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RESEARCH PAPER

Evaluation of Radiosterilized Glycerolated Amniotic Membranes as a Substrate for Cultured Human Epithelial Cells

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ABSTRACT

Human amniotic membrane (HAM) is a biomaterial with biological properties beneficial to tissue repair, serving as a substrate for cell cultivation. Irradiation is used for tissue sterilization, but can damage the HAM structure. The objective of this paper was to construct a skin substitute, composed of human keratinocytes cultured on glycerolated HAMs, and to evaluate the influence radiation on subsequent cell culture growth. Four batches of HAMs were glycerolated, and half of them were radio-sterilzed with 25 kGy. Non-irradiated glycerolated HAM (ni-HAM) and irradiated glycerolated HAM (i-HAM) samples were then de-epithelized and analyzed using optical microscopy (Picrossirius staining), immunofluorescence and electron microscopy. Subsequently, keratinocytes were cultured on ni- and i-HAMs, and either immersed or positioned at the air-liquid interface. The basement membranes of the ni-HAM group remained intact following de-epithelialization, whereas the i-HAM group displayed no evidence or remnant presence of these membranes. Concerning the keratinocyte cultures, the ni-HAM substrate promoted the growth of multi-layered and differentiated epithelia. Keratinocytes cultured on i-HAM formed epithelium composed of three layers of stratification and discrete cell differentiation. The glycerolated HAM was compatible with cultured epithelia, demonstrating its potential as a skin substitute. Irradiation at 25 kGy caused structural damage to the amnion.

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Amnion; radiation; ionizing; glycerol; keratinocytes; skin; artificial; basement membrane

Introduction

In regenerative medicine, some biomaterials can repair or replace damaged tissues, stimulating the endogenous mechanisms of tissue healing.[1] In this context, the human amniotic membrane (HAM) is a biomaterial with great potential for repair, serving as a regeneration matrix or substrate for the transplantation of cultured autogenous and allogeneic cells.[2]

Histologically, amniotic membranes are very similar to cutaneous tissue, which also originate from the embryonic ectoderm. When applied to wounds, a barrier against bacterial invasion is formed, the loss of body fluids and proteins is reduced, pain is decreased and growth factors and modulators of wound healing are delivered to the wound bed. In other words, they reestablish ideal conditions so that the endogenous healing processes progress satisfactorily.[3–6]

With the evolution of cell culture techniques, the use of HAM as a substrate for the culture of limbic cells has been reported.[7] Additionally, its utility as a substrate for the human keratinocyte cultures has also been tested for use in cutaneous substitutes.[8–12] However, it should be pointed out that previous studies have employed several techniques for HAM preparation, mainly cryopreservation. In fact, it has been shown that the specific HAM preparation method can affect epithelial limbic cell expansion.[13]

The process of glycerolization is performed by serially bathing the tissue in high (>85%) concentrations of glycerol, and is a proven technique for tissue preservation.[14,15] Compared to other methods, it presents low cost, technical feasibility and, although highly toxic to cells, preserves the structure of the tissue protein matrix.[16] Due to its cytotoxicity, this technique can effectively inactive viruses and bacteria, and is considered to be a decontamination method. However, it cannot be considered a sterilization method, since bacterial and viral strains have been isolated from tissues stored in glycerol for extended periods of time.[17,18]

In order to reduce the risk of transmitting infectious diseases, complementary radiosterilization can

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be used to guarantee the sterility of the biomaterial. The ionizing radiation presents high penetrability, with great efficiency in destroying microorganisms, generating almost no heat and affords the ability to sterilize products in the final packaging.[19] However, exposure to radiation is not completely inert to biological tissues. In fact, some types of tissues undergo substantial structural changes when exposed to high doses of radiation.[20–22]

Despite these histological changes, the clinical use of irradiated membranes for the treatment of burns and wounds shows results similar to those obtained with non-irradiated forms, with both promoting the re-epithelialization of the lesions.[23,24] Concerning the ability of HAM to serve as a substrate for cell culture following irradiation little is known, since most of these types of studies were performed with non-irradiated cryopreserved HAM.

This study sought to create an *in vitro* skin substitute formed by culturing human keratinocytes on de-epithelized glycerolated HAMs, and to evaluate the influence of radiosterilization on the ability of this biomaterial to support epithelial cell culture growth.

Methods

This study was approved by the Ethics Committee for Research Projects Analysis – CAPPesq of the Clinical Directorate of Hospital das Clínicas at the University of São Paulo Faculty of Medicine, under no. 0309/08. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Human amniotic membrane preparation

Four HAMs were collected in a sterile form, using digitiform chorion separation. The amnion was washed and cleaned with physiological saline solution. After the remaining residues were removed, the membranes were exposed to a glycerol solution of >85% concentration and stirred at 90 rpm for three hours at 37°C. After this interval, the >85% glycerol solution was exchanged for a fresh one and maintained for 24 hours at 4°C. The amnion was then affixed to filter paper, with the brighter epithelial portion facing upwards, cut into rectangles measuring 8 × 7 cm, packed and stored at 4°C. Each of these packages was considered a batch. Half of the batches of each HAM preparation were sent out for radiosterilization. The samples were irradiated in Cobalt 60 sources at a dose of 25 kGy at 4°C and then the material was transported back to the laboratory.

Human amniotic membrane deepithelization

To determine which de-epithelization technique was best suited for HAM preparation, ni-HAMs were rehydrated in physiological saline solution for 30 minutes, completely removing any residual glycerol. Each of these 8 × 7 cm batches was cut into three fragments. Thereafter, each was exposed to a different enzymatic method of epithelial removal: 0.04% dispase (Protease Neutral – grade II, Boehringer-Manheim, Germany) for 45 minutes at 37°C; 0.02% EDTA for 2 hours at 37°C, followed by mechanical friction for the removal of epithelial cells; or 0.05% trypsin/0.02% EDTA (GIBCO®) for 20minutes at 37°C. They were stained with HE and the efficiency and utility of each de-epithelization technique were evaluated. Each slide was analyzed using an Olympus BX2 optical microscope, and based on this analysis, the best method of de-epithelialization was determined using the following criteria: complete removal of the epithelium and maintenance of the remainder of the histological structures of the amnion.

Comparative analysis of de-epithelialized nonirradiated and irradiated HAM samples

Four batches of ni-HAM and i-HAM were de-epithelialized and evaluated by: optical microscopy (Picrosirius staining), immunofluorescence, confocal microscopy and transmission electron microscopy.

Picrosirius staining

De-epithelized ni- and i-HAM samples were preserved in 10% formaldehyde, embedded in paraffin, sliced into 4-μm sections, stained with Picrossirius and observed using an Olympus BX2 polarized light microscope.

Immunofluorescence using confocal microscopy

De-epithelialized ni- and i-HAMs were embedded in paraffin, cut and prepared on aminosilane microscopy slides. Subsequently, they were dewaxed in xylol and washed in decreasing concentrations of ethanol. Immunogenic sites were exposed by digesting the samples with bovine pepsin (10,000/units of dry tissue-UTD) (Sigma Chemical Co.) in acid buffer (pH = 2.2) (2 mg/mL) for 30 min. Then, the samples were washed three times with phosphate buffered saline (PBS) and incubated with 5% Molico® milk in phosphate buffer pH = 7.0. The slides were incubated overnight at 4°C with monoclonal anti-human type IV antibody (Sigma Diagnostics, St Louis, USA), obtained in mouse, and polyclonal human antilaminin (Dako A/S Copenhagen, Denmark), obtained in goat, diluted in PBS at concentrations of 1:100.

The slides were then washed with PBS in 0.05% Tween three times and incubated with anti-mouse or goat anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma Chemical Co.) at concentrations of 1:50, washed five times in PBS with 0.05% Tween and the coverslips were then placed in buffered glycerin solution. The reaction was evaluated using an Olympus BX2 fluorescence microscope, with a magnification of 400 × .

For three-dimensional analysis by confocal microscopy, the samples were incubated with anti-mouse or goat anti-mouse IgG secondary antibody conjugated with Alexa 633 or 488 (Invitrogen), diluted 1:500 in PBS solution for 60 minutes and the coverslips with buffered glycerin solution. The reaction was observed using an LSCM laser microscope (LSM 410; Carl Zeiss; Jena, Germany) using a 400× magnification.

Electron microscopy

Samples of de-epithelized ni- and i-HAMs were exposed to 2% glutaraldehyde for two hours and transferred to 1% osmium solution. After two washes in 0.9% saline and 8% sucrose, they were submerged for 18 hours in 0.5% uranyl acetate. The material was dehydrated by means of baths in 70% ethanol (2 × 10 minutes), 95% ethanol (2 × 15 minutes), absolute ethanol (4 × 15 minutes) and acetone (2 × 15 minutes). Slices with 75 nanometer thickness, in resin blocks, were transferred to 200 mesh copper plates, covered with 0.25% FormVar film (Ladd Research Industries, USA). The material was then visualized using a Philips Tecnai 10 transmission electronic microscope.

Primary keratinocyte cultures

Keratinocytes were isolated from cutaneous fragments of reductive mammoplasties. Skin fragments smaller than 1 mm^2 were immersed in a 0.05% trypsin/0.02% EDTA (GIBCO®) enzyme solution with agitation at 37°C. Every 30 minutes, the supernatant (enzyme solution and detached cells) was removed and transferred to a conical tube. The action of the trypsin enzyme was blocked by the addition of calcium ions in DMEM medium enriched with 10% fetal bovine serum (GIBCO®) in a ratio of 1:1. The cell suspension was centrifuged at 1500 rpm for five minutes (centrifuge Marathon 8k – Fisher Scientific). The supernatant was then discarded and the pellet resuspended. Typically, for the primary cultures, cells from the 2^{nd} to the 5^{th} half hour of the enzymatic separation process were utilized. We opted for the high density cultivation system.

The medium used for culturing the keratinocytes was based on the one proposed by Rheinwald and Green.[25] The culture bottles were placed in an incubator with 5% CO_2 atmosphere and a constant temperature of 37°C (Fisher Scientific). The culture medium was changed every 48 hours until the semi-confluence of the cells was reached.

Construction of the skin subsitute

Four glycerolated HAM samples (1 × 2 cm) from each group (ni- and i-HAM) were rehydrated in 0.9% physiological saline solution for 30 minutes and submitted to the previously selected and described de-epithelization protocol. Subsequently, each was placed in six-well multiwell plates, totaling four samples. The experiments were performed in duplicate. Then 1 cm diameter stainless steel rings were placed in the center of each sample and incubated in an oven, with 5% CO_2 atmosphere at 37°C, for 15 minutes. After this period, 1.5 × 105 cells were suspended in culture medium, as described by Rheinwald and Green[25] and seeded inside each ring. After 48 hours, the ring was removed. Around the 7th day, two of the four skin substitutes (from

each group) were suspended in stainless steel grids so that only the amniotic membrane base was in contact with the culture medium and the keratinocytes were situated at the air-liquid interface. Cultures were maintained in an incubator with 5% CO_2 atmosphere, at a constant temperature of 37°C, and the culture medium was changed every two days until the 14th day of the study and daily until the 21st day, when the experimental protocol was stopped. After 14 and 21 days of culture, one skin substitute from the immersed group and another from the air-liquid interface group were removed and sent out for histological analysis. As control, we used an *in vitro* skin equivalent,[26] previously published by our group, based on keratinocytes cultured on rat tail collagen I lattices povoated by human fibroblasts.

Histological analysis using HE staining and optical microscopy

Epidermal compounds cultured on ni-HAM, i-HAM and control (collagen lattices) for 14 and 21 days, in an immersed or air-liquid interface situation were sent out for inclusion in paraffin, sectioned, stained with HE and analyzed by light microscopy.

Quantitative analysis

For a quantitative analyses, we choose to count the number of keratinocytes in each epithelium cultivated in MA-ni, MA-I and control, at 14 and 21 days, in immerse and interface air-liquid situation. The number of keratinocytes was established by counting cell nucleus in ten adjoining fields under 200x magnification of each specimen. Mean values of the difference between number of cells at 14 and 21 days were calculated and one-way ANOVA and Tukey test applied to evaluated significant statistical differences in cell proliferation between groups.

Electron microscopy

Epidermal compounds cultured on ni- and i-HAM substrates for 21 days, at the air-liquid interface were processed according to the protocol of preparation of material for electron microscopy described above and analyzed using a Philips Tecnai 10 transmission electron microscope.

Results

HAM de-epithelization

The HAM were successfully glycerolated, and were stored at 4oC. Complete removal of the epithelium was best achieved by immersing the HAMs in 0.05% trypsin/0.02% EDTA for 20 minutes. In fact, this methodology inflicted the least amount of damage to the amniotic architecture, when compared to the other techniques tested. Thus, this technique was chosen as the standard method for HAM de-epithelization.

Comparative analysis of de-epithelized ni- and i-HAM

Picrosirius staining

Following de-epithelization, under polarized light, picrossirius staining of ni-HAM samples appeared orange-reddish birefringence in most of the tissue structure, while on its surface, this birefringence showed to be more yellowish-green, indicating the existence of fibers of different sizes (Figure 1a). On the other hand, images of samples from the i-HAM group only showed the existence of thick orange-reddish fibers (Figure 1b), and on the surface of the tissue structure, no birefringence is observed.

Immunofluorescence and confocal microscopy

To evaluate the basement membrane of each preparation, indirect immunofluorescence was used to detect collagen IV and laminin, in the lucid and dense layers, respectively, following de-epithelization. Images of samples from the ni-HAM group, showed that there is the clear green phosphorescent expression of collagen IV (Figure 1) and laminin (Figure 1e) on the entire surface of the amniotic structure. In contrast, images of samples from the i-HAM group, did not have any observable type of fluorescence on the surface of the membrane (Figure 1d,f). Thus, confirming that the radiosterilzation process removed these potential antigens.

In order to assess the three-dimensional structure of the residual basement membrane in relation to the rest of the stroma, collagen IV and laminin were also analyzed using confocal microscopy. Similar to what was observed in Figure 1, both collagen IV and laminin were detected in the ni-HAM samples, as

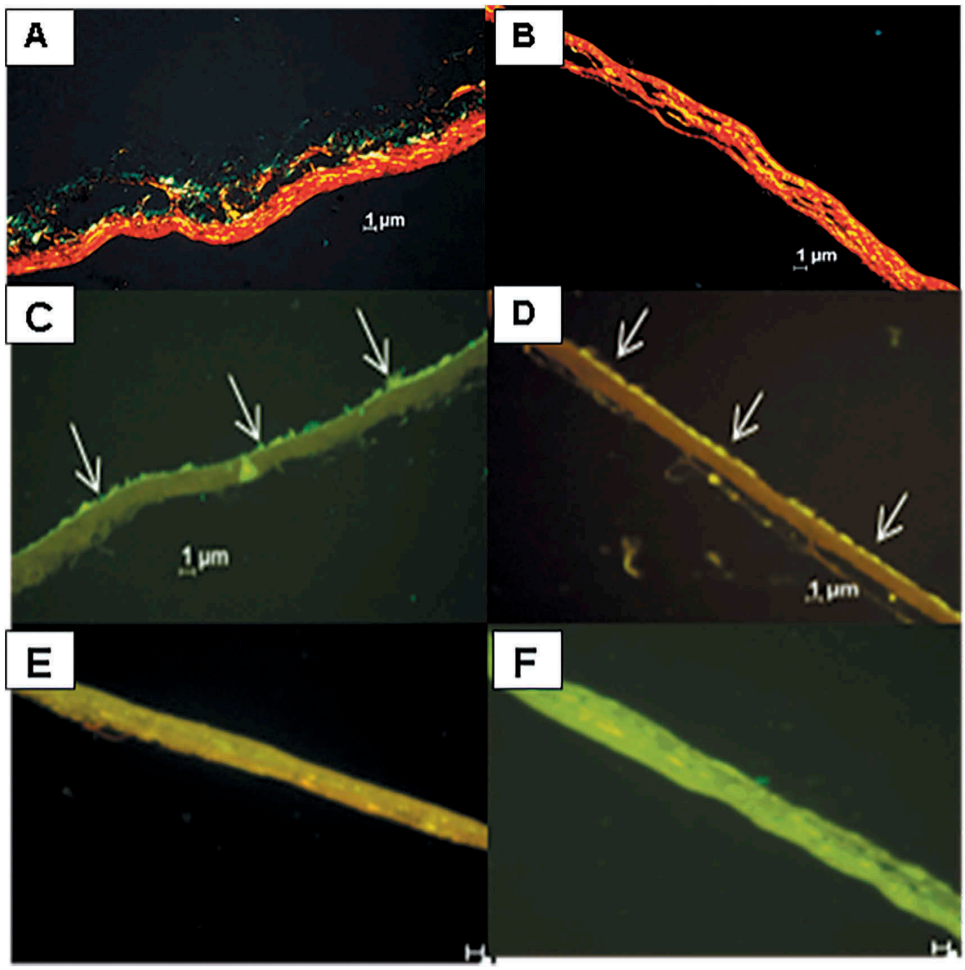

Figure 1. De-epithelized ni-HAM and i-HAM samples stained with Picrossirius and observed under polarized light: a – De-epithelized ni-HAM: on the surface there is a yellow-green birefringence indicating the existence of fine fibers. The orange-birefringent birefringence shows a firmly compacted structure. (Picrossirius: 400× magnification); **b**- De-epithelized i-HAM: absence of yellow-green coloration on the surface, reddish orange birefringence with signs of delamination of the wefts. (Picrossirius, 400× magnification). Immunofluorescence of in-HAM and i-HAM for collagen IV and laminin: in the photos, clear green phosphorescent staining indicates the expression of the studied antigen – collagen IV (c and e); laminin (d and f). Photos presented in c and d represent the de-epithelized HAM-ni group, the white arrows indicate the antigen (collagen IV and laminin, respectively) expression site, and in the e and f, the de-epithelized i-HAM group, where no reaction occurred. (400× magnification).

evidenced by the formation of a green phosphorescent line on the surface. Moreover, this signal can be viewed at different three-dimensional angles (Figure 2a–d). As expected, the green phosphorescent line, due to the presence of collagen IV and laminin was not observed in i-HAM samples (Figure 2e,f).

Transmission electronic microscopy

In de-epithelized ni-HAMs, the cleavage and release point of epithelial cells appears to have occurred at a point above the dense lamina (Figure 3a). A line of continuous electrodense material is observed on the surface of the structure and, above it, there is a small amount of electroluscent material arranged heterogeneously along this line (Figure 3b). In de-epithelialized i-HAM, these structures are not detected. In fact, the continuous line of electrondense material ceases to exist, accompanied by the exposure of collagen-like structures of the amniotic connective tissue (Figure 3c,d).

Skin substitute analysis

Histological analysis of HE stained HAMs observed with optical microscopy

On the 14th day of cell culture on immersed ni-HAMs (Figure 4a), the beginning of the epithelial stratification process was detected, as evidenced by

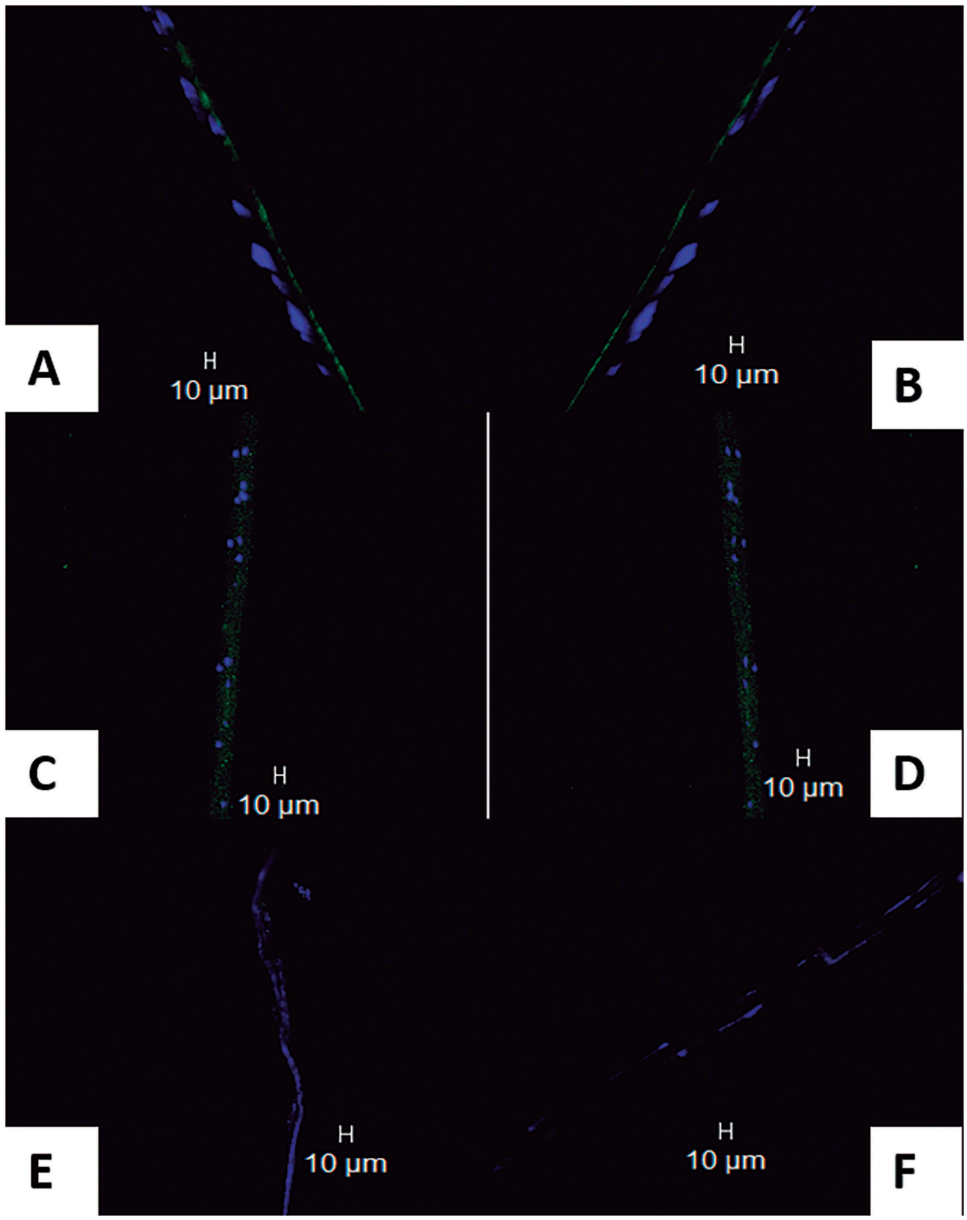

Figure 2. Evaluating collagen IV and laminin presence in ni-HAM and i-HAM samples using confocal microscopy: In panels a and b, a two-angle view demonstrates collagen IV expression on de-epithelized ni-HAMs. In c and d, laminin expression is noted on de-epithelized ni-HAMs, at different angles. In e and f, collagen IV and laminin, respectively, are not present on i-HAMs.

the formation of three to four layers of epithelial cells. The architecture was disorganized, with randomly arranged cells. On the 14th day of cell culture on ni-HAM located at the air-liquid interface (Figure 4b), a more stratified epithelium was observed, which was fairly organized when compared to the immersed condition (Figure 4a). In fact, positioning the cultures at the air-liquid interface resulted in epithelial cones that were well-formed, composed of keratinocytes juxtaposed with each other in layers, and the occurrence of clear cellular differentiation. In the layers closest to the amnion, a more cuboidal cell morphology was detected, which is typical of basal layers. It is known that during the stratification process, cells acquire a flattened conformation with a dense nucleus, and this characteristic was also identified on the surface some keratinized cells.

On the 21st day, epithelial cells cultured on immersed ni-HAM (Figure 4c), displayed a substantial increase in cellular stratification, but the epithelial architecture remained somewhat disorganized. The more apical cells of the epithelium are more elongated and keratinized, but the formation of

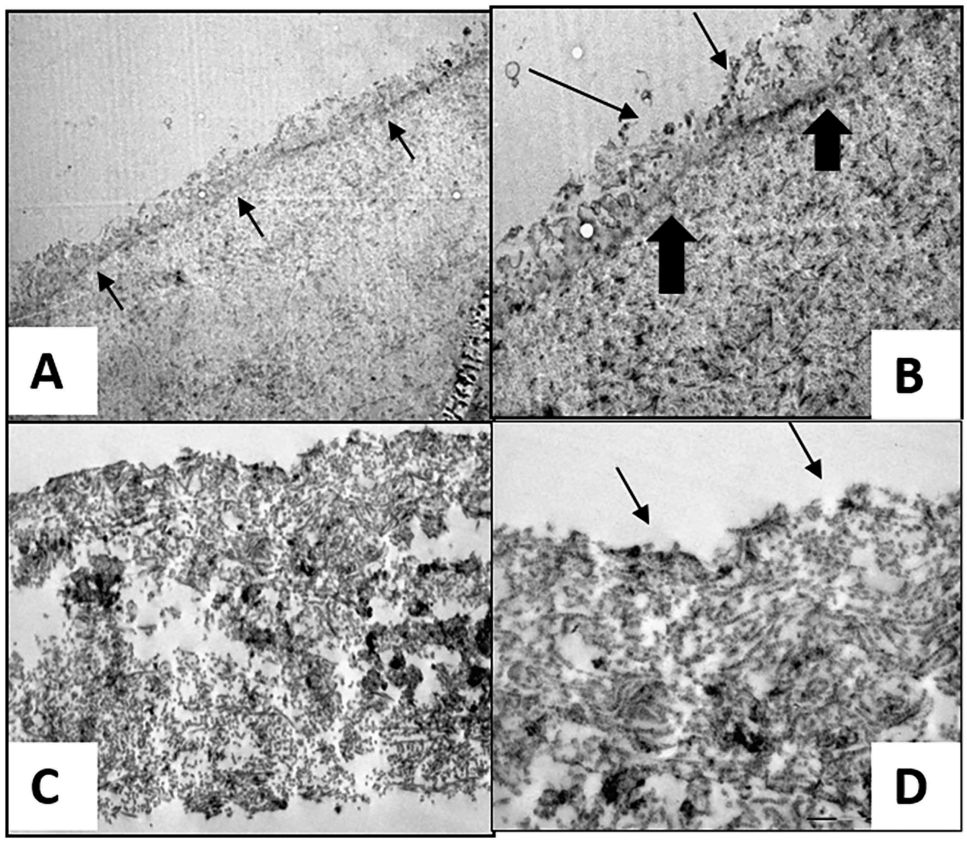

Figure 3. Evaluating de-epithelialized ni-HAM-ni and i-HAM samples using electron microscopy: **a** – De-epithelialized ni-HAM. It is observed the existence of an electrodense structure (indicated by the red arrows) continuous suggestive of presence of basement membrane. (2,550× magnification). **b –** At higher magnification, the presence of an electrodense structure (red arrows) and above the electroluscent material (black arrow) is observed in the ni-HAM group. (6,600× magnification). c – In the HAM-i group, the electrodense material on the surface of the structure is not visualized. (6,600× magnification). d – At the highest magnification, we can see the extravasation of collagen fibers from the conjunctival stroma (black arrows) (15,000× magnification).

a corneal layer, per se, is not observed. On the 21st day of cell culture on ni-HAMs positioned at the air-liquid interface (Figure 4d), more stratified and differentiated epithelia were observed, when compared to the immersed condition (Figure 4c). Additionally, there is the formation of lamellae of strongly keratinized eosinophilic material, indicating the onset of stratum corneum formation.

Comparing the obtained results with cell cultures grown on ni- and i-HAM substrates, it appears as though radiosterilization inhibited the growth of the cell cultures. On the 14th day of cell culture on immersed i-HAMs (Figure 4e), the formation of a thin epithelium is verified and almost all cells present an elongated and scattered appearance. In a few regions of the structure, there is some stratification with two cell layers. On the 14th day cells cultured on i-HAMs positioned at the air-liquid interface (Figure 4f), presented a more stratified epithelium than in the immersed situation, but had with fewer layers when compared to the ni-HAM (Figure 4b). In most of its areas, the epithelium has two to three layers of cells, along with monolayers of keratinocytes. Moreover, the cellular morphology is elongated and some keratin granules can be observed.

On the 21st day of cell culture on immersed i-HAM-i (Figure 4g), stratification of the epithelium becomes more homogeneous throughout its structure, but remains restricted to two or three cell layers. Keratinocytes have a more elongated appearance. In contrast, on the 21st day of cell culture with i-HAMs positioned at the air-liquid interface (Figure 4h), a more stratified epithelium is observed; however, the degree of stratification is much less than obtained with ni-HAMs (Figure 4d). The cell layout is shown in three or four layers at some points, with elongated nuclei containing keratin granules. The epithelial

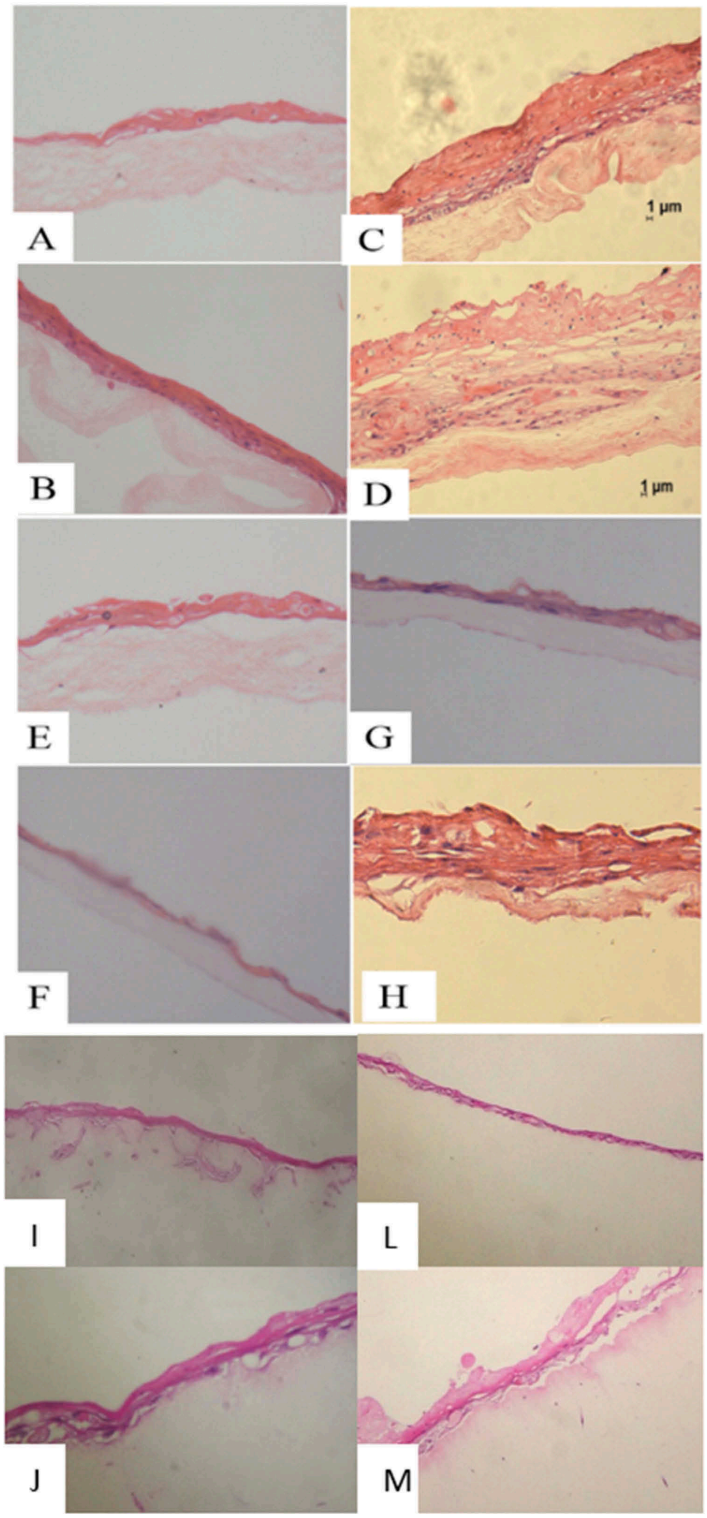

Figure 4. **a- 14th day** – **Optical Microscopy of keratinocytes cultured on immersed ni-HAMs**: Culture of keratinocytes grown on immersed ni-HAM- optical microscopy: disorganized epithelium with little stratification (3 to 4 cell layers). (HE staining, magnification 200×) **b- 14th day** – Culture of keratinocytes grown on **ni-HAM** positioned at the air-liquid interface – optical microscopy:

architecture is quite disorganized, and it is not possible to identify well-defined layers.

In comparison with control (Figure 4i,j,l,m), the epithelium cultured on ni-HAM shows better results in all periods of the study (14 and 21 days) and situations of culture (immerse/interface air/liquid), with better stratification and cellular organization.

Quantitative analysis

In Figure 5, we can observe a large number of epithelial cells on ni-HAM in comparation with control and i-HAM groups, in both situations: immerse and air-liquid interface. In one way ANOVA, we can observe a statisitical significance (p = 0,0002) difference between the groups, and Tukey test shows that only ni-HAM differs from other groups (i-HAM- p = 0,000001 and control p = 0,00004). This findings indicate that the epithelium formed in ni-HAM is different (more stratified) than other groups.

Skin substitute- electron microscopy

In the ni-HAM group, it is noticed that the plasma membranes of the epithelial cells are packed together more tightly, without any observable gaps between them. It was also possible to visualize the stratum corneum on the epithelial surface in this group (Figure 6a). In fact, at higher magnifications, the existence of numerous well-formed keratinocytes was observed (Figure 6b,c). Inside the cells, vacuoles with the formation of lamellae of keratin inside were identified (Figure 6d). In some situations, this keratin is released by the cells, remaining in the intercellular spaces or, sometimes, forming the so-called epithelial pearls (Figure 6e). In the longitudinal section, a certain thickening of the existing basement membrane can be perceived (Figure 6f).

In the i-HAM group, there is a less stratified epithelium, and reduced cohesive cell adhesion, which was evidenced by the presence of intercellular gaps (Figure 7a). Additionally, keratin production was attenuated (Figure 7b), only being detected in a few areas of the intercellular space, with no detectable corneal pearls, per se. The cells were found to have a cylindrical morphology (Figure 7c). Differently from the other group, the formation of desmosomes (Figure 7d) between the cells is practically not observed, and there are gaps between cells. Additionally, the presence of a few desmosomes among some keratinocytes can only be observedat higher magnification (Figure 7d).

Discussion

Skin substitutes are a heterogeneous group of materials that temporarily or permanently occlude wounds, and their employment depends on both the characteristics of the wound bed and the product itself. Unfortunately, to date, there is no skin substitute that is considered perfect.[27]

epithelium with a certain organization, cells of basal layer with cuboid appearance, differentiating into cylindrical cells in the upper layers of the epithelium. (HE staining, magnification 200×) **c- 21º day**- Culture of keratinocytes grown on immersed **ni-HAM** -optical microscopy: epithelium with some disorganization, but multistratified with apical keratinocytes with fusiform appearance (HE staining, magnification 200×). **d- 21º day** – Culture of keratinocytes grown on **ni-HAM** positioned at the air-liquid interface- optical microscopy: epithelium with better stratification and organization, noting the presence of epithelial pearls. Formation of stratum corneum. (HE staining, magnification 200×). **e- 14th day** – Culture of keratinocytes grown on immersed **i-HAM**- optical microscopy: monolayer epithelium, with few foci of stratification. (HE staining, magnification 200×) **f- 14th day** – Culture of keratinocytes grown on **i-HAM** positioned at the air-liquid interface- optical microscopy: two- to three-layer laminated epithelium, elongated cells and keratin granules. (HE staining, magnification 200×) **g- 21º day** – Culture of keratinocytes grown on immersed **i-HAM** – immersed situation – optical microscopy: minimal stratification (HE staining, magnification 200×) **h- 21º day** – Culture of keratinocytes grown on **i-HAM** positioned at the air-liquid interface- optical microscopy: epithelial layering in three to four layers, disorganization of the architecture with horny pearls Primitive corneal layer formation (HE staining, magnification 200×) **i-14th day- Control**- Culture of keratinocytes grown on type I collagen matrix with fibroblast- immersed situation and **j- 14th day- Control**- Culture of keratinocytes grown on type I collagen matrix with fibroblast- interface air-liquid: in both situations, stratified epithelium with two or three layers (HE staining, magnification 100x) **l-21th day- Control**- Culture of keratinocytes grown on type I collagen matrix with fibroblast- immersed situation: more organized epithelium without stratification (HE staining, magnification 100x) **m- 21th day- Control**- Culture of keratinocytes grown on type I collagen matrix with fibroblast- interface air-liquid: a more organized epithelium with initial stratification, but not so organized like culture on MA-ni (HE staining, magnification 100x). **i,j,l and m** extracted from Paggiaro A. et al. Construction of an *in vitro* equivalent skin. Rev. Bras. Cir. Plast. 2007;22(3):153–157.

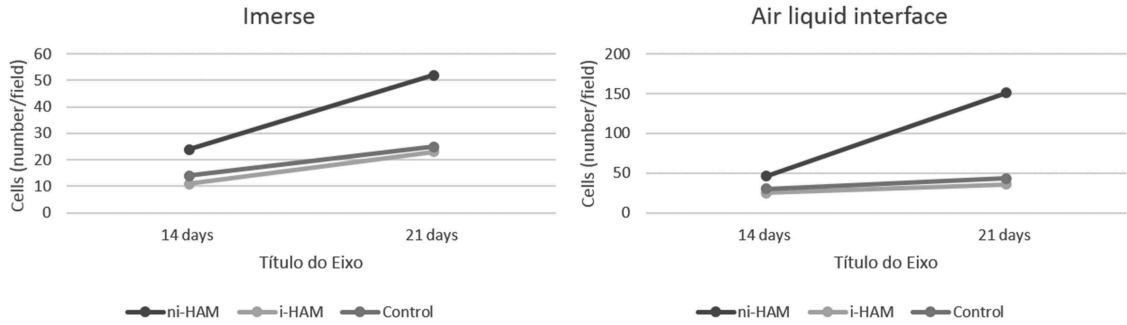

Figure 5. Cell numbers observed in cultivated epithelium on ni-HAM, i-HAM and control in immerse and air-liquid interface cultures.

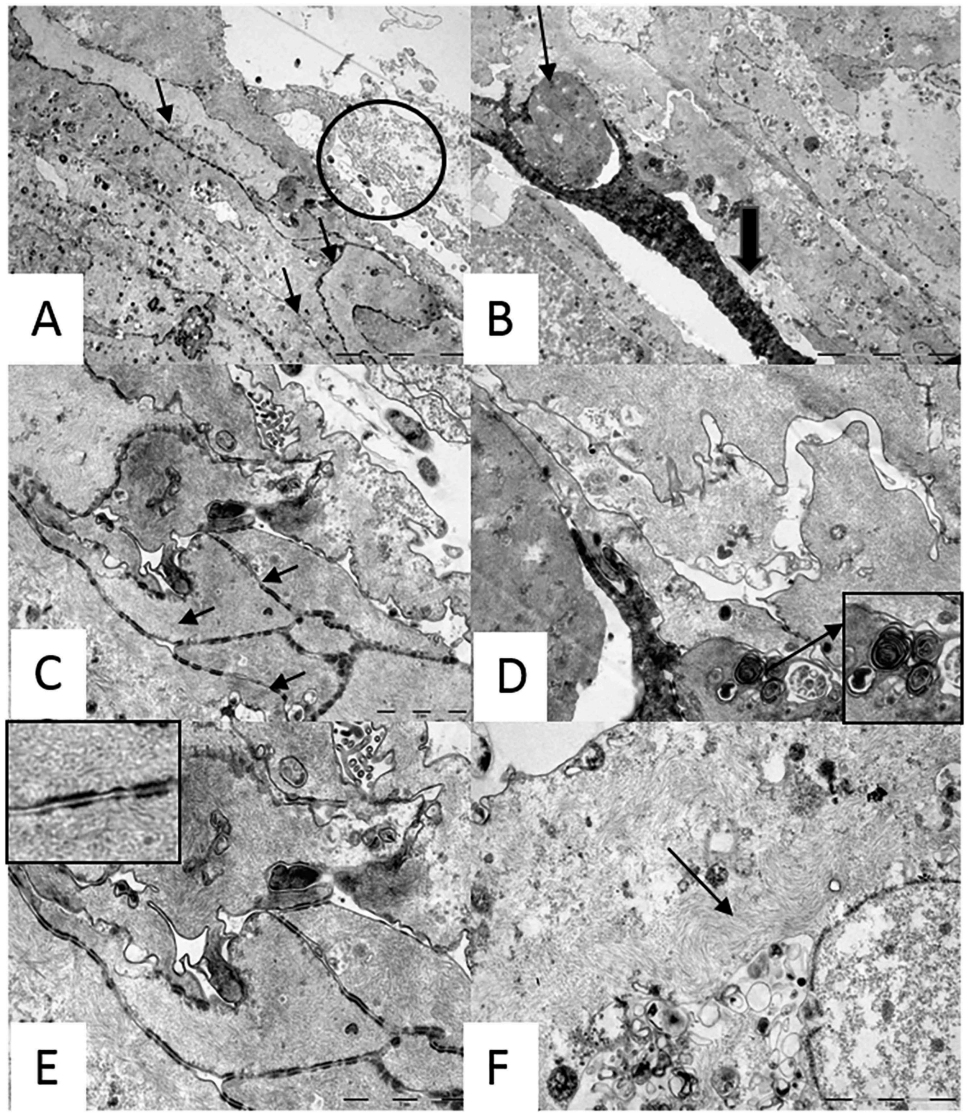

Figure 6. Electron microscopy results of keratinocytes cultured on ni-HAM at the air-liquid interface for 21 days.: a – Visualization of plasma membranes of cohesively clustered keratinocytes (arrows indicating good adhesion between cell plasma membranes), stratum corneum formation (indicated in the circle) (2,550× magnification). b – presence of keratin lamella (black arrow) in the intercellular space and formation of the epithelial pearl (red arrow) (2,550× magnification). **c** – cellular cohesion marked by the presence of desmosomes along the entire plasma membrane of the cells (red arrows), joining them together (8,900× magnification). **d** – In the cytoplasm of the cell, the formation of a vacuole with the presence of lamellar keratin inside (highlighted in greater increase in the red square in the left corner); keratin released into the intercellular space (black arrow) (8,900× magnification). **e** – Greater increase showing strong cellular adhesion by the existence of desmosomes between the cells. In the red square, better desmosome visualization (12,500× magnification) **f** – basement membrane with thickening signals (red arrow) (12,500× magnification).

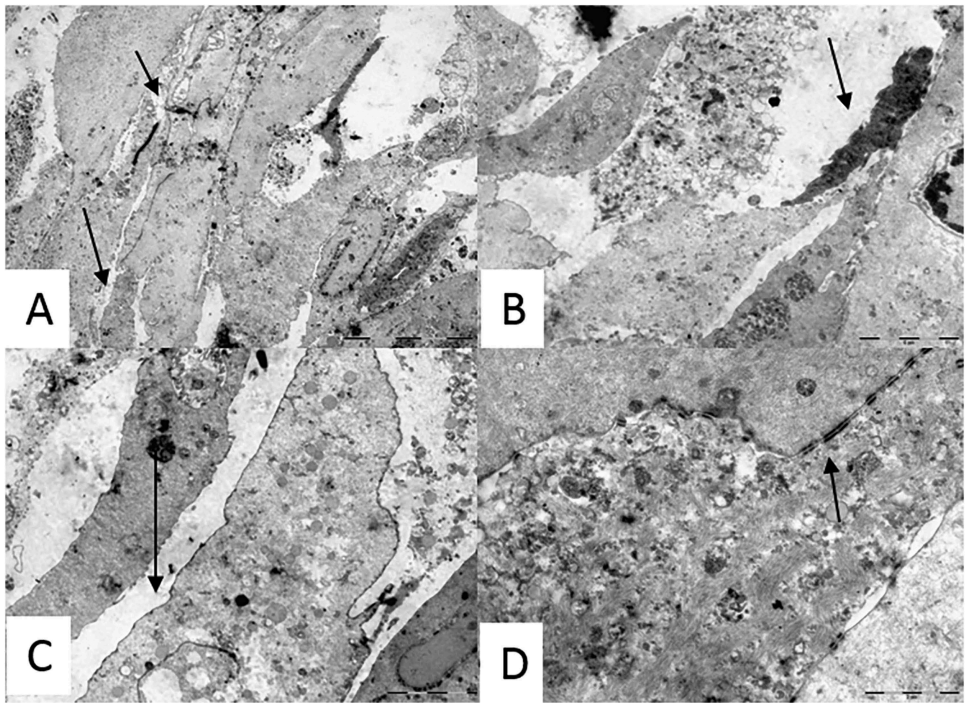

Figure 7. Electron microscopy results of keratinocytes cultured on i-HAM at air-liquid interface for 21 days. a – Keratinocytes with a cylindrical morphology and presence of intercellular gaps (red arrow). (1,250× magnification). g – Keratin in the intercellular space (red arrow) (3,700× magnification). c– Keratinocyte with a cylindrical appearance, gap between cells without the presence of desmosomes (indicated by red arrow indicating). (3,700× magnification) d – Few desmosomes between cells (indicated by arrow) (8,900× magnification).

In general, HAMs are used as a temporary biological cutaneous substitute, due to the ease of use and wide availability, they also present a good adhesive capacities, restoring epidermal and dermal components.[28,29] The results of the present study, demonstrate that de-epithelialized HAMs are a biomaterial that can potentially serve as the substrate for the cultivation of autologous keratinocytes, which could permanently integrate into the wound bed.

Some epidermal substitutes, based on stratified epithelial cultures grown on HAMs, have been described in the literature using a variety of membrane processing techniques.[8–12,30] In this study, HAMs were processed by first glycerolating the membranes in bathes containing high concentrations of glycerol (>85%). Glycerolation effectively destroys the cellular components rendering them nonviable, and, as a result, less antigenic and more biocompatible.[31]

Koizumi et al.[32] compared limbic cells cultured on intact or de-epithelized HAMs. They showed that epithelial cells migrate more rapidly on the de-epithelized membrane and the final epithelium is more stratified and organized. The standardized de-epithelization technique in this study proved to be efficient in the removal of all the epithelium in both ni- and i-HAM preparations. Furthermore, it was found that an intact basement membrane was only present in samples from the ni-HAM group. In fact, it was determined that irradiation caused significant damage to the basement membrane, resulting in its loss during de-epithelialization. For all of the experiments performed the observed differences, at the cellular level, were repeatedly reproduced.

Samples of ni-HAM stained with Picrossirius group showed a yellow-green birefringence, indicating the presence of a thinner collagen layer, typical of that present in the basement membrane. In contrast, i-HAM samples did not display these birefringences, suggesting that the basement membrane was absent. Furthermore, immunofluorescence experiments only detected collagen IV and laminin in the ni-HAM group. These two proteins are typical markers for the basement membrane, and are normally present in the dense and lucid layers, respectively. Furthermore, electron microscopy conclusively demonstrated the presence of

the basement membrane in ni-HAM and its absence in i-HAM

These findings indicate that we established a model of de-epithelialization that maintains the basement membrane of ni-HAMs. For the construction of skin substitutes, it is known that the preexistence of components of the basement membrane contribute to an improvement in the stratified epithelium morphology, and plays a fundamental role in the formation of hemidesmosomes and in the development of the dense lamina.[33] In fact, the electron microscopy experiments showed the existence of electrondense material on the surface, corresponding to the dense lamina and, above that, an electroluscent material indicative of the lucid layer of the electronic basement membrane. Therefore, trypsinization of the non-irradiated group probably digested the membrane at the level of the lucid lamina, preserving the collagen IV and part of the laminin.

Laminin is one of the major components of the basement membrane and contributes to cell differentiation and movement, with a variety of isoforms, including: types 2, 4, 5, 6, 7, 10 and 11.[34] Laminin type 5 has been shown to be critical for epithelial adhesion. Moreover, the association of laminins 6 and 7, promoting binding between $\alpha_6\beta_4$ integrins present in epithelial cells and connective anchor fibers, mainly type IV collagen,[8] and forming the dense lamina.

On the other hand, in the irradiated group, the electron microscopy results showed no sign of the basement membrane after de-epithelization, resulting in a surface only containing amorphous connective tissue. Glycerolation is considered to be the best preservation method when irradiating a tissue, since glycerol functions as a cryoprotectant that removes water, and prevents the indirect effects of irradiation produced by the formation of reactive oxygen species and free radicals.[35] In fact, Von Versen-Hoynck et al. reported the presence of an integral basement membrane in glycerolated HAMs irradiated with a 32 kGy dose of radiation.[36] However, it should be noted, that the irradiated glycerolated HAMs were not de-epithelizized beforehand. In our experiments, we noticed that after the enzymatic bath the basement membrane did not resist and completely disengaged from the amnion, in stark contrast to what was observed with the non-irradiated group. It is possible that the ionizing radiation caused structural damage to collagens IV and VII. Interestingly, when the epithelial cells were attached to the tissue, this damage could not be observed. However, with the loss of tissue architecture, the damaged collagen eventually loosened, leading to complete loss of the basement membrane.

At days 14 and 21, the epithelium of cells cultured on the ni-HAM was more stratified and differentiated than the irradiated counterpart. The comparison with control also showed better stratification results in group ni-HAM. Probably, the absence of a previous basement membrane on control group harmed the development of a more mature stratified epithelium, and in i-HAM, the irradiation destroyed the preexisting basement membrane.

Cultures of keratinocytes grown on immersed ni-HAMs showed a stratified and unorganized epithelium with little cell differentiation and no stratum corneum formation. On the other hand, when the non-irradiated group was positioned at the air-liquid interface, epithelial development was much more exuberant, displayed better stratification and cellular differentiation, and began to form a stratum corneum around the 21st day. Therefore, the culture situation (immersed vs. air-liquid interface) on non-irradiated substrates influenced the dermo-epidermal properties, with cells cultured at the air-surface interface yielding a more clinically acceptable cutaneous substitute. Indeed, it has been reported that human epithelia cultures at air-liquid interface display accelerated epithelial maturation.[37]

When we compared our results, using glycerolated HAM substrates, with studies that used cryopreserved HAM[8,9,11], it was found that the epithelial formation and stratification for these methods were quite similar. Thus, glycerolation proves to be an efficient preservation mechanism, which maintains the ability of HAM to serve as substrate for human epithelia cultures.

On the other hand, the substitutes constructed on glycerolated and irradiated HAMs had extremely unsatisfactory results. On the 14th day, the epithelia almost formed a monolayer and, over time, there was a small stratification with the formation of three to four additional layers of cells. Even exposure to air was not sufficient to cause a stratification and differentiation of the epithelia,

further reinforcing the importance of the presence of the basement membrane to the cell culture.

The International Atomic Energy Agency (IAEA) recommends using a 25 kGy dose of radiation for the sterilization of biological materials. However, this dose inflicts greater physical, chemical and biomechanical damage to tissues, including the loss of basement membrane density,[38] which was verified in the present study. While glycerol cannot be used for sterilization, high concentrations of this compound have a disinfecting effect, as a consequence glycerolated tissue has a reduced bioburden. This may be an advantage of this type of HAM preservation, since subsequent radiosterilzation may require lower doses of irradiation, inflicting less structure damage to the HAM substrate, and guaranteeing a safe product to future recipients of this cutaneous subsititute.

The glycerolated membrane was shown to be a biomaterial compatible with culturing allogeneic and autogenous cells with potential for clinical use in the treatment of burns and wounds. Future studies sterilizing the HAMs with doses of radiation of less than 25 kGy should be performed, to determine the minimal amount of radiation necessary for adequate sterilization, while maintaining the structural integrity of the HAM substrate.

Conflicts of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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