Rapid Expansion and Auto-Grafting Efficiency of Porcine Full Skin Expanded by a Skin Bioreactor *Ex Vivo*

Man-Il Huh^{1,2}, Sun Hee An¹, Hwi-Gang Kim³, Yun-Jeong Song³, Eun-Chang Choi³, Sang-Hyun An⁴, Woo-Sung Choi⁴, Jeung Soo Huh⁵, Jeong Ok Lim^{1*}

¹Biomedical Research Institute, Joint Institute for Regenerative Medicine, Kyungpook National University Hospital, Daegu, Korea

²Department of Ophthalmology, School of Medicine, Kyungpook National University, Daegu, Korea

³Medical IT Convergence Research Section Daegu-Gyeongbuk Research Center, Electronics and Telecommunications Research Institute, Daegu, Korea

⁴Daegu-Gyeongbuk Medical Innovation Foundation, Daegu, Korea

⁵Department of Materials Sciences and Metallurgy, College of Engineering, Kyungpook National University, Daegu, Korea

Full skin auto-grafts are required for reconstruction of skin burns and trauma scars. However, currently available clinical approaches such as sheet skin graft, mesh skin grafts, artificial skin graft, and *in vivo* skin expansion have limitations due to their potential danger for secondary damage and scar formation at the donor site, and discomfort during skin expansion. We developed an advanced bioreactor system and evaluated its function in skin expansion using porcine full skin. The reactor was designed as a pneumatic cylinder type, was programmed to adjust the pressure and the operating time. The system was composed of culture chamber unit, environmental control unit, and monitoring unit. Skins were expanded at 200 kPa pneumatic force and the expanded skins were analyzed by immunohistochemistry and histology. Furthermore we carried out auto-grafting experiment of the expanded skins *in vivo* using Yucatan pigs and skins were harvested and histologically analyzed after 8 weeks. The results showed that the bioreactor expanded skins to 160% in 4 hours. Histological analysis of the expanded skins revealed that epidermal cells and dermal fibroblasts were viable and remained integrity. The results of auto-grafting experiment indicated that fibrosis and scars were not detected in the grafted skins. This study demonstrates that the newly developed skin bioreactor enabled to obtain large sized full skin rapidly and successful grating.

Tissue Eng Regen Med 2016;13(1):31-38

Key Words: Skin expansion; Bioreactor system; Full skin graft

INTRODUCTION

Skin grafts are required in numerous clinical procedures, such as reconstruction after skin removal, and correction of contracture or scarring because of burns, accidents, and trauma. The current standard for skin defect replacement procedures is the use of autologous skin grafts. However, donor-site tissue availability, which remains a major obstacle to the successful replacement of skin defects, often limits this option. Because of this limitation, other approaches are commonly employed to

cover skin defects. These include commercially available skin products based on biomaterials and tissue engineering, allografts, and xenografts [1-3].

In addition, *in vivo* subcutaneous tissue expanders or meshed split thickness skin grafts are being used clinically to generate larger segments of autologous skin when donor-site tissue is limited. Subcutaneous tissue expanders are balloon implants that are sequentially filled with increasing amounts of saline to increase the amount of overlying skin. The mechanical stress of the tissue expander results in biologic creep, greater mitotic activity, and greater vascularity, which ultimately leads to expanded skin. Subsequently, the expanded skin can be used as a tissue flap or harvested for use as a skin graft. However, the shortcomings of this method lie in the requirement of additional surgical procedures, which may increase morbidity, and extensive waiting times to obtain appropriate sized tissue. Moreover, discom-

Received: October 15, 2015

Revised: October 27, 2015

Accepted: October 29, 2015

***Corresponding author:** Jeong Ok Lim, Biomedical Research Institute, Joint Institute for Regenerative Medicine, Kyungpook National University Hospital, 130 Dongdeok-ro, Jung-gu, Daegu 41944, Korea.

Tel: 82-53-420-6950, Fax: 82-53-427-5447, E-mail: jolim@knu.ac.kr

fort associated with the increasing expander volume and frequent tissue fibrosis remains as major limitations [4-14]. Currently, numerous reports supported that stem cells and their production have a great potential for accelerating wound healing and regeneration of damaged skin, however these approaches need further research to obtain healthy full skin for transplant [15-17].

In the present study, we investigated whether these difficulties could be overcome by developing an automated skin bioreactor which enables effective skin expansion *ex vivo* while maintaining tissue viability. In 2009, Ladd et al. [18] at the Wake Forest University in the United States reported the first full skin expansion using a computer-controlled bioreactor system, which resulted in skin expansion of 110.7% under culture conditions for 7 days. Following the work, Jeong et al. [19] in Korea published a paper in 2014 on a clinical trial with ten patients for investigation of whether tissue expansion using a bioreactor is effective and safe for reconstruction of large skin lesions and scars. The findings are average skin expansion rate of $10.54 \pm 6.25\%$ for 10 days; take rate of $88.89 \pm 11.39\%$; and contraction rate of $4.2 \pm 2.28\%$ after 6 months, which confirmed the safety and applicability of the *ex vivo* skin expansion.

We developed an advanced bioreactor system by adding functions of real time monitoring, remote-control, optimized grip structure, and creating pores to skin for efficient tissue expansion. We evaluated the morphological, ultra-structural, and mechanical properties of the expanded skin before and after expansion using histology, immunohistochemistry (IHC), and tensile testing. We further carried out grafting study *in vivo* using Yucatan pigs to investigate the feasibility of this method for clinical application.

MATERIALS AND METHODS

Bioreactor components

The system consists of three parts and additional components; the culture chamber, environment, and expansion control parts and components including skin grippers and expansion clamps. The skin is expanded in the chamber with culture medium and its temperature is maintained approximately 37°C by circulating water around the chamber using environment controller. The grippers are designed to hold the skin using micro-needles in order to avoid the skin necrosis due to excessive pressure. The grippers are connected to clamps which play a role for expanding skin under designated tension (Fig. 1).

Expansion study

Porcine full-thickness skins were obtained from local slaughterhouse (Farm pig, Yeongcheon, Korea). The skins were submerged in 70% EtOH for 10 sec, and washed with sterile phosphate buffered saline (PBS). The skin was expanded by 200 kPa of pneumatic pressure in Dulbecco's modified eagle's medium containing 10% fetal bovine serum and 2% penicillin-streptomycin for 4 hrs. Real time monitoring of the expanding skin for entire 4 hrs was carried out using IP camera. The area of the skin was calculated by Image J software (National Institute of Health, USA).

Grafting study

To evaluate the grafting efficiency of expanded skins, we used two female Yucatan pigs after getting approval from the Institutional Animal Care and Use Committee at DGMIF (Daegu-Gyeongbuk Medical Innovation Foundation, Daegu, Korea). For donor skin, the inguinal one was used. Full-thickness skin (5×5 cm) was obtained under anesthesia and subse-

Figure 1. The skin bioreactor. The skin bioreactor was composed of a reacting chamber (37°C) and a control system. Harvested skin was harnessed in the reacting chamber (A) by skin grippers embedded with micro-needles (B). The skin was expanded by operating arms (C) controlled with adjustable pneumatic pressure. All procedures were controlled by automatic control system.

quently expanded for 4 hrs using the bioreactor. The expanded skin was removed from the reactor and grafted on the back site where full-thickness skin of size as 6×6 cm was removed. Wound sites were treated with dry dressing at daily basis, and

allowed to heal. The animals were observed daily for 8 weeks and at the end of the period they were euthanized, and tissues including inguinal, back, and grafted skin tissues were harvested for histological and immunohistochemical analysis. All grafting experiments were conducted in Laboratory Animal Center (Daegu-Gyeongbuk Medical Innovation Foundation, Daegu, Korea).

ICG fluorescence angiography by the SPY fluorescent imaging system

To evaluate the vascularity or blood circulation at the recipient site after skin grafting, laser-assisted indocyanine green (ICG) angiography using the SPY imaging system was used. The SPY imaging system (Novadaq Technologies Inc., Toronto, Canada) provides real-time assessment of tissue perfusion and viability and serves as a tool for non-invasive perfusion assessment with a high degree of sensitivity and specificity. The ICG molecule binds strongly to plasma proteins, causing it to remain in the intravascular space and enables fluorescent detection. Evaluation of blood flow assessment was performed on 2, 4, and 8 week.

Histology

Retrieved skin tissues were fixed with 4% paraformaldehyde in PBS (pH 7.4) for overnight at 4°C. Tissues were dehydrated

Figure 2. Porcine skin expansion by skin bioreactor. The inguinal full-thickness skin was expanded by 200 kPa of pneumatic pressure in DMEM containing 10% PBS and 2% penicillin-streptomycin for 4 hrs. (A) Pre-, (B) post-expanded skin. The bioreactor expanded an average expansion rate of $156.7 \pm 1.7\%$ ($n=4$) (C). * $p<0.001$. DMEM: Dulbecco's modified eagle's medium, PBS: phosphate buffered saline.

Figure 3. Expanded skin revealed the tissue viability and intact structure of skin. For analyzing tissue viability and structural integrity, pre-expanded (A, B, and C) and post-expanded skins (D, E, and F) were performed with TUNEL assay (A and D), H&E (B and E), Masson's Trichrome staining (C and F). Nuclease treated section was used as positive control (A'). Scale bar indicates 200 μm.

through a graded ethanol series, cleared with xylene, and mounted in paraffin. Slides were made with 2 μm sections, and hematoxylin and eosin (H&E) and Masson's trichrome were performed routinely. For immunofluorescence (IF), sections

were de-waxed, rehydrated, and labeled with proliferating cell nuclear antigen (PCNA), E-cadherin, and α -smooth muscle actin (SMA) and FITC-conjugated secondary antibody, and counterstained with 1 $\mu\text{g}/\text{ml}$ 4',6-Diamidino-2-Phenylindole

Figure 4. Expanded skin showed proliferation capacity and integrity of epidermis. For IF staining, pre-expanded (A, C, E, I, G, and K) and post-expanded skins (B, D, F, J, H, and L) were probed with anti-PCNA (E and F) or anti-E-cadherin (G and H). DAPI was used as counterstain (A–D) and images were merged (I–L). Scale bar indicates 400 μm . PCNA: proliferating cell nuclear antigen, DAPI: diamidino-2-phenylindole.

Figure 5. Assessment of blood circulation at the graft site. The grafted skins were captured at 2 (A and D), 4 (B and E), and 8 (C and F) weeks after operating. A, B and C are camera captured images, and D, E and F are images obtained by SPY laser system.

(DAPI). Fluorescence images were taken by using Eclipse 80i (Nikon, Japan). When IHC, HRP-conjugated secondary antibodies were used, they were visualized with diaminobenzidine substrate.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

To determine whether skins went on apoptosis during expansion on the reactor, we used TACS[®] 2 TdT Fluorescein Kit (Trevigen, Gaithersburg, MD, USA). The procedure was performed according to manufacturer's protocol and counterstained with DAPI (1 $\mu\text{g/mL}$). For positive control, sections were treated with TACS-Nuclease[™].

Statistical analysis

Data presented in this study are means $\pm$ standard error of the mean. As all data were shown to be not normally distributed, the non-parametric Mann-Whitney U test was used to determine significant differences between samples. $p < 0.05$ was taken as indicating statistical significance.

RESULTS

The skins expanded by 200 kPa for 4 hrs resulted in an average expansion rate of $156.7 \pm 1.7\%$ ($n=4$) (Fig. 2). Tunnel assay

revealed no positive signal in the pre- and post-expanded skins (Fig. 3A and D). Histological examinations performed pre- and post-expansion demonstrated a significant increase in the porosity of the dermal layers and a uniaxial arrangement of the collagen fibers. Especially, there was thinner epidermis and straightened arrangement in post-expanded skin (Fig. 3B, C, E, and F). PCNA staining showed that PCNA (+) cells were predominantly located at the basal layer of epidermis, just above basement membrane, in both pre- and post-expanded skin. The post-expanded skin showed proliferating epithelial cells more than pre-expanded skin (Fig. 4E and F). To determine epidermal integration, E-cadherin distribution was visualized with FITC. The IF results showed the expression of E-cadherin in the whole epidermis area except superficial layer. Despite physical expansion, cell-cell adhesion was well preserved (Fig. 4G and H).

Blood flow of the auto-grafted skin was evaluated by ICG fluorescence angiography using SPY system. The grafted skin was macroscopically brighter than its vicinity when examined on 2, 4, and 8 weeks following surgery, and intensity of ICG fluorescence was decreasing through wound healing process (Fig. 5).

For bio-compatibility of an expanded skin, we examined skin auto-graft from an inguinal skin to dorsal site after expansion and skins were allowed healing for 8 weeks. After 8 weeks,

Figure 6. Histological images of grafted skin. Auto-grafted skin expanded by the bioreactor showed similar arrangement of collagen fibrils, and no fibrogenesis. Full-thickness inguinal skin (A-D), dorsal skin (E-H), and skin of grafted site (I-L) were collected and analyzed. H&E (A, E, and I), Verhoeff's stain (B, F, and J: Each insert figure in B, F, and J shows enlarged image), Masson's Trichrome (C, G, and K), and α -SMA (D, H, and L). Scale bar indicates 200 μm . α -SMA: α -smooth muscle actin.

skins were collected and subjected to histology. Histological data showed cleaved elastin fibers in an auto-grafted skin (Fig. 6B, F, and J) and decreased intensity of the ECM staining (Fig. 6C, G, and K). Apparently skins did not show α -SMA positive signals (Fig. 6D, H, and L).

From the grafting data analysis, proliferative cells were present more in dermal layer than other areas of auto-grafted skins, whereas less present in epidermis layer (Fig. 7).

DISCUSSION

Although there are various methods for the treatment of skin defects, such as skin grafting, artificial skin, and skin expander inside of body, these have limitations such as discomfort, high cost, immune response, and long term treatment. We attempted to expand skin *ex vivo* using a newly developed skin expander, and performed grafting study using female Yucatan pigs.

In the preliminary experiments, data showed that maximum expansion rate was observed at 4 hrs after the expansion was begun under 200 kPa of pneumatic pressure (data not shown). According to this observation, the expansion pressure and expansion period were determined. In previous studies by Ladd et al. [18] and Jeong et al. [19], *ex vivo* skin expansion using skin bioreactors showed expansion rates of 110.7% and $110.54 \pm 6.25\%$ [15,16]. The expansion period of these experiments lasted 7 to 10 days. In contrast, the skin expansion rate turned out to be $156.7 \pm 1.7\%$ ($n=4$) in spite of short time period using the newly designed reactor. Apoptosis was not detected during expanding skin and histological results showed that porous area were increased in the expanded skin dermis (Fig. 3) [18,20]. During skin expansion, internal micro-structure of skin looks loosened as the arrangements of collagen and elastin were altered.

In the Figure 4, the expanded skins had proliferating activity

Figure 7. Proliferating cells were increased in the grafted site. Proliferating cells were visualized with PCNA antibody (B, E, and H). Skins of inguinal (A, B, and C), grafted (D, E, and F), and dorsal site (G, H, and I) showed proliferating activity in the basal epidermis. All sections were counter-stained with DAPI (A, D, and G) and images were merged (C, F, and I). Scale bar indicates 200 μ m. PCNA: proliferating cell nuclear antigen, DAPI: diaminido-2-phenylindole.

and epidermal integrity with positive staining of PCNA and E-cadherin. PCNA positive represented that cell growth is in progress [21]. In addition, unimpaired staining pattern of E-cadherin after expansion indicated the preservation of cell-cell interaction and the integrity of skin epidermis [22,23]. These data showed expanded skin maintained natural cell activity and genuine characteristics.

Bio-compatibility of expanded skin was examined by auto-grafting. Grafted skins were observed every day for 8 weeks, and collected skins were subjected to histology and IHC. The grafted site was scanned SPY and evaluated by intensity of ICG fluorescence. In Figure 5, intensity was decreased by a time-dependent manner, and it implies that decrease of blood flow in early wound healing stage. Histological data showed that low intensity of ECM deposits and short elastin fibrils are present in the skin of 8 weeks after grafting. At 8 weeks, the wound healing was still on progress. All tissues did not show secondary contraction, the reason is speculated due to no occurrence of fibrogenesis which is hyperplasia of α -SMA positive cells (Fig. 6). In addition, numerous PCNA positive cells were detected in the basal layer of epidermis and dermal layer in the grafted skins. That means that the cells possess proliferating activity in the grafted skin [21].

Conclusively, the experimental findings demonstrated that full thickness skins can be expanded to 160% in a very short period of less than 6 hours which is considered as a golden time in auto-transplantation. Histological analysis of the expanded skins showed that epidermal cells and dermal fibroblasts were viable and remained integrity. The results of the current investigations indicated that substantially large sized viable full thickness skin can be obtained *ex vivo* for soft tissue reconstruction using advanced type of skin bioreactor.

Acknowledgements

This work was supported by ETRI R&D Program [15ZC3110, Local-based medical device/robot development & medical IT convergence for small and medium enterprises revitalization project] funded by the Government of Korea, Kyungpook National University Research Fund, 2014 and Regenerative Medicine Research Grant by Daegu Metropolitan city.

Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

Performance standards approved by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and the American Association for Animal Sciences (AALAS) must be followed. The experimental protocol was approved by

the Institutional Animal Care and Use Committee of Daegu-Gyeongbuk Medical Innovation Foundation Laboratory Animal Center (IACUC approval number; DGMIF-15060403-01).

REFERENCES

1. Hansen SL, Mathes SJ, Young DM. Skin and subcutaneous tissue. In: Brunickardi FC, Anderson DK, Billiar TR, Dunn DL, Hunter JG, Mathews JB, et al., editors. Schwartz's Principles of Surgery. Columbus, USA: The McGraw-Hill Companies, Inc.; 2008. p.103-110.
2. Holmes JH, Heimbach DM. Burns. In: Brunickardi FC, Anderson DK, Billiar TR, Dunn DL, Hunter JG, Mathews JB, et al., editors. Schwartz's Principles of Surgery. Columbus, USA: The McGraw-Hill Companies, Inc.; 2005. p.55-62.
3. Shenaq SM, Kim JY, Bienstock A, Yuksel E. Plastic and reconstructive surgery: soft tissue reconstruction. In: Brunickardi FC, Anderson DK, Billiar TR, Dunn DL, Hunter JG, Mathews JB, et al., editors. Schwartz's Principles of Surgery. Columbus, USA: The McGraw-Hill Companies, Inc.; 2007. p.319-324.
4. Atiyeh BS, Hayek SN, Gunn SW. New technologies for burn wound closure and healing--review of the literature. Burns 2005;31:944-956.
5. Ehrenreich M, Ruszczak Z. Update on tissue-engineered biological dressings. Tissue Eng 2006;12:2407-2424.
6. Hansbrough JF, Mozingo DW, Kealey GP, Davis M, Gidner A, Gentzkow GD. Clinical trials of a biosynthetic temporary skin replacement, derma-graft-transitional covering, compared with cryopreserved human cadaver skin for temporary coverage of excised burn wounds. J Burn Care Rehabil 1997;18(1 Pt 1):43-51.
7. Lang EM, Eiberg CA, Brandis M, Stark GB. Biobrane in the treatment of burn and scald injuries in children. Ann Plast Surg 2005;55:485-489.
8. Machens HG, Berger AC, Mailaender P. Bioartificial skin. Cells Tissues Organs 2000;167:88-94.
9. Radovan C. Breast reconstruction after mastectomy using the temporary expander. Plast Reconstr Surg 1982;69:195-208.
10. Spence RJ. Clinical use of a tissue expander--enhanced transposition flap for face and neck reconstruction. Ann Plast Surg 1988;21:58-64.
11. Tausche AK, Skaria M, Böhlen L, Liebold K, Hafner J, Friedlein H, et al. An autologous epidermal equivalent tissue-engineered from follicular outer root sheath keratinocytes is as effective as split-thickness skin autograft in recalcitrant vascular leg ulcers. Wound Repair Regen 2003; 11:248-252.
12. Wainwright DJ. Use of an acellular allograft dermal matrix (AlloDerm) in the management of full-thickness burns. Burns 1995;21:243-248.
13. Waymack P, Duff RG, Sabolinski M. The effect of a tissue engineered bilayered living skin analog, over meshed split-thickness autografts on the healing of excised burn wounds. The Apligraf Burn Study Group. Burns 2000;26:609-619.
14. Augustine R, Kalarikkal N, Thomas S. An in vitro method for the determination of microbial barrier property (MBP) of porous polymeric membranes for skin substitute and wound dressing applications. Tissue Eng Regen Med 2015;12:12-19.
15. Chung E, Choi H, Lim JE, Son Y. Development of skin inflammation test model by co-culture of reconstituted 3D skin and RAW264.7 cells. Tissue Eng Regen Med 2014;11:87-92.
16. Hong HS, Son Y. Substance-p-mobilized mesenchymal stem cells accelerate skin wound healing. Tissue Eng Regen Med 2014;11:483-491.
17. Huh MI, Kim MS, Kim HK, Lim JO. Effect of conditioned media collected from human amniotic fluid-derived stem cells (hAFSCs) on skin regeneration and photo-aging. Tissue Eng Regen Med 2014;11:171-177.
18. Ladd MR, Lee SJ, Atala A, Yoo JJ. Bioreactor maintained living skin matrix. Tissue Eng Part A 2009;15:861-868.
19. Jeong C, Chung HY, Lim HJ, Lee JW, Choi KY, Yang JD, et al. Applicabil-

- ity and safety of in vitro skin expansion using a skin bioreactor: a clinical trial. *Arch Plast Surg* 2014;41:661-667.
20. Fung MA, Sharon VR, Ratnarathorn M, Konia TH, Barr KL, Mirmirani P. Elastin staining patterns in primary cicatricial alopecia. *J Am Acad Dermatol* 2013;69:776-782.
21. Ouhtit A, Muller HK, Davis DW, Ullrich SE, McConkey D, Ananthaswamy HN. Temporal events in skin injury and the early adaptive responses in ultraviolet-irradiated mouse skin. *Am J Pathol* 2000;156:201-207.
22. Aberle H, Schwartz H, Kemler R. Cadherin-catenin complex: protein interactions and their implications for cadherin function. *J Cell Biochem* 1996;61:514-523.
23. Jamora C, Fuchs E. Intercellular adhesion, signalling and the cytoskeleton. *Nat Cell Biol* 2002;4:E101-E108.