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A new alternative method for testing skin irritation using a human skin model: A pilot study ☆

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ABSTRACT

Background: Studies assessing skin irritation to chemicals have traditionally used laboratory animals; however, such methods are questionable regarding their relevance for humans. New *in vitro* methods have been validated, such as the reconstructed human epidermis (RHE) model (Episkin®, Epiderm®). The comparison (accuracy) with *in vivo* results such as the 4-h human patch test (HPT) is 76% at best (Epiderm®). There is a need to develop an *in vitro* method that better simulates the anatomo-pathological changes encountered *in vivo*.
Objectives: To develop an *in vitro* method to determine skin irritation using human viable skin through histopathology, and compare the results of 4 tested substances to the main *in vitro* methods and *in vivo* animal method (Draize test).
Methodology: Human skin removed during surgery was dermatomed and mounted on an *in vitro* flow-through diffusion cell system. Ten chemicals with known non-irritant (heptylbutyrate, hexylsalicylate, butylmethacrylate, isoproturon, bentazon, DEHP and methylisothiazolinone (MI)) and irritant properties (folpet, 1-bromohexane and methylchloroisothiazolinone (MCI/MI)), a negative control (sodiumchloride) and a positive control (sodiumlaurylsulphate) were applied. The skin was exposed at least for 4 h. Histopathology was performed to investigate irritation signs (spongiosis, necrosis, vacuolization).
Results: We obtained 100% accuracy with the HPT model; 75% with the RHE models and 50% with the Draize test for 4 tested substances. The coefficients of variation (CV) between our three test batches were <0.1, showing good reproducibility. Furthermore, we reported objectively histopathological irritation signs (irritation scale): strong (folpet), significant (1-bromohexane), slight (MCI/MI at 750/250 ppm) and none (isoproturon, bentazon, DEHP and MI).
Conclusions: This new *in vitro* test method presented effective results for the tested chemicals. It should be further validated using a greater number of substances; and tested in different laboratories in order to suitably evaluate reproducibility.

1. Introduction

Occupational dermatoses are a significant health problem. They represent more than 20% of occupational diseases in Europe and are costly for the health care system (Lawrence, 1997). To reduce the prevalence of dermatoses due to occupational exposure, an appropriate assessment of the sensitizing, irritant or corrosive potential of chemicals is of major importance. In this way, each substance are subject to regulations (before selling and using), aimed at properly classifying and labeling potentially dangerous chemicals (1967). Dermal irritation has been assessed using the Draize test since 1944 (Draize et al., 1946). It is based on the application of a single dose of the tested chemical on the shaved skin of an albino rabbit for 4 h. After 24 h to 48 h the level of irritation is determined via a visual inspection of the rabbit's skin for any erythema or edema. Despite these tests being less expensive and easy to carry out, they rapidly became the subject of criticism from both scientific and ethical viewpoints. A 2004 study by US Scientific Advisory Committee on Alternative Toxicological Methods analyzed the modem Draize skin test. They found that the test would misidentify a serious irritant as safe (0–0.01%), a mild irritant as safe (3.7–5.5%) and a serious irritant as a mild irritant (10.3–38.7%). Due to the lack of specific definitions for the concepts of irritant and non-irritant testing chemicals on rabbits might not predict the effects on humans (Wilhelmus, 2001), there is a significant level of subjectivity introducing a large variability and

☆ Each author has participated sufficiently to take public responsibility for appropriate portions of the work.

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difficulty in result interpretations (Weil and Scala, 1971). Furthermore, these tests exhibit a weak predictive value compared to the true potential for the chemical to induce irritation of the human skin. Several clinical tests carried out in the 1990s used 4-h human patch tests (HPT); each HPT contains a chemical substance and is applied directly onto human volunteers. Comparing results from these HPT tests showed that Draize tests tended to under- and sometimes over-estimate irritation. (Nixon et al., 1975; Patil and Patrick, 1998; Phillips et al., 1972; Zuang et al., 2002). Rabbit skin is more sensitive than the human skin so usually the irritation is overestimated, giving an extra protective degree. Subsequently, much effort was put into finding a replacement for the Draize test, especially by the European Centre for the Validation of Alternative Methods (ECVAM). Several *in vitro* methods were developed and evaluated, for instance on pig or mouse skin (Fentem et al., 2001; Heylings et al., 2003; Kandarova et al., 2004; Portes et al., 2002; Roguet, 1999; Zuang et al., 2002). The best results are obtained from methods using reconstructed human epidermis (RHE). Such models comprise a matrix of the main 3 types of collagen; these constitute a dermis, on it a differentiated, layered epidermis made up of human keratinocytes. Several reconstituted human skin exists (Episkin® and Epiderm®). Chemicals to be tested are deposited directly on the stratum corneum. The viability of exposed cells is evaluated by measuring their mitochondrial activity (the enzymatic conversion of MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), a yellow tetrazole into blue formazan dye in comparison to those of unexposed control cells. Chemicals are classified as irritant if they make cell viability drop below a predetermined threshold (Cotovio et al., 2005; EC_ECVAM, 2009; Eskes et al., 2007; Kandarova et al., 2005; Spielmann et al., 2007). In 2009, two of RHE methods (Episkin® and Epiderm®) were validated by the European Union (EU) and in 2010 the Organization for Economic Co-operation and Development (OECD) published international guidelines on the use of these new *in vitro* methods.

The Episkin® and Epiderm® methods were also compared to the *in vivo* 4-h HPT method. Their sensitivity and their accuracy on irritant classification were higher than expected (Jirova et al., 2010). The Episkin® model exhibited 76% accuracy with the *in vivo* 4-h HPT, whereas it only showed 56% accuracy with the Draize test. The Epiderm® model showed 70% accuracy with the *in vivo* 4-h HPT model, and 56% accuracy with the Draize test (20). Nonetheless, when undergoing validation, RHE methods were still compared with the Draize test. Despite evidence of its weak specificity affects its sensitivity and poor confirmed results with those from RHE models. Many chemicals were reclassified on the basis of erroneous reference tests while having been correctly classified originally. The main disadvantage with the RHE models is that they use artificial skin. We hypothesized that if human skin, freshly excised during a surgical procedure, would give better results, i.e. results that were even closer to the *in vivo* 4 HPT model.

To our knowledge, no clear *in vitro* model using fresh excised human skin exists. The first aim of our study was to develop an *in vitro* model to assess chemicals'irritation properties using fresh excised human skin. The second aim was to compare the efficiency of this new model with published RHE and 4-h HTP models for four chemicals assessed for irritation. These chemicals were chosen among a list of 81 chemicals studied using the *in vivo* 4-h HPT model and proposed for use in a database for the evaluation of new *in vitro* methods for predicting skin irritation (Basketter et al., 2012). Finally, to assess our results, we included isoproturon (250 μg), bentazon (basagran®), the phthalate DEHP, folpet and the mixture of 2 isothiazolinones, methylchloroisothiazolinone (MCI/MI) and methylisothiazolinone (MI), both preservatives and biocides known for their allergenicity, present in many industrial fluids and cosmetics. MCI/MI was gradually removed from cosmetics, because of its strong allergenicity. It shows an important sensitization/exposure quotient (SEQ) (Schnuch et al., 2011).MCI/MI and MI are both irritant at higher concentrations. MI alone is increasingly used as a preservative since the early 2000s. Despite its lower allergenic potential, the concentrations used for biocide purpose are significantly higher than for the MCI/MI. The consequences are an increase in contact allergy-related to MI with a current prevalence level similar to other preservatives (Lundov et al., 2010). However, the irritation potential at lower concentrations (below 750 ppm) of these two preservatives/biocides has been poorly studied.

A list stands under EU directive 67/548/EEC which determines chemical substances as irritants with the following label "Irritating to skin" (R38). The R-phrases (short for Risk Phrases) are defined in Annex III of European Union Directive 67/548/EEC: Nature of special risks attributed to dangerous substances and preparations.

2. Materials and methods

2.1. Chemical products

From this list we selected those chemicals, which had given the most equivocal results in different studies using RHE models (Cotovio et al., 2005; Eskes et al., 2007; Spielmann et al., 2007); we also required that chemicals be classed as merely irritants (non corrosive), and that they be readily available and easy to handle. The ten chemicals were heptylbutyrate (CAS n°5870-93-9, purity >95%), hexylsalicylate (CAS n°6259-76-3, purity >98%), butylmethacrylate (CAS n°97-88-1, purity >99%), isoproturon (CAS n°34123-59-6, purity >99%, at 250 μg), bentazon (CAS n°25057-89-0, basagran®), bis-2-ethylhexylphtalate (DEHP, CAS n° 117-81-7), methylisothiazolinone (CAS n°2682-20-4, aqueous solution, purity >98%, 500 pm), N-trichloromethylthiophtalimide (folpet; 6.3 mg in 4.5 ml; CAS n° 133-07-3), 1-bromohexane (CAS n°111-25-1, purity >98.5%), methylchloroistothiazolinone (CAS n° 26172-55-4, purity >98%, 100/25 ppm, 250/75 ppm, 375/125 ppm, 750/250 ppm). Sodium chloride (NaCl) at 0.9% prepared by dissolving 18 g of NaCl in purified water was used as a negative control. The positive control was sodiumlaurylsulphate (SLS; CAS n°151-21-3, purity >95%). SLS was prepared at 10% in MilliQ water. All chemicals were purchased as reference standards in Sigma Aldrich (Sigma–Aldrich (Buchs, St. Gallen, and Switzerland). Except for SLS, the four chemicals were applied as neat on the skin.

2.2. Fresh human skin preparation

Human abdominal full thickness skin was obtained as surgical waste from the Department of Plastic and Reconstructive Surgery at the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne, Switzerland). All human donors were Caucasian females between 35 and 48 years old and had given their full consent. They had no known previous dermatological conditions. The skin samples were de-identified for use in this study. Skin, still attached to its subcutaneous fatty layer, brought directly to our laboratory in an ice-box and rinsed with saline solution at room temperature (NaCl 0.9% at 25 ± 2 °C). The skin was dermatomed to a thickness of 0.8 mm (Acculan II, B.Braun/Aesculap, Sempach, Switzerland), allowing the conservation of the entire epidermis and superficial dermis. A series of 2.5 cm^2 discs were cut out of the length of each skin sample using a scalpel and mounted in an *in vitro* flow-through diffusion cell system (Permgear® obtained from SES Analytical System, Bechenheim, Germany). This system consisted of a series of six diffusion cells; each one was divided into a donor chamber (upper compartment) above the skin and a receptor chamber (lower compartment) below the skin, and kept together