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SkinEthic™ RHE for *in vitro* evaluation of skin irritation of medical device extracts

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ABSTRACT

According to ISO 10993 standards for biocompatibility of medical devices, skin irritation is one of the three toxicological endpoints to be always addressed in a biological risk assessment. This work presents a new protocol to assess this endpoint *in vitro* rather than *in vivo*. The protocol was adapted to medical devices extracts from the OECD TG 439 with the SkinEthic™ RHE model as test system. It was challenged with irritant chemicals, Sodium Dodecyl Sulfate, Lactic Acid and Heptanoic Acid spiked in polar solvents, sodium chloride solution or phosphate buffer saline and non-polar solvent, Sesame Oil. Cell viability measured by MTT reduction after 24 h exposure was used as readout. Quantification of IL-1α release as secondary readout did not increased performance. Samples of heat-pressed polyvinyl chloride (PVC) and silicone sheets infused with or without known irritant (4% Genapol-X80, 6% Genapol-X100 and 15% SDS) were tested after extraction in polar and non-polar solvents. Medical device extracts are classified irritant when the cell viability is inferior or equal to 50%, compared to the negative controls tissues, in at least one extraction solvent. The correct classification of all the samples confirmed the good performance of this new protocol for *in vitro* skin irritation of medical devices extracts with the SkinEthic™ RHE model. Seven naïve laboratories were trained in prevision of the Round Robin Study to evaluate Reconstructed Human Epidermis (RhE) models as *in vitro* skin irritation test for detection of irritant potential in medical device extracts.

1. Introduction

Medical devices cover a wide range of health or medical instruments used in the treatment, mitigation, diagnosis or prevention of a disease. Since the early 1990's, biocompatibility testing and evaluation to ensure safety of medical devices are driven by the ISO 10993 standards. According to ISO 10993 Part 1 for evaluation and testing within a risk management process (ISO, 10993-1 Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process, 2009), skin irritation is one of the three toxicological endpoints to be always addressed in a biological risk assessment whatever the category of the device. Irritation testing of medical devices can be performed with the finished product and/or extracts thereof. The reference test method listed in the ISO10993-10 (ISO, 10993-10 Biological Evaluation of Medical devices - Part 10: Tests for irritation and skin sensitization, 2010) is based on *in vivo* testing even if this standard mentions previous validation by ECVAM Scientific Advisory Committee (ESAC) of an *in vitro* method using reconstructed human epidermis. Indeed, this *in vitro* test for skin irritation has so far been validated only for neat chemicals and not for medical device extracts where potential leached irritants are diluted in a solvent. In order to apply these assays for the testing of irritant potential of medical devices, further validation for this specific area is essential.

Turning toward alternatives to animal testing is driven by ethical consideration but also by scientific and economic reasons. In recent years, new cosmetics regulations, first in Europe (EU, 2003) and then in other parts of the world, have progressively banned the use of animals to ensure the safety of ingredients and cosmetics products. In parallel with these regulatory developments, toxicology is undergoing a profound revolution, with a shift from toxicology based on the observation of effects in animals to mechanistic approaches based on *in vitro* and *in silico* tests to predict potential adverse effects in humans. Significant progress has been made with successful replacements, especially for acute toxicological endpoints, even if systemic endpoints are more complex to replace. Several validated *in vitro* test methods were adopted as OECD test guidelines for these endpoints and Integrated

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Approaches to Testing and Assessment (IATA) provides guidance on how to integrate and use existing test and non-test data for the assessment of health hazards. Even if successful steps from chemical and cosmetics industry need to be adapted, previous experience from these sectors will definitely accelerate the move in medical devices area (Casas et al., 2013; Coleman et al., 2015).

The purpose of this paper is to present the development and preliminary results of a new *in vitro* method for evaluating the skin irritation of medical device extracts. This new protocol is adapted from the OECD test guideline for skin irritation (OECD TG439) of chemicals and uses the SkinEthic™ RHE model as experimental system. The SkinEthic™ RHE model is reconstructed from human primary keratinocytes and mimics human epidermal morphology and physiology. This model is validated as a full *in vitro* replacement method to animal testing to assess the skin corrosion and the skin irritation potential of chemicals (Alépée et al., 2010) (EC-ECVAM, 2006). The protocol for medical device extracts was developed in preparation of an international Round Robin Study conducted under the umbrella of the workgroup eight of the ISO technical committee 194 (ISO/TC 194/WG 8) irritation and sensitization.

2. Materials and methods

2.1. Functional reconstructed human epidermis model SkinEthic™ RHE

The SkinEthic™ Reconstructed Human Epidermis (RHE) model (EPISKIN Laboratories, France) consists of normal, human-derived keratinocytes, cultured to form a fully differentiated three-dimensional epidermis on a 0.5 cm^2 surface inert polycarbonate filter at the air–liquid interface in a chemically defined medium (Rosdy and Clauss, 1990; Rosdy et al., 1993) (Fig. 1). SkinEthic™ RHE is produced under ISO 9001 certification. Quality control data sheet is provided with every batch of tissue including histology, viability and safety data.

2.2. Exposure protocol

The day of receipt the SkinEthic™ RHE inserts are transferred into a 24-wells plates filled with 0.3 mL fresh medium of culture and maintained a minimum period of 2 h in an incubator (37 °C, 95% humidity, 5%CO_2). This pre-incubation step could be extended to 24 h by using 6-well plates filled with 1 mL of fresh medium. For the experiment, 100 µL of each sample is topically applied concurrently on three tissues replicates (n = 3) for 18 ± 2 h or for 24 ± 2 h at 37 °C. Attention should be made to ensure a correct distribution of the solution onto the surface of the tissue. The surface of the stratum corneum being hydrophobic, surface tension mechanisms can induce peripheral repartition of polar solvent. This can be solved by slightly tapping the insert on the bottom of the well. Solutions of SDS 1% (w/v) in polar solvent, sodium chloride (NaCl 0.9%) or phosphate-buffered saline without Ca ++ and Mg++ (PBS) and non-polar solvent (sesame oil) are used as positive controls (PC). Negative control (NC) are PBS-treated tissues. The solvents alone are tested as vehicle extraction controls (VC). At the end of the exposure step the SkinEthic™ RHE inserts are rinsed 25 times with PBS (1 mL by 1 mL) using a multistep pipette and are manually dried. Samples of the incubation medium are collected and frozen at −20 °C for potential future cytokines quantification.

2.3. Cell viability

The cell viability is determined by measuring in the reconstructed epidermis the formation of insoluble blue formazan crystals by the dehydrogenase enzyme after addition of 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma–Aldrich) to the medium of culture (Mosmann, 1983; Korting et al., 1994). The tissues are incubated with 0.3 mL MTT solution (1 mg/mL) for 3 h (± 15 min). The formazan crystals are extracted using 1.5 mL isopropanol for 2 h (± 15 min) at room temperature. Then 200 µL are transferred three times per tissue into a 96-well plates and the concentration of formazan is quantified by measuring the optical density (OD) through

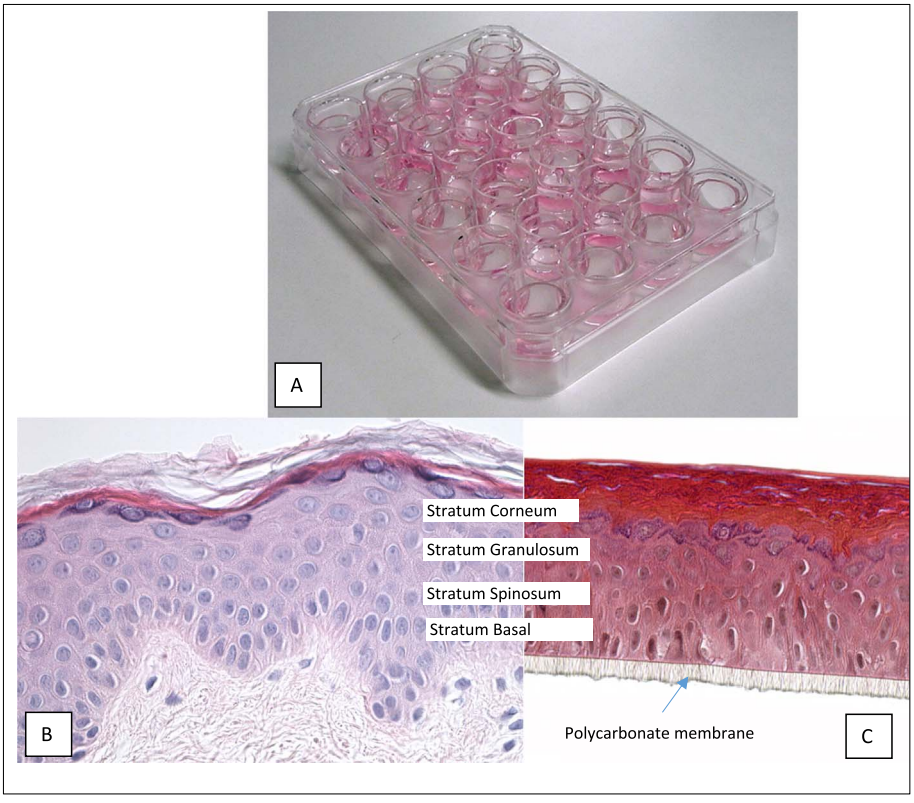

Fig. 1. SkinEthic™ RHE model.
SkinEthic™ RHE model is produced and shipped every week in 24 well plates (A). Hemalun Eosine-Saffron staining (HES) show in the reconstructed epidermis (C) the typical layers of human epidermis (B).

spectrophotometry at 570 nm wavelength. Isopronanol is used as blank. After subtracting the blank OD from all raw data, mean OD values and standard deviations (SD) are calculated. The percentage of cell viability for each tissue is expressed relative to negative control as: 100 × mean $OD_{treated}$ / mean $OD_{negative\ control}$. By default, the negative control cell viability value is set at 100%. For each test substance the mean ± SD of the triplicate tissues is calculated. When the cell viability value is under or equal to 50% the sample is classified as irritant.

2.4. Spiked solvents with lactic acid and heptanoic acid

SkinEthic™ RHE tissues in triplicate (n = 3) were exposed 24 h to 100 µL of 0.1%, 1%, 1.25%, 1.5%, 1.75%, 2% and 4% solutions (w/v) of lactic acid (LA; CAS No.: 50-21-5; 85% purity) in 0.9% sodium chloride solution (NaCl 0.9%) or Phosphate Buffer Saline (PBS) solution without Ca++ and Mg++ (polar solvent) or 0.1%, 0.25%, 0.5%, 0.75%, 1%, 2% and 4% solutions (w/v) of heptanoic acid (HA; CAS No.: 111-14-8; P99% purity) (Sigma–Aldrich Company) in pharmaceutical grade sesame oil (SO) (non-polar solvent). SDS 1% in SO and in NaCL were used as positive controls (PC) and PBS alone as negative control (NC). Cell viability and IL-1α release were measured after 24 h of exposure.

2.5. Extraction of medical devices (ISO 10993-12)

Polymer was provided by Arthrex (Naples, USA), Nelson Laboratories Inc. (Salt Lake City, USA) and the National Institute of Health Sciences (Tokyo, Japan). Samples were extracted according to ISO 10993-12:2012 using polar solvent (PBS) and non-polar (Sesame Oil of pharmaceutical grade, SO) extraction vehicle at a ratio of 3 cm^2/mL, 6 cm^2/mL, or 0.2 g/mL at 37 ± 1 °C for 72 ± 2 h with stirring in a borosilicate tube with tight closure. Vehicle extraction control (VC) is exposed to the same process, 37 ± 1 °C for 72 ± 2 h. All the extracts are used maximum 24 h after end of extraction process.

2.6. Cytokine quantification

The inflammatory response was evaluated by quantifying the specific human cytokine, interleukin-1 alpha (Coquette et al., 1999) using a commercial enzyme linked immuno-absorbent assay (ELISA) kit (Quantikine Human IL-1α/IL-1F1 Immunoassay, from R&D systems, UK). The analyses were performed according to manufacturer's instructions. The detection limit of the assay was 1 pg/mL. Mean concentrations (pg/mL) ± standard deviations (SD) were calculated (one replicate per tissue and three tissues per assay).

3. Results

3.1. SkinEthic™ RHE quality controls and reproducibility

Quality and reproducibility of the tissues, batches after batches, years after years is essential for data interpretation. EPISKIN performs quality controls for each SkinEthic™ RHE batch by assessing MTT reduction viability test using negative control tissues. The mean optical density (OD) value from 2014 to 2017 is 1.3 ± 0.2, demonstrating the reproducibility of this assay over the years (Table 1). A resulting OD value threshold acceptance limit of 0.7 has been established. Prior testing, the barrier function property of the tissue should also meet the historical acceptability range defined by EPISKIN. This property is estimated by the exposure time required to reduce cell viability by 50% (ET-50) upon application of 1% Triton X-100. The reproducibility of the ET-50 on SkinEthic™ RHE model evaluated on the same period has an overall mean for ET-50 TritonX-100 values of 5.8 ± 1.5 h. An upper ET-50 threshold acceptance limit has been established for the ET-50 TritonX-100 criteria ranging from 4 to 10 h. The third EPISKIN quality control criteria, is based on histological examination. The tissues exhibit four to seven viable layers comprising at least basal, suprabasal, spinous and granular cell layers, and a stratum corneum. The overall mean of living cell layers over this 4 years' period is 5.9 ± 0.7.

Table 1
Mean quality controls (2014–2017).

Number of batches (n)	Viability (OD)	ET50	Viable layers
215	1.3 ± 0.2	5.8 ± 1.5	5.9 ± 0.7

Mean of SkinEthic™ RHE Quality Control batches on years 2014, 2015, 2016 and 2017 for 215 batches. Viability of the tissues is expressed by formation of formazan measured by optical density (OD) after MTT reduction. Barrier function property of the tissue is estimated by the exposure time required to reduce cell viability by 50% (ET-50) upon application of 1% Triton X-100. The third EPISKIN quality control criteria, is based on histological examination. The tissues should exhibit four to seven viable layers comprising at least basal, suprabasal, spinous and granular cell layers, and a stratum corneum.

3.2. Solvents spiked with irritant

We observed a dose response decrease of cell viability of SkinEthic™ RHE exposed to increasing concentrations (0.1% to 4%) of the known irritants, Lactic acid and Heptanoic acid, in saline and sesame oil solvents, respectively (Fig. 2). The solvents alone have no effect compared to reference negative control, PBS. The concentration at which the spiked chemical reduces the viability of the tissues by 50% (IC50) after a fixed exposure time was calculated. The IC_{50} for Lactic Acid and for Heptanoic acid are 1.9% and 0.5%, respectively.

3.3. Reference materials extracted in polar and non-polar solvents

The same protocol used for solvent spiked with irritants is performed on extracts of different samples of heat-pressed polyvinyl chloride (PVC) and silicone sheets infused with or without known irritant (Table 2). Polyvinyl chloride (PVC) and silicone are plastics widely used in medical devices industry and, thanks to partners, we were provided with several samples with or without irritants added during the process of fabrication. Y1 and Y-4 were produced by National Institute of Health Sciences (NIH, Japan) and provided by Nelson Laboratories (USA). Silicone-SDS and PVC-Genapol came from Arthrex (Naples, USA). Using the prediction model of the OECD TG439, ie classification in category 2 when cell viability is below 50%, the Y-1 medical device extracts is non-irritant and the Silicone-SDS, PVC-Genapol and Y-4 are irritants (Fig. 3). The Silicone-SDS, containing 15% SDS is classified irritant only when the material is extracted in the polar solvent (SO). PVC-Genapol and Y-4 materials, containing Genapol are irritants with the two extraction solvent even if a slight increase is observed in the non-polar solvent (SO) compared to the polar one (PBS).

Histological observations were performed for a run with two polymers, Y-1 and Y-4, compared to NC and PC (Fig. 4). It confirms the strong effect of the PC (SDS1%) especially in the polar solvent (PBS). The death of the tissues is accompanied by IL-1α release in the medium with a 10 and 67 fold increase compared to NC for SDS in PBS and in SO, respectively. Histological observations of NC in PBS and SO revealed no significant effects and there was no increase in IL-1α level for these conditions. Y-1 exposed tissues are slightly damaged but this is not correlated to decrease in cell viability nor increase in IL-1α. At 24 h post-exposure to Y-4 extracts the poor histology of the tissues correlates with the decrease in cell viability measured with MTT. A 10 fold increase in the IL-1α level was also observed for that condition compared to NC.

1% SDS as positive control induces a loss of tissue integrity with sometime detachment of the tissue from the membrane during the rinsing step. This problem is not observed at a lower concentration (0.5% SDS) while causing a significant decrease in viability and a high

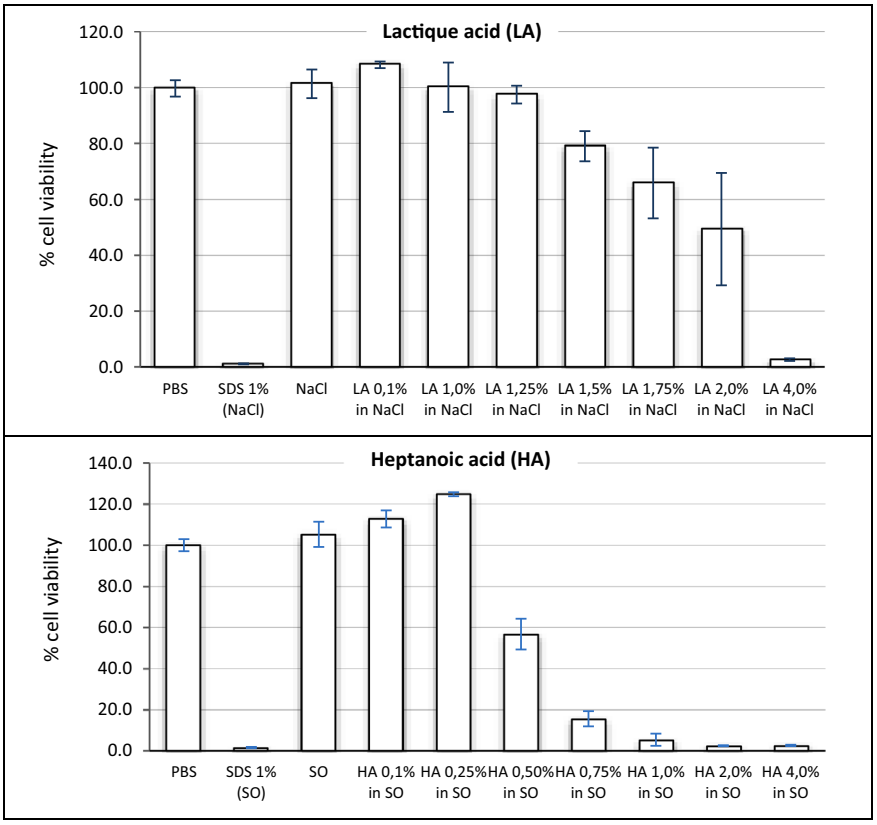

Fig. 2. Dose response assays.
Dose response curve of cell viability of SkinEthic™ RHE exposed to increasing concentrations (0.1% to 4%) of known irritant, Lactic acid and Heptanoic acid, in respectively saline and sesame oil solvents (n = 6 tissues). The solvents alone have no effect compared to reference negative control, PBS. The concentration at which the spiked chemical reduces the viability of the tissues by 50% (IC50) after a fixed exposure time have been calculated. The IC50 for Lactic Acid and for Heptanoic acid are 1.9% and 0.5%, respectively.

Table 2
Composition of polymers.

	Composition	Provider
Silicone-SDS	Solid SDS (15%)in MED 4210 silicone	Arthrex (USA)
PVC-Genapol	Liquid Genapol (4%, X-100) in PVC	Arthrex (USA)
Y-1	PVC	Nelson Lab (USA), NIH (Japan)
Y-4	PVC w/Genapol (6%, X-80)	Nelson Lab (USA), NIH (Japan)

Polyvinyl chloride (PVC) and silicone are plastics widely used in medical devices industry. Y1 and Y-4 have been produced by National Institute of Health Sciences (NIH, Japan) and provided by Nelson Laboratories (USA). Silicone-SDS and PVC-Genapol came from Arthrex (Naples, USA).

release of IL-1α.

3.4. 18 h versus 24 h exposure time

To assess the effect of the duration of exposure, SkinEthic™ RHE tissues were exposed to NC, PC, VC, Y-1, Y-4 samples and a 0.5% SDS solution for 24 h and 18 h (Fig. 5). No difference between the two exposure times was observed for classification of the different samples (Fig. 3). PC, SDS 0.5% and Y-4 are classified irritants after both 18 h and 24 h exposure.

3.5. Cytokine as secondary endpoint

The cytokine IL-1α, which is a marker of skin inflammation, was quantified in the supernatant of SkinEthic™ RHE models after exposure to different samples (Fig. 6). The amplitude of measured level goes from 1.7 pg/mL to 184 pg/mL. Results representation for 44 samples with IL-1α in abscissa and cell viability in ordinate show two main clusters. A first cluster of 26 irritant samples, with a cell viability lower than 50%, and a IL-1α level higher than 10 pg/mL. A second cluster of 14 samples classified non-irritant by the assay, with a cell viability higher or equal to 50% and a IL-1α level below 10 pg/mL. For these two clusters, the complementary IL-1α quantification is well correlated to classification from cell viability.

Four plots are outside these two clusters. Two samples, Silicone 15% SDS in saline and Heptanoic acid 4% in SO are classified irritant due to their low cell viability despite a IL-1α level below 10 pg/mL. The low level of IL-1α, while cell viability is low is surprising and difficult to explain. We may hypothesize that this could be linked to different mechanisms such as degradation of the cytokine by the irritant or a fast kinetic of cell death which prevents a *de novo* synthesis of cytokine and accumulation in the medium.

The two other points outside the clusters are in the upper right quadrant of the figure. The samples, Lactic acid 1% in PBS and Silicone 15% SDS extracted in SO, present a cell viability around 107% but an IL-1α higher to the cut-off value, 16 pg/mL and 20.7 pg/mL, respectively. The Silicone 15% SDS is clearly irritant in the polar solvent, but the low solubility of SDS in non-polar solvents can explain the higher cell viability measured when extracted in SO. In this case, Il-1α seems to be helpful to detect presence of a potential irritant in SO extraction solvent. For the sample containing 1% LA in polar solvent, the concentration is lower to the calculated IC_{50} and cell viability correctly classified it as non-irritant. The slight elevation of IL-1α maybe an indicator of a low the inflammatory effect of LA on the living epidermis.

4. Discussion

The SkinEthic™ RHE model is a reconstructed human epidermis produced on an industrial scale for 25 years. Quality controls confirm the high level of reproducibility and stability of this model over time. Characterization of SkinEthic™ RHE model demonstrating that this test system reproduces many features of normal human epidermis (Rosdy

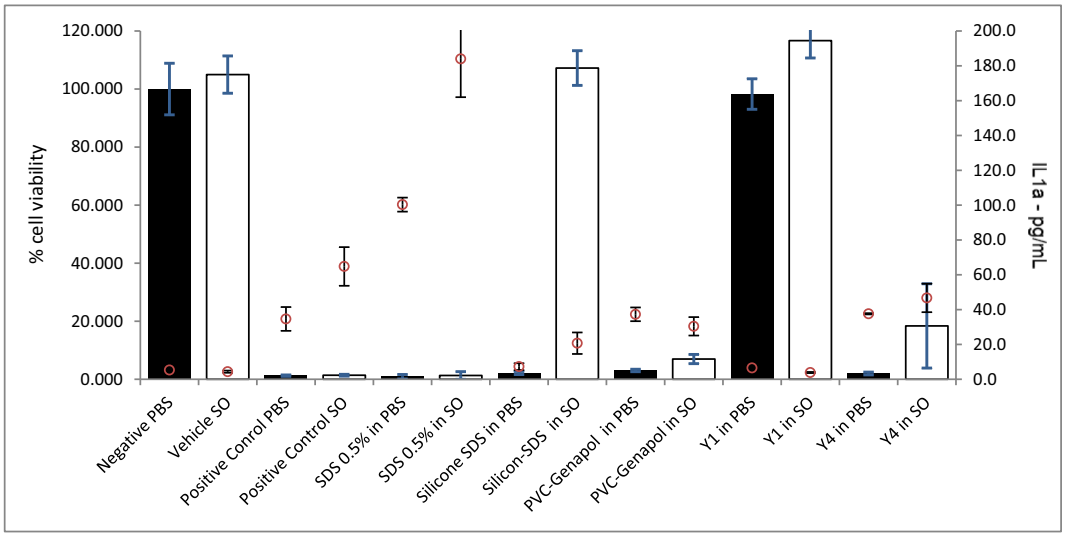

Fig. 3. Tests of medical devices polymer extracts.
The same protocol used for solvent spiked with irritants is performed on extracts of different samples of heat-pressed polyvinyl chloride (PVC) and silicone sheets infused with or without known irritant (n = 6 tissues). Using the prediction model of the OECD TG439, i.e. classification in category 2 when cell viability is below 50%, the Y-1 medical device extracts is non-irritant and the Silicone-SDS, PVC-Genapol and Y-4 are irritants. The Silicone-SDS, containing 15% SDS is classified irritant only when the material is extracted in the polar solvent (SO). PVC-Genapol and Y-4 materials, containing Genapol are irritants with the two extraction solvent even if a slight increase is observed in the non-polar solvent (SO) compared to the polar one (PBS). Interleukin-1 alpha (IL-1α) released in the medium is represented with red circle. The death of the tissue reflected by cell viability decrease is accompanied by a release of IL-1a in the medium with a 10 to 67 fold increase compared to NC respectively for SDS1% in PBS and in SO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and Clauss, 1990; Rosdy et al., 1993; Ponec et al., 2000). The assay developed with the SkinEthic™ RHE model for skin irritation of medical devices is derived from the validated protocol used for chemicals (OECD TG439). It was adapted to extracts of medical device from our experience regarding safety assessment of both ingredients (Alépée et al., 2010; Tornier et al., 2010) and cosmetics finished products (De Brugerolle de Fraissinette et al., 1999; Roguet et al., 1998; Damour et al., 1998; Faller et al., 2002) that are also complex mixtures of diluted ingredients. The paper of Casas et al. (Casas et al., 2013) was also helpful to design the assay. Compared to the OECD TG439, changes were made to take into account the dilution of potential irritants and to maximize their effects: The volume of tested product increase from 16 to 100 µL and the time of exposure was extended from 42 min to 24 h. The extended duration of exposure showed to be enough not to require additional post-exposure step. The negative control, PBS, and positive controls, SDS, are classical references used in *in vitro* and *in vivo* skin irritation assays (Lee and Maibach, 1995; Coquette et al., 2003; Basketter et al., 2012). The cut-off value of 50% under which a product is classified irritant is inherited from the OECD TG439. The same acceptance criteria was also kept for the positive control with a cell viability < 40% and a standard deviation between the SkinEthic™ RHE triplicate inferior or equal to 18%.

Using this protocol, we first reproduced the experiments of Casas et al. with solvents spiked at known concentrations of irritants. From dose response curves, the calculated IC_{50} for LA (1.9%) and HA (0.5%) are close to those observed by Casas et al. The lower IC_{50} measured with SkinEthic™ RHE compared to their data may reflect a higher sensitivity of the model for low concentration of irritants. For further training of naive laboratories, we decided to use LA and HA as reference irritants at a concentration higher than the IC_{50}: 4% of LA in saline and 2% of HA in sesame oil.

After demonstrated capacity of the *in vitro* method to detect low concentrations of irritants in extraction solvents we verified performance in conditions as close to a realistic scenario with polymer materials used in medical devices. The Y-4 polymer is a heat-pressed PVC sheets spiked with 5.8% of Genapol X-080, a known irritant. Y-1 presents the same composition but without Genapol. Y-1 and Y-4 were developed as positive reference materials for hemolysis testing (Haishima et al., 2014) of medical devices (ISO, 10993-4 Biological evaluation of medical devices - Part 4: Selection of tests for interactions with blood, 2017). Silicone-SDS is a medical grade silicone elastomer (MED 4210 silicone) spiked with 15% of SDS. PVC-Genapol, is a PVC containing 4% of Genapol X-100. These four samples belong to the set of reference materials used during the Round Robin Study to evaluate two reconstructed human epidermis models as *in vitro* skin irritation test for detection of irritant activity in medical device extracts.

The SkinEthic™ RHE assay classified Y-1 as non-irritant and Y-4 as irritant. This findings are in agreement with their respective composition, only Y-4 contents an irritant, and with previous data, including intra-cutaneous reactivity test in the rabbit (Olsen et al., 2016). Silicone-SDS and PVC-Genapol are classified irritant in the assay which is also in agreement with the presence of irritants in their composition. Yet, there is a discrepancy between the two solvents for Silicone-SDS: the extract is classified irritant in the polar solvent and non-irritant in the non-polar solvent. This discrepancy between extraction conditions is not observed with the Genapol containing material (Y-4) even if cell viability is slightly higher in SO. The difference observed in cell viability between extracts for a given material might be linked to the solubility of the irritant chemical into the selected solvent leading to variable extraction efficiency. Genapol and SDS are amphiphilic surfactants that can be characterized by the hydrophilic-lipophilic balance (HLB) index calculated from their relative ratio of polar and non-polar groups (ICI Americas Inc., 1976). The lower the HLB value the more lipophilic the molecule is and *vice versa*. With an HLB index of 13–14 (Table 3), Genapol is compatible for extraction in polar and non-polar solvents and the sample is classified irritant in the two solvents used. Quite the opposite, SDS has an HLB of 40, reflecting a strong hydrophilic component compared to its lipophilic part. The resulting lower solubility in lipophilic solvents could results in a poor extraction efficiency explaining the non-irritant classification of this sample extracted in SO. This point clearly illustrates the importance of using both polar and non-polar solvents during the extraction process. The prediction model of the assay integrates this parameter by classifying irritant a medical device when the cell viability is inferior or equal to 50% in at least one solvent or both.

The low solubility of SDS in non-polar solvent is also problematic

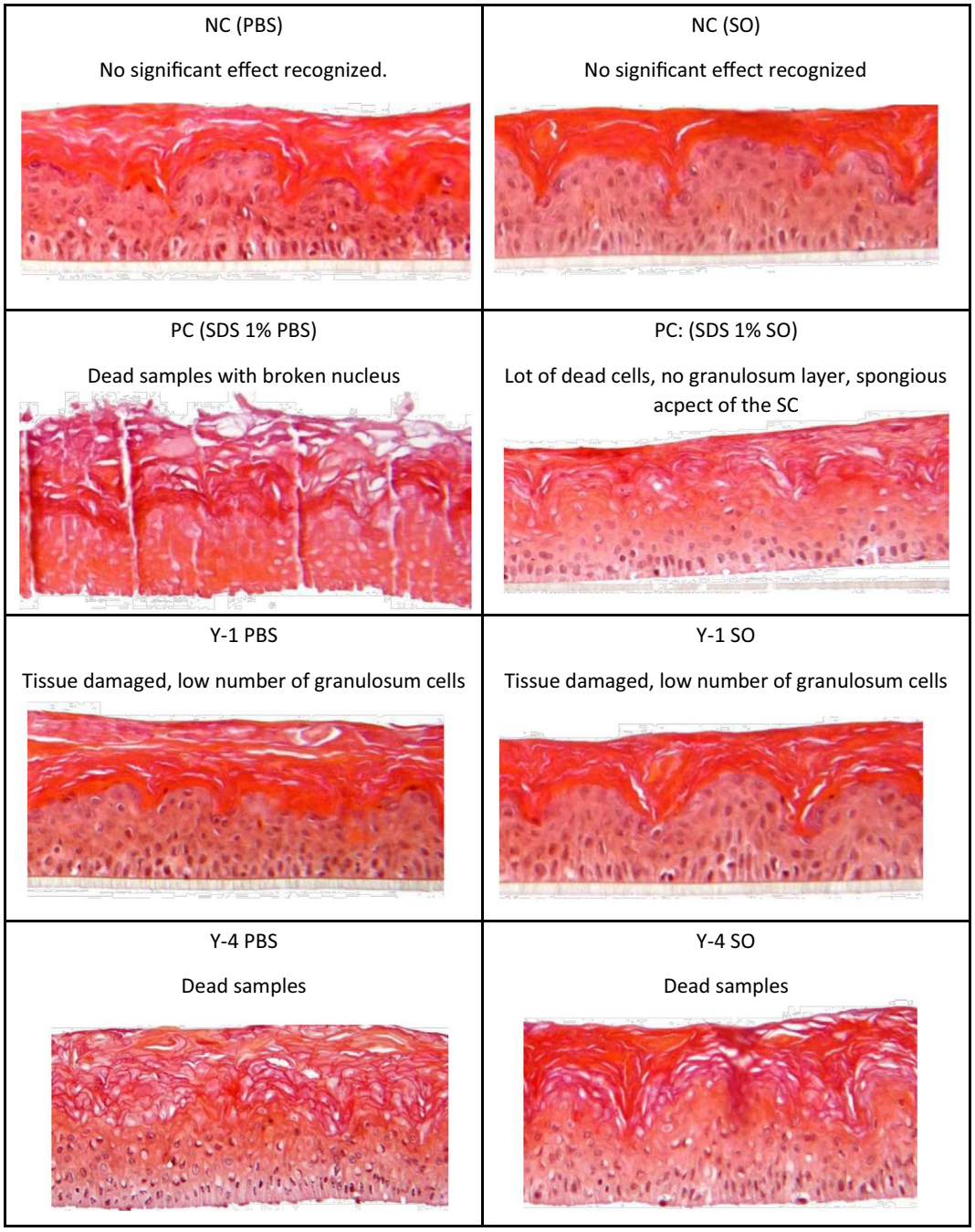

Fig. 4. HES histology of SkinEthicTM RHE model.
Hemalun Eosine-Saffron (HES) staining of the tissues after exposure to medical devices polymers extracts. The histology confirms the strong effect of the PC (SDS1%) especially in the polar solvent (PBS). No significant effects are observed after exposure to PBS and SO alone. Y-1 exposed tissues exhibit slight damages that are not detected by MTT cell viability assay. Y-4 extracts induce the death well correlated to the high decrease of cell viability.

when preparing the 1% SDS positive control (PC) in SO. At this concentration, the PC is a suspension of SDS rather than a solution. To limit the risk of variability related to non-homogeneous distribution of SDS in the preparation it is recommend to prepare the positive control from a stock solution of 20% SDS in water and to vortex it just prior to application. The concentration of SDS used as positive control is also questionable. 1% SDS, recommended in the OECD TG439 for a 42 minute exposure time, induces 24 h post-exposure an intense denaturation of the tissues with occasional detachment and elimination of it during the rinsing step. We have tested various concentrations and 0.5% SDS in saline or in SO gives the same cell viability reduction without the side effect observed with 1%. However, discussions with the workgroup members regarding Round Robin Study execution led to upholding 1% SDS concentration as positive control since this latter provided satisfactory and unambiguous results.

During the ISO congress in 2015 in Lund, the duration of the exposure time was discussed by the workgroup and a shorter time exposure of 18 h, was compared to 24 h used in our protocol (Fig. 3). No significant difference was observed for the classification of the different samples with the two exposure times. In the non-polar solvent, the cell viability of 0.5% SDS and Y-4 extracts were slightly higher after 18 h exposure compared to 24 h. According to the fact that the shorter time does not present clear advantages in term of results or organization the 24 h protocol was kept for the following Round Robin Study. We

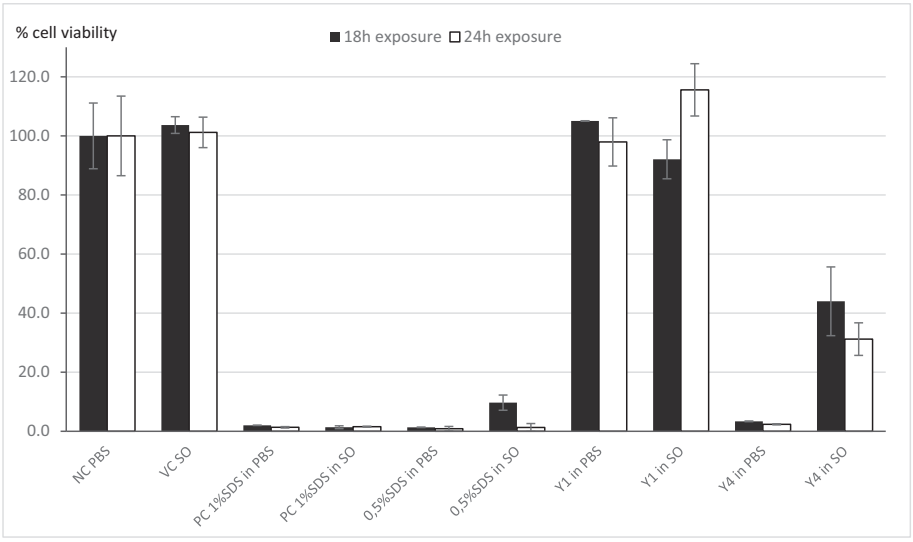

Fig. 5. 18 h *versus* 24 h exposure time.
Effect of a shorter time of exposure, 18 h, compared to 24 h on the classification of different samples (n = 2 tissues for 18 h and n = 3 tissues for 24 h). SkinEthic™ RHE tissues were exposed to NC, PC, VC, Y-1, Y-4 and a 0.5% SDS solutions during 24H and 18 h. No significant difference between the two exposure times has been observed for classification of the different samples (Fig. 3). PC, SDS 0.5% and Y-4 are classified irritants after both 18 h and 24 h exposure.

thought that this condition could maximize the effects of low concentrations of irritants to obtain clear results for potential border line materials.

Quantification of IL-1α release in the culture medium was also assessed as a potential complementary endpoint to cell viability for classifying the tested extracts. In 2007, the ECVAM validation for skin irritation of chemicals with EpiSkin model (Griesinger et al., 2009) and more recently the catch-up validation of the SkinEthic™ RHE model (Alépée et al., 2010) stated that the accuracy was not improved by measuring IL-1α release even if this endpoint might be considered to confirm negatives and being of help to better classify the mild to moderate irritancy potential of some chemicals. From the set of samples we tested in the medical device protocol, no evidence of added value of this secondary endpoint was observed. There was one example where a sample, Silicone 15% SDS, wrongly classified as negative (false negative) based upon cell viability, was correctly classified as irritant with IL-1α results. Nevertheless, the concerned polymer was already correctly classified in the second extraction solvent (NaCl) without the need of IL-1α quantification. Since results provided in this study showed that IL-1α release measurements did not improve the performances of the assay, no further evaluation of this endpoint was

Table 3
Hydrophilic-lipophilic balance (HLB) of surfactants.

Surfactant	HLB value	Ref
SDS	40	Stavroudis (2009)
Genapol X-100	14,1	MERCK http://www.merckmillipore.com/FR/fr/product/GENAPOL-X-100%2C-PROTEIN-GRADE-Detergent%2C-10%25-Solution%2C-Sterile-Filtered—Calbiochem,EMD_BIO-345798?bd=1#anchor_PDS
Genapol X-80	13,1	Clariant Produkte (Deutschland) GmbH http://www.acarchemicals.com/Assets/Documents/Genapol_X_080_20150608_224444.pdf

Genapol X-80, X-100 and SDS, used as irritant in the polymer samples, are amphiphilic surfactant that can be characterized by the hydrophilic-lipophilic balance (HLB) calculated from their relative ratio of polar and non-polar groups.

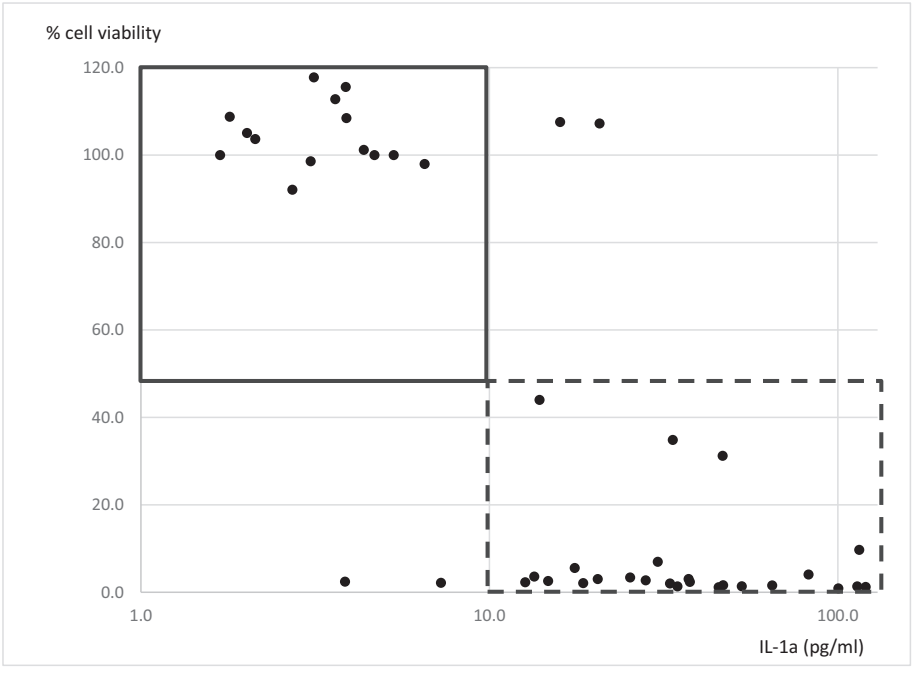

Fig. 6. IL-1alpha *vs.* cell viability.
Inflammatory response evaluated by the quantification of the cell viability measured by MTT reduction and expressed in percentage of control (N = 44) represented in function of the specific human cytokine, interleukin-1 alpha (IL-1α) on a logarithmic scale. A cut off value of 50% for cell viability 10 pg/mL for IL-1α allow to identify two main clusters were prediction based onto IL1-α correlates with that onto cell viability: A cluster of 26 samples classified irritants, with a cell viability lower than 50%, and a level of IL-1α higher than 10 pg/mL. A cluster of 14 samples classified non-irritant, with a cell viability superior or equal to 50% and Il-1α level inferior to 10 pg/ml. 4 dots are outside these clusters. 2 in the upper right with a viability higher than 50% and a IL-1α higher than 10 pg/mL. And 2 in the lower left part with a viability lower than 50% and IL-1α level lower than 10 pg/mL.

performed.

To prepare the Round Robin Study performed in 2016, 7 naïve laboratories were trained to this protocol. The good results of these trainings reflect the transferability of this protocol in different types of laboratories (CRO, medical devices manufacturer and academics laboratories) and different countries (France, Italy, Germany, USA, and Korea). The robustness of such type of assay and its simplicity of implementation were already proved in the context of OECD with the spread around the world of the TG431 and TG439 for *in vitro* skin corrosion/irritation of chemicals. This is an important point for future deployment of this assay in medical devices industry.

In conclusion, this study confirms the good performance of this new protocol for *in vitro* skin irritation of medical devices extracts with the SkinEthic™ RHE model. This assay is able to detect low concentrations of irritant spiked in different solvents and in extracts of medical devices materials containing known concentration of the irritants SDS and Genapol. In our conditions, cell viability alone appears to be sufficient for classification without the need to IL-1α as a secondary readout. The results of the training of 7 naïve laboratories reflect the robustness and transferability of this assay in preparation of the Round Robin Study to evaluate Reconstructed Human Epidermis models as *in vitro* skin irritation test for detection of irritant activity in medical device extracts (De Jong et al. 2018).

Transparency document

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