Keratinocyte Culture: Siriraj's Experience

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ABSTRACT

Objective: Cell-based therapy is gaining increasing prominence in medicine, where it has the potential to replace or repair damaged tissue using new engineered cells. Skin cell engineering, also known as keratinocyte culture or cultured epithelial autograft (CEA), is a promising field in cell-based therapy. CEA is now used in many parts of the world as an alternative treatment for some diseases that require large defects to be covered, such as severe and major burn patients and congenital melanocytic nevus. The use of CEA in conjunction with acellular skin substitution is rapidly expanding.

Materials and Methods: This study is an initiative aimed at supporting the production and use of keratinocyte cultures at Siriraj Hospital. This is the first stage of developing sheet keratinocyte culture *in vitro*.

Results: Our study yielded very promising results. As feeder cells, we used irradiated 3T3 murine fibroblasts, as per the standard protocol for keratinocyte culture. The growth duration was four weeks: 2 weeks for the 3T3 murine fibroblasts and 2 weeks for the keratinocytes. The keratinocytes grew rapidly and formed sheets with irradiated 3T3 murine fibroblasts. The retrieval of the cell sheets was straightforward thanks to the temperature-response cell culture dish and halo-ring cell recovery sheet. Flow cytometry revealed that the cells had a very high viability and purity. H&E staining revealed the sheets comprised two to four layers of stratified epithelial tissue.

Conclusion: From this study, our method of manufacturing the CEA can offer a promising result. This can be use in the treatment which require large skin coverage. However, we aim to initiate animal and human trial phase next.

Keywords: Keratinocyte culture; keratinocyte culture in Siriraj Hospital; cultured epithelium autograft; CEA; cultured epithelium autograft in Siriraj Hospital; CEA in Siriraj Hospital (Siriraj Med J 2022; 74: 274-283)

INTRODUCTION

The treatment workhorse for covering large wounds, such as in burn victims or after cancer resection surgery, is the skin graft. Skin grafts are classified into three categories based on the origin of the tissue: autografts (from the patient), allografts (from another person), and xenografts (from other species, such as pigs).

Generally, using autologous tissue is the best option; however, in some cases, such as severe burns or after the removal of a large tumor, an autologous graft may not be sufficient. As a temporary dressing, an allograft or xenograft may be used, but must be later removed due to graft rejection.

The field of regenerative medicine and tissue engineering has grown in recent years. In North America and some European countries, autologous skin culture (keratinocyte culture) is now commercially available. This keratinocyte culture is extremely useful in covering

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large skin defects.[1-3] There are three types of keratinocyte culture[4]: sheet, suspension, and spray. However, none of these cultures are available yet in Thailand.

This study is an initiative to support the production and use of keratinocyte cultures at Siriraj Hospital. This is the first stage of developing sheet keratinocyte culture *ex vivo*. Animal and human phases will follow later.

MATERIALS AND METHODS

This study was conducted at the Plastic and Reconstructive Surgery Unit Department of Surgery, and Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand. This study protocol (Si 122/2020) was approved by Ethics Committee of the Siriraj Institutional Review Board. The subjects understood the protocol and gave informed consent prior to the participation.

Preparation of human skin

After receiving informed consent, the human skin used in this study was obtained from patients who had surgical debridement or from a skin graft that was left over after skin graft transplantation. The skin was harvest from thigh using Zimmer dermatome (Zimmber biomet company, Ohio, USA) with 0.010 inches thickness. The sample skin was cleansed with 100 ml of normal saline and wrapped in a sterile gauze soaked in normal saline. The skin was then transferred to a laboratory room in a sterile plastic bag. The skin was washed in phosphate-buffered saline (PBS) with 50 µg/mL streptomycin and 50 unit/mL penicillin G before being transferred to a new sterile 10.0 cm diameter dish (Fig 1).

Isolation of human skin keratinocytes

The sample skin was finely chopped into small pieces (approximately 2 × 2 mm^2). The pieces were then transferred into a 50 mL tube. The tube was then incubated in a water bath for 20 minutes with 5 mL of 0.25% trypsin-EDTA at 37°C. The mixture was washed twice through centrifuged at 1000 rpm for 5 minutes at room temperature. The supernatant was discarded, and the cell pellets were reconstituted in 5 mL of Keratinocyte culture medium (KCM).

Irradiated 3T3 fibroblast preparation (Feeder cell)

The frozen cryotube of 3T3 fibroblasts (murine fibroblast) was then removed from the cryopreserved tank, and 70% ethanol was used to clean the outside of the tube. The frozen cryotube of 3T3 fibroblasts was thawed in a 37°C water bath. When the cell-preservative medium had nearly completely defrosted, the cell suspension was quickly mixed into 5 ml pre-warmed Fibroblast derived matrix (FDM) in a 15 ml tube. The cell suspension solution (approximately 6 ml) was then divided equally and added to each of the two 75 cm^2 flasks. The next day, the culture medium was changed was completely changed to remove the remaining cryoprotectant (Fig 2). Note, the more 75 cm^2 flasks there are, the larger the cell expansion possible.

All of the cultured cells from the 75 cm^2 flasks were collected and placed in a 50 mL tube after two passages. The tube was transferred for two cycles of 34 Gy radiation. Note, the preparation of the irradiated 3T3 fibroblasts took about one to two weeks, and so must be planned ahead of time when needed.

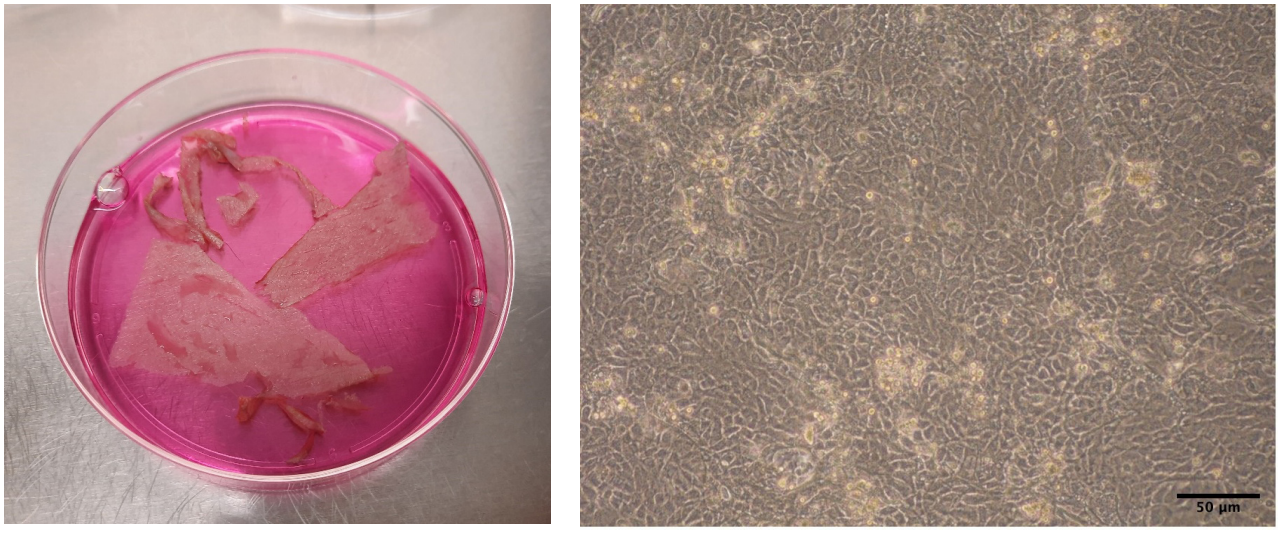

Fig 1. Skin sample was retrieved from a split-thickness skin graft leftover (1A), Primary keratinocytes prepared from the skin samples on day 7 were visualized under 10× objective lens (1B)

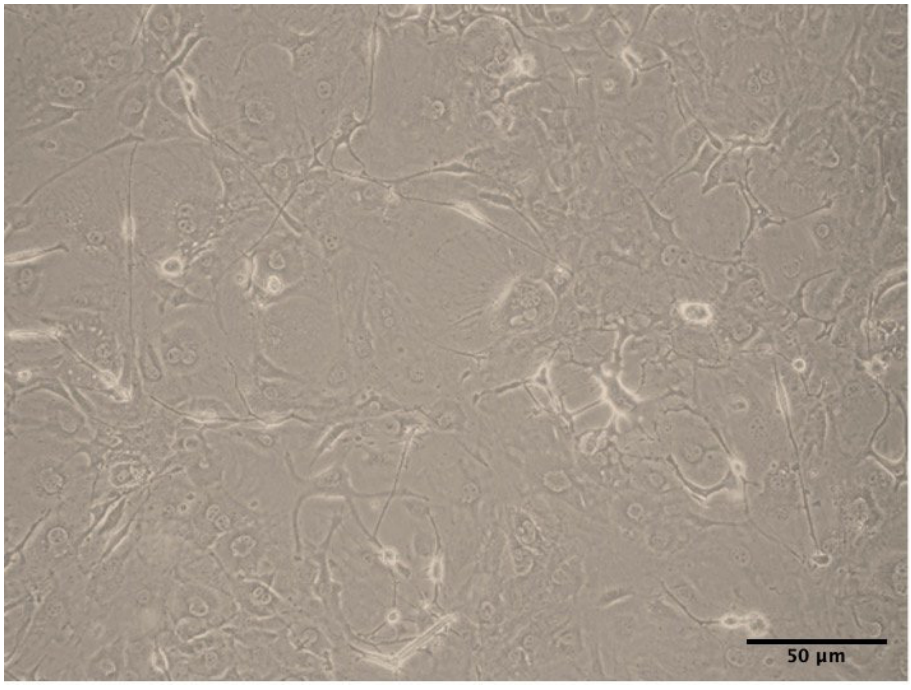

Fig 2. 3T3-murine fibroblasts (feeder cell) were visualized under 10× objective lens.

Plating the human keratinocytes over irradiated 3T3 fibroblasts (Feeder layer)

We used an UpCell dish, which is a specialized culture dish. This UpCell dish has the unique property that when the temperature is reduced, the cultured cells automatically lift off the surface.

Irradiated 3T3 fibroblasts were seeded onto the dish first, covering the entire surface overnight. The irradiated-fibroblasts were then seeded with a suspension of human keratinocytes at concentrations 2.0×10^5 and 4.0×10^5 cells in a 3.5 cm UpCell dish. The dish was then placed in a CO_2 incubator and incubated at 37°C.

Keratinocyte sheet lifting

Every day, the culture medium [keratinocyte medium (KCM)] was changed. Also, the cultured cells were examined every day under a microscope. The keratinocyte sheet was ready to be lifted off once the cell confluence reached 100%, which took about one to two weeks. The keratinocyte sheet was lifted from the dish's surface by lowering the temperature from 37°C to 20°C over 30 minutes. (Fig 3)

A specialized doughnut-shaped paper called a halo-ring cell recovery sheet was used to retrieve the keratinocyte sheet. The halo-ring sheet's outer diameter was smaller than the dish's diameter, so that when the halo-ring sheet was placed over the keratinocyte sheet, the keratinocyte sheet's edge was larger than that of the halo-ring sheet's. Next, by folding the keratinocyte sheet's edge over the edge of the halo-ring sheet, the halo-ring sheet and the keratinocyte sheet could be lifted off the surface of the dish together (Fig 4).

RESULTS

Duration of keratinocyte sheet culture

The preparation of the irradiated 3T3 fibroblasts took about one to two weeks in this study. It then took two weeks from the time the keratinocytes were seeded to the formation of a keratinocyte sheet. As a result, the entire process took three to four weeks overall.

Characteristics of the cultured keratinocyte sheets

Keratinocyte cells were found to grow on irradiated 3T3 fibroblasts in explant culture. At days 5, 7, and 14, the confluence rates were 20%, 80%, and 100%, respectively (Fig 5). Keratinocytes with typical morphological features, such as a polygonal cobblestone shape, were observed to have proliferated.

Histological examination revealed that all the manufactured cell sheets with a 2-4 stratified structure were made up of epithelial cells (Fig 6). The results showed that the keratinocyte cells could be cultured on temperature-responsive cell culture inserts and that the cell sheets could stratify (Table 1).

Lifting the cultured keratinocyte sheets

After 14 days of culture on the temperature-responsive cell culture dish, all the cells were successfully harvested

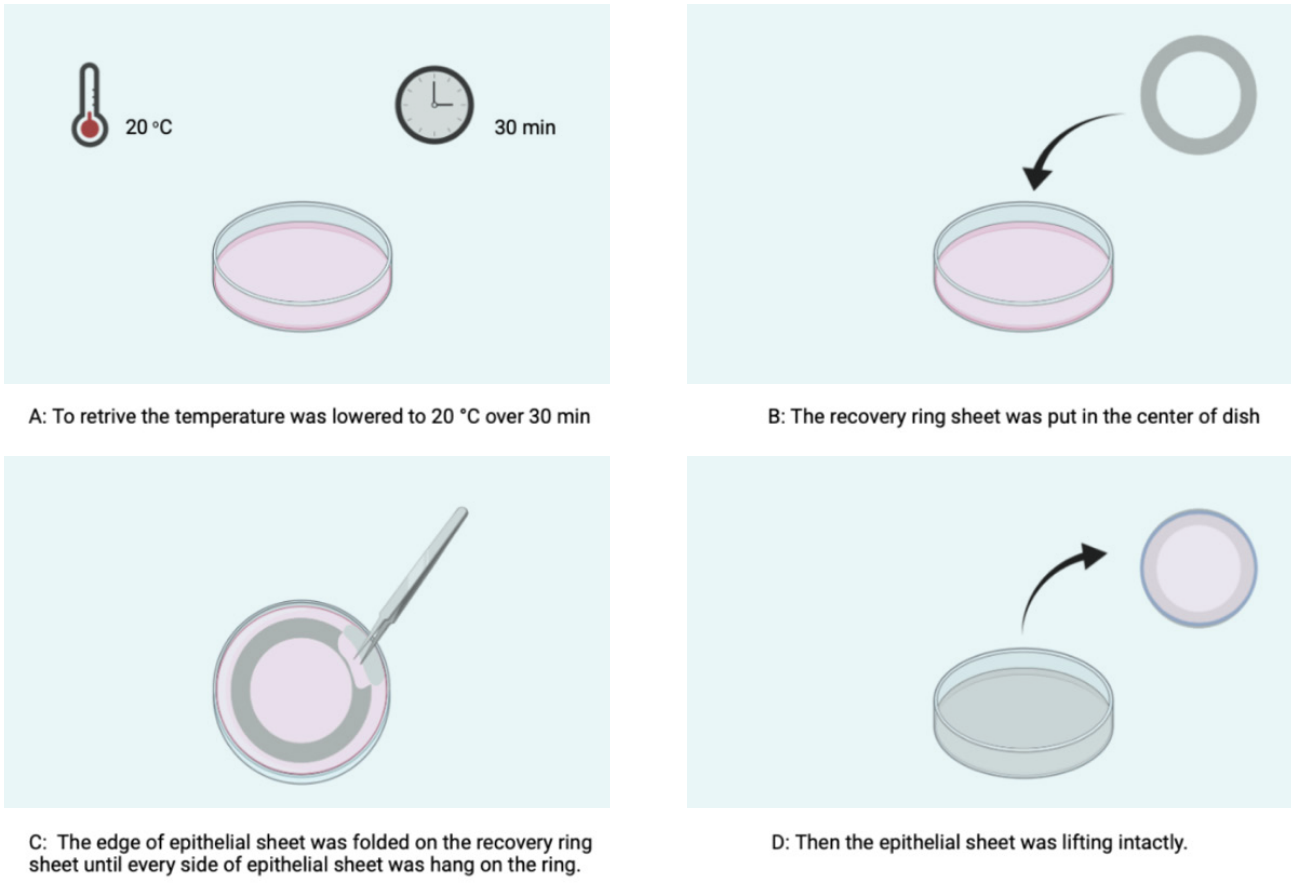

Fig 3. The culture epithelial sheet retrieving method

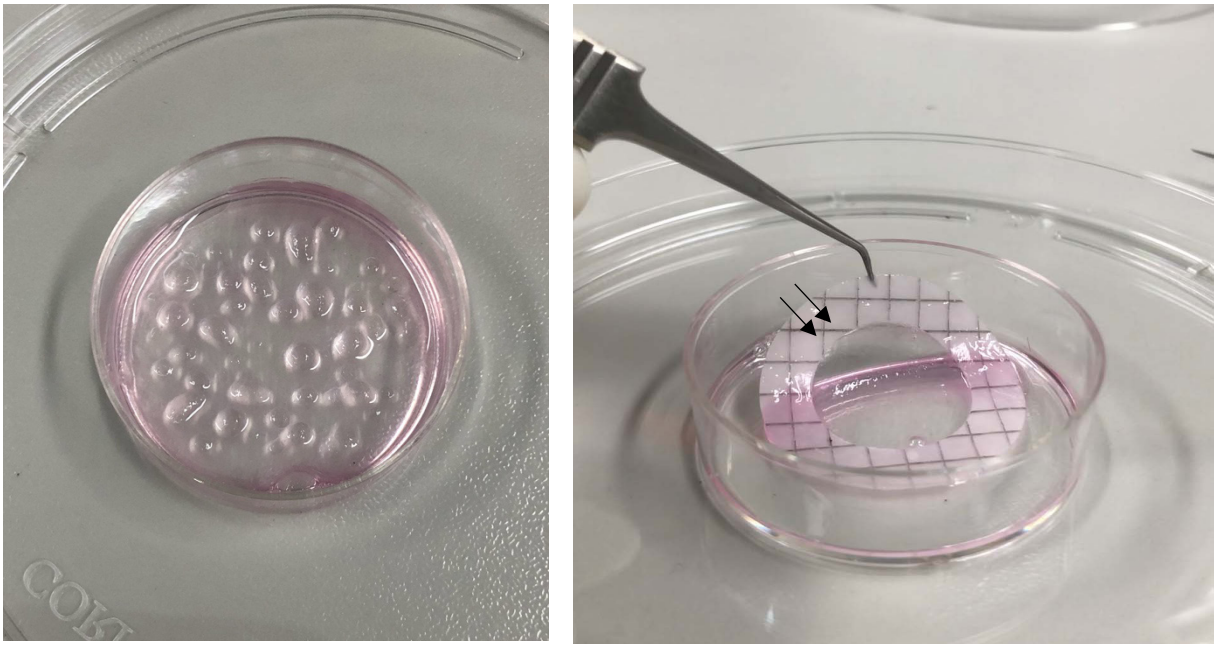

Fig 4. After 14 days, the culture epithelium sheet was growing all over the temperature-responsive UpCell dish (left). For the lifting, the temperature was lowered from 37°C to 20°C over 30 min; then a recovery ring sheet was used for lifting the cultured epithelial cell sheets (right), culture epithelial sheet (arrow).

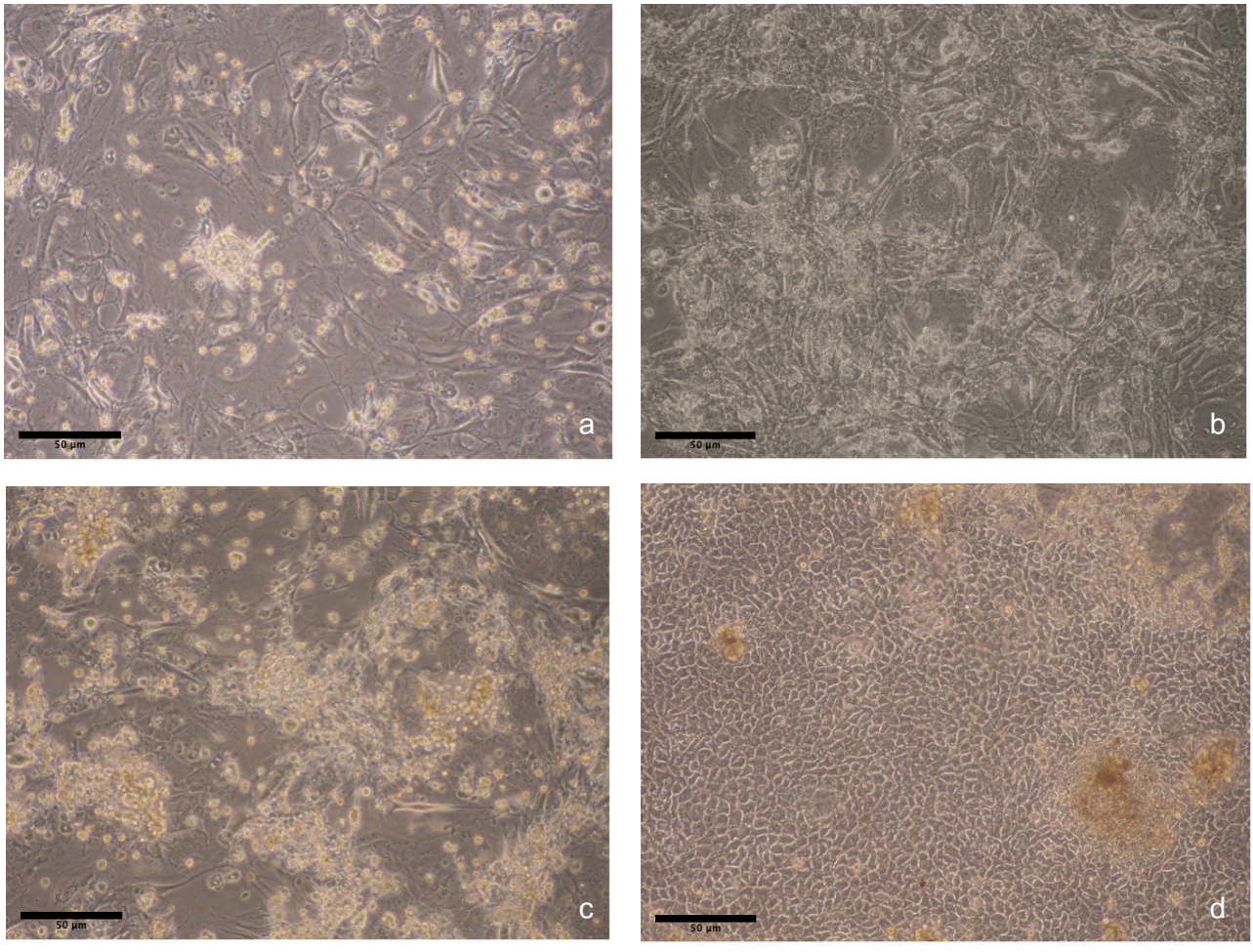

Fig 5. Culture dish at 10x microscopic view on the 1st day showing a low keratinocyte : fibroblast ratio (a). During the culture, the keratinocytes continuously grew in number while the fibroblasts decreased, as can be seen on the 5th day (b), 7th day (c), and finally, on the 14th day, by which time the keratinocytes were confluence. (d)

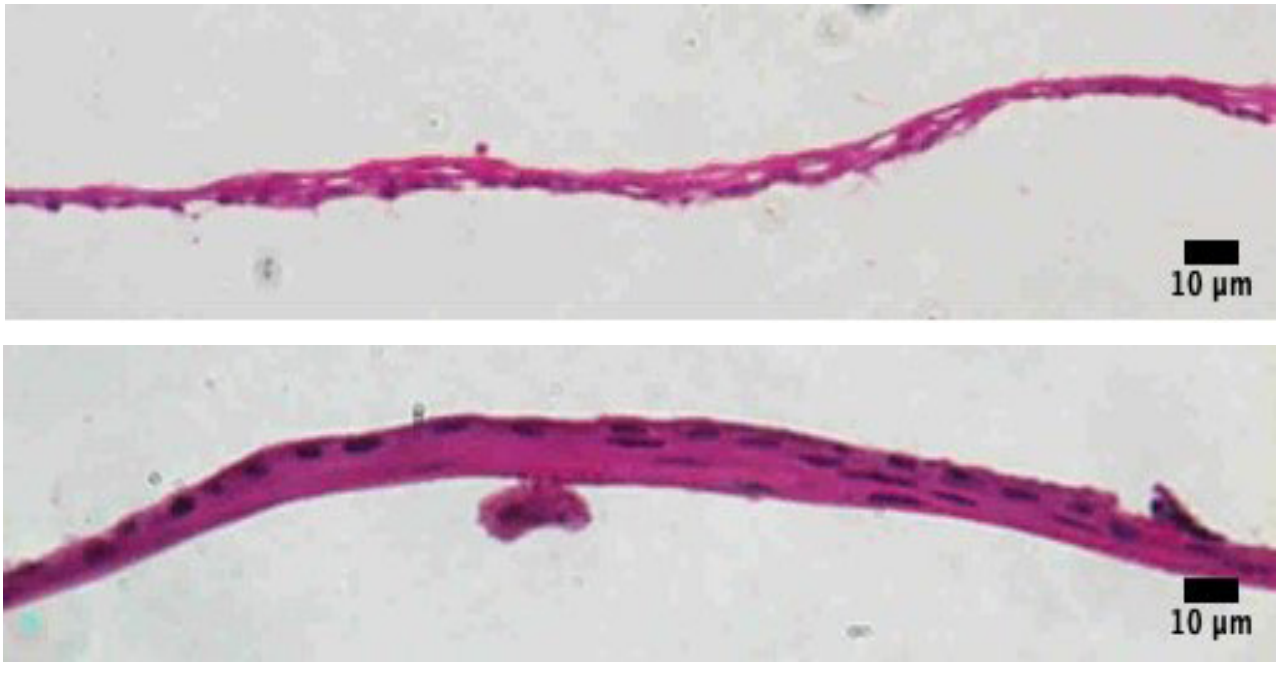

Fig 6. The Cross section of the cell sheet was stained with H&E and viewed under 10× objective lens: keratinocyte at 2.2×10^4 cells/cm^2 (above), and 4.4×10^4 cells/cm^2 (below).

TABLE 1. Physical characteristics of the cultured keratinocyte sheet seeding at 2.2×10^4 cells/cm^2 and 4.4×10^4 cells/cm^2.

Cell sheet		2.2×10^4 cells/cm^2	4.4×10^4 cells/cm^2
Cell morphology	Full confluence keratinocytes and cobble stone-like morphology	Confirmed	Confirmed
Cell sheet recovery	Harvesting w/o any damage	Confirmed	Confirmed
Total cell number	Over 1.0×10^5 cells	8.9×10^5 cells	17.3×10^5 cells
Cell viability	Over 60.0%	92.8%	95.6%
Karatinocytes purity	Over 80.0%	96.5%	98.2%
Degree of stratification	More than 2 layers	More than 2–4 layers	More than 2–4 layers

as contiguous transplantable cell sheets by lowering the incubation temperature from 37°C to 20°C over 30 minutes and by using a halo-ring cell recovery sheet.

Validation of the viability of the culture

Flow cytometry was used to validate the cultured cell sheets. The results showed that the total cell counts in the cell sheets using keratinocyte at cell seedings of 2.0×10^5 (2.2×10^4 cells/cm^2) and 4.0×10^5 (4.4×10^4 cells/cm^2) were 8.9×10^5 and 17.3×10^5 cells, respectively. The viability rates were 92.8% and 95.6%, respectively (Figs 7&8).

Purity of the cultured keratinocyte sheets

Cell purity was 96.5% and 98.2%, respectively, in the above cultures.

DISCUSSION

Cultured epithelial autograft (CEA) was first developed 30 years ago by Green and Rheinwald based on murine 3T3 fibroblasts.[5,6] Because of the high cost and time required for processing, subsequent progress in this field has been very slow.

In the new millennium, cell-based therapy has gained increasing prominence in medicine; particularly in the fields of tissue engineering, regenerative medicine, and stem cell therapy, and is widely recognized to offer the potential to replace or repair damaged tissue using new engineered cells.

Skin cell engineering, also known as keratinocyte culture or cultured epithelial autograft (CEA), is a promising field in cell-based therapy. CEA is now used in many countries as an alternative treatment for large wounds.[1] The indication is still within the controversy, such as major burn greater than 30% of total body surface area.

The lack of skin donor is still a major problem in numerous cases such as severe burn, large post-oncologic resection, or congenital melanocytic nevus in pediatric. In these cases, we can use mesh or meek technique for expand the graft tissue 2 to 6 times. However, the wider mesh/meek is needed to facilitate larger areas of cover, result in the poorer donor site's scar outcome. Re-harvesting of the donor sites normally used, but is associated with a delay overall healing time, as the donor sites require time to heal between procedure. The CEA may play an important role in these cases. This technology has capability to expand the tissue more than the previous strategy we utilized in the past and use fewer tissue donor.

There are currently three types of CEA available: the sheet, suspension, and spray forms.[1,4,7-9]

Morimoto et al.[2,3] demonstrated the use of CEA for accelerating wound healing in neonates with complicated wounds.

The ReCELL spray-on skin system[4,10,11] offers the use of a spray form of CEA combined with an animal-derived enzyme for less complicated wounds.

Nowadays, the sheet form of CEA is classified as a skin substitute. Skin substitution is divided into two types: cellular (composed of living cells, such as CEA) and non-cellular or acellular (composed of biocompatible or biodegradable materials). Acellular skin substitution is further subdivided into allogenic (made up of a decellularized extracellular matrix from the

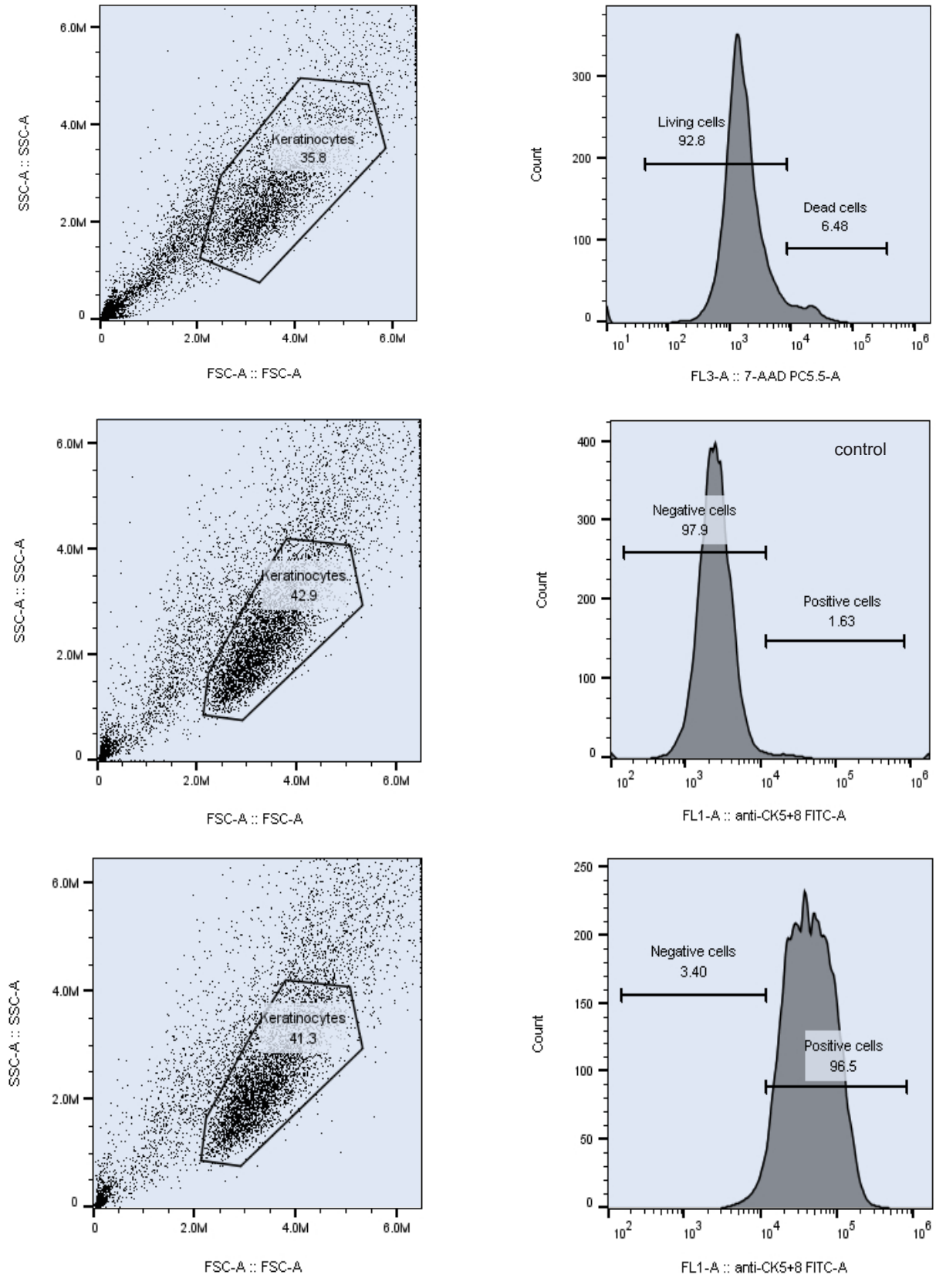

Fig 7. The flow cytometry was used to characterize cultured keratinocytes sheet seeding at 2.2×10^4 cells/cm^2. The cluster of putative keratinocytes was gated based on front scattering and side scattering (left panels). The viability was assessed using 7-amino actinomycin D (7-AAD) assay in PerCP-Cy5.5 channel. The histogram illustrated 92.8% live cells and 6.48% dead cells (top). The purity was assessed using anti-cytokeratin 5 + 8 in FITC (fluorescein isothiocyanate) channel to stain keratinocytes. The histograms illustrated minuscule autofluorescence (1.63%) in the unstained group (middle), and overwhelmingly 96.5% positive cells in the stained group (bottom).

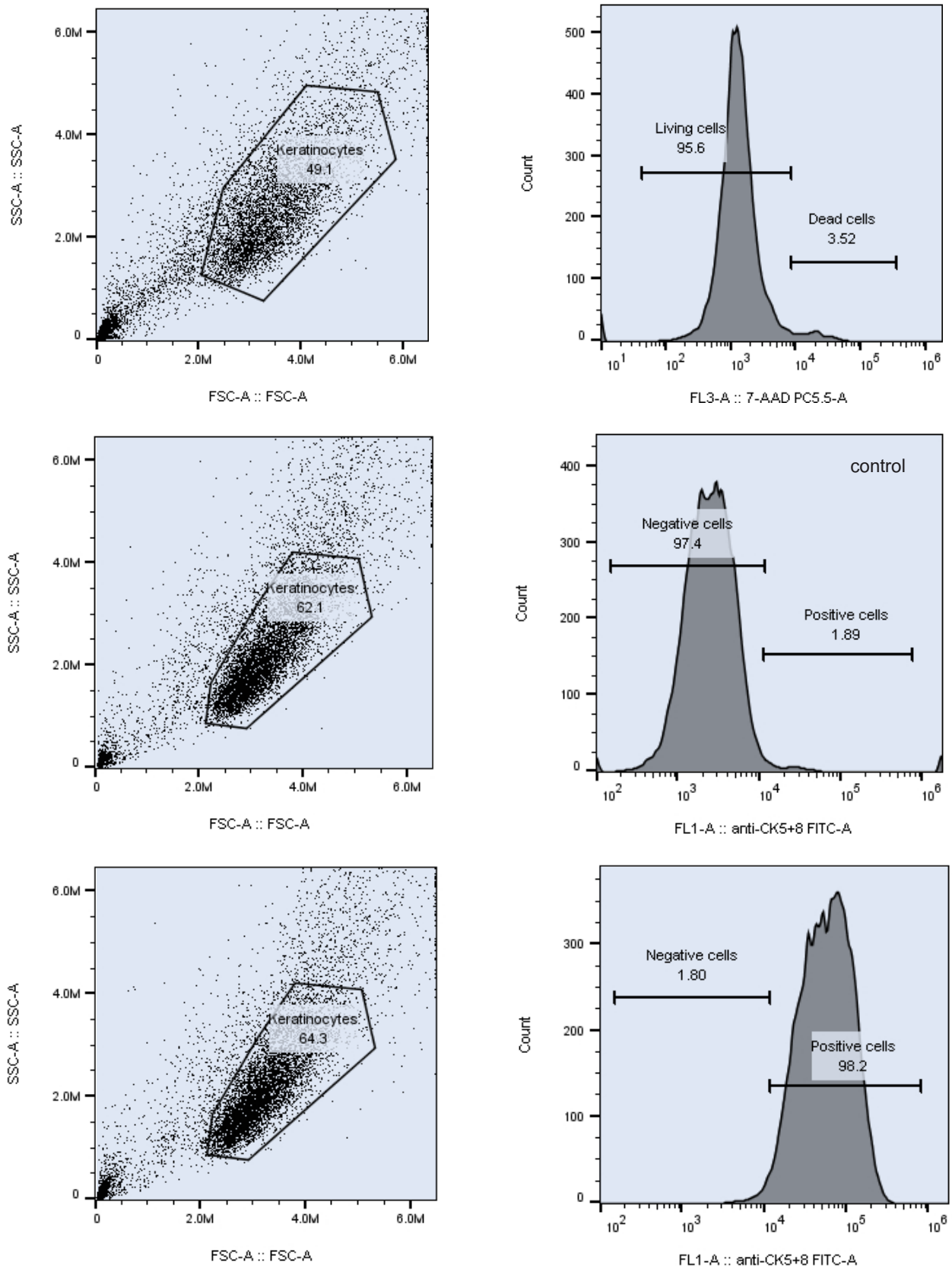

Fig 8. The characteristics of cultured keratinocyte sheet seeding at 4.4×10^4 cells/cm^2 based on flow cytometry were similar to those with lower cell density. There were 95.6% live cells and 3.52% dead cells (top). The autofluorescence in FITC channel was 1.89% (middle). The purity of keratinocytes was 98.2% (bottom).

same species, such as a human cadaver) and xenogenic (composed of a decellularized extracellular matrix from different species, such as bovine or porcine).

The disadvantage of CEA is the thinning of the tissue, as it consists with a couple layers of stratified keratinocyte. In case the wound is deeper to subcutaneous tissue, utilizing this CEA alone will result in loss contour of the area. In the deep wound, acellular skin substitution is very useful, as it is designed to stimulate neodermis formation for 3-4 weeks resulting in the tissue fullness. It can be used as an intermediate step for split- or full-thickness grafting in patients with both small and large defects. Additionally, it can be used in the wound that exposed bone or tendon which cannot be grafted primarily.

There have been numerous reports on these acellular skin substitutes being used as scaffolds in conjunction with the sheet form of CEA for complex wounds.[7,12-15] Matsumura et al.[16] reported the successful use of combined CEA and acellular skin substitution in severe burn patients.

Our research yielded very promising results. As feeder cells, we used irradiated 3T3 murine fibroblasts, as per the standard protocol for keratinocyte culture. In our protocol, we use partial thickness of skin (around 0.010 inches) for isolate human keratinocytes instead of full thickness skin donor. We found out that it can shorten time in cell isolation process and reducing cell damage, as normally it must use thermolysin and incubation overnight for separating epidermis from dermis. The overall growth duration was four weeks: 2 weeks for the 3T3 murine fibroblasts and 2 weeks for the keratinocytes. The keratinocytes grew rapidly and formed sheets with irradiated 3T3 murine fibroblasts. In the retrieval of the cell sheets, we used the temperature-response cell culture dish and halo-ring cell recovery sheet. Normally, enzymatic treatment (for example: dispase) is typically used in the collection of epithelial keratinocyte sheets, but it tends to break the adhesion and basement membrane proteins. We assume that using harvesting technique by temperature dish can lowering cell damage result in improve the survival outcome of epithelial sheet. The flow cytometry revealed that the cells had very high viability and purity. H&E staining revealed two to four layers of stratified epithelial tissue. Following these promising results, animal and human trial phases will be initiated.

In our practice, we usually use acellular skin substitutes in conjunction with the split-thickness skin graft especially in cosmetic area or exposed bone or tendon wound. It will take times approximately 3-4 week for the tissue to be vascularized and good adhere to wound bed. Next, the patient will undergo the second operation for skin grafting. In our perspective, this research is giving more benefit to the patient. During the time for waiting the revascularized process, we aim to prepare the culture keratinocyte sheet and utilize for the second stage operation.

CONCLUSION

The future of CEA is very promising in the treatment of some diseases that require large defects to be covered, such as severe and major burn patients and congenital melanocytic nevus. The use of CEA in conjunction with acellular skin substitution is rapidly expanding globally, and will hopefully be an option in Thailand soon too.

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REFERENCES

1. Chrapusta A, Nessler MB, Drukala J, Bartoszewicz M, Madry R. A comparative analysis of advanced techniques for skin reconstruction with autologous keratinocyte culture in severely burned children: own experience. Postepy Dermatol Alergol 2014;31(3):164-9. DOI: 10.5114/pdia.2014.43190.
2. Morimoto N, Kakudo N, Kako A, et al. A case report of the first application of culture epithelial autograft (JACE((R))) for giant congenital melanocytic nevus after its approval in Japan. J Artif Organs 2018;21(2):261-264. DOI: 10.1007/s10047-017-1007-0.
3. Morimoto N, Jinno C, Sakamoto M, Kakudo N, Yamaoka T, Kusumoto K. An Exploratory Clinical Trial of a Novel Treatment for Giant Congenital Melanocytic Nevi Combining Inactivated Autologous Nevus Tissue by High Hydrostatic Pressure and a Cultured Epidermal Autograft: Study Protocol. JMIR Res Protoc 2016;5(3):e162. DOI: 10.2196/resprot.6195.
4. Ter Horst B, Chouhan G, Moiemen NS, Grover LM. Advances in keratinocyte delivery in burn wound care. Adv Drug Deliv Rev 2018;123:18-32. DOI: 10.1016/j.addr.2017.06.012.
5. Green H, Rheinwald JG, Sun TT. Properties of an epithelial cell type in culture: the epidermal keratinocyte and its dependence on products of the fibroblast. Prog Clin Biol Res 1977;17:493-500. (https://www.ncbi.nlm.nih.gov/pubmed/928463).
6. Rasmussen C, Thomas-Virnig C, Allen-Hoffmann BL. Classical human epidermal keratinocyte cell culture. Methods Mol Biol 2013;945:161-75. DOI: 10.1007/978-1-62703-125-7_11.
7. Kljenak A, Tominac Trcin M, Bujic M, et al. Fibrin gel as a scaffold for skin substitute - production and clinical experience. Acta Clin Croat 2016;55(2):279-89. DOI: 10.20471/acc.2016.55.02.15.
8. Gerlach JC, Johnen C, Ottomann C, Bräutigam K, Plettig J, Belfekroun C, et al. Method for autologous single skin cell isolation for regenerative cell spray transplantation with non-cultured cells. Int J Artif Organs 2011;34(3):271-9. DOI: 10.5301/ijao.2011.6508.
9. Ramos MG, Ramos DG, Ramos CG. Evaluation of treatment response to autologous transplantation of noncultured melanocyte/keratinocyte cell suspension in patients with stable vitiligo. An Bras Dermatol 2017;92(3):312-318. DOI: 10.1590/abd1806-

4841.20175700.
10. Holmes JHt, Molnar JA, Shupp JW, Hickerson WL, King BT, Foster KN, et al. Demonstration of the safety and effectiveness of the RECELL((R)) System combined with split-thickness meshed autografts for the reduction of donor skin to treat mixed-depth burn injuries. Burns 2019;45(4):772-82. DOI: 10.1016/j.burns.2018.11.002.
11. Peirce SC, Carolan-Rees G. ReCell((R)) Spray-On Skin System for Treating Skin Loss, Scarring and Depigmentation after Burn Injury: A NICE Medical Technology Guidance. Appl Health Econ Health Policy 2019;17(2):131-41. DOI: 10.1007/s40258-018-00457-0.
12. Haldar S, Sharma A, Gupta S, Chauhan S, Roy P, Lahiri D. Bioengineered smart trilayer skin tissue substitute for efficient deep wound healing. Mater Sci Eng C Mater Biol Appl 2019;105:110140. DOI: 10.1016/j.msec.2019.110140.
13. Cubo N, Garcia M, Del Canizo JF, Velasco D, Jorcano JL. 3D bioprinting of functional human skin: production and in vivo analysis. Biofabrication 2016;9(1):015006. DOI: 10.1088/1758-5090/9/1/015006.
14. Horch RE, Wagner G, Bannasch H, Kengelbach-Weigand A, Arkudas A, Schmitz M. Keratinocyte Monolayers on Hyaluronic Acid Membranes as "Upside-Down" Grafts Reconstitute Full-Thickness Wounds. Med Sci Monit 2019;25:6702-6710. DOI: 10.12659/MSM.915649.
15. Matsumura H, Gondo M, Imai R, Shibata D, Watanabe K. Chronological histological findings of cultured epidermal autograft over bilayer artificial dermis. Burns 2013;39(4):705-13. DOI: 10.1016/j.burns.2012.10.004.
16. Matsumura H, Matsushima A, Ueyama M, Kumagai N. Application of the cultured epidermal autograft "JACE((R))") for treatment of severe burns: Results of a 6-year multicenter surveillance in Japan. Burns 2016;42(4):769-76. DOI: 10.1016/j.burns.2016.01.019.