Biomimetic model: skin equivalent model with inflammation induction

Bibiana Matte*, Helouise Medeiros*, Petra Reinher*#, Luiza Brand*#

*Núcleo Vitro, Porto Alegre, Brazil

#Institue of Health Sciences, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Abstract

The human skin covers the entire external surface of the body and performs several fundamental functions, including thermoregulation. The global prevalence of inflammatory skin disorders is on the rise, with conditions like acne vulgaris ranking among the ten most common diseases worldwide. Preclinical studies on cutaneous inflammatory processes pose significant challenges. Therefore, utilizing an equivalent skin model becomes a vital alternative for research in this field, as it facilitates cellular interaction with the tissue's extracellular matrix, yielding results comparable to those observed in humans.

The objective of this study was to develop a skin equivalent model capable of reproducing the characteristics of inflamed human skin. The equivalent skin was constructed using a collagen matrix with fibroblasts and keratinocytes on the surface. To induce inflammation in the model, the following experimental groups were evaluated: 1) Control group; 2) Lipopolysaccharide (LPS) group of bacteria; 3) Sodium lauryl sulfate (SLS) group.

To assess inflammation in the experimental groups, a reverse transcriptase reaction test followed by reverse transcription polymerase chain reaction (RT-PCR)

was performed to analyze the expression of interleukins IL-1, IL-6, and IL-8, as well as tumor necrosis factor alpha (TNF-α). Additionally, histological analysis was conducted to compare the experimental groups with the control group. Initially, it was determined the concentration of LPS and SLS that induces a strong inflammatory response. The concentration of 50 µg/mL of LPS and 100 µg/mL of SLS demonstrated to induce interleukin expression significantly. For IL-6, it was observed that SLS increased by a 2.09 (± 0.6) fold-change in comparison to the control ($p<0.05$), and LPS increased by a 5.63 (± 0.78) fold-change in comparison to the control ($p<0.01$). In the histological analysis, it was observed that the treatments induced modification in the epidermal layer.

The different protocols used to induce inflammation in the equivalent skin model resulted in a significant increase in the expression of the interleukins tested. Ultimately, gene expression analysis confirmed the successful reproduction of an equivalent skin model with induced inflammation. This study demonstrated the possibility of studying inflammatory processes with a skin equivalent model that can be further studied for the evaluation of products targeting skin inflammatory disorders.

Keywords: skin equivalent model; inflammatory disorders; acnes vulgaris;

Introduction

Human skin, covering the entire external surface of the body, performs several fundamental functions, including protection against external aggressions, body temperature regulation (thermoregulation), sensory perception, and maintenance of water homeostasis. As the first line of defense against pathogens, toxins, and ultraviolet radiation, the skin is frequently subject to damage and inflammation. The

global prevalence of inflammatory skin disorders is on the rise, with conditions such as acne vulgaris ranking among the ten most common diseases worldwide. Other examples include atopic dermatitis, psoriasis, and eczema, which affect millions of people and significantly impact patients' quality of life.

Preclinical studies on cutaneous inflammatory processes present significant challenges due to the complexity of human skin and the need for experimental models that can accurately mimic the physiology and pathology of the skin. Animal models are often used but have important limitations, including differences in skin composition and immune response between humans and animals. Furthermore, ethical issues related to the use of animals in research have led to the search for more suitable and humane alternatives.

The use of a skin equivalent model thus becomes a vital alternative for research in this area. Skin equivalent models are constructed to replicate the structure and function of human skin, facilitating cellular interaction with the tissue's extracellular matrix. These models can mimic the architecture of the epidermis and dermis, including the presence of keratinocytes, fibroblasts, and collagen as an extracellular matrix. As a result, they produce results comparable to those observed in humans, allowing for a more accurate analysis of the molecular and cellular mechanisms involved in cutaneous inflammatory processes.

Advances in tissue engineering and biotechnology have enabled the development of increasingly sophisticated skin models that can be used to study inflammatory responses, tissue regeneration, and the effects of therapeutic agents. These models not only provide valuable insights into the pathogenesis of skin

diseases but also allow for the evaluation of new treatments and interventions more efficiently and ethically.

Therefore, the development and use of skin equivalent models are essential for advancing dermatological research, providing a powerful tool to explore the complex biological processes occurring in inflamed skin and effectively and safely evaluate new therapeutic approaches. The objective of this study was to develop a skin equivalent model capable of reproducing the characteristics of inflamed human skin. The equivalent skin was constructed using a collagen matrix with fibroblasts and keratinocytes on the surface. To induce inflammation in the model, the following experimental groups were evaluated: 1) Control group; 2) Lipopolysaccharide (LPS) group of bacteria; 3) Sodium lauryl sulfate (SLS) group. It was then evaluated cytokines release to correlate with inflammation potential.

Materials and Methods

Cell culture

Two cell types were used to compose the equivalent skin: primary human fibroblasts (approved by the UFRGS Ethics Committee CAE#59124916.6.0000.5327) and human keratinocytes (HaCaT - BCRJ® CRL – 0341™). Both were grown in their own culture medium and in a controlled environment at 37°C in an atmosphere with 5% CO2. DMEM culture media (Gibco, Thermo Fischer Scientific, Massachusetts, USA) were supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin, in addition to the presence or absence of glucose, which will be taken into account. cell line used.

Equivalent skin model

The conventional equivalent skin model has already been validated and served as a control group in this work. The model consists of mimicking the two layers of the skin (dermis and epidermis). The dermis is made from fibroblasts embedded in a collagen matrix. In the upper part, there are human keratinocytes that are stimulated to form multiple layers. For the experimental model, two different ways of inducing inflammation were evaluated. The experimental groups were divided into:

1. Control group: equivalent skin with conventional culture medium;
2. Lipopolysaccharide group of bacteria (LPS): equivalent skin with the addition of LPS (50 μg/ml) in the conventional culture medium;
3. Sodium lauryl sulfate group (SLS): equivalent skin with the addition of SLS (100 μg/ml) in the conventional culture medium;

The equivalent skin model was created according to the already validated conventional model, with a stratification period of 14 days. To induce inflammation, the skins were treated in the respective groups by changing the culture medium every 2 days after 14 days of stratification. These groups were defined due to the fact that the factors described are known to induce inflammation.

Evaluation of experimental groups by RT-PCR

To evaluate inflammation in the experimental groups, RNA extraction was carried out after the defined period of treatment in the experimental and control groups. This extraction was carried out using Trizol and the purity of the total RNA obtained was verified using absorbance (A280/A260). Next, conversion into complementary DNA strand (cDNA) was carried out. cDNA was generated by adding random hexamer

primers and Super Script III Reverse Transcriptase (Thermo) to 2µg of RNA. Finally, the reverse transcriptase reaction test followed by polymerase chain reaction (RT-PCR) was used to analyze the expression of the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α). Quantitative RT-PCR was performed (45 cycles, 95°C for 15 seconds followed by 60°C for 1 min) using a 7900HT Fast Real-Time PCR System (Thermo) with the primer sets described in the table below and SYBR Green Supermix. All quantification cycle (Cq) values were normalized to βactin and analysis was performed by 2-^ΔΔCt.

Statistical analysis

The results were evaluated using Microsoft Excel and Graphpad Prism software. For the analysis of data obtained by RTq-PCR, analysis based on 2^-ΔΔCt was used to graphically represent the relative expression by fold-change and statistical analysis based on ΔCt data. The control group was normalized to 1.0 and the other groups compared to it. Bar graphs are represented as mean ± standard deviation. Statistical analysis for comparison between groups was performed using T test. The level of statistical significance considered was less than 0.05.

Histological Analysis

The distribution of cells in the matrix structure was analyzed through histological section carried out after a period of 14 days of stratification and 5 days of exposure to the specific treatment of each group (SLS 100 μg/ml, LPS 50μg/ml and control group culture medium only). After this period, the samples were fixed in formalin, embedded in paraffin and then sectioned using a microtome. The slides were deparaffinized, hydrated and finally stained with hematoxylin and eosin (HE). The analysis was carried

out on a BX51 microscope coupled to an Olympus Q-color 5 RTV with 20x and 40x magnification.

Results

The development of an equivalent skin model with inflammation was evaluated based on the exposure of two different inducers known to stimulate inflammation: LPS and SLS. For SLS, a concentration of 100 μg/ml was selected. After exposure for 14 days, gene expression analysis was performed for the interleukins IL-1, IL-6, IL-8 and TNF-a. It was observed that exposure to SLS led to an increase in all cytokines evaluated compared to the control group. For IL-1, an increase of 69.5% (±4.3) in expression was observed in relation to the control group (Figure 1A). In relation to IL-6, exposure to SLS led to a 2.09-fold increase (±0.69) compared to the control group (Figure 1B). For IL-8, a 3.09-fold (±0.59) increase in expression was observed in relation to the control group (Figure 1C). In relation to TNF-α, exposure to SLS led to an increase of 3.97 times (±0.1) compared to the control group (Figure 1D). In the statistical analysis, all cytokines evaluated led to a significant increase after exposure to SLS in relation to the control group with exception of IL-6.

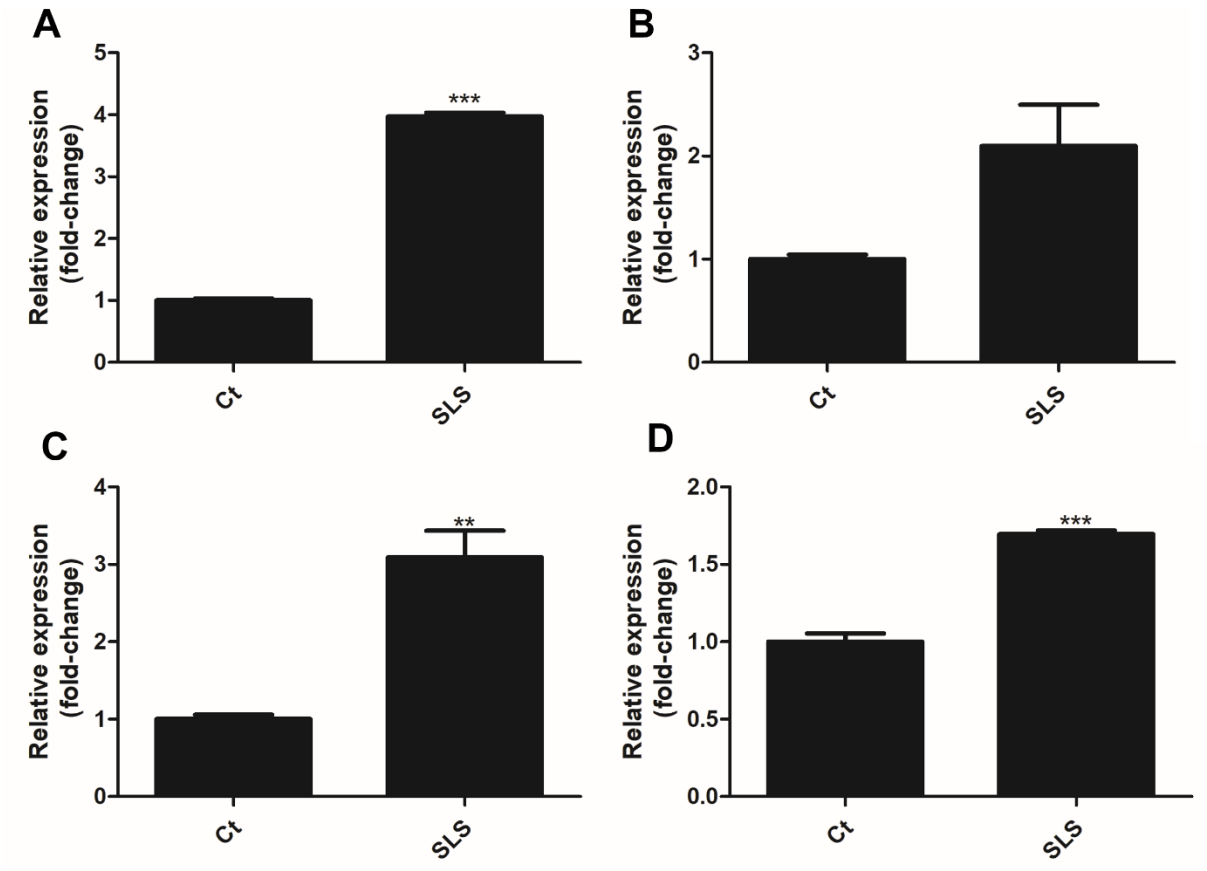

Figure 1. Relative expression results of the equivalent skin model treated with SLS for the control group in relation to the evaluated markers (A) IL-1; (B) IL-6; (C) IL-8; (D) TNF-a.

LPS was evaluated at a concentration of 50μg/mL. It was observed that exposure to LPS led to an increase in all cytokines evaluated compared to the control group. For IL-1, a 6.05-fold (±0.08) increase in expression was observed in relation to the control group (Figure 2A). In relation to IL-6, exposure to LPS led to a 5.63-fold increase (±0.14) compared to the control group (Figure 2B). For IL-8, a 1.82-fold (±0.04) increase in expression was observed in relation to the control group (Figure 2C). In relation to TNF-α, exposure to LPS led to a 4.2-fold increase (±0.83) compared to the control group (Figure 2D). In the statistical analysis, all cytokines evaluated led to a significant increase after exposure to LPS in relation to the control group.

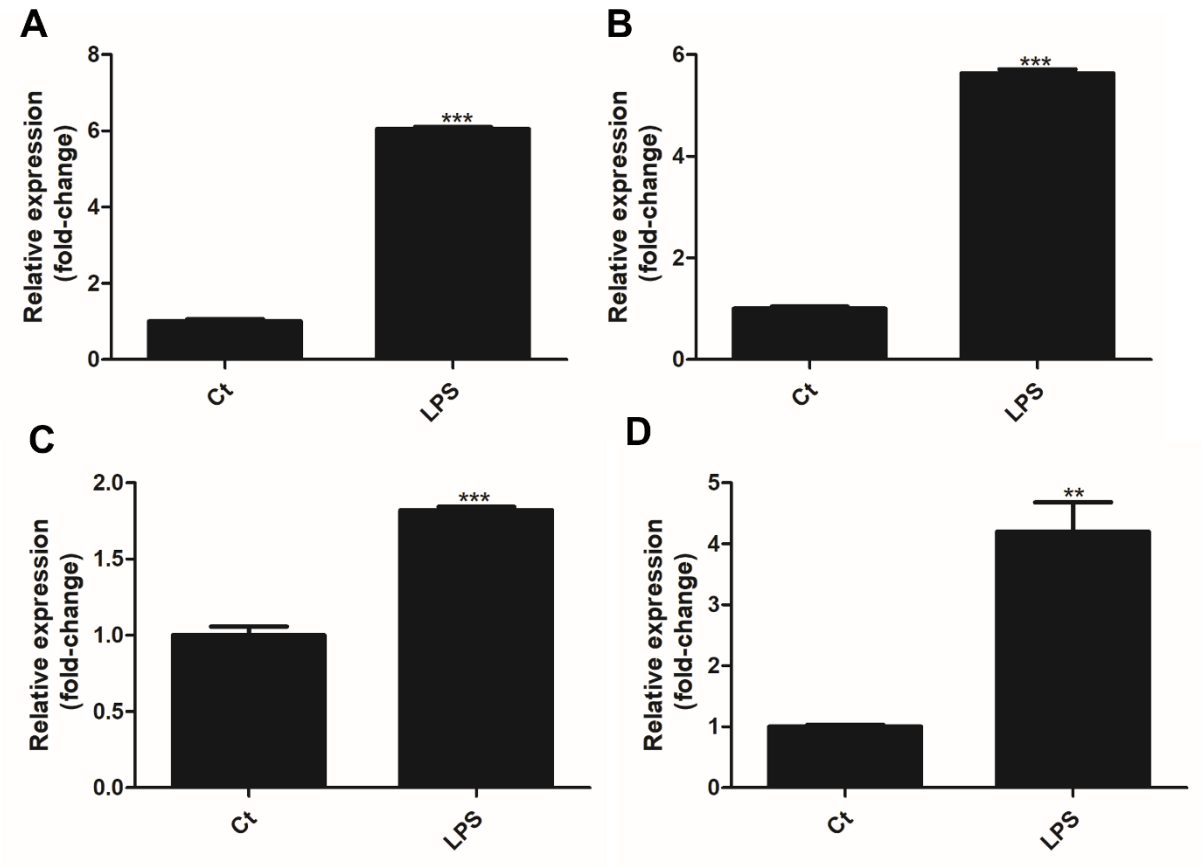

Figure 2. Relative expression results of the equivalent skin model treated with LPS for the control group in relation to the evaluated markers (A) IL-1; (B) IL-6; (C) IL-8; (D) TNF-a.

In order to evaluate the histological characteristics of conventional equivalent skin compared with the treatment groups (LPS 50 ug/ml and SLS 100 ug/ml), histological sections were made (Figure 3). In both groups, the organization of elongated dermal fibroblasts embedded in the extracellular matrix and the stratification of keratinocytes in the epidermis can be observed, reproducing the two main layers of the skin. According to the morphological observation of the histological sections, we can evidence the elongated and similar morphology of the fibroblasts in the dermal layer and the stratification of the keratinocytes in the epidermis. In the model treated with SLS, we observed damage to the barrier between the epidermis and dermis, as

well as a reduction in the thickness of the stratum corneum. The model treated with LPS shows a thicker stratum corneum, demonstrating hyperproliferation of keratinocytes due to the induction of the agent used.

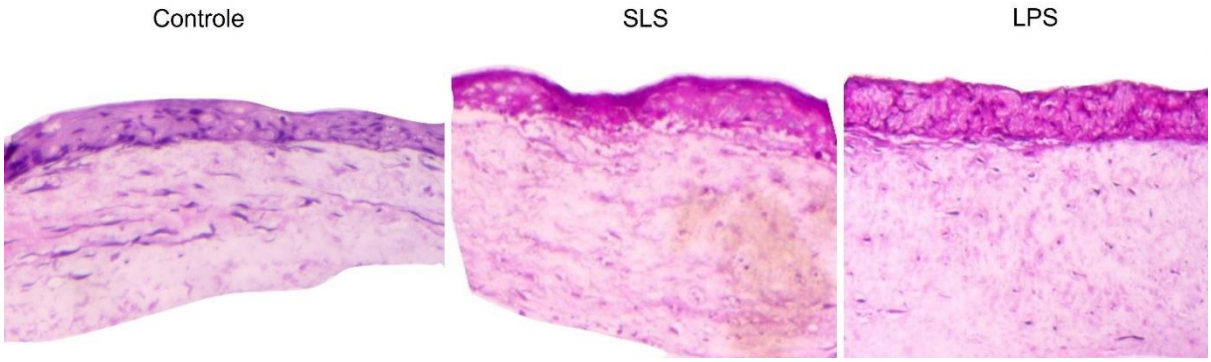

Figura 3. Histological sections stained with HE from conventional equivalent skin models, exposed to SLS 100μg/ml and LPS 50 μg/ml.

Discussion

The inflammatory process in human skin is complex and can be induced by several factors such as pathogens, drugs and mainly by the activation of cytokines. With the development of equivalent skin models, it became possible to mimic human skin, including the activation of pro-inflammatory cytokines, compensating for the lack of immune cells in the model. This way, it is possible to carry out in-depth studies on the physiology and pathology of different skin conditions as well as evaluate the effects of different active ingredients on the skin in laboratory models.

To develop an equivalent skin model with inflammation induction, we evaluated the use of LPS and SLS. These agents were selected because they are involved in triggering inflammatory reactions. To evaluate their potential, we selected four cytokines (TNF-α, IL-6, IL-8 and IL-1) and evaluated the gene expression of each

group. It has been observed that exposure to LPS and SLS has the potential to trigger the release of inflammatory cytokines by skin cells.

LPS is the main component of the cell walls of gram-negative bacteria, making it a circulating environmental toxin that is generally linked to chronic diseases and low-grade inflammation. In the study by Morris MC et al. (2014) the role of LPS modulation in the expression of inflammatory mediators such as the IL-6 and TNF-α genes became clear depending on the concentration of the exposed dose. In the study on acne vulgaris by Wu J et al. (2021) LPS was used at a concentration of 100 ng/ml, for 24 hours, to induce the state of inflammation in cell culture with the purpose of playing an inflammatory role similar to that of acne, significantly increasing the expression of inflammatory factors IL -1β, IL-6 and TNF-α in the acute phase, corroborating the results observed in this study.

Furthermore, we demonstrated that there was a significant increase in the inflammatory cytokines TNF-α and IL-6 in the SLS group. This is also seen in studies showing the use of SLS to mediate inflammation in fibroblast and keratinocyte cultures *in vitro.* SLS is a surfactant widely used in skin irritation studies, as it causes skin sensitivity, as its penetration increases when the skin's barrier function is impaired. Due to its action on the skin, this agent has been used in studies involving dermatitis, as it is an inflammatory skin disease caused by exposure to contact allergens and irritants. An example is the study by De Jongh (2006) who, in order to determine the inflammatory cytokines present in dermatitis, used volunteers to be exposed to 1% SLS for 24 hours and after repeated exposure to 0 .1% SLS over a 3 week period. Barrier impairment and inflammation have been reported with expression of the cytokines IL-1 and IL-8.

Dermatological disorders induced by pro-inflammatory cytokines are characterized by the secretion mainly of TNF-α, IL-6, IL-8 and IL-1, which develop a condition of abnormal proliferation of keratinocytes and desquamation, in line with the literature on acne vulgaris and psoriasis. Both agents evaluated (LPS and SLS) for inducing inflammation achieved a better increase in cytokine expression.

The continued development of *in vitro* models that mimic the characteristics of inflammatory skin diseases such as psoriasis, acne vulgaris and dermatitis is essential, as there are currently no methodologies that completely match the pathology of these diseases. In view of the models present in the literature, the equivalent skin model containing a co-culture of the dermis and three-dimensional epidermis contributes fundamentally to mimicking the modeling of inflammatory diseases of skin development, since this model has cell-cell interaction, mechanisms of barrier function, demonstrates skin cell differentiation and signaling, thus highlighting several advantages over previous models.

Therefore, this study demonstrates two protocols that can be used to establish an equivalent skin model, with dermal and epidermal layers, that mimics the induction of inflammation. However, it is still necessary further studies that validate the protocol developed with the use of cosmetic products whose anti-inflammatory effect is already well established in the literature, so that they can later be used to evaluate the safety and efficacy of anti-inflammatory dermocosmetic agents *in vitro.*

Conclusion

It was successfully developed an *in vitro* skin equivalent model that has an inflammatory profile. Therefore, this can be useful to evaluate and understand new compounds efficacy in more sensitive-type skin.

Acknowledgments

We thank Prof. Márcia Wink for the support during this work.

Conflicts of Interest Statement

None to state.

References

ALBANESI, C.; PASTORE, S. Pathobiology of chronic inflammatory skin diseases: interplay between keratinocytes and immune cells as a target for anti-inflammatory drugs. Current Drug Metabolism, v. 11, n. 3, p. 210-227, 2010.

ARDA, Oktay; GÖKSÜGÜR, Nadir; TÜZÜN, Yalçın. Basic histological structure and functions of facial skin. Clinics in dermatology, v. 32, n. 1, p. 3-13, 2014.

AREND, William P. et al. Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. The Journal of clinical investigation, v. 88, n. 5, p. 1445-1451, 1991.

BARKER, Carol L. et al. The development and characterization of an *in vitro* model of psoriasis. Journal of investigative dermatology, v. 123, n. 5, p. 892-901, 2004.

BRAGAZZI, Nicola Luigi et al. Fasting and its impact on skin anatomy, physiology, and physiopathology: A comprehensive review of the literature. Nutrients, v. 11, n. 2, p. 249, 2019.

BISWAS, Subhra K.; LOPEZ-COLLAZO, Eduardo. Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends in immunology, v. 30, n. 10, p. 475-487, 2009.

BOJAR, R. A.; CUNLIFFE, W. J.; HOLLAND, K. T. The short-term treatment of acne vulgaris with benzoyl peroxide: effects on the surface and follicular cutaneous microflora. British Journal of Dermatology, v. 132, n. 2, p. 204-208, 1995.

CHAE, Byeong Suk. Pretreatment of low-dose and super-low-dose LPS on the production of in vitro LPS-induced inflammatory mediators. Toxicological research, v. 34, n. 1, p. 65-73, 2018.

COLOMBO, Irma et al. HaCaT cells as a reliable *in vitro* differentiation model to dissect the inflammatory/repair response of human keratinocytes. Mediators of inflammation, v. 2017, 2017.

De JONGH, Cindy M. et al. Stratum corneum cytokines and skin irritation response to sodium lauryl sulfate. Contact dermatitis, v. 54, n. 6, p. 325-333, 2006.

DESMET, Eline et al. *In vitro* psoriasis models with focus on reconstructed skin models as promising tools in psoriasis research. Experimental biology and medicine, v. 242, n. 11, p. 1158-1169, 2017.

FRANKOVA, J. et al. Influence of silymarin components on keratinocytes and 3D reconstructed epidermis. Toxicology *in Vitro*, v. 74, p. 105162, 2021.

HAY, Roderick J. et al. The global burden of skin disease in 2010: an analysis of the prevalence and impact of skin conditions. Journal of Investigative Dermatology, v. 134, n. 6, p. 1527-1534, 2014.

HE, Bing; CHEN, Guomin; ZENG, Yi. Three-dimensional cell culture models for investigating human viruses. Virologica Sinica, v. 31, n. 5, p. 363-379, 2016.

HENNIES, Hans Christian; POUMAY, Yves. Skin Disease Models *In Vitro* and Inflammatory Mechanisms: Predictability for Drug Development. 2021.

HÜBNER, Griseldis; WERNER, Sabine. Serum growth factors and proinflammatory cytokines are potent inducers of activin expression in cultured fibroblasts and keratinocytes. Experimental cell research, v. 228, n. 1, p. 106-113, 1996.

JEAN, Jessica et al. Development of an *in vitro* psoriatic skin model by tissue engineering. Journal of dermatological science, v. 53, n. 1, p. 19-25, 2009.

KARASEK, Marvin A. *In vitro* culture of human skin epithelial cells. Journal of Investigative Dermatology, v. 47, n. 6, p. 533-540, 1966

KANWAR, Indu Lata et al. Models for acne: A comprehensive study. Drug discoveries & therapeutics, v. 12, n. 6, p. 329-340, 2018.

KATSAMBAS, Andreas D.; LOTTI, Torello M. European handbook of dermatological treatments. Clio, v. 2, p. 3, 2003.

KHAVKIN, Jeannie; ELLIS, David AF. Aging skin: histology, physiology, and pathology. Facial Plastic Surgery Clinics, v. 19, n. 2, p. 229-234, 2011.

KÜCHLER, Sarah et al. Hallmarks of atopic skin mimicked *in vitro* by means of a skin disease model based on FLG knock-down. Alternatives to Laboratory Animals, v. 39, n. 5, p. 471-480, 2011.

LI, Yan; LI, Linfeng. Contact dermatitis: classifications and management. Clinical Reviews in Allergy & Immunology, v. 61, n. 3, p. 245-281, 2021.

MA, Xiao; WANG, Feifei; WANG, Bo. Application of an in vitro reconstructed human skin on cosmetics in skin irritation tests. Journal of Cosmetic Dermatology, v. 20, n. 6, p. 1933-1941, 2021.

MIAO, Xiao et al. TRIM27 promotes IL-6-induced proliferation and inflammation factor production by activating STAT3 signaling in HaCaT cells. American Journal of Physiology-Cell Physiology, 2020.

MORRIS, Matthew C. et al. Dynamic modulation of innate immune response by varying dosages of lipopolysaccharide (LPS) in human monocytic cells. Journal of Biological Chemistry, v. 289, n. 31, p. 21584-21590, 2014.

NETZLAFF, Frank et al. The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: An evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. European Journal of Pharmaceutics and Biopharmaceutics, v. 60, n. 2, p. 167-178, 2005.

PAIK, Seung Hwan et al. Skin equivalent assay: an optimized method for testing for hair growth reconstitution capacity of epidermal and dermal cells. Experimental dermatology, v. 28, n. 4, p. 367-373, 2019.

Passeron T, Zouboulis CC, Tan J, Andersen ML, Katta R, Lyu X, Aguilar L, Kerob D, Morita A, Krutmann J, Peters EMJ. Adult skin acute stress responses to short-term environmental and internal aggression from exposome factors. J Eur Acad Dermatol Venereol. 2021

PONTES, MARCOS; COSTA, RENATA MAZARO E. Conselho Nacional de Controle de Experimentação Animal.

POUMAY, Yves; COQUETTE, Alain. Modelling the human epidermis *in vitro*: tools for basic and applied research. Archives of dermatological research, v. 298, n. 8, p. 361-369, 2007.

PROST-SQUARCIONI, Catherine. Histology of skin and hair follicle. Medecine Sciences: M/S, v. 22, n. 2, p. 131-137, 2006.

RADEMACHER, Franziska et al. Skin microbiota and human 3D skin models. Experimental dermatology, v. 27, n. 5, p. 489-494, 2018.

RISUEÑO, I. et al. Skin-on-a-chip models: General overview and future perspectives. APL bioengineering, v. 5, n. 3, p. 030901, 2021.

ROGER, Mathilde et al. Bioengineering the microanatomy of human skin. Journal of anatomy, v. 234, n. 4, p. 438-455, 2019.

SIVAPALARATNAM, Suthesh et al. Identification of candidate genes linking systemic inflammation to atherosclerosis; results of a human in vivoLPS infusion study. BMC medical genomics, v. 4, n. 1, p. 1-8, 2011.

SHI, Jihong et al. IL-10 alleviates lipopolysaccharide-induced skin scarring via IL-10R/STAT3 axis regulating TLR4/NF-κB pathway in dermal fibroblasts. Journal of cellular and molecular medicine, v. 25, n. 3, p. 1554-1567, 2021.

Soltanipoor M, Stilla T, Riethmüller C, Thyssen JP, Sluiter JK, Rustemeyer T, Fischer TW, Kezic S, Angelova-Fischer I. Specific barrier response profiles after experimentally induced skin irritation in vivo. Contact Dermatitis. 2018

TAN, Cher-Han; RASOOL, Sarah; JOHNSTON, Graham A. Contact dermatitis: allergic and irritant. Clinics in dermatology, v. 32, n. 1, p. 116-124, 2014.

TJABRINGA, Geuranne et al. Development and validation of human psoriatic skin equivalents. The American journal of pathology, v. 173, n. 3, p. 815-823, 2008.

VAN DEN BOGAARD, Ellen H. et al. Crosstalk between keratinocytes and T cells in a 3D microenvironment: a model to study inflammatory skin diseases. Journal of Investigative Dermatology, v. 134, n. 3, p. 719-727, 2014.

VIDAL YUCHA, Sarah E.; TAMAMOTO, Kasey A.; KAPLAN, David L. The importance of the neuro-immuno-cutaneous system on human skin equivalent design. Cell proliferation, v. 52, n. 6, p. e12677, 2019.

VOWELS, Benjamin R.; YANG, Shijun; LEYDEN, James J. Induction of proinflammatory cytokines by a soluble factor of Propionibacterium acnes: implications for chronic inflammatory acne. Infection and immunity, v. 63, n. 8, p. 3158-3165, 1995.

WANG, Xiaoxiao; DONG, Shiyang; WU, Yaojiong. Isolation and cultivation of skin-derived precursors. Methods Mol. Biol., v. 1879, p. 149-152, 2019.

Wu J, Guo R, Chai J, Xiong W, Tian M, Lu W, Xu X. The Protective Effects of Cath-MH With Anti-Propionibacterium Acnes and Anti-Inflammation Functions on Acne Vulgaris. Front Pharmacol. 2021

YANG, Hongming et al. Effect of lipopolysaccharide on the biological characteristics of human skin fibroblasts and hypertrophic scar tissue formation. IUBMB life, v. 65, n. 6, p. 526-532, 2013.

YAP, Wei Hsum et al. Fibroblast-derived matrices-based human skin equivalent as an *in vitro* psoriatic model for drug testing. Journal of biosciences, v. 46, n. 3, p. 1-13, 2021.

YOUSEF, Hani; ALHAJJ, Mandy; SHARMA, Sandeep. Anatomy, skin (integument), epidermis. 2017.