



Assessment of test method variables for *in vitro* skin irritation testing of medical device extracts

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ABSTRACT

Skin irritation is an important component of the biological safety evaluation of medical devices. This testing has typically been performed using *in vivo* models. However, in an effort to reduce the need for *in vivo* testing, alternative methods for assessing skin irritation potential *in vitro* have been developed using a Reconstructed Human Epidermis (RhE) model. During the development of the protocol for the round robin validation of *in vitro* irritation testing for medical device extracts, it became clear that there were three points in the procedure where different options may be validated within each laboratory for routine testing: sample exposure time (18 vs 24 h), SDS positive control concentration, and cytokine (IL-1 α) release testing. The goal of our study was to evaluate the effect of these variables. EpiDerm™ tissues were exposed to extracts of three plain polymer samples, and four polymers embedded with known irritant chemicals. Exposures were performed for 18 and 24 h. Resulting tissue viability was assessed by MTT reduction and IL-1 α release was assessed by ELISA. Testing was also performed using various concentrations of SDS ranging from 0.5 to 1% (w/v). Overall, results were similar for samples tested and 18 and 24 h, but the 18 h exposure time has the potential to have an impact on the results of some sample types. IL-1 α testing was shown to be useful to clarify conflicting tissue viability results. Use of a lower concentration of SDS as a positive control can help prevent issues that arise from excessive tissue damage often caused by 1% SDS.

1. Introduction

Skin irritation is an important component of the biological safety evaluation of medical devices. Irritation testing for medical devices has typically been performed using *in vivo* models (ISO, 2014). However, in an effort to reduce the need for *in vivo* testing, alternative methods for assessing skin irritation potential *in vitro* have been developed (Corp., 2014; Eskes et al., 2007; Portes et al., 2002; Spielmann et al., 2007). Because of this, there is a general move in the industry towards the use of a Reconstructed Human Epidermis (RhE) model for the assessment of skin irritation. The currently accepted *in vitro* irritation procedure for the testing of neat chemicals, detailed in OECD 439 (OECD, 2013), has been adapted for the testing of medical devices (Casas et al., 2013; ISO, 2016). This *in vitro* method is based on exposing RhE tissues to medical device extracts and then measuring percent tissue viability through the conversion of the yellow tetrazolium dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a purple formazan salt by cellular reductase enzymes present in viable cells (Mosmann, 1983). Round robin testing has been performed on medical device materials using the adapted procedure (ISO, 2016).

As this alternative method becomes more common, laboratories will begin to validate the assay and develop internal procedures for performing the testing. There are multiple variables in the method that have the potential to affect the results of the assay and should be considered during such validation processes. In the present study, we examined the effect of three such variables: sample exposure time, positive control concentration, and use of the secondary endpoint testing of interleukin 1 alpha (IL-1 α).

1.1. Sample exposure time

The sample exposure time outlined in OECD 439 is 1 h. This method and exposure time are designed for the testing of pure chemicals and mixtures. These samples are tested neat, regardless of their intended final concentration and use. However, because medical devices are extracted in a manner that simulates patient exposure, any irritant compounds found in medical device extracts will generally be at very low concentrations. Therefore, a longer exposure period is needed for the assessment of irritation potential for medical devices. Both 18 h and 24 h exposure periods have commonly been used. It is possible that the

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Table 1
Test samples.

Base polymer material	Spiked chemical (final concentration)	Format	Thickness	Extract ratio	Sample ID
Polymer test samples					
Polyvinyl chloride	N/A	Sheet	< 0.5 mm	6 cm ² /mL	PVC
Silicone	N/A	Molded	> 0.5 mm	3 cm ² /mL	Silicone
80A Polyurethane	N/A	Molded	> 0.5 mm	3 cm ² /mL	PU
Spiked polymer test samples					
Silicone	Sodium dodecyl sulfate (15%)	Molded	> 0.5 mm	3 cm ² /mL	SDS
Silicone	Heptanoic acid (25%)	Molded	> 0.5 mm	3 cm ² /mL	HA
Polyvinyl chloride	Genapol X-100 (4%)	Molded	> 0.5 mm	3 cm ² /mL	X-100
Polyvinyl chloride	Genapol X-80 (10%)	Pellets	N/A	0.2 g/mL	X-80

length of the exposure period for *in vitro* irritation test methods may have an effect on the test results.

In order to evaluate the effect of exposure duration, *in vitro* irritation testing was performed using three polymer materials and four polymers spiked with known irritants (Table 1). Each sample was tested with both an 18 h and a 24 h exposure time.

1.2. Positive control concentration

The positive control and concentration outlined in OECD 439 is 5% sodium dodecyl sulfate (SDS) (OECD, 2013). The concentration used in the adapted procedure for medical devices is 1% SDS (ISO, 2016). This reduction in concentration is necessary because of the increase in exposure time from 1 h for neat chemicals to 18–24 h for medical devices. The acceptance criterion for the positive control in the adapted procedure is $\leq 20\%$ tissue viability. Although the use of 1% SDS as a positive control consistently meets the acceptance criteria, severe tissue damage often results. This damage can lead to partial or complete detachment of the tissues from the filter on which they are grown and tested, resulting in a loss of replicates for statistical analysis.

OECD 439 states that tissues treated with a positive control “should reflect their ability to respond to an irritant chemical under the conditions of the test method” (OECD, 2013). In addition, ISO 10993-10 guides that a positive control should be selected to avoid complete “knock out” of the test model (ISO, 2014). Use of 1% SDS as a positive control consistently results in complete cell death. A lower concentration of SDS that is capable of demonstrating the tissue's ability to respond to an irritant without completely damaging the tissue would be more appropriate for use as the positive control.

In order to evaluate the effect of positive control concentration, *in vitro* irritation testing was performed using various concentrations of SDS, ranging from 0.5 to 1% (w/v).

1.3. IL-1 α release testing

IL-1 α has been identified as a key cytokine released from keratinocytes during an irritation response (Coquette et al., 1999; Lee et al., 2013). It was originally analyzed in method development testing in 2009 but the dataset was too small to enable a full evaluation of the endpoint (Kandarova et al., 2009). The concentration of IL-1 α released into the culture media during the sample exposure period is measured and can be used alongside MTT assay results for the prediction of irritancy. Although the MTT assay has been shown to generally be sufficient for predicting irritation (Alepee et al., 2010; Griesinger et al., 2009), various implementations of IL-1 α as a secondary endpoint have been used in the industry. These can include using IL-1 α results to verify borderline MTT assay results or using IL-1 α to verify all non-irritant results in the MTT assay.

In order to evaluate the usefulness of IL-1 α data as a supplement to MTT assay results, IL-1 α release was also measured for the test samples used in the exposure time evaluation studies previously mentioned.

2. Materials and methods

Testing was performed according to the officially approved protocol for the *in vitro* skin irritation round robin, “Evaluation of a Method to Detect Skin Irritation of Medical Device Extracts using Reconstructed human Epidermis (RhE)” (ISO, 2016). Unless otherwise indicated, all incubations were performed in a humidified incubator with standard cell culture conditions of 37 °C and 5% CO₂.

2.1. Reconstructed human epidermis model

EpiDerm™ reconstructed human epidermis tissues (EPI-200-SIT) were obtained from MatTek, Corp. (Ashland, Massachusetts). This *in vitro* model consists of normal, human-derived epidermal keratinocytes (NHEK) cultured on specially prepared tissue culture inserts. EpiDerm exhibits human epidermal tissue structure and cellular morphology consisting of organized and proliferative basal cells; spinous and granular layers; and cornified epidermal layers.

2.2. Test sample preparation

For each round of testing, a polar and a non-polar extract were prepared for each sample. The polar extraction solvent used was physiological saline (0.9% NaCl) and the non-polar extraction vehicle was sesame oil (Spectrum Chemical Manufacturing Corp., New Brunswick, NJ). Test samples are shown in Table 1. Samples were prepared according to ISO 10993-12 (ISO, 2012). Samples were either polymer sheets cut into 3 cm² pieces, molded polymers formed into 3 cm² pieces, or in pellet form (Table 1). All test samples were extracted in 1 mL of extraction vehicle using the appropriate surface area or weight (Table 1). Test samples along with vehicle controls, consisting of 1 mL of each extraction vehicle without sample, were extracted in a non-humidified incubator at 37 °C for 72 h with agitation.

2.3. Chemical controls

The negative control was phosphate buffered saline (PBS). The positive control was a 1% (w/v) solution of SDS in water. For the positive control concentration study, this 1% SDS solution was then diluted to the following additional concentrations: 0.05%, 0.1%, 0.125%, 0.25%, 0.5%.

2.4. Tissue preconditioning

Upon receipt, the EpiDerm™ tissues were inspected for damage according to manufacturer instructions. The tissues were then preconditioned by transferring the tissues to 6-well plates prefilled with 0.9 mL of assay medium (MatTek Corp., proprietary media provided with EpiDerm™ tissues) and incubating for 1 h. After the initial incubation, the tissues were transferred to fresh media and incubated overnight.

2.5. Tissue exposure

After overnight incubation, the tissues were transferred to new 6-well plates containing 0.9 mL of fresh assay medium per well. Then 100 μ L of each sample extract and control was added to the apical surface of the tissues in triplicate and incubated for either 18 or 24 h. After exposure, the tissues were thoroughly rinsed with PBS, blotted on an absorbent pad, and dried with a sterile cotton-tipped swab to remove any remaining liquid on the surface of the tissue.

2.6. MTT viability assay

Cell viability after sample exposure was determined using the MTT viability assay. A 1 mg/mL solution of MTT (provided with tissues) in assay medium was prepared. Following exposure and rinsing, the tissues were placed in a 24-well plate containing 0.3 mL per well of the MTT medium and incubated for 3 h. After the incubation, residual MTT medium on the outside of the tissue inserts was blotted on an absorbent pad. The resulting formazan salt was then extracted from the tissues by placing them in a 24 well plate containing 2 mL of 99% isopropanol per well. The plates were placed in a sealed plastic bag to prevent evaporation and set on an orbital shaker for 2 h at room temperature. The formazan extracts were then mixed thoroughly and 200 μ L was transferred in duplicate to a 96 well plate. The optical density at 570 nm was measured on a spectrophotometer with isopropanol used as a blank. The percent viability of each sample was calculated relative to negative control using the following equation:

$$\% \text{Viability}_{\text{Sample}} = [\text{OD}_{\text{Sample}} / \text{Mean OD}_{\text{NC}}] \times 100\%$$

2.7. IL-1 α assay

The concentration of IL-1 α released from the RhE tissues into the assay medium during the exposure period was measured by ELISA (Thermo Fisher, Waltham, MA), according to the manufacturer's instructions. Assay medium for each sample was collected immediately following the exposure period and was stored at -20°C until ELISA testing was performed. The concentration of IL-1 α for each sample was calculated and a two-fold increase or greater, compared to the appropriate extraction vehicle control, was considered a positive irritation response.

3. Results

3.1. Sample exposure time

To determine the effect of exposure of time, tissues were exposed to test sample extracts and controls for 18 and 24 h. Three individual rounds of testing were performed with each sample tested in triplicate each round. The MTT assay results for all samples tested showed a lower overall mean percent viability after 24 h exposure than after 18 h exposure (Fig. 1). However, in most cases, the end classification of irritant vs. non-irritant was the same for both exposure times. The two exceptions were the 25% heptanoic acid samples extracted in sesame oil and the X-100 samples extracted in sesame oil. In addition, the IL-1 α assay resulted in the same irritant/non-irritant classification for each sample at both 18 and 24 h exposure times (Figs. 4–5).

The 25% heptanoic acid samples extracted in sesame oil displayed a wide variation in response. The overall average percent viability for these samples was 50.9% (non-irritant) at 18 h and 39.0% (irritant) at 24 h (Fig. 2). However, the results from individual test runs varied; with two out of the three runs resulting in an irritant classification and one run resulting in a non-irritant classification. It is interesting to note that although the classification for these samples varied between test runs (Irritant: Runs 1 and 3, Non-Irritant: Run 2), the classification was the

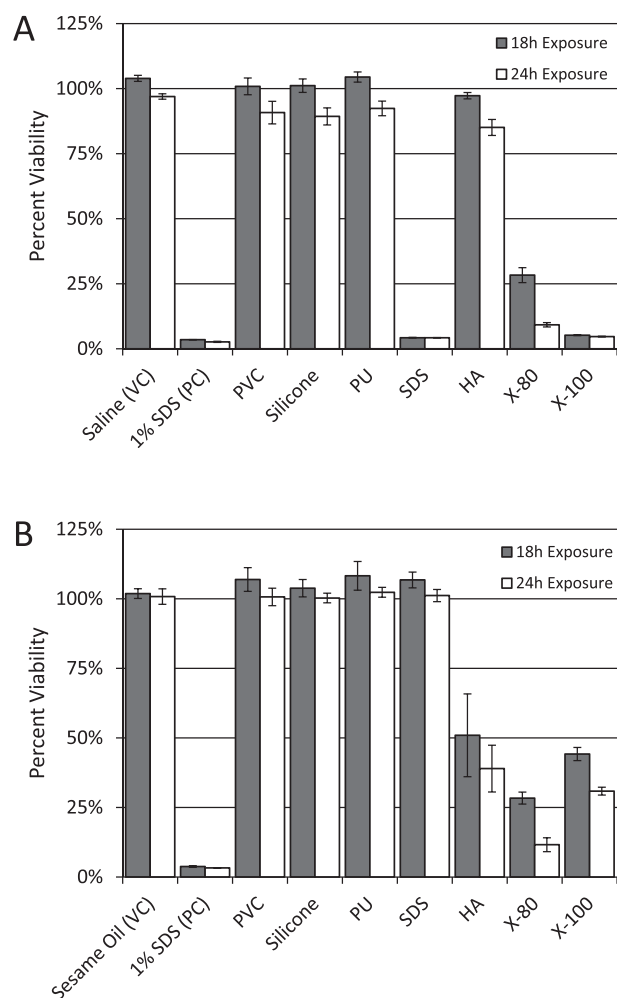


Fig. 1. EpiDerm™ tissues were exposed to controls and sample extracts for 18 and 24 h. Extraction vehicles were saline (A) and sesame oil (B). Tissue viability was assessed by MTT and is expressed as a percent of the negative control. Data is shown as the mean of 9 replicates tested over 3 independent experiments. Error bars represent standard error (SEM).

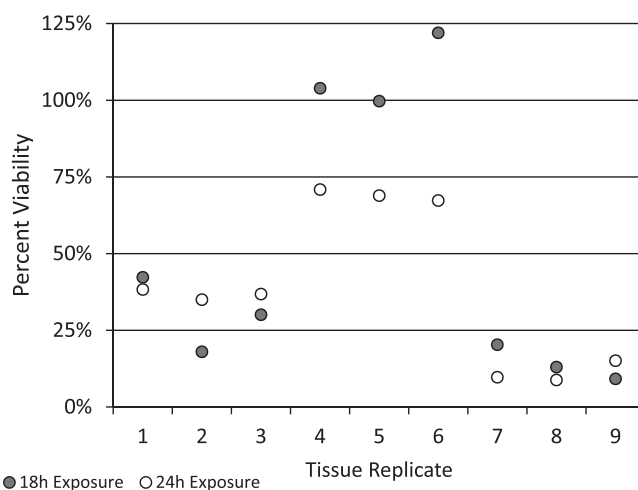


Fig. 2. EpiDerm™ tissues were exposed for 18 and 24 h to HA samples extracted in sesame oil. Tissue viability was assessed by MTT and is expressed as a percent of the negative control. Data is shown as the individual results for each of 9 tissue replicates tested over 3 independent experiments (Experiment 1: Replicates 1–3, Experiment 2: Replicates 4–6, Experiment 3: Replicates 7–9).

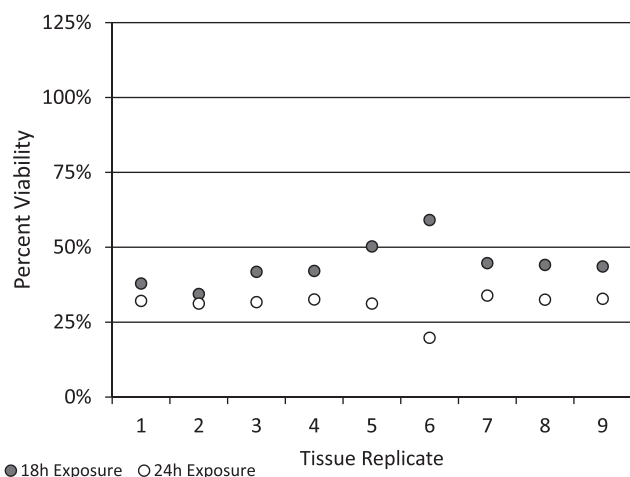


Fig. 3. EpiDerm™ tissues were exposed for 18 and 24 h to X-100 samples extracted in sesame oil. Tissue viability was assessed by MTT and is expressed as a percent of the negative control. Data is shown as the individual results for each of 9 tissue replicates tested over 3 independent experiments (Experiment 1: Replicates 1–3, Experiment 2: Replicates 4–6, Experiment 3: Replicates 7–9).

same at both exposure times in each individual test run (Fig. 2). This is an indication that the variability in these results is more likely due to inconsistency in the sample itself than in the test method. In fact, similar variability was observed for this sample in multiple laboratories participating in the round robin validation.

The X-100 oil extract samples were classified as irritants overall at both 18 h and 24 h exposure times with overall percent viabilities of 44.2% and 39.0%, respectively (Fig. 1B). However, the results for the second round of testing showed borderline results at 18 h. The average percent viability for these samples in the second round of testing was 50.5% at 18 h and 27.9% at 24 h (Fig. 3), resulting in inconsistent classification between exposure times (non-irritant at 18 h and irritant at 24 h). At the 18 h exposure time, one tissue replicate was below the irritation classification threshold of 50% viability while the other two tissue replicates were above 50% viability. At 24 h, all three replicates clearly showed a positive irritation response.

IL-1 α assay results confirmed the irritant classification of X-100 oil extract samples at both 18 and 24 h exposure times with all replicates tested resulting in high levels of IL-1 α release (Fig. 5; Range: 5.2–58.1 fold increase over extraction vehicle control). In addition, the IL-1 α assay results are consistent with the corresponding MTT assay results; Samples resulting in low viability in the MTT assay also have increased levels of IL-1 α release (Fig. 6). The results are also generally clustered into distinct groups of double positives (samples classified as irritants by both assays) and double negatives (samples classified as non-irritants by both assays). Importantly, there were no instances where a sample resulted in high tissue viability and increased release of IL-1 α . These results are consistent with previous reports that the addition of IL-1 α data does not tend to increase specificity beyond that of the MTT assay alone (Alepee et al., 2010; Griesinger et al., 2009; Kandarova et al., 2009). However, the addition of IL-1 α data does appear to be beneficial in cases where the MTT assay results in tissue viability very close to the irritant categorization threshold of 50% viability; such as with the X-100 oil extract samples.

3.2. Positive control concentration

In order to identify a positive control concentration that would consistently demonstrate an irritation response while avoiding excessive tissue damage, multiple concentrations of SDS were tested with a 24 h exposure time. As expected, the results show a consistent dose response with a higher concentration of SDS resulting in lower percent

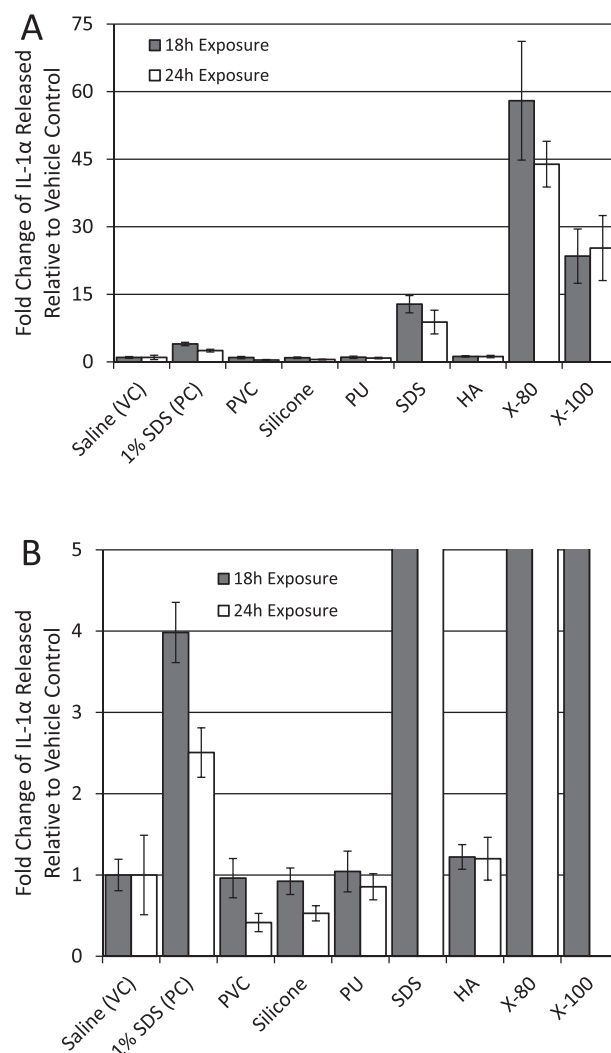


Fig. 4. EpiDerm™ tissues were exposed for 18 and 24 h to controls and samples extracted in saline. The concentration of IL-1 α released into the basal assay medium during the exposure period was measured by ELISA. Data is shown as the mean fold change, relative to the vehicle control, of 9 replicates tested over 3 independent experiments. Error bars represent standard error (SEM). (B) displays the same data as (A), but with an expanded scale on the y-axis.

viability (Fig. 7). The tissues exposed to 0.05 and 0.1% SDS were above the 20% viability threshold required in the test method acceptance criteria for the positive control. The tissues exposed to 0.125% SDS had an average viability below 20%, however the results between replicates varied widely; ranging from 5.4% to 37.8% viability. The results for the tissues exposed to 0.25% and 0.5% SDS showed consistently reproducible values below 20% viability and the tissues remained intact throughout the study.

Tissue detachment was only observed in tissues exposed to 1% SDS. This tissue damage ranged from partial detachment, in which a portion of the tissue was separated from the plastic insert or polycarbonate filter; to full detachment, in which the tissue was completely separated from the insert and filter (Fig. 8). In order to estimate the frequency at which tissue detachment occurred after exposure to 1% SDS, testing was performed in which 24 tissues were treated with 1% SDS for 24 h. Thirteen of the 24 tissues (54.3%) were either partially or fully detached from the tissue insert housing or filter (Table 2).

4. Discussion

Skin irritation is an important part of the safety assessment required

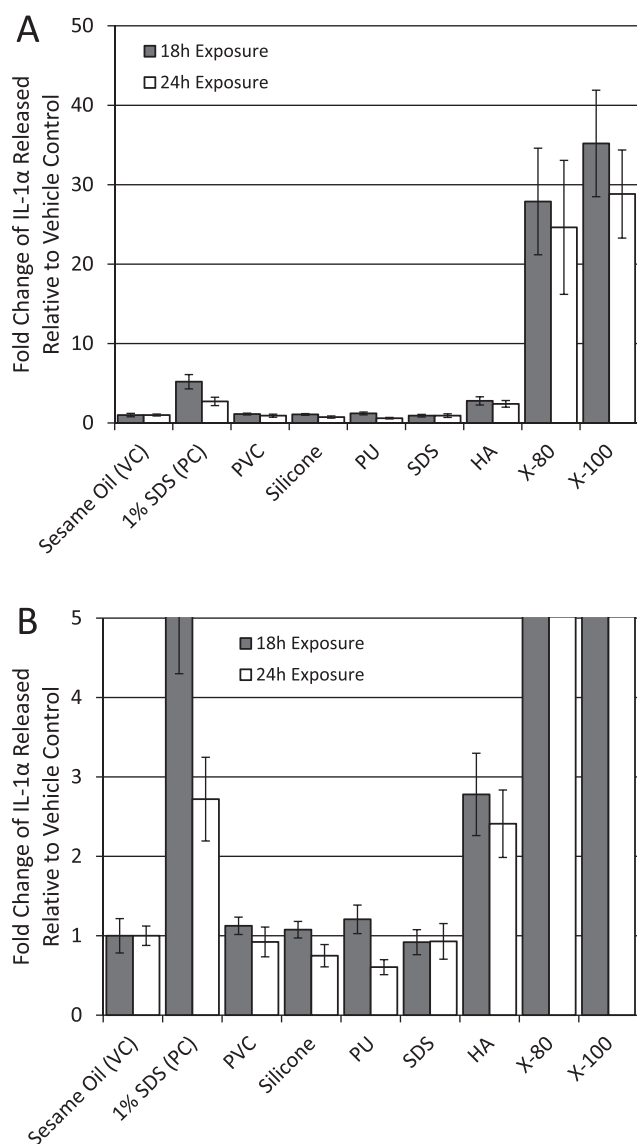


Fig. 5. EpiDerm™ tissues were exposed for 18 and 24 h to controls and samples extracted in sesame oil. The concentration of IL-1α released into the basal assay medium during the exposure period was measured by ELISA. Data is shown as the mean fold change, relative to the vehicle control, of 9 replicates tested over 3 independent experiments. Error bars represent standard error (SEM). (B) displays the same data as (A), but with an expanded scale on the y-axis.

for medical devices. While this testing has been performed in animal models, the development of *in vitro* alternative methods has been a high priority. Skin irritation testing using RhE tissues has been shown to be an effective and reliable alternative to traditional *in vivo* methods. The purpose of this study was to address some of the potential variables within the test method that should be considered as laboratories validate the method and develop internal procedures for conducting the test.

One such variable is the length of time that the tissues are exposed to the test article. In many cases, the variation in exposure times used by different testing facilities is a result of various factors affecting the overall testing logistics (e.g. availability and delivery time of RhE tissues used for testing, availability of facilities and technicians performing testing, etc).

To assess the impact of exposure time on the test results, testing was performed on tissues exposed to identical test article extracts for either 18 or 24 h. Although there was little overall change in the end result (classification as either irritant or non-irritant) for each sample as a

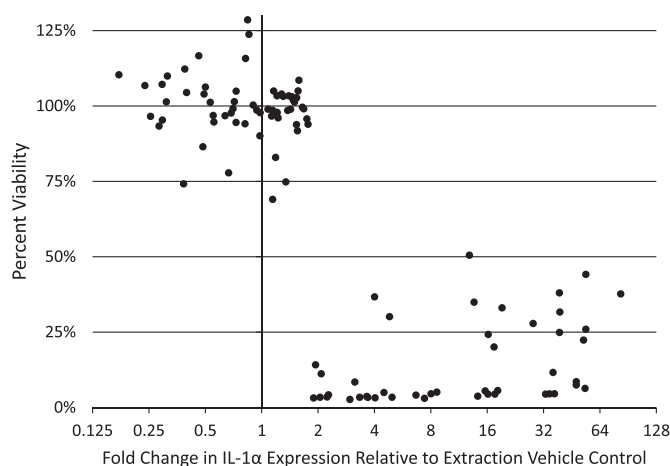


Fig. 6. EpiDerm™ tissues were exposed for 18 and 24 h to controls and sample extracts. Extraction vehicles were saline and sesame oil. Tissue viability was assessed by MTT and is expressed as a percent of the negative control. The concentration of IL-1α released into the basal assay medium during the exposure period was measured by ELISA and is expressed as the fold change in IL-1α concentration, relative to the vehicle control. Each data point represents the mean results of three tissue replicates tested in one of three independent experiments.

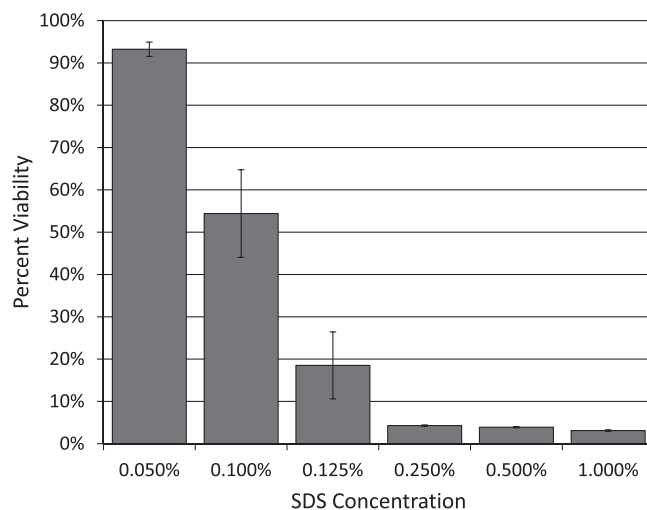


Fig. 7. EpiDerm™ tissues were exposed to six concentrations of SDS for 24 h. Concentrations tested were: 0.05% (n = 3), 0.1% (n = 5), 0.125% (n = 4), 0.25% (n = 8), 0.5% (n = 9) and 1% (n = 9). Tissue viability was assessed by MTT and is expressed as a percent of the negative control. Bar height represents the mean. Error bars represent standard error (SEM).

result of the difference in exposure time, there was a consistent trend of lower viability in the 24 h samples compared to those exposed for 18 h (Fig. 1). While in most cases, the 18 h exposure is sufficient to detect irritation, there is an increased possibility for false negative results with an 18 h exposure compared to a 24 h exposure, especially for samples containing a low concentration of irritants.

This is demonstrated in the results for the X-100 oil extracts, which were classified as both irritants and non-irritants in different rounds of testing at the 18 h exposure time, but were clearly classified as irritants in all testing at 24 h (Fig. 3). This type of conflicting result is a distinct possibility in the routine testing of laboratories which regularly work with a wide variety of test samples. Testing laboratories should carefully consider how to address such situations, whether it is through testing of additional replicates of the sample, confirmatory testing using the secondary endpoint of IL-1α, or some other means. In this case, the IL-1α results identified that the correct classification of these samples at 18 h exposure was as an irritant.

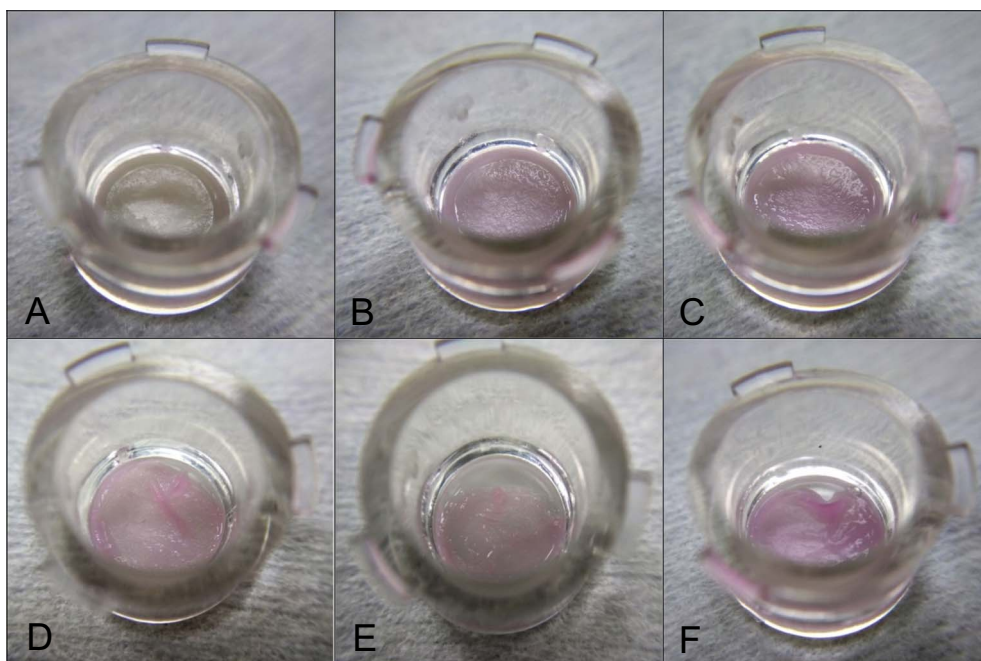


Fig. 8. Representative photos of tissue condition following 24 h exposure to negative control or SDS. Examples of intact tissues following exposure to (A) PBS negative control, (B) 0.1% SDS and (C) 0.125% SDS. Examples of partially (D) and fully (E–F) detached tissues after exposure to 1% SDS.

Table 2
Tissue condition after exposure to 1% SDS for 24 h.

Tissue condition	Number	Percent occurrence
Intact	11	45.8%
Partially detached	4	16.7%
Fully detached	9	37.5%

In this case, the usefulness of IL-1 α release as a secondary endpoint for *in vitro* skin irritation assessment is demonstrated. While it has been shown that, in most cases, the tissue viability measured by the MTT assay is sufficient to accurately predict the irritation potential of a test sample, use of IL-1 α as a secondary endpoint can provide valuable additional data in certain circumstances. Testing of additional replicates of a sample in order to confirm conflicting results may not always be a feasible approach; particularly with respect to complex medical devices that are difficult or expensive to produce. The additional data from the IL-1 α assay in such situations can help ensure the correct classification of the test sample.

In addition to the effect of exposure time on the test sample results, it is important to consider the effect of the concentration of positive control chemical used. The standard positive control used for this *in vitro* method is a 1% solution of SDS. However, when the tissues are exposed to 1% SDS, the resulting tissue damage is often so extensive that the tissue becomes partially or fully detached from the polycarbonate filter on which it is grown and can be washed away during the rinsing portion of the test. Data regarding the frequency of observed issues with the positive control tissues was not formally captured in the round robin testing. However, through discussions with other technicians performing the assay, we have confirmed that this finding was not uncommon among many of the labs that participated in the round robin validation. A variety of strategies have been implemented to reduce the loss of tissue, including gentler rinsing of the positive control tissues or placing a filter-housing into the rinse container to catch the detached tissue so that it can still be analyzed. While these methods can be effective to retain the tissue, there are concerns with any adaptation to the testing process that would bias treatment of one tissue over another.

In order to identify an appropriate positive response while still preserving the integrity of the tissue, we have tested various concentrations of SDS. Tissue detachment was only observed at a

concentration of 1% SDS. The lower concentrations tested, 0.05% and 0.1%, consistently demonstrated viability results above 20% indicating that the SDS concentration was too low to be used as a positive control. The viability results for the 0.125% SDS tissues were not reproducibly below 20% viability. The 0.25% and 0.5% SDS concentrations consistently produced results below 20% viability without excessive damage to the tissues (Fig. 7). These concentrations are, therefore, suitable for use as a positive control for this test method.

Testing laboratories performing alternate *in vitro* irritation methods should carefully consider the positive control concentrations used in order to prevent excessive tissue damage as this can result in a loss of replicates and ability to perform robust statistical analysis during test method development and validation. We recommend using reduced SDS concentrations (below 1%) to meet the acceptance criterion of $\leq 20\%$ cellular viability through proper validation.

5. Conclusion

In the development and validation of any test method, there are numerous variables that must be considered. For the *in vitro* skin irritation test for medical device extracts using RhE tissues, we have addressed a few of these variables; sample exposure time, usefulness of IL-1 α cytokine testing and positive control concentration. Overall, results were similar for samples tested at 18 and 24 h, but the 18 h exposure time has the potential to have an impact on the results of some sample types. IL-1 α testing was shown to be useful under certain circumstances. Use of a lower concentration of SDS as a positive control can help prevent issues that arise from excessive tissue damage often caused by 1% SDS. It is not our intent to say that there is a definitive best option for each of these points. However, we do wish to draw attention to them as they each have the potential to impact the success of this test method and are important points to consider during the development and internal validation process of this test method in individual testing facilities.

Conflict of interest

The authors do not have any conflicts of interest.

Transparency document

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Transparency document

The [Transparency document](#) associated with this article can be found in online version.

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