

Effect of a lipid-rich emollient containing ceramide 3 in experimentally induced skin barrier dysfunction

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In the present study we compared the effect of a ceramide 3-containing emollient (Locobase® Repair) with a control emollient (vaselinum album/cremor lanette ana) and untreated damaged skin using clinical, bioengineering and immunohistochemical methods in two different models of experimentally induced skin barrier dysfunction. In model A ($n = 13$) skin barrier dysfunction was inflicted at three investigation sites by tape stripping. In model B ($n = 13$) the volunteers were patch tested at three investigation sites with sodium dodecyl sulphate (0.2%) for 4 h a day for 4 consecutive days. The investigation sites were treated once a day with the above-mentioned agents. Irritant reaction was assessed daily by erythema scoring and measurements of transepidermal water loss (TEWL). After 5D, punch biopsies were taken from all sites. Immunohistochemical assessment was carried out with respect to epidermal proliferation, epidermal differentiation and Langerhans cells. Tape stripping resulted in an erythematous reaction and an increase of TEWL associated with up-regulation of cycling cells, involucrin and expression of cytokeratin 16. At day 4, ceramide 3-containing emollient significantly decreased ($p < 0.03$) the erythema score, TEWL and cycling cells in comparison with the untreated site. Repetitive exposure to SDS induced a variable degree of erythema, gradual increase of TEWL, an increase of cycling cells, and up-regulation of involucrin, E-FABP and SKALP. The treatment with the control emollient significantly prevented erythema, increase of TEWL and cycling cells at day 4 compared to the untreated site. In summary, the present study demonstrated that both tested emollients improve skin barrier in different conditions compared to the untreated skin. There is some indication that formulations containing skin-related lipids might be of benefit in barrier disruption following tape stripping. Different models and clinical trials are needed to establish the usefulness in specific conditions of emollients containing skin-related lipids.

Key words: ceramides; emollients; skin barrier; skin lipids; sodium dodecyl sulphate; tape stripping. © Blackwell Munksgaard, 2002.

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Emollients have been used in the treatment of dry, scaly skin conditions for over 2000 years. Although we are starting to understand how emollients work, only a few trial data have been published demonstrating their efficacy (1–4).

Theoretically, emollients may act on three different levels of the epidermis:

- an immediate effect by adding a lipid mixture to the skin surface (occlusion);
- an intermediate effect by adding a lipid mixture to the intercellular spaces;
- a delayed effect by providing lipids to the epidermal cells, enabling these to enhance the natural lipid production and lipid release (5).

Most emollients act on the first two levels listed. An ideal emollient would be one that possesses all

three of these qualities. A promising approach in this respect may be the incorporation of lipids chemically related to the physiological content of the intercellular domain of the horny layer in a topical formulation.

Recently, findings on the composition of intercellular lipids and the effects of well-defined preparation of these lipids on the skin barrier have made this approach feasible.

Firstly, it was shown that the topical application of an equimolar three-component system of ceramides, cholesterol and fatty acid allows normal barrier repair in acetone-treated murine skin as compared to delayed barrier repair by lipids alone or by other combinations. In these animal studies it has also been demonstrated by fluorescence and electron microscopy that the mixture of cholesterol, fatty acid and ceramides rapidly

traversed the stratum corneum with subsequent uptake into the epidermal nucleated layers (6, 7). Secondly, it was shown that acceleration of barrier repair can be achieved if the relative contribution of any of these ingredients is increased (8).

Finally, it has been suggested that cholesterol, ceramides, palmitate and linoleate in the composition 4.3:2.3:1:1.08 is the optimal ratio for barrier recovery. This lipid formulation causes a markedly accelerated skin barrier recovery following barrier disruption in mice (9). To correlate animal data with human skin, different lipid formulations containing varying molar ratios of these lipids were tested in limited numbers of patients. In some combinations, a noticeable improvement in skin suppleness was observed (10).

These experimental studies have provided evidence that emollients containing certain combinations of key physiologic lipids may have therapeutic benefits in conditions involving skin barrier dysfunction (11). However, studies that document the efficacy of such lipid compositions in barrier disturbance in humans either clinically or in models are scarce. The limited number of available human studies using bioengineering methods to assess barrier function are contradictory (10, 12–17).

In the present study we investigated the efficacy of a ceramide 3-containing emollient (Locobase® Repair). It is claimed to be a formulation which contains an optimal composition of skin-related lipids in barrier recovery. We studied this claim by comparing Locobase Repair with vaselinum album/cremor lanette I ana (control emollient) and untreated damaged skin in barrier recovery capacity. We investigated skin barrier function by measuring transepidermal water loss. We also performed clinical erythema scoring and (immuno)histological studies of experimentally induced skin barrier dysfunction. The skin barrier dysfunction was inflicted by tape stripping or exposure of the skin to a low concentration of sodium dodecyl sulphate (SDS) in a cumulative exposure model.

The beneficial effects of both emollients using clinical, bioengineering and immunohistochemical methods were observed depending on the type of injury.

Materials and Methods

Participants

A total of 26 healthy volunteers without past or present history of skin diseases (11 male, 15 female, mean age 38 years, range 20–61) were included in the study. Written informed consent was obtained from all participants and the study was approved by the local Ethics committee. The study was conducted in January 2000.

Methods

Experimentally induced barrier dysfunction was studied in two different models:

- (A) single tape stripping and
- (B) repetitive exposure to low concentrations of sodium dodecyl sulphate (SDS) 0.2% g/v.

Model A: mechanical irritation by single tape stripping. On baseline (day0) a plastic template with 3 windows (2.5 cm diameter) was applied to 3 sites of the lower back of 13 participants. The exposed areas were at least 2.5 cm apart. Adhesive tape (TesaFilm®) was applied with gentle pressure to the exposed areas (sites 1, 2, 3) and then removed. This procedure was repeated until the skin was glistering.

Model B: repetitive exposure. In all, 13 participants were patch tested with 0.2% SDS once daily for 4 h on each of 4 consecutive days from Monday to Thursday (D0–3). 200 µL of SDS was applied to our self-made patches consisted of a 1.5-cm² piece of absorbent, non-woven fabric fitted into a flat round silver chamber with an internal diameter of 30 mm. The chambers were applied on the three sites of the lower back (min. distance 2.5 cm) and held in place with tape (Fixomull® stretch, Beiersdorf AG, Hamburg, Germany). After the removal of all SDS chambers, the skin was washed with cold running water and gently patted dry with a paper towel.

Topical application of the emollients. The exposed areas were treated once daily for 7D in model A and for 5D in model B according to the following scheme:

Site 1: control emollient (vaselinum album/cremor lanette I ana, Dutch pharmacopoeia).

Site 2: untreated.

Site 3: ceramide 3-containing emollient (Locobase Repair).

Evaluation

Clinical assessment. Erythema was evaluated by the investigator daily according to the scoring system: 0, no response; 1, slight, patchy redness; 2, diffuse mild redness; 3, moderate redness; 4, intense redness.

Transepidermal Water Loss (TEWL) measurements. TEWL was measured by TEWAMETER TM 210 (Robinson Electronic Ltd.) according to the guidelines from the Standardization Group of the European Society of Contact Dermatitis (18).

Measurements were performed following a waiting period of 30 min. Measurements were taken more than 12 h after application of the emollients. Room temperature varied from 19°C to 22°C and relative humidity from 40% to 50%.

Model A: measurements were performed at day 0 – before (baseline TEWL) and at various moments after tape stripping: postexposure (day0), and 24 h (day1), 48 h (day2), 96 h (day4), 168 h (day 7) after barrier disruption.

Model B: measurements were performed at day 0 – before SDS exposure (baseline TEWL), after 24 h (day1), 48 h (day2), 96 h (day4). The measurements at 24 h, 48 h and 96 h were performed before the following exposure of SDS.

Biopsy procedures

Model A. 24 punch biopsies (3mm) were taken under local anaesthesia at 48 h (day2) and 96 h (day4) after tape stripping from all investigated sites of 8 participants. The biopsy specimens were embedded in Tissue TEK II OTC compound, immediately snap-frozen in liquid nitrogen and stored at -80°C until further use. Sections of $6\text{ }\mu\text{m}$ were cut and fixed during 10 min with acetone-ether (60/40 vol.%) for the MIB-1 staining and for the other staining with cold acetone only.

Model B. 24 punch biopsies (3mm) were taken under local anaesthesia at 96 h (day 4) after repetitive application of SDS from all investigated sites of 8 participants. After 24 h fixation in formalin, the samples were embedded in paraffin, sectioned at $6\text{ }\mu\text{m}$ and prepared for staining.

Cell biological methods

Biopsy sections were evaluated light microscopically using haematoxylin-eosin staining and immunohistochemical stainings. For the immunohistochemical stainings, a panel of monoclonal and polyclonal antibodies dependent on model was used.

Model A. To assess epidermal proliferation, an antibody directed against the Ki-67 antigen was used (MIB-1, 1:50, Immunotech, SA, Marseilles, France). To assess epidermal differentiation, monoclonal antibodies against involucrin (MON-150, 1:15, Department of Clinical Chemistry, St. Elisabeth Hospital, Tilburg, the Netherlands) and monoclonal antibody against cytokeratin 16 (LL025, 1:10, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) were used. Using monoclonal antibodies DAKO-T6 (1:100, DAKO A/S, Denmark), analysis of the presence of Langerhans cells was done. For all monoclonal antibodies, an indirect immunoperoxidase technique was used.

Model B. All markers except the marker for Langerhans cells were used. Besides that, we used two other differentiation markers: a polyclonal antibody against skin-derived-antileukoproteinase

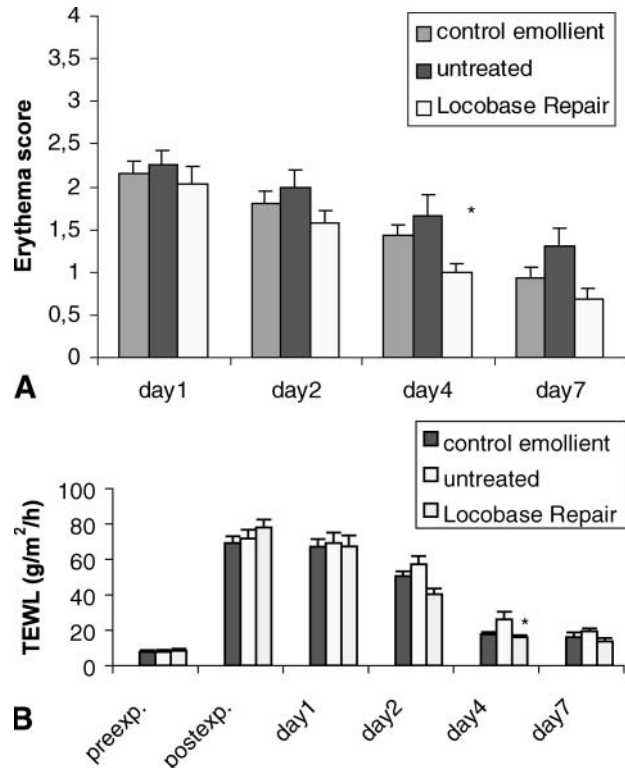


Fig. 1. a: Erythema score in tape stripping model. Significant difference between the site treated with Locobase Repair vs. the untreated site ($p < 0.03$). b: TEWL measurements ($\text{g/m}^2/\text{h}$) in tape stripping model. *Significant difference between the site treated with Locobase Repair and the untreated site ($p < 0.03$) and Locobase repair vs. control emollient ($p < 0.03$).

(anti-SKALP, 1:500) and a polyclonal antibody against epidermal-fatty-acid-binding-protein (anti-E-FABP, 1:100). For all antibodies, except for MIB-1 and anti-E-FABP, an indirect immunoperoxidase technique was used. An avidin-biotin immunoperoxidase technique was carried out for MIB-1 and anti-E-FABP.

Statistical analysis

The differences were analysed by Student's *t*-test for TEWL and by Wilcoxon's test for erythema score and immunohistochemical scoring.

$p < 0.05$ was chosen as the level of significance.

Results

Clinical response and TEWL measurements

Tape stripping of human skin resulted in an erythematous reaction. After tape stripping, TEWL strongly increased and then gradually decreased to day7. The time course of the visual grading of erythema and TEWL measurements is shown in Fig. 1(a) and Fig. 1b, respectively. The erythema score and TEWL value were significantly decreased ($p <$

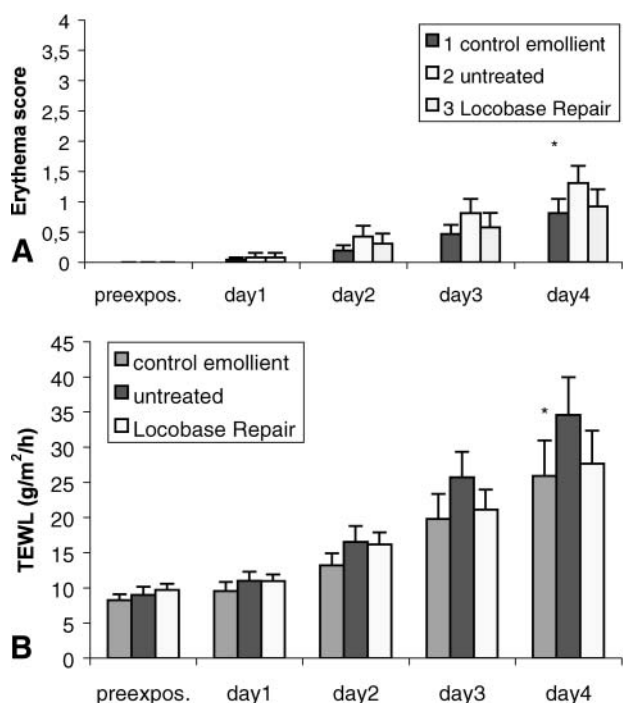


Fig. 2a: Erythema score in model of repetitive application of SDS (0.2%). *Significant difference between the site treated with control emollient and the untreated site ($p < 0.03$). b: TEWL measurements in model of repetitive application of SDS (0.2%). *Significant difference between the site treated with control emollient and the untreated site ($p < 0.05$).

0.03) at day4 on the site treated with ceramide 3-containing emollient but not on the site treated with the control emollient.

Repetitive exposure of human skin to SDS induced a variable degree of erythema. The erythematous response increased in intensity up to day4 in 9 participants (Fig. 2a). No strong erythematous reactions were observed. 4 participants did not have any erythematous reaction at all. However, a gradual increase of TEWL values with a maximum at day4 was observed in all participants (Fig. 2b). Treatment with both the ceramide 3-containing emollient and the control emollient prevented erythema and the increase of TEWL values. The treatment with the control emollient significantly prevented erythema and increase of TEWL value ($p < 0.05$) at day4 compared to the untreated site.

Immunohistochemistry

Epidermal proliferation. We observed an increase in the number of cycling cells at 48h and 96h after tape stripping at all sites (Fig. 3a). At the site treated with ceramide 3-containing emollient the number of cycling cells was significantly reduced in comparison to the untreated site.

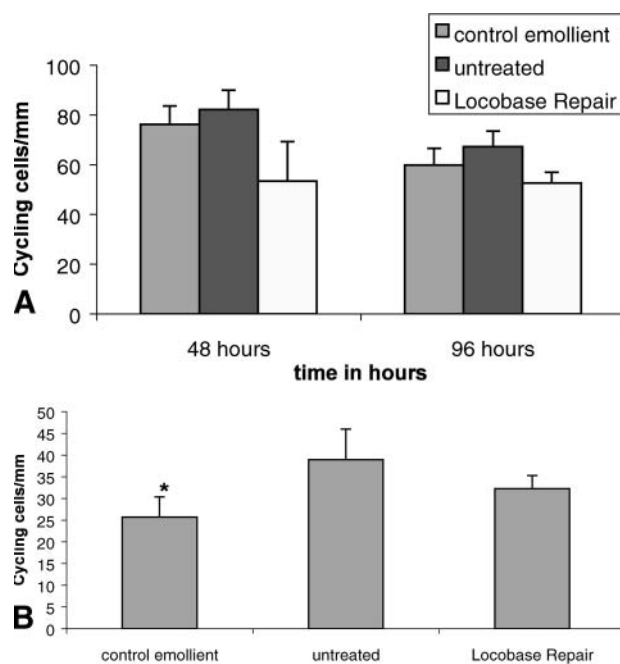


Fig. 3a: Cycling cells (Ki-67) 48h and 96h after tape stripping. Significant effect of Locobase repair compared to control emollient ($p < 0.03$) and the untreated site ($p < 0.01$). The sampling time was not significant. b: Cycling cells (Ki-67) 96h after tape stripping. Significant difference between the site treated with control emollient and the untreated site ($p < 0.03$).

Repetitive exposure to SDS induced an increased number of cycling cells after 4D at all investigated sites (Fig. 3b). The site treated with control emollient showed a significant lower number of cycling cells at day4 than the untreated site.

Epidermal differentiation. In the present study, tape stripping induced clear expression of cytokeratin 16. However, no significant effect of any treatment was noticed. We did not observe the induction of cytokeratin 16 expression after SDS application.

Expression of involucrin was slightly up-regulated in both models. However, treatment with neither of the emollients significantly altered involucrin expression (data not shown).

With respect to E-FABP and SKALP, we observed up-regulation of E-FABP and expression of SKALP exclusively in patients with slight erythema after SDS application. We did not see any effects of the treatment on these markers.

Langerhans cells (LCs). We investigated the expression of Langerhans cells 48h and 96h after tape stripping. At all sites we observed an increase of epidermal LCs after epidermal injury. No significant differences were found in the exposed treated and untreated skin.

Tables 1, 2 and 3 summarize the responses using various clinical, physiological and immunohistochemical parameters.

Table 1. Interference of Locobase Repair and vaselinum/cr. lanette with tape stripping-induced changes

Parameters	Locobase Repair P1- value	Vaselineum/cr. lanette P2-value
Erythem	↓↓	
↓	0.018	
TEW	↓↓	
↓	0.012	0.020
Ki 6	↓↓	
↓	0.018	0.003
Involucrin	↔	↔
Cytokeratin 16	↔	↔
Langerhans cells	↔	↔

↓↑ = tendency of an inhibition or stimulation, ↓↓↑↑ = statistically significant inhibition or stimulation, ↔ = no effect.

P1 is the *p*-value for difference between the site treated with Locobase Repair and the untreated site P2 is the *p*-value for difference between the site treated with Locobase Repair and vaselinum/cr. lanette.

p-values are only mentioned if significant.

Discussion

In both models, following a single course of tape stripping and repeated applications of SDS, skin barrier repair was accelerated by the two lipid formulations (Locobase Repair and vaselinum album/cremor lanette I ana). In the acute model of barrier disruption (tape stripping) Locobase Repair accel-

erated repair of barrier damage significantly. In the cumulative SDS-model, vaselinum album/cremor lanette I ana showed a significant recovery of damaged skin barrier.

In the tape stripping model we observed a significant suppression of proliferating cells at the site treated with Locobase Repair, whereas in the SDS models we observed a significant suppression of proliferating cells on the site treated with the emollient-control. The decrease of proliferating cells correlated with suppression of erythema score and TEWL. We did not observe any modulation of cytokeratin 16 (hyperproliferation), involucrin, E-FABP, SKALP (accelerated differentiation) or Langerhans cells.

The results are in line with the results of an animal study using a comparable lipid mixture. In the animal experiment, the tested lipid mixture accelerated barrier recovery after tape stripping but not after SDS related damage (9). Several different mechanisms can explain the discrepancy of results between the models.

Firstly, horny layer damage after detergent application differs from tape stripping-induced injury. Tape stripping disrupts barrier function by removing the stratum corneum mechanically; detergents disrupt barrier function by removing and rearranging stratum corneum lipids. It is suggested

Table 2. Interference of Locobase Repair and vaselinum/cr. lanette with SDS-induced changes

Parameters	Locobase Repair	vaselinum/cr. lanette	<i>p</i> -value
Erythem	↓		
↓↓	0.027		
TEW	↓		
↓↓	0.032		
Ki 6	↓		
↓↓	0.013		
Involucrin	↔	↔	
Cytokeratin 16	↔	↔	
SKALP	↔	↔	
E-FABP	↔	↔	

↓↑ = tendency of an inhibition or stimulation, ↓↓↑↑ = statistically significant inhibition or stimulation, ↔ = no effect.

P is the *p*-value for difference between the site treated with vaselinum/cr. lanette and the untreated site.

p-values are only mentioned if significant.

Table 3. Characteristic clinical, TEWL and immunohistochemical changes following repetitive application of SDS and tape stripping in our study

Parameters	Repetitive application of SDS (0.2%)	Tape stripping
Erythema	gradual increase	↑↑
TEWL	gradual increase	↑↑
Ki 67	↑	↑↑
Cytokeratin 16	negative	↑↑
Involucrin	increased expression	increased expression
SKALP	slight expression	ND
E-FABP	slight expression	ND
Langerhans cells	ND	slightly increased in epidermis

ND = not done.

that the quantity and spectrum of lipids removed from the stratum corneum, is detergent-specific (9). It is possible that detergents such as SDS may cause damage to the nucleated layers of the epidermis that cannot be corrected by providing lipids alone (9). The difference in efficacy of tested emollients between the tested models supports the hypothesis that the ability of the lipid mixture to accelerate barrier repair depends on the specific damage, requiring damage-specific correction (9).

Secondly, the limited efficacy of Locobase Repair in the SDS model compared to control emollient could be a matter of the ratio of the lipid components. It is known that not all types of topical formulations that contain lipids chemically related to the physiological content of the intercellular domain of the stratum corneum, are effective. Out of a number of preparations, those with a lipid ratio most comparable to that present in the human skin barrier is considered to be effective (16). It is unknown whether the lipid ratio of Locobase Repair, which consists of cholesterol, semisynthetic ceramide 3, oleic acid and palmitic acid, mimics the lipid ratio in the human skin. Furthermore, ceramide 3 is the exclusive ceramide incorporated in Locobase Repair. Ceramides represent a unique, heterogeneous group of at least 6 ceramides that differ from each other by the head group architecture and by the mean fatty acid chain length. Other ceramides in the stratum corneum are probably needed to provide the stratum corneum with a water-resistant lipid multilayer. It is assumed that especially ceramide 1 is of major importance for proper stratum corneum barrier function (19). Because of its unusual structure, which contains linoleic acid linked to the long chain ($C > 30$) omega hydroxy fatty acid, ceramide 1 is of great importance for the formation of the lateral lipid packing as well as the long range lamellar ordering in stratum corneum (19, 20). Studies of lipid composition of atopic skin showed a lowered level of ceramides, especially ceramide 1, suggesting its important role in barrier function (21–23). Concerning SDS-induced irritation, only a few references can be found with respect to the correlation between stratum corneum abnormalities and SDS irritation (24, 25); Nevertheless, the data imply that ceramide 1 is an important ceramide, and it is unknown whether emollients with ceramide 3 could sufficiently replace the role of ceramide 1 and other natural occurring ceramides.

In human studies the beneficial effect of emollients on the barrier function in SDS-induced skin irritation and tape stripping using clinical and bio-engineering methods has been established (15–17, 26). In one of these studies the effect of a ceramide 3-containing emulsion was investigated in which

no additional positive effect was observed on barrier repair in SDS-induced skin irritation. However, the authors did find positive effects on TEWL when damaged skin was treated with o/w emulsions enriched with mixtures of ceramides, cholesterol and fatty acids (16). In two other studies, a lipid mixture containing ceramide 3, cholesterol and fatty acids (Locobase Repair) was compared with different emollients which contain non-physiological lipids on SDS-induced skin irritation. In these studies, Locobase Repair was of no advantage compared to non-physiological lipids containing emollient with respect to acceleration of barrier recovery after SDS application, which is in line of our results (15, 17); To the best of our knowledge there is only one clinical study on the beneficial effect of emollients containing skin-related lipids on tape stripped human skin (17). In this study Locobase Repair was not superior to petrolatum, in contrast with our results. It is probable that the methods used to inflict barrier dysfunction were dissimilar. In our study the barrier dysfunction after tape stripping was more severe, which may explain the lack of the superior effect of Locobase Repair in their study.

In addition to the clinical assessment and TEWL measurements, we investigated cell biological changes using markers for epidermal proliferation (MIB-I), epidermal differentiation (LL025, MON 150, anti-E-FABP, anti-SKALP) and Langerhans cells (T6). The disturbances in epidermal proliferation, epidermal differentiation and Langerhans cells can be associated with impaired skin barrier function in some skin diseases and experimentally induced skin barrier irritation (27–33). Using these immunohistochemical markers, which are supposed to be associated with skin barrier dysfunction, we wanted to compare the cell biological effects of emollient treatment on experimentally induced skin barrier dysfunction. In our models of skin barrier dysfunction we observed differences in the dynamics of proliferating cells after treatment with emollients. With regards to the other markers, we observed down-regulation after skin barrier dysfunction, but we did not see any significant differences in the expression of these markers after application of the investigated emollients.

According to some authors, the accelerated recovery of barrier disruption after emollients could be due to a semioclusive effect of the emollient, which restricts water evaporation from the skin, thereby allowing the skin to recover (15). However, the role of occlusion has been questioned (34, 35). We hypothesize that the limited efficacy of Locobase Repair as compared to other more occlusive emollients may not be related to limited barrier

recovery but rather to limited protection against repeated exposure to a detergent. The fatty components of the emollient-control may form a better physical barrier than the components of Locobase Repair. These results support the hypothesis that the optimal barrier repair therapy should be based on the specific damage and recovery (17, 36); We suggest that an ideal emollient does not exist and that a specific emollient will not be appropriate for all clinical purposes.

In summary, the present study demonstrates that emollients improve skin barrier in different conditions compared to untreated skin and that there is some indication that formulations containing skin-related lipids might be of benefit in barrier disruption by tape stripping. To demonstrate a beneficial effect of emollients containing skin-related lipids, clinical studies are mandatory. An emollient protecting against all kinds of physical and chemical injuries is probably not realistic.

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