Comparative study of the skin tolerability of hand disinfectants using the BUS model

Summary

Background: No objective in vivo method has yet been established for evaluating the skin compatibility of hand disinfectants, in particular with respect to a realistic, comparative, objective and practically relevant test of the two important constituents of potential skin irritation, cell irritancy and cytotoxicity. Therefore the standardized in vitro BUS (bovine udder skin) test system was implemented, enabling experiments with a mature, viable skin with intact skin barrier and reservoir functions.

Method: In the BUS skin compatibility test five approved pharmaceutical formulations of hand disinfectants (coded 01-01, 01-03, 01-05, 01-06 und 01-07) were openly applied to the skin. Distilled water was used as negative control and SLS 10% as positive control. Full-thickness skin biopsies were taken from treated as well as from untreated skin after three exposure times (30 min, 1 h and 5 h). These biopsies were then biochemically analysed with regard to their inflammatory (prostaglandin E2 concentration) and their cytotoxic (MTT-assay) potentials. In order to determine skin compatibility, the total score was computed from the sum of cytotoxicity and cell irritancy values.

Results: Regarding the irritant potential of the hand disinfectants no significant differences between the formulations were observed at any exposure time. Also, there were no differences in the cytotoxic potential after 30 minutes of exposure. However, after 1 h and 5 h of exposure, significant formulation-specific differences were found. The positive control SLS 10% and also three of the hand disinfectants reached a skin-critical score of 3.0 at one exposure time (1 h: 01-07, 5 h: 01-06) or at two exposure times (1 h and 5 h: 01-05). In contrast, neither the formulations 01-03 and 01-01 nor the negative control reached a critical cytotoxic level of a total score of 3.0.

Conclusion: The significantly better skin compatibility of the formulations 01-03 and 01-01 could possibly be explained by the lower total alcohol concentration but is primarily achieved through the use of an effective moisturizing and lipogeneous agent systems.

Introduction

Besides proof of the microbicidal efficacy of hand disinfectants, good skin compatibility is an important criterion for compliance with hand hygiene [11]. Hence the “Save lives: clean your hands” campaign organized by the World Health Organisation (WHO) also uses subjective evaluation methods to investigate compliance and acceptance of hand disinfectants [2].

Whereas a precise method has been defined for furnishing proof of the efficacy of a hand disinfectant, no test method has been explicitly stipulated to demonstrate compatibility, i.e. to exhibit an acute skin reaction or a reaction induced by repeated contact. To date there is no method known that allows parallel and comparative test-
ing, and quantification, of the skin compatibility of different formulations.

In vitro models represent the quality of the horny layer determines the degree and extent of the skin reaction [4]. In 1994, based on the existing scientific data the European Centre for the Validation of Alternative Methods (ECVAM) Workshop XIII, with German co-authorship, defined a ranking order between in vivo testing (humans) and other skin models [5]. The authors placed the “perfused skin” immediately after the in vivo situation and before full-thickness or partial-thickness skin models, since perfused skin models per se are viable (Figure 1). In the present study the isolated perfused Bovine Udder Skin (BUS) model was used for objective evaluation of the skin compatibility of hand disinfectants. This offers the advantage of ethical acceptance, practicability (open application) combined with the economic benefits of an in vitro method as well as good applicability to the in vivo situation thanks to the use of a viable horny layer endowed with active, aerobic skin metabolism.

In general, hand disinfectants are presented as ready-to-use, alcohol-based solutions that are intended for repeated use in hand hygiene (hygienic and surgical hand disinfection). In Germany, they are placed on the market as medical devices and in the course of the licensing process are subjected to clinical and dermatological expert testing. However, often this does not include the biochemical parameters for skin compatibility, such as skin irritation and damage. Therefore, one aspect of this study was to investigate whether good efficacy of hand disinfectants was equated with deterioration of these parameters in the BUS model [3].

A chemical product or formulation that elicits no perceptible cellular reaction of the skin can be designated as being skin compatible. However, one must bear in mind that it is only in the viable epidermis and dermis after penetration of the horny layer that metabolizing, and possibly a skin reaction, can be induced. Experimental demonstration of skin irritation calls for, inter alia, invasive test methods which, for ethical and technical reasons, are not at all, or only to a very limited extent, possible under in vivo conditions. Hence it is of paramount importance to choose a suitable skin model, as in our case the BUS model.

Materials and Methods

BUS model

The isolated perfused Bovine Udder Skin (BUS) model was used to investigate the skin compatibility of hand disinfectants [6]. The BUS model was introduced into the experimental dermatology and pharmacology setting by Kietzmann et al. in 1993 to investigate the skin penetration and compatibility of drugs and active ingredients, without having to resort to animal experiments [7]. This ex vivo method has been adapted for use in the chemicals and cosmetics industry (skin compatibility, penetration) as well as for occupational skin protection [8–13].

In the BUS model bovine udders are isolated immediately after slaughter) and then continuously perfused via the left and right external pudendal arteries with an oxygenated nutrient solution to maintain aerobic metabolism as well as the barrier and reservoir function of the naturally integrated and viable udder skin (Figure 2a). As such, the BUS model is directly comparable with living skin. In this way, the udder and udder skin can be kept viable for more than eight hours.

The functional state of the BUS model includes transepidermal water loss (TEWL; water loss via the skin) at a rate that is on a par with that exhibited in the in vivo situation [14]. Interactions between a test substance and the horny layer, as may be occur in TEWL-based test methods, are ruled out in the BUS model due to the fact that...
full-thickness skin biopsies are used. The two components of skin irritation, i.e. cell irritancy and cell damage, can be biochemically determined – as a function of the absorption of the test substance by the horny layer [6].

The horny layer, as the outermost boundary layer, does not overall serve as an absolute barrier since it must assure water loss (approximately 85 % in the epidermis and 15 % in the horny layer) in order to be able to function as a reservoir and barrier endowed with a particular moisture content. Besides, there are the openings for the skin appendages (hair, sebaceous and sweat glands), the most notable being the hair follicle (Figure 2b). Hence a certain amount may always penetrate [15].

Topical application of a test substance is followed by direct transcellular penetration of hydrophilic components, during and after evaporation, as well as by rather slow intercellular penetration of lipophilic components. This property of the horny layer is demonstrated for the BUS model (Figure 2b).

The use of the BUS model to investigate penetration of a rinse-off product (product applied for a limited time, e.g. shampoo) showed that penetration takes place very rapidly and in a well-differentiated manner [11].

Further testing of leave-on products (products left for an unlimited time e.g. hand disinfectants) confirm that fact. The natural, mature horny layer reacts to specific formulations of constituent substances [8, 9, 12]. Therefore the BUS model is eminently suitable for investigation of ready-to-use products.

Conversely, no such distinctions can be made in skin cell cultures and 3D skin models of varied configuration when emulsions are present since, inter alia, they have a very low physiological reservoir and barrier function and do not possess a moisture gradient. This in turn means that cell cultures are very sensitive to surfactants (e.g. 0.1 % sodium lauryl sulphate), emulsifiers, alcohols, biocide products and to problems related to contaminated products.

Test products
In the standardized BUS compatibility test, five different disinfectants, licensed as medical devices, were tested, encoded as 01-01, 01-03, 01-05, 01-06 and 01-07 and applied undiluted (Table 1). Distilled water was used as negative control, and sodium lauryl sulphate (SLS; SLS p.a., L-4509, Sigma-Aldrich), which had been diluted with tap water to 10 %, was used as positive control.

Standard test plan for BUS skin compatibility test
Skin compatibility of hand disinfectants was tested using a standardized test plan, which has also been used in studies for other medicinal, cosmetic and biocide products (Table 2). Here, the test substances were openly applied in each case with a glass spatula (quantity: 2 g/100 cm²) and the skin areas kept moist for 5 min. Onset of cell damage and cell irritancy is a function of current penetration through the horny layer. Therefore the BUS compatibility test was designed in a standardized manner over three exposure times (30 min, 1 h and 5 h). After expiry of the exposure time full-thickness skin biopsies were taken with a 6 mm diameter (skin punches, manufacturer: Stiefel, Offenbach). As a control, full-thickness skin biopsies of untreated skin were taken after 30 min, 1 h and 5 h. From the full-thickness skin biopsies the biochemical parameters were then determined for cell irritancy and cell damage.

Determination of parameters for skin irritation
Onset of skin irritation following product application is marked by activation of skin tissues and reversible structural changes to the cell membrane (cell irritancy). The final stage of this process is irreversible cell damage (cytotoxicity). Determination of the test parameters for cell damage (MTT) and cell irritancy (PGE₂) is a standard feature of in vitro methodology and the relevant details have been published [6, 16, 17].

Cell damage
Cell damage (cytotoxicity) is directly correlated with inactivation of mitochondria and was determined in the MTT assay. The underlying principle of this test derives from the fact that only undamaged mitochondria in viable cells of the epidermis and dermis are able to convert the yellow water-soluble tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into the blue-violet water-insoluble formazan dye. Formation of formazan is directly proportional to the number of viable cells. The loss of mitochondria, i.e. of cells, is irreversible, hence progression over three measuring points can at most come to a halt.

Cell irritancy
To determine cell irritancy, the tissue concentration of the proinflammatory mediator prostaglandin E₂ (PGE₂) is measured. Increased formation of PGE₂ is driven by the proinflammatory potential of the applied test substance. Following activation of phospholipase A₂, arachidonic acid me-
in vivo to vascular dilation and in-
to swell-
leads in vivo to vascular dilation and in-

The data obtained from the MTT test and PGE₂ determination for treated skin were compared with those related to untreated skin (=100 %) and expressed as a percen-
tage. The results (Column 1 for the MTT assay, Row 1 for PGE₂ tissue concentration) are correlated with a “score value” (Column 2 for the MTT assay, Row 2 for PGE₂ tissue concentration). The total score (MTT + PGE₂) is composed of the individual score values and set against a bright blue or bright grey background. A total score of approximately 3.0 is classified after repeated contact as “skin critical” and as “incompatible” and is set against a bright grey background.

Table 4: Differences (expressed as percentage (%) ± standard deviation) in MTT and PGE₂ between “treated” and “untreated” skin after expo-

<table>
<thead>
<tr>
<th>Test substance</th>
<th>MTT</th>
<th>PGE₂</th>
<th>MTT + PGE₂</th>
<th>MTT</th>
<th>PGE₂</th>
<th>MTT + PGE₂</th>
<th>MTT</th>
<th>PGE₂</th>
<th>MTT + PGE₂</th>
</tr>
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<tbody>
<tr>
<td>01-01</td>
<td>-6.0 ± 2.2 [0.3]</td>
<td>0.1 ± 4.1 [0.0]</td>
<td>[0.3]</td>
<td>-10.0 ± 1.9 [1.0]</td>
<td>16.7 ± 4.4 [1.3]</td>
<td>[2.3]</td>
<td>-13.1 ± 0.3 [1.6]</td>
<td>8.2 ± 5.4 [0.3]</td>
<td>[1.9]</td>
</tr>
<tr>
<td>01-03</td>
<td>-5.2 ± 3.4 [0.3]</td>
<td>-2.7 ± 7.7 [0.0]</td>
<td>[0.3]</td>
<td>-10.2 ± 2.2 [1.0]</td>
<td>16.2 ± 6.6 [1.3]</td>
<td>[2.3]</td>
<td>-12.5 ± 2.8 [1.5]</td>
<td>3.3 ± 1.8 [0.0]</td>
<td>[1.5]</td>
</tr>
<tr>
<td>01-05</td>
<td>-1.9 ± 4.6 [0.2]</td>
<td>1.9 ± 2.7 [0.0]</td>
<td>[0.2]</td>
<td>-13.8 ± 2.7 [1.8]</td>
<td>14.1 ± 10.5 [1.3]</td>
<td>[3.1]</td>
<td>-23.3 ± 1.7 [3.7]</td>
<td>6.7 ± 5.2 [0.2]</td>
<td>[3.9]</td>
</tr>
<tr>
<td>01-06</td>
<td>-7.0 ± 3.8 [0.5]</td>
<td>1.0 ± 4.5 [0.0]</td>
<td>[0.5]</td>
<td>-9.5 ± 2.6 [0.9]</td>
<td>14.4 ± 5.8 [0.9]</td>
<td>[1.8]</td>
<td>-25.9 ± 4.4 [4.2]</td>
<td>5.4 ± 7.3 [0.2]</td>
<td>[4.4]</td>
</tr>
<tr>
<td>01-07</td>
<td>-8.7 ± 2.8 [0.7]</td>
<td>-2.3 ± 2.3 [0.0]</td>
<td>[0.7]</td>
<td>-15.5 ± 1.4 [2.1]</td>
<td>14.7 ± 1.3 [0.9]</td>
<td>[3.0]</td>
<td>-16.3 ± 0.9 [2.3]</td>
<td>2.8 ± 3.3 [0.0]</td>
<td>[2.3]</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-3.7 ± 2.6 [0.1]</td>
<td>0.5 ± 1.8 [0.0]</td>
<td>[0.1]</td>
<td>-3.8 ± 2.4 [0.1]</td>
<td>1.4 ± 1.8 [0.0]</td>
<td>[0.1]</td>
<td>-6.2 ± 2.0 [0.3]</td>
<td>1.4 ± 6.2 [0.0]</td>
<td>[0.3]</td>
</tr>
<tr>
<td>SLS 10 %</td>
<td>-15.9 ± 6.9 [2.2]</td>
<td>5.8 ± 4.3 [0.0]</td>
<td>[0.2]</td>
<td>-22.7 ± 5.2 [3.5]</td>
<td>18.7 ± 8.0 [1.8]</td>
<td>[5.3]</td>
<td>-32.1 ± 2.4 [5.4]</td>
<td>11.2 ± 6.3 [0.6]</td>
<td>[6.0]</td>
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Determinations of predictability

The results of the full-thickness skin punch-

The differences between the treated and untreated skin in % per parameter were also statistically evaluated using the univariate analysis of variance and Fisher’s LSD (least significant difference) test for multiple comparisons. The significance lev-
el in the Fisher’s LSD test for multiple com-
parisons was defined (p ≤ 0.05 (weakly sig-
nificant), **p ≤ 0.01 (significant), ***p ≤ 0.001 (highly significant). The statistical data refer to paired comparison of the for-
mulations 01-01 and 01-03 with all other

The early phase eicosanoids (products of arachidonic acid metabolism) initiate, drive and reinforce the inflammatory process un-
til, under in vivo conditions, inflammatory cells migrate into the tissues. The media-
tors released secondarily to PGE₂ underpin this process. Therefore the tissue concen-
tration of PGE₂ is a decisive parameter to begin measuring the cellular, inflamma-
tory and reversible reaction [18]. Elevation of the PGE₂ concentration in the tissue leads in vivo to vascular dilation and in-
creased permeability, and in turn to swelling, tension and reddening of the skin (ery-
thesma).
hand disinfectants tested as well as with distilled water and 10 % SLS.

**Results**

**Cell damage and cell irritancy**

Evaluation of the full-thickness skin biopsies of untreated skin showed regular perfusion for all isolated perfused bovine udders. The physiological conversion rate for formazan in the MTT test as well as the PGE₂ tissue concentration corresponded to the historic controls i.e. in all cases the entire udder, including the skin, was maintained in a viable state by perfusion with oxygenated, heated Tyrode’s solution (100 ml/min/side) (data not illustrated).

As regards the treated skin, no change in PGE₂ tissue concentration was seen after 30 min (Table 4, Figure 3). Maximum cell irritancy occurred in general after 1 h, and with reversibility noted after further exposure over 4 h. For the hand disinfectants tested, the maximum difference versus the untreated skin ranged between 14 % and 17 %, and in most cases was completely reversible after a further 4 hours. Reversibility was least pronounced after application of the positive control. No differences were seen in the test results for skin treated with the negative control. As regards the PGE₂ tissue concentration, i.e. onset of inflammation and erythema, there were no significant differences between the various hand disinfectants. However, a significant difference was observed after 30 min and 5 h in the paired comparison for formulations 01-01 and 01-03 with the positive control, and after 1 h a significant difference versus the negative control (Figure 3) was observed.

The results for the property of the various hand disinfectants implicated in cell damage revealed marked formulation-specific differences after 1 h and 5 h (Table 4, Figure 4). Application of the negative control did not give rise to any significant differences, whereas application of the positive control induced, as expected, severe progressive cytotoxicity from the start of application. For all hand disinfectants, the cell-damaging potential after 30 min was still less than 10 %. The positive control penetrated especially rapidly because of changes to the horny layer, going on to cause 15.9 % moderate cell damage already after 30 min. Only after an exposure time of 1 h were marked differences demonstrated between the formulations. After 30 min this difference, amounting to almost 9 %, was highest for 01-07, and lowest for 01-05 (< 2 %).

The results for 01-05 and 01-07 progressively exceed the 10 % mark. But whereas for 01-07 cytotoxicity did not progress any more after a further 4 h, the 01-05 application damaged more than one out of every five skin cells (23.3 %). That mark was exceeded only by 01-06 (25.9 %) as well as by the positive control (32.3 %). The differences for 01-01, 01-03 and 01-06 were highly significant (Figure 3).

**Figure 3**: Difference in PGE₂ tissue concentration between treated and untreated skin, including the statistical result. The statistical data refer to paired comparisons of formulations 01-01 and 01-03 with all other hand disinfectants tested as well as with distilled water and SLS 10% (*p ≤ 0.05 (weakly significant), **p ≤ 0.01 (significant), ***p ≤ 0.001 (highly significant)).

**Figure 4**: Difference in MTT concentration between treated and untreated skin in %. The statistical data refer to paired comparisons of formulations 01-01 and 01-03 with all other hand disinfectants tested as well as with distilled water and SLS 10% (*p ≤ 0.05 (weakly significant), **p ≤ 0.01 (significant), ***p ≤ 0.001 (highly significant)).
after the 1 h exposure time was around 10%. After a further 4 h pronounced differences were discerned between these hand disinfectants. The difference in progression for 01-01 and 01-03 versus the control continued to be markedly less than 15% and significantly less cell damage was observed compared with the other hand disinfectants and the positive control.

Predictability

Figure 5 illustrates the individual and total score values listed in Table 4 for the various hand disinfectants, distilled water (negative control) and 10% SLS (positive control). Cell damage and cell irritancy developed independently of each other: after 30 min the potential of certain formulations to inflict cell damage becomes clear, and additionally the inflammatory potential after 1 h. No positive correlation was seen between these two parameters in the ensuing course either.

As regards cell irritancy, essentially no differences were discerned between the hand disinfectants: whereas the inflammatory potential of the products was not detectable after 30 min, it reached its maximum peak 1 h after application and was reversible after a total of 5 h.

However, the skin-critical mark of a total score of approx. 3.0 was reached or exceeded by not only the positive control, but also by hand disinfectants 01-07 and 01-06 at one exposure time (1 or 5 h) and by 01-05 at two exposure times (1 h and 5 h). After 1 h both components of skin irritation, were contributing to the critical total score, whereas after 5 h only the cell-damaging potential did so.

The total score of 3.0 was not exceeded by either of the two formulations 01-01 and 01-03 or by the negative control.

Discussion

In addition to the scientific approach, the tried and tested biochemical methods applied to determine irreversible cell damage and reversible inflammation, equally important is the issue of predictability, i.e. how relevant are the results in terms of their extrapolation to medical practice. To answer that, the differences noted were expressed as score points. The total score value (MTT+PGE₂) is calculated from the sum of both individual score values [17].

Each instance of skin irritation begins after penetration through the horny layer with (biochemically) measurable cellular reactions. The absorption and toxicity potential of the formulation constituent substances or chemicals determine, as a function of the internal and external exposure conditions, the ensuing course. If the extent of cell damage and/or cell irritancy is too great and complete regeneration is possible only to a limited degree, the entire range of symptoms as reflected in the diagram according MALTEN are manifested, depending on individual factors, despite the fact that the single insults implicated were subclinical (Figure 6) [19, 20].

Based on the, in the meantime, more than 15 years’ experience gleaned from laboratory studies, market and operational applications, the three exposure periods are sufficient to achieve reliable predictability. There is no probability of skin irrita-
tion after repeated application up to a skin-critical total score of approximately 3.0, but the profile of the partial score values should be taken into account.

A functional horny layer in the skin model is a precondition for this evaluation and applicability. This means that it must be possible to realistically measure in the viable skin layers the toxicological potential under rinse-off and leave-on conditions (limited time and unlimited time periods) for hydrophilic and lipophilic constituents. Only if this is assured can constituents with recognized lipid replenishing or protective properties be detected under in vitro conditions as being effective – to that effect, they must establish a physiological connection to the horny layer. Since practical relevance is called for when testing hand disinfectants, the test plan was based on open application, keeping wet for 5 min and a very short (30 min), medium and prolonged (1 h and 5 h) exposure time.

Products 01-01 and 01-03 are declared by their respective manufacturers to possess a balanced formulation with relatively low alcohol concentration and a lipid replenishing system that has proved itself in the cosmetics industry. The latter is active in particular in the horny layer and makes an important contribution to the positive skin compatibility. Since we do not have detailed information on the other constituents in products 01-07, 01-06, 01-05, it is not possible at this juncture to engage in any discussion of the reasons for the different performances of the formulations.

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**Remark**

This article is based on a lecture “Comparative study of the skin compatibility of anti-
sptic formulations using the isolated per-
fused udder model (BUS model), method, results and evaluation” (11th German Soci-

ey of Hospital Hygiene (DGKIH) Congress, Berlin 2012).

**Conflict of Interest**

The author Dr. rer. med. Claudia Hilde-

brandt is an employee of Antiseptica chem-

pharm. Produkte GmbH in Pulheim, Ger-

many. The author Dr. rer. nat. habil. Anke

Prinz is an employee of Dr. Schumacher

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