monolayers, their thin nature makes handling difficult. During the course of transfer, from the media to the wound, they tend to fold or tear. Second, it is necessary to minimize the time between sheet harvesting and transplantation. After harvesting, the survival rate of cells might decrease with time. Further studies are needed to optimize the delivery system, to ensure that thin sheets can be transferred to the wound without damage, and to develop storage methods than can sustain sheet viability for as long as possible for clinical applications.

V. CONCLUSION

*h*ASCs are good sources for MSC therapy. Photothermal cell sheet harvesting techniques enable cultured cells to be harvested non-invasively while minimizing cellular damage and maintaining contact between neighboring cells. *h*ASC sheets harvested using this technique were shown to preserve their intrinsic characteristics and multi-lineage differentiation capacities. Compared with conventional stem cell delivery strategies, they showed superior growth factor secretion and enhanced wound healing ability for both acute and chronic wounds, and this effect was enhanced during the initial phase.

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ABSTRACT (IN KOREAN)

시트형 지방유래 줄기세포의 창상 치유 효과

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양 채 은

지방유래 줄기세포는 분화유연성(plasticity)과 측분비효과(paracrine effect)에 의해 피부의 창상 치유를 촉진시키는 것으로 알려져 있다. 세포의 치료 효과를 극대화하기 위해서는 고농축 세포가 창상 표면에 적절하게 전달되어야 한다. 기존에 줄기세포를 창상에 전달하기 위해서는 배양기에서 배양된 세포를 트립신과 같은 효소를 이용하여 탈착시킨 후 이 현탁액을 창상에 주사하거나 분사하는 방법을 이용하였는데, 효소를 처리하는 과정에서 세포 손상이 불가피하고, 이식 과정 중에 소실되는 세포가 많은 것으로 그 효과를 극대화 시키는데 한계가 많았다.

본 연구에서는 인간 지방유래 줄기세포(*human* Adipose-derived Stem Cells, *h*ASCs)를 지방조직으로부터 배양한 후 비침습적 광열기술을 이용하여 시트 형태로 수확하여 창상치유 효과를 기존의 세포 전달 방법과 비교하였다.

In vitro 실험에서, 전자현미경으로 단층으로 세포간 접촉을 유지하고 있는 시트를 시각화 할 수 있었으며 유세포 분석을 통하여 시트 수확 후에도 시트 내 세포들이 지방유래 줄기세포의 고유 특성을 유지하고 있음을 확인하였다. 시트 수확 후 세포의 생존율은 시간이 지남에 따라 감소했지만, 이들 세포는 개별화된 세포보다 bFGF, TGF-β1, EGF, HGF, PDGF 및 VEGF를 포함하는 성장 인자를 더 활발하게 분비함을 확인하였다.

In vivo 실험은 SKH-1 hairless mouse를 이용하여 급성 창상과 만성 창상으로 나누어 진행하였다. 급성창상에서의 치유 효과를 알아보기 위하여 마우스의 등배부에 8mm지름의 전층 피부결손을 만든 후 지방

유래 줄기세포를 시트, 주사, 스프레이 형태로 적용하였고, 이를 대조군과 비교하였다. (10마리/군) 육안 비교와 H&E 염색에서 대조군에 비하여 줄기세포를 적용한 실험군에서 빠른 상피화를 보였으며, 특히 시트형태로 적용한 그룹에서 초기에 신속한 재상피화가 진행되었다. 또한 면역조직화학검사에서도 실험군, 특히 시트군에서 혈관형성이 활발함을 확인하였다. 만성 창상에서의 줄기세포시트의 창상 치유 효과를 확인하기 위해서 마우스의 등에 피부 깊이로 20Gy의 방사선을 조사하고 6주 후에 같은 창상을 만들었다. (12마리/군) 줄기세포시트를 이식한 그룹에서 대조군에 비하여 초기에 빠른 상피화를 관찰할 수 있었다.

시트형태로 줄기세포를 이식하는 방법은, 기존의 방법과는 달리 단백분해효소를 사용하여 세포를 개별화하지 않기 때문에 세포 표면의 단백질이나 세포간의 접촉이 보존되어 줄기세포가 더 많은 성장인자를 분비할 수 있었고, 창상에도 효과적으로 이식할 수 있다. 이를 바탕으로 지방유래 줄기세포를 비침습적 광열효과를 이용하여 시트형태로 수확하여 실제 창상에 적용한다면 기존의 방법보다 빠르고 효과적인 치유를 기대할 수 있을 것이다.

핵심되는 말 : 지방유래 줄기세포, 광열기반 세포시트, 창상 치유