Cell based models for validation of safety of cosmetic products and ingredients

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Abstract- The use of cosmetic products has grown many folds in recent years. The consumer and regulatory bodies are more concerned about the hazardous outcome of these products on skin health. The development of alternative and effective safety assessment methodologies to avoid animal testing is an urgent need due to restrictions and ethical considerations set by various regulatory bodies such as OECD, COLIPA, and BIS. The new integrated approaches like *in-vitro* assays (Cell-based and Cell-free assays) are indispensable for assessing the safety of cosmetic products. *In-vitro* testing systems provide advantages over animal testing as they are more cost-effective, and less time-consuming. In this article, we present the details of some of the important *invitro* assays that are routinely used by different laboratories for validation of the safety of cosmetic ingredients.

Key words: Safety and toxicity, Skin irritation, Skin sensitization, Eye irritation, Phototoxicity, Chromosomal aberration.

Introduction

In today's highly competitive personal care and cosmetic product market, evidence that the product is safe and effective can mean the difference between getting noticed and getting lost in the crowd. The EU defines a cosmetic product as "any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odors and/ or protecting them or keeping them in good condition[1]. Personal care products may contain some ingredients which can potentially cause adverse reactions when applied locally or systemically. Regulatory authorities worldwide have made it mandatory to prove the safety of any products intended for human use. In earlier years various toxicology testing methods and guidelines were developed by different agencies across the globe and most of them involved wide use of different animals; for example, Draize test for eye irritation was done in rabbits, acute oral and reproductive toxicity is done in mice etc. In recent years, there was a large outcry led by organizations like PETA and Humane Society International against the widespread use of animals for cosmetic testing. In a historical decision, the European Cosmetic and Perfumery Association (Colipa) in its 7th Amendment to the Cosmetics Directive (2003/ /15/EC) introduces a progressive ban on animal testing and a marketing ban on cosmetic products and their ingredients that are tested on animals[2]. The Government of India prohibited the testing

of cosmetics on animals vide G.S.R. 346(E) in 2014 by inserting the following rule in the D & C Rules (Drugs and Cosmetics Act 1940 and Rules 1945), "148-C: Prohibition of testing of cosmetics on animals- No person shall use any animal for testing cosmetics"[3]. Further, it was specified that when there is a need to demonstrate the absence of adverse reaction, the manufacturer shall submit the safety data based on alternative non-animal test methods. In China where pre-market animal tests for cosmetic ingredients were previously mandatory, it's National Medical Product Administration (NMPA) has approved two new non-animal tests (for skin sensitization and eye irritation) for cosmetics ingredient testing.

Development of relevant in-vitro tests requires selecting appropriate endpoints and also analysis and appreciation of the key initiating events that occur in vivo in the progression of the toxic damage. In 1992 Colipa created the Steering Committee on Alternatives to Animal Testing (SCAAT) to coordinate the efforts of the cosmetics industry in the development, validation, and acceptance of alternatives to animal testing for evaluating the safety of products and ingredients[4]. In Europe, alternative testing methods developed by different laboratories are scientifically validated by the European Centre for the Validation of Alternative Methods (ECVAM), an official body appointed for this purpose by the European Union[5]. The ECVAM has proposed a list of validated cell-based in vitro models for predicting the safety and toxicity of cosmetic ingredients[6]. On an international level, Colipa works with the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in the United States, as well as with partners from Canada and Japan[7]. OECD has developed Guidelines for the testing of chemicals which are a collection of the most relevant internationally agreed testing methods used by governments, industry, and independent laboratories to assess the safety of chemicals. They adhere to the concept of 3R's (Reduce, Replace, and Refinement) for animal usage and have validated protocols for in vitro genotoxicity, skin corrosion, skin absorption, phototoxicity, ocular severe irritation, and corrosion, as well as for screening potential endocrine disrupters. OECD countries and full adherents have agreed that a safety test carried out in accordance with the OECD Test Guidelines and Principles of Good Laboratory Practice in one OECD country must be accepted by other OECD countries for assessment purposes. In wake of a growing concerns of product safety amongst the consumers across the world and need for adopting alternative methods for animal testing, establishing in vitro assay methods to evaluate the safety of personal care products is of utmost importance for several laboratories. Several cosmetic laboratories, including ours, are progressively adopting various in vitro methodologies for ensuring the safety of personal care products before taking them to clinical studies and marketing. Different safety parameters such as skin and eye irritation/corrosion, phototoxicity, skin sensitization etc have been addressed in the array of tests that we have adapted according to our product portfolio. Different mammalian cell lines are used in accordance with the internationally validated and approved protocols (OECD guidelines) to establish the safety profile of personal care products. The in-vitro tests to check the adverse reactions such as skin irritation (OECD TG439), eye irritation (OECD TG491, 492), phototoxicity (OECD TG432), and skin sensitization (OECD TG442C&E) have been established in our laboratory.

In-vitro assays and models to assess the safety of cosmetic products

In-vitro assays and models are used to assess the toxicity and safety of test substances in biological systems under artificial conditions without the involvement of animals. There are a variety of *in-*

vitro assays developed to test the safety of cosmetic ingredients, and identification of hazardous ingredients. In the sections below, we discuss some of the routinely used cell based assays. **Figure-1** summarizes the types of the assay systems discussed in this article.

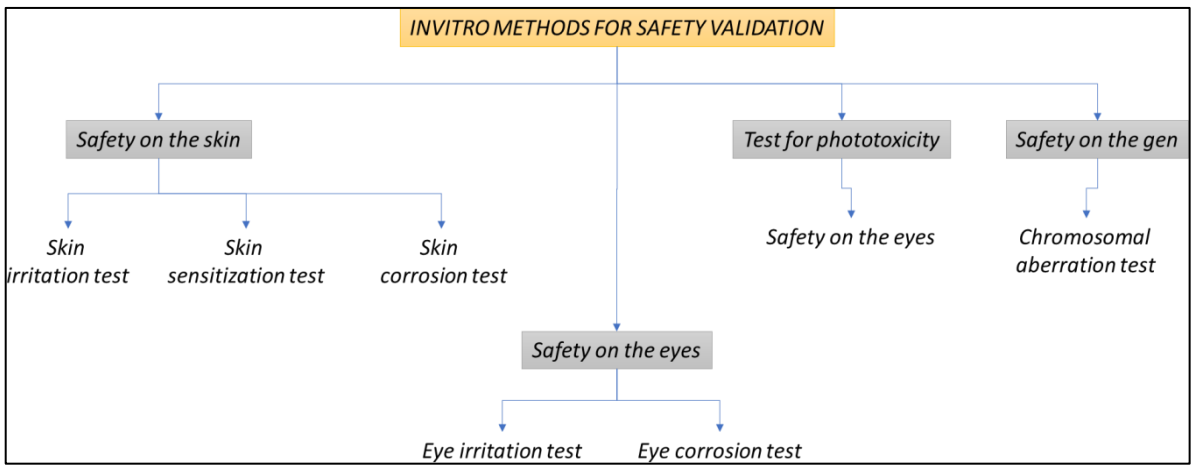

Figure-1 Methods for safety validation

In vitro Skin-irritation using Reconstructed Human Epidermis is test method

As per the United Nations (UN) Globally Harmonized System of Classification and Labeling of Chemicals (GHS), the reversible damage to the skin caused by exposure to a mixture or substance refers to skin irritation (https://unece.org/ghs-rev8-2019). The skin irritation potential of ingredients in the formulation is a critical factor in cosmetic safety assessment. In this test, the reconstructed RhE is used, which resembles the human epidermis and mimics its biochemical and physiological nature. The non- transformed human keratinocytes are used to create RhE, the final model is analogous to the In-vivo conditions as it consists of multiple layers of human epithelial cells[8]. Various concentrations of the test compound are directly applied to 3D-RhE model and cell viability is quantitatively assessed by enzymatic conversion of yellow dye MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide into a blue formazan salt[9-10]. **Figure-2** outlines the steps involved in, this assay.

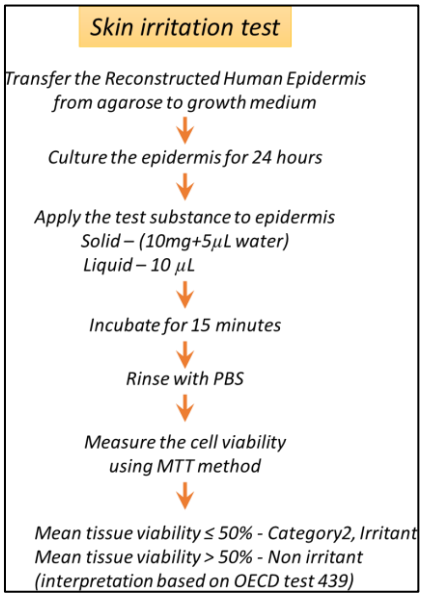

Figure-2 Skin Irritation Test

In- vitro 3T3 phototoxicity testing

Phototoxicity refers to the toxic elicited by photoreactive chemicals upon exposure to response environmental light. Cosmetic products may contain ingredients that are photo reactive and elicit an adverse response by the host. This is taken into account in an in vitro model system for assessing phototoxicity. As described in OECD test number – 432, 3T3 (Immortalized mouse fibroblast) cell lines are used for testing the phototoxic effects of the cosmetic ingredients. Cells are treated with test substances under two different conditions – one set of cells is not irradiated and the other set is irradiated. Further, neutral red dye is used to measure the viability of the cells. The IC_{50} values (the concentration of the test substance that causes 50% cell death) are calculated under these two conditions. The weak cationic neutral red dye is used to measure the viability as it can readily enter the membrane and accumulates in the lysosomes. Phototoxins induce cellular damage through reactive oxygen species and other mechanisms which alters the permeability of lysosomal membrane resulting decreased uptake and binding of Neutral red. The UV/vis spectrum of test chemical can be determined before testing *in-vitro*. Photo-cytotoxicity induced by test chemical is expressed quantitatively as reduction in the uptake of neutral red dye by cells in presence and absence of light with respect to solvent controls[11]. The chemical found to be photocytotoxic *in vitro* in this test can be phototoxic *in-vivo*. Some of the steps involved in this assay are outlined in **Figure-3.**

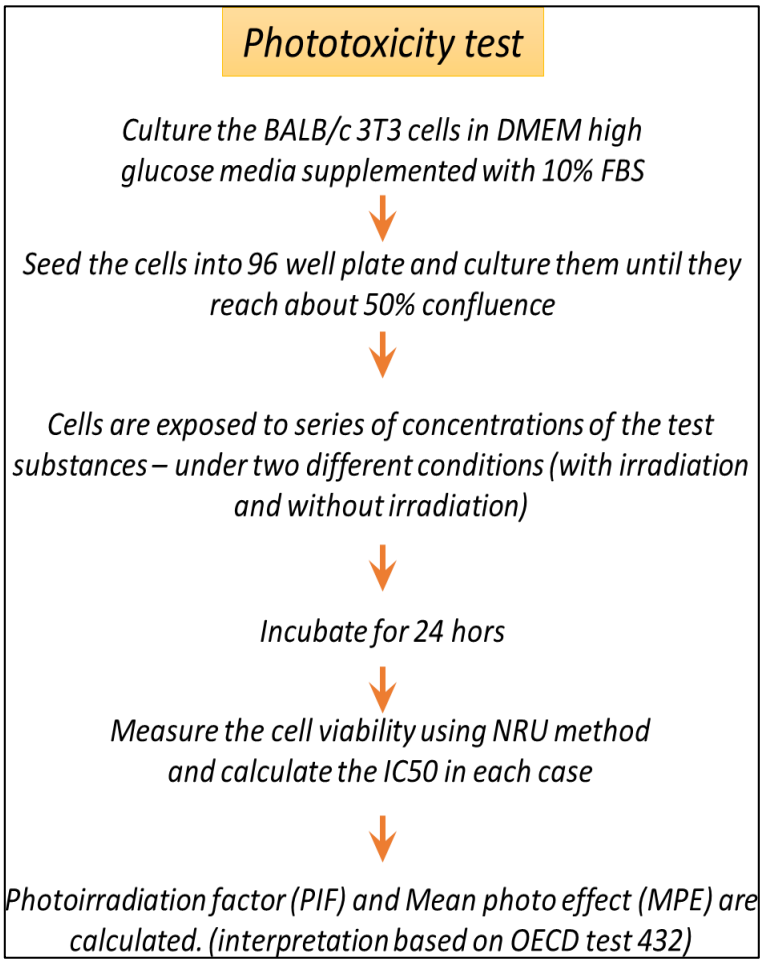

Figure-3 Phototoxicity test

In-vitro skin sensitization (Human Cell Line Activation Test, h-CLAT)

A skin sensitizer is any substance which induces allergic response following skin contact. This *in-vitro* test supports the discrimination between skin sensitizer and non-sensitizers. In h-CLAT, the sensitizer causes change in expression of cell surface markers resulting in activation of monocytes and dendritic cells h-CLAT method is useful to test the potential chemical with variety of functional groups, reaction mechanisms, and skin sensitization potency. As summarized in Figure-4, this test quantifies the change in expression of CD86 and CD54 on Human monocytic cell line (THP-1) following treatment with the test chemical. Flow cytometric analysis following cell staining with fluorochrome-tagged antibodies is used for measurement of changes in expression of cell surface markers.

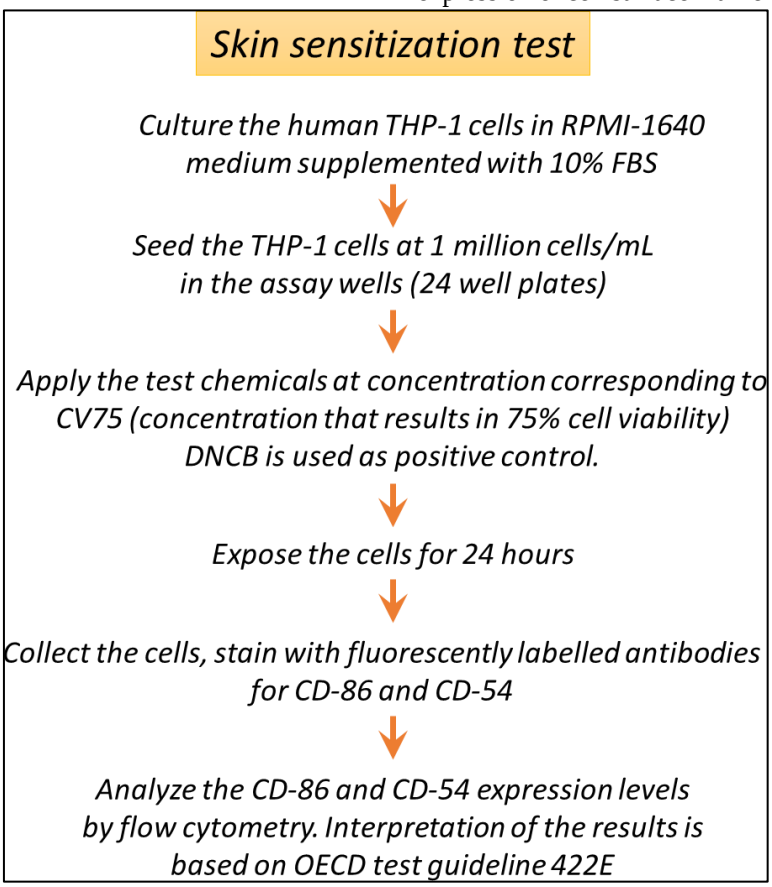

Figure-4 Skin Sensitization test

In -vitro Skin Corrosion Test

In this test, the 3D reconstructed human epidermis is used to identify potential skin corrosives following exposure time points of 3, 60, and 240 minutes. The human skin model assay works on the assumption that corrosive chemicals can penetrate the stratum corneum via diffusion or erosion and are cytotoxic to the underlying cell layers. The test material (solid or liquid) is applied topically and uniformly to a three-dimensional human skin model that includes at least a reconstructed epidermis with a functional stratum corneum. For each treatment (exposure time) and control, two tissue replicates are used. Corrosive materials are distinguished by their ability to reduce cell viability below defined threshold levels during specified exposure times. The cell viability is assessed

using MTT assay as end point. If cell viability is below 35% after 3 minutes of exposure time, the test chemical is corrosive as per sub-category 1A. The chemical will be considered as corrosive as per sub-category 1B and 1C if cell viability ≥ 35% after 3 min exposure AND < 35% after 60 min exposure OR ≥ 35% after 60 min exposure AND < 35% after 240 min exposure. In case cell viability remains ≥ 35% after minutes of exposure, the test chemical is considered non-corrosive[14].

In vitro eye irritation test (Short time exposure)

In this test, the sample is evaluated for its eye irritation potential using a confluent monolayer of Statens Serum institute Rabbit Cornea (SIRC) cells[12]. The cytotoxicity can be quantitively measured as the relative viability of SIRC cells following a five-minute exposure to test chemical. The reduction in cellular viability in SIRC cells following treatment with the test chemical is indicative of an adverse effect which may cause ocular damage. The cell viability is quantitatively assessed by enzymatic conversion of yellow dye MTT 3-(4, 5- Dimethylthiazol-2-yl) -2, 5-diphenyltetra-zolium bromide into a blue formazan salt. The hazardous potential of test chemical can be estimated by comparing the viability of treated cells with their respective solvent controls. The recommended exposed concentration of test chemical to the cells is 5%, 0.5%, and 0.05%. If the cellular viability at both 5% and 0.05% is less than or equal to 70% then the test chemical may have eye irritant properties and falls under category 1 chemical of the United Nations Globally Harmonized System of Classification and Labeling of Chemicals (UN GHS). The test chemical can be predicted as U N GHS No Category when the cellular viability is greater than 70% at both 5% and 0.05% concentration. Figure-5 outlines the steps involved in this assay.

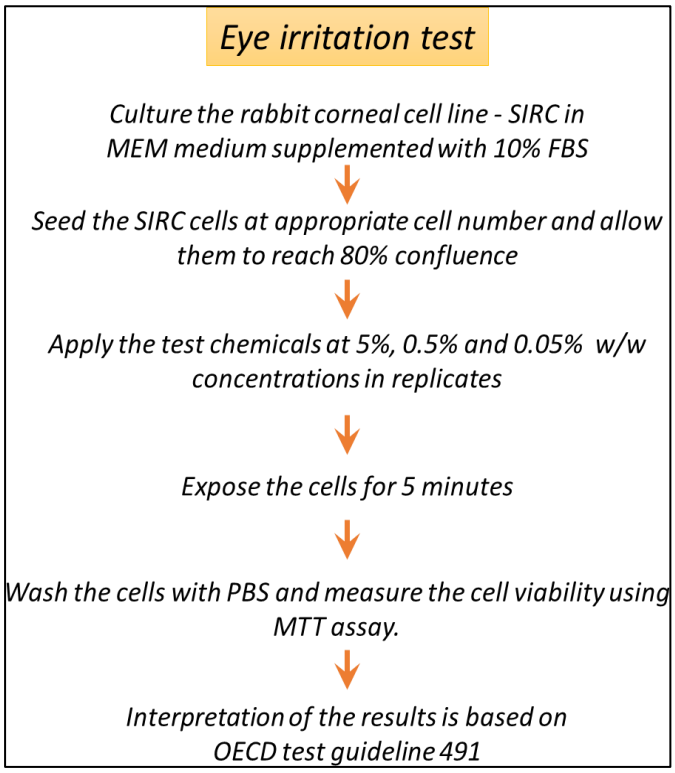

Figure-5 Eye irritation test

In vitro Fluoresce in Leakage Test Method

In this *in vitro* assay, the test chemical is assessed for its potential ocular corrosive nature and irritant effect[13]. This assay is performed on a confluent monolayer of Madin-Darby Canine Kidney (MDCK) cells grown on semi-permeable inserts. The MDCK cell line forms tight and desmosomal junction like the conjunctiva and corneal epithelia. The alteration in the permeability through these junctions, is the key event in chemical induced ocular damage. The leakage of fluoresce in through the junctions following test chemical treatment can be measured spectrofluorometrically. The amount of fluoresce in leakage is proportional to damage to the junctions and calculated with reference to fluorescence intensity of blank control and maximum leakage control. The concentration of test chemical (mg/ml) which produces 20% Fluoresce in leakage (FL_{20}) is considered as ocular corrosive and irritant. The chemical is ocular corrosive/severe irritant if the 20% Fluoresce in leakage (FL_{20}) is less than or equal to 100mg/ml.

In Vitro Mammalian Chromosomal Aberration Test

This test is to identify substances that cause structural chromosomal aberrations in cultured mammalian cells[15]. Structural aberrations are of two types: Chromosome and Chromatid. Primary cell cultures or the established cell lines of human or rodent origin can be used for this *in vitro* test. The stability of karyotype, capacity for cultured growth, and the frequency of chromosomal abnormalities are important factors while choosing the cells. Cell cultures are exposed to the test substance (liquid or solid) both with and without metabolic activation throughout roughly 1.5 typical cell cycle lengths. There should be at least three separate analyzable concentrations of the test substance used. It is recommended to utilize duplicate cultures for each concentration. Following exposure of cell cultures to the test drug, the cells are treated with a compound that stops the metaphase, collected, and stained. Cells that have been arrested in metaphase are examined under a microscope to see whether chromosomal aberration is present (Figure-6)

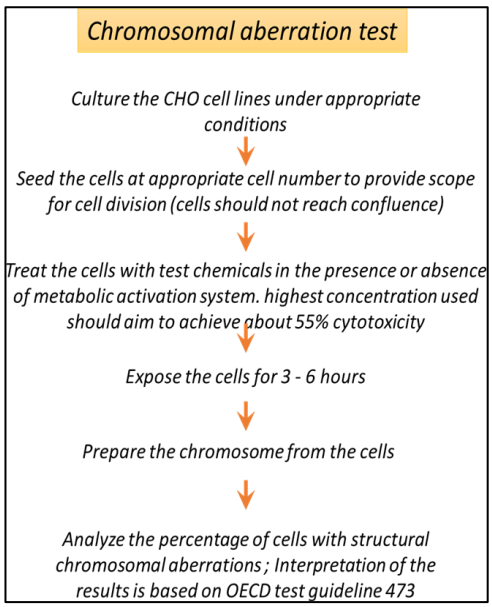

Figure-6 Chromosomal aberration test

Conclusion

Safety validation of the cosmetic products and ingredients is critical during the product development. As a matter of internal policy and owing to regulatory requirements, animal-based tests are not conducted in most of the industrial set ups for evaluating the safety of the cosmetic products. This necessitates extensive use of cell based systems. Several laboratories have been working on developing state of the art *in-vitro* methods that best mimics the physiological conditions. However, the non-animal based testing systems do have some limitations and some of the aspects of drug metabolism, absorption and toxico kinetics cannot be completely mimicked by these systems. In addition, the isolated cell based systems may lack physiological milieu which might be essential for capturing the effects of test substances. These aspects need to be carefully considered while setting up the safety assays for the cosmetic products. More than one assay system may be necessary before drawing the conclusion. With the advent of 3D skin technology, several drawbacks of the isolated cell based system can be overcome.

Disclaimer Statement

Authors declare that no competing interest exists. The products used for this research are commonly used products in research. There is no conflict of interest between authors and producers of the product.

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