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Skin compatibility of cutting fluids: in vitro / in vivo test strategy

Abstract
Three water-based cutting fluids containing different biocide concentrations and different emulsifier systems were tested for skin compatibility using *in vivo* and *in vitro* systems. Used and unused samples of one of the cutting fluids were compared using the *in vitro* system. With the *in vivo* test, a 24 h Patch Test revealed good skin compatibility for all the cutting fluids tested, with no significant differences between the three samples. In contrast the *in vitro* tests using human epidermal or human cornea skin models revealed significant differences between the samples. Differences were observed between the three unused cutting fluids, and between the used and the unused samples. To summarise, *in vitro* test systems using human skin models provide useful option for a sensitive test of the skin compatibility of cutting fluids in long term contact with the skin.

Key words
Skin compatibility, cutting fluid, skin model, *in vitro* test, contact dermatitis.

Introduction

Contact dermatitis in metal workers is considered to be the most relevant occupational illness in the metal working industry. Different studies report a 20-25% prevalence of hand eczema in metal workers (1,2). Irritant contact dermatitis is more often diagnosed than the allergic contact dermatitis (3). Several different chemicals with which metal workers have skin contact contribute to the high prevalence of contact eczema, including cleaning detergents, solvents, degreasers and metalworking fluids. Though rarely harmful on short-term exposure, metalworking fluids may evolve their harmful attributes after long term and repeated contact with the skin. Since several hours of contact on each working day is typical in many working environments, even a low irritant capacity contributes to the high incidence of contact eczema in this work group. The use of cutting fluids with good skin compatibility is therefore an important factor in preventing occupational hand dermatitis in metal workers. In recent studies, both *in vitro* and *in vivo*, the irritancy of different cutting fluids was compared. Both strategies have advantages, but also limitations.

In vivo studies on human volunteers are of the highest relevance for predicting skin irritancy. However, when looking at chronic skin irritancy induced by mild irritants, established *in vivo* studies often fail to predict the irritant potential. For example in a study by de Boer et. al. (4) different metal working fluids induced only minimal skin irritation, even after stripping of the stratum corneum and repeated application to the forearm skin over 5 days. Differentiation between the tested fluids and water was only possible for one of three cutting fluids tested. In addition, in *in vivo* tests the safety of volunteers has the highest priority. Hence, some products may not be tested. A study director should take into account the higher penetration rate of components and a higher risk of sensitisation under the conditions of a repeated patch test design. This holds especially true for all used cutting fluids for which the exact composition is not known.

For *in vitro* tests correlation to the *in vivo* situation has to be verified before they can be used as a predictive tool. On the other hand, *in vitro* test procedures can be used with any sample and at any concentration. The analysis of the irritancy potential is based on the measurement of inflammation mediators or on the quantification of cell damage. This allows a relatively sensitive analysis of the irritant potential of test substances, since the biochemical parameters increase before clinical symptoms are manifest. However, careful examination of the test results is necessary due to the model nature of *in vitro* tests. Correlation with the *in vivo* situation can only be assumed when validated test designs are used. In previous studies, the BUS model was accepted as an *in vitro* model for testing the skin compatibility of cutting fluids (5). Although it served for some time as a suitable test system, it also has some disadvantages such as the lack of standardisation and insufficient data for correlation with the *in vivo* situation.

Today, 3-dimensional cell cultures from human skin cells, so called skin models, have gained increasing importance as alternative to *in vivo* test systems (6). Due to great efforts of the cosmetic and raw material industry to find alternative methods to replace animal testing, the development of skin models has made fast improvements in the last years. The reconstituted human skin model, cultivated from human epidermal skin cells on a collagen matrix, was validated for the toxicological endpoint skin corrosion by ECVAM (European Centre for the Validation of Alternative Methods) (7). The validation study was performed in three independent laboratories and proved that this human skin model was able to correctly predict the corrosion potential of 12 OECD reference chemicals. The validation procedure led to a new OECD Guideline for the testing of chemicals for skin corrosion based on skin models (8). So far, three different skin models have been validated according to the OECD TG 431 as suitable test systems for skin corrosion: EPISKIN™, EpiDerm™ and SkinEthic™. Skin corrosion, defined as the production of irreversible tissue damage of the skin following the application of a test material, is certainly not induced by the application of metal working fluids. The damage to skin by long term contact with this product category is a mild irritation, with irritation being defined as the production of reversible tissue

Lube-Tech
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damage of the skin. However, to date no validated *in vitro* assay for testing and classification of skin irritation exists. However, in a pre-validation study by ECVAM for the testing of skin irritation and skin compatibility, human skin models turned out to be the most promising of several different *in vitro* methods (9). In addition to the investigations on alternative skin irritation models for classification purposes, several positive investigations on the testing of consumer products with low irritation potential on skin models also exist (10). In the experiments presented here we used an established *in vivo* study, the 24h Patch Test and an accepted *in vitro* test procedure on human skin models to test the skin compatibility of three cutting fluids. The aim was to find out whether these test systems are able to differentiate between the three test samples.

Experimental

Materials

Table 1: Summary of the characteristics of the products A, B and C

Product	Emusifier	Biocide	pH	Use concentration
A	I	low concentration	~ 9,2	5%
B	I	high concentration	~ 9,2	5%
C	II	low concentration	~ 9,2	5%

Cutting Fluids

Table 1 lists the cutting fluids referred to in this paper. Product A, B and C are based on formulations of the MULTAN® series (Henkel technologies). General information on fields of application, components, pH, and use concentrations are listed.

Skin Models

SkinEthic human epidermis model (RHE) and SkinEthic human cornea model (HCE) were purchased from SkinEthic Laboratories (Nice, France). On arrival, cultures were transferred to fresh culture medium and incubated overnight at 37°C before application of the test samples.

Methods

Treatment of skin models

A 30µl aliquot of test sample was applied directly onto the surface of the epithelial culture. Each product was tested in triplicates (minimum) against negative (water) and positive (0.5% or 1% SDS in water) controls.

Membrane Integrity Assay

Media were sampled at the specified time intervals and tested for lactate dehydrogenase (LDH) activity. (Cytotoxicity Detection Kit LDH, Roche Diagnostic Corporation, Mannheim, Germany).

24 h Patch Test

The test was carried out on 20 healthy female and male volunteers in a mixed panel representing the average spectrum of the normal skin type. Volunteers were informed about the aim of the study, test procedure, test substances, and any possible health risks or discomforts before the start of the study. Written consent was signed by all volunteers before participating in the study.
The test substances (5% dilution in demin. water) were applied side by side on the back of the volunteers over a period of 24 hours in a volume of 70ml under occlusive plasters (Finn Chamber on Scanpor, 12 mm). Any irritant effects observed were recorded and documented 6, 24, 48 and 72 hours after removal of the plasters, divided into erythema, edema, squamation and fissuration parameters according to the scale of Frosch (11). The resulting score values are evaluated numbers reflecting the strength of the individual reactions. Individual score values were summed for all recorded time points and divided by the number of volunteers. The resulting total irritation score was calculated for each test substance, for erythema and a parameter combination of erythema + edema + squamation + fissuration (shown in tables). SDS 0.5% and demin. water were used reference substances for this test. For statistical comparison of the visual scores the "area under curve" (AUC) was calculated. A Many to One Comparison of product A and product C versus product B was performed. The Friedman test was used to clarify whether there were significant differences between the groups. The significance level was $p<0.05$.

Results and Discussion

Three water soluble cutting fluids, A, B and C were analysed with regard to their skin compatibility in a 24h Patch Test and in skin models respectively. Differences in product formulas are listed in Table 1.

In the 24h Patch Test the positive control (0,5% SDS) led to a total irritation score of 9.16. The negative control demin. water induced a total irritation score of 0.37. Products A, B and C induced irritation scores between 1.05 and 1.47. The irritation score for erythema was between 0.68 and 1.05 for products A, B and C. All irritation scores and related standard deviations are shown in Table 2.

Table 2: Total irritation score, irritation score erythema and standard deviation found in the 24h Patch Test (number of volunteers n = 20).

Substance	Total Irritation Score	Standard Deviation	Irritation Score Erythema	Standard Deviation
Product A, 5%	1,47	2,57	1,05	1,87
Product B, 5%	1,05	1,65	0,68	1
Product C, 5%	1,16	2,01	0,84	1,46
Positive control: 0,5% SDS	9,16	4,35	6	2,94
Negative control: Aqua demin	0,37	1,38	0,26	0,93

The 5% product dilution used in the patch test resembles the recommended usage concentration for the products. At this concentration all products were well tolerated with irritation scores low above that of demin. water. Under these test conditions differences in skin compatibility between product A, B and C were not detectable. The Friedman test yielded no statistical significant differences in the score values of the three products. This finding corresponds with that of other investigators, who found patch tests on human volunteers insufficient to detect compatibility differences between well tolerated formulations (5).

In the *in vitro* tests, human epidermal skin models and human cornea models respectively, the products were tested with 5% and 10% product dilutions. Table 3 and Figures 1 and 2 show the LDH release in epidermis models after treatment with products A, B and C and with the positive control 1% SDS. An increase in LDH release indicates damage to the cell membrane leading to the leakage of enzymes from the cell. Not surprisingly, the 10% product dilution induced higher LDH-release compared to the 5% product dilution for all products tested. Clear differences between the three

Lube-Tech
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No.52

products in the amount of LDH release they induced were observed at both concentrations. At both concentrations product B induced the highest cell damage with product C producing the lowest.

Table 3: Mean values and standard deviation of LDH-release from epidermis models after treatment with products A, B and C in different concentrations. Values are given as % of negative control. Each value is a mean of four measurements.

	Mean value		Standard deviation	
Time [h]	5	15	5	15
Product A, 1%	32	161	7	22
Product B, 1%	26	162	7	14
Product C, 1%	26	162	5	50
Product A, 5%	161	632	100	93
Product B, 5%	170	729	59	36
Product C, 5%	77	534	22	96
Product A, 10%	558	1038	156	38
Product B, 10%	840	1359	58	132
Product C, 10%	206	728	45	132
Positive control: 0,5% SDS	n.e.	1098	n.e.	4
Negative control: Aqua demin	30	100	13	27

Figure 1: Course of LDH-release from epidermis models after treatment with products A, B and C in 5% product dilution. Values are shown as % of negative control (n=4).

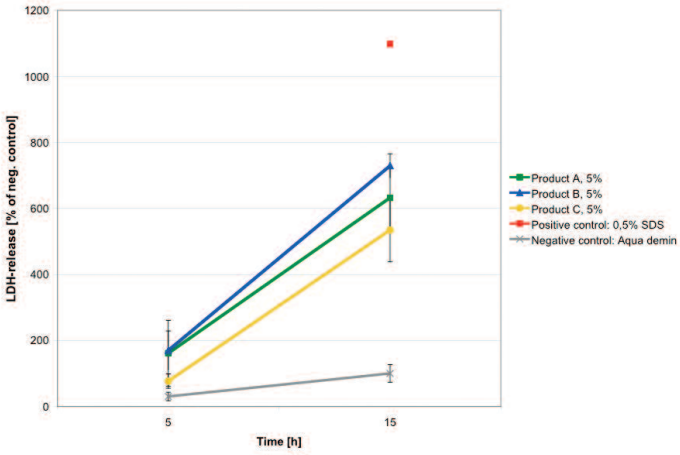
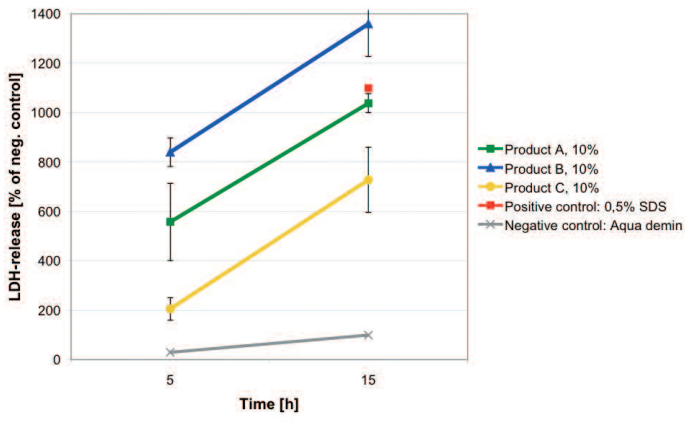


Figure 2: Course of LDH-release from epidermis models after treatment with products A, B and C in 10% product dilution. Values are shown as % of negative control (n=4).



A similar picture was seen after application of the products to cornea models. Since cornea models are more sensitive to chemical noxes, a shorter application time was chosen before measuring LDH in this test system. The data generated with cornea models is summarized in Table 4 and Figures 3 and 4. Again, as expected the 10% product dilution induced a higher LDH-release as a measure of cell damage compared to the 5% product dilution for all products tested. Clear differentiation between product A, B and C on cornea models was only seen after application of the products at 10% dilution. At 5% product dilution no significant difference was detected in the LDH released by the three products.

Table 4: Mean values and standard deviation of LDH-release from cornea models after treatment with products A, B and C in different concentrations. Values are given as % of negative control. Each value is a mean of four measurements.

	Mean Value			Standard Deviation		
Time [h]	1	2	3	1	2	3
Product A, 5%	179	396	998	35	52	151
Product B, 5%	143	367	699	42	30	255
Product C, 5%	161	467	515	25	91	189
Product A, 10%	291	929	2328	24	383	481
Product B, 10%	327	1589	3334	52	214	128
Product C, 10%	252	518	745	12	145	362
Positive control: 0,5% SDS	2936	n.e.	n.e.	151	n.e.	n.e.
Negative control: Aqua demin	100	n.e.	165	18	n.e.	20

Figure 3: Course of LDH-release from cornea models after treatment with products A, B and C in 5% product dilution. Values are shown as % of negative control (n=4).

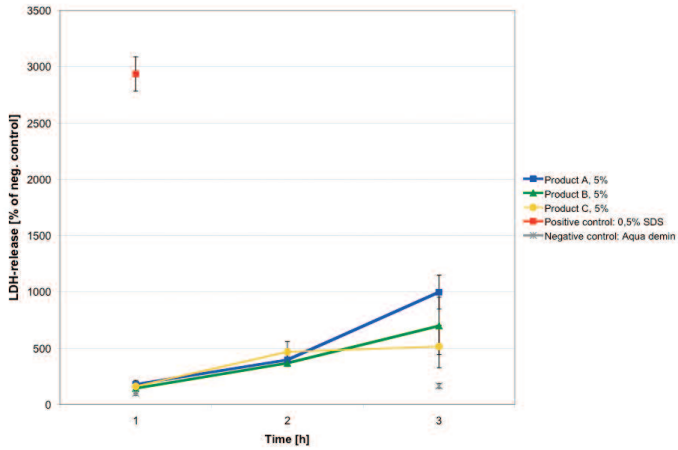
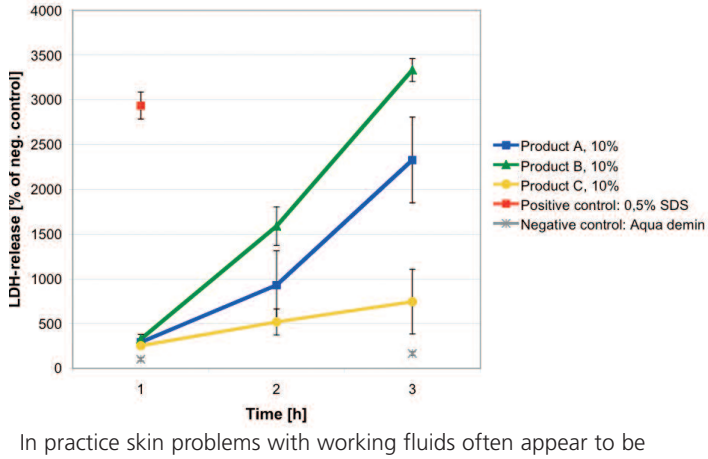


Figure 4: Course of LDH-release from cornea models after treatment with products A, B and C in 10% product dilution. Values are shown as % of negative control (n=4).



In practice skin problems with working fluids often appear to be due to contamination or chemical changes in the product during

Lube-Tech
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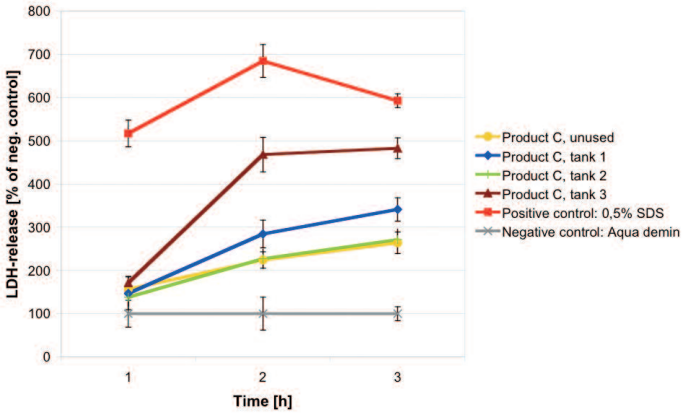
No.52

use. Testing used cutting fluids on human volunteers is untenable for ethical reasons since the precise composition of a used working fluid is not known. With *in vitro* systems, where such concerns do not exist, tests were performed in order to differentiate between used and unused cutting fluids. Product C was tested as a 5% product dilution on cornea models. In parallel, used samples of product C taken from different tanks in an Italian factory were tested. The results are summarised in Table 5 and Figure 5. Product from tank 1 and tank 2 induced a LDH release comparable or only slightly higher than that induced by the unused product C. In contrast product C from tank 3 induced a higher LDH release, heralding upcoming problems in skin compatibility.

Table 5: Mean values and standard deviation of LDH-release from cornea models after treatment with used and unused samples of product C in 5% product dilution. Values are given as % of negative control. Each value is a mean of four measurements.

	Mean Value			Standard Deviation		
Time [h]	1	3	5	1	3	5
Product C, unused	157	224	264	7	19	25
Product C, tank 1	147	284	341	38	32	27
Product C, tank 2	138	227	271	21	22	24
Product C, tank 3	171	468	483	15	40	24
Positive control: 0,5% SDS	517	684	593	31	38	16
Negative control: Aqua demin	100	100	100	48	37	32

Figure 5: Course of LDH-release from cornea models after treatment with used and unused products in 5% product dilution. Values are shown as % of negative control (n=4).



Conclusion

The data presented here shows that epidermis models and cornea models with cells of human origin are useful for investigating the skin compatibility of cutting fluids. Differentiation between metal working fluids with low irritation potential is possible. We propose the skin models as test method that is able to differentiate the skin compatibility of products that are indistinguishable from another and scored as having good skin compatibility in human 24 h Patch Tests. These *in vitro* tests are particularly suitable for comparing different formulations, e.g. in the development of new products. Used cutting fluids can be tested in these models without any ethical concerns.

We can assume that the differentiation in the *in vitro* method is relevant under the extensive skin contact of the cutting fluids in working conditions. More experience is needed to define the limit of LDH release below which a cutting fluid can be considered skin compatible. In addition, due to the model nature of *in vitro* tests an additional human *in vivo* test should be performed for each new product to verify the absence of any irritancy potential.

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