IN VITRO SAFETY TESTING STRATEGY FOR SKIN IRRITATION USING THE 3D RECONSTRUCTED HUMAN EPIDERMIS

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One of the most recent trends in skin irritation safety testing of personal care and cosmetic products, discussed in detail in this review, is the use of reconstructed human epidermis models. Some of the commercially-available models have undergone a series of validation trials, which successfully advanced them as valuable tools for industry use. On a broader perspective, the 3D models are now part of the integrated decision-tree testing strategies used in regulatory settings for skin irritation and/or corrosion registration and labeling of newly designed formulations. Furthermore, the reconstructed human epidermis models are also widely used by industry for product development, safety testing, and ranking of personal care and cosmetic products’ irritation potential. Although the specific protocols used for meeting either legislation-driven regulatory safety testing or non-regulatory, industrial safety screening may differ considerably, the in vitro tissue systems and approaches are aligned to meet the newest legislation or corporate policies prohibiting the use of animals for safety testing.

Key words: skin irritation, in vitro testing, 3D reconstructed human epidermis, cytokines.

INTRODUCTION

The skin represents the first defense barrier of the human body against the physical and chemical factors to which it is routinely exposed. Skin irritation is a complex phenomenon that involves a host of cell populations acting in a coordinated fashion and focused on locally containing the effect of these physical and chemical factors which are potentially harmful to the body. Keratinocytes, the main cell type of the outer layer of skin play a critical role in the initiation and perpetuation of skin inflammatory reactions through the release of, and responses to a network of cytokines, of which interleukin-1 alpha (IL-1α) is considered the main switch in the induction of the inflammatory cascade.

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Whenever new personal care or cosmetic products are about to be launched for public use, or when a new active substance is to be used as a medicinal product in humans, critical safety testing approaches need to be employed with the purpose of limiting the risks for the population using those products. While \textit{in vivo} (animal) testing is still used particularly in the pharmaceutical industry, the personal care and cosmetic industry is currently adopting alternative \textit{in vitro} methods, which reduce, refine, and replace the use of animals. The use of validated \textit{in vitro} assays is of particular interest to personal care and cosmetic industries in their effort to comply with the most recent legislative measures banning the marketing of the products that contain ingredients tested in animal models (i.e., the 7\textsuperscript{th} Amendment of the European Union Directive 76/768/EEC [Cosmetics Directive]). One alternative to animal testing for skin irritation is represented by the use of reconstructed human epidermis models, which are commercially available from several suppliers internationally. Several examples of specific \textit{in vitro} protocols for these reconstructed human skin models utilized in our laboratory (Institute for In Vitro Sciences, Inc., Gaithersburg, USA) will be detailed herein.

The focus of this review is to provide an integrated view of the current understanding of skin irritation testing, based on the available clinical, \textit{in vivo} and alternative \textit{in vitro} methods used particularly by personal care and cosmetic companies in their effort to establish the safety of their newly designed products.

**HUMAN SKIN BARRIER FUNCTION**

The skin is a complex, integrated and dynamic organ that plays a critical protective role against harmful environmental hazards, such as exposure to exogenous noxes (corrosive chemicals, irritants etc.) or ultraviolet (UV)-irradiation, as well as against endogenous water loss. Being the body’s first line of defense against external insults, the skin’s surface is therefore routinely exposed to chemicals and thus may inadvertently serve as a portal of entry for topical aggressive agents that can induce acute or chronic systemic reactions (1). Based on its biological sophistication, skin performs a wide range of functions, participating directly in thermal, electrolyte, hormonal, metabolic and immune regulation and thus, to the maintenance of internal homeostasis of the body (1).

The human skin is anatomically divided into three layers: the epidermis, which is the outermost layer, the underlying dermis and the deeper localized hypodermis. The epidermis is composed of several distinct cell populations, of which the keratinocytes represent the majority. As shown in Fig. 1a, the human epidermis consists of four layers (basal layer, \textit{stratum spinosum}, \textit{stratum granulosum} and \textit{stratum corneum}) constantly undergoing an orderly pattern of proliferation, differentiation, keratinization, and exfoliation. Keratinocytes of the
basal layer comprise the germinative compartment. When a basal cell divides, one of the progeny detaches from the basal layer and migrates outward (2); as cells move toward the skin surface, they undergo a remarkable program of terminal differentiation and start to gradually produce and accumulate keratin proteins. At the granulosum layer, the cells undergo a striking morphological transformation, becoming flattened; at this level, lipid granules fuse with the plasma membrane at the stratum granulosum/stratum corneum interface, filling the intercellular spaces with lipids, as opposed to the aqueous intercellular solution in the viable epidermis. Concurrently, the plasma membranes of these cells become permeable, resulting in the loss of their reducing environment and, consequently, in extensive disulfide bonding among keratin proteins. Cellular organelles are degraded, while a protein envelope is synthesized immediately beneath the plasma membrane. The membrane is altered characteristically by the loss of phospholipids and the addition of sphingolipids (2).

Fig. 1. – a) Structure of human skin epidermis showing the different component layers; b) Possible molecular mechanisms of skin irritation; c) Sequence of events and symptoms induced by the interaction of skin with a potentially irritating agent. Abbreviations: ERK – extracellular signal-regulated kinase; IL – interleukin; MAPK – microtubule-associated protein kinase; PLA2 – phospholipase A2; TNF-α – tumor necrosis factor alpha.
This program of terminal differentiation produces the outermost layer of the skin, the *stratum corneum*, which contains nonviable but biochemically active cells, called cornified keratinocytes, also known as corneocytes, embedded in extracellular lipids. This epidermal barrier reduces transepidermal water loss from within and provides a unique barrier that prevents invasion by infectious agents and noxious substances from without. The *stratum corneum* is an anatomically unique structure with an unusually high resistance arising from its robust brick (corneocytes) and mortar (intercellular lipid bilayers)-like structural organization (3). Structurally, the epidermal permeability barrier is conferred by the intercellular lipids, arranged into lamellar sheets and which make the *stratum corneum* the rate-limiting barrier to the absorption of most topically applied chemicals (4). However, there is a shift in the lipid components of the skin, from nonpolar and neutral lipids in the *stratum corneum* to the polar lipids in the lower layers of the epidermis (from basal layer to *stratum granulosum*) that determines the different pathways molecules follow to diffuse across the skin. As such, most topically applied substances, particularly nonpolar or hydrophobic compounds, are absorbed by diffusion across the *stratum corneum* and epidermis through the intercellular corridors, while the hydrophilic compounds prefer the transcellular absorption route. It is the biphasic composition of the *stratum corneum* that is the primary determinant of skin permeability and thus regulates the potential of topically-applied irritants to induce irritation or corrosion (5).

**SKIN IRRITATION/CORROSION**

Among the cutaneous inflammatory reactions, skin irritation is one of the most common adverse effects in humans and thus presents a major health problem, with serious social and occupational impacts (6). The presence of erythema, edema, dryness of the skin, fissures, desquamation, itching and pain are common symptoms of skin irritation and ultimately represent the physiological manifestation of a complex chain of biochemical, neural, vascular and cellular responses following the initial single or repeated irritation signal (7). While skin irritation is characterized by reversible damage of the tissue following exposure to certain products, skin corrosion is irreversible and is characterized by visible necrosis of the epidermis and dermis.

**IRRITANTS**

Every pure chemical or final formulation, including even water after long-term exposure, has an irritation potential for skin (8). Since the skin is often exposed, either intentionally or unintentionally, to cosmetic products, the potential for a particular ingredient or final product to cause skin irritation needs to be
thoroughly evaluated as part of the overall safety assessment process before the new products are launched on the market (9).

For example, molecules consisting of a hydrophobic tail and a hydrophilic head, like surfactants and emulsifiers, are commonly used in the composition of cosmetics and cleaning agents. Both are able to reduce the surface tension of and to form micelles in solution, which makes them essential ingredients in many formulated products. However, they can also interact with lipids of the skin, fluidizing lipid-based membranes and thus leading to adverse effects (10). Furthermore, solutions with lipid dissolving properties (such as aromatic and aliphatic hydrocarbons and their derivatives) can lead to skin irritation; inorganic chemicals like acids, bases and salts, and numerous pharmaceutical compounds can also trigger irritation of the skin.

Since many different classes and chemical structures of substances have irritant potential, it seems highly probable that different pathways must be involved in skin irritation. For a compound to be an irritant, it has to first penetrate the stratum corneum and reach the living epidermal layers underneath. Although some chemicals may have limited penetration through the stratum corneum, those of high toxic potency may still be very dangerous. Damage to viable cell membranes and release of pre-stored pro-inflammatory cytokines, such as IL-1α, initiate the cytokine/chemokine cascade, which in turn triggers an immune reaction (11–15). Therefore, the strength of an irritant is dependent on both its ability to penetrate the stratum corneum and its cytotoxic effect on living cells.

**MOLECULAR MECHANISMS OF SKIN IRRITATION**

In general, it is accepted that chemicals can follow at least two distinct pathways to initiate and modulate skin irritation (16): firstly, via damage to the barrier function of the stratum corneum and secondly, by direct effects on cells of the skin. Both pathways may lead to irritation, either alone or in combination.

Structurally, the stratum corneum is the outermost layer of the epidermis; the dead, keratin-rich corneocytes are tightly connected by desmosomes, and the intercellular space is filled with various lipids, mainly ceramides and neutral lipids, such as free cholesterol, cholesterol esters and free fatty acids (17). This composition gives rise to an effective barrier against exogenous influences and endogenous water loss. Some irritants entering the stratum corneum may lead to delipidation and protein denaturation. During delipidation, the composition or balance of the various lipids is disturbed, subsequently resulting in an altered lipid composition or changes in lipid organization and loss of the barrier function (18). These routes to barrier damage lead to increased penetration of irritants to the living keratinocytes and enhanced transepidermal water loss.
Despite numerous published reports on the effect of irritants on keratinocytes, it is still far from clear how substances lead to irritation. However, the most detailed mechanism by which chemicals lead to skin irritation is that triggered by surfactants. This understanding is mainly based on the widespread use of surfactants in products frequently exposed to skin, and particularly the evaluation of effects of sodium lauryl sulphate (SLS) as a reference substance in numerous in vivo and in vitro experiments (19). Surfactants can be especially disruptive to cell membranes, resulting in cell death and the concomitant release of keratinocyte cytoplasm (20, 21) containing the pro-inflammatory cytokine IL-1α.

Keratinocytes represent the major cell type in the epidermis and their primary function is to provide the structure and barrier function of the epidermis. However, keratinocytes play an important role in the initiation and perpetuation of skin inflammatory and immunological reactions (22). In an intact epidermis, the IL-1α reservoir is naturally eliminated by desquamation, due to the fact that IL-1α has no hydrophobic leader sequence for transmembrane secretion. A variety of environmental stimuli, such as tumor promoters, UV light and chemical agents, can induce epidermal keratinocytes to up-regulate the release of inflammatory cytokines (22); therefore, the vast reservoir of sequestered IL-1α is released from leaky cells following cell injury or membrane perturbation (23) and initiates the inflammatory cascade upon damage to the skin, as a primary event in skin defense. IL-1α is considered the main switch in the induction of the inflammatory cascade (24-26) because it induces the expression of further (secondary) cytokines, such as IL-6 and IL-8. Furthermore, IL-1α activates phospholipase A2 (PLA2), which is a key enzyme in the arachidonic acid signaling cascade (27) (Fig. 1b). In response to physical or chemical stresses, keratinocytes produce and release other inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), growth promoting factors (IL-6, IL-7, IL-15), transforming growth factors, cytokines regulating humoral versus cellular immunity (IL-10, IL-12) and other signaling factors which rapidly generate cutaneous inflammation (28) (Fig. 1b). TNF-α is stored in dermal mast cells (29), but following stimulation it may be produced by keratinocytes (30) and Langerhans cells as well (31).

The keratinocyte cytokines IL-1α, IL-1β and TNF-α activate a sufficient number of effector mechanisms to independently trigger cutaneous inflammation, and thus they are considered primary cytokines. In addition to being directly chemotactic for leukocytes, IL-1α induces the expression of intercellular adhesion molecules on the surface of endothelial cells and fibroblasts (32) that contribute to the healing of the affected tissue. Thus, by cytokine cascades and networks, an inflammatory response can be rapidly generated following exposure to a skin irritant. Overall, the inflammatory cascade involves specific interactions between the cells of the immune system, which are initiated by the release of cytokines from initial target cells in the skin epithelium, following the exposure to an irritant. The
cytokines then activate the endothelial cells of the blood vessels to undergo vasodilation and permit an influx of fluid and associated inflammatory responding cells (neutrophils, leukocytes), leading to erythema and edema, respectively (Fig. 1c). Given the IL-1α role in the inflammatory pathways, it has been suggested that the measurement of keratinocyte responses with respect to cytokine production may allow the evaluation of toxicological properties of chemicals in order to identify irritants.

**HUMAN CLINICAL TRIALS**

Few experiments focused on skin irritation of potentially dangerous compounds have been performed on humans because of the direct risk of lesions, overt skin irritation or corrosion, and intoxication of the subjects. However, testing of mild or non-irritating materials on humans using ethically approved protocols is done routinely. Among the available human data on chemical toxicity, some derive from chemical insults with severe irritants due to accidents at home or at work, or due to repeated skin exposure to moderate irritants. The other human data for skin irritation testing were obtained by patch testing on relatively high numbers of volunteers (33–35). At least in the United States, compounds were tested pure or diluted, for different exposure times, but the experiments were stopped when moderate to severe reactions to the test compound were observed (36). However, due to concerns of inducing sensitization reactions, the European Union has more stringent regulations restricting the repeat dose studies on human subjects.

The fact that the use of animals has been recently banned in the European Union for testing skin irritation potential of cosmetics ingredients and products is not a problem for the safety assessment, since testing of expected mild cosmetics products and ingredients is easily and ethically conducted in volunteer human subjects. In most instances, the hazard of the test substance has already been characterized in previous *in vivo* or *in vitro* studies; therefore, the use of volunteers provides the completion or verification of the risk assessment by performing skin compatibility and exposure studies and to confirm the absence of any harmful effects (37). Furthermore, human volunteer testing might be applicable to the risk assessment of industrial chemicals under the Registration, Evaluation and Authorization of Chemical initiative (REACH) system in specific circumstances, particularly when occupational exposures are anticipated (38).

The acute irritancy of a substance can be studied by the open or closed patch test in healthy human volunteers. Typically, the substance is applied for 24 hours, and the test area is evaluated after 24, 48, and 72 hours. In order to evaluate substances with low irritancy, a minimum 100 volunteers are necessary. The cumulative irritancy can be assessed with the 21-day cumulative patch test (also
called the human repeated insult patch test, HRIPT) (39). In order to increase the sensitivity of the method, special volunteer panels of persons with sensitive skin may be used. In both the acute and the repeated irritancy tests, modern skin bioengineering equipment may be used to increase objectivity and sensitivity of the readings. Of the many methods available, mainly the measurement of transepidermal water loss for the assessment of barrier function and the measurement of skin color and blood flow for the assessment of inflammation have been used (40).

As described on the website of one large personal care products manufacturer (41), the Procter & Gamble Company, human clinical trials can be designed to comprise several phases:

“The single application patch tests are short-term tests conducted to confirm that a product is not a skin irritant even under highly exaggerated exposure conditions. In the single application patch test, undiluted test material is moistened and applied to the upper arm using a small patch (smaller than the pad of a standard plaster). The patch stays in place for 4 hours, then the test site is observed for signs of irritation over the next 3 days. If the test material is irritating, the patch site will appear red, and may also look dry or rough. Typically, a minimum of 12 volunteer panelists will participate in a single test.

Once a product or material has been shown to be non-irritating in a single application patch test, the next step is the 3-patch application test conducted to confirm that the product will not be irritating with exposures of even longer duration; the 3-Patch Application Test (3-PAT) is commonly used for this purpose. The exposure conditions of the 3-PAT are highly exaggerated over any exposure that would be encountered in real-life circumstances. Therefore, it is considered a very rigorous test of the potential to cause irritation. In the 3-PAT, a dilution of the test material is applied to the upper arm using a small patch, and left in place for 24 hours and then removed. After a 24 hour recovery period, it is replaced by a fresh patch. This is repeated for a total of 3 applications (i.e., three 24 hour applications in a five day period). The test site is observed for signs of irritation throughout the test, and at 24 hours after removal of the final patch. If the test material is irritating, the patch site will appear red, and may appear to be dry or rough. Typically, 12 volunteer panelists are used in the 3-PAT” (41).

Finally, the 21-day cumulative irritation test was developed as a means of evaluating any irritant effects that may be cumulative with repeated exposure. “In this test, the materials to be tested are applied to the upper arms of the panelists five times a week for three weeks. Each application stays in place for 24 hours. The
sites are observed for signs of irritation throughout the test. If the test material is
irritating, the patch site will appear red, and may also look dry or rough” (41).

Special practical interest is focused on detergents and their interaction with the *stratum corneum* barrier in the context of the irritation process. However, the exposure model and the evaluation methods are important variables influencing the outcome in irritancy testing (42, 43). For example, combining detergents and physical irritants creates an additive effect on *stratum corneum* barrier impairment (44, 45). Tandem repeated irritation tests (TRITs) demonstrate that combined mechanical irritation, SLS and occlusion cause complementary effects on the epidermal barrier disruption (44).

Application of such TRITs in irritants testing resembles to a greater extent the real conditions in everyday life and occupational settings than single-irritant exposure models. Different mechanisms of barrier impairment are involved in the action of various irritating stimuli. Organic solvents extract the lipids in the *stratum corneum*, thus disrupting the epidermal barrier (46). The anionic surfactant SLS was shown to damage protein structures such as keratin, which exposes new water-binding sites and causes hyperhydration of the *stratum corneum* and disorganization of the lipid bilayers (46, 47).

Overall, the human clinical trials represent the key step in the risk assessment of final formulations, particularly in the personal care and cosmetic industry. Together with the *in vivo* and *in vitro* data, the clinical studies provide critical information regarding the irritation potential of a final product designed for human use.

**ANIMAL TESTS**

For purposes of human safety assessment, new chemicals are still evaluated for their irritant potential by application to animals followed by the evaluation of visible changes such as erythema and edema. The *in vivo* skin irritation test in rabbits was introduced by Draize in the 1940s to predict hazardous effects of substances and formulations coming into contact with human skin (48). The Draize test for skin irritation involves the application of a test substance to a shaved area of the experimental animal (usually albino rabbits). The test substance is held against the skin with a gauze patch for 4 hours, after which the patch is removed and the degree of irritation is regularly assessed for a period of up to 14 days (38).

Although rabbit data were for long considered as reference to determine the irritant potential of chemicals to humans, the Draize test presents several major disadvantages. The rabbit and human skin have different physiological properties and responses to environmental and chemical agents (49-51) and, with the exception of rare publications (34, 49, 52), few studies have compared data obtained both on animals and humans. Some compounds are more toxic for rabbits
than for humans and vice versa (34, 49). Therefore, the Draize rabbit skin irritation test has frequently been criticized for its poor quality historical data and over-prediction of human skin irritation (53). Moreover, Draize test lacks reproducibility (54, 55); the scoring of skin damage in the Draize test is highly subjective, and therefore, different laboratories – and even different tests within the same laboratory – often yield different results. Finally, a debated ethical issue of the in vivo test concerns the animals’ suffering and discomfort.

The poor quality of this standard in vivo assay has confounded efforts to validate alternative, thoroughly-standardized in vitro assays based on cell lineages of human origin that may more accurately predict adverse effects in humans induced by exposure to chemicals, drugs and other commonly used personal care and cosmetic products.

TEST MODELS TO EVALUATE SKIN IRRITATION/CORROSION IN VITRO

In vitro alternative methods for skin irritation/corrosion testing have gained increasing attention in recent years, not only in response to the need to find ethical alternatives to the animal-based tests, but also because many in vitro methods allow one to obtain more relevant, objective test results. Therefore, several in vitro test systems have been proposed to replace skin irritation tests in rabbits, and to avoid experiments in human volunteers (56). These tissue models serve many testing purposes, such as for hazard identification and labeling of chemicals, transport of dangerous substances (industrial chemicals), labeling of finished products (cleaning agents, households cosmetics), occupational safety/industrial hygiene, and safety testing and risk assessment of raw ingredients or final formulations of personal care and cosmetic products.

Protocols using three commercially developed three-dimensional (3D) epidermal culture models, EpiDerm™ (MatTek Corporation, Ashland, USA), EPISKIN™ (SkinEthic Laboratories, Nice, France) and the reconstructed human epidermis (RHE) (SkinEthic Laboratories, Nice, France) have been validated by the European Centre for the Validation of Alternative Methods (ECVAM) as in vitro alternatives for testing the irritant and corrosive capacity of chemicals (57) for regulatory labeling purposes. Other protocols using these tissue models allow for the rank ordering of the irritant potential of formulations such that the researcher can use the test methods to differentiate among similar product candidates with a high level of precision here-to-fore not possible with animal models. The 3D reconstructed models represent the most promising alternative to animal testing for the screening of potential skin irritants and corrosives since they model the barrier effects of native skin and allow for cytotoxic effects to be measured in the underlying viable epidermis.
Several technical and performance aspects need to be taken into account regarding the use of tissue-engineered models to investigate irritancy. The strength of an irritant is dependent on both its ability to penetrate the stratum corneum and its cytotoxic effect on living cells (58). Although all epidermal equivalents have a stratum corneum, the barrier properties of the cultures vary model to model, and they do not yet exhibit the degree of competency found in native healthy skin (59–62). Variations in the barrier function between different epidermal equivalent models might become particularly relevant when testing chemicals with different physical properties. To this end, several protocols using the EpiDerm™, EPISKIN™ and RHE reconstructed models have been used depending on the final goal of the testing. In general, assays designed for regulatory settings often rely on a single exposure time/dose and are defined for a specific regulatory query such as if a chemical is corrosive or not. The typical protocol for skin corrosion testing of chemicals is based on the experience that corrosive substances are cytotoxic after a short-term exposure to the stratum corneum. Indeed, the tissues are exposed to the chemicals for 3 and 60 minutes, modeling in vivo exposure times, and their viability is assessed by measurement of mitochondrial activity using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) endpoint that will be further detailed.

The skin irritation assays used for regulatory purposes (hazard identification and labeling) are also designed to use single exposure times, which vary from 60 minutes, for EpiDerm™, to 15 minutes, for EPISKIN™, and 42 minutes, for the RHE model. In these assays, the % viability of the tissues is calculated and a chemical is classified as irritant if the viability decreases below 50% of the negative control. The skin corrosion (63–65) and skin irritation (66–68) protocols using each of these three models are now validated and available for use. They enable an irritant chemical to be distinguished from a non-irritant chemical, but have the major limitation in that they generally cannot rank the potency of the irritant chemicals. For example, the assays cannot determine whether the chemical is an extreme or moderate irritant. Similarly, the assays cannot resolve differences among materials found to be non-irritant in the context of a Globally Harmonized System (GHS) of classification and labeling of chemicals scheme. To address the irritancy ranking and to meet the typical needs of product development groups charged with developing increasingly milder products, protocols using the time-to-toxicity approach are recommended and are based on the exposure time it takes to reduce tissue viability by 50% (ET₅₀), as measured by the tissue’s ability to reduce MTT. The method can readily be enhanced to increase resolution to measure modest improvements in candidate formulations by modifying the test system exposure kinetics (exposure times, doses, exposure concentrations). The time-to-toxicity approach that will be further detailed has not been validated yet for regulatory purposes, but it is in current use by personal care and cosmetic industry as a useful screening tool for their newly formulated products.
STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF RECONSTRUCTED HUMAN SKIN

As an example of the general structure and functional characteristics of commercially available reconstructed human skin, we describe here the EpiDerm™ (EPI-200) skin model, produced by MatTek Corporation (Ashland, USA). This model consists of normal, human epidermal keratinocytes (NHEK) cultured to form a multilayered, highly differentiated model of the human epidermis in vitro (69, 70). EpiDerm™ consists of organized basal, spinous, granular and cornified layers (Figure 2a) analogous to those found in vivo (Figure 2b). The EpiDerm™ tissues with a surface area of 0.63 cm² are cultured on specially prepared cell culture inserts, are shipped as kits containing 24 tissues on agarose and are cultured in the Dulbecco’s Modified Eagle medium (DMEM)-based culture medium (71). Since the tissue construct has a functional stratum corneum, the test articles are applied directly to the culture surface, at air interface, so that undiluted and/or end use dilutions can be tested directly. The tissue model can accommodate the testing of those types of test articles that could not otherwise be tested in tissue culture, such as creams, lotions, etc.

![EpiDerm model (Mattek Corporation)](image1)

**Fig. 2.** – Histology of (a) EpiDerm™ model from MatTek (http://www.mattek.com) and (b) native human skin showing the different epidermal layers.

*Treatment of Reconstructed Skin Tissues (Time-to-Toxicity Protocol).* We describe here an approach to irritation assessment that has been used by our laboratory (IIVS) with the EpiDerm™ skin model. On the day of the experiment, the tissues are aseptically removed from the transport agarose bag and conditioned for at least 1 hour incubation in 0.9 ml assay medium (at 37°C in 5% CO₂/95% air, with saturated humidity) (standard culture conditions) in 6-well plates, in order to release transport stress-related compounds and any debris accumulated during shipment (72). The tissues are then transferred to 0.9 ml fresh assay medium and exposed topically to the test articles; usually, the test articles are tested by treating two EpiDerm™ tissues per exposure times, for at least four exposure times, which are determined based upon the expected toxicity of the test articles. One hundred microliters of the liquid test article or approximately 30 mg of a solid test article are applied to each EpiDerm™ tissue. One hundred µl of sterile water are used to
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dose the negative control when undiluted liquid or solid test articles are tested. Duplicate tissues are treated with the negative control for at least one exposure time. However, additional negative control exposure times are recommended, to address the range of test article exposure times. The positive control, 100 µl of 1% Triton®-X-100, is tested in duplicate cultures for 4 and 8 hours. The cultures are then incubated in standard culture conditions for the appropriate exposure times. After the appropriate exposure time, each EpiDerm™ tissue is rinsed with Ca++-Mg++-Free-Dulbecco’s phosphate buffered saline (CMF-DPBS). The tissues are finally assessed for viability, using the MTT assay (66, 73).

Cell Viability Measurement by MTT Reduction. The typical assay used to assess viability of 3D cultures is the MTT assay, which is based on the reduction of yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide to the purple formazan dye by mitochondrial succinate dehydrogenase in viable cells. If an irritant substance induces cytotoxicity, it will result in a corresponding decrease in mitochondrial activity, as measured by a decrease in the amount of MTT reduction. Topical application of potential irritants to epidermal and skin equivalents followed by the MTT assay is now becoming a well recognized assay for determining cytotoxicity and is the endpoint for the ECVAM validation (57).

A 10X stock of MTT prepared in phosphate buffered saline (PBS) (filtered at time of batch preparation) is thawed and diluted in warm MTT addition medium to produce the 1.0 mg/ml solution no more than two hours before use. Three hundred µl of the MTT solution are added to each designated well of a pre-labeled 24-well plate. After the appropriate exposure times, each tissue is blotted on a sterile paper towel and transferred to the appropriate well containing 0.3 ml of MTT solution. The 24-well MTT plates are incubated at standard culture conditions for 3 ± 0.1 hours.

After the 3 ± 0.1 hour incubation, the EpiDerm™ tissues are blotted dry on absorbent paper, cleared of excess liquid and transferred to a pre-labeled 24-well plate containing 2.0 ml of isopropanol in each designated well. The plates are covered with parafilm, protected from exposure to light and shaken for at least 2 hours at room temperature to extract the MTT. If necessary, the plates may be stored in a refrigerator (2–8ºC) overnight (or up to 20 hours after the last tissue is harvested) prior to extracting the MTT. At the end of the extraction period, the liquid within the Millicell® inserts is decanted into the well from which the Millicell® insert was taken.

The extract solution is mixed and 200 µl are transferred to the appropriate wells of a 96-well plate. Two hundred µl of isopropanol are added to the wells designated as blanks (72). The absorbance at 550 nm (OD_{550}) of each well is measured using a 96-well spectrophotometric microtiter plate reader.

The mean OD_{550} value of the blank wells is calculated. The corrected mean OD_{550} value of the negative control(s) is determined by subtracting the mean OD_{550}...
The viability of the tissues exposed to the test articles is calculated and expressed as a percentage relative to the viability of the negative control-treated tissues using the following calculation:

\[
\text{Corrected OD}_550 \text{ of test article exposure time} \\
\times 100 \\
\text{Corrected mean OD}_{550} \text{ of negative control}
\]

The individual % viability values are then averaged to calculate the mean % viability per exposure time. Test article and positive control viability calculations are performed by comparing the corrected OD\textsubscript{550} values of each test article or positive control exposure time to a relevant negative control.

Exposure time response curves are plotted with the % of control on the ordinate and the test article or positive control exposure time on the abscissa. The ET\textsubscript{50} value is interpolated from each plot. To determine the ET\textsubscript{50}, the two consecutive points are selected, where one exposure time results in a relative survival greater than 50%, and one exposure time results in less than 50% survival. The two selected exposures are used to determine the slope and the y-intercept for the equation \( y = mx + b \). To determine the ET\textsubscript{50}, the equation is solved for \( y = 50 \). When all of the exposure time points show greater than 50% survival, the ET\textsubscript{50} value is presented as greater than the longest test article exposure time. When all of the exposure time points show less than 50% survival, the ET\textsubscript{50} value is presented as less than the shortest test article exposure time.

The Use of Killed Controls for Assessment of Residual Test Article Reduction of MTT. A false negative result can occur in the MTT assay if the test article itself is able to reduce MTT and if the test article remains on the tissue or in the insert supporting membrane after the rinsing step. In such a situation, the amount of MTT directly reduced by the test article would be evaluated as if the MTT signal was generated by viable cells. Thus, the total MTT reduced would appear to be notably higher (implying that the test article is less cytotoxic) when potentially significant cytotoxicity may have been induced. The ability of a test article to directly reduce MTT is assessed by adding the test article to 1 ml of MTT solution (1mg/ml) prepared in DMEM medium. The test articles are added to the MTT solution and the mixtures are incubated for at least 60 minutes at standard culture conditions. If the MTT solution turns to blue/purple, it is assumed that the test article had reduced the MTT (64).
To evaluate whether test article residues may directly reduce the MTT in the tissue-based assay, a functional check is performed using intact freeze-killed tissues in parallel to the assay using viable tissues (64). Freeze-killed tissues are prepared by placing untreated EpiDerm™ tissues in the –20°C freezer at least overnight, thawing to room temperature, and then refreezing. Once frozen, the tissues may be stored indefinitely in the freezer. To test for residual test article reduction, killed tissues are treated with the test article just as in the treatment of viable tissues. All assay procedures are performed in the same manner as for the viable tissue. At least one killed control (KC) treated with sterile deionized water (negative killed control) is tested in parallel since a small amount of MTT reduction is expected from the residual nicotinamide adenine dinucleotide (NADH) and associated enzymes within the killed tissue.

If little or no MTT reduction is observed in the test article-treated killed control, the MTT reduction observed in the test article-treated viable tissue may be ascribed to the viable cells. If there is appreciable MTT reduction in the treated killed control (relative to the amount in the treated viable tissue), additional steps must be taken to account for the chemical reduction or the test article may be considered un-testable in this system.

If killed controls are used, the following additional calculations are performed to correct for the amount of MTT reduced directly by test article residues. The raw OD$_{550}$ value for the negative control killed control is subtracted from the raw OD$_{550}$ values for each of the test article-treated killed controls (at each appropriate exposure time), to determine the net OD$_{550}$ values of the test article-treated killed controls.

\[
\text{net OD}_{550}\text{ for each test article KC} = \text{raw OD}_{550}\text{ test article KC} - \text{raw OD}_{550}\text{ negative control KC}
\]

The net OD$_{550}$ values represent the amount of reduced MTT due to direct reduction by test article residues at specific exposure times. In general, if the net OD$_{550}$ value is greater than 0.150, the net amount of MTT reduction is subtracted from the individual corrected OD$_{550}$ values of the viable treated tissues, at each corresponding exposure time, to obtain a final corrected OD$_{550}$ value. These final corrected OD$_{550}$ values are then used to determine the % of control viabilities at each exposure time.

\[
\text{final corrected OD}_{550} = \text{corrected test article OD}_{550}\text{ (viable)} - \text{net OD}_{550}\text{ test article (killed control)}
\]

Finally, the following % of control calculations are made:

\[
\%\text{ viability} = \frac{\text{final corrected OD}_{550}\text{ of test article or positive control}}{\text{corrected mean OD}_{550}\text{ of negative control}} \times 100
\]

and the ET50 is calculated as described above.
An example of the exposure-time continuum based on determined ET₅₀ values for different products tested is shown in Fig. 3.

**IL-1α Immunoassay.** Considering that cytotoxicity represents the basis for skin irritation, the 3D reconstructed human epidermis models can be used to measure changes in the tissue viability, using the MTT vital dye, and also the released inflammatory cytokines, such as IL-1α, IL-6, IL-8, TNF-α. The MTT endpoint has been demonstrated to be a suitable parameter to extrapolate on the irritancy potential of chemicals in human reconstructed epidermis models and therefore provides the first measure of irritancy in the validated skin irritation protocols used for legislation-driven regulatory safety testing as well as in the time-to-toxicity assays. However, IL-1α may be considered a (secondary) cell membrane disruption endpoint, and the measure of its up-regulation and release could potentially provide further information on irritation potential of raw ingredients, chemicals or final formulations (58, 74).

Briefly, the assay medium collected from underneath the tissues exposed to the test articles (and further post-incubated for 24 hours to allow cytokine expression and release) are analyzed for IL-1α using a Quantikine human immunoassay kit (R&D Systems, Minneapolis, MN, USA) based on a quantitative sandwich-enzyme immunoassay technique. A monoclonal antibody specific for
human IL-1α is coated on a 96-well microtiter plate; human IL-1α standards (0–250 pg/ml) and the assay medium samples are pipetted (200 µl) into 96-well, pre-coated plates, according to the kit instructions for cell culture medium. Standards and samples are incubated at room temperature for 2 hours, after which the unbound proteins are removed from the plate by washing three times with approximately 250 µl of wash solution. After washing, 200 µl of a polyclonal antibody against IL-1α conjugated to horseradish peroxidase are added to all wells for a 1 hour incubation at room temperature. After this incubation, the solutions are removed from the wells and the plate is washed three times with approximately 250 µl of wash solution. Two hundred µl of chromogenic substrate (stabilized hydrogen peroxide and tetraethylbenzidine) are added to each well and the plate is then incubated for 20 minutes at room temperature, protected from light, without shaking; the blue color develops in proportion to the amount of IL-1α bound in the initial step. The color development is stopped with 50 µl of 2N sulfuric acid and the plate is read at 450 nm, subtracting the absorbance at 540 or 570 nm (OD₄₅₀–₅₇₀), within 30 minutes of stopping the reaction.

The OD₄₅₀–₅₇₀ value of each test sample and IL-1α standard is determined. The corrected OD₄₅₀–₅₇₀ value for the test samples and each IL-1α standard are determined by subtracting the mean OD₄₅₀–₅₇₀ value of the blank wells. The standard curve is plotted as the concentration of the standards (y-axis) versus the corresponding corrected average absorbance (x-axis). The amount of IL-1α released by the test sample groups (controls and test articles as appropriate) is mathematically interpolated from the standard curve (quadratic).

CONCLUSIONS

The use of reconstructed human tissues for skin irritation/corrosion testing represents a major milestone for the efforts focused on finding a standardized and reliable alternative *in vitro* assay for the *in vivo* rabbit Draize skin irritation test – long the only accepted test by regulatory agencies. It has been demonstrated that the *in vitro* skin reconstructed models provide good correlation with human patch test data (75), thus suggesting that data obtained from highly standardized human patch tests may provide a more relevant and reliable regulatory standard, as well as a better future standard for the development and validation of *in vitro* methods (76). In this context, it is worth pointing out that considerable information has already been gained from past testing, and therefore many companies are utilizing the results of tests done years ago to obtain information on the safety of similar ingredients in their new products without having to carry out new animal tests.

Several lines of investigation have shown human skin equivalents to be good candidates for pre-clinical skin irritation screening to aid safety assessment for chemicals and product formulations and to facilitate design of safe and efficient
human studies. Studies by Perkins (77) using EpiDerm™ model showed that for surfactants, dose-response curves of MTT cell viability data clearly distinguished strongly-irritating from milder surfactants and rank-ordered irritancy potential in a manner similar to repeat-application patch test results. For the antiperspirant and deodorant products, the same study showed that all the in vitro endpoints correlated well with consumer reported irritation; furthermore, IL-1α release showed the greatest capacity to distinguish irritancy over a broad range and provided the best prediction of human scores from 14-day cumulative irritancy tests of cosmetic products (77). These results confirm the potential value of the EpiDerm™ model used in that study as a valuable in vitro pre-clinical tool for prediction of human skin irritation responses.

In conclusion, in vitro testing using human skin models enhances the ability of cosmetics and chemicals producers to effectively and efficiently replace the use of animals for the screening of their products’ safety and efficacy and to comply with the European Union legislations. Used for pre-clinical studies, the in vitro 3D reconstructed human skin models provide accurate predictions of human skin responses to potentially irritating products. The skin irritation/corrosion assays based on these models and currently in use represent the result of the efforts of animal welfare organizations corroborating with academic research and industry support towards replacing the in vivo testing and to designing well-standardized in vitro alternatives.

REFERENCES


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In vitro testing for skin irritation


