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## INVESTIGATIVE REPORT

# Effects of Topical Corticosteroid and Tacrolimus on Ceramides and Irritancy to Sodium Lauryl Sulphate in Healthy Skin

Jakob Mutanu JUNGERSTED<sup>1,3</sup>, Julie K. HØGH<sup>2</sup>, Lars I. HELLGREN<sup>2</sup>, Gregor B.E. JEMEC<sup>1</sup> and Tove AGNER<sup>3</sup>

<sup>1</sup>Department of Dermatology, Roskilde Hospital, University of Copenhagen, Roskilde, <sup>2</sup>Department of System Biology and Centre for Advanced Food Science, Technical University of Denmark, Lyngby, and <sup>3</sup>Department of Dermatology, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark

The skin barrier, located in the stratum corneum, is influenced mainly by the lipid and protein composition of this layer. In eczematous diseases impairment of the skin barrier is thought to be of prime importance. Topical anti-inflammatory drugs and emollients are the most widely used eczema treatments. The aim of this study was to examine the effects of topically applied corticosteroid, tacrolimus and emollient on stratum corneum lipids and barrier parameters. Nineteen healthy volunteers participated in the study. Both forearms of the subjects were divided into four areas, which were treated twice daily for one week with betamethasone, tacrolimus, emollient, or left untreated, respectively. After one week each area was challenged with a 24 h sodium lauryl sulphate patch test. The lipids were collected using the cyanoacrylate method and evaluated by high performance thin layer chromatography. For evaluation of the skin barrier, transepidermal water loss, erythema and electrical capacitance were measured. The ceramide/cholesterol ratio was increased in betamethasone- ( $p=0.008$ ) and tacrolimus-treated ( $p=0.025$ ) skin compared with emollient-treated skin. No differences in ceramide subgroups were found between treatment regimes. Pretreatment with betamethasone ( $p=0.01$ ) or with tacrolimus ( $p=0.001$ ) causes a decreased inflammatory response to sodium lauryl sulphate compared with emollient. In conclusion, treatment with betamethasone and tacrolimus has a positive effect on the ceramide/cholesterol ratio and susceptibility to irritant reaction compared with an emollient. **Key words:** ceramides; corticosteroid; emollient; lipids; skin barrier; tacrolimus.

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Jakob Mutanu Jungersted, Department of Dermatology, Bispebjerg Hospital, Copenhagen University, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark. E-mail: Jungersted@gmail.com

Skin barrier impairment is thought to be of prime importance in eczematous diseases and has been studied extensively in atopic dermatitis (AD) and irritant eczema (1, 2). The barrier function of the skin is located in the stratum corneum (SC) and is influenced mainly by the

lipid and protein composition of this layer (3, 4). The protein, filaggrin, has been shown to play a major role, while for lipids, the ceramide profile in particular has been reported to be affected. In patients with AD, a decreased amount of ceramide 1 and 3 is characteristically found (5–7). Recent data, however, suggest that ceramide profiles are not correlated with filaggrin mutations, suggesting that the two mechanisms appear to be mutually independent (8).

Topical application of anti-inflammatory drugs and emollients is frequently used for treatment of eczema. Traditionally, topical corticosteroids have been used, but in recent years non-steroidal alternatives, such as tacrolimus and pimecrolimus, have been used increasingly, in particular in the treatment of AD. The effect of topical corticosteroids on inflammatory dermatoses has been well documented (9), as has the effect of topical tacrolimus on AD and, to a lesser degree, on other eczematous diseases (10). Whereas the effects of these drugs on inflammation are well established, their effect on barrier integrity is less well described. Irrespective of the anti-inflammatory effect of these drugs, any added iatrogenic impairment of barrier function would be undesirable from the point of view of the current understanding of the disease pathogenesis of AD and irritant eczema. However, it has been suggested that the use of topical corticosteroids potentially damages the skin barrier further, and hence is counterproductive in the treatment of skin diseases where barrier impairment is a core element of the pathogenesis (11, 12). The use of corticosteroids may lead to a decrease in the number of lamellar bodies, and thereby, a decrease in SC lipids, which is of particular concern when treating AD (12). A recent study has examined consequences of the use of betamethasone treatment on people with AD, and found that it led to inconsistent extracellular lipid bilayers (11). In contrast, it has been speculated that tacrolimus could normalize the abnormalities in the lipid composition of AD (13). The effect of tacrolimus on the SC lipids in AD patients has been studied in an uncontrolled study, and no effect found (13).

The positive clinical effect of emollients on eczema is well known, and their use is generally recommended as adjuvant therapy of both AD and irritant eczema. In spite of their widespread use, the effect of emollients on the lipid composition of human skin, in both healthy

and diseased skin, is comparatively poorly described (14–19) and further investigation is required.

The aim of this study was to identify the effect of topical betamethasone and tacrolimus on skin barrier function, structurally with respect to SC lipids, and functionally through the susceptibility to challenge with an irritant.

## MATERIALS AND METHODS

Nineteen healthy participants with no former history of any major skin diseases were included in the study (6 men and 13 women, median age 25 years, age range 18–51 years). Participants were enrolled after responding to posters at the local educational centres and the library. The study was approved by both the local ethics committee (SJ-7-17583-3) and the Eudra CT (2008-000819-15) for the use of drugs.

Four areas ( $4 \times 7 \text{ cm}^2$ ) on the volar forearms on each participant, two on each forearm, were randomized for treatment twice daily with betamethasone-17-valerate (Betnovat<sup>®</sup>; GlaxoSmith-Kline Pharma), tacrolimus (Protopic<sup>®</sup>; Astellas) or emollient (Olefin<sup>®</sup>, per 100 g: white Petrolatum 15 g, Parafine oil 6 g, Cetomacrol 1000 I, 8 g, Cetostearyl alcohol 7.2 g, Chlorocresol 0.1%, Glostrup apotek, Glostrup, Denmark) respectively, and one area was left as untreated control. The distances between the treated areas were approximately 4 cm. The participants were instructed in how much ointment (approximately 1.5 g/day) to apply on the skin (20), and treatment was performed twice daily for 7 consecutive days. In the morning the topical treatments were applied under supervision of a physician, and in the evening, by the participants themselves, after being reminded by a text message (SMS).

The topical treatment was applied for the last time in the evening of day 7. On day 8 all participants were examined with non-invasive measurements (see below). On each of the four areas a 1% sodium lauryl sulfate (SLS) (Sigma-Aldrich >99.0% purity, Sigma Aldrich, St Louis, USA) patch was placed, using Large Finn-Cambers<sup>®</sup> (12 mm) with filter discs (60  $\mu\text{l}$ ) (21), and adjacent to this a cyanoacrylate sample was taken for lipid analysis (22). SLS patches were removed after 24 h (day 9), and on day 10 the non-invasive measurements trans-epidermal water loss (TEWL) and erythema were repeated. Cyanoacrylate samples were obtained from 16 of the 19 patients, due to practical difficulties.

For non-invasive measurements, TEWL was measured using a Dermalab<sup>®</sup> (Cortex Technology, Hadsund, Denmark), following recommended guidelines (23), two measurements were performed and the mean value used for further calculations.

Erythema was assessed by a chroma meter (Minolta chroma Meter CR-300, Minolta Camera Co., Osaka, Japan), which expresses colour in the  $L^*a^*b$  system, where erythema is expressed as  $a^*$ , in accordance with guidelines (24), two measurements were taken and the mean value used for further calculations.

Capacitance was measured using a corneometer (CM825PC, Courage + Khazaka, Köln, Germany), which expresses an arbitrary unit, representing the water content of the SC. Three measurements were performed and mean values used for further calculations (25).

For the cyanoacrylate method, a method recently found best suited for the use on human skin (22), the chosen area was wiped with acetone to eliminate contamination from surface lipids. A drop of cyano-acrylate tissue-glue (LiquiBand<sup>®</sup>) was placed at one end of a glass-slide and held tight against the skin, until it dried, and was then removed. The samples were kept at  $-80^\circ\text{C}$  until lipid extraction and analysis by high performance thin layer chromatography (HPTLC). Lipids were extracted from the cyanoacrylate and separated on silica-gel HPTLC plates. Development of the plates was

carried out in chloroform:methanol:acidic acid (190:9:1(v:v:v)). The plates were stained with primuline and lipids were quantified based on fluorescence intensity (Desaga TLC Densitometer CD (Desaga GmbH, Wiesloch, Germany), using standard curves made from ceramide 5 and cholesterol (Matrega, LLC, Pleasant Gap, PA, USA). For details, this method is described thoroughly elsewhere (22). This set-up describes relative differences in ceramide subgroups and ceramide/cholesterol ratio.

## Statistics

Friedman's test was used as an omnibus test for more than two groups, including parameters from all four areas. If this test achieved statistical significance, the Wilcoxon matched pairs test was applied for pair-wise comparison.  $p < 0.05$  was chosen as the level of significance. Data were analysed using SPSS statistics 17.0.

## RESULTS

### *Skin lipids in stratum corneum*

Ceramide/cholesterol ratio and ceramide profile from the four test areas after 7 days of topical treatment are given in Fig. 1. Statistical significant difference between the treatment regimes was found for the ceramide/cholesterol ratio. The highest median ratio was found for the area treated with betamethasone, and statistically significant higher than emollient ( $p = 0.008$ ), but also tacrolimus had statistically significant higher ceramide/cholesterol ratio than emollient ( $p = 0.025$ ). No statistically significant difference was found for the ceramide/cholesterol ratio between untreated control and the different treatment regimes. For ceramide 1–9, no statistical significant differences were found between test areas, and no obvious tendencies between any of the treatment regimes.

### *Functional evaluation of skin barrier function*

TEWL, erythema and capacitance measurements after different treatment regimes are presented in Table I.

With respect to TEWL values from the four test areas after 7 days of topical treatment, a statistically significant difference between the different areas was found, with the lowest values in the betamethasone-treated area, followed by the tacrolimus-treated area. However, no pair-wise statistically significant difference was found between any of the treated areas or for the untreated area.

With respect to TEWL values after the SLS-irritation performed after the different treatment regimes, no statistically significant difference was found between the different treated areas. The betamethasone-treated area, however, still had the lowest TEWL values (Table I).

For erythema measurements from the four test areas after 7 days of topical treatment, we found statistically significant differences for  $a^*$ , with betamethasone showing statistically lower levels of erythema than both tacrolimus and untreated control (Table I). Decreased

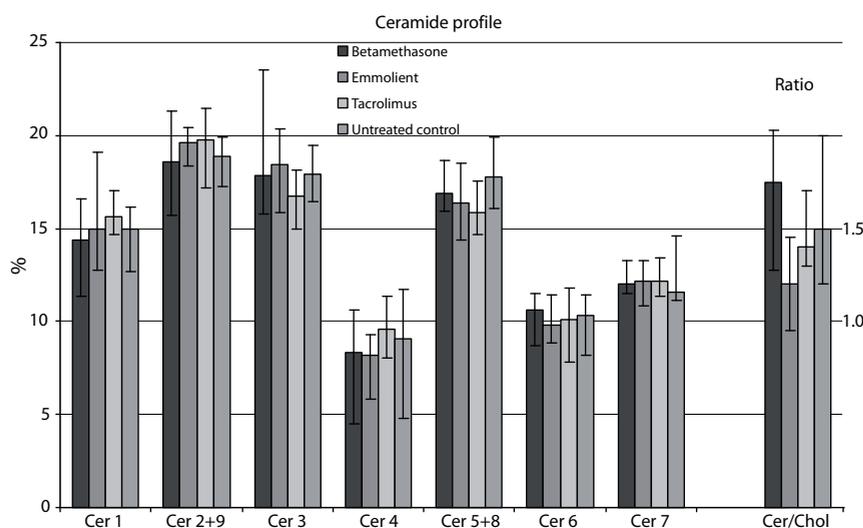


Fig. 1. Percentages of median ceramide values from four different treatment regimes. Note that ceramide/cholesterol is not in percentages but illustrates a ratio. Error bars illustrate 1<sup>st</sup> and 3<sup>rd</sup> quartiles.

erythema after SLS-irritation was found in betamethasone- and tacrolimus-treated skin compared with emollient-treated skin. Increased erythema after SLS-irritation was found in emollient-treated skin compared with untreated control.

For capacitance evaluated 24 h after the last application of the topical treatment regimes, no statistical significance was found (Table I).

## DISCUSSION

Topical treatment regimes with corticosteroids and tacrolimus are used widely for eczematous diseases, and improved understanding on their influence on the skin barrier function is therefore needed.

It is well known that the skin barrier is impaired in eczematous diseases, and that the barrier function is in-

Table I. Median values and p-values from barrier measurements as well as ceramide/cholesterol ratio

	Median values (percentiles 1 and 3)		Significance using Friedman's test	Significance using Wilcoxon's paired test
TEWL after treatment			$p=0.027$	No significance
Betamethasone	2.98	(2.43–4.25)		
Emollient	3.67	(2.81–4.69)		
Tacrolimus	3.18	(2.63–4.14)		
Untreated control	3.40	(3.13–4.55)		
TEWL after SLS			$p>0.05$	Not performed
Betamethasone	14.95	(11.60–21.53)		
Emollient	18.20	(15.00–30.45)		
Tacrolimus	18.25	(12.25–27.65)		
Untreated control	15.45	(13.38–23.73)		
Erythema after treatment			$p=0.026$	Significance between betamethasone vs. tacrolimus ( $p=0.016$ ) and betamethasone vs. untreated control ( $p=0.004$ )
Betamethasone	7.86	(6.87–8.13)		
Emollient	8.55	(7.18–8.70)		
Tacrolimus	8.30	(7.58–8.62)		
Untreated control	8.30	(7.47–13.33)		
Erythema after SLS			$p=0.044$	Significance between emollient vs. betamethasone ( $p=0.01$ ) and emollient ( $p=0.024$ ) vs. untreated control and emollient vs. tacrolimus ( $p=0.001$ )
Betamethasone	11.37	(9.85–13.08)		
Emollient	12.49	(11.6–14.27)		
Tacrolimus	11.54	(9.58–13.72)		
Untreated control	11.57	(9.85–13.33)		
Capacitance after treatment			$p>0.05$	Not performed
Betamethasone	33.50	(28.25–38.63)		
Emollient	32.25	(29.50–34.86)		
Tacrolimus	35.50	(32.13–39.63)		
Untreated control	31.50	(27.75–34.86)		
Ceramide/cholesterol ratio			$p=0.050$	Significance between emollient vs. betamethasone ( $p=0.008$ ) and emollient vs. tacrolimus ( $p=0.025$ )
Betamethasone	1.75	(1.25–2.03)		
Emollient	1.20	(0.95–1.45)		
Tacrolimus	1.40	(1.30–1.53)		
Untreated control	1.50	(1.20–2.00)		

TEWL: trans-epidermal water loss, in g/m<sup>2</sup>/h; SLS: sodium lauryl sulphate.

Erythema: colour from green to red measured by a cromometer (range: 0–60).

Capacitance: electrical resistance in the skin and expressed in arbitrary units that correlates to the water level in the stratum corneum (range: 0–120).

fluenced mainly by the lipid and protein composition of the SC (3, 4). Very few studies have, however, focused on the SC lipids in relation to topical treatment regimes, and to the best knowledge of the authors, this is the first human study to describe the consequences of the use of topical corticosteroids on SC ceramides. One previous study has examined the SC lipids in a mouse model, as well as *in vitro* in human keratinocytes. In both models corticosteroid was applied topically, and a decrease in lamellar body generation and epidermal lipid synthesis in mice ( $n=4$ ) and in *in vitro* human keratinocytes ( $n=5$ ) were found (16). Another study examining the skin barrier structure using transmission electron microscopy, found an inconsistency in the extracellular lipid bilayers after treatment with betamethasone in patients with AD (11). These findings suggested a negative barrier effect from topical corticosteroids.

The actual effect of betamethasone and tacrolimus can be seen when comparison with the emollient-treated skin is made, since emollient-treated skin represents the changes from treatment without active ingredients, but with the effects from the emollient itself. The emollient Olefin® was chosen for its simplicity; however, it should be noted that the present study does not examine whether other emollients would have the same effects on the skin barrier.

By using volunteers with healthy skin, we ensured that the skin of each individual was homogeneous. Examining patients with AD or psoriasis, might make it impossible to use four areas on the forearms that were affected the same, and thereby make interpretation of the results more difficult. However, the results from the present study should be interpreted keeping in mind that patients with AD and psoriasis are known to have a low ceramide/cholesterol ratio, especially on lesional skin (5–8, 26).

We found a statistically significant higher ceramide/cholesterol ratio for betamethasone compared with emollient-treated skin, and a tendency towards a higher ceramide/cholesterol ratio for betamethasone compared with the untreated control area. For people with AD, an increased ceramide/cholesterol ratio is usually interpreted as a barrier-positive increase, based on the decreased ceramide/cholesterol ratio found in AD skin (6). We found no difference in the subgroups of ceramides.

With respect to values after SLS irritation, the TEWL values were found to be in agreement with the positive barrier hypotheses from the ceramide/cholesterol ratio, since the betamethasone-treated area presents the lowest TEWL (Table I). However, the reservoir effect on the skin from the topical drugs could result in vasoconstriction and might also contribute to the reduction in TEWL and erythema values.

Topical treatment with tacrolimus caused a significantly higher ceramide/cholesterol ratio compared with emollient, suggesting an overall positive effect on the

skin barrier function similar to that of betamethasone. For tacrolimus, only one human study on SC lipids has been published previously. It was speculated that topical treatment with tacrolimus might change the ceramide content of SC (13). Therefore, the ceramide profile for seven patients with AD was compared before the start and after 28 days of treatment with tacrolimus. The authors found a decrease in ceramide 4, but no comparisons with other topical treatment regimes were made (13).

The present study analysed healthy individuals, and found no difference in ceramide subgroups after treatment with tacrolimus, compared with betamethasone, emollient or untreated control. We were not able to confirm the formerly described decrease in ceramide 4 described in seven patients with AD, who had an abnormal ceramide subgroup composition from the onset (13).

For the water content of the SC, none of the treatment regimes changed the capacitance significantly compared with untreated skin. This result is in agreement with a study comparing five different moisturizers, of which they found no change in capacitance, but in contrast to longer-term studies suggesting that the use of a simple moisturizer can increase capacitance (14, 27).

Our results indicate that the active ingredients in betamethasone and tacrolimus have a positive effect on ceramide/cholesterol ratio compared with treatment with emollient, as well as diminish the response to application of an irritant (SLS).

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