UNIVERSIDADE FEDERAL DO PARANÁ


VIVIANA STEPHANIE COSTA GAGOSIAN

# APLICAÇÃO DE UM NOVO MODELO DE EPIDERME HUMANA RECONSTITUÍDA *IN VITRO* PARA AVALIAÇÃO DE IRRITANTES CUTÂNEOS

CURITIBA
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Dissertação apresentada ao curso de Pós-Graduação em Genética , Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Genética.

Orientadora: Profa. Dra. Daniela Morais Leme

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CURITIBA

2021

# TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **VIVIANA STEPHANIE COSTA GAGOSIAN** intitulada: **Aplicação de um Novo Modelo de Epiderme Humana Reconstituída in vitro para Avaliação de Irritantes Cutâneos**, sob orientação da Profa. Dra. DANIELA MORAIS LEME, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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CURITIBA, 29 de Março de 2021.

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## AGRADECIMENTOS

À minha orientadora, Profª Dani, por ter me dado a oportunidade de fazer parte do Laboratório de (Eco)Toxicologia *In Vitro,* antes mesmo do mestrado. Obrigada pela liberdade e confiança que depositou em mim desde o começo, para realizar os experimentos e atividades do laboratório. Agradeço por sempre estar disponível para discutir novas ideias e por sempre me incentivar a seguir em frente, mostrando nossos trabalhos ao mundo. Posso dizer que cresci muito profissionalmente com sua orientação e levo comigo todos esses aprendizados para seguir nessa carreira incrível de pesquisadora. Na Universidade ou no Shopping você sempre será a PROFESSORA, como uma forma de admiração por quem me ensinou muitas coisas, por isso te chamo de assim.

A mis padres, Carlos y Susy, y a mi hermano Carlitos, que siempre me apoyaron mucho en todas mis decisiones personales e profesionales. Agradezco a mi Papa, porque mismo estando en otro país siempre me escucha, me da excelentes consejos y nunca me deja desistir de mis sueños. Por ponerse orgulloso cuando conquisto algo, quedándose hasta más feliz que yo. Agradezco a mi Mama que mismo no estando conmigo físicamente en todos estos momentos importantes de mi vida, siempre siento su apoyo. A mi hermano por estar siempre a mi lado ayudándome a cada tomada de decisión, por hacerme reír cuando más necesito y por soñar conmigo en las locuras de la vida. España ponto estaremos ahí, esperen por nosotros. Muchas gracias a los tres por todos los consejos, cariño y amor, porque todo lo que he conquistado fue principalmente por todo el soporte que me dieron. Los quiero mucho.

À Ema, que fez uma previsão no começo do mestrado, que nos tornaríamos *bests* de mestrado e acabamos nos tornando melhor amigas não só de mestrado, mas fora dele também, com uma parceria incrível. Obrigada por toda a ajuda nos experimentos e pelas correções de muitos textos em inglês (o que seria de mim sem vc nesse mestrado?). Agradeço por sempre estar ao me lado me apoiando e me dando conselhos, por ser muito paciente nos meus surtos pré-apresentação, e por estar comigo em todos os momentos da minha vida, dentro e fora da UFPR, vibrando a cada conquista. Obrigada por todas as risadas, idas ao shopping, almoços, e todos os momentos de descontração. Com certeza esta amizade

maravilhosa levarei para o resto da vida e espero que possamos trabalhar juntas novamente algum dia.

À minha coorientadora Dra. Cynthia, pela oportunidade de poder trabalhar em convênio com a empresa TECAM Tecnologia Ambiental, por me coorientar e por sempre contribuir com novas ideias.

A mi Abuela Rosa, a mis tías Flávia y Mary, a mis tíos Panos y Samuel, a mis primos Nátaly y Gustavo, a mis padrinos Rúben y Cristina y a mi cuñada Patricia, por haber estado siempre a mi lado en los momentos buenos y malos apoyándome. Les agradezco, pues siempre fueron muy pacientes, comprensivos y me supieron entender cuando no podía ir a reuniones, por tener que estudiar o escribir, y mismo así siempre estuvieron de brazos abiertos. Los quiero mucho.

À Ana Carolina (Anazoca), que fez parte do grupo por pouco tempo, mas se tornou uma amigona. Obrigada pela parceria, companhia, amizade, por toda a ajuda nos experimentos e por me incentivar a te seguir na Europa. Obrigada por todos os momentos sérios e de risada no laboratório, com certeza o começo do mestrado foi mais leve por sua causa e você foi essencial para completar este trabalho.

À Tota, Laís, Mariana, Antônio, Laercio, Gê e Taynah, principalmente a Laís e a Tay por me proporcionarem momentos divertidos no laboratório e por me fazerem companhia quando ficava até às 23h no laboratório fazendo experimento. Obrigada Laís por me ajudar nos primeiros passos da padronização da RHE. Obrigada a todos pelas risadas, momentos descontraídos, vídeos chamadas e por sempre me incluírem em tudo desde o primeiro dia no laboratório.

Aos doadores de tecido para o isolamento dos queratinócitos. Obrigada pela participação na pesquisa, sem vocês não seria possível executar os experimentos.

Ao Dr. Ayrton Aranha que realizou todas as cirurgias de fimose, para a coleta de tecido.

Aos meus melhores amigos Mallu, Luquinhas, Pah e Daniel, muito obrigada por toda a parceria, por estarem sempre ao meu lado desde a graduação, pelos ótimos conselhos de vida, por me darem força, principalmente no último ano horrível e pandêmico, pelas muitas risadas, pela comilança, pelas brincadeiras, enfim por todos os bons momentos vividos.

A todos meus amigos, que sempre choram, gritam, dançam, bebem, jogam e comemoram junto comigo, que essa pandemia termine pra gente poder festejar e comemorar tudo o que não pudemos comemorar em 2020.

Às minhas quatro coorientadas de Iniciação científica, Elisa, Júlia, Luísa, Mariana, que sempre se mostraram dispostas a aprender e me proporcionaram muita aprendizagem neste ultimo ano, está sendo uma experiência incrível treinar e ensinar vocês, meninas.

À PETA International Science Consortium Ltd. pelo prêmio “Award for Innovative Approaches in Science” concedido a mim.

A todo o pessoal do Laboratório de (Eco)Toxicologia *In Vitro,* que contribuíram de alguma maneira na minha formação, muito bom fazer parte do grupo com vocês. E a professora Dra. Marta Margarete Cestari, por estar sempre disposta a ajudar.

Ao departamento de Fisiologia da UFPR e a Débora, por nos permitir usar o leitor de microplacas TECAN e o criostato para o corte dos materiais.

Ao Centro de Tecnologias Avançadas em Fluorescência (CTAF) da Universidade Federal do Paraná, pelos excelentes serviços prestados. Agradeço principalmente ao Israel, pelas imagens de microscopia de fluorescência.

Ao CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) pela bolsa de Mestrado concedida.

À FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) pelo auxílio financeiro a esse projeto mediante o Convênio UFPR-TECAM.

À CAPES, pois o presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001

Ao Programa de Pós-Graduação em Genética e a todos os professores que fazem parte do departamento, obrigada pelos conhecimentos passados e experiências divididas.

À Universidade Federal do Paraná, aos servidores e funcionários que proporcionaram um ambiente agradável de trabalho.

## RESUMO

O desenvolvimento de metodologias alternativas tem se mostrado cada vez mais importante para a aplicação do princípio dos 3 Rs (*Replacement*, *Reduction* and *Refinement*). Dentro desse contexto, a Epiderme Humana Reconstruída (RHE – *Reconstructed Human Epidemis*) é um modelo capaz de simular as características morfológicas, bioquímicas e fisiológicas da epiderme humana, podendo ser utilizado para diversos fins científicos, por exemplo, na avaliação da toxicidade de substâncias químicas. Apesar de esse modelo ter elevada aceitação no contexto da toxicologia regulatória, ele é ainda pouco adotado em pesquisas acadêmicas, principalmente devido aos altos custos, dificuldades de aquisição e limitações de informações acerca do protocolo de construção. Frente a isso, nosso objetivo foi desenvolver um novo modelo de RHE, aqui referido como Skinvitro-RHE, por meio da análise de fatores que potencialmente influenciam culturas de RHE (*i.e.*. recobrimento dos insertos, placa de cultivo, densidade de poros dos insertos, meio de cultura, suplementos do meio de cultura, tempo de condição submersa, umidade e idade dos doadores de queratinócitos). A partir dessa análise, elaboramos um protocolo aberto, a fim de aumentar a acessibilidade do modelo de RHE para laboratórios acadêmicos e estudos que não se enquadram nas condições de Boas Práticas de Laboratório (BPL). Além disso, como segundo objetivo do trabalho, avaliamos a aplicabilidade desse modelo no teste de irritação dérmica *in vitro*, seguindo o método OECD TG 439. A melhor condição de construção da RHE foi a geração de um modelo construído com queratinócitos neonatais, plaqueados em insertos de alta densidade de poros e revestidos com colágeno IV. Por meio desse protocolo de construção, obtivemos uma RHE altamente similar à epiderme humana com as quatro camadas de diferenciação e estrato córneo bem definido (~ 7 camadas, 66,7 µm de espessura). Além disso, quando exposta a marcadores de diferenciação (Queratina-10, Queratina-14, Filagrina, Involucrina), apresentou padrão de marcação semelhante à epiderme humana. Este modelo também se enquadrou nos parâmetros de controle de qualidade preconizados pela OECD TG 439 e, assim, demonstrou boa viabilidade celular (densidade óptica média em 570 nm de 1,7 ± 1,08) e função de barreira ($IC_{50}$ de 2,77 ± 0,53 mg/mL - Dodecil Sulfato de Sódio). A Skinvitro-RHE foi, então, avaliada quanto ao seu potencial de uso para avaliação de irritação dérmica por meio de 15 substâncias de referência (7 irritantes e 8 não irritantes), usualmente empregadas em estudos de avaliação. Os resultados mostraram que a Skinvitro-RHE foi capaz de discriminar substâncias irritantes de não irritantes, apresentando especificidade de 85,71 %, sensibilidade de 100 % e precisão de 93,33 %. Em conclusão, esses resultados demonstram que a Skinvitro-RHE é um modelo promissor, acessível e altamente reprodutível, que pode ser utilizado para teste de irritação dérmica, bem como outras finalidades científicas por meio de seu protocolo aberto, contribuindo para a disseminação e implementação de métodos alternativos, principalmente em setores em que esse modelo 3D ainda não é bem difundido.



**Palavras-chave:** métodos alternativos, sistemas de cultivo 3D, Epiderme Humana Reconstruída (RHE), teste de irritação dérmica.

## ABSTRACT

The development of alternative methodologies has been shown to be increasingly important for the application of the principle of 3 Rs (Replacement, Reduction and Refinement). Within this context, the Reconstructed Human Epidemic (RHE) is a model capable of simulating the morphological, biochemical and physiological characteristics of the human epidermis, and can be used for various scientific purposes, for example, in the evaluation of the toxicity of chemical substances. Although this model is highly accepted in the context of regulatory toxicology, it is still little adopted in academic research, mainly due to high costs, difficulties in acquisition and limitations of information about the construction protocol. In view of this, our objective was to develop a new model of RHE, here referred to as Skinvitro-RHE, through the analysis of factors that potentially influence cultures of RHE (*i.e.,* coverings of the inserts, cultivation plate, pore density of the inserts, culture medium, culture medium supplements, time in submerged condition, humidity and age of keratinocyte donors). From this analysis, we developed an open protocol in order to increase the accessibility of the RHE model for academic laboratories and studies that do not fit the Good Laboratory Practices (GLP) conditions. In addition, as a secondary objective of the study, we evaluated the applicability of this model in the dermal irritation test *in vitro*, following the OECD TG 439 method. The best construction condition for the RHE was the generation of a model constructed with neonatal keratinocytes, plated in high-density pore inserts and coated with collagen IV. Through this construction protocol, we obtained an RHE highly similar to the human epidermis with the four layers of differentiation and well-defined *stratum corneum* (~ 7 layers, 66.7 µm thick). In addition, when exposed to differentiation markers (Keratin-10, Keratin-14, Filaggrin, Involucrin), it presented a marking pattern similar to the human epidermis. This model also fits the quality control parameters recommended by OECD TG 439 and, thus, demonstrated good cell viability (mean optical density at 570 nm of 1,7 ± 1,08) and barrier function ($IC_{50}$ of 2,77 ± 0,53 mg/ml - Sodium Dodecyl Sulfate). The Skinvitro-RHE was then evaluated for its potential use for the evaluation of dermal irritation using 15 reference substances (7 irritants and 8 non-irritants), usually used in evaluation studies. The results showed that the Skinvitro-RHE was able to discriminate between irritating and non-irritating substances, with a specificity of 85.71%, sensitivity of 100% and precision of 93.33%. In conclusion, these results demonstrate that the Skinvitro-RHE is a promising, accessible and highly reproducible model, which can be used for skin irritation testing, as well as other scientific purposes through its open protocol, contributing to the dissemination and implementation of alternative methods, especially in sectors where this 3D model is not yet widespread.



**Keywords**: alternative methods, 3D culture systems, Reconstructed Human Epidermis (RHE), skin irritation test.

## LISTA DE FIGURAS

**3.1 CAPÍTULO I - The evaluation of factors influencing cultures of the reconstructed human epidermis (RHE) and a proposal of a detailed open-source RHE protocol to study skin toxicity and biology.**



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**3.1 CAPÍTULO I - The evaluation of factors influencing cultures of the reconstructed human epidermis (RHE) and a proposal of a detailed open-source RHE protocol to study skin toxicity and biology.**



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**SUMÁRIO**

# 1 INTRODUÇÃO

O desenvolvimento de novos medicamentos, compostos químicos e produtos de higiene pessoal requerem do setor industrial a identificação prévia do perigo e risco à saúde humana decorrentes do uso destes produtos/compostos (DANIEL et al., 2018; PRIDGEON et al., 2018). Diferentes vias de efeitos adversos devem, então, ser investigadas por meio de testes de toxicidade, que eram, até recentemente, conduzidos prioritariamente por experimentação animal (HARTUNG, 2010; PRIDGEON et al., 2018). Contudo, fortes pressões sociais quanto ao uso de animais em testes de toxicidade, principalmente voltados a indústria de cosméticos, levaram ao movimento de desenvolvimento de metodologias alternativas enquadradas no princípio dos 3 Rs (*Refinement, replacement and reduction of animal testing*) (RUSSELL; BURCH, 1959; TÖRNQVIST et al., 2014). Além disso, estímulos ao desenvolvimento e uso de testes alternativos também se deram pela problemática de extrapolação de dados entre espécies, que acarretam em falhas na predição de toxicidade ao homem, devido a particularidades fisiológicas e diferenças de complexidades biológicas entre espécies (GARATTINI, 1985; RAM, 2019; RÉGNIER et al., 1992).

As medidas destinadas ao uso de métodos alternativos para a certificação da segurança toxicológica de produtos e componentes de formulações foram iniciadas e consolidadas principalmente em países da União Europeia e Estados Unidos (EUA) (LEE, 2016). No Brasil, o movimento de implementação de métodos alternativos iniciou-se em 2014 com a primeira resolução normativa do CONCEA (Conselho Nacional de Controle de Experimentação Animal) nº. 18 de 24.09.2014, a qual reconhecia a implementação de 17 metodologias alternativas validadas. A utilização de métodos alternativos no Brasil foi se tornando cada vez mais necessária, visto que as metodologias reconhecidas em 2014 pelo CONCEA tiveram um prazo de 5 anos para implementação (DE OLIVEIRA et al., 2011;GUIMARÃES, FREIRE, MENEZES, 2016; BRASIL, 2016; BRASIL, 2014).

Frente a essa situação, o uso de métodos alternativos no Brasil focou primeiramente no desenvolvimento de modelos biológicos similares aos já reconhecidos pelas normas da Organização para a Cooperação e Desenvolvimento Econômico (OECD – *Organisation for Economic Co-operation and Development*)

que pudessem, desta forma, atender à demanda nacional de regulamentação de produtos.

A Epiderme Humana Reconstituída *in vitro* (RHE) é um modelo de cultura tridimensional (3D) de queratinócitos humanos primários empregada para diversas finalidades científicas, principalmente para estudos de exposição dérmica, como avaliação de substâncias irritantes (GROEBER et al., 2016; PEDROSA et al., 2017; POUMAY et al., 2004) e sensibilizantes (COQUETTE et al., 1999, 2003; SAITO et al., 2013) cutâneos, mas também para análises metabólicas de produtos (BERNARD et al., 2000; JIA et al., 2019; VICKERS et al., 1995), análise de respostas imunotoxicológicas cutâneas (PEDROSA et al., 2017; RHEINS et al., 1994) e avaliação da fototoxicidade da pele (BERNARD et al., 2000).

Até o momento, existem seis RHEs validadas, reconhecidas pela OECD e amplamente comercializadas na Europa e EUA, sendo elas a EpiDerm® (MatTek Corporation) (CHAPMAN et al., 2014), a EpiSkin® (L'OREAL) )(ALÉPÉE et al., 2014), a SkinEthic® (L'OREAL) (ALÉPÉE et al., 2016), a LabCyte EPI-MODEL (Japan Tissue Engineering Co., Ltd.) (KATOH et al., 2009), a epiCS® (CellSystems®) (ESAC, 2009; OECD, 2019) e a Skin+® (Sterlab) (OECD, 2019). No entanto, nossas barreias alfandegárias não permitem a aquisição destes produtos biológicos em tempo hábil que garanta a qualidade dos mesmos (viabilidade celular, estrutura da epiderme *in vitro*) (GROEBER et al., 2016; DE WEVER; KURDYKOWSKI; DESCARGUES, 2015). A L'OREAL é a única empresa com modelo de RHE validado presente no Brasil, sendo a responsável por atender toda a demanda nacional. Tais fatos reforçam a necessidade de desenvolvimento e validação de novos modelos de epiderme *in vitro* e metodologias alternativas ao uso de animais em geral para os mais diversos usos na ciência (PIERSMA et al., 2018).

Adicionalmente, mesmo com o aumento das pressões sociais e grandes esforços para o desenvolvimento de métodos alternativos, o uso de animais utilizados em pesquisas científicas ainda é muito alto, chegando a cerca de duzentos milhões de animais por ano (TAYLOR; ALVAREZ, 2019). Grande parte do uso de animais ainda é destinado principalmente para obtenção de conhecimentos de ciências básicas e para o desenvolvimento de medicamentos (TAYLOR; ALVAREZ, 2019). Esse número ressalta a eminente necessidade de desenvolvimento e uso de metodologias alternativas ao uso de animais, de acesso aberto, principalmente em

áreas onde a acessibilidade a essas novas metodologias são limitadas (*e.g.*, pesquisas acadêmicas), visando à redução significativa do uso de animais em laboratórios.

## 1.1 OBJETIVOS

### 1.1.1 Objetivo Geral

Este trabalho teve como objetivo desenvolver um novo modelo RHE e elaborar um protocolo aberto de RHE detalhado e acessível a diferentes setores de pesquisa. Além disso, esse trabalho também objetivou verificar a aplicabilidade do modelo de RHE gerado (Skinvitro-RHE) no teste de irritação dérmica *in vitro*, seguindo diretrizes da OECD TG 439.

### 1.1.2 Objetivos Específicos

a. Avaliar a influência de fatores que potencialmente interferem em culturas de RHE, a fim de desenvolver um novo modelo de RHE que possa ser utilizado para diversos fins científicos;
b. Comparar a similaridade dos modelos de RHE gerados pela análise dos fatores com a epiderme humana por meio de análise histológica e de marcadores de diferenciação por imunofluorescência;
c. Elaborar um protocolo detalhado de construção do novo modelo de RHE;
d. Verificar a reprodutibilidade da metodologia definida para a construção da Skinvitro-RHE quanto às características funcionais deste sistema *in vitro* (morfologia da epiderme e viabilidade celular), utilizando queratinócitos primários de diferentes doadores;
e. Verificar a aplicabilidade da Skinvitro-RHE no teste de irritação dérmica *in vitro* segundo o protocolo padrão OECD TG 439 em termos de controles de qualidade e avaliação com 15 substâncias de referência (7 irritantes e 8 não irritantes).

# 2 REVISÃO BIBLIOGRÁFICA

## 2.1 MÉTODOS ALTERNATIVOS AO USO DE ANIMAIS

Há anos, diferentes espécies de animais vêm sendo utilizadas em pesquisas de desenvolvimento de medicamentos, produtos cosméticos e vacinas, sendo, assim, ferramentas úteis para a compreensão de efeitos causados pelo uso/exposição destes produtos (DOKE, DHAWALE, 2015; HENDRIKSEN, 2009). O número de animais utilizado em pesquisa no mundo vinha crescendo rapidamente e, com isso, William Russell e Rex Burch propuseram em 1959 o princípio dos 3 Rs (*Refinement, Replacement, Reduction*) objetivando: (1) reduzir o número de animais em experimentos (*Reduction*); (2) refinar os testes com animais de forma a minimizar o estresse e dor destes organismos (*Refinement*); (3) substituir, sempre que possível, testes *in vivo* por alternativas metodológicas não baseadas em animais (*in vitro* ou *in silico*) (*Replacement*) (RUSSELL; BURCH, 1959).

A proposta de implementação do princípio dos 3 Rs teve baixo impacto inicial e apenas ganhou força a partir de meados dos anos 80, quando a Comissão Europeia proibiu o uso de animais em experimentos científicos que já apresentassem alternativas de substituição (EC, 1986). Desde então, vários atos e leis foram aprovadas, resultando em um aumento do rigor da experimentação animal regulamentada por comitês de ética do mundo (FLECKNELL, 2002).

Na indústria, a redução do uso de animais iniciou-se na União Europeia a partir da proibição da experimentação animal para avaliação toxicológica de produtos cosméticos e seus ingredientes, regulação que foi totalmente implementada em 2013. Essa medida estendeu-se rapidamente para outros países que passaram adotar legislações semelhantes, como Israel, Índia, Noruega e Nova Zelândia (LAQUIEZE; LORENCINI; GRANJEIRO, 2015) e impactou também outros setores, como a indústria farmacêutica (LEE, 2016).

No Brasil, a iniciativa de utilização de métodos alternativos ao uso animais iniciou-se com a aprovação da Lei 11.794/2008, conhecida como Lei Arouca, que normatizou os procedimentos para uso de animais na ciência (BRASIL, 2008). Com isso, vários comitês de ética em pesquisa utilizando animais (CEUAs) foram criados em instituições de pesquisa (FLECKNELL, 2002).

Além disso, a Resolução Normativa Nº 18/2014 estabelecida pelo CONCEA reconheceu 17 normas baseadas em métodos *in vitro* validados pela OECD, as quais deveriam ser implementadas no país em um período de 5 anos (GUIMARÃES, FREIRE, MENEZES, 2016). A criação da Rede Nacional de Métodos Alternativos (Renama) e o Centro Brasileiro de Validação de Métodos Alternativos (BraCVAM) também vieram com o intuito de ajudar na implementação de métodos alternativos em laboratórios no país, utilizando os 3 Rs como filosofia (GUIMARÃES, FREIRE, MENEZES, 2016).

Para um melhor controle do número de animais utilizados nas pesquisas científicas, vários países, incluindo o Brasil, requerem o envio de dados estatísticos sobre a utilização de animais experimentais (EC, 2020). Esses relatórios abordam dados sobre o número de animais criados para fins científicos e para a manutenção de linhagens (animais convencionais/selvagens ou geneticamente modificados), e os detalhes do uso de animais para pesquisa. De acordo com os dados de 2015 a 2017, cerca de 10 milhões de animais experimentais são utilizados por ano na União Europeia, sendo as espécies mais visadas os camundongos, peixes, ratos e aves. Além disso, as principais áreas de uso de animais são: pesquisa básica (45%), correspondendo à maioria dos usos, seguidos por pesquisa translacional e aplicados (23%) e uso regulatório (23%) (EC, 2020).

## 2.2 TESTE DE IRRITAÇÃO DÉRMICA

A pele humana é um órgão complexo que tem como principal função a proteção contra dessecação e age como uma barreira protetora ao ambiente (MACLEOD, HAVRAN, 2011). Ela é composta por duas camadas, sendo elas a derme e a epiderme (COMBADIERE, LIARD, 2011).

A epiderme é um epitélio estratificado não vascularizado, composto principalmente por queratinócitos e que apresenta constante renovação celular. Os queratinócitos desempenham um papel significativo na manutenção da integridade bioquímica e física da pele, especialmente por meio da produção de citoqueratinas, lipídios e mucopolissacarídeos que evitam a perda excessiva de água e mantêm a correta hidratação da epiderme (BARKER et al., 1991; BOER et al., 2016; SUN; GREEN, 1976). Os lipídios secretados por essas células na matriz intercelular da

camada córnea também contribuem para regular a adsorção de vários componentes da superfície da pele (BARKER et al., 1991). Além disso, os queratinócitos participam ativamente das respostas imunológicas da pele ao interagir com células mononucleares infiltrantes, seja por meio da expressão de moléculas de adesão intercelular, como ICAMs (*Intercellular Adhesion Molecule)*, ou por meio da liberação de diferentes citocinas pró-inflamatórias, que fornecem um sistema de detecção precoce para patógenos e contribuem para os processos que caracterizam várias doenças inflamatórias da pele (BARKER et al., 1991; BONNET et al., 2011; NESTLE et al., 2009). Além disso, os queratinócitos sofrem um processo de queratinização, no qual as células diferenciam-se da camada basal em direção à superfície, formando o estrato córneo, camada mais externa da epiderme. Esta camada é fundamental para a proteção do organismo, uma vez que age como uma barreira contra a penetração de substâncias químicas e microorganismos (PROKSCH, BRANDNER, JENSEN, 2008).

Por ser um órgão que está em contato direto com o ambiente, a pele é suscetível à exposição de diversos agentes químicos/físicos, que podem danificar este órgão ou, ainda, desencadear efeitos adversos via exposição dérmica. Frente a isso, há uma necessidade de identificar agentes químicos com potencial de causar danos a pele, como irritação dérmica (HOFFMANN et al., 2005; WORTH et al., 2014).

Segundo a Agência Nacional de Vigilância Sanitária (ANVISA), a irritação dérmica é um processo inflamatório que ocorre quando uma substância química com potencial irritante entra em contato com a epiderme e ultrapassa a barreira cutânea, gerando uma reação de desconforto local com variações de intensidade, podendo desencadear ardor, coceira, vermelhidão, ou até mesmo corrosão e destruição do tecido (ANVISA, 2012).

Antes da comercialização, os produtos cosméticos devem passar por um processo de avaliação de segurança, que visa identificar os possíveis efeitos adversos causados por esses produtos (*e.g.,* irritação dérmica), a fim de garantir a proteção do consumidor (DANIEL et al., 2018).

A homeostase da pele é complexa e envolve uma multiplicidade de interações entre diferentes componentes do tecido, que foram extensivamente estudadas em sistemas *in vivo*. Modelos animais, particularmente camundongos,

tendem a ser o padrão ouro para a compreensão de patologias relacionadas à pele e avaliação do efeito de vários medicamentos para o tratamento de doenças (LÖWA et al., 2018).

Em 1944, o teste de irritação dérmica *in vivo* de Draize foi desenvolvido para avaliar o potencial irritante de substâncias químicas, na qual mudanças visíveis na pele de coelhos eram avaliadas (DRAIZE; WOODARD; CALVERY, 1944). Entretanto, esse ensaio causava dor e desconforto nos animais, além de apresentar resultados que muitas vezes não poderiam ser extrapoladas ao ser humano. A forte pressão social quanto ao uso de animais em testes toxicológicos levou ao desenvolvimento de métodos alternativos para testes de irritação dérmica, que requerem o uso de modelos que mimetizem a epiderme humana (LEE, HWANG, LIM, 2017).

O modelo de Epiderme Humana Reconstituída *in vitro* (RHE) reproduz a estrutura e complexidade da epiderme *in vivo.* Dessa forma, este modelo proporciona uma promissora abordagem para testes de avaliação de produtos com potencial de irritação dérmica (GROEBER et al., 2016; KIM et al., 2016; THAKOERSING et al., 2013; ROGUET et al., 2000). Além disso, a RHE apresenta uma ampla aplicabilidade para os mais diversos estudos, principalmente toxicológicos (GROEBER et al., 2016; PEDROSA et al., 2019), bioquímicos (JIA et al., 2019), e de doenças (KLICKS et al., 2017).

## 2.3 EPIDERME HUMANA RECONSTITUÍDA

O cultivo de células 2D envolve o crescimento de células em monocamadas, e é amplamente utilizado em diversos ensaios para compreensão de processos biológicos. Entretanto, as principais características fenotípicas e funcionais são perdidas (*e.g.,* diferenciação das células das camadas da epiderme), uma vez que é um modelo que não se assemelha ao encontrado *in vivo*. Já os modelos de cultura 3D são sistemas complexos que mimetizam as características morfológicas e funcionais dos tecidos *in vivo*, podendo ser monotípicos, ou seja, modelos que utilizam um único tipo celular, ou modelos multitípicos (cocultura) (PANPALONI, REYNAUD, STELZER, 2007; BARRILA et al., 2010).

A Epiderme Humana Reconstituída (RHE) *in vitro* é um modelo de cultura 3D, que mimetiza a epiderme humana e pode variar em sua composição celular, podendo ser monotípica (queratinócitos isolados de origem neonatal ou adulta) ou multitípica (co-cultura com melanócitos para modelos pigmentados ou com células de Langerhans para modelos imunológicos) (ALÉPÉE et al., 2014; ALÉPÉE et al., 2016).

A construção deste modelo é dificultada pela falta de interação celular. Sabe-se que, *in vivo*, os fibroblastos apresentam uma influência significativa na estruturação da epiderme pela atuação na diferenciação dos queratinócitos (FRANKART et al., 2012). Desta forma, a reprodução de um modelo epidérmico *in vitro* (RHE) requer o uso de meio de cultivo com adição de diferentes suplementos, que irão promover a proliferação e diferenciação de queratinócitos, associados aos sistemas de cultivo tradicional e à interface ar-líquido. Esta condição promoverá a diferenciação celular, sendo possível obter um modelo epidérmico contendo as 4 camadas da epiderme *in vivo*: camada basal, camada espinhosa, camada granulosa e o estrato córneo (FRANKART et al., 2012).

A RHE *in vitro* representa melhor a função da epiderme *in vivo* do que modelos 2D de cultivo celular utilizando queratinócitos, pois é possível realizar aplicação tópica de substâncias químicas na camada mais externa da pele (estrato córneo) (BARRILA et al., 2010).

## 3 DESENVOLVIMENTO

As sessões a seguir compreendem a descrição dos métodos e resultados obtidos nos experimentos de desenvolvimento da Skinvitro-RHE e da avaliação da aplicabilidade do modelo no teste de irritação dérmica *in vitro*. As sessões foram elaboradas no formato de artigos científicos, como segue.

## 3.1 CAPÍTULO I

**The evaluation of factors influencing cultures of the reconstructed human epidermis (RHE) and a proposal of a detailed open-source RHE protocol to study skin toxicity and biology**

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(Potenciais revistas para submissão: Journal of Biotechnology – fator de impacto 3.5, Tissue and Cell – fator de impacto 1.87)

**Abstract**

The reconstructed human epidermis (RHE) can be used for a wide range of scientific purposes. However, the use of this model is limited in academia compared to the regulatory environment. Limitations of use mainly lie in the manufacturer's availability, which depends on commercial interest, and the lack of a detailed open-source protocol to generate good quality RHE models. To increase the accessibility of RHE models, especially for academia, this study evaluated factors influencing epidermal reconstructions to develop a detailed RHE protocol for different scientific applications. Keratinocytes from adult donors (38-years-old) strongly influenced the quality of the RHE models, displaying disorganized layers and impaired differentiation. On the other hand, RHE models from neonatal keratinocytes cultured in inserts with high pore density and coated with collagen IV (named Skinvitro-RHE) showed a well-stratified epidermis (*i.e.*, multiple layers of viable epithelial cells – 6 to 7 layers and functional *stratum corneum*). The Skinvitro-RHE models also showed histological patterns (H&E) and protein expression markers (Keratin-10, Keratin-14, Filaggrin, Involucrin) similar to those of native human epidermis, as well as high cell viability and effective barrier function when compared to the commercial RHE models. At last, the Skinvitro-RHE models demonstrated reproducibility among batches for all quality criteria evaluated. Unlike other published RHE protocols, this open-source protocol describes in detail the construction of a fully differentiated RHE developed from neonatal keratinocytes and without the use of undefined or unstable components. By presenting this open-source preparation procedure of the RHE model, we expect to contribute to animal testing alternatives in basic and applied research, which accounted for substantial use of animals according to statistic reports of 2020.

**Keywords:** *in vitro* models, reconstructed human epidermis, culture condition, skin research.

## 1. Introduction

The epidermis is an essential tissue of the human body, which has the primary roles of preventing skin dehydration and loss of nutrients and protecting the body against physical and chemical agents in contact with our skin (CAPALLERE et al., 2018; POUMAY; COQUETTE, 2007). Epidermis also has an important role in the immunological process, providing the first line of defense of our body against potentially pathogenic microorganisms or toxic chemicals (BOER et al. 2016; ELIAS, 2007; NESTLE et al., 2009). The epidermis is composed of different cell types and organized in a multilayered structure. Keratinocytes are the major cells of this tissue, making up around 95% of the cell mass of the human epidermis. Thus, keratinocytes take place in many skin biological processes, being target cells of most skin research. For instance, keratinocytes are involved in maintaining the biochemical and physical integrity of the skin, especially via the production of cytokeratins, lipids, and mucopolysaccharides that prevent the excessive loss of water and keep the correct hydration of the epidermis (BARKER et al., 1991; BOER et al., 2016; SUN; GREEN, 1976). The lipids secreted by these cells in the intercellular matrix of the *stratum corneum* also contribute to regulating the adsorption of various components from the surface of the skin (BARKER et al. 1991). Moreover, keratinocytes actively participate in skin immune responses by interacting with infiltrating mononuclear cells either via expression of intercellular adhesion molecules or via the release of different pro-inflammatory cytokines, which provide an early detection system for pathogens or hazardous chemicals and contribute to the processes that characterize several inflammatory skin diseases (BARKER et al., 1991; BONNET et al., 2011; NESTLE et al., 2009).

A variety of keratinocyte-based models are available and can be used in many ways for performing skin research, ranging from safety assessments to efficacy determinations of new drugs or cosmetics (ODRASKA et al., 2011; CAPALLERE et al., 2018 e PEDROSA et al., 2017). Two-dimensional (2D) monolayer models of keratinocytes have the advantages of not requiring intensive handling, and they are low-cost and good models to understand basic mechanisms of the skin (MORROW; LECHLER, 2015). However, cells grown in 2D monolayers cannot capture the relevant complexity of the *in vivo* microenvironment (BROHEM et al., 2010), and thus, bioengineered approaches for developing three-dimensional (3D) epidermal

models gained attention and investments during the last years. The reconstructed human epidermis (RHE) model uses human-derived non-transformed keratinocytes as a cell source to reconstruct an epidermal model with histology and cytoarchitecture similar to those of the human epidermis (POUMAY; COQUETTE, 2007). Several RHE models have been made commercially available, and the most common models are the EpiSkin® (EpiSkin, L'Oréal, Lyon, France), developed from adult keratinocytes isolated from women breast skin (ROGUET et al.,1994), and the EpiDerm™ (MakTek Corporation, Ashland, MA, USA), developed from neonatal foreskin-derived keratinocytes (CANNON et al., 1994). RHE models have been used in a wide variety of studies, including metabolic analyses of diseases and pharmaceutical products (BERNARD et al., 2000; JIA et al., 2019; VICKERS et al., 1995), cutaneous immunotoxicity responses (PEDROSA et al., 2019; RHEINS et al., 1994), determination of absorption properties (GYSLER et al., 1999; SCHÄFER-KORTING et al., 2008), as well as the assessment of skin phototoxicity (BERNARD et al., 2000), irritation (DE BRUGEROLLE DE FRAISSINETTE et al., 1999; GROEBER et al., 2016; PEDROSA et al., 2017) and sensitization (COQUETTE et al., 1999, COQUETE et al., 2003; SAITO et al., 2013). However, the use of RHE models is currently more pronounced in the regulatory context and the industry than for other scientific purposes, such as basic and applied research, in which the use of a high number of animal models is still in place. Statistic reports from the European Union showed that basic and applied research accounts for 69% of the total number of experimental animals used in Europe, which is much higher than the regulatory area (23%) that is now strongly engaged in promoting alternatives to animal testing (EC, 2020).

The lack of adoption of RHE models lies in the fact that commercially available models are rarely affordable by small and medium-companies or academic laboratories to conduct their investigations (POUMAY et al., 2004). Furthermore, dependence on the availability of the model, the production scale, and discontinuation of production are some of the limitations that can put at risk the work of laboratories that rely on such models for their analyses (SOUTHEE et al., 1999). Difficulties in controlling specific culture parameters (including culture conditions, the origin of cells, supplementation, and other requirements) also represent a problem for the researcher to conduct the experiments since only partial information of the

preparation of the skin model is provided by the manufacturers, and the information is never complete enough to allow extensive and reproducible production of the tissues (COQUETTE et al., 2000; GROEBER et al., 2016; POUMAY et al., 2004). Therefore, extensive efforts in developing accessible RHE models for different scientific purposes are needed to reduce the use of animals and improve the quality of research.

For this study, we have investigated factors reported to influence RHE cultures to develop a detailed open-source RHE protocol accessible for any laboratory and potentiality to be applied for different scientific purposes (*e.g.*, from skin biology to skin toxicity studies). The evaluated factors were the age of keratinocyte donors (adult or neonatal donors), type of coating of the inserts (recombinant type-1 collagen or collagen IV), pore density of inserts ($1x10^6$ pore/$cm^2$ or $1x10^8$ pore/$cm^2$), type of plate (standard plate or deep well plate), type of culture media and composition of supplements (low or high amount of supplements), time in submerged culture (24-96 h). Similarity with the native human epidermis of the epidermal reconstructions was evaluated by parameters of morphology (H&E) and patterns of protein expression markers (Keratin-10, Keratin-14, Filaggrin, Involucrin). Cell viability (optical density acquired by MTT assay), the integrity of barrier function (determination of $IC_{50}$ with a cytotoxic benchmark chemical), and reproducibility among production batches were also quality criteria parameters considered in this study to define the best RHE model and, consequently, the best protocol of construction.

## 2. Material and Methods

### *2.1. Cell culture*

Cell culture medium, medium supplements, and reagents were obtained from Gibco® unless otherwise indicated.

#### 2.1.1 *Human primary keratinocytes culture*

For this study, both neonatal and adult human keratinocytes (HEKn and HEKa, respectively) were used to compare the quality of RHE models generated by healthy young and adult donors. HEKn were isolated from foreskin samples (1 to 4-year-old donors) (Local Ethical Committee Approval –

CAAE: 84000016.9.3001.0097) according to the protocol of Aasen and Belmonte (2010), with some modifications. Foreskin samples were cut into pieces, and the tissue fragments were incubated in a 6 ml of cold dispase II solution (1 mg/ml) overnight, at 4 ºC, and under 0.1 g orbital rotation. The epidermis was then separated from the dermis, and epidermis fragments were incubated in 0.05% v/v trypsin solution for 28 min to obtain a single-cell suspension. The cell suspension was placed in culture flasks containing EpiLife medium supplemented with Human Keratinocyte Growth Supplement (HKGS) and incubated for seven days in a humidified atmosphere with 5% $CO_2$ at 37 °C. After the seven days, cells were subcultured, controlling the trypsinization time to remove any melanocyte from the HEKn cultures. Pure HEKn cultures at passages 2 to 5 were used in the construction of the Skinvitro-RHE models.

HEKa from 38-year-old donors (Gibco®) were grown in EpiLife medium, with a medium change every two days until reaching 80% confluence. HEKa cultured until passage three was used for RHE constructions. The number of passages was determined according to the recommendations of the manufacturer (Gibco®)

### *2.1.2. Reconstructed human epidermis (RHE)*

3D epidermal cultures can be affected by several factors that may comprise the quality of RHE models. Thus, in our study, we selected a set of factors and investigated their influence on the RHE quality, providing, in the end, an accessible, easy and reproducible RHE protocol. Factors selected for this study are listed in Table 1; and the development of our RHE protocol was based on modifying published protocols (KUNINGER; GOPINATH; NEWMAN, 2014; LEMPER; DE PAEPE; ROGIERS, 2014; PEDROSA, et al., 2017; POUMAY et al., 2004), especially for avoiding the use of unstable and undefined components that can affect the model reproducibility. The final Skinvitro-RHE protocol description (referred to as open-source RHE protocol) can be verified below.

**Table 1**. Factors evaluated to define the open-source protocol for generating RHE models.

| Category of factors | Factors evaluated | The best alternative for the open-source RHE protocol |
|---|---|---|
| Age of Keratinocyte Donors | Adult keratinocytes (HEKa)<br>Neonatal keratinocytes (HEKn) | HEKn<br>Neonatal (1 to 4 years old) |
| Type of coating of the inserts | Recombinant type-1 collagen<br>Collagen IV | Collagen IV |
| Type of culturing plate | Standard plate<br>Deep well plate | Deep well plate |
| Type of culture media | EpiLife<br>DMEM + Ham's F-12 | DMEM + Ham's F-12 |
| Composition of supplements in the culture media | Low amount of supplements<br>High amount of supplements | High amount of supplements |
| Time in submerged culture | 24 h<br>48 h<br>72 h<br>96 h | 96 h |
| Humidity condition of the $CO_2$ incubator | Low humidity<br>Normal humidity | Normal humidity |
| Pore density of the inserts | Low pore density ($1x10^6$ pores/cm²)<br>High pore density ($1x10^8$ pores/cm²) | High pore density |

*2.1.2.1. Open-source RHE protocol*

The construction of RHE models was divided into three stages: (1) The preparation of the acellular layer, (2) The preparation of the cellular layer and submerged culture, (3) Air-liquid interface culture. All stages of construction are described in detail below.

*(1) Preparing the acellular layer*

First, a sterile solution of 0.03 mg/ml collagen IV (Sigma-Aldrich, C5533) was prepared in DMEM high glucose medium with sodium pyruvate. This solution needs

to be freshly prepared, and thus, it is was prepared on the same day as the initial assembly of the organotypic culture. After the collagen IV solution was prepared, 150 µl of the solution was added to each cell culture insert (polycarbonate membrane, 0.4 µm pore size, pore density of $1\times10^8$ pores/$cm^2$). The inserts were incubated for at least 3 h at room temperature (RT) on a flat, non-vibrating, sterile surface, and then the remaining collagen was removed from the inserts using a micropipette.

*(2) Preparing the cellular layer and the submerged culture*

The cellular layer was prepared by seeding $4\times10^5$ of HEKn, in 376 µl of the growth medium, inside each insert and adding 1400 µl of the growth medium outside each insert. The inserts were incubated in a humidified atmosphere with 5% $CO_2$ at 37 ºC for 96 h. The culture medium was replaced every day of the submerged culture (96 h).

*(3) Air-liquid interface culture*

After the submerged culture, the whole culture medium was removed from inside the insert to start the air-liquid interface culture. The culture media was aspirated with a micropipette, and the outermost part of the organotypic culture was gently dried using a sterile filter paper. Inserts were incubated for ten days, replacing the culture media every two days. The RHE models were fully formed and ready to be used on the fourteen-day after the initial organotypic culture.

### *2.2. Quality parameters of RHE models*

The quality of RHE from young and adult donors was evaluated by verifying similarities with native human epidermis in terms of morphology and protein expression markers. RHE quality was also verified by parameters of cellular viability and epidermal barrier function. In addition, all of these parameters were used to demonstrate that each batch of RHE models generated by the open-source RHE protocol met defined production release criteria.

#### *2.2.1. Morphology and protein expression markers*

##### *2.2.1.1 H&E staining*

A histological evaluation of epidermal morphology was performed on the RHE models generated. To do so, RHE models were fixed in 2% paraformaldehyde solution (2 h at RT), cryoprotected in 15% and 30% sucrose solutions (2 h in each solution at RT), then embedded in Tissue-Tek® (Fisher Scientific) and frozen at -80 ºC. 5 µm thick sections were obtained using a cryostat (Leica Biosystems) at -23 ºC. The sections were placed onto slides coated with Biobond Tissue Section Adhesive (EMS). Later, the slides were gently washed with PBS to remove the Tissue-Tek and stained by placing the slides first in hematoxylin for 30 s and then in eosin for another 30 s. Slides were washed twice with water after incubation in hematoxylin and eosin solutions to remove dye residues. The tissue sections were dehydrated in several washes of alcohol and xylene (alcohol 70% to alcohol 100%, 1-min washes in each solution, absolute alcohol: xylene: 1:1-v/v and xylene PA, 3-min washes in each solution), and then slides were mounted with Entellan® mounting medium (Merck). The tissue sections were analyzed under a light microscope (BX51 Olympus) at 100 x magnitude.

*2.2.1.2 Immunodetection of protein expression markers*

Marker expression of Keratin 10 (CK10), Keratin 14 (CK14), Involucrin and Filaggrin were evaluated by immunofluorescence. RHE models were fixed, embedded in Tissue-Tek, and cut in 5 µm sections as above described. The tissue sections were placed on top of slides, the slides were fixed with 2% paraformaldehyde solution for 40 min at RT, and then slides were washed 3x with PBS. The slides were incubated first in a 0.1 M glycine solution containing 0.01% saponin (5 min at RT) and then in a 1% BSA solution containing 0.01% saponin (20 min at RT) to block the free aldehyde sites and permeabilize cell membranes. After that, the tissue sections were incubated overnight in the dark at 4 ºC with the primary antibodies (Involucrin, CK10 and CK14 1:200 dilution (all from Invitrogen), Filaggrin 1:100 dilution (Sigma)) diluted in antibody dilution buffer (1% BSA with 0.01% saponin). The next day, the slides were washed 3x with PBS and incubated for 1 h in the dark at 4 ºC with the secondary antibodies (Alexa fluor 488 goat anti-IgG1 mouse and Alexa fluor 633 rabbit anti-IgG goat, both 1:400 dilution and from Invitrogen) diluted in 0.01% saponin solution. PBS was the dilution vehicle of all solutions. The slides containing the labeled tissue sections were mounted with DAPI Fluoromount-

G® (Invitrogen) mounting medium, and slides were analyzed under confocal fluorescent microscopy (ECLIPSE Ti-E Nikon Inverted Microscope) at 60x of magnification. Images were processed and compiled using ImageJ software (FIJI, Image J version for 3D images).

### *2.2.2. Cellular viability*

At the end of the RHE construction (fourteen-day after the initial organotypic culture), cellular viability was measured by the MTT colorimetric assay. Fully-formed RHE models were washed 25x with PBS and transferred to a 6-well plate containing 2 ml of DMEM:Ham's F12 (3:1-v/v) without supplementation (maintenance medium). The plate was incubated for 42 h at 37 ºC in a 5% $CO_2$ incubator, and then the *in vitro* models were incubated with 1 mg/ml of MTT solution for 3 h at 37 ºC in the dark. To extract the formazan from the tissues, RHE models were placed in a 24-well plate containing isopropanol and incubated for 2 h under orbital shaking (120 x g). 200 µl of each extraction solution were transferred into a 96-well plate (triplicate/extraction), and the optical density (OD) was measured at 570 nm using Tecan microplate reader (Tecan Infinity, M200). The acceptability range for the OD values of non-exposed RHE models was defined in this study by OD ranges of commercial RHE models (*e.g.*, EpiSkin®, SkinEthic®) (lower acceptance limit ≥0.6, upper acceptance limit ≤3.0).

### *2.2.3. Barrier function*

The epidermal barrier function evaluation verifies if the *stratum corneum* contains the essential lipid profile to produce a functional skin barrier. The epidermal barrier function can be evaluated by exposing RHE models to a cytotoxic benchmark chemical followed by the determination of the concentration at which a benchmark chemical reduces the tissue viability by 50% ($IC_{50}$) at a fixed exposure time (ALÉPÉE et al., 2010; ESAC, 2008). Thus, we evaluated the effect of the benchmark chemical Sodium Dodecyl Sulfate (SDS) at concentrations of 0.625, 1.25, 2.5 and 5 mg/ml and exposure time of 18 h. Cell viability was quantified by MTT reduction assay, as described above, to determine the $IC_{50}$. The barrier function test was performed in triplicate.

## 3. Results and discussion

RHE model represents a significant improvement compared to the use of monolayer cultures for skin research. However, its use is not widely adopted and it is limited for some scientific purposes, such as basic and applied research. In general, limitations in the use of RHE models are related to high costs, market restrictions of commercial RHE and restricted information of production, as knowledge is essential depending on the scientific application of the model. This study aimed to evaluate factors that can affect RHE cultures and develop an accessible RHE protocol for different types of laboratory, especially academic laboratories, in which more efforts are needed to foster alternatives to animal testing. Important issues to avoid problems in the quality and reproducibility of RHE models are discussed.

A crucial aspect of obtaining good quality 3D epithelial tissues is the adequate condition of the air-liquid interface (ALI) culture. The proper maintenance of ALI is required to generate well-structured *in vitro* human tissues because epithelial cell differentiation strictly depends on the contact with high oxygen levels (NGO et al., 2007). Pore density of cell culture inserts, type of cell culture plate, type of matrix protein coating, and humidity of $CO_2$ incubator are factors that significantly influence ALI culture. In general, published RHE protocols use inserts with 0.4 µm pore size. However, protocols for epithelial models vary in the use of inserts with low ($1x10^6$ pores/cm²) (Fig. 1a, c) or high ($1x10^8$ pores/cm²) (Fig. 1b, d-h) pore density, as well as types of culture plate (standard or deep well plates) (ABDAYEM et al., 2015; POUMAY et al., 2004). Although the low pore density of inserts contributes to avoiding moisture in the inner part of inserts at ALI cultures, we verified it did not promote proper nutrition of the epidermal tissue, resulting in a thin epidermal reconstruction (24.4 µm of thickness and 1-2 layers), which is far from resembling the native human epidermis. The standard cell culture plate is the main type of plate used to reconstructed *in vitro* skin tissues. However, the insert position into the plate not only favors extending the time interval of medium exchange during tissue growth but also contributes to achieving a drier condition in the ALI cultures (SCOTT et al., 2016).

Culturing keratinocytes on top of cell culture inserts usually requires pre-coating with matrix proteins to adequately support cell proliferation and growth. The matrix proteins mostly recognized to promote keratinocytes growth onto inserts are

animal-derived matrices. Cell-based 3D systems are seen as alternative *in vitro* models in compliance with the replacement of animal testing. To better promote animal testing alternatives, it has been advisable to eliminate animal-derived products in cell culture (CARVALHO et al., 2013; EL GHALBZOURI et al., 2008). Thus, we analyzed whether an animal-free matrix would be as useful in developing the RHE models. For this study, the coating matrices used were recombinant human type-1 collagen (Fig. 1a) and collagen IV (Fig. 1b-h). RHE reconstructions with type-1 collagen coating presented only basal and corneal layers of the epidermis, indicating that the coating impaired cell growth, especially under submerged conditions, which compromised the later differentiation and stratification of keratinocytes in culture. Differences in collagen types may have influenced the keratinocyte growth in our 3D cultures. However, the successful use of the selected collagens (I and IV) in developing 3D epidermal tissues is well documented in the literature (NETZLAFF et al., 2007; PEDROSA, et al., 2017; RASMUSSEN et al., 2010; ROGUET et al., 1994; WHA KIM et al., 2002). We also verified the necessity of ensuring the full coverage of the inserts with the matrix protein to obtain good quality RHE models. The inappropriate covering of the inserts increases moisture in the tissue during ALI culture and stimulates cell proliferation at stages in which differentiation should prevail. Thus, the improper insert covering results in RHE models with proliferating cells located above corneal layers, showing a completely inappropriate epidermis structure (Fig. 1a).

Chemically defined cell culture media are relevant in ensuring tissue reproducibility for a wide variety of toxicological and other skin research. Some RHE protocols reported using conditioned medium from primary fibroblasts as a supplement of the 3D epidermal culture media (PEDROSA, et al., 2017). Unstable reagents may also affect the reproducibility of 3D epidermal models. Thus, it is desirable that cells are cultured under conditions that minimize the presence of undefined or unstable components to ensure good quality among the production batches of RHE models. Also, reducing the number of supplements is desirable to reduce costs. As the composition of RHE culture media vastly varies among published protocols, we aimed to identify and evaluate components that may bring uncertainties in experimental data acquired using RHE models. We also aimed to achieve a medium formulation with as few supplements as possible. For that, we

tested two final media formulations composed by: (1) EpiLife™ supplemented with HKGS (Human Keratinocyte Growth Supplement), KGF (Keratinocyte Growth Factor) (10 ng/ml), calcium chloride ($CaCl_2$) (140 µM: submerged condition; 1.7 mM: air-liquid condition) and ascorbic acid (50 µg/ml) (medium 1 – low supplementation); and (2) DMEM and Ham's F-12 (3:1 vol/vol) supplemented with cholera toxin (0.1 nM), insulin (5 µg/ml), apo-transferrin (5 µg/ml), hydrocortisone 21-hemisuccinate (0.4 µg/ml), EGF (1 ng/ml) and TGF-α (2 ng/ml) (medium 2 – high supplementation), both of them not including any undefined component. However, medium 1 presented an unstable reagent, ascorbic acid, which is reported as a vitamin that improves the barrier characteristics of 3D skin tissues (POUMAY et al., 2004). Although epidermal reconstructions with medium 1 displayed a well-formed corneal layer, fewer layers of epidermis were observed (Fig. 1c) compared to RHE constructed using medium 2 (Fig. 1d-h). Media with low supplementation probably do not stimulate the adequate cell proliferation level in submerged conditions required to achieve multilayered epidermis. In the development of this protocol, we also verified that an increase in the time of culturing cells in submerged conditions to 96 hours resulted in a higher confluence of monolayer cells on top of the inserts, which promoted a higher degree of differentiation/stratification of keratinocytes and resulted in an RHE with 66.7 µm of thickness (Fig. 1h). These results are in contrast to RHE of 48-hour submerged culture, which resulted in an RHE with 50.2 µm of thickness (Fig. 1d, e, g). Extending the culturing time in submerged conditions for a period longer than usually recommended by published RHE protocols (48 hours) increases the number of layers. This improvement is probably due to the high growth rate of keratinocytes, which later favored cellular differentiation and resulted in an RHE model with higher similarity to native human skin (CARLSON et al., 2008).

Another issue about published RHE protocols is the consensus of normal $CO_2$ incubator humidity or lowered humidity at ALI culture when constructing the epidermal model (PEDROSA, et al., 2017). Some studies have shown that lowering humidity by 50% enhances the barrier properties of 3D epidermal tissues (CAU et al., 2017; SUN et al., 2014). Since not all types of incubators have humidity module control, the water pan can be removed or partially covered with plastic film to reduce water evaporation and lower the humidity of the $CO_2$ incubator (SUN et al., 2014). The way of lowered humidity without a humidity module control cannot ensure equal

humidity conditions in all tissue reconstructions, affecting tissue batch reproducibility. In our RHE reconstructions under normal and 50% lowered humidity, no significant differences in the corneal layer formation were observed. Recently, Mieremet et al., (2019) demonstrated that, although external relative humidity influences epidermal morphogenesis, the lipid barrier formation is similar in conditions of normal and lowered humidity during *in vitro* development of human skin tissues (MIEREMET et al., 2019).

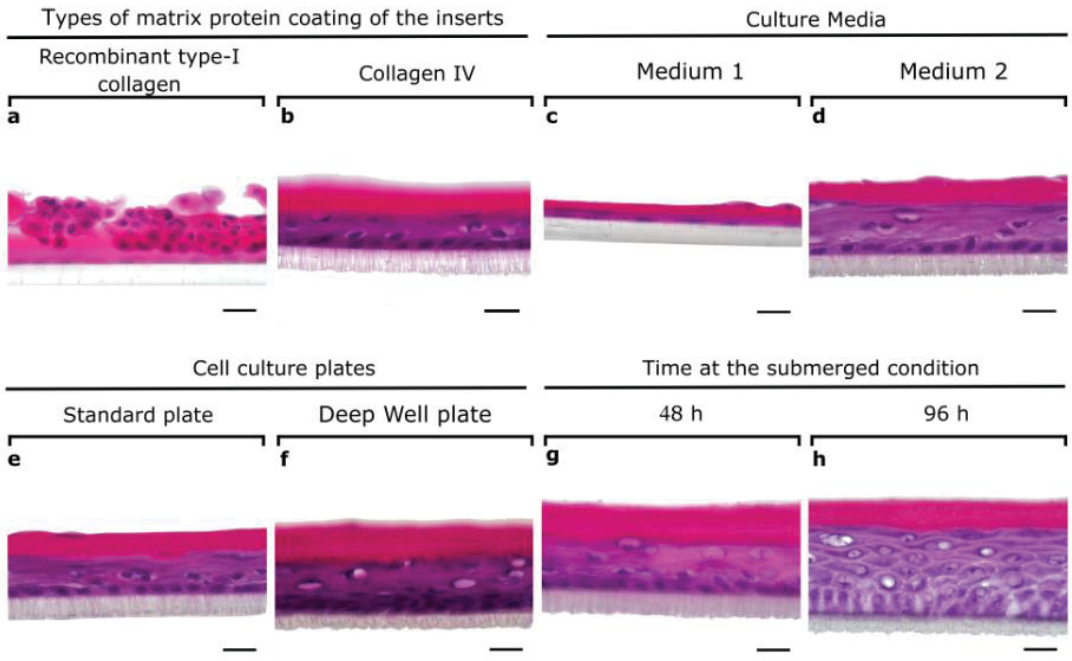


**Figure 1. The influence of culturing variations on the construction of reconstructed human epidermis (RHE).** (a-b) Variations in the types of matrix protein coating of the inserts. (a) Insert covered with recombinant type-1 collagen, RHE with low stratification, and presenting proliferating cells above the corneal layer. (b) Insert coated with collagen IV, RHE showing a higher number of epidermal layers and absence of proliferating cells in inappropriate locations. (c) RHE constructed using culture media with low supplementation (medium 1: EpiLife™ supplemented with HKGS, KGF (10 ng/ml), $CaCl_2$ (140 µM: submerged condition; 1.7 mM: air-liquid condition) and ascorbic acid (50 µg/ml)). (b, d-h) RHE constructed using culture medium with high supplementation (media 2: a mixture of DMEM and Ham's F-12 culture media supplemented with cholera toxin (0.1 nM), insulin (5 µg/ml), apo-transferrin (5 µg/ml), hydrocortisone 21-hemisuccinate (0.4 µg/ml), EGF (1 ng/ml)

and TGF-α (2 ng/ml)). (e-f) Evaluation of cell culture plate types, (e) standard plate, and (f) deep well plate. (g-h) Evaluation of the influence of culturing time at the submerged condition, (g) 48 hour-submerged culture and (h) 96 hour-submerged culture. Hematoxylin and eosin staining. Scale bar, 20 µm.

Good barrier function is required for the scientific use of RHE models. The barrier function can be assessed by determining the median inhibitory concentration ($IC_{50}$) in cell viability using a cytotoxic benchmark chemical (SDS) (MOLINARI et al., 2013; PORTES et al., 2002; RÉGNIER et al., 1992). Under normal humidity conditions, the Skinvitro-RHE model showed values (average $IC_{50}$ of 2,77 mg/ml, Fig. 2a) similar to the upper levels of optical density obtained to commercial RHE models for skin irritation and corrosion tests (KATOH et al., 2009, 2010).

The optical density of the negative control of all lots was evaluated, and the average OD of the Skinvitro-RHE obtained was 1.7 with lower (LL) and upper (UL) acceptance limits determined statistically among ten batches of RHE models, of 0.62 and 2.88, respectively, in accordance with the viability intervals acceptable by OECD 439 ($0.6 \leq x \leq 3.0$) and conforming to commercialized RHE (Fig. 2b).

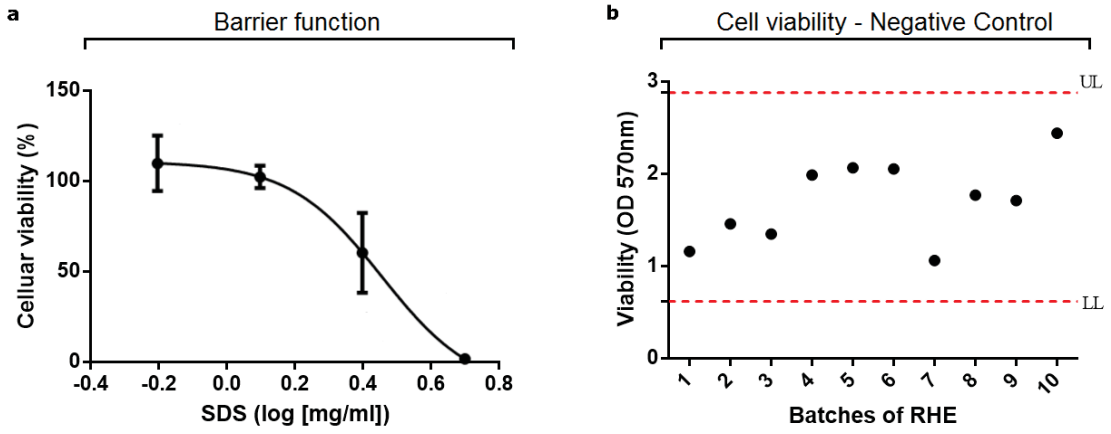


**Figure 2. Quality control tests.** (a) The barrier function integrity test of the Skinvitro-RHE showed an IC50 of 2,77 mg/ml. (b) Optical Density (OD) of the negative control. Each point represents the OD of one tissue (n = 10) treated with PBS and measured at 570 nm in MTT assay. UL: Upper acceptance limit; LL: Lower acceptance limit (by

skin irritation and corrosion tests). Our analysis showed average viability of 1,7 OD for the Skinvitro-RHE negative controls.

We also performed several epidermal reconstructions under the same protocol condition (optimized protocol) with keratinocytes from donors of ages 1-4 years old (yo) (neonatal cells) and 38 yo (adult cells). H&E histological staining of Skinvitro-RHE cross-sections showed a well-stratified epidermis in the tissues sourced from donors until age 4 yo (Fig. 3a). In this model constructed with neonatal cells, 7-8 layers of epidermis were observed and included a basal layer with columnar cells, a spinous layer, a granular layer and a corneal layer. This model showed extreme similarity when comparing the structure of the neonatal-derived RHE model with the native human epidermis (Fig. 3b). In contrast, RHE models from cells of adult donors displayed thinner structures with disorganized layers and impaired differentiation. The pattern of differentiation expression markers of keratinocytes did not demonstrate the proper development of the epidermal equivalent constructed with adult cells contrarily to the neonatal-derived RHE model. In the neonatal-derived RHE model, these markers had the same expression pattern as the native human epidermis, confirming the high similarity between this *in vitro* model with its *in vivo* counterpart. CK 14 showed exclusive expression at the basal layer, while CK 10, an early differentiation marker, was expressed in suprabasal layers. Involucrin was mainly expressed in the upper spinous and granular cell layers, and Filaggrin was found in the granular cell layer (Fig. 3c). Donor age can influence keratinocyte cultures in the quantity of skin senescent cells. Cellular senescence alters the expression of key molecules involved in cell growth. In older individuals, the epidermis is thinner than the epidermis from younger individuals because the population of proliferating keratinocytes at the basal layer is significantly reduced, and cell junctions are suffering from flattening (JENKINS, 2002; LIAO et al., 2013). Thus, age directly impacts skin characteristics, and the use of adult keratinocytes in RHE constructions may limit the number of end uses of this model. Nevertheless, reconstructions of epidermal tissues from different donor ages are of interest for aging studies.

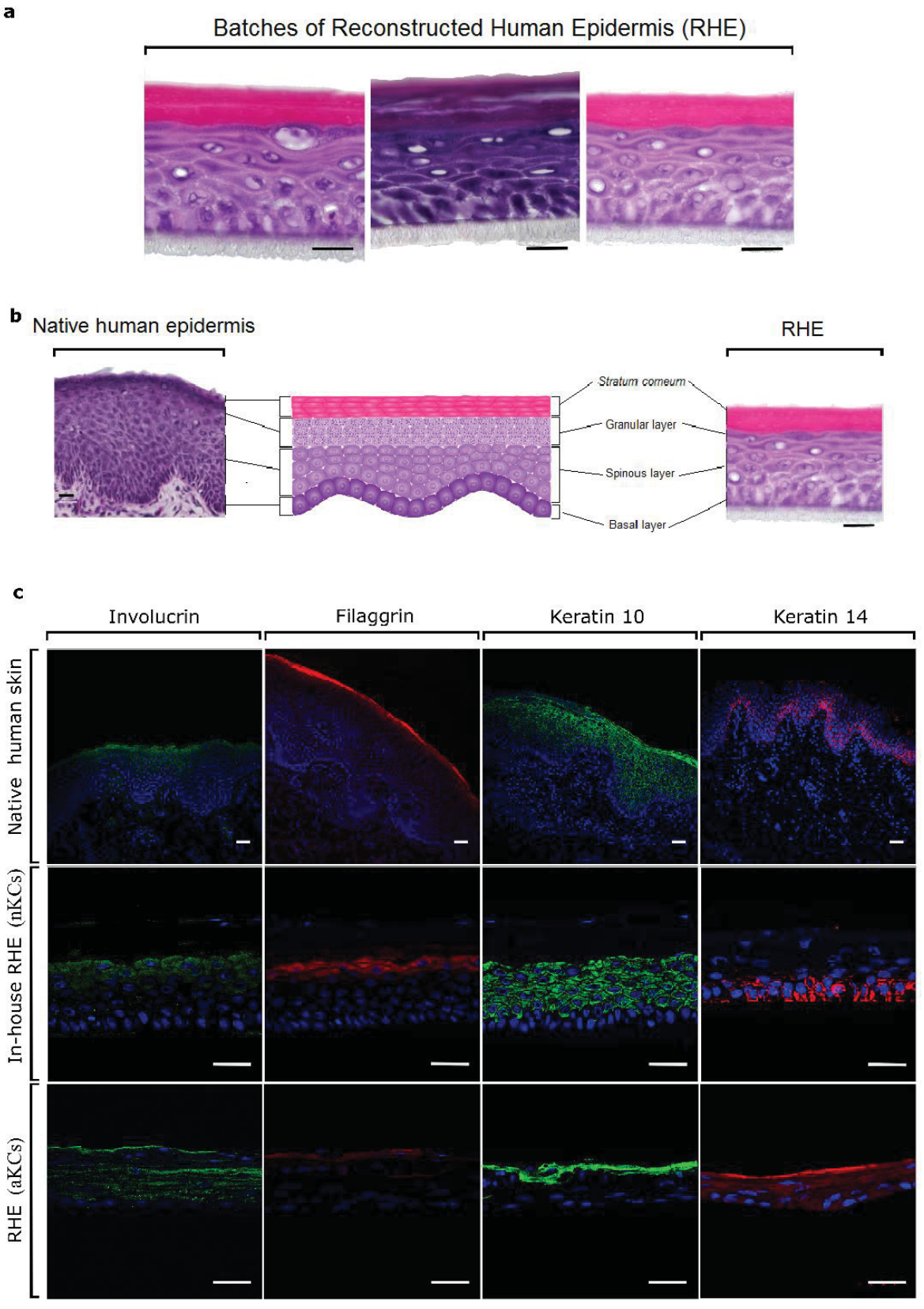


**Figure 3. Verification of the Skinvitro-RHE quality by the morphological similarity with the human epidermis (*in vivo*), histological images stained with H&E**. (a) Skinvitro-RHE models built from neonatal keratinocyte cultures from different donors that show the reproducibility of the standardized construction

protocol. (b) Representative epidermis scheme and histological images of human native skin and Skinvitro-RHE stained with H&E, showing all epidermal layers (basal layer, spinous layer, granular layer, and *stratum corneum*). (c) Immunostaining of frozen sections of epidermal differentiation markers in the native human epidermis, Skinvitro-RHE model generated from neonatal keratinocytes and RHE model generated from adults keratinocytes, harvested on day 11 after air-liquid condition. The differentiation markers Involucrin (green) is expressed in the upper part of the spinous layer and granular layer; Filaggrin (red) is expressed in the granular layer; Keratin 10 (green) is expressed suprabasally, and Keratin 14 (red) is expressed in the basal layer. Blue color represents the staining of cell nuclei with DAPI. The panels show merged confocal images of DAPI and secondary antibody fluorescence (Alexa 488 or Alexa 633 conjugated secondary antibodies). Black scale bar, 20 µm (H&E) and white scale bar, 40 µm (Immunofluorescence).

## 4. Conclusion

In conclusion, we demonstrated that several factors significantly influence RHE cultures, and optimization of the 3D culture condition is fundamental to obtain RHE models for various types of skin research. We identified the best conditions to generate good quality RHE models and developed an accessible and detailed protocol for obtaining 3D epidermal models. After only 14 days (4 days of submerged culture and 10 days of ALI culture), the Skinvitro-RHE developed from neonatal keratinocytes displayed a multilayered epidermis comparable to the native human epidermis, both morphologically and biochemically. To our knowledge, this article is the first to describe in detail a method of constructing an RHE model in order to widen its use for those laboratories that do not have access to the commercial epidermal tissues or those researchers who need transparency in the information of RHE construction to perform their scientific studies. This robust open-source model may serve as a valuable tool for performing a variety of skin researches.

**Acknowledgments**

The authors would like to thank the National Council for Scientific and Technological Development (CNPq) and the São Paulo Research Foundation (FAPESP), for the scholarships (2017/12506-7 and 2019/07710-0) and financial support (2016/06985-7), and the Advanced Fluorescence Technologies Center (CTAF) at Federal University of Paraná (Curitiba-PR, Brazil) for technical assistance in images acquisition.

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## 3.2 CAPÍTULO II

**A novel 3D epidermal model for evaluating skin irritants: performance as skin irritation model and comparison with validated models**

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(Potencial revista para submissão: Journal of Pharmacy and Pharmacology – fator de impacto 2.57)

**Abstract**

**Objectives** Chemical exposure is associated with skin irritation, an innate inflammatory process in response to direct injury to the skin; thus, most chemicals need to be tested regarding their irritancy potential to human skin. While commercial reconstructed human epidermis (RHE) models have regulatory acceptance to test skin irritation, Skinvitro-RHE models can add value for *in vitro* skin irritation testing under non-GLP conditions. This study reports the performance of a novel open-source Skinvitro-RHE as a skin irritation model by using reference chemicals and comparing the results with validated RHE models of the OECD Test Guideline (TG) 439.

**Methods** We used an Skinvitro-RHE model developed by our group and published as an open-source protocol. This complies with the quality criteria of morphology, cell viability and barrier function. To validate it as a skin irritation model, we evaluated the irritation potential of 15 reference chemicals listed in the OECD TG 439 and used the validated reference method (VRM) SkinEthic® RHE.

**Key findings** The Skinvitro-RHE model correctly identified the known skin irritants and non-irritants, showing the specificity of 85,71 %, sensitivity of 100 %, and accuracy of 93,33 %. Comparing these results with validation studies of the OECD TG 439 RHE models, our Skinvitro-RHE has similar or superior performance as a skin irritation model than the validated models.

**Conclusion** Our Skinvitro-RHE model performed well for the *in vitro* skin irritation testing with similar results to those models listed in OECD TG 439. Therefore, this model can be used to conduct irritant testing under non-GLP conditions, contributing to better dissemination of alternative testing for scientific research and reduction of the usage of experimental animals worldwide.

**Key-words:** alternative test method, open-source RHE model, skin irritation model, OECD TG 439.

## 1. Introduction

The human skin is constantly exposed to different types of chemicals, including cosmetics, pharmaceuticals, and personal care products. Before these

products are launched into the market, critical safety testing approaches need to be employed in order to evaluate the potential of a particular ingredient or final formulation to cause dermal irritancy (ZUANG et al., 2005; COSTIN; RAABE; CURREN, 2009). Skin irritation is one of the most common adverse effects following dermal exposure to chemical and physical agents (GOH et al., 1984; COSTIN; RAABE; CURREN, 2009). It is characterized by the reversible damage of the tissue following exposure to certain compounds (UNITED NATIONS, 2017) and involves a series of complex biochemical and cellular responses that act to locally contain the effect of substances potentially harmful to the body (WELTFRIEND et al., 1996; COSTIN; RAABE; CURREN, 2009). Typically, irritant chemicals damage the barrier function of the *stratum corneum*, the outermost layer of the epidermis, and subsequently affect the viability of the living keratinocytes in the epidermal layers underneath (COSTIN et al., 2009).

Dermal irritancy was originally assessed via the Draize *in vivo* rabbit test introduced in the 1940s (DRAIZE; WOODARD; CALVERY, 1944), in which a test substance is applied to the eye or skin of a non-anesthetized rabbit, followed by the subsequent evaluation of signs of irritation such as erythema and edema. This approach was initially used to predict the hazardous effects of cosmetic formulations coming into contact with the human skin and was further extended to evaluate other types of chemicals such as insecticides, sunscreens and antiseptics (LEE et al., 2017). Although previously considered a reliable and internationally accepted standard for skin irritation (OECD, 2002), the Draize test has been largely criticized over the past decades for several reasons. Differences in the physiological properties of the rabbit and the human skin lead to different responses to environmental and chemical agents, causing certain formulations to be more toxic for rabbits than for humans and vice-versa (NIXON et al., 1975; MARZULLI et al., 1975; SCOTT et al., 1991). The scoring of skin damage is highly subjective and often leads to different results even in experiments performed within the same laboratory, limiting the reproducibility and reliability of the method (WEIL et al., 1971). Moreover, the Draize test represents a cruel and invasive procedure in which experimental animals are imposed to severe suffering and discomfort (YORK et al., 1998; CHRISTIAN et al., 1996; COSTIN et al., 2009; LEE et al., 2017).

The increased concern for animal welfare throughout the world led to ethical debates and political measures aiming at reducing animal experimentation, following the 3Rs concept (Reduce, Refine, Replace) proposed by Russell and Burch, (1959). For evaluation of potential skin irritants and corrosives, the most promising alternatives to animal testing validated by ECVAM (European Centre for the Validation of Alternative Methods) are the three-dimensional (3D) epidermal culture models, such as the reconstructed human epidermis (RHE) (ALÉPÉE et al., 2010; COSTIN; RAABE; CURREN, 2009; LEE et al., 2017). These *in vitro* models are developed by culturing non-transformed human keratinocytes over a matrix to reconstruct an epidermal model that mimics the histological and biochemical properties of the human epidermis (LEE et al., 2017). Using human cells that represent the *in vitro* target organ of the species of interest, these models often provide more accurate predictions of human skin responses to potentially harmful chemicals (COSTIN et al., 2009). The test substances are topically applied onto the RHE model, and irritant chemicals can be identified by measuring the decrease in the barrier properties and cell viability via the MTT assay, which is based on the reduction of the 3-[4,5-dimethythiayol-2-yl]-2,5-diphenyltetrazolium bromide salt to the purple formazan dye by mitochondrial succinate dehydrogenase in viable cells (ALÉPÉE et al., 2010; COSTIN; RAABE; CURREN, 2009; LEE et al., 2017).

Animals are still widely used in scientific research, mainly to obtain knowledge of basic science, drug development, drug safety testing, product development, environmental research, among others. Even with social pressures and the availability of some alternative methods, the number of animals used in scientific research in the world is still very high, being around two hundred million animals used per year (TAYLOR; ALVAREZ, 2019). Therefore, the development of alternative methods and predictive models for various scientific purposes is increasingly necessary for a significant reduction in the use of animals in laboratories. Currently, several RHE models are commercially available and have been validated for skin irritation testing according to OECD TG 439, including the EpiSkin® (EpiSkin, L'Oréal, Lyon, France) (ROGUET et al., 1994), EpiDerm TM (MakTek Corporation, Ashland, MA, USA) (CANNON et al., 1994), SkinEthic® (EpiSkin, L'Oréal, Lyon, France), LabCyte EPI-MODEL24 SIT (Labcyte, Gamagori, Japan), epiCS® (CellSystems, Troisdorf, Germany), and Skin+® (SterlabStore,

Vallauris, France). Other models have also been developed by several companies, such as OS-Rep (Open Source Reconstructed Epidermis) (Henkel, Dusseldorf, Germany), StratiCELL (Straticell, Les Isnes, Belgium), StrataTestR (Stratatech, Madison, WI, USA); and academic institutions (PEDROSA et al., 2017; POUMAY et al., 2004; JUNG et al., 2014). In Brazil, due to customs problems, the availability of RHE models is severely restricted to a single manufacturer (L'Oréal), which often makes it difficult to achieve the *in vitro* test demands throughout the country. Moreover, the high cost of commercial kits, the dependence on the availability of the model, and difficulties in controlling cell culture parameters are some factors that limit the work of companies and academic laboratories that rely on these models to conduct their scientific research (COQUETTE et al., 2000; POUMAY et al., 2004; SOUTHEE et al., 1999). Therefore, open-source protocols that allow the development of Skinvitro-RHE models are critical to enabling autonomy and progress towards reducing the use of experimental animals in cutaneous toxicity assays (PEDROSA et al., 2017).

Consistent with this discussion, our work aimed at evaluating a new open-source RHE model for acute skin irritation according to OECD Test Guideline 439, "*In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method" (OECD, 2019). The Skinvitro-RHE model for this study was developed by our research group and published as an open-source RHE protocol. This model meets quality controls of morphology, cell viability, barrier function, and reproducibility (GAGOSIAN et al., 2021 – under publication), all parameters required by the OECD guideline to predict skin irritancy (OECD, 2019). The skin irritation model was evaluated using 15 reference chemicals and the validated reference method (VRM) SkinEthic® RHE, one of the six validated methods of the OECD guideline. Results of this study were compared to validated RHE models listed in the OECD TG 439.

## 2. Material and Methods

### *2.1. Cell culture and Skinvitro reconstructed human epidermis (RHE)*

The construction of the Skinvitro-RHE model here presented was developed by our research group and was published as an open-source protocol (Gagosian et al., 2021 – submitted to publication). The characterization of this 3D model was previously published as an abstract (GAGOSIAN et al. 2019; GAGOSIAN et al., 2021

– submitted to publication). This Skinvitro-RHE model consists of a well-differentiated tissue with at least 5-6 layers, the basal, spinous and granular layers, and multilayered *stratum corneum*. Compared to the human epidermis, it showed characteristics similar to that of the native epidermis in terms of morphology and pattern of the protein expression markers Keratin 10, Keratin 14, Involucrin and Filaggrin. Furthermore, this 3D model demonstrated high viability, good barrier function and reproducibility among production batches, turning out as a suitable model for skin research and toxicity testing. The construction of this Skinvitro-RHE model with human neonatal keratinocytes (HEKn) is briefly described below.

HEKn were isolated from foreskin samples (1 to 4-year-old donors) after receiving informed consent and ethical approval (Local Ethical Committee Approval – CAAE: 84000016.9.3001.0097). HEKs were cultured in EpiLife medium supplemented with HKGS (Human Keratinocytes Growth Supplement) at 37 °C and in a humidified atmosphere containing 5% $CO_2$. For the epidermal reconstructions, second- to third-passage proliferating HEKs were used. The epidermal reconstructions were done by seeding HEKn on pre-coated collagen IV inserts (Thermo Scientific™ Nunc™, 141002) (4 x $10^5$ cells/insert) in a deep well plate (Thermo Scientific™ Nunc™, 141002). Cells were kept submerged in a 3:1 DMEM and Ham's F12 mixture supplemented with cholera toxin (0.1 nM), insulin (5 µg/ml), apo-transferrin (5 µg/ml), hydrocortisone 21-hemisuccinate (0.4 µg/ml), EGF (1 ng/ml) and TGF-α (2 ng/ml)), for 96 h. After that, cells were exposed to the air-liquid interface (ALI) by removing the culture medium in the upper compartment of the insert. Cells were then cultured under ALI condition for ten days, replacing the culture medium every two days (GAGOSIAN et al., 2021 – under publication).

### *2.2. Quality control of the Skinvitro-RHE model*

The OECD TG 439 requires quality control evaluation in order to ensure accuracy in predicting skin irritants which include parameters of morphology, cell viability and barrier function. Tissues of all batches need to be evaluated in accordance with the release criteria.

Histological sections stained with Hematoxylin and Eosin (H&E) were used to evaluate the morphology of the Skinvitro-RHE models on day 15 after the initial assembly of the organotypic culture. RHE models were fixed in 2% (v/v)

paraformaldehyde, and samples were embedded in Tissue-Tek and sections of 5 µm were performed. Tissue sections were deposited on glass slides, and they were stained with H&E, according to Schmitz et al., (2010) with modifications. The slides were examined using optical microscopy (BX51 Olympus) at 100 x magnitude, and pictures were acquired with an Olympus DP72 camera.

Viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (MOSMANN, 1983). On day 15 after the initial assembly of the organotypic culture, the Skinvitro-RHE models were exposed to 16 µl of PBS for 42 minutes, washed with PBS and incubated for 42 h in growth medium (*i.e.*, culture medium with addition of supplements). After the incubation time, the inserts were exposed to MTT 1 mg/ml solution (Sigma-Aldrich) for 3 hours at 37 ºC, in a 5% $CO_2$ incubator, and protected from light. After that, the tissues were washed with PBS to remove the excess of MTT, and they were incubated with isopropanol P.A. for 2 h and under orbital agitation to formazan extraction. Each extract (200 µl) was transferred to a 96-well plate, and optical density (OD) was measured at 570 nm using the Tecan microplate reader (Tecan Infinity, M200) (OECD, 2019).

Finally, the barrier function was evaluated by the determination of $IC_{50}$ using the cytotoxic benchmark chemical Sodium Dodecyl Sulfate (SDS) (OECD, 2019; SEBAUGH, 2011). The Skinvitro-RHE models were exposed to different concentrations of SDS (0.625, 1.25, 2.5 and 5 mg/ml), PBS (Negative Control) and 5% SDS (Positive Control), for 18 h (single exposure time), and then cell viability was determined by the MTT assay (above described) (OECD, 2019; PEDROSA et al., 2017). Data were acquired by running three independent experiments.

### *2.3. In vitro skin irritation test and reference chemicals*

The performance of the Skinvitro-RHE model as a skin irritation model was done by exposing the models to 15 reference chemicals listed in OECD TG 439 (OECD, 2013, 2019), followed by the evaluation of their irritancy potential to human skin using the VRM SkinEthic® RHE, which is one of the six validated methods of the OECD TG 439 (OECD, 2019). The reference chemicals (8 skin irritants and 7 non-irritants) used in this study are listed in Table 1, and the skin irritation test method used is next described.

**Table 1.** List of reference chemicals for determination of accuracy and reliability values for Skinvitro-RHE skin irritation testing.

| Test substance name | CAS number | Supplier | Physical State | VRM Category based on *in vitro* | Skinvitro-RHE viability | UN GHS Cat. based on *in vivo* results |
|---|---|---|---|---|---|---|
| Isopropanol | 67-63-0 | Merck | Liquid | NI | 89.56 | No Cat. |
| 1-bromo-4-chlorobutane | 6940-78-9 | Sigma-Aldrich | Liquid | I | 45.82 | No Cat. |
| diethyl phthalate | 84-66-2 | Sigma-Aldrich | Liquid | NI | 76.98 | No Cat. |
| naphthalene acetic acid | 86-87-3 | Sigma-Aldrich | Solid | NI | 105.27 | No Cat. |
| heptyl butyrate | 5870-93-9 | Sigma-Aldrich | Liquid | NI | 78.28 | No Cat. |
| hexyl salicylate | 6259-76-3 | Sigma-Aldrich | Liquid | NI | 83.31 | No Cat. |
| allyl phenoxy-acetate | 7493-74-5 | Sigma-Aldrich | Liquid | NI | 78.38 | No Cat. |
| 1-decanol | 112-30-1 | Sigma-Aldrich | Liquid | I | 1.13 | Cat. 2 |
| 2-chloromethyl-3,5-dimethyl-4-methoxypyridine HCl | 86604-75-3 | Sigma-Aldrich | Solid | I | 0.54 | Cat. 2 |
| 1-methyl-3-phenyl-1-piperazine | 5271-27-2 | Sigma-Aldrich | Solid | I | 1.97 | Cat. 2 |
| tetrachloroethylene | 127-18-4 | Sigma-Aldrich | Liquid | I | 10.62 | Cat. 2 |
| cyclamen aldehyde | 103-95-7 | Sigma-Aldrich | Liquid | I | 2.08 | Cat. 2 |
| 1-bromohexane | 111-25-1 | Sigma-Aldrich | Liquid | I | 28.74 | Cat. 2 |
| Heptanal | 111-71-7 | Sigma-Aldrich | Liquid | I | 2.06 | Cat. 2 |
| potassium hydroxide (5% aq.) | 1310-58-3 | Merck | Liquid | I | 1.90 | Cat. 2 |
| Negative control (PBS) | / | Sigma-Aldrich | Liquid | NI | 100 | / |
| Positive control (SDS 5%) | 151–21-3 | Merck | Liquid | I | 1.95 | / |

Validation of the skin irritation test, using 15 reference substances suggested by OECD 439, in the Skinvitro-RHE. irritant (I); non-irritating (NI). All compounds were applied to three independent epidermis. The classification of validated reference methods (VMR) was determined according to the following cut-off point: Irritant (I) (category 2) ≤50% of cell viability and Non-irritant (NI) (without category)> 50% of cell viability. The *in vivo* score is the reference value in the Draize test *in vivo* (irritating substances have an *in vivo* score greater than or equal to 2.3 and non-irritants less than or equal to 2). United Nations - Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS) is the hazard classification of substances.

*2.3.1. Skin irritation test* – VRM SkinEthic® RHE

For the skin irritation test, SOP of SkinEthic® was used. Fully formed Skinvitro-RHE (models from day 15 of culture) were transferred to plates containing maintenance culture medium (*i.e.*, culture medium without addition of supplements), and they were incubated for 2 hours (pre-incubation) at 37 °C and 5% $CO_2$. The models were topically exposed to PBS (negative control – NC), 5% SDS (positive control – PC) and the reference chemicals for 42 min at 37 °C and 5% $CO_2$. The liquid substances were exposed in 16 µl and the solid substances in 16 mg for all treatments. After the exposure time, RHE models were carefully rinsed 25 times with PBS and transferred to a 6-well plate containing the growth medium (2 ml) for 42 h at 37 °C and 5% $CO_2$ (post-exposure incubation). The cell viability was determined by the MTT assay (above described), and the tissue viability of treated models was normalized to the NC. Substances with a tissue viability percentage lower or equal (≤) 50% were considered irritants (I), while substances in which the percentage of tissue viability was higher than (>) 50% were considered non-irritants (NI). Data were acquired by running three independent experiments.

The analysis was performed using Graphpad Prism software. The potential for irritation of each of the 15 reference substances was established based on the mean viability values of three independent experiments. Parameters were analyzed to identify false positives, false negatives, accuracy, specificity and sensitivity, as well as reproducibility performance within our laboratory.

The analyses of tissue viability in the barrier function and skin irritation tests were performed by subtracting blank values and considering the negative control as being 100% viable, provided that the optical density value was within the values recommended by OECD 439 (OD $0.6 \leq X \leq 3.0$). The viability of each condition analyzed was then calculated (in %) proportionally to the negative control.

## 3. Results

### *3.1 Quality control of the Skinvitro-RHE model*

All the Skinvitro-RHE models from the different production batches met the quality control of morphology, cell viability and barrier function integrity required to

perform the *in vitro* skin irritation test method OECD TG 439. The histological observation (H&E stained frozen tissue sections) of the Skinvitro-RHE models showed the establishment of the expected cellular layers (basal, spinous and granular layers and multilayered *stratum corneum*), with a number of layers between 5 to 6 layers. No significant histological abnormalities were verified in models (Fig. 1). Thus, even in the models constructed with HEKn from different donors, reproducibility among production batches in terms of morphology was verified.

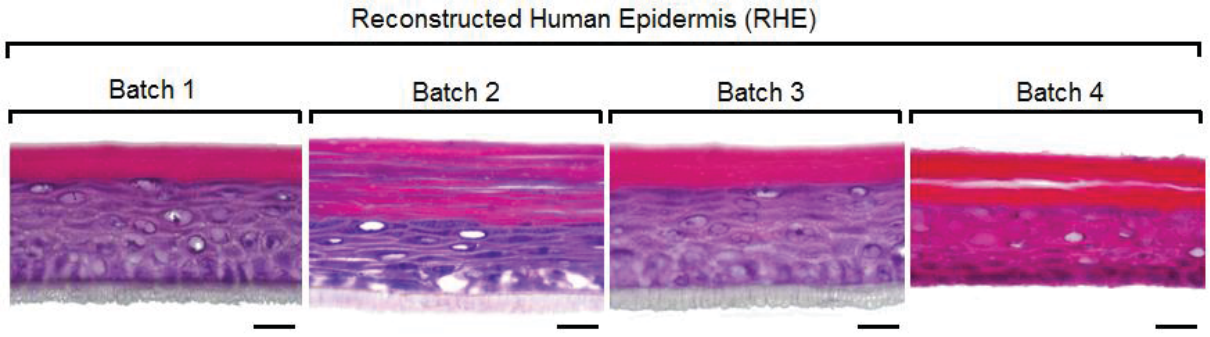


**Figure 1. Reproducibility between Skinvitro-RHE batches produced.** Histological observation of Skinvitro-RHE models showed 6 to 7 layers of the epidermis and the absence of significant histological abnormalities. Reproducibility among production batches in terms of morphology was verified. Tissue cross-sections stained with Hematoxylin and Eosin (H&E). Scale bar, 20 µm.

Tissue viability was another quality control demonstrated for Skinvitro-RHE models from different production batches as specified by the OECD TG 439. OD values higher than 0.6 (MTT assay, 570 nm) indicate acceptable cell viability for the test method. Our RHE models showed an average OD value of 1,7, which agrees with the viability range described in the OECD TG 439 (OECD, 2019) (Table 2). Upper (UL) and lower (LL) acceptance limits were statistically determined among ten batches of RHE models. The values obtained were LL of 0.62 and UL of 2.88. Additionally, each experiment included a PC (5% SDS solution) in order to demonstrate the sensitivity of the tissue model to a known irritant (Table 2). The average viability of the PC tissue replicates ranging from 0,36% to 2,25% was always clearly below the maximum acceptable value of 40% as defined for the SkinEthic® – validated RHE and the model adopted for VRM chosen for this study.

The barrier function integrity of the Skinvitro-RHE model was evaluated by determining $IC_{50}$ values using the MTT assay with the benchmark cytotoxic chemical

SDS. Our results demonstrated a reduction in cell viability of the RHEs exposed to higher SDS concentrations compared to the negative control (PBS). The value of $IC_{50}$ was determined at 2,77 mg/ml (Table 2). The two models validated at the OECD that use the $IC_{50}$ as parameters for assessing the barrier function are EpiSkin® and LabCyte EPI-MODEL24 SIT, so our results were compared with these two models, in agreement with the parameters required by OECD TG 439 (OECD, 2019) (Table 2).

**Table 2.** Acceptability ranges for negative control OD values in the MTT assay and barrier function (IC50) required by OECD 439 (OECD 439).

| RHEs | Negative control OD | | Barrier Function ($IC_{50}$) | | |
|---|---|---|---|---|---|
| | Lower acceptance limit | Upper acceptance limit | $IC_{50}$ | Lower acceptance limit | Upper acceptance limit |
| Skinvitro-RHE | ≥ 0.6 | ≤ 2.8 | 2,77 mg/ml | 1,79 mg/ml | 4,19 mg/ml |
| EpiSkin® | ≥ 0.6 | ≤ 1.5 | / | 1 mg/ml | 3 mg/ml |
| EpiDerm™ SIT (EPI-200) | ≥ 0.8 | ≤ 2.8 | / | / | / |
| SkinEthic® RHE | ≥ 0.8 | ≤ 3.0 | / | / | / |
| LabCyte EPI-MODEL24 SIT | ≥ 0.7 | ≤ 2.5 | / | 1,4 mg/ml | 4,0 mg/ml |
| epiCS® | ≥ 0.8 | ≤ 2.8 | / | / | / |
| Skin+® | ≥ 0.8 | ≤ 2.5 | / | / | / |

### *3.2 Evaluating the performance of the Skinvitro-RHE model as a skin irritation model*

The Skinvitro-RHE model was submitted to a complete run of three independent experiments with 15 reference chemicals (each run was performed in triplicate) to verify its predictive capacity for the *in vitro* skin irritation test. It well-discriminated irritants from non-irritants and correctly classified all tested irritants (*i.e.*, eight irritant substances were identified out of eight), showing a sensitivity of 100%. The Skinvitro-RHE specificity was 85,71% (six non-irritant substances were identified out of seven). The overall accuracy was 93,33% since fourteen reference substances out of fifteen were correctly classified when compared to the *in vivo* classification (Draize test) (Fig. 2) (Table 3).

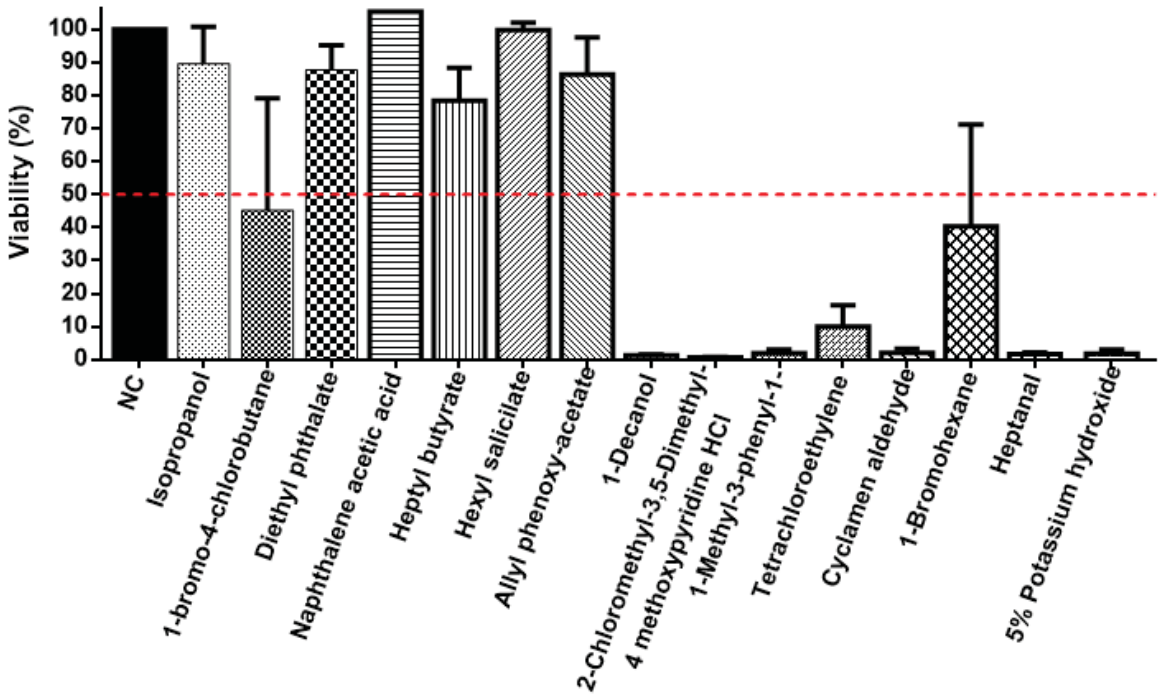


**Figure 2. Skin irritation test with 15 reference substances used to demonstrate the performance as a skin irritation model of the Skinvitro-RHE model.** The reference substance is classified as an irritant when cell viability is below 50%, and it is a non-irritant when cell viability is greater than 50%.

**Table 3.** Predictive capacity of the Skinvitro-RHE to discern skin irritants from non-classified (according to UN GHS). Minimal required sensitivity (false negative), specificity (false positive) and accuracy required by OECD

| Predictive capacity | OECD Acceptance limits | Results of Skinvitro-RHE (OECD, 2013) | Results of Skinvitro-RHE (OECD, 2019) |
|---|---|---|---|
| Specificity | ≥70% | 85,71 % (6/7) | 100 % (4/4) |
| Sensitivity | ≥80% | 100 % (8/8) | 100 % (5/5) |
| Accuracy | ≥75% | 93,33 % (14/15) | 100% (9/9) |

The skin irritation test results with the Skinvitro-RHE model were compared to the results of the validation studies of the RHE models listed in the OECD TG 439. Data comparison is shown in Table 4. Our RHE model showed similar predictivity capacity as the validated RHE models.

**Table 4.** Comparison between the Skinvitro-RHE model and published data of validated RHE models listed in the OECD TG 439 (EpiSkin®, EpiDerm ™ SIT, SkinEthic®, LabCyte EPI-MODEL24 SIT) with regard to their capacity to discriminate between skin irritating and non-irritating substances.

| Reference Substance - OECD 439 | CAS number | Skinvitro-RHE (GAGOSIAN et al., 2021 - submitted to publication) | EpiSkin® (SPIELMANN et al., 2007) | EpiDerm™ SIT (SPIELMANN et al., 2007) | SkinEthic® (ALÉPÉE et al., 2010) | LabCyte EPI-MODEL24 SIT (KOJIMA et al., 2012) |
|---|---|---|---|---|---|---|
| | | Relative viability values (%) | | | | |
| Isopropanol | 67-63-0 | 89.56 | 89.27 | 92.29 | 96.47 | 84.36 |
| 1-bromo-4-chlorobutane | 6940-78-9 | 45.82 | 4.79 | 55.51 | 2.73 | 17.62 |
| diethyl phthalate | 84-66-2 | 76.98 | 87.63 | 100.09 | 93.43 | 82.75 |
| naphthalene acetic acid | 86-87-3 | 105.27 | 92.31 | 100.22 | 97.81 | 101.92 |
| heptyl butyrate | 5870-93-9 | 78.28 | 105.95 | 97.14 | 98.86 | 108.45 |
| hexyl salicylate | 6259-76-3 | 83.31 | 98.3 | 93.17 | 95.87 | 104.15 |
| allyl phenoxy-acetate | 7493-74-5 | 78.38 | 98.45 | 100.23 | 77.313 | 79.28 |
| 1-decanol | 112-30-1 | 1.13 | 6.9 | 9.88 | 1.52 | 11.38 |
| 2-chloromethyl-3,5- dimethyl-4- methoxypyridine HCl | 86604-75-3 | 0.54 | 4.75 | 6.14 | / | 2.88 |
| 1-methyl-3-phenyl-1- piperazine | 5271-27-2 | 1.97 | 10.39 | 45.1 | / | 3.93 |
| tetrachloroethylene | 127-18-4 | 10.62 | / | / | / | / |
| cyclamen aldehyde | 103-95-7 | 2.08 | 16.45 | 18.75 | 1.73 | 10.80 |
| 1-bromohexane | 111-25-1 | 28.74 | 28.30 | 97.57 | 1.3 | 68.28 |
| heptanal | 111-71-7 | 2.06 | / | / | 1.32 | 12.8 |
| potassium hydroxide (5% aq.) | 1310-58-3 | 1.90 | / | / | / | 1.43 |

## 4. Discussion

The *in vitro* skin irritation RHE test method (OECD TG 439) is accepted as a replacement for the rabbit skin irritation test (Draize test), and it brings reliability to the prediction of irritation classification. By using validated RHE models, the skin irritation test method can be performed under GLP conditions. However, they are costly and have limited availability due to the suppliers' commercial interest, and their manufacturing process is not openly accessible. Such facts can hinder scientific progress and dissemination of animal-free test methods, especially in laboratories that have restricted budgets for research and perform mostly non-GLP studies.

To overcome these issues, open-source concepts of RHE models have been encouraged, and this study presents a novel Skinvitro-RHE model for testing the irritancy potential of chemical substances to human skin.

The Skinvitro-RHE model used in this study was developed by our group and published as an open-source RHE protocol (GAGOSIAN et al., 2021 – submitted to publication), and this model, even from different production batches, meets the quality control criteria of the OECD TG 439 (morphology, viability and barrier function integrity). The Skinvitro-RHE model showed a human epidermis-like structure with a number of layers greater than the acceptable limit (≥ 4 layers). The tissue viability was also in accordance with the TG requirements, and the lower and upper acceptance limits of OD values were in the same range as for the validated RHE models, particularly closer to the EpiSkin® model (SPIELMANN et al., 2007). The Skinvitro-RHE showed an excellent barrier function with a mean $IC_{50}$ of 2,77 ± 0,53 mg/ml, complying with the OECD TG 439 acceptance limits (OECD, 2019). Thus, this Skinvitro-RHE model is highly comparable to RHE models listed in the OECD TG 439, and it is potentially applicable for skin irritation testing.

After the Skinvitro-RHE model comparison with quality control criteria established by the OECD TG 439, we evaluated its performance as a skin irritation model. For that, 15 reference substances from different chemical categories and physical states were used. These reference substances represent the full range of Draize irritation scores (*i.e.*, non-irritants to strong irritants), and they are often used in validation studies to demonstrate sensitivity, specificity, and accuracy of RHE models for skin irritation testing

(OECD, 2019). Of the 15 reference substances, seven are categorized as non-irritating substances and eight as irritating substances by the *in vivo* Draize test. The skin irritation test using the Skinvitro-RHE model correctly classified all irritants but falsely classified one non-irritant as an irritant (1-bromo-4-chlorobutane). This misclassification was also verified with other validated RHE models, such as EpiSkin®, SkinEthic®, LabCyte EPI-MODEL24 SIT, as well as with OS-Rep (CAPALLERE et al., 2018; GROEBER et al., 2016). The relative tissue viability of the Skinvitro-RHE model exposed to 1-bromo-4-chlorobutane was slightly lower (45.19%) than the threshold limit (<50% viability) for classifying a substance as an irritant, while the validated RHE models EpiSkin®, SkinEthic® and LabCyte EPI-MODEL24 SIT showed a much lower relative viability (4.79, 2.73 and 17.62%, respectively) for 1-bromo-4-chlorobutane (ALÉPÉE et al., 2010; KOJIMA et al., 2012; SPIELMANN et al., 2007).

By performing a statistical analysis on the skin irritation data with the Skinvitro-RHE model, we could determine its sensitivity, specificity and accuracy. The minimal requirements of the OECD TG 439 for the predictive capacity of RHE models are specificity ≥70%, sensitivity ≥80% and accuracy ≥75%. Our RHE model demonstrated to have a specificity of 85.71%, a sensitivity of 100%, and an accuracy of 93.33%. This predictive capacity can still be improved if we consider only the proficiency substances listed in the last version of the OECD TG 439 (updated in 2019), among which 1-bromo-4-chlorobutane is not included. Thus, by excluding 1-bromo-4-chlorobutane, we could achieve 100% specificity, sensitivity and accuracy. In comparison with the validated RHE models, the Skinvitro-RHE model presented a comparable or even exceeding predictive capacity. With respect to other open-source RHE models, our model showed better results in predictive ability to identify irritating and non-irritating substances than the Skinvitro-RHE model of Capallere et al. (2018) and Groeber et al. (2016). However, it was shown to be similar to the results of the predictive capacity of Pedrosa et al. (2017).

## 5. Conclusion

In conclusion, this study demonstrates the capacity of the Skinvitro-RHE model to discriminate between skin irritating and non-irritating substances with a good overall accuracy of 93.33%. The predictive capacity for skin

irritation of the Skinvitro-RHE model was comparable to the validated OECD TG 439 RHE models, and in general, our model had better performance than other skin irritation open-source models. Therefore, the open-source Skinvitro-RHE model present here was demonstrated to be a valid alternative to the commercially available RHE models for skin irritation testing, contributing to the reduction of animal usage, especially for non-GLP studies.

## Conflicts of interest

The authors declare that they have no competing financial interests.

## Acknowledgments and Funding

This work was supported by National Council for Scientific and Technological Development (CNPq) and the São Paulo Research Foundation (FAPESP, scholarships: 2017/12506-7 and 2019/07710-0, financial support: 2016/06985-7). The authors would like to thank the Advanced Fluorescence Technologies Center (CTAF) at the Federal University of Paraná (Curitiba-PR, Brazil) for the technical assistance in image acquisition.

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