An evaluation of skin lesion treatment in mice using keratinocyte-like cell sheets consisting of mesenchymal stem cells and a collagen scaffold

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Abstract

The amniotic membrane has recently garnered attention as a potential scaffold for regenerating damaged tissues such as burns, corneal surfaces and orthopedic injuries. Our study utilized collagen membranes derived from amniotic membranes as a scaffold for mesenchymal stem cells obtained from umbilical cord blood to produce keratinocyte-like cell sheets. These cell sheets were employed to treat skin loss lesions in a mouse model. Analysis of the results indicated that the group treated with cell sheets exhibited the most rapid tissue healing, with wound sizes healed at 83.60%, while the untreated group showed the slowest tissue healing at 77.63%.

Histological examination revealed that utilizing cell sheets for regenerating damaged skin tissue resulted in superior healing, with the structure of the regenerated tissue closely resembling that of mouse skin epithelium, surpassing the outcomes of the other two groups of mice. This research underscores the potential of keratinocyte like cell sheets as a viable graft for autologous keratinocyte sheets or autologous skin grafts in treating patients with skin loss lesions. Particularly beneficial for patients lacking a sufficient supply of autologous skin grafts or requiring immediate intervention, cell sheets can serve as a tissue graft product, offering a viable alternative to conventional grafts.

Keywords: Mesenchymal stem cells, collagen scaffolds, keratinocyte-like cell sheets, NIS-Elements AR.

Introduction

Skin tissue possesses regenerative capabilities; however, in cases of deep damage or extensive burns, the impaired epidermis impedes the natural regeneration of skin tissue. The established method for addressing such challenges is autologous skin grafting. Nevertheless, this approach is beset by limitations, notably the necessity to procure healthy skin tissue from alternative locations on the patient's body. This process can entail treatment prolongation and discomfort and may be unfeasible in cases of widespread burns. Exploring alternative modalities such as autologous keratinocyte sheets or tissue-engineered skin substitutes, holds promise in surmounting these obstacles, potentially yielding more efficacious and less invasive treatments for damaged skin tissue[11,14].

Researchers have developed autologous keratinocyte sheets, a significant advancement in medicine for the treatment of patients who lack skin grafts. In this method, a small amount of healthy skin tissue is collected from the patient and then used to isolate, culture and increase keratinocytes under *in vitro* conditions. These cells are then transferred to a suitable scaffold to create cell sheets for transplantation[1,3]. The only drawback of this solution is the time it takes to develop grafts from isolation, culture, proliferation and cell sheet creation. However, it is crucial to remember that for skin-loss patients, the sooner they receive treatment, the more influential the treatment will be and there will be fewer complications for the patient[5].

From the above difficulties, we have conducted this study with the potential to revolutionize the treatment of skin loss. Our unique approach involves creating keratinocyte-like cell sheets using tissue engineering, a method that overcomes the limitations of traditional methods[9]. This method not only eliminates the need for surgery to harvest healthy skin tissue from patients but also uses only mesenchymal stem cells (MSCs) from umbilical cord blood, which has immature characteristics and immunomodulatory capabilities[7,11,20]. This reduces the immune response barrier of tissue transplantation[10,18]. The resulting keratinocyte-like cell sheets, which closely resemble autologous keratinocyte sheets in structure and function, are always ready for transplantation, thereby increasing treatment effectiveness and minimizing patient complications[11].

Before considering using these cell sheets in patients, we conducted a comprehensive trial of treating skin lesions in a mouse model with these cell sheets to evaluate their therapeutic efficacy. We compared the grafting results with the negative control groups (mice with skin lesions) and the control group (mice grafted with collagen membranes). The thoroughness of our evaluation ensures the robust scientific evidence we present, demonstrating the therapeutic efficacy of these cell sheets in skin loss and their potential future applications.

Material and Methods

Research subjects: Human umbilical cord blood and amniotic membrane were collected under sterile conditions in the operating room, with negative tests for HIV, HBV, HCV and VDRL. The pregnant women had no accompanying diseases, no complications during labour and the fetuses did not have fetal infections and congenital malformations. White mice (Mus musculus var. albino) were collected from the Pasteur Institute in Ho Chi Minh City. They were male, healthy mice weighing 30 grams or more, kept in cages and cared for under the same experimental conditions. The selection criteria for both human subjects and mice were rigorous, ensuring the reliability of the study.

Amniotic membrane into collagen membrane as a cell culture scaffold: Use a sterile, cold PBS solution to clean the amniotic membrane. Then, incubate in a mixture of trypsin (0.25%) - EDTA (0.02%) for 30 minutes at 37^0C, ensuring gentle shaking at 200 rpm to ensure effective enzyme activity. After incubation, remove the surface epithelial cell layer of the amniotic membrane and wash with a sterile cold PBS solution. The results of collagen membrane collection were evaluated by staining the collagen membrane with Giemsa dye, Hematoxylin - Eosin (H and E) staining and observing the surface structure of the collagen membrane by Transmission electron microscopy (TEM).

The collagen membranes, after collection, were cut into small pieces with a size equivalent to the surface area of the Corning 6-Well Plate (*Costar Snapwell inserts*, diameter 24 mm) and stretched onto the surface of these plates so that the collagen bottom membrane surface was facing up. Then, the plates with collagen membranes were left to dry naturally and underwent a sterilization process with Gamma rays at 22-25 kGy, ensuring the safety of all subsequent experiments. We then stored the plates at room temperature[17].

Collection, isolation and identification of mesenchymal stem cells: Umbilical cord blood was divided equally into sterile 50 ml centrifuge tubes (*Corning*), approximately 40 ml of cord blood/tube, centrifuged at 3000 rpm for 20 minutes (Buffycoat). Collect approximately 3 ml of mononuclear cell suspension and transfer to a new sterile 15 ml tube (*Corning*). Next, dilute this mononuclear cell suspension with a sterile PBS solution (ratio 1:1, v/v). Place the cell suspension mixture on top of the Ficoll-Paque™ premium solution layer (1.077 g/ml, *Cytiva Sweden A8*) at a ratio of 2 volumes of cell suspension: 1 volume of Ficoll-Paque solution (ratio 2:1, v/v). Then, centrifuge at 2000 rpm for 20 minutes at 4^0C.

Collect the white mononuclear cell layer between the plasma layer and the Ficoll-paque layer. Rinse with PBS solution to clean the cell suspension by centrifuging at 2000 rpm for 10 minutes at 4^0C (repeat two times). Finally, the cell sediment is collected at the bottom of the tube. Determine the cell density with a cell counter (Vi-CELL-XR, Beckman Coulter, USA).

Cells were cultured in DMEM/F12 medium (*Gibco™*), supplemented with 15% FBS (*Gibco™*), 100 IU/ml penicillin (*Gibco™*) and 100 µg/ml streptomycin (*Gibco™*) at 37^0C, 5% CO_2. The culture medium was changed every three days. When the cells grew to 70 - 80% of the surface area of the T25 cell culture flask, we subcultured them to increase the number of cells.

In the second subculture, the candidate cells were rigorously identified as MSCs according to the stringent standards of The International Society for Cellular Therapy (ISCT)[13]. These standards, known for their reliability and strict adherence to scientific principles, ensure the quality and reliability of our research. According to ISCT standards, MSCs must satisfy the following three minimum criteria: First, the cells adhere to the bottom of the culture flask and have the typical morphology of MSCs. Second, these cells can differentiate into adipocytes, chondroblasts and osteoblasts under *in vitro* conditions[7]. The third criterion for identifying MSCs is their expression of certain markers, particularly CD73, CD90, CD105 (with a positive expression of over 95%) and their lack of expression of negative markers such as CD34, CD45 and HLA-DR (with an expression of less than 5%)[15].

After identifying MSCs that meet the requirements, we embarked on the crucial phase of our research - the differentiation of these cells into keratinocyte-like cells[2,6,7]. This process was facilitated by a mixture of cell differentiation induction medium, including Defined Keratinocyte-SFM (*Gibco™*) and Human Keratinocyte Growth Supplement (HKGS - *Gibco™* S0015). The differentiated cells were then rigorously evaluated by morphological changes and expression of the p63 marker was determined by immunohistochemical staining.

Pioneering the creation of a keratinocyte-like cell sheet from mesenchymal stem cells and collagen membranes: The MSCs in the third subculture were collected and transferred to culture on collagen membranes inside previously prepared insert plates at approximately 10^5 cells/cm^2 density. The cells were cultured in DMEM/F12 medium, supplemented with 15% FBS, antibiotics at 37^0C and 5% CO_2. After stable cell growth (about 3-5 days), the cell culture medium was replaced with a differentiation induction medium to induce MSCs into keratinocyte-like cells on the collagen membranes. The cells were cultured in the differentiation medium for two weeks and then induced to form cell layers using the Airlifting technique for another two weeks. This procedure, meticulously designed and executed, has been shown to be effective in producing cell sheets[8,11].

Testing the effectiveness of keratinocytes-like cell sheets in treating skin loss lesions on mice model, a potential

breakthrough in skin regeneration: We systematically divided mice into three experimental groups: Group 1 (Mice only modelled with lesions - negative control group), group 2 (mice modelled with lesions and grafted with collagen membrane - control group) and group 3 (mice modelled with lesions and grafted with keratinocyte-like cell sheets - research group). The process of grafting the keratinocyte-like cell sheets onto the mice was conducted under strict ethical guidelines and according to the approved ethical standards in medicine (N.22.06-001, in the Ethical Approval section). Each experimental group consisted of 12 mice, divided into four monitoring time points (1 week, two weeks, three weeks and four weeks); each time point had three mice (the experiment was repeated three times).

Mice were fixed on the surgical stand, anesthetized with Zoletil 50 (*Virbac*) at a precise dose of 5 mg/kg. Then clean the fur on the back of the mouse where the surgery would be performed, disinfect the surgical area with Betadine solution and create a wound with a size of about 1.5 x 1.5 cm^2. The area of the removed back skin tissue must ensure complete removal of the epidermis down to the muscle tissue area. Then, we grafted collagen membrane (Group 2) and keratinocyte-like cell sheets (Group 3) at the damaged area for the experimental mice. The steps of grafting ensured aseptic performance. After surgery, the mice were raised separately in a cage, cared for and fed under the same nutritional conditions throughout the experiment.

We conducted evaluations at four specific time points (1 week, two weeks, 3 weeks and 4 weeks) by capturing images of the surgical area for all three groups of mice. Subsequently, these images underwent processing with the NIS-Elements software, enabling precise observation and assessment of wound healing. The results were then recorded and a comparative table was developed based on the wound healing size measurements obtained from the software. Following the image capture for wound size measurement, tissue samples will be obtained from the grafting site. These samples are integral to creating histological slides, which are crucial for evaluating the structural aspects of the skin tissue during the wound healing progression. Our tissue biopsy must ensure accurate identification of the host and graft regions on the histological slide (Figure 1).

Results

Collagen membrane collection from amniotic membrane: The collagen membrane, with its intact structure and preserved extracellular matrix, is a testament to the quality of our collection process. The quantity of the collected collagen membrane, enough to create the scaffold, further demonstrates the efficiency of our process. This result shows that our group established the collagen membrane collection process which is effective and achieves the desired results of collecting collagen membranes used as scaffolds for cell culture in tissue engineering.

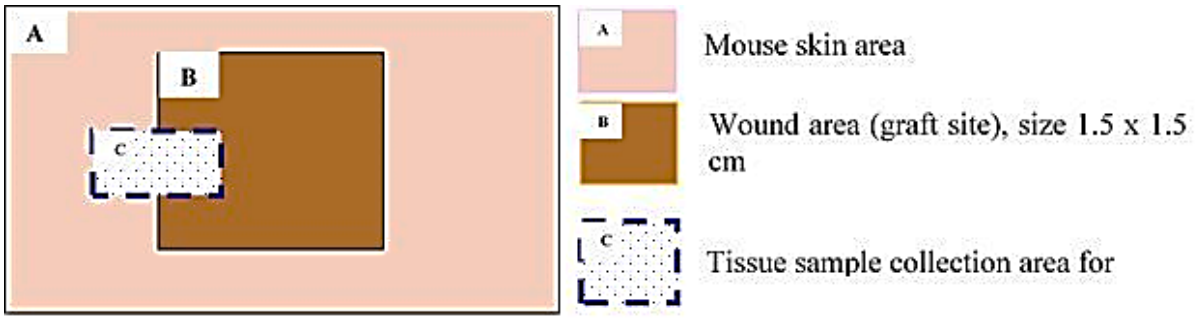

Figure 1: Illustration of skin tissue sample collection for H and E staining. (A) Mouse skin area. (B) Wound area (graft site), size 1.5 x 1.5 cm. (C) Tissue sample collection area for H and E staining.

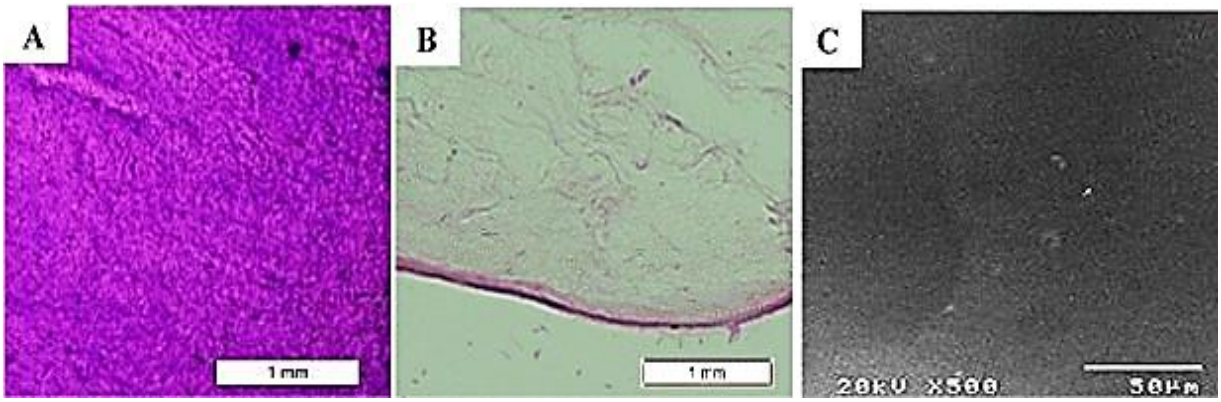

Figure 2: Results of processing the amniotic membrane to obtain a collagen membrane. (A) Following processing, the collagen membrane was stained with Giemsa. (B) Histological staining with H and E was performed on the collagen membrane. (C) SEM imaging confirmed the complete removal of the epithelial layer from the collagen membrane.

The results of our research are promising, showing that the treated amniotic membrane completely removed the epithelial layer when observed fresh under an inverted microscope and after Giemsa staining (Fig. 2A). The H and E histological staining results further demonstrated the collagen membrane, which showed that the surface epithelial cell layer of the amniotic membrane had been removed (Fig. 2B). This result was also confirmed when observing the collagen membrane under Scanning electron microscopy (SEM) (Fig. 2C). We removed the amniotic membrane epithelial layer but preserved the extracellular matrix's intact collagen structure.

The thinner collagen membrane was suitable for stretching onto insert plates to create cell sheets, a potential breakthrough in skin tissue engineering and regenerative medicine that could significantly impact the field. The next step involves carefully placing the collagen membrane onto the 6-well insert plates, ensuring that it is evenly spread, as shown in fig. 3. These specially prepared plates will be the foundation for cultivating MSCs to form keratinocyte-like cell sheets.

Identification of MSCs and differentiation of MSCs into keratinocyte-like cells: In the initial phase of primary cell culture, the cells exhibited adhesion to the bottom of the flasks. Subsequently, after three days of cultivation, spindle-shaped cells, reminiscent of fibroblasts, became evident and adhered to the flask surface, conforming to the primary criterion of the ISCT standard for MSCs identification[13]. These cells are amenable to further cultivation, allowing for an increase in cell mass within 2-4 weeks following the primary culture period (Figure 4A).

Our research group rigorously adhered to the ISCT standards and conducted comprehensive tests to assess the differentiation of umbilical cord blood stem cells into osteoblasts, chondroblasts and adipocytes in the second passage under *in vitro* conditions (Fig. 4 B-D)[7]. These results align with the second criterion of the ISCT standard used for identifying MSCs[11] and highlight our unwavering commitment in upholding the highest quality standards in our research endeavors.

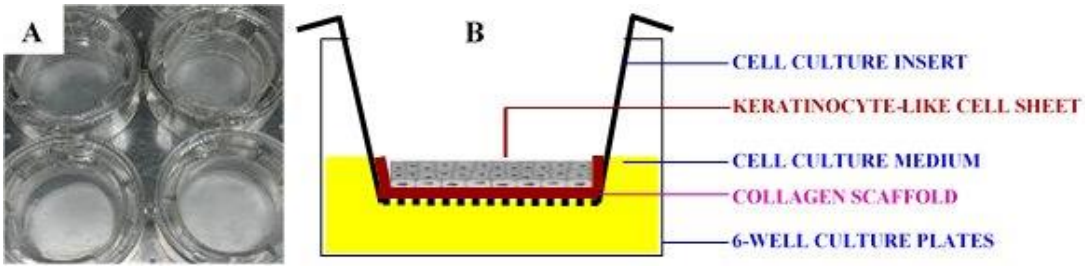

Figure 3: Cell sheet model utilizing collagen membrane. (A) The collagen membrane is placed on the insert plate in a 6-well culture plate. (B) Model for the formation of keratinocyte-like cell sheet.

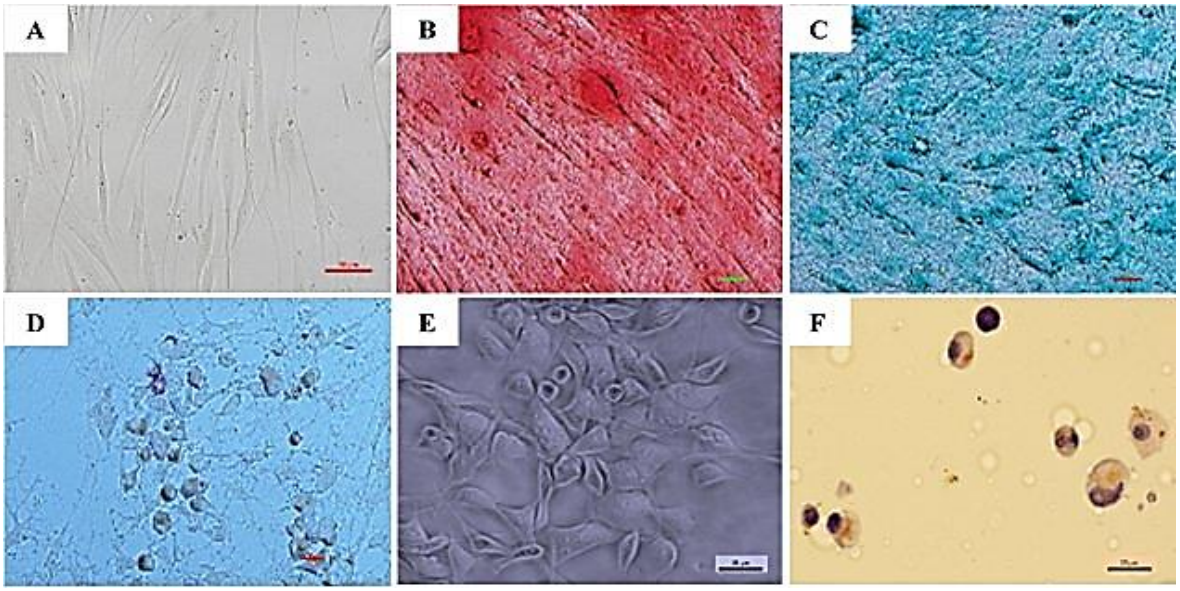

Figure 4: Culture results, identification of mesenchymal stem cells and differentiation assay of MSCs into keratinocyte-like cells. (A) Candidate stem cells cultured without induced differentiation, magnification 20X. (B) Cellular differentiation into osteoblasts, stained with Alizarin Red, magnification 20X. (C) Cellular differentiation into chondroblasts, stained with Alcian Blue, magnification 20X. (D) Cellular differentiation into adipocytes, stained with Oil Red O, magnification 20X. (E) Cellular differentiation into keratinocyte-like cells, magnification 20X. (F) Keratinocyte-like cells stained with marker p63 by immunohistochemistry, magnification 20X.

The results of the flow cytometry, displayed in figure 5, play a crucial role in identifying MSCs. Notably, CD73, CD90 and CD105 are expressed on the surface of MSCs, while CD19, CD34, CD45 and HLA-DR are not typically expressed on these cells. These results confirm the positive expression of CD73, CD90 and CD105 markers in the umbilical cord blood stem cell population, showing very high expression levels (≥ 98%), meeting the essential identification criteria as per the ISCT standard. CD19, CD34, CD45 and HLA-DR markers also showed negative expression levels (< 2%). This outcome fulfills the third criterion of the ISCT, providing a solid foundation for our research[15].

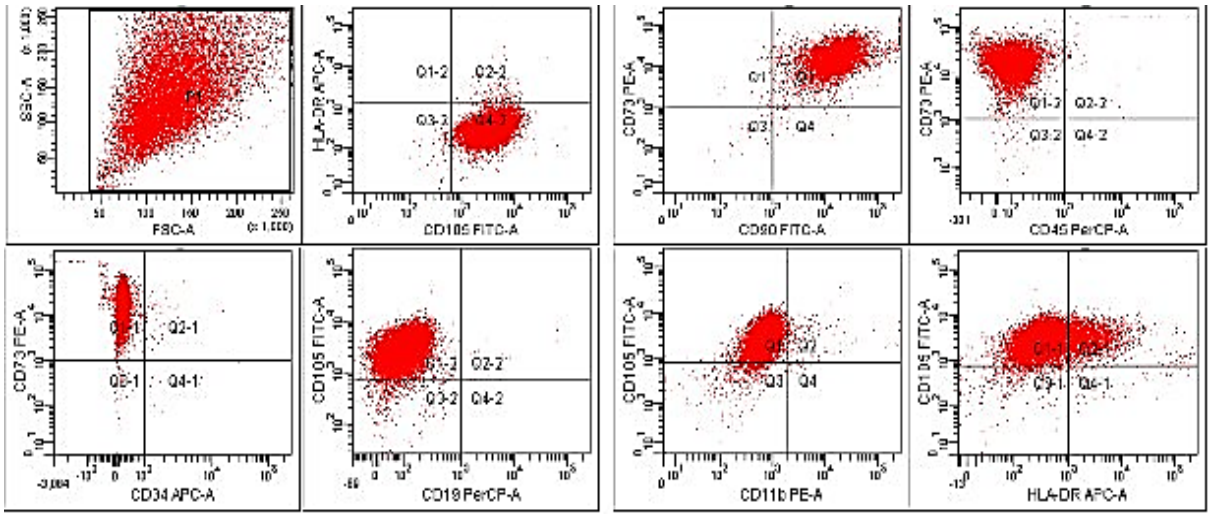

Figure 5: Illustrates the precision of flow cytometry in identifying MSCs from umbilical cord blood. The assay revealed that the MSCs were positive for CD73, CD90 and CD105, while negative for CD19, CD34, CD45 and HLA-DR.

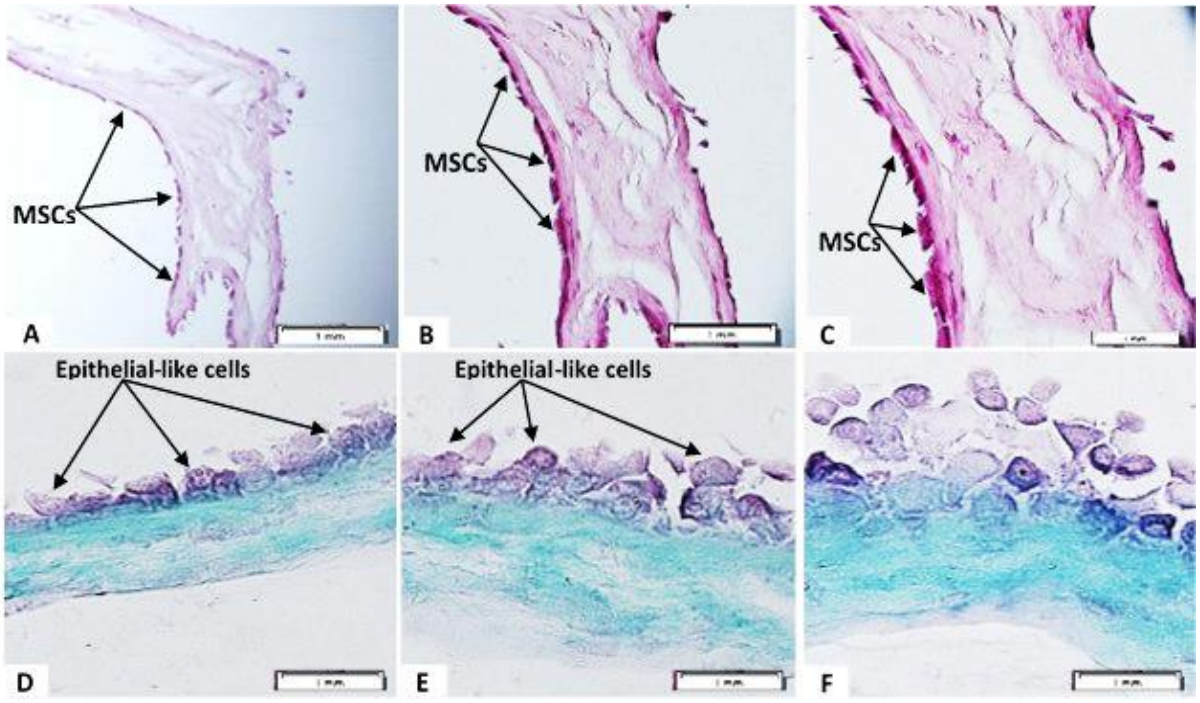

Figure 6: The outcomes of generating keratinocyte-like cell sheets on a collagen scaffold. (A), (B) and (C): The results of our cultivation of MSCs on H and E-stained collagen membranes were captured at 10X, 20X and 40X magnifications respectively. The histological examination revealed that the MSCs maintained their elongated morphology, adhered firmly to the surface of the collagen membrane and interconnected to form a monolayer of cells on the surface. (D), (E) and (F): H and E stained keratinocyte-like cell sheets observed at 10X, 20X and 40X magnifications, respectively. The histological analysis demonstrated that the cells adhered to the collagen membrane no longer exhibited the characteristic shape of MSCs. Instead, they displayed the typical squamous morphology of epithelial cells. These cells adhered together to form multiple layers on the collagen membrane's surface, resembling the skin epithelium's structure (Fig. 6F).

Upon conducting cell isolation, subculture and identification, we verified that the cells in our study fulfilled the minimum criteria for being classified as MSCs according to ISCT guidelines. Additionally, our differentiation experiments using the K-SFM + HKGS kit have demonstrated that these cells underwent a noticeable morphological change after cell culture for two weeks, transitioning from the characteristic elongated shape of MSCs to a nearly round polyhedral shape (Fig. 4E). Furthermore, immunohistochemical staining revealed positive expression for the p63 marker (Fig. 4F), indicating the cells' capacity to differentiate into epithelial keratinocyte-like cells, consistent with recent studies by Dos Santos et al[2], Ghauri et al[6] and Yavari et al[22].

Epithelial sheet creation: The entire process of cell culture and the generation of keratinocyte-like cell sheets are described in figure 6. As the cells underwent culture and differentiation induction, we observed a gradual transition from the typical elongated shape of MSCs (Fig. 6A-C) to the formation of epithelial cells with a square or polyhedral shape (Fig. 6D). Subsequently, the airlifting culture method resulted in the development of cell layers on the collagen membrane (Fig. 6E-F), ultimately assuming the morphology and structure characteristic of the skin epithelium, featuring 2 - 4 cell layers.

Evaluation of grafting results on mouse models of skin loss: All three cohorts of mice (n = 36) not only survived but flourished during the study period, particularly in cohorts 2 and 3, where collagen membrane and cell sheet grafts were employed. No instances of graft rejection were observed. Our research team used the NIS-Elements software from Nikon Instruments Inc. to assess the progression of tissue healing. This software determines the actual size of the experimental tissue area as a percentage of the healed size compared to the baseline measurement at day 0.

We have gathered data from this software which offers insights into the wound-healing process observed in three different groups of mice. This information has been summarized in table 1, showing the average wound healing size across the three groups and the corresponding standard deviations based on three measurements (Fig. 7).

Table 1
Wound healing size in mice over the study time points.

Experimental mouse groups	Wound healing size by average value (cm^2)					
	Week 0	Week 1	Week 2	Week 3	Week 4	Wound healing rate (0 – 4 weeks) (%)
Group 1 (n = 12)	3.80	3.62	1.66	1.10	0.85	77.63
Group 2 (n = 12)	4.72	3.27	1.59	0.92	0.60	87.28
Group 3 (n = 12)	4.27	3.26	2.33	0.89	0.70	83.60

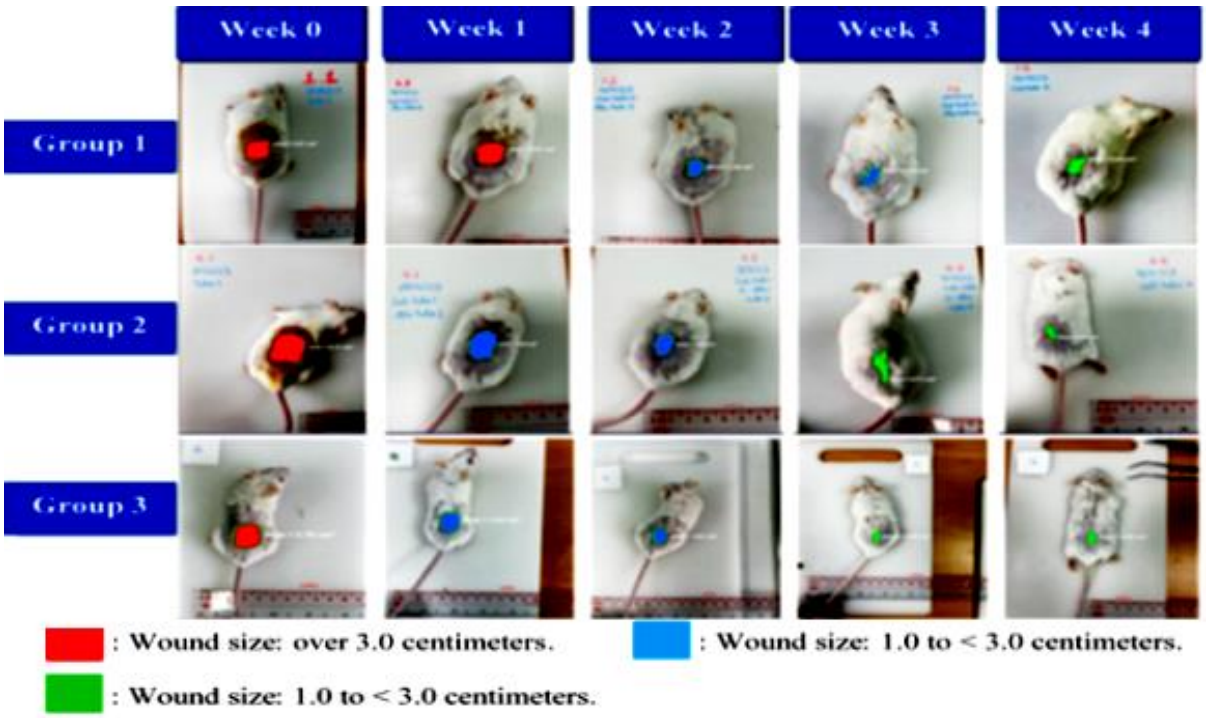

Figure 7: Results of wound size assessment using NIS-Elements software. A camera captures an image of the tissue area under study, which is then imported into the software. The software's measuring tool calculates the wound's specific size in centimeters. Consequently, the wound-healing size at the observed location will be collected for all research time points.

When comparing data from this software, it shows that the wound healing size in group 2 mice is higher than in groups 1 and 3, at 87.28%, compared to 77.63% and 83.60% respectively. This result is due to the acellular collagen membrane, which accelerates the healing process by skipping the tissue reaction stage with the graft material. The collagen membrane retains the biological components analyzed earlier, contributing to inducing the cells of the host tissue to be active and divide strongly, thereby facilitating faster healing[16]. Additionally, the collagen membrane, obtained from amniotic membranes, is thin, tough and has good elasticity, adhering tightly to the wound. These limit wound infection and promote faster healing than the other two experimental groups.

Studies have also shown that collagen membranes obtained from amniotic membranes have antibacterial properties and promote the healing process of granulation tissue in the skin[3,12]. In the first week after transplantation, in group 1 mice, the wound remained open and displayed early signs of healing, characterized by the commencement of wound cleaning and connective tissue proliferation. Histological staining revealed a notable defect on the wound surface (Fig. 8A).

In the case of group 2 mice, collagen membranes persisted and covered the damaged tissue area. In certain locations, fibroblasts invaded and migrated into the collagen membrane (Fig. 8E). For group 3 mice, the keratinocyte-like cell sheet plate persisted on the wound surface. The cells within the sheet demonstrated viability, displaying a polyhedral cell shape with 1 to 3 cell layers. Histological findings confirmed that the cell sheet was not rejected and adhered firmly to the wound surface (Fig. 8I). The histological results indicated that the healing observed in group 3 mice was better than that of the other two research groups.

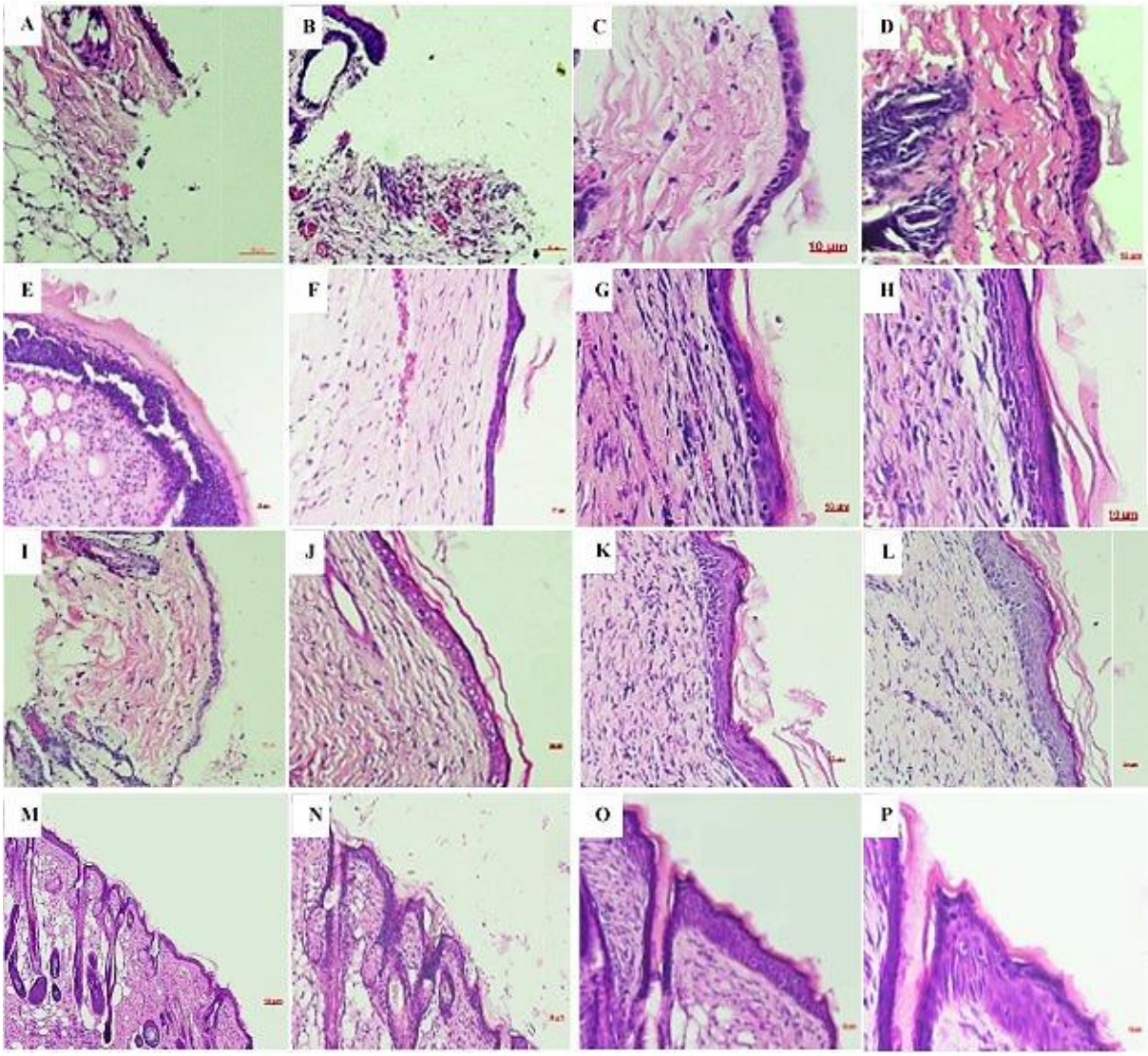

Figure 8: Histological evaluation results (H and E staining) of 3 experimental groups of mice with skin loss lesions. (A-D): Histological results for group 1 mice from week 1 to week 4. (E-H): Histological results for group 2 mice from week 1 to week 4. (I-L): Histological results for group 3 mice from week 1 to week 4. (M-P): Histological staining results for normal mouse skin tissue at 4X, 10X, 20X and 40X objectives respectively.

In the second week after transplantation, Group 1 mice showed gradual tissue healing as connective tissue papillae began to form and blood vessels developed to nourish surrounding tissues. However, connective tissue formation was slow and the wound area still had significant defects (Fig. 8B). In group 2 mice, an epithelial layer with 1-2 rows of cells began to appear. These cells were flat and round, gradually thinning from the edge to the center of the wound, marking the beginning of the epithelialization process. The collagen scaffold stimulated this process, inducing the migration of epithelial cells from the wound edge into the wound area[12,21]. Cells capable of migrating and proliferating are in the basal layer of the skin epidermis. This result is consistent with a study by Dos Santos[2] (Fig. 8F).

The evaluation results in group 3 mice were very positive. The epithelial layer formed several layers (1-3 cell layers) and the layers of the epithelium could be distinguished (basal layer, spinous layer, granular layer). At this stage, the collagen scaffold began to peel off from the forming epithelial layer below (Fig. 8J).

Three weeks after transplantation, The connective tissue in the damaged area underwent restructuring in mice from group 1. Cells from the basal layer of the healthy tissue migrated into the wound area to facilitate repair and regeneration (Fig. 8C). However, the epithelialization process in group 1 mice was slow, resulting in only 1-2 cell layers. In group 2 mice, the epithelial structure consisted of numerous layers of cells, but the distinction between the layers was unclear, with approximately 1-3 cell layers. This cell layering decreased as it approached the center of the wound (Fig. 8G). Group 3 mice exhibited the highest quality of healing, with the structure of the epithelialization part readjusted according to the biological structure of the skin epidermis.

The basal cell layer was concentrated at the edge of the wound and migrated into the tissue during the healing process. This process is essential as the cell sheet needs to be replenished from healthy tissue to aid in the repair and regeneration of the epidermis. Comparing the epidermis structure in group 3 mice (Fig. 8K), the positive control sample (Fig. 8O) revealed a similarity in histological structure.

Analysis of histological images indicates variations in the healing progression among the three groups of mice. Notably, in group 1, cell stratification was initiated, with 2-3 cell layers discernible.

Comparatively, the healing progress in group 1 was akin to that of group 2 at week 2, signifying a two-week delay in the epithelialization process in group 1 mice compared to group 2. Within group 2, the stratification and epithelial structure resembled those of group 3 by week 3; layers were discernible in the histological structure, albeit lacking clear differentiation between them. Conversely, within group 3, the epithelial structure exhibited near-complete reconstruction, evincing segments reminiscent of the normal skin tissue observed in the control group.

Discussion

Amniotic membranes are safe and effective natural biomaterials for various clinical applications such as treating epithelial surface damage in burns, ophthalmology and orthopedics[12,19]. Currently, global research groups are utilizing amniotic membranes as a scaffold for cultivating human keratinocyte cells for the development of autografted keratinocyte cell sheets for the treatment of burn injuries, cultivating corneal limbal cells to create epithelium cell sheets for treating ocular pathologies[1] and for culturing stem cells to develop therapeutically applicable grafts in regenerative medicine[3,4,21].

Stem cells, which have profoundly impacted medicine and society, encompass various types that have undergone extensive study and application recently, with MSCs being particularly notable[10,18]. Our team has successfully isolated and identified MSCs from umbilical cord blood in compliance with the International Society for Cellular Therapy (ISCT) standard[16]. Furthermore, we have differentiated MSCs into keratinocyte like cells, validated by morphological changes and functional expressions (such as p63 marker expression, figure 4F). This successful result enables the generation of keratinocyte-like cell sheets by cultivating MSCs on collagen membranes (Fig. 6D-F)[6,20,22].

We have successfully generated keratinocyte-like cell sheets with a multi-layer cellular structure (2-5 cell layers, figure 6F), with characteristics consistent with the morphology and functionality of the basal cell layer (Figure 4E-F). This outcome aligns with the research results of Horch et al[8,9], Kamolz et al[11] and Yavari et al[22].

Notably, the evaluation of the treatment results for skin loss injuries in the three groups of mice discovered significant differences in the early treatment period. Specifically, within the first week, only mice in group 3 had the presence of an epithelial cell layer (including 1-2 cell layers) in the injured skin tissue area. Conversely, no epithelial layer was evident in groups 1 and 2. The findings validate our conviction that prompt grafting is imperative for burns or skin loss patients. Early intervention offers enhanced treatment prospects, mitigates deleterious wound complications and elevates the patient's recuperation and quality of life[5].

The histological analysis conducted at week 4 revealed successful wound healing in all three groups of mice. The regeneration of the lost epidermis occurred without eliciting rejection, resulting in a structure resembling a normal epidermis. However, the limited 4-week follow-up period prevented a comprehensive evaluation of the damaged skin tissue's long-term healing and regeneration process. Certain limitations were observed including the absence of hair follicles, sweat glands and sebaceous glands. Furthermore,

complete regeneration of the dermis and hypodermis is yet to be achieved. Tran et al[20] reported that the grafted skin area appeared lighter than the adjacent normal skin tissue post-healing, lacking typical skin colouration. This occurrence was attributed to the absence of pigment-producing cells, notably melanocytes, in the autologous keratinocyte sheet.

Notably, our histological assessment also did not reveal the presence of pigment-producing cells within the healed skin area, prompting consideration for future investigation, particularly regarding the co-cultivation of additional cells such as melanocytes or fibroblasts, to enhance skin coloration and expedite the healing process.

Conclusion

Our research has yielded a cell sheet with substantial potential for clinical applications. The preliminary assessment conducted in a mouse model of skin loss injury is not only promising but also remarkable, illustrating the regenerative capacity of these cell sheets in reinstating the lost epidermis. These cell sheets demonstrate a lack of rejection and effectively promote the regeneration of damaged epithelium. Nevertheless, the 4-week duration of our follow-up has curtailed our ability to comprehensively evaluate the repair and regeneration of damaged skin tissue in the mouse model. Consequently, we have identified specific recommendations for remedy regarding the limitations inherent in this study.

In brief, cell sheets address the limitations associated with prolonged culturing periods traditionally necessary for generating autologous keratinocyte sheets. These cell sheets show promise as adjunctive therapy for individuals with skin loss lesions, particularly in cases involving severe burns and dehiscence trauma, wherein the availability of skin grafts is limited or insufficient time exists to produce autologous keratinocyte sheets for treatment. Timely treatment of skin lesions in patients is associated with accelerated recovery and reduced wound complications. Consequently, our group endeavors to achieve this objective and we consider these cell sheets a remedy, offering a solution to the aforementioned challenges.

Acknowledgement

This research was funded by Thu Dau Mot University, Binh Duong Province, Vietnam, under grant number DT.22.1-035.

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(Received 17th September 2024, accepted 18th October 2024)