Human amniotic fluid-derived stem cell patch affects the healing process of burn wound

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Directed by Professor Incheol Park

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ABSTRACT

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Early treatment of acute burn injury, especially in the case of extensive injury, requires hemodynamic maintenance, prevention of infection, promotion of wound healing processing and re-epithelization. The effectiveness of dressing treatment, which should be performed early in the emergency department, is critical to determining the patient's prognosis. Many dressing methods and materials have been proposed from the past and various cell treatment methods have been tried in the early stage of burn in order to promote wound healing and re-epithelization. Currently, cell-based, engineered skin substitutes are showing promise for the treatment of acute and chronic wounds such as deep and partial burns, ulcers resistant to conventional therapies and surgical wounds. The knowledge on the stem cell biology has been dramatically increased for last several years and attempts for applying stem cells to burn wound treatment has been introduced. Stem cell-based therapies have the potential to enhance cutaneous regeneration, largely through trophic and paracrine activity. Among various sources of mesenchymal stem cells (MSCs), amniotic fluid-derived stem cells (AFSCs) are attractive due to their high proliferation capacity, multipotency, immunomodulatory activity and lack of significant immunogenicity. In this study, we developed poly (L-lactide-co-glycolide) (PLGA) scaffold patches which have

highly porous structure and suitable for cell attachment and delivery. AFSCs are attached to these scaffold patches and their therapeutic efficiency in burn wound care was evaluated in animal models.

AFSCs were collected from a third-trimester pregnant woman. Cells were expanded and cultured on scaffold patches. Ultrastructure of scaffold patches and attached cells were evaluated by scanning electron microscopy. Full-thickness burn was induced by tripod brass comb heated by boiling water in paravertebral area of athymic nude rats. Eschar was removed one day after burn and AFSCs-laden scaffold patches (patch with cell group, n=5) and cell-free scaffold patches (patch only group, n=4) were applied to wound site. Seven days after scaffold patch application, the rats were sacrificed and whole thickness of skin surrounding wound sites was sampled. The specimen was bisected at the center of the wounds, then one half was fixed in formaldehyde for histologic evaluation and the other half was stored in deep freezer for western blotting.

In histologic evaluation, the fibrosis involves almost whole thickness of the dermis and abuts subcutaneous muscle. Microscopically, the wounds were re-epithelialized except in necrotic portion. Dermis covered by re-epithelialized squamous epithelium shows thick irregular collagen bands and Percentage of burn wound re-epithelialization was statistically significant in patch with cells group. The irregularity and size of collagen bands were more prominent in patch with cell group than patch only group. Transforming growth factor-β (TGF-β) expression was more than seven-times prominent and statistically significant in patch with cell group than in patch only group. Vascular endothelial growth factor (VEGF)-A expression was not different between two groups and VEGF-B expression showed a trend of increase in patch with cell group but did not obtain statistical significance.

Consequently, pre-prepared AFSC laden PLGA nano-patches suggested the possibility of one of the new methods that can be used immediately in the early stage of acute burn patients. In addition, this method could achieve cell treatments as well as dressing effects.

Key words: burn wound, amniotic fluid stem cell, PLGA scaffold patch, athymic rat

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I. INTRODUCTION

Early treatment of acute burn injury, especially in the case of extensive injury, requires hemodynamic maintenance, prevention of infection, promotion of wound healing processing and re-epithelization. Especially, in severe pediatric burns prompt resuscitation and management is vital to survival[1]. Although management principles for children are similar to adult, children have unique pathophysiologic responses to burn injury; thus, an understanding of the differences in fluid resuscitation requirements, airway management, burn and wound care is essential to optimize their outcomes[2]. Severe burns impact every organ system. Severity is related to the percentage total body surface area (%TBSA) burned and the burn depth, but even relatively small burns can be life-threatening and lifechanging[3]. Burns greater than 20% TBSA cause a system-wide inflammatory response. The large volume release of inflammatory mediators and cytokines into the circulation leads to leaky microvasculature, vasodilation and decreased cardiac output. Simplistically, the local response overwhelms the microenvironment and becomes systemic[4]. Careful documentation of fluid input and output, beginning in the emergency department, is important in the management of burn resuscitation. Additionally, an increased susceptibility to infection due to altered immune status may lead to sepsis, further exacerbating systemic inflammation. Sustained hypermetabolism and inflammation impair

wound healing through delayed re-epithelialization. Owing to the high lethality, suspicion of an invasive burn wound infection mandates rapid diagnosis, often by histopathology, and excision or re-excision of the wound[5]. Both small burn and large severe burn injuries initiate the wound healing process which consists of several highly integrated and overlapping phases: inflammation, cell recruitment, matrix deposition, epithelialization and tissue remodeling[6].

The effectiveness of dressing treatment, which should be performed early in the emergency department, is critical to determining the patient's prognosis. Many dressing methods and materials have been proposed in the past and various cell treatment methods have been tried in the early stage of the burn to promote wound healing and re-epithelization. Dressings allow for a protective barrier until tissue integrity is restored, or until skin grafting can take place (if necessary)[7]. Commonly used salves include silver sulfadiazine and bacitracin. Silver sulfadiazine (SILVADENE ®) provides added coverage for gram-negative and enteric bacteria[2]. Most wounds can be covered with typical dressings including petroleum gauze or iodine impregnated gauze. Wound care following debridement varies depending on the depth of the burn. Many partial or full thickness burns can be initially managed with salves, soaks, or dressings. Salves are topical ointments and creams that are applied to provide moisture to the wound bed[2]. Recently, many novel alternative dressings have become available for use in burn wound care. Biologic options include xenografts, cadaveric skin, and placenta-derived tissue[2]. Foam dressings have the ability to absorb exudate, which allows for less frequent dressing changes[8]. The non-adhesive nature of foam potentially causes less wounding and surrounding skin damage during dressing changes[8]. The use of xenografting in burns was described as early as 1880[9]. In 2013, Hermans[10] reported their modified systematic review to determine if there was a clinical difference between xenografting and allografting. He concluded that either type of skin substitute seemed to promote rapid wound healing and re-epithelialization[10].

Currently, cell-based, engineered skin substitutes are showing promise for the treatment of acute and chronic wounds such as deep and partial burns, ulcers resistant to conventional

therapies and surgical wounds[11-13]. These various engineered tissue formats include allogeneic fibroblasts and keratinocytes in a bovine collagen matrix (OrCel®, Ortec International, Atlanta, GA, USA)[14] or a bilayered living skin equivalent, Apligraf® (Organogenesis,Inc., Canton, MA, USA). Electrospinning was developed over 30 years ago but has attracted new interest in the last decade[15]. Using this technique, nano-scale fibers can be produced to mimic ECM in native human skin. A major advantage of electrospinning technology is the ability to spin both natural polymer such as silk, chitosan, gelatin, fibrinogen and synthetic materials such as Poly (ε-caprolactone (PCL), polyurethane (PU), poly (glycolic acid) (PGA) and poly (L-lactide) (PLLA)[16,17]. In a previous study, we developed polymer scaffold patches that patches contain numerous holes suitable for cell attachment and survival[18].

Many dressing methods and materials have been proposed from the past, various cell treatment methods have been tried in the early stage of burn in order to promote wound healing and re-epithelization. The study of epithelial cell culture or mesenchymal stem cells (MSCs) culture, which are representative methods using cells, are the most effective. However, due to the limitation of using autologous cells, it has limited applications in treating acute serious burn patients as they need to be treated immediately after arrival at the emergency department.

The human amniotic membrane has been used as a biological dressing material from the early 1990's and it's utility for burn wound care has been supported by several studies.[19,20] To secure safety from transmissible diseases from the amniotic fluid, several preconditioning methods have been developed and commercial products such as dehydrated human amnion/chorion membrane (dHACM) are being used.[20]

Similar to MSCs the amniotic fluid stem cells (AFSCs) contained in the amniotic fluid have the potential to provide high proliferation and lack of significant immunogenicity. The knowledge on stem cell biology has dramatically increased in the last several years, along with several attempts in applying stem cells to burn wound treatment. Stem cell-based

therapies have the potential to enhance cutaneous regeneration, largely through trophic and paracrine activity. Further, MSCs pretreating and preconditioning enhance the cell migration, proliferation, and survival rate leading to higher angiogenesis, re-epithelialization, wound closure and granulation tissue formation[21]. Bone marrow-derived stem cells and AFSCs have been applied for wound treatment and especially burn wound treatment and their therapeutic mechanism has also been investigated[22]. Liu et al.[23] performed experiments on pigs where they applied a collagen scaffold patch with seeded MSCs onto the surface of inflicted burns; this was found to induce better burn wound healing with less contraction and better vascularization and keratinization. Therefore, development of new methods to increase the efficacy of MSCs-based wound treatment is desirable developed.

In this study, we compared the therapeutic effect of AFSCs in a full-thickness burn model of athymic rats by pre-preparing AFSCs to our previous described nano-scaffold patches and testing its efficacy for immediate application to treat acute serious burn patients in the emergency department. We evaluated re-epithelialization and wound remodeling, which are potentially related to the paracrine and trophic effect of AFSCs and attempted to determine the benefits of AFSCs treatment on burn wound.

II. MATERIALS AND METHODS

1. Isolation and culture of human AFSCs

Amniotic fluid samples were obtained from a donor in the third trimester of pregnancy during her cesarean section delivery. Informed consent was obtained from the donor, and the sample collection was approved by the institutional review board of Yonsei University. The donor was cleared for hepatitis B and C, human immunodeficiency virus and syphilis. After spinning down cells from the amniotic fluid at approximately 1,200 rpm for 10 minutes, the cells were resuspended in CHANG Amnio media (Irvine Scientific, Santa Ana,

CA, USA). Meanwhile, tissue culture plates were prepared by coating with CTS™ CELLstart™ matrix (Invitrogen Life Sciences, Grand Island, NY, USA) based on the manufacturer's instruction. The cells were added to treated tissue culture plates and placed at 37 °C in 5% humidified CO_2 atmosphere. Through regular media changes, the attached cells gradually appeared as spherical colonies, and began to demonstrate the characteristics of human AFSCs. Using fluorescence activated cell sorting technique, CD117-positive cells were selected and routinely expanded in the following media: high glucose Dulbecco's modified Eagle's media containing 1% penicillin-streptomycin, 20% fetal bovine serum, 2mM glutamax, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and a growth factor supplement consisting of basic fibroblast growth factor (10 ng/ml) and epidermal growth factor (10ng/ml). The multipotency of the obtained AFSCs were verified using human MSCs functional identification kit (Catalog # SC006. R&D systems Inc., Minneapolis, MN, USA).

2. Characterization of AFSCs laden PLGA patch

AFSCs attached to the PLGA scaffold patches that were previously developed by us[18]. Briefly, the PLGA scaffold patched were placed inside cell culture inserts, and then 1×10^5 AFSCs were seeded on the PLGA patch-embedded inserts. To examine the morphological features of the AFSCs-PLGA patches, both scanning electron microscopy (SEM) and actin filament fluorescent staining were used. AFSCs in the patches were stained with rhodamine phalloidin (50ng/ml) for 20 minutes to stain the actin filaments, while DAPI (4′,6-diamidino-2-phenylindole) solution (0.4 μM) was used to counterstain the nuclear DNA of the ells. The stained cells were observed by a fluorescence microscope (OX.2053_PLPH microscope, Euromax, Netherlands).

3. Animal model of burn wound

Male athymic nude rats (Hsd:RH-*Foxn1*^{rnu}) that were 10-weeks-old were purchased from Envigo (Greenfield, IL, USA). Full thickness burn was induced by using a tripod comb (147g, POSTECH, Pohang, Korea) having three 10-mm teeth separated by two 10-mm notches (Figure 1).

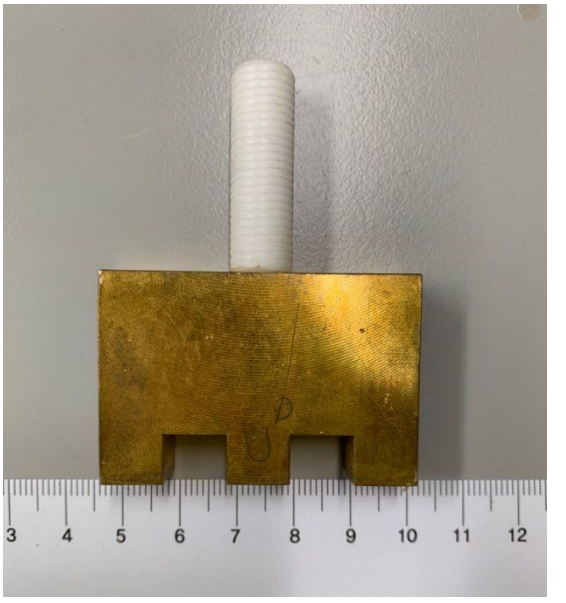

Figure 1. The tripod brass comb. This was used to induce thermal contact burns.

This tripod comb produced of three 10 × 20 mm burn wounds at the paravertebral area. Initially, the rat was anaesthetized through intraperitoneal injections of ketamine. In some instances, the rat also received 1 mL of saline subcutaneously along the spine to cushion the spinal cord from any injury[24] The tripod comb was preheated in boiling water (100 ℃) for 3 minutes and applied with minimal pressure for 1 minute on one paravertebral area. The tripod comb was reheated and similarly applied to the other side of the paravertebral area. The wounds made by the center pod were selected because surface contact pressure

and time were most well controlled at this pod site. Eschar was removed after 24 hours and scaffold patches with (treatment group, n = 5) or without (control group, n = 4) attached AFSCs were applied. Seven days after applying scaffold patches, rats were euthanized and the whole thickness of skin surrounding wound sites was sampled. The specimen was bisected at the center of the wounds, then one half was fixed in formaldehyde for histologic evaluation and the other half was stored in deep freezer for western blotting.

4. Histologic evaluation of the wounds

Formaldehyde-fixed tissues were routinely processed (paraffin embedding and microtome cutting) for histologic evaluation. They were cut into 4-μm sections and stained with hematoxylin-eosin (H&E) and trichrome. The degree of fibrosis was evaluated from the trichrome-stained sections using Image J software (Version 1.51j8, National Institutes of Health, Bethesda, Maryland, USA). Photos were taken at non-necrotic portion of the wounds nearest to the center at a magnification of 400×. Three consecutive fields were evaluated. The degree of dermal fibrosis was calculated by morphometric analysis using the Image J software. Blue color was gated after adjusting the color threshold parameters, and the area occupied by blue-stained fibrosis was measured and compared to the whole area and represented by area percentage. The amount of re-epithelialization was evaluated by measuring the width between epithelium-covered shoulders at the center of the wound.

5. Western blot analysis

Small fragments of fresh frozen tissue were obtained from the non-necrotic portion near the center of the wounds. They were lysed in radioimmunoprecipitation assay buffer (Biosesang, Inc., Seongnam, Korea). After centrifugation at 15,871×g for 30 minutes at 4 °C, protein concentration was measured using the BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's guide. Then, 30 μg

of each sample was loaded and separated on 12% SDS-PAGE for 2 hours at 100 V. Samples were transferred to a polyvinylidene fluoride membrane and nonspecific binding epitopes were blocked by 3% skim milk for 1 hour at room temperature. The membrane was incubated with primary antibodies overnight at 4 °C. Primary antibodies used were as follows: TGF-β1 (ab64715, 1:500, Abcam, Cambridge, MA, USA), VEGF-A (ab214424, 1:1000, Abcam, Cambridge, MA, USA), and VEGF-B (sc-81670, 1:200, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). An antibody against β-actin (sc-47778, 1:5000, Santa Cruz Biotechnology, Inc.) was used as a loading control. All antibodies bound to the membrane were visualized by applying secondary antibodies (mouse IgG-HRP, GTX212311-01, Genetex, Irvine, CA, USA or rabbit IgG-HRP, GTX213110-01, Genetex) for 1 hour at room temperature and Pierce Enhanced Chemiluminescence Western Blotting Substrate (Thermo Fisher Scientific, Inc., MA, USA). Densitometry analysis of the bands was performed using the Image J software.

6. Statistical analysis

Data were analyzed using SPSS version 25 (IBM-SPSS Inc, Armonk, NY). For comparison between two groups Mann-Whitney U test was used. All values were expressed as mean ± standard error of mean. Statistical significance was defined as $p < 0.05$.

III. RESULTS

To assess the efficacy of the PLGA patches in delivering AFSCs to the burn wound site, AFSCs were directly seeded onto the PLGA patches, and characterized by SEM. When seeded with AFSCs, the PLGA patch was fully covered, indicating that the AFSCs had grown on the porous nanofibrous matrix (Figure 2A). Moreover, it was found that the AFSCs were present inside of the PLGA patch consisting of multiple sub-layers of PLGA

nanofibers (Figure 2B). At the micro-scale level, several layers of a PLGA patch were occupied with AFSCs, demonstrating that the PLGA patch was a stem cell-friendly biocompatible scaffold patch. It was also observed that each layer of the PLGA patch is made of non-woven PLGA nanofibers (Figure 2C).

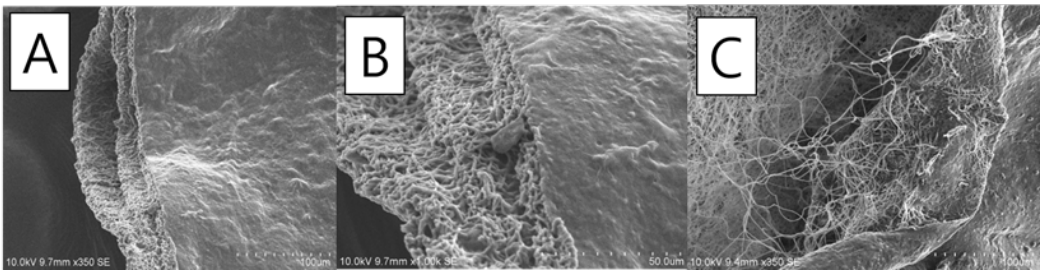

Figure 2. Scanning electron microscopy images of the human AFSCs-PLGA patch. The scale bar is located at the bottom in each image. (A) Tilted side view of the AFSCs-PLGA patch (350×). (B) Tilted side view of the same AFSCs-PLGA patch in (A) at 1000× magnification. (C) Inside view of the AFSCs-PLGA patch (350×)

Further characterization of the AFSCs-PLGA patches was done by actin filament staining. By visualizing actin microfilaments within an AFSCs-PLGA patch, it was found that the PLGA patch was a suitable wound healing patch for delivering AFSCs. Phalloidin binds filamentous actin and its counterpart, rhodamine, showed red color in fluorescent microscopy. Compared to the confluent AFSCs on a regular tissue-culture treated plate (Figure 3A), comparable AFSCs were observed in the PLGA patches. Regardless of different PLGA patches, AFSCs were well identified by actin filament staining (Figure 3B and 3C). The nanofibrous PLGA patches exhibited the stem-cell friendly substrate, increasing the cellular activity on the nanofibrous structures. Therefore, it was speculated that the AFSCs-PLGA patches have the potential to restore the normal function of the damaged skin. The AFSCs-PLGA patch may reduce severe skin inflammation associated with burning and increase the stem-cell engraftment on the damaged skin due to its porous

and nanofibrous nature.

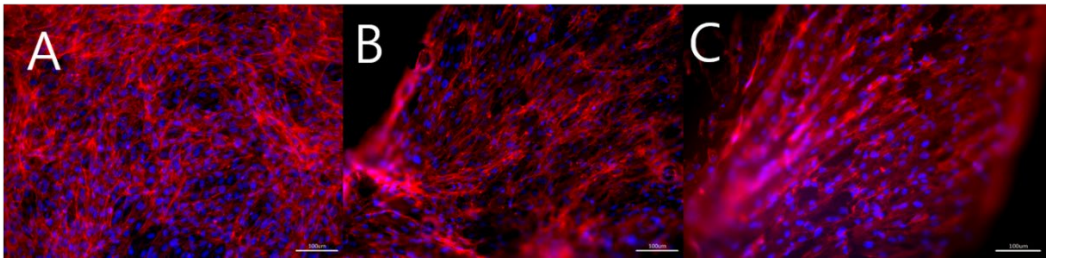

Figure 3. Rhodamine-phalloidin staining for actin filaments of the human AFSCs-PLGA patch. Filamentous actin (F-actin) are stained in red color whereas cellular DNA are shown in blue. Scale bar, 100 μm. (A) AFSCs cultured in a tissue-culture treated plate served as a control. (B) AFSC grown onto the surface of an AFSCs-PLGA patch #1. (C) AFSC grown onto the surface of an AFSCs-PLGA patch #2..

To analyze the effect of AFSC containing patches on the process of burn wound healing, we next estimated epithelialization (%), fibrosis (%), and cytokine (TGF- β, VEGF-A, VEGF-B) levels (Table 1).

Table 1. Total outcomes of AFSCs with patch group and patch only group.

Group	Epithelialization (%)	Fibrosis (%)	TGF-β	VEGF-A	VEGG-B
		Trichrome stain	Relative expression (/β-actin)		
Patch only-1	15.00	21.60	0.00	1.14	0.00
Patch only-2	13.33	14.00	0.00	0.78	0.00
Patch only-3	6.67	16.30	0.00	1.25	0.14
Patch only-4	20.00	16.40	4.00	0.82	3.86
Patch with cells-1	35.00	25.20	5.36	0.93	2.82
Patch with cells-2	33.33	30.10	5.20	0.84	2.19
Patch with cells-3	100.00	55.00	0.43	0.53	2.07
Patch with cells-4	41.67	45.20	14.51	0.56	4.74
Patch with cells-5	30.00	50.20	12.36	0.83	3.51

1. Effect of AFSCs patch on the wound healing process

A. Morphology of wound healing

Burn wound of rats was induced by using the tripod comb Eschar was removed after 24 hours and scaffold patches with (treatment group) or without (control group) attached AFSCs were applied (Figure 4A). After 1 week, the surface of the wounds was replaced by brown tan crusts in most of the rats (Figure 4B).

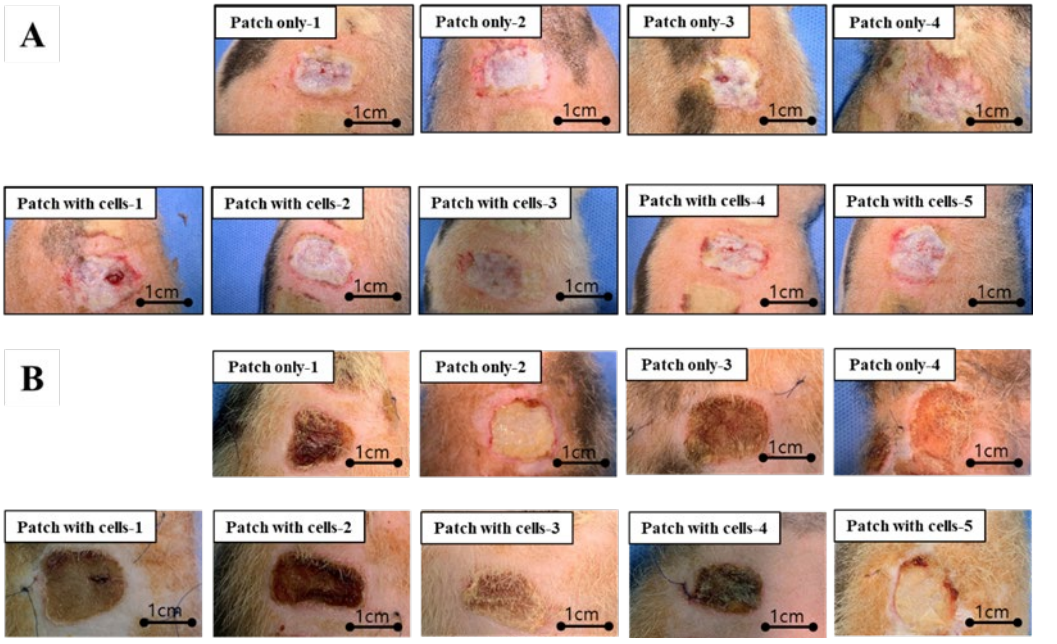

Figure 4. Burn wounds induced by tripod comb. The wound was a rectangular space consisting of three 10 × 20 mm burn wounds at the paravertebral area. The wounds made by the center pod was selected because surface contact pressure and time were most well controlled at this pod site. Eschar was removed after 24 hours and scaffold patches with or without attached AFSCs were applied (A). After 1 week, the surface of the wounds was replaced by brown tan crusts in most of the rats (B).

The cut surface of each wound revealed small central portions containing necrotic debris surrounded by dense fibrosis replacing the dermis (Figure 5A, 5B). Histological examination showed higher epithelialization rate (Figure 5C), and increased granulation tissue formulation in the treatment group compared with that in the control group. Microscopically, the wounds were re-epithelialized except in the necrotic portion. Percentage of burn wound re-epithelialization was statistically significantly higher in the treatment group compared with that in the control group (Table 2).

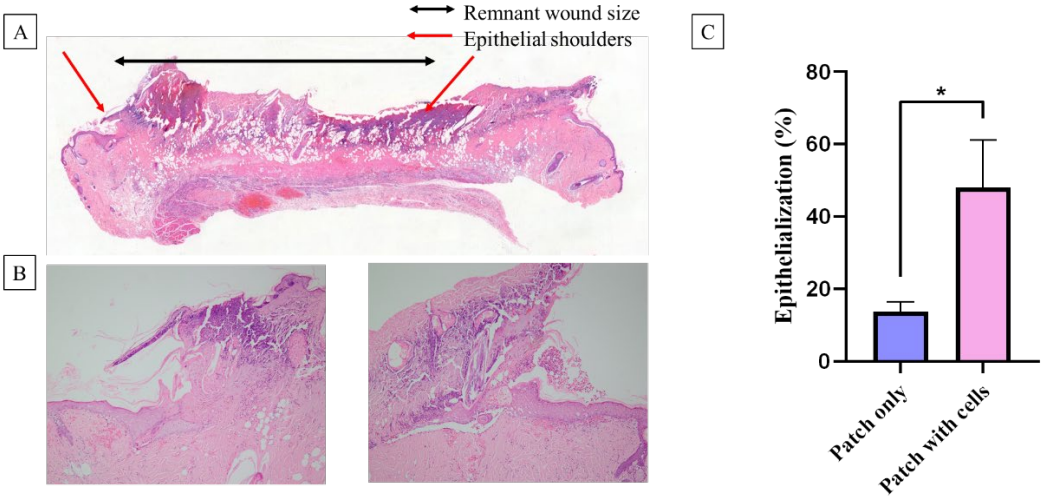

Figure 5. Histological analysis of re-epithelization after hematoxylin and eosin staining (A; 1:1 scan, B; epithelial shoulder (×100)). Re-epithelialization of bun wound was significantly higher in patch with cells group (* $p< 0.05$, C).

Table 2. Statistical analysis of Re-epithelialization of bun wound between cell with patch group and path only group

	Patch only group (n=4)	Patch with cell group(n=5)	Mann-Whitney U test (p-value)
% Re-epithelialization	13.75 ± 2.75	48.14 ± 13.14	0.02*

Mean ± S.E.M; *Significant difference ($p< 0.05$)

The fibrosis involved almost whole thickness of the dermis and abuts subcutaneous

muscle (Figure 6). Dermis covered by re-epithelialized squamous epithelium showed thick irregular collagen bands. The irregularity and size of collagen bands were more prominent in patch with cell group than patch only group. Morphometric analysis demonstrated that the area percentage of trichrome-stained collagen bands was larger in patch with cell group than in patch only group (Table 3).

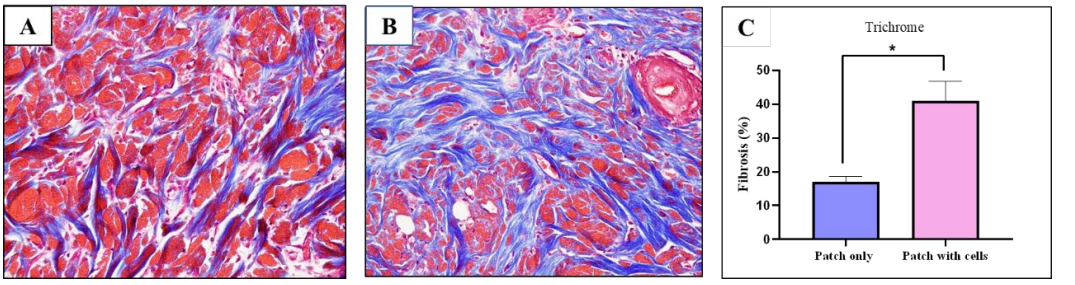

Figure 6. The wounds showing dermal fibrosis in patch only group (A). (Trichrome, 200×) Image analysis detecting blue-colored fibrosis area (B) revealed significantly larger amount fibrosis in rats treated with AFSCs-laden scaffold patches. * $p< 0.05$ (C).

Table 3. Statistical analysis of trichrome stained fibrosis area between cell with patch group and path only group

	Patch only group (n=4)	Patch with cell group(n=5)	Mann-Whitney U test (p-value)
% Fibrosis	17.08 ± 3.21	41.14 ± 12.91	0.02*

Mean ± S.E.M; *Significant difference ($p< 0.05$)

B. Expression of wound healing-related molecules

Western blot analysis of wound healing-related molecules in wound tissues revealed that TGF-β expression was more than seven-times prominent in AFSCs-laden scaffold patch group than in patch only group. VEGF-A expression was not different between the two groups. VEGF-B expression showed an increasing in patch with cell group, although the difference was not statistically significant (Figure 7) (Table 4).

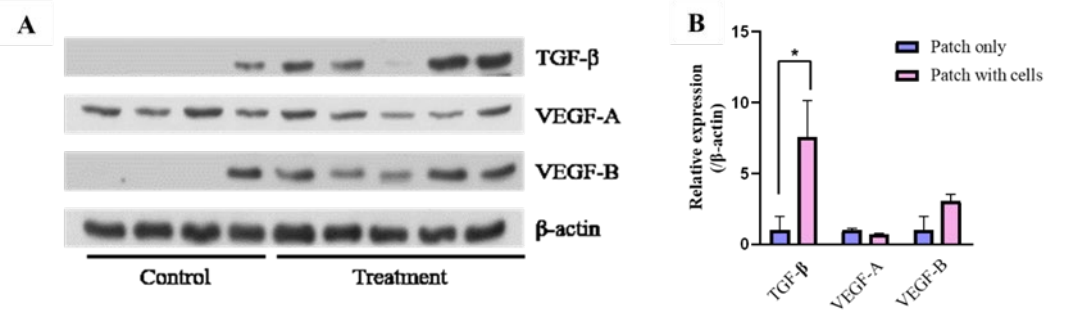

Figure 7. Western blot analysis. TGF-β showed higher expression in rats treated with cell with patch group (treatment group) than in rats treated with patch only group (control group). (A: western blot, B: densitometry analysis). * $p< 0.05$ (C)

Table 4. Statistical analysis of western blot of wound tissue between cell with patch group and path only group

	Patch only group (n=4)	Patch with cell group (n=5)	Mann-Whitney U test (*p*-value)
TGF-β	1 ± 2	7.57 ± 5.76	0.03*
VEGF-A	1 ± 0.23	0.74 ± 0.18	0.41
VEGF-B	1 ± 1.91	3.07 ± 1.10	0.18

Mean ± S.E.M; *Significant difference ($p< 0.05$)

IV. DISCUSSION

Burn treatment needs fast-action intervention. If we consider stem cells as a treatment modality, they should be modulated for instant use. Culture and preparation of the autologous stem cells take time. Allogeneic stem cells can be prepared and kept on hand for burn treatment[25]. Although there are studies showing that only autologous cells can promote rapid wound healing[26], a bank of allogeneic cells can provide a possibility to quickly treat patients suffering extensive and deep second-degree burns, and in this case, the most preferable cells are stem cells possessing hypo-immunogenicity (e.g., AFSCs) Moreover, attempts have been made to reproduce skin appendages (for instance, hair follicles and sebaceous glands) in vitro and integrated them into skin substitutes[27-29].

Stem cells are immature cells that have multi-differentiative potential which allows them to generate other cell types of various organs. There are two subtypes of stem cells: adult and embryonic. Embryonic stem cells are derived from newly fertilized egg called blastocysts[30]. They can differentiate into any kind of cell lineages and have an unlimited ability to self-renew and are named as totipotent[31]. Unlike embryonic stem cells, adult stem cells can be obtained almost throughout the body, have limited differentiation potential compared, and are called pluripotent[32]. MSCs have generated great interest in the fields of regenerative medicine and immunotherapy because of their unique biological properties. MSCs can be purified from various tissues, including bone marrow[33], adipose tissue[34] and umbilical cord blood[35]. Amniotic fluid or amniotic membrane as another source of stem cell harvest was identified relatively recently. Harvesting of MSCs from bone marrow is an invasive procedure. Especially, in small animal studies, this invasive procedure usually cannot be completed without sacrifice of animal bone marrow, as a source of MSCs are decreasing in the number, maximal life span and differentiation potential of MSCs in elderly and systemic inflammatory diseases such as major burn[36]. Another source for MSCs is adipose tissue, which provides a rich source of MSCs by simple harvesting[37]. Properties of adipose-derived MSCs are similar to bone marrow derived MSCs[38]. Isolation of adipose-derived MSCs in small animals such as rats is well defined procedure[39].

Among MSCs, the AFSCs have several characteristics that make them attractive candidates for tissue engineering cell replacement strategies[40,41]. AFSCs are not ethically controversial; in fact, the excess amniotic fluid collected during routine clinical amniocentesis that is not used for genetic research is normally discarded[42]. The amniotic fluid is an under-utilized source of stem cells, with therapeutic potential in the field of regenerative medicine. Stem cells from the amniotic fluid can be isolated and expanded easily, and can differentiate into various cell types without the risk of tumorigenesis when implanted in immuno-deficient mice[43]. AFSCs fulfill the criteria for MSCs[44] and express the pluripotency markers Nanog, Oct-4, and SOX-2,4 and the embryonic stem cell markers CD117, SSEA-4, TRA-1–60, and TRA-1–81[40,41]. AFSCs are broadly multipotent as they are able to differentiate not only into mesoderm-derived (bone, fat, cartilage, muscle, hematopoietic), but also into non-mesodermal lineages (endothelial, hepatic, neuronal)[41].

The role of stem cells in wound healing, specifically burn wound healing, and the therapeutic applicability of stem cells in wound care are being actively investigated. Depending on burn severity, the healing process may result in different consequences. Superficial burns recover within two weeks and cause minimal scarring. The re-epithelization of partial thickness burns is ensured by keratinocyte migration from skin dermal appendages within a few hours of the injury. In deeper burns, the healing starts around the edges, but not at the center because of the necessity of rapid wound closure[45-47]. The therapeutic effect of stem cells and their tentative mechanism have been proposed in several ways. Several studies have reported that MSCs promote re-epithelialization by enhancing proliferative activity of keratinocytes via paracrine activity of various growth factors.[48,49] Direct differentiation of these stem cells into keratinocytes and other MSCs such as endothelial cells and fibroblasts has also been demonstrated.[50,51]

In this study, we applied nanofiber scaffold patches with or without attached AFSCs. To our knowledge, postburn plasma TGF-β levels have been reported to increase on postburn days 6–8 [52]. Therefore, our study was designed to investigate TGF-β levels in the first week after burns. Well-formed collagenous fibrosis was observed 7 days after burn wound

induction, which is still in acute phase of wound healing. Athymic rats treated with the AFSCs containing patches showed thicker collagenous bands and showed significantly increased TGF-β expression level. In contrast, VEGF family, which is associated with neovascularization and granulation tissue formation during wound healing process tended to increase, but did not show a statistically significant compared with the patch only group.

Wound healing is a dynamic, interactive process involving soluble mediators, ECM components, resident cells (keratinocytes, fibroblasts, endothelial cells, nerve cells), and parenchymal cells[53,54]. In each type of wound, healing occurs in three overlapping phases: inflammation, proliferation, and remodeling. Each phase is regulated by growth factors secreted by cells present at the wound site, and the growth factors attract the cells necessary for the next phase of healing[55]. The onset of the inflammatory phase occurs when tissue injury causes the disruption of blood vessels and extravasation of blood constituents. Platelets aggregate at the wound site, and fibrin is deposited to form a clot that seals the wound. Numerous vasoactive mediators and chemotactic factors are generated by the activate coagulation and complement cascades and by injured or activated parenchymal cells. These substances recruit inflammatory leukocytes to the site of injury[56]. Neutrophils, and later macrophages, are responsible for destroying bacteria in the wound and phagocytosing any debris. Macrophages also secrete angiogenic factors and growth factors that stimulate fibroblast production and collagen synthesis[57]. The proliferative phase is characterized by epithelialization, granulation tissue formation, connective tissue deposition, angiogenesis, and contraction. Epithelialization is the process of replacing damaged surface tissue with new healthy tissue. Re-epithelialization of wounds begins within hours after injury[58].

In this study, western blot analysis for TGF-β and VEGF was performed in the burn wounds to determine whether AFSCs that migrate into burn injury lesions affect the levels of protein involved in wound healing. As mentioned before, TGF-β expression increased significantly but VEGF showed only an upward trend. In addition, H & E and Masson's Trichrome staining of burn wounds were performed to assess the change in epithelialization

and collagen production induced by AFSCs. Re-epithelialization of burn wounds were relatively increased by the AFSCs and collagen deposition of the ECM was increased. Recent study regarding adipose-derived stem cells (ADSCs) also reported significant increase of TGF, re-epithelialization and production of collagen-based ECM on wound healing of a diabetic rat model[25]. Ghosh et al. investigated interaction between MSCs and TGF- β1 a growth factor that plays an important role both in normal wound healing and in fibrosis. They found that TGF- β1 pretreatment contributed to more efficient propagation of MSCs and increased the wound closure rate in vivo[59].

At the wound site immediately after injury, TGF-β1 is released in large amounts from platelets[60]. This release then serves as a chemoattractant for neutrophils, macrophages, and fibroblasts These cell types further enhance TGF-β1 levels in various cell types. During this time, latent forms of TGF-βs are also produced and stored within the wound matrix. This ensures a continuous supply of TGF-β throughout the repair process. Although TGF-β is basically a potent growth factor, effects on the cells in the context of wound healing is diverse. Some researchers have shown that TGF-β was an important cytokine at the root of the development of tissue fibrosis, and critical for initiation as well as final steps of tissue repair[61]. Excessive or sustained production of TGF-β1 is a key molecular mechanism of tissue fibrosis. In contrast topical application of TGF-β accelerated wound healing. In rats, topical or limited intravenous administration of recombinant TGF-β1 normalized wound healing that was impaired by age or glucocorticoids[62]. In humans, TGF-β2 has dual effects on both tissue fibrosis and healing, therefore it could be a potential target in wound healing treatment.

Importantly, stem cell therapy on experimental burn model pays attention to some critical issues; stem cell source, therapeutic dose, delivery method and timing of stem cell delivery methods in order to get reproducible and effective outcomes and to show validity of stem cell therapy at burn wound[63]. In this study, the source was amniotic fluid obtained from a pregnant woman at 3rd trimester. We used the PLGA patch developed in the previous study[18] as a delivery method and after 24 hours of burn injury, eschars were removed and scaffold

path with or without AFSC were applied on burn wounds. Burn wounds begin to heal by contraction. Burn wound contraction involves a complex and coordinated interaction of cells, ECM and cytokines. It is the process by which the wound edges move closer together, toward the center of the wound. This reduces the healing time by inducing the amount of ECM that needs to be produced in order to fill the wound, but healing by contraction also results in more scarring[55].

To date, few studies have used stem cells and nanofiber scaffolds in burns[64,65]. Although there was animal study of burn using poly-D,L-lactic acid (PDLLA) nano-scaffold and stem cell, there was no statistically significant difference in treatments between the two groups[66].

This study demonstrated that AFSCs delivered by PLGA nanofiber scaffold patches potentiated the production of TGF-β and re-epithelialization associated dermal fibrosis during burn wound healing process. VEGF did not show significant increase, indicating that the amount of active granulation tissue formation was not significantly increased. Fibrosis in the context of wound healing may have contrasting effects. It can contribute to the early stabilization of the wound sites and also hamper the functional recovery. It is more probable that the fibrosis in our model contributed to the wound stabilization because it was associated with re-epithelialization of surface keratinocytes.

V. CONCLUSION

In summary, our study showed pre-prepared AFSC laden PLGA nano-patches as a novel method for the immediate treatment of acute burn patients. In addition, our study revealed that the associated mechanisms involved re-epithelialization of burn wounds, increased TGF-β, and subsequent fibrosis.

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ABSTRACT (IN KOREAN)

양수유래 줄기세포가 화상 치유에 미치는 효과

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급성 화상의 조기 치료, 특히 그 부위가 광범위할 경우에는 혈역학적 감시와 감염 예방, 상처 치유를 위한 재상피화가 필요하다. 특히 중증 소아 화상에서는 소생술이 신속히 이루어지는 것이 환자의 생존에 매우 중요하다. 소아에서도 치료의 원칙은 성인과 유사하지만 소아는 화상에 대한 고유한 병리 생리학적 반응을 보이므로 소생술, 기도 관리, 화상 및 상처 관리의 차이점에 대한 이해는 치료를 위한 필수적인 요소이다. 심한 화상은 모든 장기 시스템에 영향을 미치고, 화상의 중증도는 전체 신체 표면적 중 화상의 범위(%) 및 화상 깊이와 관련이 있다

다양한 화상 치료방식이 개발되었지만, 광범위한 화상 및 전체 피부층을 파괴하는 상처는 여전히 환자에게 신체적 손상뿐 아니라 심리적으로도 큰 상처를 준다. 화상 치료에서의 중간엽 줄기세포의 적용은 보다 나은 치료결과를 보일 것으로 기대된다. 사람의 신체에는 화상 상처부위에 적용될 수 있는 줄기세포의 공급원이 다양하게 있다. 중간엽 줄기세포의 다양한 공급원 중에서, 양수유래 줄기세포는 높은 증식 능력, 다능성, 다양한 면역 조절 기능 및 세포의 면역 반응의 감소로 화상 치료에 장점이 있다. 이 연구에서 우리는 많은 다공성 구조로 세포 부착 및 전달에 적합한 poly (L-lactide-co-glycolide) (PLGA) 스캐폴드 패치를 개발했다. 본 연구에서는 양수유래 줄기세포 (Amniotic Fluid Stem Cells, AFSCs)를 스캐폴드 패치에 부착시키고 화상 상처치료에서의 치료 효율을 동물 모델에서 평가하였다.

양수유래 줄기세포는 3 기 임산부로부터 얻어졌다. 스캐폴드 패치상에서 세포를 확

장시키며 배양을 진행하였다. 스캐폴드 패치 및 부착된 세포의 구조는 주사 전자 현미경에 의해 평가되었다. 무 흉선 누드 래트의 등 부위 영역에서 끓는 물에 의해 가열된 황동 삼각 화상 유발체에 의해 전체 피부에 대한 화상이 유발되었다. 화상을 입힌 다음날 에스카를 제거하고 양수유래 줄기세포 함유 스캐폴드 패치 (치료 그룹, n = 5) 및 무 세포 스캐폴드 패치 (대조군, n = 4)를 상처 부위에 적용하였다. 스캐폴드 패치 적용 7 일 후, 래트를 희생시키고 상처 부위를 둘러싼 피부의 전체 두께를 샘플링 하였다. 샘플링 된 피부를 상처의 중심에서 이등분한 다음, 절반을 조직학적 평가를 위해 포름알데히드로 고정시키고 다른 절반을 웨스턴 블롯을 위해 냉동 보관하였다.

조직 학적 평가에서, 섬유화는 진피의 거의 전체를 수반하고 피하 근육까지 접하는 것이 관찰되었다. 현미경적으로 상처는 괴사부분을 제외하고 재상피화되었다. 재상피 편평상피로 덮인 진피는 불규칙한 콜라겐 밴드를 보여주었다. 재상피화의 정도는 실험군에서 통계학적으로 유의하게 증가하였으며, 콜라겐 밴드의 불규칙성과 크기 역시 대조군보다 치료군에서 더 두드러졌다. Transforming growth factor-β(TGF-β)의 발현은 실험군에서 대조군보다 7 배 이상 증가하였으며 통계학적으로 유의하였다. VEGF-A (Vascular endothelial growth factor)발현은 두 그룹간에 차이가 없었으며, VEGF-B 발현은 실험군에서 증가하는 경향을 보였지만 통계학적 유의성을 얻지 못했다.

결과적으로, 미리 준비된 AFSCs 함유 PLGA 나노 패치는 급성 화상 환자의 초기 단계에서 즉시 사용될 수 있는 새로운 치료 방법의 가능성이 있음을 확인할 수 있었다. 또한, 이 방법은 드레싱 효과뿐만 아니라 세포 치료 효과도 달성할 수 있을 것으로 기대된다.

핵심되는 말: 화상상처, 양수유래 줄기세포, PLGA 스캐폴드 패치,무흉선 랫트