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무흉선 마우스에서 저용량 레이저 치료를 이용한 개 지방 유래 중간엽줄기세포의 창상 치유 효과 증진

Enhanced Wound Healing Effect of Canine Adipose-Derived Mesenchymal Stem Cells with Low-Level Laser Therapy in Athymic Mice

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Enhanced Wound Healing Effect of Canine Adipose-Derived Mesenchymal Stem Cells with Low-Level Laser Therapy in Athymic Mice

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Abstract

Adipose-derived mesenchymal stem cells (ASCs) are attractive cell source for skin tissue engineering. However, one obstacle to this approach is that the transplanted ASC population

can decline rapidly in the recipient tissue.

The aim of this study was to investigate the effects of low-level laser therapy (LLLT) on transplanted canine ASCs in a skin wound animal model.

LLLT, ASC transplantation (ASCs group) and ASC transplantation with LLLT (ASCs + LLLT group) were applied to the wound bed in athymic mice. Wound healing was assessed by gross evaluation and by hematoxylin and eosin staining. The survival, differentiation and secretion of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) of the ASCs were evaluated by immunohistochemistry and western blotting.

The ASCs and ASCs + LLLT groups stimulated wound closure and histological skin regeneration. The ASCs + LLLT group enhanced the wound healing, including neovascularization and regeneration of skin appendages, compared with the ASCs group. The ASCs contributed skin regeneration via differentiation and secretion of growth factors such as VEGF and bFGF. In the ASCs + LLLT group, the survival of ASCs was increased by the decreased apoptosis of ASCs in the wound bed. The secretion of VEGF as well as bFGF was stimulated in the ASCs + LLLT group compared with the ASCs group.

These data suggest that LLLT is an effective biostimulator of ASCs in wound healing that enhances the survival of ASCs and stimulates the secretion of VEGF as well as bFGF in the wound bed.

Keywords: Wound healing, Skin regeneration, Adipose-derived mesenchymal stem cells, Low-level laser therapy, Athymic mice

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CONTENTS

I. Introduction

Skin wound healing can be delayed because of the wound location, personal history of disease or large area of skin defect resulting from trauma, burn or surgery. To accelerate skin regeneration, many skin tissue engineering techniques, such as the use of various scaffolds, cells and growth factors, have been researched and a number of artificial skin substitutes have reached commercialization. However, the effect of this materials fall short of the autologous skin graft. The ultimate goal for skin tissue engineers is to regenerate skin such that the complete structural and functional properties of the wound area are restored to the levels before injury (Metcalfe et al. 2007). Regeneration of skin appendages is very important for the maintenance of skin homeostasis. Recovery of skin strength is also main issues in wound healing. However, only a subset of the functions can be restored with existing tissue engineering techniques.

Mesenchymal stem cells (MSCs), which are found in many adult tissues, are an attractive cell therapy source for the regeneration of damaged tissues because they are able to self-renew and are capable of differentiating into various cells and tissues (Kern et al. 2006). A recent paradigm shift has emerged suggesting that the beneficial effects of stem

cells may not be restricted to cell restoration alone, but also due to their transient paracrine actions (Baraniak et al. 2010). Bone marrow, umbilical cord blood and aipose tissue have been the sources for the isolation of MSCs. Adipose tissue-derived mesenchymal stem cells (ASCs), which have characteristics similar to bone marrow-derived mesenchymal stem cells, can be acquired in large quantities with a simple surgical procedure relatively (Kern et al. 2006).

In cutaneous repair, MSCs significantly accelerate wound closure, exhibiting increased re-epithelialization, neovascularization and regeneration of skin appendages by differentiation and secretion of growth factors (Wu et al. 2010). However, several studies claim that the effects of stem cell therapy are not dramatic in the absence of scaffolds or stimulators (Cha et al. 2007, Wu et al. 2010). Recently, various scaffolds or growth factors were studied to increase the skin regeneration effect of stem cells (Li et al. 2006, Nakagawa et al. 2005, Perng et al. 2008).

Low-level laser therapy (LLLT) has been used for various purposes, such as relief of pain and inflammation and improvement in the local circulation, for a long time. Recently, many studies have demonstrated positive biostimulatory effects of LLLT on cells. The

results of LLLT vary according to the applied energy density and wavelengths to which the target cells are subjected. Generally, an energy density value of 0.5 to 4.0 J/cm^2 and a visible spectrum ranging from 600 to 700 nm of LLLT are helpful in enhancing the viabilaty of various cell lines. LLLT can stimulate cellular responses and promote the migration and proliferation of various cells by stimulating mitochondrial activity and maintaining viability (Alghamdi et al. 2012, Hawkins et al. 2006, Hu et al. 2007). The proliferation, growth factor secretion and differentiation of MSCs were also enhanced by LLLT without alteration of the expression of stem cell markers in vitro (Hou et al. 2008, Mvula et al. 2008, Peplow et al. 2011). However, little is known about the effects of LLLT on transplanted stem cells in animal models.

This study was performed to determine the effect of LLLT on transplanted ASCs in a skin wound animal model. The ASC transplantation and LLLT were applied in the wound beds of athymic mice. I compared the skin regeneration effects between the ASC transplantation group (ASCs group) and the ASC transplantation with LLLT group (ASCs + LLLT group).

II. Materials and Methods

1. Culture of ASCs

I use canine ASCs that were previously characterized (Ryu et al. 2009). The ASCs were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco®, Life Technologies, NY, USA) with 10% fetal bovine serum (FBS, Gibco®, Life Technologies, NY, USA), 100 units/ml penicillin and 100 μg/ml streptomycin at 37.0˚C, in a 5% CO_2 incubator. At passage 4 or 5, green fluorescent nanoparticles (Cell Stalker™ Ⅱ-CSF, Biterials, Seoul, Korea) were used for to label the ASCs. The nanoparticles were added to the culture medium at a final concentration of 0.2 mg/ml. After 24 hours, the nanoparticle-containing medium was removed from the culture flask. The ASCs were washed 3 times with phosphate buffered saline (PBS) and incubated with culture medium for 1 day prior to injection.

2. Preparation of the experimental animal model

Thirty-six athymic mice (BALB/cSlc-*nu/nu*, six weeks old, female, 15-17 g, Japan SLC Inc., Shizuoka, Japan) were anesthetized by intraperitoneal injection of a tiletamine/zolazepam combination (30 mg/kg, Zoletil50®, Virbac Korea, Seoul, Korea) and

xylazine (10 mg/kg, Rompun®, Bayer Korea, Seoul, Korea). After preparing the surgical site aseptically, two-full thickness skin wounds were created on the dorsal part using a 6 mm biopsy punch. To inhibit wound contraction, 0.5 mm thickness of silicone splint was applied as described previously (Galiano et al. 2004). The splint was fixed by instant adhesive and six simple interrupted sutures around the wound with Nylon 6-0. The wounds were randomly classified into four groups: control, LLLT, ASCs and ASCs + LLLT. In the ASCs and ASCs + LLLT group, 1 x 10^6 ASCs in 20 μl of PBS were transplanted intradermally at four injection sites on the border between the wound and the normal skin. Tegaderm (3M Health Care, MN, USA) was used for wound protection. All of the procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-110609-4), and the animals were housed in a specific pathogen-free room at Seoul National University.

3. Low-level laser therapy

In vitro study, viable cell number of ASCs significantly increased in the group irradiated with the 1 J/cm^2 compared with 0 and 3 J/cm^2 ($P<0.05$) (data is not shown). Upon this result, a helium-neon laser (He-Ne cold laser stimulator model PDT-A2, Lead Medical Science, Tokyo, Japan) was applied for 20 seconds daily from day 0 to 20. The distance

from the laser tip to the wound bed was approximately 10 mm. This laser model exhibited an irradiated wavelength of 632.8 nm and output energy of 17.0 mW. The energy density of each wound site was 0.34 J/defect and approximately 1.2 J/cm^2.

4. Gross evaluation of the wound area

The wounds were photographed using a digital camera at 0, 3, 7, 10, 14 and 21 days after surgery. The wound area was measured by tracing the wound margin and calculated using an image analysis program (ImageJ, NIH, MD, USA). The wound area was analyzed as a percentage of the original wound area. The wound was considered to be completely closed when the wound area was grossly equal to zero.

5. Histological examination

The mice were euthanized at 7, 14 and 21 days from the time of wounding. The wounds were harvested, including the surrounding normal tissue. The samples were fixed in a 10% formalin solution and embedded in paraffin. The skin samples underwent routine histological processing with hematoxylin and eosin (H&E). Histological examination was performed in a blinded fashion under light microscopy. The criteria used for the histological scores of wound healing were modified from previous reports (Wu et al. 2007) and are

summarized in Table 1. The histological parameters were re-epithelialization, dermal regeneration, granulation tissue formation, and angiogenesis. The regeneration of skin appendages was assessed by counting the number of hair follicles or sebaceous glands in the wound bed. For evaluation of angiogenesis, we perfomed immunohistochemistry for endothelial cells with an anti-CD31 antibody (1:200, ab28364, Abcam, MA, USA). Neovascularization was assessed by manually counting the number of vasculatures at the high-power field (HPF: x400) using 3 randomly selected fields from each skin section.

Table 1. Histological scoring system (a modification of previous study (Wu et al. 2007).

Scores	Re-epithelialization	Dermal regeneration	Granulation tissue formation	Angiogenesis
1	Minimal epidermal regeneration (<50%)	No skin appendage formation	Thin granulation around wound edges only	Little angiogenesis (<10 vessels/HPF[a])
2	Moderate epidermal regeneration (≥50%)	A few skin appendage formation (<5 appendages/ wound area)	Moderate granulation in the wound bed	Moderate angiogenesis (10-20 vessels/HPF)
3	Complete epidermal regeneration (100%)	Considerable skin appendage formation (≥5 appendages/ wound area)	Thick granulation in 100% of the wound bed	Marked newly formed and well-structured capillary vessels (>20 vessels/HPF)

[a] HPF (High-power field; original magnification x400)

For immunofluorescence, the tissue sections were blocked in 5% normal goat serum for 30 minutes at room temperature and incubated overnight at 4˚C with the following primary antibodies: pan-cytokeratin (1:200, sc-58826, Santa Cruz Biotechnology, CA, USA), vascular endothelial growth factor (VEGF), (1:200, ab1316, Abcam, MA, USA), basic fibroblast growth factor (bFGF), (1:800, ab8880, Abcam, MA, USA), caspase 3 (1:50, ab4051, Abcam, MA, USA) and Ki67 (1:200, ab15580, Abcam, MA, USA). Subsequently, the sections were rinsed with PBS and incubated for 1 hour at room temperature with Alexa 594-conjugated goat anti-rabbit or anti-mouse IgG secondary antibody (1:500, Invitrogen™, Life Technologies, NY, USA). Subsequently, 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. The numbers of ASCs and caspase 3 or Ki67-positive ASCs were counted using 3 randomly selected confocal images at the x200 field (Olympus IX70 Inverted Microscope, NY, USA). Each positive cell was counted only if clearly defined borders could be detected around the DAPI-positive nuclei.

6. Western blotting

Tissue proteins were extracted in protein extraction buffer (T-PER®, Thermo Fisher scientific, NY, USA) and protease inhibitor cocktail (Sigma-Aldrich, Seoul, Korea). The protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, CA, USA) (Bradford 1976). Aliquots of 30 μg protein were mixed with Laemmli's sodium dodecyl sulfate (SDS)-sample buffer (GenDEPOT, TX, USA), and boiled for 5 minutes before loading. The proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk for 60 minutes and incubated overnight at 4˚C with primary antibodies against VEGF (1:1000, ab1316, Abcam, MA, USA), bFGF (1:1000, ab8880, Abcam, MA, USA) and β-actin (1:5000, sc-47778, Santa Cruz Biotechnology, CA, USA). As a secondary antibody, goat anti-mouse or anti-rabbit IgG-HRP (1:5000, Invitrogen™, Life Technologies, NY, USA) was administered for 1 hour at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence kit (ECL kit, Invitrogen™, Life Technologies, NY, USA) and exposed to X-ray film for 30 seconds. The optical densities were quantified using NIH image analyzer software.

7. Statistical analysis

The data were presented as the means ± standard deviation, and the results were analyzed by one-way analysis of variance (ANOVA). Turkey's test was performed for comparisons between the groups. Student's t-test was used for the survival of the ASCs in the wound bed. A value of $p < 0.05$ was considered to be statistically significant. The statistical analyses were performed using SPSS, version 19.0 (SPSS Inc. DE, USA).

III. Results

1. Wound closure

The excisional wound splinting model was prepared, and the silicon splints remained tightly adherent to the skin and restricted wound contraction during the experimental period (Fig. 1A). Our data showed that the ASCs and ASCs + LLLT groups accelerated wound closure (Fig. 1B). At 3, 7, 10 and 14 days after surgery, the ASCs and ASCs + LLLT groups exhibited significantly smaller wound areas than did the other groups. At 10 days, the ASCs + LLLT group showed a significantly smaller wound area than the ASCs group. No significant difference was observed between the control and LLLT group at any time (Fig. 1C). At 21 days, all of the wounds of the ASCs and ASCs + LLLT groups had achieved complete wound closure, but not all of the wounds of the control (50%: 3/6) and LLLT (66.7%: 4/6) groups had completely closed. Distinctively, many hairs observed in regenerated skin especially in ASCs + LLLT group compared with other groups in gross evaluation (Fig. 1B).

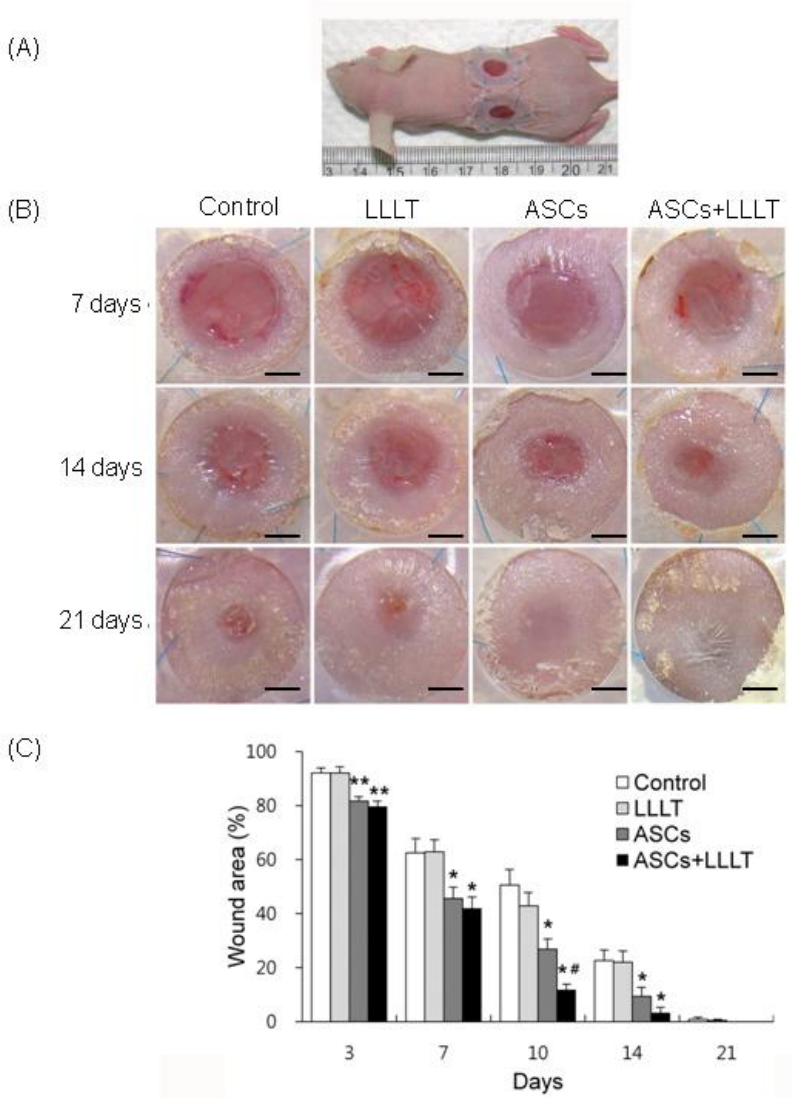
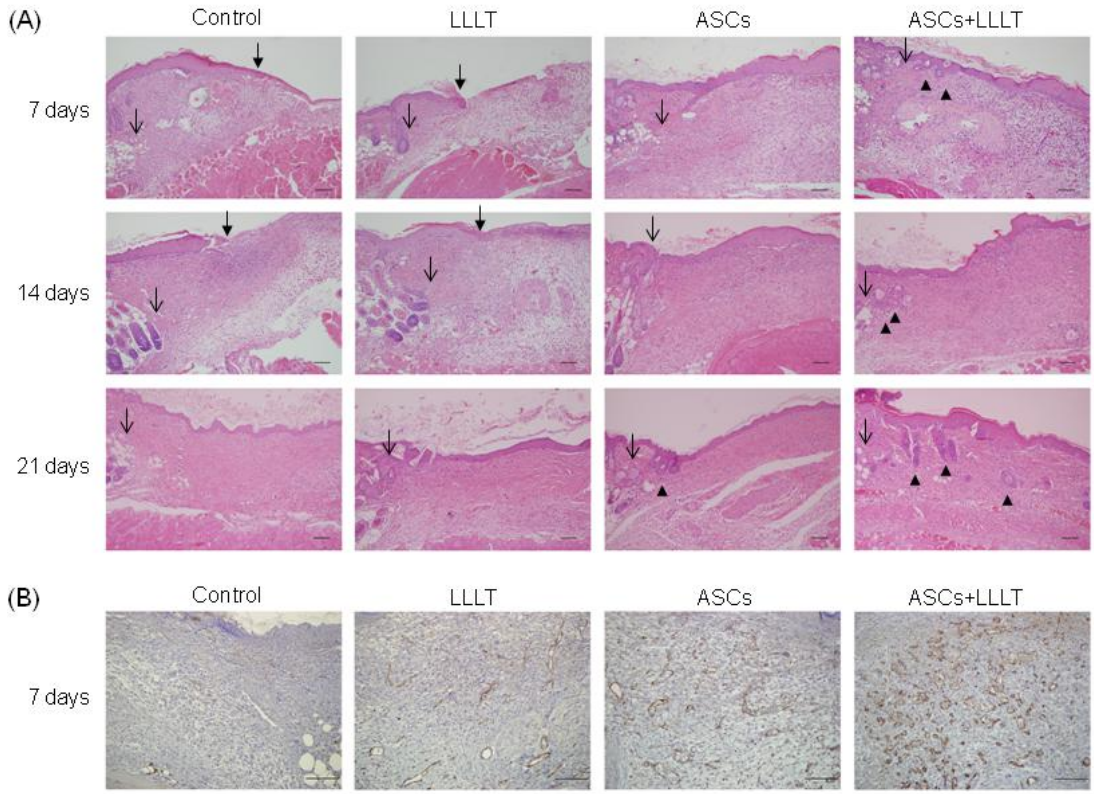


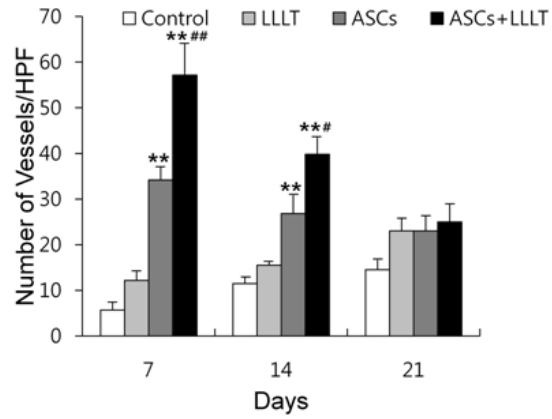
Fig. 1. Evaluation of wound closure. (A) The excisional wound splinting model was prepared. (B) Photographs of the wounds. (C) The percentage of wound area was calculated using photographs of the wounds at 3, 7, 10, 14 and 21 days. *$p < 0.05$, **$p < 0.01$ versus the control group; #$p < 0.05$ versus the ASCs group. Scale bar = 2 mm.

2. Wound re-epithelialization and dermal reaction

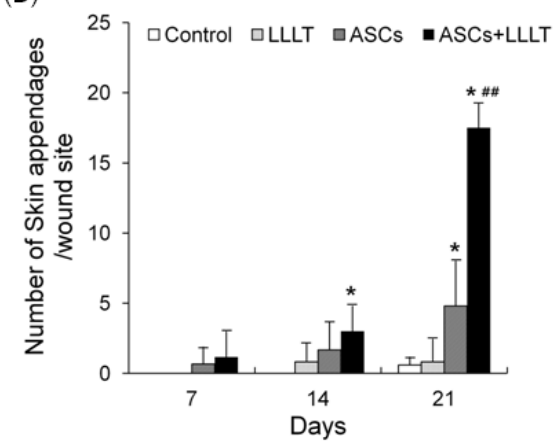
The histological observation showed that the skin regeneration was much greater in the ASCs and ASCs + LLLT groups compared with the control group. Our data indicated that the ASCs and ASCs + LLLT treatments enhanced re-epithelialization and granulation at 7 and 14 days (Fig. 2A). At 7 and 14 days, an analysis of the neovascularization indicated that the ASCs (34.17 ± 2.81 and 26.83 ± 3.94/HPF) and ASCs + LLLT (57.17 ± 6.73 and 39.83 ± 3.57/HPF) groups had significantly increased vasculature relative to the other groups (control: 5.67 ± 1.67 and 11.5 ± 1.31, LLLT: 12.17 ± 1.89 and 15.5 ± 0.76/HPF). Also, the ASCs + LLLT group showed significantly increased number of vasculatures compared with the ASCs group (Fig. 2B and C). Furthermore, the ASCs and ASCs + LLLT groups appeared to have increased numbers of skin appendages. In the ASCs and ASCs + LLLT groups, skin appendages were first observed in the wound edge at 7 days. The ASCs + LLLT group displayed significantly increased numbers of hair follicles and sebaceous glands (3 ± 1.79 and 17.5 ± 1.73/wound bed, respectively) at 14 and 21 days (control: 0 ± 0 and 0.6 ± 0.55, LLLT: 0.83 ± 1.33 and 0.83 ± 1.6, ASCs: 1.67 ± 1.97 and 4.8 ± 3.19/wound bed). These skin appendages were found more centrally in the regenerated dermis (Fig. 2A and D).


(A)
Control
LLLT
ASCs
ASCs+LLLT
7 days
14 days
21 days
(B)
Control
LLLT
ASCs
ASCs+LLLT
7 days

(C)


Control
LLLT
ASCs
ASCs+LLLT
Number of Vessels/HPF
70
60
50
40
30
20
10
0
**##
**
**
**#
7
14
21
Days

(D)


Control
LLLT
ASCs
ASCs+LLLT
Number of Skin appendages /wound site
25
20
15
10
5
0
*
*
*##
7
14
21
Days

(E)

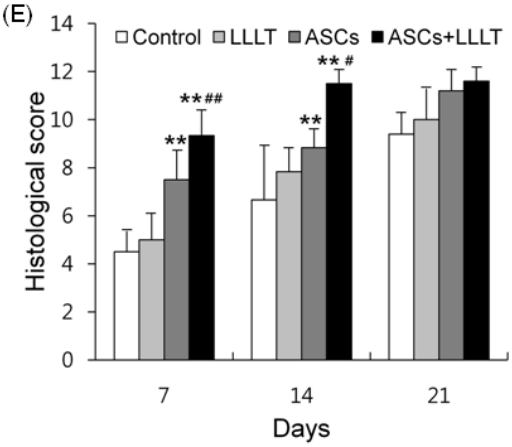

Control
LLLT
ASCs
ASCs+LLLT
Histological score
14
12
10
8
6
4
2
0
**
**
**##
**#
7
14
21
Days

Fig. 2. Histological analysis of the wound bed. (A) Wounds were stained with H&E at 7, 14 and 21 days. The wound edges are indicated by open arrows. The epithelial margins are indicated by closed arrows. The arrowheads indicate skin appendages. (B) Immunohistochemistry for endothelial cells at 7 days. (C) Neovascularization was determined by counting the number of vasculatures per HPF. (D) The regeneration of skin appendages was investigated by counting the number of skin appendages per wound section. (E) Histological scoring was performed using the criteria in Table 1. *$p < 0.05$, **$p < 0.01$ versus the control group; #$p < 0.05$, ##$p < 0.01$ versus the ASCs group. Scale bar = 100 μm.

In our study, the number of vessels was not directly proportional to granulation tissue volume. This result means there was difference in the quality of granulation tissue between groups. So, the criteria used for the histological scores of wound healing were modified from previous report, including re-epithelialization, dermal regeneration, granulation tissue volume and angiogenesis (Wu et al. 2007). The histological scores of the ASCs and ASCs + LLLT-treated wounds at 7 and 14 days were significantly higher than those of the control (Fig. 2E). Compared with the ASCs group, the ASCs + LLLT group showed significantly higher scores at 7 and 14 days. No significant difference was observed between the control and LLLT groups in H&E staining.

3. Survival of ASCs in the wound bed

At 21 days, ASCs, caspase 3-positive ASCs and Ki67-positive ASCs were identified using fluorescence microscopy throughout the wound bed to determine whether locally transplanted ASCs were incorporated into the healing wounds. In the ASCs and ASCs + LLLT groups, ASCs labeled with green fluorescent nanoparticles were observed in the regenerated skin tissue (Fig. 3A), but no ASCs were found in the control or LLLT groups. The ASCs + LLLT group exhibited significantly increased numbers of ASCs (ASCs group: 20.00 ± 1.51, ASCs + LLLT group: 33.75 ± 2.56/HPF) (Fig. 3A and B) and decreased proportions of caspase 3-positive ASCs (ASCs group: 39.86 ± 5.90%, ASCs + LLLT group: 9.21 ± 6.45%) (Fig. 3A and C). However, there was no significant difference between two groups in the proportions of Ki67-positive ASCs (ASCs group: 1.11 ± 1.11%, ASCs + LLLT group: 3.4 ± 1.7%) (Fig. 3A and D).

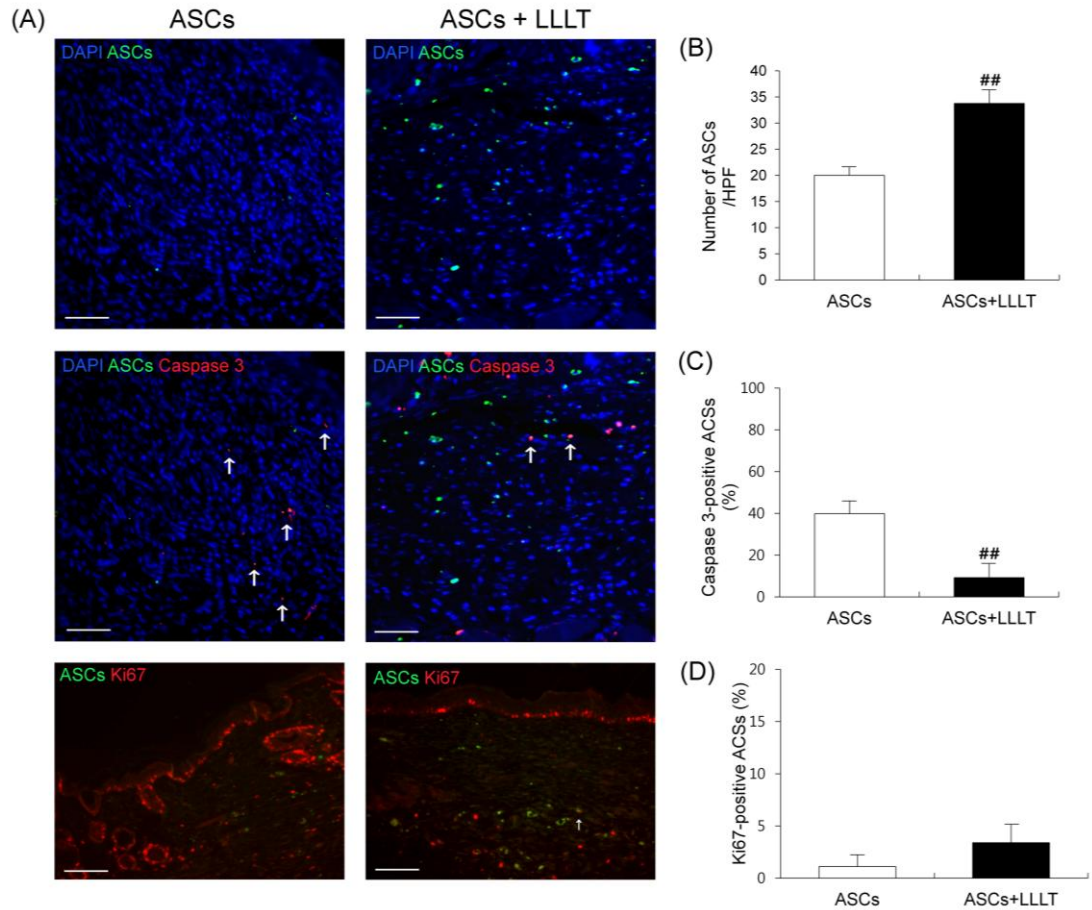


Fig. 3. Survival of transplanted ASCs in the wound bed. (A) In the ASCs and ASCs + LLLT groups, DAPI (blue), ASCs (green) and caspase 3 or Ki67 (red)-positive cells were detected after immunostaining at 21 days. Caspase 3-positive ASCs (yellow) or Ki67-positive ASCs were indicated by arrows. (B) The surviving ASCs were determined by counting the number of ASCs. (C) The caspase 3-positive ASCs were counted in wound beds. (D) The Ki67-positive ASCs were counted in wound beds. The results were analyzed in percentage terms. ##$p < 0.01$ versus the ASCs group. Scale bar = 50 μm.

4. Differentiation of ASCs into epithelial cells

To determine whether the ASCs could contribute to the epidermal structure, immunohistochemistry for pan-cytokeratin was performed at 14 days. The ASCs were found especially in the newly formed dermis close to the epidermis or hair follicles (Fig. 4). Some cytokeratin-positive ASCs were found in the epidermis, hair follicles or sebaceous glands in the ASCs and ASCs + LLLT group. There is no obvious difference in the number of cytokeratin-positive ASCs between the ASCs and ASCs + LLLT groups (Fig. 4A, B, C and D).

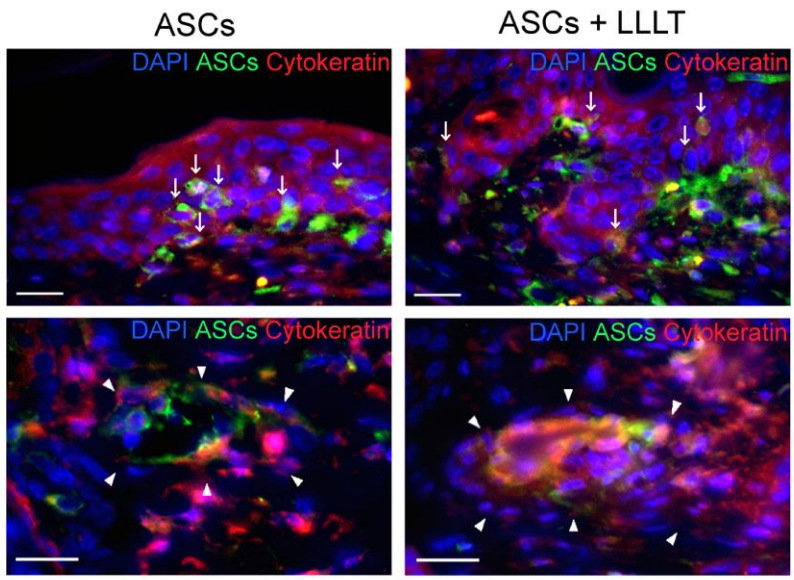


Fig. 4. Differentiation of ASCs into epithelial cells. Immunofluorescence images show cytokeratin-positive epithelial cells (red) at 14 days. Cytokeratin-positive ASCs (yellow, indicated by arrows) were observed in the regenerated epidermis or hair follicles in the ASCs and ASCs + LLLT groups. The regenerated skin appendages are indicated by arrowheads. Scale bar = 20 μm.

5. Secretion of growth factors in the wound bed

To identify whether a correlation existed between particular growth factor levels and the wound healing process, I performed immunohistochemistry and western blotting at 14 days. In the ASCs and ASCs + LLLT groups, VEGF-positive ASCs were mostly found in the dermis close to the epidermis. The bFGF-positive ASCs were mostly found in the newly formed dermis. Compared with the ASCs group, more growth factor-positive ASCs were observed in the ASCs + LLLT group (Fig. 5A). Western blotting showed that significantly higher levels of VEGF and bFGF were secreted by the ASCs and ASCs + LLLT groups than by the control group, and greater amount of growth factors were observed in the ASCs + LLLT group than in the ASCs group. However, there is no significant difference between LLLT-treated wounds and control wounds (Fig. 5B and C).

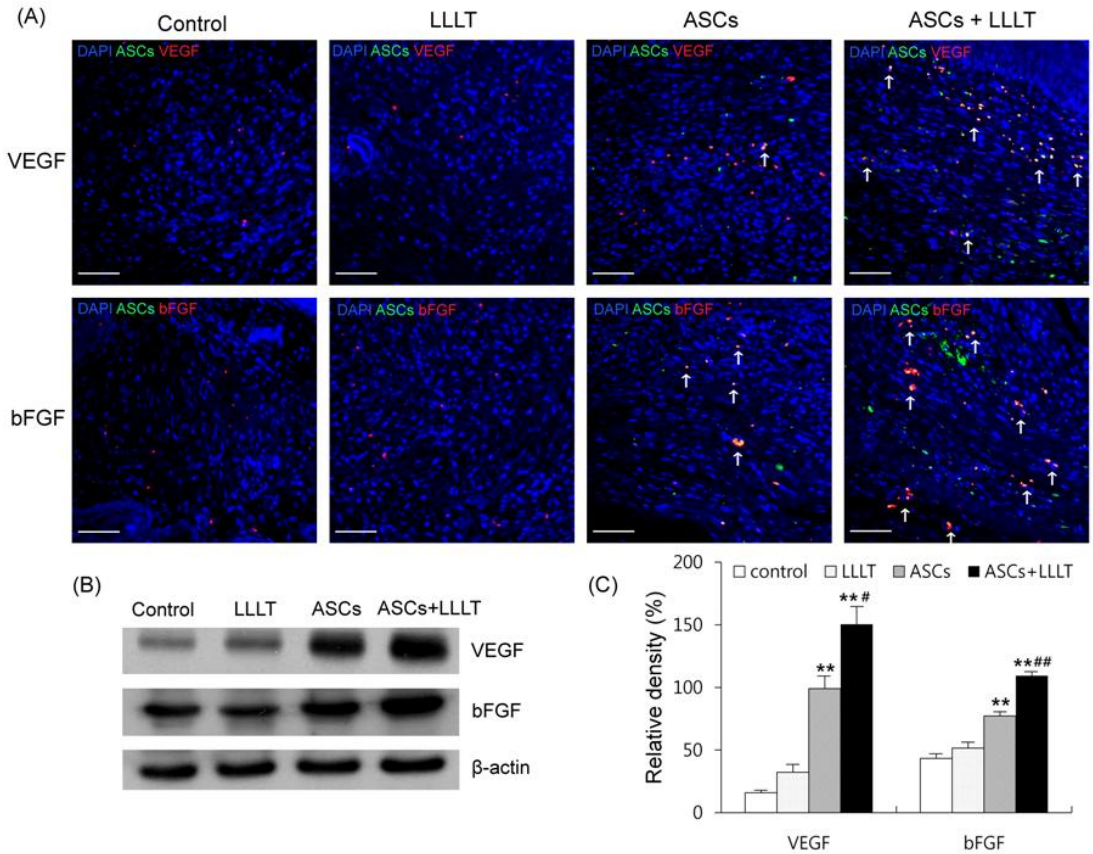


Fig. 5. Evaluation of growth factor levels. (A) Immunostaining was performed with anti-VEGF or anti-bFGF antibody (red) at 14 days. VEGF- or bFGF-positive ASCs (yellow) are indicated by arrows. (B) Western blotting shows the expression of VEGF and bFGF at 14 days. (C) The results of western blotting were analyzed as relative density. $^{**}p < 0.01$ versus the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus the ASCs group. Scale bar = 50 μm.

IV. Discussion

In this study, ASCs accelerated wound closure with increased re-epithelialization, granulation, neovascularization and regeneration of skin appendages. In addition to the hair follicles and sebaceous glands, some cytokeratin positive-ASCs were observed in regenerated epidermis. Additionally, some VEGF or bFGF positive-ASCs were detected in the wound bed. VEGF is most effective and specific growth factor that regulate angiogenesis (Nie et al. 2009). bFGF is important growth factor in wound healing because of that affect migration and proliferation of fibroblast, angiogenesis, and matrix deposition (Lee et al. 2012). Consistent with our findings, several recent studies reported that ASCs enhanced wound repair by differentiation and paracrine effects. Previous studies reported that the MSCs migrate into the wound area (Smith et al. 2010) and differentiate into keratinocytes, endothelial cells, sweat glands, sebaceous glands and hair follicles (Kataoka et al. 2003, Nie et al. 2011, Wu et al. 2007). Additionally, several studies have shown that MSCs secrete paracrine factors, such as VEGF, bFGF, epidermal growth factor, keratinocyte growth factor, insulin-like growth factor and hepatocyte growth factor (Baraniak et al. 2010, Chen et al. 2008, Lee et al. 2011, Nie et al. 2009, Nie et al. 2011, Wu et al. 2007) and stimulate the deposition of extracellular matrix (Kim et al. 2007).

Recent studies showed that a significant decline in MSC number was observed in the skin wound bed, bone defect or infarcted myocardium within the initial 2 weeks (Levi et al. 2010, Noiseux et al. 2006, Wu et al. 2007). Our data revealed an increased number of ASCs and a decreased percentage of caspase 3-positive ASCs in the ASCs + LLLT group compared with the ASCs group at 21 days. However, no significant difference was observed between the ASCs and ASC + LLLT group in percentage of Ki67-positive ASCs. These data suggest that LLLT enhanced the survival of ASCs by the inhibition of apoptosis. Furthermore, in the ASCs + LLLT group, more VEGF- or bFGF-positive ASCs were observed in the regenerated dermis, and greater amounts of growth factors were found in the wound bed than in the ASCs group. These data suggest that LLLT enhanced not only the survival, but also the functionality of the transplanted ASCs in the wound bed. Several studies have found that LLLT is effective for the enhancement of the viability and proliferation of stem cells with appropriate energy density and wavelengths (Alghamdi et al. 2012, Hou et al. 2008, Mvula et al. 2008). In addition, LLLT increases the gene expression and release of several growth factors, including VEGF and nerve growth factor, from stem cells (Hou et al. 2008, Peplow et al. 2011, Saygun et al. 2008). It is possible that LLLT enhances cellular responses in terms of gene expression and the secretion of growth factors

and cell proliferation via increases in the mitochondrial membrane potential and ATP and cAMP levels (Hu et al. 2007).

The differentiation of ASCs into epithelial cells had no obvious difference between the ASCs and ASCs + LLLT groups. In previous studies, LLLT stimulated the differentiation of stem cells into myocytes or osteoblasts (Abramovitch-Gottlib et al. 2005, Hou et al. 2008, Kim et al. 2009). However, another study suggested that LLLT could avert the premature differentiation of these cells into other tissue types, such that stem cells could maintain their characteristics for longer periods (Mvula et al. 2008). Therefore, the effect of LLLT in the differentiation of stem cells is not fully understood, and further study is necessary.

In this study, the ASCs + LLLT group was evaluated as exhibiting rapid wound closure and a higher histological score compared with the ASCs group. In skin bioengineering, the ultimate aim is to rapidly produce a construct that offers the complete restoration of functional skin, ideally involving the regeneration of all of the skin appendages and layers (Metcalfe et al. 2007). Athymic mice are not hairless, but instead show an abortive reduced hair growth on different sites of the integument. There are no obvious structural differences between normal and athymic mice in the appearance of the outer root sheath, dermal papilla,

bulb and sebaceous glands. In the athymic mice the hairs are bent and coiled in the upper dermis and they usually fail to penetrate the epidermis (Militzer 2001) . So, athymic mice used for the studies about hair growth effect of new therapy (Udagawa et al. 2001, Vegesna et al. 2002). Interestingly, my results showed that the ASCs + LLLT group had significantly increased numbers of hair follicles and sebaceous glands in the wound bed in the ASCs + LLLT group than in the ASCs group. Previous studies suggest that hair follicle growth and development were controlled by several growth factors (Messenger 1993). VEGF is a major mediator of hair follicle growth and cycling by improving angiogenesis. The transgenic overexpression of VEGF in hair follicles strongly induced perifollicular neovascularization, resulting in accelerated hair regrowth after depilation and in increased size of hair follicles and hair shafts (Yano et al. 2001). The gene expression of FGF ligand and FGF receptor increased in the hair follicle during the hair growth cycle (Rosenquist et al. 1996). These data suggest that the ASCs + LLLT treatment increased the regeneration of skin appendages due to the enhanced secretion of growth factors.

In summary, this study suggested that ASC transplantation accelerates wound healing through differentiation and growth factor secretion. Furthermore, my results demonstrated that LLLT enhanced the wound-healing effect of the ASCs by enhancing survival of the

ASCs and stimulating secretion of growth factors in the wound bed. In particular, the ASCs + LLLT treatment enhances the functional recovery of the wound area with respect to the regeneration of skin appendages. These results may provide therapeutic approaches for the treatment of delayed wound healing.

V. References

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요약(국문초록)

무흉선 마우스에서 저용량 레이저 치료를 이용한 개 지방 유래 중간엽줄기세포의 창상 치유 효과 증진

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지방 유래 중간엽줄기세포 (adipose-dirived mesenchymal stem cells, ASCs)는 피부 조직 공학에 유용한 재료이다. 하지만 앞선 연구를 통해 ASCs가 이식한 조직내에서 빠르게 감소하여 그 효과가 감소한다는 것이 보고되고 있다. 본 연구는 피부 창상 동물 모델에서 ASCs를 이식한 후, 저용량 레이저 치료 (low-level laser therapy, LLLT)를 적용하여 ASCs의 창상 치유 효과에 어떠한 영향을 미치는지에 대하여 조사하였다.

32마리의 무흉선 마우스에 피부 창상을 유발한 후, 대조군, LLLT 적용군 (LLLT군), ASCs 이식군 (ASCs군), ASCs 이식 후 LLLT 적용군 (ASCs+LLLT군)으로 무작위로 나누어 처치를 적용하였다. 육안적인 창상 면적 평가 및 hematoxylin & eosin 염색, CD31에 대한 면역화학염색 후 실시한 조직학적 평가를 통해 창상 치유 과정을 분석하였다. 활성화된 caspase 3, Ki67에 대한 면역화학염색을 통해 조직 내 이식한 ASCs의 생존성을 평가하였고, cytokeratin, VEGF, bFGF에 대한 면역화학염색 및 Western blot을 통해 ASCs의 분화, 성장인자 분비에 대해 평가하였다.

ASCs군과 ASCs+LLLT군에서 대조군에 비해 육안적으로 빠른 창상 면적 감소 효과를 보였고, 조직학적으로 빠른 육아조직 형성, 혈관 신생, 피부부속기 신생, 상피화 효과가 확인되었다. ASCs군과 비교하여 ASCs+LLLT군에서 혈관 신생, 피부부속기 신생을 포함한 창상 치유 과정이 더욱 촉진된 것으로 평가되었다. 또한, ASCs는 이식된 피부 창상면에서 상피세포로의 분화, 성장인자 분비를 통해 피부 재생을 촉진시킨 것으로 확인되었다. ASCs와 LLLT를 함께 적용한 군에서는 ASCs를 단독으로 적용한 군과 비교하여 ASCs의 세포자멸사 억제로 인해 생존성이 증가하였으며, 성장인자 분비가 촉진되었다.

이상의 결과를 토대로, LLLT가 이식한 조직 내에서 ASCs의 생존성 및 성장인자 분비능을 향상시켜 ASCs의 창상 치유 효과를 증진시킨다는 결과를 확

인할 수 있었다.

주요어: 창상 치유, 피부 재생, 지방 유래 중간엽줄기세포, 저용량 레이저 치료, 무흉선 마우스

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