Skin barrier disruption by acetone – observations in a hairless mouse skin model

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Abstract
The stratum corneum (SC) comprises the main barrier function of the skin. To disrupt the skin barrier function, different in vivo methods have been established, e.g. by acetone wiping or tape stripping. In this study, the acetone-induced barrier disruption of hairless mice was investigated in order to establish a reliable model to study beneficial, long-term effects on barrier recovery after topical application. For this purpose we focused on morphological features and transepidermal water loss (TEWL) after acetone-induced barrier disruption of hairless mice. In comparison, the same features were examined after disrupting the barrier by tape stripping. For both treatments (i.e. acetone treatment and tape stripping) the TEWL directly after disruption and the subsequent barrier recovery profile were similar. Histological assessment showed significant lower number of corneocyte layers in acetone-treated and tape stripped skin compared to untreated skin, while there was no statistical difference between the two treatments. Lipid analysis revealed that predominantly nonpolar lipids were extracted by acetone. Importantly, the ratio of the barrier lipids, i.e. cholesterol, free fatty acids and ceramides, remained similar between control and acetone-treated skin. This reflects the undisrupted lipid organization, as determined by small-angle X-ray diffraction measurements: the long-periodicity lamellar phase was still present after acetone treatment. These results contradict earlier studies reporting no mechanical SC removal, a substantial extraction of lipids and disruption in lipid organization. In contrast, we observed clearly the removal of corneocytes, the presence of a lamellar lipid organization and only little extraction of lipids by acetone. In conclusion, our results demonstrate that acetone treatment is a less defined method to induce barrier disruption compared to tape stripping.

Introduction
The uppermost layer of the epidermis, the stratum corneum (SC), comprises the main barrier function of the skin. The SC is composed of protein-enriched, non-viable corneocytes embedded in a lipid-rich extracellular matrix. It has been established that the lipids account for the main epidermal permeability barrier [1, 2]. These lipids are organized in lamellar stacks with characteristic short and long periodicity phase (LPP) being 6 and 13 nm in size, respectively [3, 4]. Three main lipid classes participate in the formation of these stacks: cholesterol (CHOL), free fatty acids (FFA) and ceramides (CER).
To reduce the skin barrier function different in vivo models have been established. Commonly used laboratory techniques are removal of SC cell layers by sequential tape stripping, treating the skin with the surfactant sodium dodecyl
sulphate or wiping the SC surface with organic solvents such as acetone [5-8]. Typically, these studies are combined with monitoring the skin barrier (recovery) by measuring the transepidermal water loss (TEWL). To gain more insight into the mechanism of the barrier perturbation with acetone, additional methods to TEWL measurements were applied such as analysis of the SC lipids to monitor the extraction of the lipids [9]. To visualize remaining lipophilic regions in the SC, fluorescence microscopy of Nile Red stained cross-sections [10] was employed and electron microscopy with RuO₄ post-fixation visualized the lipid organization in the SC [11]. The results of the applied techniques showed that after acetone treatment I) barrier lipids are removed to a substantial extent [9], II) virtually all Nile Red staining of nonpolar lipids is removed [10] and III) a reduced number of intercellular lamellae are observed [11].

Our aim is to establish a reliable barrier disruption method to study the long-term effects on barrier recovery after topical application of pharmaceuticals. In a previous study, we thoroughly investigated different disruption levels by tape stripping [12]. In most cases a very fast recovery (i.e. 3 days), as monitored by TEWL, was observed. Only when the highest degree of barrier disruption was applied, it took approximately 8 days to completely restore the skin barrier function. Aim of the present study was to evaluate the acetone-induced barrier disruption and its possible utilization to study long-term effects on barrier recovery. Barrier disruption and lipid extraction of hairless mouse skin was achieved by wiping with acetone-soaked cotton sticks, a method described in detail previously [6, 8, 10, 11]. After disruption, we examined the degree of skin barrier disruption (by TEWL), lipid composition (by high-performance thin-layer chromatography (HPTLC)), lipid organization (by small angle X-ray diffraction (SAXD)) and the morphology (confocal laser scanning microscopy and light microscopy). As comparison, the morphological features and TEWL of tape stripped skin were also explored.

Materials & Methods

Materials

Black D-squame tapes (rectangles from 70 mm x 25 mm) were obtained from CuDerm (Dallas, USA). Gelatin capsules were provided by Spruyt-Hillen (IJsselstein, The Netherlands). Tissue-Tek® O.C.T. Compound was obtained from Sakura Finetek Europe B.V. (Zoeterwoude, The Netherlands). Safranin O was purchased from Sigma (Schnellendorf, Germany). Absolute acetone (HPLC-grade) was supplied by J.T.Baker (Deventer, The Netherlands) and the cotton sticks were provided by Hartmann (Heidenheim, Germany). Nile Red was manufactured by Molecular Probes Europe BV (Leiden, The Netherlands).
Skin Barrier Disruption

Male hairless mice (SKH-hr1), 7-9 weeks old and 28 ± 2 g in weight, were purchased from Charles River Laboratories (St-Aubin-les-Elbeuf, France). All animal experiments were conducted in conformity with the Public Health Service Policy on use of laboratory animals and had been approved by the Research Ethical Committee of Leiden University. The mice were maintained in the animal care facility of the Gorlaeus Laboratories, Leiden University, with temperature- and humidity-controlled rooms and fed standard laboratory chow and tap water ad libitum. During anaesthesia [12] the skin was gently washed with deionised water. Subsequently, one of the flanks of the back was treated with approximately 10 acetone wipes until a TEWL of ~60 g/m²/h was obtained. One wipe consisted of rolling a delipidized, acetone-soaked cotton stick for 4 times back and forth over the skin surface. The contra lateral flank was left untreated. As reference for barrier disruption, sequential tape stripping in alternating direction of the stretched skin [12] was performed until a TEWL of ~60 g/m²/h was reached (7 strips). The level of barrier disruption was assessed by measuring the transepidermal water loss (TEWL) using a Tewameter TM 210 (Courage + Khazaka, Cologne, Germany). The TEWL of the disrupted skin sites was measured by holding the probe lightly against the test area until a constant TEWL value was obtained. Skin biopsies were taken directly after disruption from the sacrificed animals and processed for the different techniques.

Histology

Histological assessment was performed as described in a previous study [12]. In brief, the biopsies (n=3) were fixed in Tissue-Tek, frozen in liquid nitrogen and cut into 5 µm thick sections. These fresh-frozen cryostat sections were stained with 1% safranin solution and subsequently treated with 2% KOH solution to allow swelling of the corneocytes [13]. Digital pictures of the light-microphotographs were captured using a Carl Zeiss axioskop (Jena, Germany) and the average of the SC layers was determined from 20 random locations of the cross-sections. Statistical differences between the groups (i.e. untreated, acetone treated and tape stripped) were determined by an one-way ANOVA with a Bonferroni post-test.

In order to visualize the lipid matrix of the cross-sections, a staining method with the lipophilic dye Nile Red was performed. Prior to addition of a droplet Nile Red solution (2.5 µg/ml (w/v) in acetone) on the frozen sections [10], the SC was swollen by alkaline expansion (see above). Subsequently it was visualized by confocal laser scanning microscopy on a Bio-Rad Radiance 2100 system with a Nikon Eclipse TE2000-U (Nikon, Japan) inverted microscope.
Analysis of lipid composition and organization

For lipid analysis, the biopsies (n=4) were placed on cover slips and exposed for 3 min to 60°C. Subsequently, the epidermis was peeled off from the underlying tissue and extracted sequentially by different mixtures of chloroform/methanol according to a modified protocol of Bligh and Dyer [14]. Lipid composition was determined by HPTLC as described previously [15].

In order to determine the lipid organization, SC sheets of the biopsies (n=3) were obtained after overnight trypsin incubation as described for human skin [16]. Then, SAXD measurements were conducted at station BM26B at the European Synchrotron Radiation Facility in Grenoble, France [17]. The dried SC sheets were transferred in a special sample holder, which was subsequently mounted into the X-ray beam. The diffraction data were collected on a two-dimensional gas-filled area detector with a 1.5 m sample-detector distance at room temperature. The spatial calibration of this detector was performed using silver behenate and CHOL. The periodicity of the lamellar phase was calculated from the positions of the various orders diffraction peaks attributed to this phase using the equation $d=\frac{2n\pi}{q_n}$ with $n$ being the order of the diffraction peak and $q$ the scattering vector. The latter is defined as $q=\frac{4\pi\sin\theta}{\lambda}$ in which $\theta$ is the scattering angle and $\lambda$ is the wavelength of the X-rays.
Results & Discussion

Untreated hairless mouse skin exhibited a TEWL of 9.7 ± 1.5 g/h/m² whereas the TEWL after acetone treatment was 65 ± 10 g/h/m². In order to compare the two disruption methods, a similar barrier disruption was achieved by sequential tape stripping (TEWL of 66 ± 3 g/h/m²). The TEWL measurements of the barrier recovery showed similar profiles for both disruption methods and complete recovery was observed 3 days post-disruption (data not shown). The histological features of Nile Red stained cross-sections of the skin, obtained directly after disruption, are depicted in figure 1 and compared to untreated skin. Untreated skin clearly showed the presence of the lipophilic dye between the alkali-expanded corneocytes (Fig. 1A). Interestingly, a lower number of SC layers was discernable directly after acetone treatment. However, no changes in the Nile Red staining was observed, indicating that no alteration in the lipid distribution within the SC occurred (Fig. 1B). On the contrary, in previous studies it had been reported that the lipids were extracted from the SC by acetone while no damage of the SC was observed by microscopy [9, 10, 18]. Our light microscopic observations confirmed the reduced amount of corneocyte layers after acetone treatment (Fig. 1E) compared to untreated (Fig. 1D).

Figure 1. Different disruption methods cause abrasion of SC layers. Photomicrographs of frozen cross-sections of hairless mouse skin before (A), directly after disruption with acetone (B) and tape stripping (C) are shown. The lipophilic fluorescent dye Nile Red stains for nonpolar lipids. The KOH expanded corneocytes can easily be distinguished. Alkali expanded safranine stained cross-sections of mouse epidermis are depicted in panel D (untreated), E (acetone treated) and F (tape stripped).
Tape stripped skin (Fig. 1C and 1F) also exhibited a lower number of SC layers than untreated skin (Fig. 1A, 1D) while the lipid distribution was maintained. When quantifying the SC layers of both disruption methods, a similar trend was observed in the light photomicrographs: a significant reduced number of SC layers between untreated and both disruption methods could be detected (Fig. 2). This indicates that during the gentle acetone-wipe a significant amount of corneocytes was removed, which accounts for a substantial barrier perturbation. The cross-sections of tape stripped skin showed the trend to a more pronounced reduction of the SC thickness compared to acetone-treatment, however no significant difference between both treatments was determined.

After acetone treatment, the lipid composition in the acetone wipe and the remaining epidermis was explored. The lipid analysis of the epidermis by HPTLC is shown in figure 3A. The lipid extracts (from 10 mm²) of the untreated (lane 1) and acetone treated epidermis (lane 2) showed very similar lipid distribution: the nonpolar skin surface lipids, i.e. SE and TG are the predominant lipid classes. Although some lipids were removed by acetone (lane 3), the HPTLC technique was not discriminative enough to determine differences in the profiles of neither the nonpolar surface lipids nor the more polar barrier lipids (i.e. CHOL, FFA and CER) of the epidermis. Importantly, the composition of the barrier lipids of the control and acetone-treated site remained very similar (comparison of lane 1 and 2), as mainly nonpolar surface lipids were removed (lane 3). This observation is in contrast to previous studies reporting that barrier lipids were extracted by acetone-soaked cotton balls in substantial amounts [9].
This is of major importance as the barrier lipids play a crucial role for the SC permeability barrier [1,2]. Now, the question rises whether the SC lipid organization was disturbed. The results obtained with SAXD on isolated mouse SC are depicted in figure 3B. The upper two patterns (i.e. control and acetone-treated site) showed similar diffraction patterns: a very weak 1\textsuperscript{st} \((q=0.46 \text{ nm}^{-1})\) and a clear 2\textsuperscript{nd} \((q=0.94 \text{ nm}^{-1})\) and 3\textsuperscript{rd} order \((q=1.42 \text{ nm}^{-1})\) of the LPP with a repeat distance of 13.4 nm were discernable for this mouse. The 2\textsuperscript{nd} donor (lower two patterns) exhibited similar diffraction profiles. Although a slight donor-to-donor variation was observed, the higher order reflections clearly indicated the presence of the LPP with and without acetone treatment.

**Figure 3.** Only little changes in lipid composition and organization after barrier disruption with acetone. A HPTLC of lipid extracts from untreated mouse epidermis (lane 1), acetone treated epidermis (lane 2) and lipids extracted by the acetone wipe (lane 3) are depicted in panel A. The amount of lipids applied per lane, represent a skin surface of 10 mm\(^2\). Legend on the left indicates the \(R_f\) values of different compounds. SE/WE – sterol esters / wax esters, TG – triglycerides, CHOL – cholesterol, FFA – free fatty acids, CER – ceramides, acyl-GSL – acyl-glycosphingolipids, PE – phosphoethanolamine. The scattered intensity (arbitrary units) is plotted as function of scattering vector \((q)\) in panel B. SC sheets of two donors (upper two and lower two patterns) before (untreated) and after acetone treatment were directly mounted into the X-ray beam. The roman numerals indicate the various orders of the LPP: 1\textsuperscript{st} order (I) located at \(q=0.46 \text{ nm}^{-1}\) \((d=13.7 \text{ nm})\), 2\textsuperscript{nd} order (II) at \(q=0.94 \text{ nm}^{-1}\) \((d=6.7 \text{ nm})\) and 3\textsuperscript{rd} order (III) at \(q=1.42 \text{ nm}^{-1}\) \((d=4.4 \text{ nm})\).
Therefore, a very similar lipid organization was observed prior to and after acetone treatment. This is in agreement with the presence of the remaining barrier lipids in a similar composition after acetone treatment because the lipid composition is the determining factor for the lipid phase behaviour. However, the increased TEWL clearly indicates a perturbed barrier that can neither be explained by the absence of the characteristic lamellar phase nor by lipid extraction. Evidently, it might be attributed to a large extent to the removal of corneocytes. Compared to tape stripped skin, the acetone treated site showed a similar TEWL value and also a (slightly) reduced amount of SC layers (Fig. 2). The residual loss in the barrier property was presumably due to a change in the lipids: during wiping, (barrier) lipids partially dissolve in acetone. As acetone evaporates rapidly, the dissolved lipids may rearrange by re-crystallisation of the (barrier) lipids and lead to a possible formation of the LPP in little domains. The increased intensity of the 1st order (Fig. 3B, lowest pattern) could also be explained by this reformation of the LPP in little domains. We already demonstrated previously that the LPP can be formed after evaporation of solvents with distinct sample history, i.e. shear stresses and temperature [19].

In comparison with previous studies, the following differences were found concerning acetone-treatment: I) Virtually all Nile Red staining from the nonpolar lipids of SC was reported to be removed [10], while our confocal images showed a homogeneous distribution of this fluorescent probe within the SC (Fig. 1A and 1B). II) No removal of SC layers up to a TEWL of 40 g/m²/h was observed [11, 18], whereas we detected a significant reduced amount of SC layers at a TEWL of 60 g/m²/h (Fig. 2). III) The removal of the bulk lipids from the SC was shown to correlate directly with the degree of barrier perturbation [10], whereas in this study only little extraction of the lipids was observed (Fig. 3A). IV) A decrease in intercellular lamellae and consequently a lipid disorganisation was reported [11], while we could not detect changes in lipid organization by SAXD, which is a bulk method (Fig. 3B). The difference in the outcome can hardly be due to the barrier disruption method, as only a slight variation exists in the application method (cotton sticks vs. cotton swabs). However, the different results show that a clear definition of ‘gently wiping’ is of importance since also the mechanical wiping has influence on the skin barrier.

When comparing the acetone-induced barrier disruption with tape stripping, as methods for studying long-term effects after topical application of pharmaceuticals, the tape stripping offers several advantages: I) A clear correlation between cells removed and increased TEWL could be observed [12]. II) The number of tape strips can be defined beforehand, which is advantageous for the degree of barrier disruption under practical conditions. III) Although
different levels of acetone-induced barrier perturbation have been established [10], no slow, long-term recovery was reported. Although the acetone-induced barrier disruption might not be the most appropriate method to study barrier recovery after topical application, its suitability for other mechanistic investigations e.g. the epidermal lipid synthesis during barrier homeostasis [20] or the response of lamellar bodies upon barrier disruption [6] is inevitable. However, this short communication provides discussion points, which need to be considered when using the acetone-induced barrier disruption method. We found that the loss in barrier function is mainly due to the removal of corneocytes, whereas changes in lipid composition and organisation only play a minor role. Importantly, the ratio of the barrier lipids remained similar between control and acetone-treated skin. This provides also a basis for the explanation of the undisrupted lipid organization after acetone treatment. However, our study strongly indicates that due to evaporation of the solvent, barrier lipids might re-crystallize in the same structure, i.e. the LPP, as prior to the acetone-treatment.

In conclusion, acetone treatment predominantly extracts nonpolar lipids, while the ratio of barrier lipids and the lipid organisation remains similar. The removal of the corneocyte layers mainly accounts for the barrier disruption as was also observed for tape stripping. In future, the tape stripping method will be employed to investigate beneficial effects of topical applications on barrier recovery.
Observations in the acetone-induced barrier disruption model

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