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A Successful Example

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Safety Assessment Without Animal Testing: A Successful Example

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ABSTRACT

Since 2013 new animal tests are banned for safety assessment of cosmetic ingredients in the EU. However, there is still a lack of sufficiently predictive in vitro assays for observation of some of the toxicological endpoints, to observe e.g. a considerable rate of so-called 'false' or 'misleading' positives has been reported for in vitro genotoxicity assays. This situation requires not only additional time-consuming and cost-intensive investigations of the genotoxic hazard but also bears the risk of excluding harmless chemicals from the market due to an overestimation of the toxicological potential. Therefore, partners from the industry and academia joined forces for the development and validation of new in vitro methods to prepare for their regulatory acceptance.

Here we report a new in vitro genotoxicity test, the 3D Skin Comet assay, which was developed in a joint effort to supplement the current in vitro test batteries for genotoxicity in order to achieve a better overall predictivity. Initial data from the ongoing validation studies provide promising results with regard to reproducibility and predictivity. In parallel, data obtained with the new method have already been used for the safety assessment of a cosmetic ingredient in a weight-of-evidence approach, which has been accepted by the regulators.

INTRODUCTION

In recent years the European legislator intensified its efforts to reduce animal testing in toxicological safety assessments. Specifically, the chemical legislation REACh [1] considers animal experiments as a last resort, while the 7th Amendment to the Cosmetics Directive even bans marketing of cosmetic ingredients that were assessed in animal experiments after specific deadlines [2].

However, a safety assessment of chemicals exclusively based on *in vitro* test results is still challenging. While *in vitro* testing strategies are available for a meaningful investigation of certain toxicological endpoints (e.g. skin irritation), this is clearly limited for others. *In vitro* test methods are available for genotoxicity or eye irritation, but often exhibit a limited predictivity. In the case of systemic or reproductive toxicity, there are still no *in vitro* assays available that could support a toxicological evaluation. This situation, which could be seen as a hurdle for future innovations, fostered strong engagement of scientists from industries, academia as well as regulators in the development, validation and regulatory acceptance of Alternatives to Animal Testing (AAT). Multinational programs were implemented which aimed at optimizing existing *in vitro* methods to improve their predictivity, e.g. with regard to a genotoxic or eye irritation potential [3, 4].

In addition, programs were implemented to develop new *in vitro* assays. They were intended either to supplement existing *in vitro* toolboxes to improve the respective safety assessment, e.g. genotoxicity [5], or to provide new methodologies for endpoints like systemic toxicological effects leading to repeated dose toxicity that so far can only be addressed *in vivo* [6].

In the following, we report the implementation of a new *in vitro* genotoxicity test, the 3D Skin Comet assay. It was developed to supplement existing test batteries for genotoxicity to improve their predictivity by mirroring the dermal route of exposure. Data obtained with the new methodology have been used successfully to support the safety assessment of a cosmetic ingredient.

Genotoxity

The safety assessment of cosmetic products in the EU is based on the safety data of their ingredients according to the Cosmetic Directive [2]. This document includes positive lists of ingredients approved as safe for use in cosmetics, namely coloring agents, preservatives and UV filters. Addition of an ingredient to one of these lists requires mandatory testing [7] that is reviewed by the Scientific Committee on Consumer Safety (SCCS), an independent expert panel of the European Commission.

An important requirement within this process is the assessment of genotoxicity, i.e. the potential of a chemical to interfere

with cellular DNA, as accumulation of persistent DNA damage is associated with a variety of adverse health outcomes, such as the development of cancer or neurodegenerative conditions.

Compounds that exhibit a genotoxic potential are divided into three classes: (1) mutagens, which induce changes in the nucleotide sequence of the DNA; (2) clastogens, which interfere with the chromosomal structure; and (3) aneugens, which induce numerical chromosome aberrations. Due to the diverse nature of mechanisms, no single *in vitro* or *in vivo* assay is able to detect all types of genotoxins. Therefore, specific batteries of tests are recommended in a variety of industry sectors, including the cosmetics sector [8].

Limited Predictivity of Current Test Batteries

In vitro test batteries have been shown to identify *in vivo* genotoxins and rodent carcinogens to a great extent. However, their ability to correctly identify compounds without a genotoxic potential, i.e. their specificity, can be as low as 5-25% [9, 10]. This especially holds true when results of two or more tests are combined, as required for cosmetic ingredients, leading to a high rate of so-called 'false' or 'misleading' positives. As a consequence, further investigation of the genotoxic potential is needed, sometimes including mechanistic studies in order to further evaluate questionable results [5, 11]. However, these additional time-consuming and cost-intensive investigations may not always be afforded, thus leading to exclusion of harmless chemicals from the market and having a serious impact on businesses when innovations are blocked. This is not limited to cosmetic ingredients, which are assessed with *in vitro* tests only, but also includes chemicals within the scope of legislation that allow or demand animal experiments to further investigate positive *in vitro* findings.

Reasons for limited predictivity

Several reasons have been identified for this low specificity of classic *in vitro* genotoxicity tests. The majority of assays are based on monolayer cultures of cells from different species, which do not possess a normal cell cycle control in all cases. Furthermore, the cells exhibit a limited metabolic capacity compared with the xenobiotic metabolism of the liver as the major detoxifying organ. Usually an external metabolically active supplement, the so-called S9 mix, is added to cope with this effect. However, compared with the metabolic capacity of an intact organ, the added mix provides only a reduced and rather imbalanced spectrum of metabolic enzymes. Consequently, this approach is limited in its ability to mirror normal human liver metabolism or to account for the intended route of exposure.

Strategy to Address Limited Predictivity

The skin functions as the first site-of-contact facing maximum exposure to many products, including cosmetics and household products. Thus, for the development of new *in vitro* genotoxicity assays 3D human reconstructed skin models were selected as promising test systems to investigate effects after dermal exposure. As they are composed of human skin cells, no species-specific differences have to be considered. The cells are derived from biopsies of healthy human donors assuming normal cell control. They are cultured in a three-dimensional environment, which generally better reflects the characteristics of human native skin [12, 13].

In addition, topical application of test compounds helps to overcome solubility issues observed with classical monolayer cultures. Moreover, the bioavailability of a given test compound in the 3D tissues is influenced by both the barrier function of the skin model and its organ- and species-specific metabolism, i.e. properties which are missing or underrepresented in monolayer cultures. Thus, 3D human reconstructed skin models support the application of doses relevant for the intended use, which might be higher than noncytotoxic doses tolerated by monolayer cultures.

Based on these considerations, 3D human skin tissues were combined with classical toxicological readout parameters like the quantification of micronuclei, which appear as small extra nuclei upon DNA damage, to establish the Reconstructed Skin MicroNucleus test, the RSMN [14]. In another approach, the determination of the DNA strand breaks measured with the comet assay were transferred to reconstructed human skin tissues to develop the 3D Skin Comet assay in a joint research

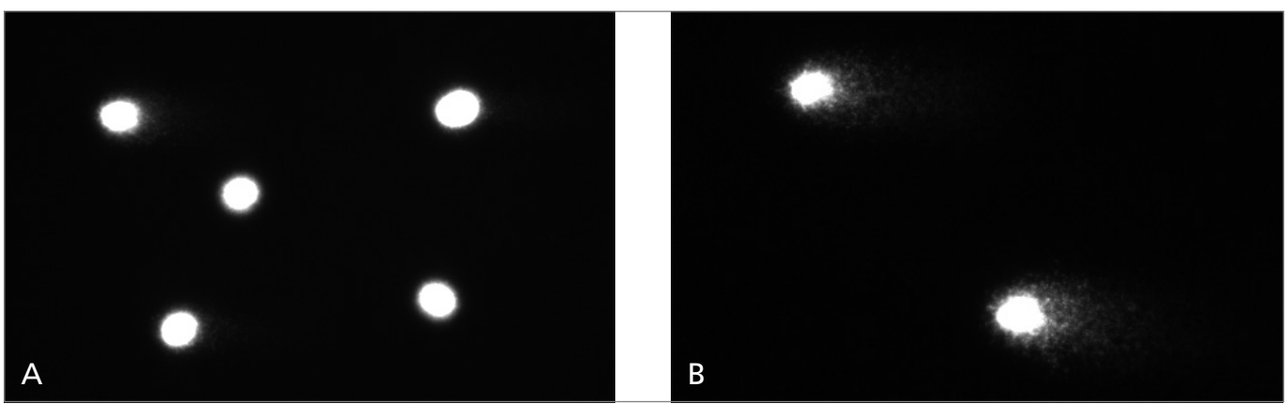

Figure 1 Picture of (A) comets representing normal nonfragmented chromosomes, which remain in the position of the nuclear DNA under the chosen electrophoresis conditions, while fragmented DNA migrates towards the anode forming a comet tail (B).

project funded by Cosmetics Europe and the German Federal Ministry for Education and Research.

3D Skin Comet Assay

In contrast to the micronucleus test, the comet assay does not rely on proliferating cells. Therefore, any cell culture or tissue that can be subjected to single cell isolation can be investigated, which makes the comet assay a versatile tool in different areas like ecological and human biomonitoring or research on DNA damage and repair. Its increased acceptance in regulatory testing was recently documented by the release of the '*In Vivo* Mammalian Alkaline Comet Assay' OECD Testing Guideline [15].

To investigate DNA damage with the comet assay, isolated cells are suspended in liquid agarose and transferred to glass slides to form a gel on top of the slides in which single cells are dispersed [16]. Afterwards cellular and nuclear membranes are disintegrated and proteins are removed by incubating the slides in buffer containing detergents and high salt concentrations. Subsequently, the condensed DNA is allowed to unwind under high alkaline conditions. The glass slides are transferred to an electrophoresis chamber in which the negatively charged DNA migrates according to its size in the electric field. Afterwards DNA is stained and investigated under a fluorescence microscope. Intact DNA, which was unable to migrate under the electrophoresis conditions used, appears as round cell nuclei ***(Figure 1)***. In contrast, DNA fragments that evolved after treatment with a test compound were able to migrate. They become visible as the tail of a comet behind the comet head formed by non-migrated DNA. The fluorescence intensity in the tail relative to that of the comet head is measured semi-automatically and finally used to assess the DNA-damaging properties of a test compound.

Two types of DNA damage can be investigated with the alkaline comet assay described above. First, clastogenic effects are detected, e.g. double strand breaks, which evolve upon direct interaction of a test compound with DNA. In addition, DNA damage that may give rise to gene mutations can be detected. As an example, certain chemicals are able to modify single nucleotides which may transfer incorrect information during upcoming DNA doubling and cellular division leading to gene mutation. Generally, such modified nucleotides are recognized and DNA is repaired by specific enzymes, which induce transient single strand breaks to excise them. In these cases, the high alkali conditions used with the comet assay also liberates fragments evolved during the DNA repair processes amplifying DNA migration and comet formation.

Recently, the alkaline comet assay has successfully been transferred to the Phenion® Full-Thickness (FT) Skin Model (Henkel, Germany; ***Figure 2***). The tissue consists of a well-built dermis based on a collagen matrix in which fibroblasts are cultivated in a reconstructed but natural environment [17]. Primary keratinocytes, which originate from the same human donor as the fibroblasts, form a fully differentiated epidermis, which is characterized by all the layers observed in human native skin including the *stratum corneum*, which pivotally mediates the barrier function of the skin ***(Figure 2)***. In addition, it could be shown that the Phenion® FT Skin Model possesses metabolic competency mirroring human native skin appropriately [18, 19].

The reproducibility and predictivity of data obtained with the method are being evaluated in a validation study funded by Cosmetics Europe and the German Federal Ministry for Education and Research using 30 coded compounds. The outcome of the first phase in which eight chemicals were investigated was promising: four laboratories predicted all chemicals correctly and the fifth laboratory obtained a predictivity of 80% [20]. In parallel to the validation study the 3D Skin Comet assay has been used successfully to support the safety assessment of a hair dye ingredient, namely Basic Brown 17.

Use of the 3D Skin Comet Assay for Regulatory Purposes

The current assessment of the genotoxic potential of chemicals foresees the use of an initial battery of *in vitro* tests in many industry sectors, including the cosmetics sector [8]. It generally starts with the Bacterial Reverse Mutation Test (Ames test, OECD TG 471) [21] to identify mutagenic lesions, typically followed by the *in vitro* mammalian cell micronucleus test (MNvit;

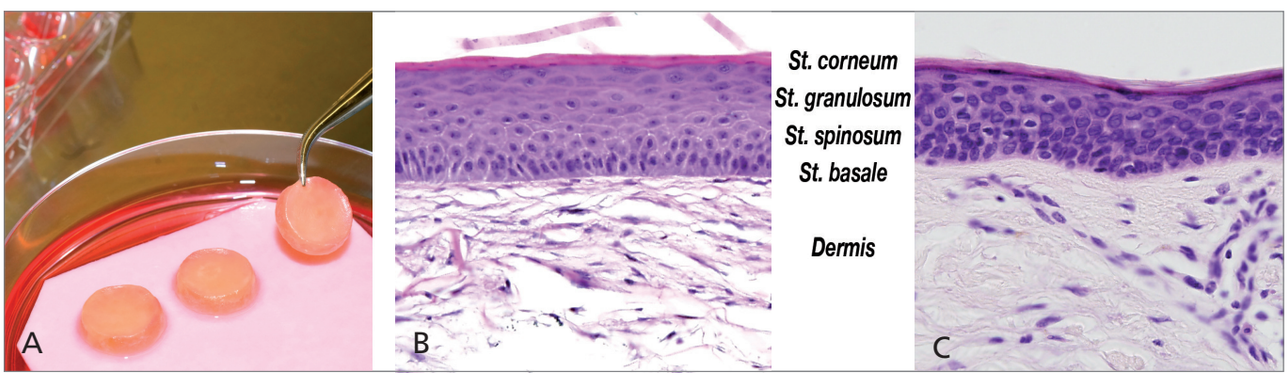

Figure 2 (A) Macroscopic picture of Phenion® Full-Thickness Skin Tissues. (B) Cross-sections of a paraffin-embedded and hematoxilin/eosin-stained (B) skin tissue and (C) human native skin. All strata of human native skin can be observed as the palisade-shape basal membrane, and the stratum corneum. The dermis beneath was generated based on a collagen matrix.

OECD TG 487) [22] for the detection of clastogenic and aneugenic effects. The relevance of possible positive findings is further analyzed in follow-up tests, which have to rely exclusively on *in vitro* methods when investigating cosmetic ingredients. Following these prerequisites, the hair dye ingredient Basic Brown 17 was investigated first in the Ames test and in the MNvit. The substance was found to be negative (favorable) in the MNvit but positive (unfavorable) in the Ames test [23]. Historical *in vivo* data performed when *in vivo* testing was allowed according to EU Cosmetic legislation and requested by the SCCS to address positive *in vitro* findings were not available. Therefore, *in vitro* experiments were conducted to further evaluate the positive Ames result, namely, two *in vitro* mammalian cell gene mutation tests that were negative (favorable). Subsequently, the 3D Skin Comet assay using the Phenion® FT Skin Model was performed. The negative findings obtained confirmed the absence of both mutagenic and clastogenic effects. After a thorough review of the data package prepared in a joint effort of different partners, the SCCS affirmed the absence of a genotoxic hazard of Basic Brown 17 for the given dermal exposure scenario [23].

In addition to the assessment of specific dossiers, the SCCS provides guidance on testing of cosmetic ingredients and recently revised the genotoxicity testing guideline to reflect progress made with the characterization and validation of the reconstructed skin model-based assays [8]. In its 'Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation' the SCCS calls these assays a 'good alternative to bridge the gap between *in vitro* and *in vivo* testing in terms of final hazard assessment' [8] and recommends using both *in vitro* assays introduced the 3D Skin Comet assay and the RSMN, to follow-up on unfavorable results from the *in vitro* standard testing battery.

With this statement, a clear step towards regulatory acceptance was achieved. Both assays, the 3D Skin Comet and the RSMN, were developed to supplement the test battery to address the problem of 'false' or 'misleading' positive results for dermally exposed compounds. The objective of the new methods is to increase the specificity of the respective toolbox while retaining or even increasing its high sensitivity. Applied together, they are able to address the three different types of DNA damage: The RSMN detects clastogens and aneugens, whereas the 3D Skin Comet assay identifies clastogens and DNA lesions, which give rise to gene mutations. Finally, with the incorporation of 3D reconstructed human skin tissues, the relevant route of exposure for dermally applied compounds is taken into account following recent recommendations of OECD Testing Guidelines (OECD TG 474; OECD TG 489) [23, 24].

In future, additional industries may also profit from these approaches when the assays have gained broader regulatory acceptance.

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