ENDOTHELIALIZED BIOPRINTED SKIN MODEL AS A PLATFORM TO ASSESS UV-INDUCED DAMAGE

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Abstract

UV radiation induces biological damage in skin layers, contributing to photo-carcinogenesis and photoaging. 3D skin models can replicate the organization of fully differentiated epidermis and populated dermis in order to assess UV-induced damage, elucidating new photoprotection pathways. To improve these models, we incorporated endothelial cells into the dermal layer to enhance physiological accuracy; using a pneumatic extrusion-based bioprinting technique we provided a more comprehensive platform to investigate the complex impacts of UV radiation on human skin. The bioink formulation comprised fibrinogen, alginate and gelatin, where fibroblasts and HUVEC were seeded. The 3D bioprinting process was followed by consolidation procedures, keratinocyte seeding and air-liquid interface. Histological and immunohistochemical procedures were conducted. For UV-induced damage assessment, the $DCFH_2$-DA probe was used, with tissues exposed or not to UVA radiation to increase ROS production. An antioxidant compound, a derivative of avobenzone and octyl methoxycinnamate, was tested to protect against UVA-induced intracellular ROS generation. The endothelialized bioprinted human skin model presented well-organized cells and microvasculature in the hydrogel, with collagen I production and EN4 staining in the dermis, and cytokeratin staining in the epidermis, mimicking distinct cellular characteristics and functions. This model was used to assess UV radiation's harmful effects. Following UVA exposure, a significant difference in ROS production was observed between irradiated and non-irradiated tissues. The antioxidant compound effectively protected the skin models, reducing ROS generation by 69.5%. These results highlight the model's potential to respond to UV radiation and its promising role for assessing solar radiation effects on human skin.

Keywords: bioprinting; skin model; UV damage; endothelial cell; immunostaining.

Introduction.

Despite the benefits of ultraviolet (UV) radiation, excessive exposure induces biological damages in the skin layers that contribute to photo-carcinogenesis and photoaging, due to cellular matrix disorganization and fibroblast alteration. Exposure to UV radiation increases the generation of Reactive Oxygen Species (ROS), causing cellular function alterations and DNA damage, along with a depletion of the endogenous antioxidant system [1].

In the last two decades, there has been significant progress in studies using monolayer cells and reconstructed human skin models, for evaluating the efficacy and toxicity of active compounds and drugs in cosmetic and pharmaceutical research. The use of 3D skin models can reproduce the right cutaneous organization with fully differentiated epidermis

and populated dermis, and can be used as tools to assess the UV-induced damage and elucidate new pathways of photoprotection. The vascularization of reconstructed tissues is one of the remaining obstacles to be considered to improve both the functionality and viability of skin models and the relevance of *in vitro* applications.
To improve existing 3D skin models, we introduced endothelial cells (HUVEC, human umbilical vein endothelial cells) into the dermal layer, promoting the formation of a connected microvascular network. This improvement not only enhances the model's physiological accuracy but also offers a more comprehensive platform for investigating the intricate impacts of UV radiation on skin tissues.
Recently, even more advanced models have been developed, integrating elements such as hair follicles, aiming to create skin models closer to *in vivo* human skin [2]. Furthermore, the technique of cell printing has received attention as an attractive biofabrication platform due to its ability to precisely position living cells in pre-defined spatial locations. With that in mind, as a way to improve 3D skin models, a bioprinted human skin model containing endothelial cells into the dermal layer was developed, in a hydrogel matrix composed of fibrinogen, alginate and gelatin, obtained through pneumatic extrusion-based bioprinting technique, aiming to evaluate its potential to respond to UV radiation and its use for assessing the effects of solar radiation on human skin.

Materials and Methods.
The endothelialized human skin models were bioprinted through a pneumatic extrusion technique, and the hydrogel matrix was composed of a solution of gelatin, alginate, and fibrinogen, dissolved in DMEM/EGM2 culture medium (50/50). After adding the fibroblasts and HUVEC, the bioink solution was homogenized and transferred to a sterile syringe where it was kept at 21°C to reach the required rheological properties [3]. The bioprinting was then performed using the BioAssemblyBot® 400 robotic 6-axis bioprinter (Advanced Solution LifeScience, USA). After bioprinting, the tissues were consolidated in a solution composed of $CaCl_2$, thrombin, and transglutaminase, and incubated at 37°C, 5% CO_2 for 7 days in a 6-well plate containing inserts (0.4 µm pore membrane), with medium changes every 2 days [4,5]. After this period, keratinocytes were added to the model, and the tissues were cultured at the air-liquid interface with the appropriate culture medium for 7 days, ensuring epidermal stratification and differentiation [6,7].
Skin samples were subjected to routine histological procedures followed by hematoxylin-eosin staining for histological analysis of the morphological development of the models. Collagen I production and the presence of endothelization (EN4) was assessed using an immunohistochemical procedure (ABCAM, AB214417 and AB8087). The presence of the epidermal layer was confirmed using a mix of cytokeratins 4, 5, 6, 8, 14, and 16 (27114, ThermoFisher Scientific). Cell proliferation within the tissues was estimated from fluorescent images after staining with calcein-AM green (C1430, ThermoFisher Scientific, France).
After the models were fully differentiated on day 16, they were placed into new 6-well plates and the measurement of intracellular ROS production began incubating them in the dark with the $DCFH_2$-DA probe. The fluorescence intensity obtained for each skin model was analyzed by ImageJ software and normalized to area/pixels. The final results were expressed as percent fluorescence compared to the untreated irradiated control (NT + UV), considered 100% of ROS production [8,9]. An antioxidant compound was used (200 µg/mL), an avobenzone derivative, hybridized with octyl methoxycinnamate molecule, aiming to protect the skin models against the UVA-induced intracellular ROS production.

Results.

After 16 days of *in vitro* culture, the endothelialized bioprinted models exhibited complex three-dimensional structures, fully populated by cells, organized into microstructures, and with the presence of an epidermal layer, as demonstrated by HE histology (Figure 1a). Immunostaining with EN4 (Figure 1b) indicates the presence of a microvascular organization distributed throughout the hydrogel core, suggesting angiogenesis, which was also observed through calcein fluorescence (Figure 1c). Dense type I collagen staining was found in the entire tissue, indicating its neo synthesis by the fibroblasts (Figure 2a), while the presence of an epidermal layer was confirmed by cytokeratin mix labeling (Figure 2b).

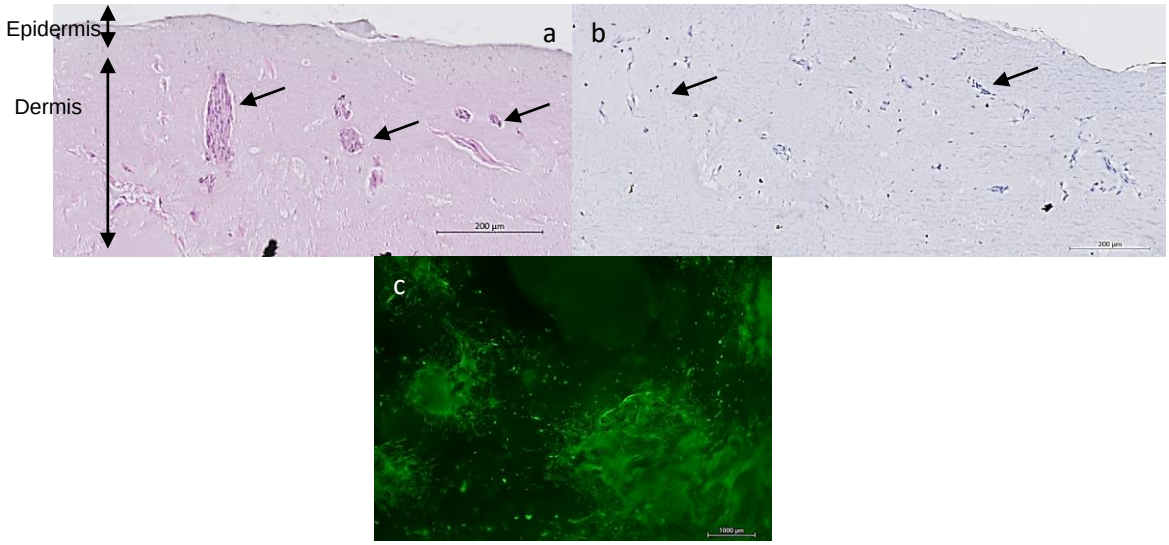

Figure 1. Characterization of the endothelialized bioprinted skin models after 16 days of *in vitro* culture. a) Histological characterization by hematoxylin and Eosin staining. Scale bar 200 µm. b) Immunofluorescent labeling with EN4. Scale bar 200 µm. c) Characterization by fluorescence microscopy with calcein-AM. Scale bar 1000 µm.

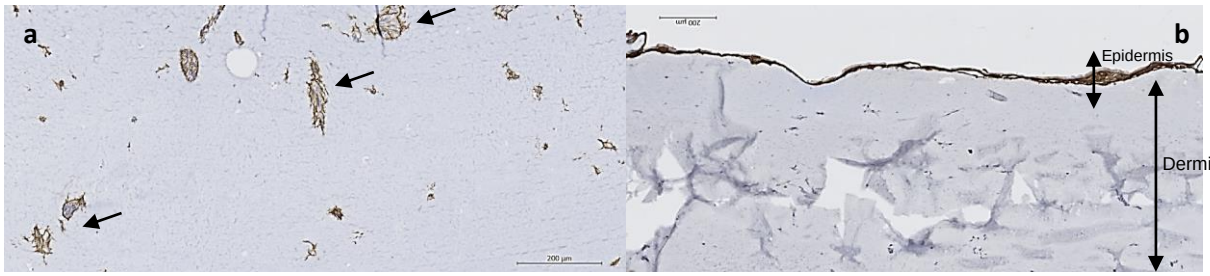

Figure 2. Characterization of the endothelialized bioprinted skin models after 16 days of *in vitro* culture. a) Immunohistological characterization by labeling of human collagen type I and b) by staining with a cytokeratin mix. Scale bar 200 µm.

After the first step of tissue characterization, skin models were subjected to an UVA exposure, and it was shown that the UVA radiation induced an enhancement of 498.1% of ROS generation in the untreated irradiated tissue (100%) when compared to the untreated non-irradiated tissue ($p < 0.05$) (Figure 3), indicating that the endothelialized bioprinted model is able to assess the UV-induced damage. The antioxidant compound tested was able to protect the skin models, demonstrating a reduction of 69.5% in ROS generation, when compared to the untreated irradiated tissues ($p < 0.05$) (Figure 3 and 4).

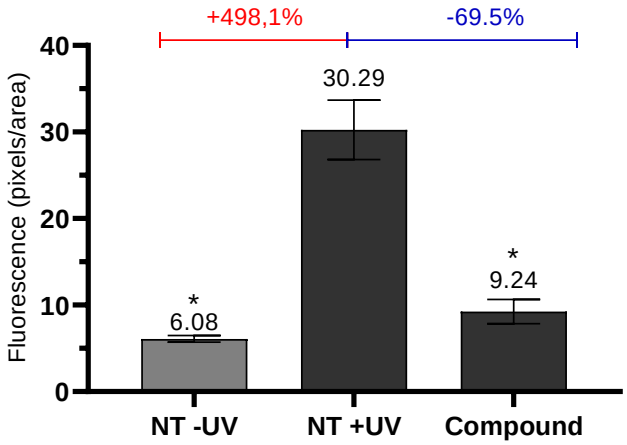

Figure 3. UVA-induced intracellular ROS production in the endothelialized bioprinted skin model. The results are expressed as the pixel/area of fluorescence in comparison to the NT +UV. Untreated non-irradiated control (NT -UV); Untreated irradiated control (NT +UV); Compound (derivative of avobenzone, hybridized with octyl methoxycinnamate molecule (200 µg/mL). Results are expressed as mean ± standard errors of the mean of three independent experiments. *: Significantly different from the untreated irradiated control (NT +UV) ($p < 0.05$).

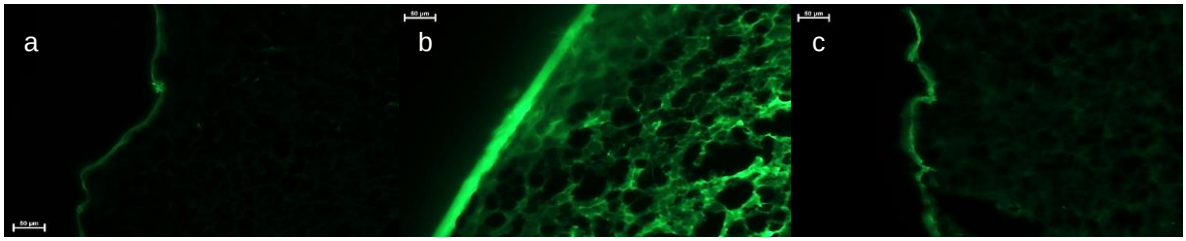

Figure 4. Fluorescence images obtained with confocal microscopy for each treatment: (A) Untreated non-irradiated tissue (NT -UV); (B) Untreated irradiated tissue (NT +UV); (C) Antioxidant compound (200 µg/mL). Green fluorescence corresponds to the presence of ROS in the tissue. Scale bar 50 µm.

Discussion.

Reconstructed skin models are widely used in the evaluation of the safety and efficacy of new cosmetic products, mimicking *in vivo* biological processes [10]. However, these models face challenges, such as the lack of functional blood vessels, which limit their ability to simulate complex processes like angiogenesis, immune response and skin metabolism. The technique of bioprinting has emerged as a promising solution to further enhance these models, allowing the creation of more sophisticated and physiologically relevant skin models with multiple cell types and more complex 3D structures [2]. Therefore, to improve the *in-house* reconstructed model, a human skin model containing fibroblasts and HUVEC was developed and standardized in a hydrogel matrix composed of alginate, fibrinogen and gelatin, obtained through the pneumatic extrusion-based bioprinting technique.

The characterization of the endothelialized bioprinted skin model showed that the endothelial cells were capable of self-organizing into micro vessels and creating complex morphologies with patterns similar to natural tissues. HUVEC are the most commonly used type of endothelial cells in laboratory research, as they form the inner layer of blood

vessels, participating in various physiological processes such as vascular development and remodeling, nutrient transport, and response to mechanical stimuli [11].
The presence of fibroblasts during the bioprinting of the dermis containing endothelial cells is crucial for the phenomenon of angiogenesis, as well as for the production and maintenance of the extracellular matrix [3]. The development of vascularization depends on the close communication between fibroblasts, endothelial cells, and their microenvironment. In the developed bioprinted skin model, dense collagen I staining was found throughout the tissue, indicating its production by the fibroblasts present in the tissue, while the epidermal layer was confirmed by staining with the cytokeratin mix, indicating the different stages of cellular differentiation within the epithelium and the phases of keratinocyte development or maturation [12].
The results for the UVA-induced intracellular ROS production in the endothelialized bioprinted skin model demonstrated the potential of the developed bioprinted model to effectively respond to UV radiation and can be further explored as a tool to assess UV damage in the skin.
This study is unprecedented, as to our knowledge, there is no prior research involving the evaluation of protection of UVA-induced ROS production in endothelialized bioprinted human skin–models. The most common use of UV radiation in the bioprinting of human skin models is in their construction, which is the photopolymerization stage of biomaterials [13]. The use of bioprinted skin models and the findings obtained, add relevance to the evaluation of the safety and efficacy of new cosmetic product derivatives and provide important insights for the development of topical materials intended to protect the skin against UV-induced damage.

Conclusion.
The endothelialized bioprinted human skin model exhibited good cellular and microvascular organization throughout the hydrogel, with collagen I production and EN4 staining in the dermis and cytokeratin staining in the epidermis, mimicking the distinct characteristics and functions of each cell type. This model was employed as a tool to evaluate the harmful effects of UV radiation. After UVA exposure, there was an increase in ROS production, when irradiated and non-irradiated models were compared. The antioxidant compound used (a hybrid between avobenzone and octyl methoxycinnamate molecules) was able to protect the skin models, demonstrating a reduction of 69.5% in ROS generation. These findings indicate the potential of the developed model to respond to UV radiation and its promising use for assessing the effects of solar radiation on human skin.

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Conflict of Interest Statement.
NONE.

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