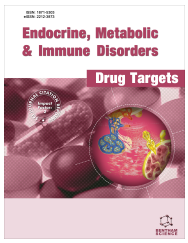
RESEARCH ARTICLE

Human-like Skin Tissue Graft Generated Using Human Umbilical Cord Blood Mesenchymal Stem Cells and Acellular Amniotic Membrane: A New Approach in Reconstructive Medicine



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Abstract: ***Background*:** Due to its outstanding biological properties, the Human Amniotic Membrane (HAM) has been a topic of medical research for many years to treat, repair, and regenerate damages related to the epithelial surface, corneal epithelial, orthopedic, and infected body parts. The HAM shows crucial anti-inflammatory, anti-bacterial, and anti-viral properties and immunomodulating qualities. Therefore, the HAM is one of the most used natural biomaterials in tissue engineering to generate bio-scaffolds.

***Objective*:** This study used HAM as a raw material to produce a collagen membrane that fully meets the criteria for creating a bioscaffold with suitable mechanical strength and limited risk of graft rejection. This collagen membrane would be a bioscaffold to carry Human Umbilical Cord Blood Mesenchymal Stem Cells (hUCB-MSCs) to generate human epithelial-like cell sheets. Shortly, these cell sheets can repair and regenerate the epithelial layer in trauma cases, severe damage from accidents, burns, and other post-operational conditions.

***Methods*:** Among the varieties of stem cells currently being researched, MSCs are the most studied and applied due to their outstanding bio-qualities and regenerative features. We used International Society for Cells and Gene Therapy (ISCT) standards to identify and characterize the hUCB-MSCs obtained from a donor's umbilical cord blood who provided consent. We used an inverted phase contrast microscope to evaluate morphological changes and epithelial stratification of cultured growing cells on the collagen scaffold. Further, staining techniques, such as H&E and Trichrome, were used to assess the morphology and structure of the new epithelial construct; histochemical staining with p63 and ck 1/10 markers was used to evaluate the cellular characteristics and specific traits. Transmission electron microscopy was used to validate cell junctions.

***Results*:** This study successfully obtained human epithelial-like cell sheets with multiple cell layers, morphology, and characteristics comparable to human epithelial tissue.

***Conclusion*:** The results of this study will constitute a potential alternative to autologous skin grafting in heavy skin loss and deep lesions.

Keywords: Human umbilical cord blood mesenchymal stem cells (hUCB-MSCs), human amniotic membrane (HAM), collagen scaffold, 3D epithelial tissue, transmission electron microscope (TEM), ISCT.

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1. INTRODUCTION

The skin is the largest organ in the body and performs essential functions as follows: (i) protection and defense against pathogens, (ii) regulation of body temperature, (iii) and maintenance of fluid balance [1, 2]. The healing process of damage proceeds in the following stages: hemostasis, inflammation, proliferation, and remodeling. In injuries that involve loss of skin tissue, such as deep ulcers and burns, the protective function of the tissue surface is automatically lost, increasing the risk of life-threatening risks. To treat these types of damage, autologous skin grafting is considered the "gold standard" procedure to regenerate the epithelial surface of damaged skin [1]. However, autologous skin harvesting procedures have disadvantages. In cases of burns or extensive skin loss, harvesting enough skin tissue for a new graft becomes difficult, if not impossible, in addition to the invasiveness of the operation, which involves pain and possible infections [1-4].

Therefore, developing a viable alternative to replace autologous skin tissue has become crucial. Currently, many products of both biological and chemical origin are used to support the skin healing process [5-10]. Again, the disadvantage is that these materials do not contain living cells and lack all the factors and molecules necessary for natural and effective integration with the natural skin tissue of the host [10-15].

Nowadays, tissue engineering and regenerative medicine allow the possibility of generating more sustainable and compatible biografts extremely close to human tissues; current technology has developed grafts capable of including almost all types of living cells within extracellular matrices that support tissue repair and regeneration [16-20]. To this end, the restoration and repair of damaged skin tissue require the presence of materials that are qualitatively and biologically capable of meeting very high standards. The HAM has unique pleiotropic and biological characteristics [21, 22]. It is a type of highly biocompatible, biodegradable, acellular, and easy-to-handle tissue, suitable for the allocation of essential stem cells in the repair and regeneration processes. It easily overcomes the immune barrier of the host tissue and integrates well into the surrounding tissue and microenvironment [23-29].

The HAM constitutes the innermost part of the placenta adjacent to the fetus. It comprises a single layer of epithelium, a thick basement membrane, an avascular layer, and a stromal matrix with a thickness of 0.02–0.5 mm [10-12]. The epithelial layer comprises metabolically active cuboidal and columnar cells in direct contact with the amniotic fluid [13, 14]. The basement membrane is composed of various components, including those that form the extracellular matrix, collagen, elastin, laminin, fibronectin, and vitronectin; glycosaminoglycans, such as hyaluronic acid; natural inhibitors (matrix metalloproteinases (MMPs); and cytokines and growth factors that are crucial for wound healing and tissue regeneration are also produced here [14, 15]. Several growth factors, Basic Fibroblast Growth Factor (b-FGF), Epidermal Growth Factor (EGF), Keratocyte Growth Factor (KGF), And Hepatocyte Growth Factor (HGF), are known to modulate the wound healing response [16]. Three components form the stromal matrix or mesenchymal layer, the most important of which is the external layer, mainly composed of mesenchymal cells similar to fibroblasts. The collagen-rich content of the mesenchymal layer not only increases its elasticity but also makes it an excellent tissue that can even replace skin [14-16].

While pluripotent MSCs are exceptional candidates for tissue regeneration, umbilical cord blood is an important source of pluripotent MSCs capable of differentiating into many cellular phenotypes [5, 9]. It is known that these stem cells, capable of differentiating into epithelial cells, become the optimal solution for tissue engineering to create cell sheets with a basic structure similar to human skin tissue. Furthermore, previous *in vitro* and *in vivo* experiments, confirmed the capacity of these cells to secrete proteins that promote epidermal self-repair, growth, and development [7, 30-33].

Therefore, the combination of HAM and MSC (HAM-MSC) forms a unique solution capable of generating an extraordinary biocomposite with characteristics highly similar to the basal layer of the skin epithelium [30, 31]. Furthermore, it was observed that HAM-MSCs sustain gene stability, which is essential for large-scale clinical applications while regulating wound healing, immunomodulation, angiogenesis, cell differentiation, and cell motility. *In vivo*, the results confirmed the anti-inflammatory and tissue remodeling properties of HAM-MSCs with high healing effects in both acute and chronic skin wounds, owing to the high presence of Lysyl Oxidase-2 (LOXL2), which promotes wound healing by activating the c-Jun N-terminal Kinase (JNK) signaling pathway [30-33].

2. MATERIALS AND METHODS

2.1. Collection of HAM and hUCB-MSCs

Tissue samples (30 control samples, 30 experimental samples) were obtained with prior consent from the donors for *in vitro* research. They were collected under sterile conditions in the hospital operating room. This study was approved according to the decision of the Ethics Board of Thu Dau Mot University with decision number N. 22.06-001. All tissue samples were transported to the laboratory, and microbiological tests were performed for the screening of HIV, HBV, HCV, and VDRL. Umbilical cord blood samples were used to isolate mesenchymal stem cells. HAM was used to produce collagen membranes.

2.2. Processing of the HAM into a Collagen Membrane used as a Bio-scaffold

After obtaining HAM from donors, we used a sterile PBS (*Gibco*^{TM}) solution to rinse it. Then, the sample was incubated in a mixture of Trypsin (*Gibco*^{TM})–EDTA (*Sigma*) (0.25%-0.02%) solution for 30 minutes at 370^{o}C with shaking at 200 rpm. Each sample was gently scraped off to remove the amniotic epithelial cell layer and rinsed with a ster-

ile, cold PBS solution. We evaluated the presence of collagen membrane by examining it directly under an inverted phase contrast microscope to observe the effects of epithelial cell layer separation; Giemsa (*Merck*) and Hematoxylin & Eosin (H&E) (*Merck*) staining methods were performed to evaluate the structure and presence of the collagen. The membrane was then cut into small pieces to be inserted in a container for cell culture inserts (about 24 mm in diameter) (*Gibco*^{TM}). Each sample was let dry in an operating sterile cabinet overnight. The final collagen membrane was then transferred into 6-well cell culture plates, packaged, sterilized by gamma rays at 25 kGy, and stored at room temperature. Afterward, it was ready to be used as a scaffold to culture adherent cells.

2.3. Procedure for Collection, Isolation, and Identification of hUCB-MSCs

Umbilical cord blood was collected by syringe from the umbilical vein into a sample bag containing Heparin (5,000 Units/ml) (*Pfizer*). Blood samples were stored at 4°C while awaiting test results and preparation for isolating candidate umbilical cord blood stem cells.

The blood was divided equally into 50ml conical centrifuge tubes (*Falcon*^{TM}), with approximately 30 mL of blood-/tube. Next, the samples were centrifuged at 3000 rpm for 20 minutes to collect the mononuclear cell layer (4ml of mononuclear cell fluid). This fluid was then transferred into new 15 mL conical centrifuge tubes (*Falcon*™). The cell solution was subsequently diluted with sterile PBS in a 1:1 ratio.

Then, the cell suspension mixture was gently transferred onto a layer of Ficoll-Paque™ Premium (*1.077 g/ml, Cytiva Sweden A8*) in a ratio of 2:1. The mixture was then centrifuged at 2000 rpm for 20 minutes, at 4°C; the mononuclear cell layer between the Ficoll-Paque layer and the red blood cell layer were collected by pipette and poured into 15ml conical centrifuge tubes. The mononuclear cell solution was washed with PBS solution at a ratio of 1:9 and centrifuged at 2000 rpm for 10 minutes at 4°C. The cell-washing step was repeated, and sedimented cells were finally collected. Cell density was performed using an automated cell counter (*Vi-CELL-XR, Beckman Coulter, USA*). Mononuclear cells were cultured at a density of 10^3 cells/ml in DMEM/F12 medium (*Gibco*^{TM}) supplemented with 15% FBS (*Gibco*^{TM}), 100 IU/ml penicillin (*Gibco*^{TM}), and 100 µg/ml streptomycin (*Gibco*^{TM}) in a T-25 cell culture flasks (*Nunc*^{TM}) at a temperature of 37°C, with 5% CO_2. The cell culture medium was changed every two days. When the cells in the primary culture grew to a density of about 70 - 80% of the surface area of the flasks, cells were harvested and moved into new T-25 flasks.

Second-generation cells were identified as MSCs according to the three criteria set by The International Society for Cellular Therapy (ISCT) [17]: First, MSCs are cells that adhere to the bottom of the culture bottle, with the typical morphology of elongated fibroblast mesenchymal shape. Second, MSCs can differentiate into adipocytes, chondroblasts, and osteoblasts under *in vitro* conditions [9]. Third, the MSCs can express several typical markers of multipotency and pluripotency, such as markers CD73, CD90, and CD105 (positive ≥ 95%), and should be negative for the following markers, such as CD34, CD45, CD45, and HLA-DR (negative ≤ 2%) [23].

2.4. The Generation Procedure for the New 3D

Second-generation MSCs were collected and cultured on collagen membranes at a density of about 10^6 cells/cm^2. The culture medium used was DMEM/F12, supplemented with 15% FBS and antibiotics (Pen/Strep), and the cells were grown at 37°C in a 5% CO_2. After one week, a differentiation kit consisting of K-SFM culture medium (Gibco™) supplemented with HKGS (Human Keratinocyte Growth Supplement, Gibco™) was applied to induce the differentiation of mesenchymal stem cells into epithelial-like cells. During the differentiation process, morphological changes of the cells were monitored by an inverted phase contrast microscope.

The negative control group consisted of MSCs grown on collagen membranes in DMEM/F12 medium, 15% FBS, and antibiotics (Pen/Strep)."

On day 15, cells were stained to confirm p63 and ck 1/10 markers to demonstrate the functional aspects of skin epithelial cell phenotypes. The air-liquid Interface Culture technique was then applied for two weeks to stimulate the creation of cell layers to develop a stratified 3D epithelial tissue [10, 14]. Morphological changes of cells on the epithelial sheet were then revealed by using H&E staining, while the presence of cell junctions between cells and collagen membranes was confirmed by TEM.

2.5. Statistical Analysis

Data were expressed as mean ± standard error of the mean (SE). In addition, the student's t-test was used to compare the data pairs in the results of the two groups if they were equally distributed; a p-value < 0.05, indicated that the results were statistically significant. All experiments were repeated three times.

3. RESULTS

3.1. Isolation and Evaluation of the Structure of Collagen Membranes

Our group established a procedure that effectively removed the HAM epithelial cell layer, preserving the collagen membrane structure integrity (Fig. **1**). Additionally, this membrane was found to have a thinner wall than the HAM due to the loss of the surface epithelial cell layer as well as theelimination of the cells in the extracellular matrix of the basement membrane (Fig. **1F**).

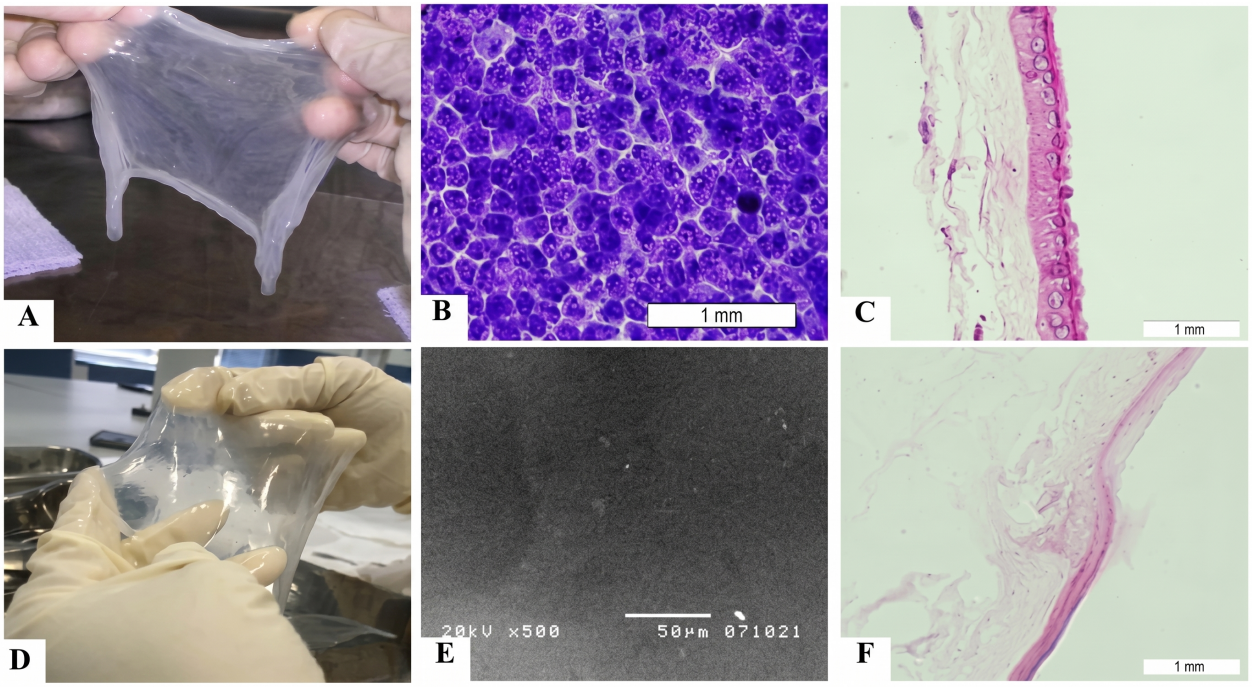


Fig. (1). Collection and processing of HAM into collagen membrane. (**A, D**). HAM before and after processing into collagen membrane. (**B, C**). Observing the HAM directly under an inverted phase contrast microscope after Giemsa staining and H&E treatment, the HAM epithelial cells are a single layer of square epithelial cells that stain dark purple (**E, F**). The collagen membrane. No epithelial cells are left on the surface of the collagen membrane when observed by SEM (1.E) and H&E (1.F). *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*.

The inner structure of the collagen membrane is revealed to be highly suitable for homing cells. Its natural flexibility and elasticity made it convenient to cultivate cells without minimal loss (Fig. **2**).

3.2. Identification of Mesenchymal Stem Cells According to ISCT Standards

Second-generation cells were also induced to differentiate into osteoblasts, chondroblasts, and adipocytes. The proper analysis and tests confirmed that the hUCB-MSCs successfully differentiated into different cell phenotypes under *in vitro* conditions, including adipocytes (oil red), osteoblasts (alizarin red), and chondroblasts (alcian blue) (Figs. **3**, **3B**, **3D**, **3F**) according to the second criterion of the ISCT standard [14]. We used the Flow Cytometry analysis procedure (Beckman Coulter's cell analysis system) with a CD panel, including CD14, CD19, CD44, CD45, CD73, CD90, and HLA-DR, to confirm surface marker expression of candidate mesenchymal stem cells (Fig. **4**).

Results showed that the MSCs were positive for markers CD44, CD73, and CD90 (≥ 98%), while they were negative for CD14, CD19, CD45, and HLA-DR (< 2.0%); overall, the results were considered indicative of the presence of MSCs (Fig. **4**) [16]. The analysis results based on the expression of these cell surface markers satisfy the third criterion of the ISCT standard [17].

Human UCB-MSC-derived epithelial-like cells were morphologically confirmed to exhibit the typical squamous/rhomboid shape characteristic of skin epithelial cells.

In addition, positive staining results for p63 and ck 1/10 confirmed the molecular phenotype of the skin epithelial cells (Fig. **5**).

The p63 marker confirmed that the stemness and the traits of the cells belong to the basal cell layer of the epithelium (Fig. **5**). Cells in the first layer of the cell sheet were positive for the p63 marker (Fig. **5B**), similar to the results obtained from the positive control sample, a human mammary tissue sample (Fig. **5C**). Cells in the basal layer of the epithelial sheet stained red-orange color, the same as the glandular epithelial cells in mammary tissue, confirming the successful differentiated process of hUCB-MSCs into epithelial-like cells. Meanwhile, cells cultured on the collagen membrane (negative control sample) were negative for the p63 marker, keeping their features and morphology unaltered (Fig. **5A**).

3.3. Characteristics and Distinctive Features of the 3D Epithelial Tissue Obtained from the Combination of hUCB-MSCs and the HAM

The results related to the 3D epithelial tissue are described in Fig. (**6**). Noteworthy, the research samples cells

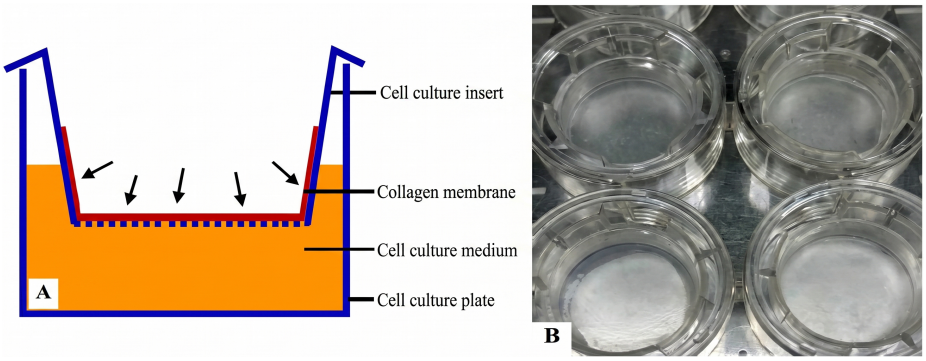


Fig. (2). Cell sheet culture model on collagen scaffold. (**A**). The cell sheet culture model was designed by our research group. Accordingly, the collagen membrane (black arrow) is spread on the surface of cell culture inserts to create a scaffold for growing mesenchymal stem cells. These cells will then be induced to differentiate into epithelial-like cells and use the air-liquid culture technique to stimulate cell sheet formation. (**B**). The actual model we used for cell sheet culture. *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

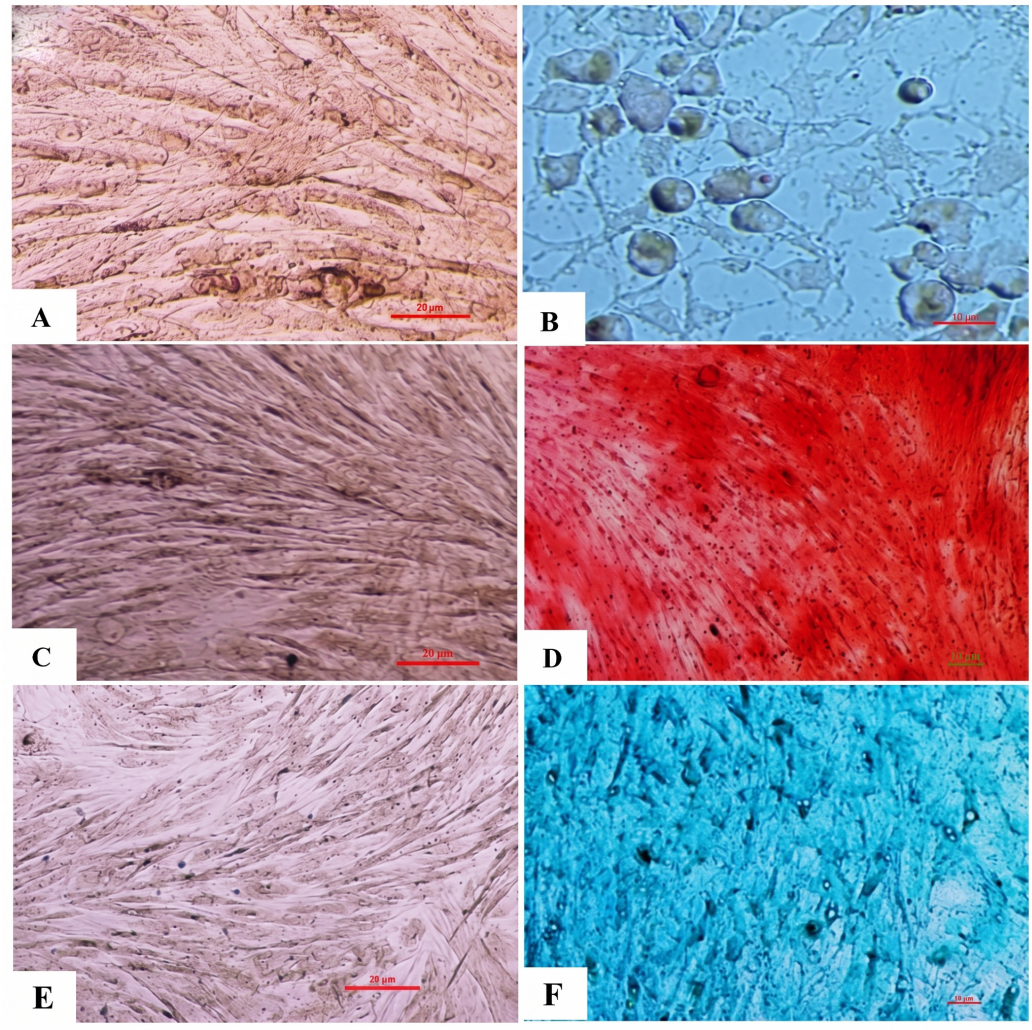


Fig. (3). Results of culturing and identifying mesenchymal stem cells using *in vitro* cell differentiation induction method. (**A, C, E**). Candidate stem cells, 40X. (**B**). Differentiation of candidate stem cells into adipocytes, Oil Red O staining, and 40X. (**D**). Differentiation of candidate stem cells into osteoblasts, Alizarin Red staining, and 40X. (**F**). Differentiation of candidate stem cells into chondroblasts, Alcian Blue staining, and 40X. *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

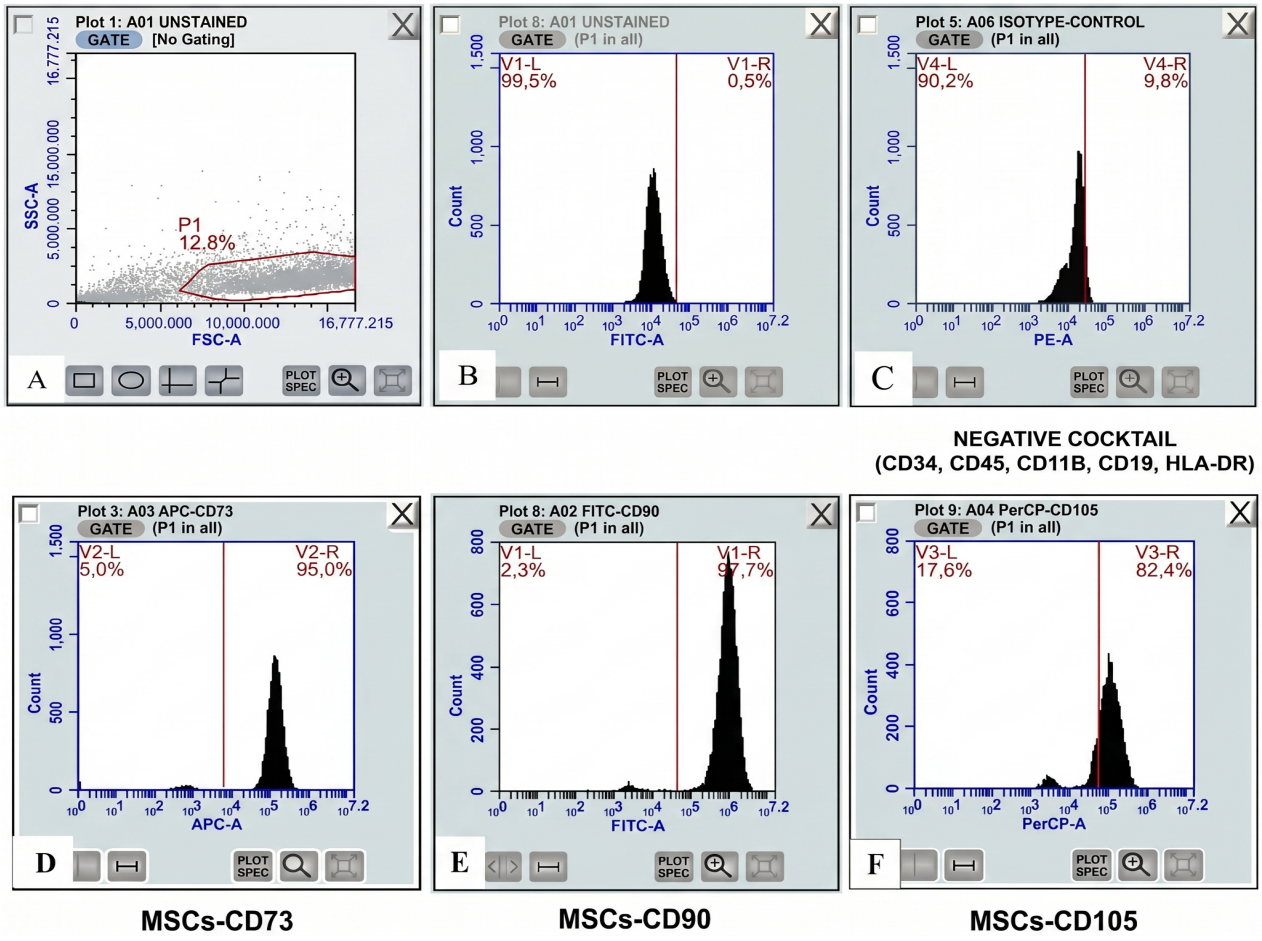


Fig. (4). Results of evaluating the expression of some specific markers used to identify MSCs according to the minimum criteria of ISCT. According to ISCT standards, MSCs must express positive markers, such as CD73, CD 90, and CD105, and not express markers, such as CD11, CD19, CD34, CD45, and HLA-DR. (**A**, **B**). Corrected results on the Flow Cytometry machine. (**C**). The MSCs candidate cell population does not express negative markers (CD11, CD19, CD34, CD45 and HLA-DR). (**D**, **E**, **F**). Meanwhile, these candidate stem cells are positive for 3 markers specific to mesenchymal stem cells: CD73, CD90, and CD105. Thus, the candidate stem cell population satisfies the criteria for expressing specific cell surface markers to identify MSCs according to ISCT standards. *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

showed a more active behavior compared to those in the control group and gradually changed their morphology from the fibroblast elongated form, a typical feature of mesenchymal stem cells, to the squamous/rhomboid shape typical of epithelial cells (p-value < 0.05) (Figs. **6D-6F**). Over time, the Air-Liquid interface cell culture allowed the cells to start forming multilayers on the collagen membrane (Fig. **6F**). After two weeks, the cells grew and created multiple cell layers on the collagen membrane (Fig. **6F**), building up a well-preserved 3D tissue highly similar to human skin epithelium. On the contrary, MSCs in the control group adhered and formed a monolayer with unchanged morphology, which was tightly attached to the collagen membrane (Fig. **6A**). The control group cells were not capable of creating cell layers, and their morphology remained almost unvaried even within the Air-Liquid interface cell culture.

Cells in the 3D epithelial tissue exhibited cell junctions that are usually formed either between cells (desmosomes) or between cells and the extracellular matrix (hemidesmosomes), as shown in Fig. (**7**). The results from TEM observation allowed the clear identification of cell junctions in epithelial sheets within the multiple layers of the 3D tissue.

4. DISCUSSION

The combination of MSCs and HAMs exhibits unique properties and functionality that make these bioscaffoldsexcellentcandidates for clinical applications. MSCs and HAMs have been used in regenerative practice for decades. They have been applied clinically in various areas to treat skininjuries and damages, including burn wound coverage, orthopedics, and corneal transplantation in ophthalmology. The results showed that MSCs and HAMs are safe and effective, and recently, both have been used as 3D biomaterials in tissue engineering to create grafts for tissue regeneration [32-37].

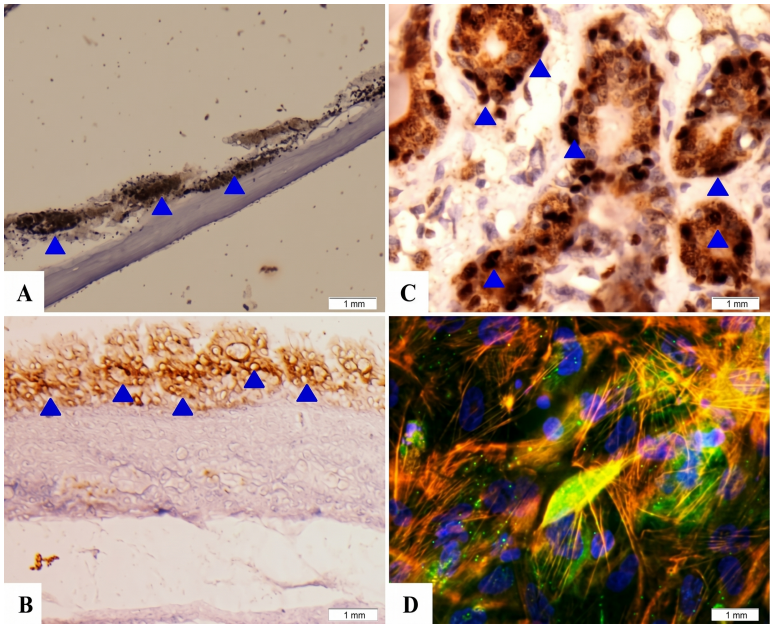


Fig. (5). Results of differentiation of mesenchymal stem cells into epithelial-like cells. (**A**). Results of immunohistochemical staining of p63 (p63, is a well-known marker of ESCs, is an indispensable factor for their biological activities during epithelial development) for mesenchymal stem cells cultured on collagen membrane that differentiated into epithelial cells (blue triangle), (negative control sample), 40X. (**B**). After differentiation, the immunohistochemical staining of the p63 marker for cells in the cell sheet showed a strong expression for the p63 marker with a characteristic red-orange morphology in the cell cytoplasm (blue triangles), 40X. (**C**). Human mammary tissue samples were immunohistochemically stained with marker p63 (positive control sample). The glandular epithelial cells showed a characteristic red-orange color in the cytoplasm (blue triangles), a typical marker of epithelial cells, 40X. (**D**). Cells positive for marker ck 1/10, a green-stained protein in the cytoplasm, a typical marker of skin epithelial basal layer cells. *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

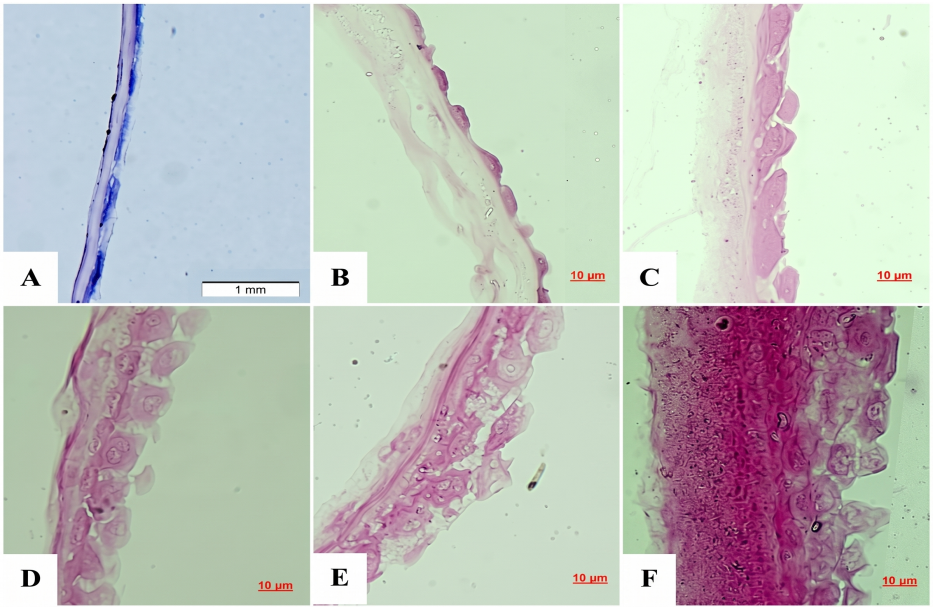


Fig. (6). Image illustrating the process of cell sheet creation. (**A**). Mesenchymal stem cells were cultured on collagen membrane after 3 days of culture and stained with H&E, 20X. (**B**) and (**C**). Cells had differentiated into epithelial cells on collagen membrane stained with H&E after 1 and 2 weeks, 20X and 40X objectives, respectively. (**D**, **E**) and (**F**). Results of cell layer formation by air-liquid interface culture technique over time. Cells divide to create many cell layers of 2 - 4 rows, and cell morphology also changes from the oval, elongated morphology of mesenchymal cells to the multifaceted morphology of epithelial cells.*(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

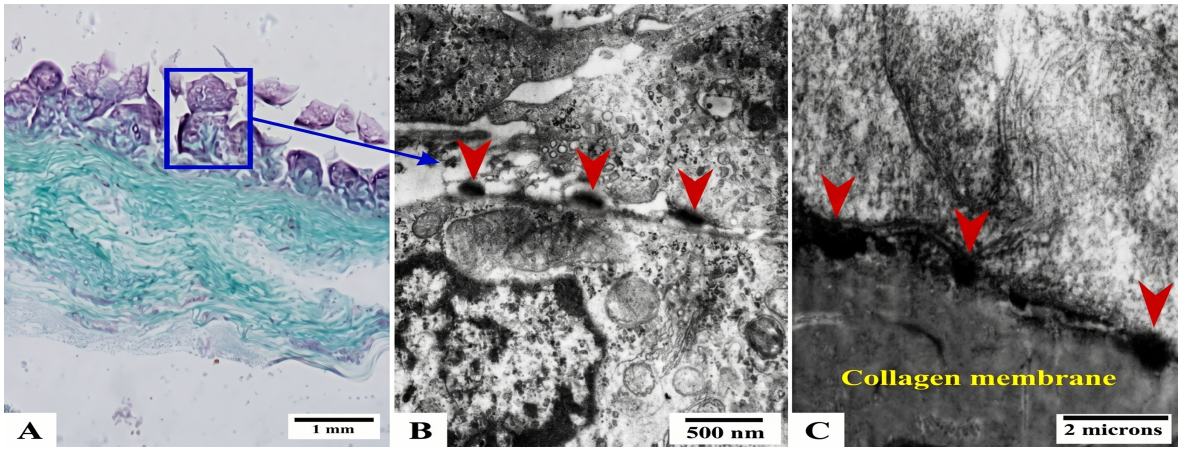


Fig. (7). Results of observing cell junctions in the epithelial sheet by TEM. (**A**). Epithelial sheets (histological staining). (**B**). Cells are linked together by desmosomes (red arrows). (**C**). Cells adhere to the collagen membrane by hemidesmosome binding (red arrow). *(A higher resolution / colour version of this figure is available in the electronic copy of the article)*

Furthermore, this sheet of cells has been shown to have many benefits, including reducing protein, electrolyte, and fluid loss while reducing the risk of infections, relieving pain, and accelerating the wound-healing process [11, 28, 31]. It is worth noting that human umblical cord blood and AM are easily accessible and obtained through non-invasive and painless procedures, which do not harm the mother or newborns, with minimal concerns for medical ethics. Furthermore, hUCB-MSCs are a source of highly versatile stem cells, which can modulate immunity, making them less likely to cause transplant rejection in the recipient [5, 9]. Our team has established a process to collect, isolate, and identify hUCB-MSCs according to ISCT standards [17].

4.1. Multiple Effects on Angiogenesis and Antibacterial Activity of hUCB-MSCs and HAM and Future Prospects

Furthermore, the HAM and MSC combination has been shown to express collagen XVIII, which exhibits anti-angiogenic properties and is an essential anti-tumor factor [38-40]. However, HAM alone revealed pro-angiogenic activity, which could be attributed to crucial promoters of angiogenesis, such as angiogenin, PDGF, and VEGF, specifically involved in tumor genesis [5, 39, 40]. A recent study confirmed that HAM could be considered an excellent matrix for generating mature vascular-like tissue owing to its ability to enhance the expression of integrin, Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1), and adhesion molecules, such as VE-cadherin in the endothelial cell culture plate [39, 40]. Therefore, as highlighted above, the combination is highly effective in repairing and regenerating damaged tissues while maintaining a potent immunomodulatory and antitumor effect. The protein content of integrin and fibronectin can interact with the growth factors PDGF, EGF, and b-FGF to activate the ERK pathway, which is linked to cell proliferation and survival [39, 40].

Therefore, we preferred to use acellular HAM as a scaffold, both to minimize the risk of immune rejection and to preserve the integrity of the biological structure, the composition of the Extracellular Matrix (ECM) of the amniotic basement membrane, and the dense layer (three-dimensional layer, 3D structure layer) [22-26, 41-46]. It should be emphasized that acellular HAM retains key properties, providing anti-scarring effects, which are highly desirable characteristics for skin substitutes [27]. Furthermore, acellular HAM can be preserved, avoiding the need for temperatures of -80C and sterile vials, thus reducing medical costs [25]. It has been confirmed that the ECM maintains a stable presence of collagen types III, IV, and V, proteoglycans, and glycoproteins, which constitute the most significant regenerative components [41].

Although it is not the main topic covered in this article, the intrinsic antibacterial and antiviral capacity of stem cells and HAMs is fundamental for their use in clinics. The aspect of possible post-traumatic bacterial infections leads us to address issues that involve emergency medicine and medical microbiology [42-46]. Due to the emergence and spread of antibiotic-resistant bacteria, the treatment of some types of infections, especially nosocomial ones, is becoming increasingly difficult [42-46]. The antibacterial properties of HAM and hUCB-MSC have been demonstrated against both gram--positive and gram-negative bacteria. For example, several results have shown that the risk of infections caused by multidrug-resistant bacterial pathogens tends to increase with the length of hospital stay of burn patients. During the first days of hospitalization after burns, the more sensitive Gram-positive bacteria predominate, while the more resistant Gram-negative bacteria appear with a longer hospitalization period [42-46].

Data showed that 7 of the 10 most frequent complications occurring in burn patients were pneumonia, Urinary Tract Infection (UTI), and cellulitis [47]. Respiratory tract infections remain the most common, with factors such as inhalation damage and the need for prolonged mechanical ventilation having the greatest impact.

Among the most common bacteria found were *Escherichia coli, Bacillus cereus, Klebsiella pneumonia,*

Streptococcus pyogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Shigella flexneri, and probiotic *Lactobacillus plantarum* [47, 48]. Several studies confirmed the validity of the antimicrobial ability of HAM by exposing it against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and two clinically isolated sensitive strains of *Escherichia coli* [49-52]. The final results demonstrated that pre-exposure of AM to IL-1β increased the secretion of antibacterial peptides, including elafin, HBD-2, HBD-3, and cathelicidin LL-37, which are well-known Antimicrobial Peptides (AMPs) [53-55]. Interestingly, cathelicidin LL-37 and human Beta-Defensin (hBD)-3, due to their antimicrobial and immunomodulatory properties, have confirmed their role in West Nile virus (WNV) infection of human primary keratinocytes. The results showed that LL-37 was able to exert a virus-inhibitory effect in the supernatant of infected keratinocytes, suggesting a direct antiviral effect of AM [52-55]. A different clinical study conducted on 33 patients with burns compared the effect of autologous skin grafts and AM. HAM gave more effective results, especially in relieving pain, accelerating healing and epithelialization, and protecting wounds from infections [54]. Finally, the antimicrobial activity of HAM may reside in the sealing function. After implantation, AM tends to interact completely with the surrounding tissues and underlying layers, forming an adherent covering shield on the wounds, not only preventing any contamination but also enabling site integrity and tissue regeneration with the involvement of local progenitor cells and the activation of new stem cells and AM extracellular matrix peptides [55].

The *in vitro* results also showed very interesting results. Experiments conducted with the use of solid agar demonstrated that HAM enhanced the solid antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) either in fresh (f-HAM) or cryopreserved (c-HAM) forms. The strain of MRSA, which is resistant to trimethoprim/sulfamethoxazole, clindamycin, erythromycin, and penicillin, but sensitive to linezolid (according to the EUCAST and CLSI disc diffusion tests), showed a pronounced zone of inhibition following the application of HAM in homogenate form [56].

Both revealed promising results: 5 and 10 µL of f-HAM homogenates resulted in zones of inhibition with an average diameter of 15.9 ± 0.5 mm and 18.53 ± 0.6 mm, respectively, while 5 and 10 µL of c-HAM homogenates produced inhibitory areas of an average diameter of 16.8 ± 0.6 mm and 19.6 ± 0.6 mm, respectively [35, 56].

CONCLUSION

Our research group created a 3D epithelial tissue from a combination of HAM and hUCB-MSCs. This new biomaterial has a morphology and cellular structure similar to the human skin epithelium. The cells of this human epithelial-like cell sheet express characteristics very similar to the epithelium of skin cells, such as the markers p63 and ck 1/10 (Fig. **5**). The combined use of HAM and hUCB-MSCs presents numerous advantages from both an ethical and purely clinical point of view. Moreover, its multiple properties make it a unique solution to promote the regeneration of damaged tissues and reduce the possibility of concomitant infections, which are very common in patients with severe burns admitted to the hospital.

Furthermore, being a biomaterial, it is easy to prepare and is economical. We want to anticipate that this 3D epithelial tissue could also be used in emergency medicine, for example, when a patient is loaded into an ambulance. This would likely save time and increase the chances of survival in many severely burned patients due to the antimicrobial effects, regenerative induction, and immune-modulating properties. On the other hand, using this new biomaterial presents challenges and limitations, such as the limited shelf life of the preparations. However, having demonstrated outstanding potential for use as antibacterial agents, further research is needed to clarify which molecules or peptides specifically cause the potent antimicrobial effect. We hope this project will promote a faster and safer translation of this new biomaterial into clinical practice.

AUTHORS' CONTRIBUTIONS

The authors confirm their contribution to the paper as follows: Conceptualization: B.T.N., T.D.H., H.T.T.N., T.T.T.T., and C.G.I.; methodology, B.T.N., T.D.H., F.I., L.S., K.C.D.N., T.D.H., C.G.I., B.T.N., and T.D.H.; formal analysis, K.T.N., B.T.N., T.D.H.; B.T.N.; and T.D.H.; investigation, T.D.H., H.T.T.N., T.T.T.T., H.K.N., and C.G.I.; resources, H.K.N., H.K.C.H., N.H.N., Q.T.M.N., F.I., and D.T.H.; writing—preparing the original draft, T.D.H. and C.G.I.; writing: review and editing, D.T.H. and C.G.I.; administration of the project B.T.N. and T.D.H.; financing acquisition, B.T.N. and L.S.; and Data Curation: H.N.N. All authors have read and accepted the published version of the manuscript.

LIST OF ABBREVIATIONS

B-FGF	=	Basic Fibroblast Growth Factor
EGF	=	Epidermal Growth Factor
KGF	=	Keratocyte Growth Factor
HGF	=	Hepatocyte Growth Factor
TEM	=	Transmission Electron Microscope
Hucb-Mscs	=	Human Umbilical Cord Blood Mesenchymal Stem Cells
ISCT	=	International Society For Cells And Gene Therapy
HAM	=	Human Amniotic Membrane

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Human study protocols approved by the Research Ethics Committee in Biomedical Research of Thu Dau Mot University with number 22.06-001.

HUMAN AND ANIMAL RIGHTS

All procedures performed in studies involving human participants were in accordance with the ethical standards of institutional and/or research committee and with the 1975 Declaration of Helsinki, as revised in 2013. The entire study was conducted according to Vietnamese Ministry of Health guidelines. The study was prepared in cooperation with SET118 of Taranto City and the University of Bari Aldo Moro.

CONSENT FOR PUBLICATION

Informed consent was obtained from all participants.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study and article are available from the corresponding author [C.G.I.], on request.

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CONFLICT OF INTEREST

Dr. Luigi Santacroce is the Senior Editor of the journal Endocrine, Metabolic & Immune Disorders - Drug Targets.

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