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A new reconstructed human epidermis for *in vitro* skin irritation testing

Tatiana do Nascimento Pedrosa[a], Carolina Motter Catarino[a], Paula Comune Pennacchi[a], Sílvia Romano de Assis[a], Fabrícia Gimenes[b], Márcia Edilaine Lopes Consolaro[b], Silvia Berlanga de Moraes Barros[a], Silvya Stuchi Maria-Engler[a,*]

[a] Clinical Chemistry & Toxicology Department, School of Pharmaceutical Sciences, University of São Paulo, Av. Professor Lineu Prestes, 580, Bloco 17, 05508-000 São Paulo, SP, Brazil

[b] Department of Clinical Analysis and Biomedicine, State University of Maringá, Avenida Colombo, 5790, CEP, 87020-900 Maringá, Paraná, Brazil

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ABSTRACT

Different models of reconstructed human epidermis (RHE) are currently validated to assess skin irritation *in vitro* and ultimately to the animal replacement of the Draize test. The development of a new RHE model is a challenge for many laboratories, representing a potential gain of autonomy and improvement of technological knowledge. The Organization for Economic Co-operation and Development (OECD) encourages the development of new models and, for this purpose, offers a thorough guideline on quality control parameters (OECD TG 439 performance standards). This work aimed to develop an RHE model (*i.e.* USP-RHE) for *in vitro* skin irritation assays, following the OECD TG 439. The developed model presents a well-differentiated epidermis similar to the Validated Reference Methods (VRM) and to native human epidermis. Quality parameters, *i.e.* optical density of negative control, tissue integrity and barrier function, were similar to VRM and in accordance with OECD TG 439. Moreover, the USP-RHE model was shown to have 85,7% of specificity (6/7), 100% of sensitivity (6/6) and 92,3% of accuracy (12/13) when compared to *in vivo* UN GHS classification. The within-laboratory reproducibility was 92.3% (12/13). Thus, we demonstrated that USP-RHE model attends to all OECD TG 439 performance standards and is ready to be used by private and public laboratories and companies for future validation studies.

1. Introduction

The development of new *in vitro* skin models and new methodologies follows a global trend to reduce or replace animal tests and it is essential for worldwide dissemination of alternative methodologies (Jung et al., 2014; Lemper et al., 2014). Since March 2013, it is no longer possible to carry out animal testing for cosmetics in the European Union; for this reason, hazard assessment, such as the skin Draize test, have been progressively replaced by models such as the reconstructed human epidermis (RHE) (Lemper et al., 2014).

Currently, the Rheinwald and Green modified culture medium (RGM), originally described in 1975, is still widely used for the *in vitro* culture and clonal expansion of primary keratinocytes (Abdel-Naser et al., 2005; Lamb and Ambler, 2013). Rheinwald and Green (1975) were the first to demonstrate that keratinocytes could grow and form stratified colonies, but failed to achieve a normal differentiation program (Rheinwald and Green, 1975). Later, Pruniéras et al. (1983) demonstrated that exposure of these cells to an air-liquid interface allowed the development of a fully differentiated epidermis (Frankart et al., 2012; Pruniéras et al., 1983).

Overall, there is an urgent need for a protocol that allows the development of an in-house RHE model for research, in order to

Abbreviations: ANOVA, one-way analysis of variance; CAS, Chemical Abstracts Service; DMEM, Dulbecco's Modified Eagle Medium; CK10, cytokeratin 10; CK14, cytokeratin 14; PBS, phosphate-buffered saline; EC50, effective concentrations at 50% viability; EGF, Epidermal growth factor, ET50, effective time at 50% viability; ELISA, Enzyme Linked Immuno Sorbent Assay; EU, European Union; HBV, human hepatitis B virus; HCV, human hepatitis C virus; HIV-1, human immunodeficiency virus-1; HIV-2, human immunodeficiency virus-2; HPV, human papillomavirus; HSV-1, herpes simplex virus 1; HSV-2, herpes simplex virus 2; HTLV-1, T-cell lymphotropic virus 1; HTLV-2, T-cell lymphotropic virus 2; HU/USP, University of São Paulo Hospital; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazoliumbromide; NaCl, sodium chloride; NC, negative control; NHEK, Normal human epidermal keratinocytes; OD, optical density; OECD, Organization for Economic Co-operation and Development; PC, positive control; PCR, polymerase chain reaction; PS, performance standards; RGM, Rheinwald and Green modified culture medium; RHE, reconstructed human epidermis; RT-PCR, Reverse transcription polymerase chain reaction; SD, standard deviation; SDS, sodium dodecyl sulfate; TG, testing guideline; TGF-α, transforming growth factor alpha; USP-RHE, reconstructed human epidermis developed by University of São Paulo; VRM, validated reference method

* Corresponding author at: Skin Biology and Melanoma Group, Dept. of Clinical Chemistry and Toxicology, School of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 580, Bl. 17, room 114, USP, São Paulo, SP 05508-000, Brazil.

E-mail address: silvya@usp.br (S.S. Maria-Engler).

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overcome the high cost of commercial kits and to obtain greater control of culture parameters (De Vuyst et al., 2014; Poumay et al., 2004; Poumay and Coquette, 2006). In this context, the Organization for Economic Co-operation and Development (OECD) encourages the production of new RHE models for skin irritation testing as described in TG 439 (Jung et al., 2014; OECD, 2015a).

The OECD provides in the document ENV/JM/MONO(2015)27 a detailed quality control (cell viability, barrier function, morphology) and performance (reproducibility) guideline which includes performance standards (PS) for the validation of similar or modified RhE for skin irritation testing as described in TG 439 (OECD, 2015b).

For cosmetic industries in developing countries such as Brazil, where customs problems often prevent the importation of commercial kits, an open-source protocol that can be used for toxicological assays is critical (De Wever et al., 2015). This tool can provide autonomy and enable progress in the establishment of alternative methods to animal testing. Thus, this work aimed at the development of a RHE model (herein denominated USP-RHE), using the RGM with modifications, that can be used for assessing skin irritation *in vitro*.

2. Material and methods

2.1. Test substances

We evaluated thirteen chemicals (commercially available) from the Minimum List for Determination of Accuracy and Reliability Values for Similar or Modified RhE Skin Irritation Test Methods listed in the OECD Performance Standards (ENV/JM/MONO(2015)27), of which, ten were also proficiency chemicals from OECD TG 439. According to OECD, laboratories should demonstrate technical proficiency, using these ten Proficiency Chemicals. The chemicals were naphthalene acetic acid (CAS No. 86-87-3), isopropanol (CAS No. 67-63-0), methyl stearate (CAS No. 112-61-8), heptyl butyrate (CAS No. 5870-93-9), diethyl phthalate (CAS No. 84-66-2), hexyl salicylate (CAS No. 6259-76-3), cynnamaldehyde (CAS No. 104-55-2), cyclamen aldehyde (CAS No. 103-95-7), 1-bromohexane (CAS No. 111-25-1), 5% potassium hydroxide (CAS No. 1310-58-3), 1-methyl-3-phenyl-1-piperazine (CAS No. 5271-27-2), tetrachloroethylene (CAS No. 127-18-4) and heptanal (CAS No. 111-71-7). Sodium chloride (NaCl) 0.9% prepared by dissolving NaCl in purified water was used as a negative control. The positive control was sodium lauryl sulfate (SLS; CAS No.151-21-3, purity > 95%).

2.2. Cell culture

Normal human epidermal keratinocytes (NHEK) were derived from donated foreskin samples obtained at the University of São Paulo Hospital (HU/USP). Cells were isolated as previously described (Pennacchi et al., 2015), under approval of the Local Ethics Committee (HU CEP Case No. 943/09 and CEP FCF/USP 534). NHEK cells were grown in KGM-Gold Bullet Kit medium (Lonza, Walkersville, MD), in a humidified atmosphere with 7.5% CO_2 at 37 °C; passages 1–3 were used for the experiments.

2.3. Primary cells quality control and contaminants evaluation

The assessment of microbiological contaminants in the primary cultures used in this study was performed as previously described (Gimenes et al., 2014). Primary cells were evaluated for the presence of *Chlamydia trachomatis*, *Treponema pallidum*, *herpes simplex* virus 1 and 2 (HSV-1 and HSV-2), *Mycoplasma genitalium*, *Trichomonas vaginalis*, *Neisseria gonorrhoeae* and human papillomavirus (HPV) using multiplex PCR technique. RT-PCR technique was used for detection of human T-cell lymphotropic virus 1 and 2 (HTLV-1 and HTLV-2), human immunodeficiency virus 1 and 2 (HIV-1 and HIV-2), human hepatitis C virus (HCV), cytomegalovirus and human hepatitis B virus (HBV). All primary cells used in this study were free of contaminants.

2.4. Reconstructed human epidermis (RHE) model

NHEK were seeded in Corning transwell® culture inserts (2.5×10^5 cells/insert) previously coated with collagen IV (0.03 mg/mL - 54 µL/cm^2) and were kept submerged in a 3:1 DMEM and HAM mixture with 5 µg/mL insulin, 0.4 µg/mL hydrocortisone, 5 µg/mL transferrin, 0.1 nM cholera toxin, 2 ng/mL TGF-α, 1 ng/mL EGF and 5% of conditioned medium obtained from primary fibroblasts cultured for 24 h at high confluence (~80%). The inserts were then raised and maintained at the air–liquid interface for 11 days. On day 9, RHE samples were transferred and maintained in a 5% CO_2 incubator with low humidity (~50%). Medium was replaced every 2 days.

2.5. Quality control of the RHE model

2.5.1. Morphology and immunohistochemistry

RHE samples were fixed in 10% formaldehyde at 4 °C, dehydrated and embedded in paraffin. Slides were stained with hematoxylin and eosin for morphological evaluation. For immunohistochemistry analysis, antigen recovery was performed twice in citrate buffer pH 6.0 for 5 min at 95 °C. The samples were incubated with primary antibodies for cytokeratin 10 (CK10–1:300) or cytokeratin 14 (CK14–1:300) (Abcam-1421 and Abcam-7800, Abcam Cambridge, UK) overnight at 4 °C in a moist chamber, and subsequently incubated for 1 h at room temperature with specie-specific secondary antibodies. All images were obtained by optical microscopy and analyzed by NIS-Elements software (Nikon Instruments, Melville, NY) according to Pennacchi et al. (2015).

2.5.2. Cell viability of negative control

The cell viability assay for the negative control was performed according to OECD TG 439 using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Tissues were exposed to phosphate-buffered saline (PBS), washed with PBS, and further incubated with 300 µL of MTT (1 mg/mL) at 37 °C in a 5% CO_2 incubator. After 3 hour incubation the excess of MTT was removed and the tissue washed with PBS. Reduced formazan was extracted by incubation with 2 mL of isopropanol for 2 h. Subsequently, 200 µL of the extraction solution were transferred to a 96-well plate, and the Optical Density (OD) of the resulting purple dye was measured at 570 nm using an ELISA plate reader (BioTek Instruments INC., USA).

2.5.3. Evaluation of barrier function - effective time at 50% viability (ET_{50}) and effective concentrations at 50% viability (EC_{50})

Evaluation of tissue integrity and barrier function of the RHE was performed by assessing the exposure time required for a cytotoxic reference chemical to reduce cell viability by 50% (ET_{50}) and the concentration at which it reduces cell viability by 50% (EC_{50}), according to OECD TG 439. For ET_{50} evaluation, tissues were exposed to the 1% Triton X-100 for 0, 4, 8, 12 and 16 h; for EC_{50} evaluation, tissues were exposed to sodium dodecyl sulfate (SDS) for 18 h at four different concentrations (0; 0.625; 1.25; 2.5; 5 mg/mL). Cell viability was assessed using the MTT assay as described above.

2.6. Applicability of the USP-RHE protocol for skin irritation testing

To evaluate the applicability of USP-RHE for skin irritation testing, we used the EpiSkin™ protocol (OECD TG 439) with some modifications (concentration of MTT and extraction time were similar to other VRM – The modifications are shown in Table 1). For this protocol, a substance was determined as irritant when the percent tissue viability accessed using MTT assay was less or equal (≤) 50% and as non-irritant when the percent tissue viability was more than (>) 50%. SDS 5% was used as positive control, as recommended by the VRM. The performance of the prediction was evaluated by testing 13 reference chemicals that