Received: 10 February 2022 | Accepted: 15 July 2022

DOI: 10.1111/ics.12803

International Journal of Cosmetic Science

REVIEW ARTICLE

Alternative in vitro models used in the main safety tests of cosmetic products and new challenges

Edith Filaire[1,2] | **Rachida Nachat-Kappes**[3] | **Camille Laporte**[4] | **Marie-Françoise Harmand**[1] | **Marina Simon**[1] | **Christian Poinsot**[1]

[1]Groupe ICARE, Saint Beauzire, France

[2]UMR 1019 INRAE-University Clermont-Auvergne, UNH (Human Nutrition Unity), ECREIN Team, Clermont-Ferrand, France

[3]InnovSkin, Chatel-Guyon, France

[4]Univ. Grenoble Alpes, CEA-Leti: Biology Division, Grenoble, France

Correspondence
Edith Filaire, Groupe ICARE, Biopôle, Rue Emile Duclaux 63360 Saint Beauzire/UMR 1019 INRAE-University Clermont-Auvergne, UNH ECREIN Team, 63000 Clermont-Ferrand, France.
Email: edithfilaire@gmail.com

Abstract

Background: Guided by ethical considerations and regulatory requirements such as the 7th Amendment to the European Cosmetics Directive N° 1223/2009, the cosmetic industry has developed and evaluated alternative test strategies such as in vitro assays, in silico approaches for toxicological endpoints and efficacy of cosmetic products and cosmetics ingredients. In consequence, the European Centre for the Validation of Alternative Methods (ECVAM) has proposed a list of validated cell-based in vitro models for predicting the safety and toxicity of cosmetic ingredients. These models have been demonstrated as valuable and effective tools to overcome the limitations of animal in vivo studies. For example, 3D human skin equivalent models are used to evaluate skin irritation potential; and excised human skin is used as the gold standard for the evaluation of dermal absorption.

Objective: This review presents, in relation to the regulatory requirements, the main alternative in vitro models used in the safety tests of cosmetic products, focusing on skin sensitization, skin corrosion, skin irritation and skin absorption, with advantages and limitations of each model. Recent innovative 3D cell technologies such as Organ-on-a-Chip (OoC) models that can bring significant improvements for toxicology and efficacy testing are also presented.

Conclusion: The development of OoC technology is promising for assessing the toxicity of substances contained in cosmetics, particularly for repeated dose toxicity, for which no alternative in vitro methods are currently available. Nevertheless, aside from the challenges, the technology needs to be validated and accepted by regulatory organizations as an effective method. Collaboration between researchers, regulatory organizations and industry would be required to achieve this validation.

KEYWORDS

chemical analysis, in vitro, skin, skin-on-a-chip, three-dimensional skin cell model, toxicology

Résumé

Contexte: Guidée par des considérations éthiques et des exigences réglementaires telles que le 7e amendement à la directive européenne sur les cosmétiques

N° 1223/2009, l'industrie cosmétique a développé et évalué des stratégies de test alternatives telles que des tests in vitro, des approches in silico pour les paramètres toxicologiques et l'efficacité des produits cosmétiques et ingrédients cosmétiques. En conséquence, le Centre Européen pour la Validation des Méthodes Alternatives (ECVAM) a proposé une liste de modèles cellulaires in vitro validés pour prédire la sécurité et la toxicité des ingrédients cosmétiques. Ces modèles ont été démontrés comme des outils précieux et efficaces pour surmonter les limites des études animales in vivo. Par exemple, des modèles équivalents de peau humaine 3D sont utilisés pour évaluer le potentiel d'irritation de la peau; et la peau humaine excisée est utilisée comme « gold standard » pour l'évaluation de l'absorption cutanée.

Objectif: Cette revue présente, en lien avec les exigences réglementaires, les principaux modèles alternatifs in vitro utilisés dans les tests de sécurité des produits cosmétiques, en se concentrant sur la sensibilisation, la corrosion, l'irritation et l'absorption cutanée, avec les avantages et les limites de chaque modèle. Des technologies cellulaires 3D innovantes récentes telles que les modèles Organ-on-a-Chip (OoC) qui peuvent apporter des améliorations significatives pour la toxicologie et les tests d'efficacité sont également présentées.

Conclusion: Le développement de la technologie OoC est prometteur pour évaluer la toxicité des substances contenues dans les cosmétiques, en particulier pour la toxicité à doses répétées, pour laquelle aucune méthode alternative in vitro n'est actuellement disponible. Néanmoins, outre les défis, la technologie doit être validée et acceptée par les organismes régulateurs comme une méthode efficace. Une collaboration entre les chercheurs, les organismes régulateurs et l'industrie serait nécessaire pour parvenir à cette validation.

INTRODUCTION

The safety assessment of cosmetic ingredients is a rapidly progressing field of research, including evaluation of alternative approaches suitable for regulatory implementation and development of both in vitro, *in chemico* and in silico methodologies avoiding any use of animals. The emerging need of new integrated approaches for testing and assessment originated from the marketing ban of cosmetics ingredients, as well as finished products, tested on animals within the European Union through the Cosmetics Regulation (EU No. 1223/2009) [1]. At the same time, the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation can impose in vivo testing of those same ingredients under its chemical testing requirements [2], even if two important REACH amendments, Regulation EU 2016/863 and Regulation EU 2016/1688, were adopted in 2016. These require in vitro methods for the assessment, respectively, of skin/eye irritation and skin sensitization unless technically infeasible. This step was taken after the Organization for Economic Co-operation and Development (OECD) approved in vitro methods for skin irritation, eye irritation and skin sensitization. It was a milestone to support the ban for animal testing.

Nevertheless, it is important to note that safety evaluation of cosmetic ingredients and products, which is listed in the 'Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation', was proposed by The Scientific Committee on Consumer Safety (SCCS) of the EU [3]. These requirements include, among others, acute toxicity, corrosion and irritation, skin sensitization, dermal absorption, chronic toxicity, mutagenicity/genotoxicity, carcinogenicity and toxicokinetic studies.

Lately, tremendous progress has been made in the field of toxicology, traditionally defined as "the science of poisons" [4], leading to the advancement of developmental toxicity assessment. Indeed, conventional animal models and in vitro two-dimensional models cannot accurately describe toxic effects and predict actual in vivo responses due to obvious inter-species differences between humans and animals, as well as the lack of a

physiologically relevant tissue microenvironment [5]. In this line, several in vitro protocols, including tissue engineering-derived ones, have been designed to increase human-like predictive response and reduce animal use, especially for toxicological testing for personal care products and related raw materials [6]. However, no validated in vitro systems are available to assess systemic toxicity, resulting in a gap in the initial assessment of molecules that become bioavailable after skin permeation or inhalation. To address these gaps, the last few years have seen the rapid development of several complex three-dimensional (3D) models mimicking both the functionality and the structure of the skin. These innovative 3D models are promising platforms for the in vitro assessment of systemic toxicity-related questions. Skin-on-a-chip platforms have also been developed mainly for cosmetics' testing as alternative test substrates to animal models. Following the experiences in this field, these constructs have also been utilized in other areas for the evaluation of cytotoxicity of several skin exposure chemical formulations used in our daily life [7].

The aim of this review was to give an overview of the alternative in vitro models used to predict the safety and toxicity of cosmetic products, focusing on dermal absorption, acute toxicity, corrosion, irritation and skin sensitization; tests validated by the European Centre for the Validation of Alternative Methods (ECVAM). The limitations of the models and their current applications have been discussed. A focus on recent innovative 3D technologies that can bring significant improvements to toxicology was also presented even if the current standards do not take them into account, this to show the perspectives in term of toxicology.

SKIN TOXICOLOGY EVALUATION

Skin is subjected to the application of cosmetic and dermocosmetic products. There are therefore essential reasons to monitor compounds that meet the skin:

1. to ensure that no absorption occurs (e.g., certain cosmetics or hazardous substances);
2. to assess possible toxicity.

The 7th Amendment to the European Cosmetics Directive banned animal testing for cosmetic products and for cosmetic ingredients in 2004 and 2009, respectively. Furthermore, European Cosmetic Regulation No. 1223/2009 and the specific Regulation No. 655/2013 specify the required data to prove the safety and support the claims [8]. Historically, the ethical dimension was put forward by Russell and Burch [9] with the rule of the three 'Rs' for Reduction, Refinement and Replacement; currently replacement is the primary goal of the 3Rs. Reduction is the application of methods that allow a reduced number of animals to be used in a protocol. Refinement refers to the application of methods that avoid animal suffering. Replacement consists of the substitution of animals with other models, such as other invertebrates, cell cultures, or organs. An 'A' is often added for accountability, recalling that animal life is required and necessary for the advancement of biology, that it must therefore be considered with respect, reflecting honesty and scientific integrity [10].

Skin absorption

Toxicokinetics refers to the time-dependent uptake, distribution and fate of a substance entering the body, including Absorption, Distribution, Metabolism and Excretion (ADME) [11]. The testing guidelines for toxicokinetics, including dermal absorption, skin absorption: in vivo method, skin absorption: in vitro method (corresponding to OECD 417, 427, 428, respectively), are designed to elucidate aspects of the fate and the potential toxicity of the substance under test [12–14].

ECVAM had validated one model to evaluate absorption properties of molecule (OECD 428) [14], this model using human skin static or flow-through diffusion cell models [15]. Franz cells are a widely used methodology to evaluate in vitro molecules permeation, which have advantages, such as (i) less handling of tissues, (ii) no continuous sample collecting and (iii) low amount of drug required for analysis.

However, a key limitation of the proposed in vitro model is that the skin has been shown to metabolize some chemicals during percutaneous absorption [16]. Moreover, ethical clearances are required. To overcome these difficulties, the miniaturization of Franz diffusion cell systems seems a promising option. This can be made possible through the devices with multi-organ platforms on a chip (OoC) also called 'microphysiological systems' (MPSs). Indeed, they offer innovative and state-of-the-art platforms essential to overcome the limitations of Franz scattering cells [7]. However, while MPSs exist for investigating pharmaceuticals, the applicability of MPS for cosmetics ingredients is yet to be evaluated.

Skin corrosion

Skin corrosion refers to the occurrence of irreversible damage to the skin manifested by visible necrosis [17]. It

is the most extreme form of skin irritation. The principle of the skin corrosion test is based on the hypothesis that corrosive chemicals can penetrate the *stratum corneum* by diffusion or erosion and are cytotoxic to the underlying cell layers. Because of animal test banned, reconstructed human epidermis (RhE) obtained from human-derived non-transformed epidermal keratinocytes is typically used. These models accurately mimic the architecture and the physiology of the human epidermis including a basement membrane, proliferating keratinocytes and a *stratum corneum* with an intact physical barrier function and xenobiotic metabolizing capacity [18]. They tend to reproduce the in vivo characteristics of the human skin in terms of epidermal morphology, differentiation and barrier function [19], and their use in toxicology has been increasing since 2010 as alternatives to in vivo models. They are a valuable tool for drug discovery, disease modelling and basic research. Primary keratinocytes derived from neonatal foreskin, abdominoplasties, but also cells from older donors, immortalized keratinocytes or keratinocytes derived from induced Pluripotent Stem Cells (iPSC) are used in these models and are cultured in cell culture inserts and then lifted to the air–liquid interphase to induce differentiation, epithelial stratification and cornification. However, it appears that working with primary cells is a key point for the correct development of a physiologically relevant model of skin [20]. It is also important to specify that the use of specific cell culture media or exposure to an air–liquid interface to induce differentiation and cornification are critical steps to obtain skin equivalents.

Four validated in vitro alternatives test methods using commercially available RhE models are validated including EpiSkin™ (L'Oreal, France), EpiDerm™, (MatTek Corporation, Massachusetts, USA), SkinEthic™ (SkinEthics, France) and epiCS® (CellSystems, Germany) [17]. Even if all these models seem to reproduce many of the characteristics of normal human epidermis, some drawbacks persist, namely the difficulty to discriminate the different strata of the viable cell layers in Episkin™ penetration models [21]. Low intra-batch and a high inter-batch variation have also been noted.

Other test methods are based on in vitro membrane barrier test method (OECD 435 [22]: not adopted in the European legislation) and excised rat skin (Rat skin Transcutaneous Electrical Resistance: OECD 430) [23].

It is important to note that the OECD 404 test [24] is not allowed for cosmetics and their ingredients. Data obtained from the in vivo skin corrosion/dermal irritation test should only be provided when already available for a test performed before the animal testing ban or if the data were obtained for the purpose to be in compliance with other (non-cosmetic) legislations.

Skin irritation

Skin irritation refers to the production of reversible damage to the skin occurring after exposure to a substance or mixture [25]. To date, seven RhE models are included in OECD 439 as validated reference methods including EPiSkin™, EpiDerm™ SIT (EPI 200), SkinEthic RHE™, LabCyte EPI-Model24 SIT, epiCS®, Skin+® f and KeraSkin™ SIT.

The in vitro RhE test system measures the cell/tissue damage using cell viability as endpoint given that corrosion and irritation assessments, rely on reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) as the primary endpoint. A designation of 'irritating' or 'non irritating' is assigned; however, it cannot define mild irritants (UN-GHS category 3) from non-irritating/no category compounds.

More sophisticated skin in vitro uses a combination of cells that best mimic the in vivo responses [26]. This may be the case with Human Skin equivalents, which are artificially reconstructed skin substitutes combining an epidermal and a dermal compartment [27]. They are developed from primary cells (keratinocytes, fibroblasts and/or stem cells) and components of the extracellular matrix (ECM) and designed to resemble native skin. The main advantage of this type of model is that it allows paracrine communication between the cells of the dermis and the epidermis. This is the case for The EPiTRI model which seems to meet the criteria of Performance Standards of OECD 439 and to be suitable for screening irritating chemicals in vitro [28, 29].

New generation in vitro 3D skin models are also available, which may reflect the skin architecture and cell composition more closely and allow more precise toxicological profiling. However, at this time, they are not accepted under the standards. They are discussed in the second part of this review.

Skin sensitization

Before a new cosmetic ingredient is placed on the European market, evaluation of its safety profile, including the assessment of skin sensitization hazards and potency is mandatory. Skin sensitizers are chemicals that have the intrinsic potential to induce specific immunological reactivity after skin contact. Repeated topical exposure may result in the development of allergic contact dermatitis (ACD).

Skin sensitization is a complex immunological process, which results from a succession of physico-chemical, chemical and biological events that lead to the activation of allergen-specific T lymphocytes, following the penetration of a sensitizing product into the skin. Different steps

have been described in this immunological process as key and have therefore been the subject of the implementation of several tests, given that information on skin sensitization is required in the safety assessment of EU Cosmetics Regulation [1].

Following the revision of Annex VII of the REACH regulation [30], as well as the transformation of the cosmetic directive into a regulation [1], traditional animal models, such as the Guinea Pig based assays (GPMT or the Buehler test) described in OECD 406 [31], the murine Local Lymph Node Assay (LLNA) described in OECD 429 [29], are no longer allowed to meet the information requirements for substances exclusively intended for use in cosmetic products. In the last years, several New Approach Methods (NAMs) have been developed, validated and regulatory accepted that address different key events of the skin sensitization Adverse Outcome Pathway (AOP).

In short, the chemical sensitizer penetrates the stratum corneum, the uppermost layer of the skin, and subsequently binds covalently to proteins (Key Event 1: KE1; OECD 442 C) [32] to form hapten–protein conjugates, which can be immunogenic. Simultaneously, keratinocytes are stimulated both to release danger signals (e.g., pro-inflammatory cytokines or ATP) and activate antioxidative response genes (Key Event 2: KE2; OECD 442 D) [33]. Dendritic cells (DCs), which are antigen-presenting cells that playing a major role in the primary immune response and can be activated by allergenic molecules, then develop a mature phenotype involving the induction of various co-stimulatory molecules and production of pro-inflammatory cytokines and chemokines with formation of hapten–protein conjugates on major histocompatibility complex molecules (Key Event 3: KE3; OECD 442E) [34]. The activated DCs are mobilized and then migrate from the skin to the draining lymph nodes to present the allergen to T cells (Key Event 4). After stimulation by DCs, hapten-peptide-specific T cells expand to elicit an adverse immune response in case of a second exposure to the chemical sensitizer.

Thus currently, three technical Test Guidelines (OECD 442 C, D and E) describe a total of seven such methods, including:

- the KE1-based Direct Peptide Reactivity Assay (DPRA), Amino acid Derivative Reactivity Assay (ADRA) and kDPRA, which is a modification of the standard DPRA [32]. In this test method, several concentrations of the test substance are incubated with the synthetic peptide for several incubation times. The reaction kinetics towards a synthetic cysteine-containing peptide is evaluated to predict the potency of the test substance
- the KE2-based assays KeratinoSens and luSens [33]
- the KE3-based assays h-CLAT, U-SENS, GARDskin and the IL-8 Luc assay [34]. GARDskin is an in vitro model that measures KE3 using gene expression profiling in the MUTZ-3 cell line. It has just been added to the tests validated in June 2022.

According to the current testing paradigm, none of the NAMs methods is considered sufficient stand-alone replacements of animal data to draw conclusions about the skin sensitization potential of chemicals or to provide information for potency sub-categorization according to the UN GHS (sub-categories 1A and 1B). Indeed, individual test methods have some known technical limitations, which may lead to false-negative results. DPRA and ADRA, for example, have no metabolic capacity and are therefore unable to identify prohaptens, sensitizers that require metabolism to be activated. Thus, these methods should be considered in the context of a tiered testing strategy, a so-called defined approach (DA), where a fixed data integration procedure is used to arrive at a final classification, based on the readout from several NAMs [8].

Novel state-of-the-art scientific methods currently in the OECD Test Guideline Program, such as the SENS-IS, which is based on the toxicogenomic analysis on 3D reconstituted epidermidis model (Episkin®) to measure skin sensitization potency, are under evaluation by EURL-ECVAM. More specifically, this test is based on the analysis of the expression of a set of genes involved in sensitization pathway using RT-qPCR method. To address all the aspects of complexity of skin sensitization and to consider the variety of different types of chemical sensitizers, SENS-IS uses a set of 62 biomarkers split into three groups: a group of 24 genes to measure skin irritation, a group of 17 genes involved antioxidant pathways (ARE genes) and a group of 21 genes involved in sensitization (SENS-IS genes).

It appears that this test is addressing the three first key events of the Adverse Outcome Pathways: the KE1 through the expression of genes under the control of «sensor» proteins, the KE2 through the expression of genes indicating keratinocytes activation and the KE3 through the expression of genes implicated in dendritic cells activation and maturation. Moreover, due to the topical application, it seems particularly adapted to formulations and represents a promising candidate for Human Repeat Insult Patch Test (HRIPT) replacement. Even if this test, derived from Draize experiments [35], constitutes a key parameter in current quantitative risk assessment for chemicals/drugs and provides data directly relating to humans, different relevant HRIPT protocols have been developed over the past century, with differences between protocols and lack of international standardization [36]. Taping and occlusion specifics, allergen quantity and concentration,

duration and schedule of patch test placement, among other considerations, have to be investigated in regard to standardization. Moreover, this test raises ethical issues and proves to be poorly representative of real exposure conditions. In addition, the great variability linked to the characteristics of the skin and the tolerance of chemical compounds are its limits [37]. However, it remains the only regulatory requirement (requested by certain authorities outside the EU) to assess the allergenic potential of a finished product before it is placed on the market.

OPPORTUNITIES FOR DEVELOPMENT OF NEW IN VITRO MODELS FOR SKIN TOXICITY

Despite the many advancements and capabilities with skin models made thus far, further assay and model development would expand investigative capabilities. Indeed, some of them reconstruct only the human epidermis like SkinEthic™ (EpiSkin, L'Oréal Lyon France), others replicate both dermal and epidermal compartments such as the Vitrolife-Skin™ model (Kyoto, Japan), the Phenion® Full-Thickness skin model (Henkel, Düsseldorf, Germany), the EpiDerm-FT™ (Mattek, Ashland, USA) [38]. Moreover, one of the main drawbacks of these skin equivalents is still their lack of a vascular system in the sense of nutrients, oxygen supply, waste removal or concentration gradient of the nutrients [20].

Some recent advances in skin engineering have produced skin equivalents that incorporate a wide variety of cell types and structures that more closely resemble the real structure of the organ. Incorporation of adipocytes, endothelial cells that give rise to vascularization, immune or Langerhans cells to reproduce immune response, chemokines to promote cell differentiation or dorsal root ganglion neurons to recreate the peripheral skin nerve system are improvements to the current in vitro skin models to better mimic its response to irritation or toxicity studies [39]. In this line, recent development in microfluidic-based cell culture technology has demonstrated the feasibility of using micro-scale in vitro physiological models 'organs-on-a-chip' models for drug screening. These Organ-on-a-chip devices (OoC) aim to mimic the architecture and function of an organ by combining 3D bioengineered constructs such as multicellular spheroids and organoids and bioprinted constructs. Their applications have been used increasingly over the last 10 years, as they represent a minimally functional unit that can replicate specific aspects of human physiology in a direct and controlled manner. These biomimetic systems are based on microfluidic systems, allowing dynamic culture and precise control of the microenvironment on cells inside [20]. Microfluidic-based cell culture systems are capable of precisely tuning the dynamic fluid flows and spatiotemporal gradients, as well as delivering nutrients, chemicals and stimuli to cells and removing of soluble molecules in a controlled manner [40]. Additionally, microfluidic systems enhance cell–cell and cell–matrix interactions and allow controlled application of mechanical stresses by fluid flow and therefore, can potentially provide a better, faster and more efficient characterization of cell interaction with the surrounding environment.

In the past decade, an increasing number of articles about the development of microfluidic devices for testing various organs, such as the lung, heart, gut, kidney or brain, have been published [41]. OoC development involving the skin is scarcer. Considering the skin complexity, many studies have simplified the skin model to single monolayers, introducing the cells manually [20]. For these latest authors, microfluidic chips for modelling skin can be created using two approaches:

1. By direct introduction of a skin fragment (derived from a biopsy or a HSE in the chip), knowing that dermo-epidermal models are more frequently used for transferred skin chips. Using this approach, Kim et al. [42] combined microfluidics and skin micro-biopsies into a skin-on-a-chip (SOC) model, allowing the study of neutrophil migration to the skin in the presence of microorganisms. This development integrated two channels separated by a red blood cell filter. Other investigations, using this transferred skin chip approach, developed a microfluidic chip for co-culturing skin and liver [43], designed a multi-organ chip including skin and hair [44] or created a four-organ system including the skin, liver, kidney and intestine [45]. More recently, Jusoh et al. [16] proposed a microfluidic platform for investigating skin irritation that permits interactions between keratinocytes and dermal fibroblasts, promoting angiogenic sprouting. Tavares et al. [46] combined a HSE model with a liver model in Chip2, consisting in two connected compartments in which skin and liver organ equivalents were cultivated. The aim of this investigation was to assess xenobiotic metabolites toxicity data.
2. In situ SOC model generating skin model directly inside the microfluidic system.

In this approach, the organ is built inside the device, the channels being used to deliver nutrients and as compartments to hold the tissue. Different functions are implemented in the systems to produce a robust skin model.

Figure 1 summarizes these two approaches.

For Klicks et al. [47], microfluidic SOC provides the best physiology and functionality. Hence, all systems have

a perfusion whereby shear stress is produced which in turn increases cell viability and proliferation in contrast to static cultures. SOC will be a useful standardized platform for molecules testing on a specific cell type to evaluate the response, or toxicity in a cell monolayer, which is easier to obtain than a 3D equivalent. It can be a platform to evaluate cell–cell interactions or immune response. In this line, 3D immunocompetent organ-on-chip models have been explored to produce an accurate model [48]. Wuefer et al. [49] developed a miniature model of human skin in a microfluidic platform consisting in epidermal, dermal and endothelial layers, each layer being separated using transparent, porous membranes to allow interlayer communication and mimic skin biology. They showed that their SOC model can potentially be used for constructing in vitro skin disease models or for testing the toxicity of cosmetics. Ramadan and Ting [50] miniaturized an immune competent in vitro model of human skin based on 3D co-culture of immortalized human keratinocytes (HaCaT) as a model of the epidermis barrier and human leukaemic monocyte lymphoma cell line (U937) as a model of human dendritic cells. The biological model was fitted in a microfluidic-based cell culture system that provides a dynamic cellular environment and mimics the in vivo environment of skin. Kwak et al. [51] created a skin chip with cocultured dermal fibroblasts and keratinocytes and matured endothelial layers in a microfluidic chip device. This model successfully mimicked the immune response of the human skin in terms of cytokine production and recruitment of immune cells to inflammatory sites, showing that the microfluidic skin chip may be useful for studying the immune response of the human. Légues et al. [52] have also developed an innovative 3D full-size bioprinted human skin models containing immune cells for the screening of cosmetic ingredients designed either to reduce inflammation or to prevent it.

These 3D skin models integrating immune components will be very useful to evaluate molecules toxicity. However, as reported by Moon et al. [53], since each model comprises of different cell types, the selection of an adequate immunocompetent 3D skin model depending on the target mechanism of the drug being tested is very

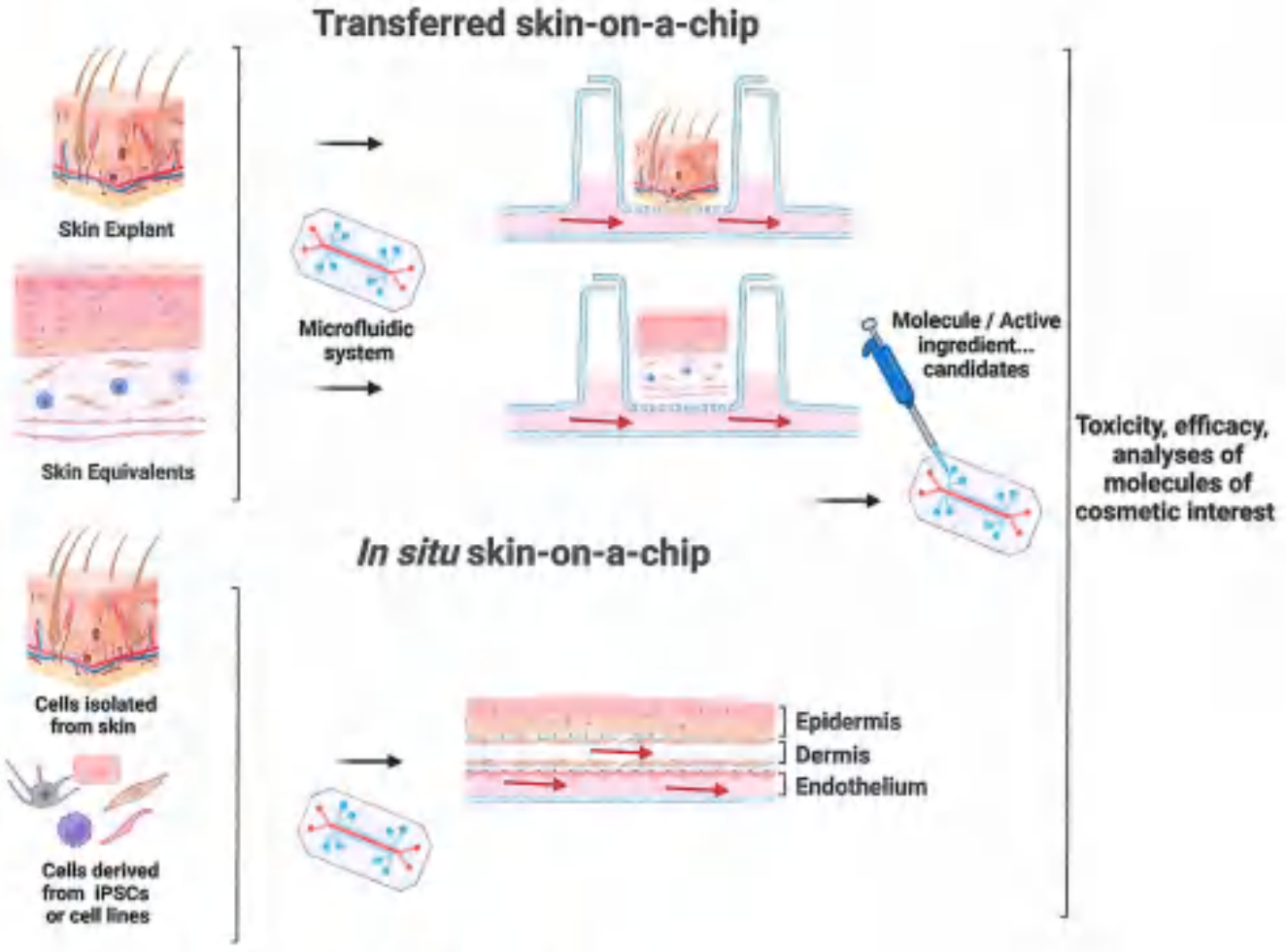

FIGURE 1 Schematic representation of approaches used for SOC models.

important. There is much work to be done to improve and refine these new 3D skin models to truly open the technology up for toxicity.

Besides, a 3D skin-on-a-chip microdevice with vascular channels coated with endothelial cells that comprises a skin equivalent fixed to a culture device connected to an external pump and tubes was proposed by Mori et al. [54]. The model can be used for the development of skin therapies and cosmetics. Few studies have developed skin coculture with other organs such as the liver or hair [55]. Recently, Ramovs et al. [56] reported successful generation of hair-bearing skin organoids from two hiPSC lines that exhibited fully stratified interfollicular epidermis. Netri™ developed a microfluidic model including human sensory neurons and keratinocytes, including two isolated channels linked by microchannels in which only axons can pass through dendrites [57]. Sensitive skin can be mimic by this model. Indeed, capsaicin can be added to axonal endings and assessing its effect on either the skin compartment or the neuronal body.

It is also important to note that microsystems applied to organs on chips make it possible to integrate a mechanical stimulation function. Indeed, as other organs, the skin is submitted to different mechanical stimuli as stretching. This mechanical stress are essentials to the functionality of the tissue in physiological and pathological way. Indeed, the implementation of a stretching function in an in vitro system has shown a real benefit of the stimulation on in situ SOC by acting on epidermal layers with an increase of the epidermis thickness and a better keratinization, at the dermal layer by increasing the fibroblasts proliferation and the ECM turnover [53]. Recently, Varone et al. [58] described their Open-top-chip that integrated a pneumatic actuation of the membrane through vacuum channel. This innovative chip combined all the functions specific to in vitro skin culture and contributes to improve the barrier function of the skin by reproducing strain and permeability properties of a native skin.

CONCLUSION

Evaluating skin toxicity is crucial in the assessment of the safety of cosmetics. Current state-of-the-art in vitro and in vivo technologies have shown limitations in accurately predicting human skin toxicity. Moreover, because animal skin models have both a different structure and different immunological responses compared to human skin, an ongoing paradigm shift in toxicity testing occurred based on expanded application of high-throughput in vitro screening and in silico methods to assess potential health risks of environmental agents.

Next-generation in vitro skin models will reflect more closely the skin architecture and cell composition to allow for more precise toxicological profiling. The development of OoC technologies is promising. Inclusion of immune components, for example, in the 3D skin model offers a new perspective. The next step is to generate multi-OoC platforms that emulate entire biological processes: incorporating immune system, organ innervation and vascularization are the keys allowing to improve these platforms. Besides these challenges, the technology needs to be validated and accepted by the regulatory organizations as an efficient method. Collaboration between researchers, regulatory organizations and the industry would be necessary to obtain this validation.

AUTHOR CONTRIBUTIONS

Conception and design: Edith Filaire, Christian Poinsot; **Design of Figures and table**: Rachida Nachat-Kappes; **Writing, review**: Edith Filaire, Camille Laporte; **Revision of the manuscript**: Marina Simon, Marie Françoise Harmand.

ACKNOWLEDGEMENT

No funding source was obtained for this review.

FUNDING INFORMATION

The authors confirm that they received no financial support. All costs were covered by the researchers themselves.

CONFLICT OF INTEREST

The authors can confirm that here are no conflicts of interest of any kind.

ORCID

Edith Filaire https://orcid.org/0000-0002-5127-7486

REFERENCES

1. European Parliament. Regulation (EC) no 1223/2009 of the European Parliament and of the council of 30 November 2009 on cosmetic products. Off J Eur Union. 2009;L396:1–1355. Available from: http://data.europa.eu/eli/reg/2009/1223/oj.
2. Knight J, Rovida C, Kreiling R, Zhu C, Knudsen M, Hartung T. Continuing animal tests on cosmetic ingredients for REACH in the EU. ALTEX. 2021;4:653–68.
3. SCCS. In: Scientific Committee on Consumer Safety Ed, editor. The SCCS's notes of guidance for the testing of cosmetic substances and their safety evaluation. Brussels: European Commission; 2015.
4. Klaassen C. In: Klaassen CD, editor. Casarett and Doull's toxicology: the basic science of poisons. 9th ed. Mc Graw Hill Education; 2018.
5. Li M, Gong J, Gao L, Zou T, Kang J, Xu H. Advanced human developmental toxicity and teratogenicity assessment using human organoid models. Ecotoxicol. Environ. Saf. 2022;235:113429.

6. Van der Worp HB, Howells DW, Sena ES, et al. Can animal models of disease reliably inform human studies? PLoS Med. 2010;7:3.
7. Öztürk AB, Miccoli B, Avci-Adali M, et al. Current strategies and future perspectives of skin-on-a-Chip platforms: innovations, technical challenges and commercial outlook. Curr Pharm Des. 2018;24:5437–57.
8. Barthe M, Bavoux C, Finot F, Mouche I, Cuceu-Petrenci C, Forreryd A, et al. Safety testing of cosmetic products: overview of established methods and new approach methodologies (NAMs). Cosmetics. 2021;8:50.
9. Russell WMS, Burch RL. The principles of humane experimental technique. London, UK: Methuen & Co Ltd; 1959.
10. Tannenbaum J, Bennett BT. Russell and Burch's 3Rs then and now: the definition and purpose. J Am Assoc Lab Anim Sci. 2015;54:120–32.
11. SCCS. In: Scientific Committee on Consumer Safety Ed, editor. The SCCS's notes of guidance for the testing of cosmetic substances and their safety evaluation. Brussels: European Commission; 2018.
12. OECD. Guidelines for the testing of chemicals, Section 4: health effects. Test N° 417. Toxicokinetics: in vitro method. Paris: OECD Publishing; 2010.
13. OECD. Guidelines for the testing of chemicals, section 4: health effects. Test N° 427. Skin absorption: in vivo method. Paris: OECD Publishing; 2004.
14. OECD. Guidelines for the testing of chemicals, section 4: health effects. Test N° 428. Skin absorption: in vitro method. Paris: OECD Publishing; 2004.
15. Almeida A, Sarmento B, Rodrigues F. Insights on in vitro models for safety and toxicity assessment of cosmetic ingredients. Intern J Pharma. 2017;15:178–85.
16. Jusoh N, Ko J, Jeon NL. Microfluidics-based skin irritation test using in vitro 3D angiogenesis platform. APL Bioeng. 2019;3:036101.
17. OECD. Guidelines for the testing of chemicals, Section 4: health effects. Test No. 431. In vitro skin corrosion: reconstructed human epidermis (RHE) test method. Paris: OECD Publishing; 2019.
18. Bakar J, Michael-Jubeli R, El Khoury R, Hamia S, Assi A, Baillet-Guffroy A, et al. Assessment of the skin barrier function in the reconstructed human epidermis using a multimodal approach at molecular, tissue and functional levels. Analyst. 2021;141:4649–58.
19. Frankart A, Malaisse J, De Vuyst E, Minner F, de Rouvroit CL, Poumay Y. Epidermal morphogenesis during progressive in vitro 3D reconstruction at the air-liquid interface. Exp Dermatol. 2012;2:871–5.
20. Risueno I, Valencia L, Jorcano JL, Velasco D. Skin-on-a-chip models: general overview and future perspectives. APL Bioengineering. 2021;5:03090.
21. Ponec M, Boelsma E, Weerheim A, Mulder A, Bouwstra J, Mommaas M. Lipid and ultrastructural characterization of reconstructed skin models. Int J Pharm. 2000;203:211–25.
22. OECD. Guidelines for testing of chemicals, Section 4: health effects. Test No. 435. In vitro membrane barrier test method for skin corrosion. Paris: OECD Publishing; 2015.
23. OECD. Guidelines for the testing of chemicals, Section 4: health effects. Test No. 430. In vitro skin corrosion: transcutaneous electrical resistance test method (TER). Paris: OECD Publishing; 2015.
24. OECD. Guidelines for the testing of chemicals, Section 4: health effects. Test No. 404. Acute dermal irritation/corrosion. Paris: OECD; 2015.
25. OECD. Guidelines for the testing of chemicals, Section 4: health effects. Test No. 439. In vitro skin irritation: reconstructed human epidermis test methods. Paris: OECD Publishing; 2021.
26. Pritsche E, Haaman-Stemman T, Kapr J, et al. Stem cells for next level toxicity testing in the 21st century. Small. 2021;17:2006252.
27. Choudhury S, Das A. Advances in generation of three-dimensional skin equivalents: pre-clinical studies to clinical therapies. Cytotherapy. 2021;23:1–9.
28. Liao CC, Wu CY, Lin MH, Hsieh FK, Hsu LT, Chang SY, et al. Validation study of a new reconstructed human epidermis model EPITRI for *in vitro* skin irritation test according to OECD guidelines. Toxicol In Vitro. 2021;75:105197.
29. OECD. Guidelines for the testing of chemicals, Section 4: Test No. 429. Skin sensitization, local lymph node assay. Paris: OECD Publishing; 2010.
30. Commission Regulation (EU). 2017/706 of 19 April 2017 amending Annex VII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards skin sensitisation and repealing Commission Regulation (EU) 2016/1688 (Text with EEA relevance.) C/2017/2369. Off. J. Eur. Union. 8-11 (2017). Available from: http://data.europa.eu/eli/reg/2017/706/oj (accessed on 7 June 2021)
31. OECD. Guidelines for testing of chemicals, section 4: health effects. Test no. 406. Skin sensitisation. Publishing; 2022.
32. OECD. Guidelines for the testing of chemicals, Section 4: No. 442C. In chemico skin sensitization: assays addressing the adverse outcome pathway key event on covalent binding to proteins. Publishing; 2022.
33. OECD. Guidelines for the testing of chemicals, section 4: test N° 442D: in vitro skin sensitisation: ARE-Nrf2 luciferase test method. Publishing; 2022.
34. OECD. Guidelines for the testing of chemicals, section 4: no. 442E. In vitro skin sensitisation assays addressing the key event on activation of dendritic cells on the adverse outcome pathway for skin sensitization. Publishing; 2022.
35. Draize JH, Woodard G, Calvert HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Therapeut. 1944;83:377–90.
36. Bormann JL, Maibach HL. Draize human repeat insult patch test (HRIPT): seven decades of pitfalls and progress. Regul Toxicol Pharmacol. 2021;121:104867.
37. Basketter DA, Gerberick GF. Skin sensitization testing: the ascendancy of non-animal methods. Cosmetics. 2022;9:38.
38. Stojic M, López V, Montero A, Quilez C, de Aranda Izuzquiza G, Vojtova L, et al. Skin tissue engineering. In: Garcia-Gareta E, editor. Biomaterials for Skin Repair and Regeneration. Woodhead Publishing; 2019:59–99.
39. Jeong KH, Park D, Lee YC. Polymer-based hydrogel scaffolds for skin tissue engineering applications: a mini-review. J Polym Res. 2017;24:1278–84.

40. Antoni D, Burckel H, Josset E, Noel G. Three-dimensional cell culture: a breakthrough in *vivo*. Int J Mol Sci. 2015;16:5517–27.
41. Li J, Chen J, Bai H, Wang H, Hao S, Ding Y, et al. An overview of organs-on-chips based on deep learning. Research. 2022:1–20.
42. Kim JJ, Ellett F, Thomas CN, Jalali F, Anderson RR, Irimia D, et al. A microscale, full-thickness, human skin on a chip assay simulating neutrophil responses to skin infection and antibiotic treatments. Lab on Chip. 2019;18:3094–103.
43. Wagner I, Materne EM, Brincker S, Süßbier U, Frädrich C, Busek M, et al. A dynamic multi-organ-chip for long-term cultivation and substance testing proven by 3D human liver and skin tissue co-culture. Lab Chip. 2013;13:3538–47.
44. Ataç B, Wagner I, Horland R, Lauster R, Marx U, Tonevitsky AG, et al. Skin and hair on-a-chip: in vitro skin models versus ex vivo tissue maintenance with dynamic perfusion. Lab Chip. 2013;13:3555–61.
45. Maschmeyer I, Lorenz AK, Schimek K, et al. A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin, and kidney equivalents. Lab Chip. 2015;15:2688–99.
46. Tavares RSN, Tao TP, Maschmeyer I, Maria-Engler SS, Schäfer-Korting M, Winter A, et al. Toxicity of topically applied drugs beyond skin irritation: static skin model vs. two organs-on-a-chip. Int J Pharmaceutics. 2020;589:119788.
47. Klicks J, von Molitor E, Torsten EF, et al. *In vitro* skin three-dimensional models and their applications. J Cell Biotechnol. 2017;3:21–39.
48. Maharjan S, Cecen B, Zhang YS. 3D immunocompetent organ-on-a-chip models. Small Methods. 2020;21:2000235.
49. Wufuer M, Lee GH, Hur W, et al. Skin-on-a-chip model simulating inflammation, edema and drug-based treatment. Sci Rep. 2016;21:37471.
50. Ramadan Q, Ting FC. *In vitro* micro-physiological immune-competent model of the human skin. Lab Chip. 2016;16:1899–908.
51. Kwak BS, Jin SP, Kim SJ, Kim EJ, Chung JH, Sung JH. Microfluidic skin chip with vasculature for recapitulating the immune response of the skin tissue. Biotechnol Bioeng. 2020;117:1853–63.
52. Légues M, Milet C, Forraz N, et al. The world's first 3D bioprinted immune skin model suitable for screening drugs and ingredients for normal and inflamed skin. IFSCC Magazine. 2020;4:8–12.
53. Moon S, Dong HK, Shin JU. *In vitro* models mimicking immune response in the skin. Yonsei Med J. 2021;62:969–80.
54. Mori N, Morimoto Y, Takeuchi S. Skin integrated with perfusable vascular channels on a chip. Biomaterials. 2017;116:48–56.
55. Kühnl J, Tao TP, Brandmair K, Gerlach S, Rings T, Müller-Vieira U, et al. Characterization of application scenario-dependent pharmacokinetics and pharmacodynamic properties of permethrin and hyperforin in a dynamic skin and liver multi-organ-chip model. Toxicology. 2021;448:152637.
56. Ramovs V, Janssen H, Fuentes I, Pitaval A, Rachidi W, Chuva de Sousa Lopes SM, et al. Characterization of the epidermal-dermal junction in hIPSC-derived skin organoids. Stem Cell Reports. 2022;17:1279–88.
57. Guichard A, Remoué N, Honneger T. *In vitro* sensitive skin models: review of the standard methods and introduction to a new disruptive technology. Cosmetics. 2022;9:67.
58. Varone A, Nguyeb JK, Leng L, et al. Novel organ-chip system emulates three-dimensional architecture of the human epithelia and the mechanical forces acting on it. Biomaterials. 2021;275:120957.

How to cite this article: Filaire E, Nachat-Kappes R, Laporte C, Harmand M-F, Simon M, Poinsot C. Alternative in vitro models used in the main safety tests of cosmetic products and new challenges. Int J Cosmet Sci. 2022;00:1–10. https://doi.org/10.1111/ics.12803