

INVESTIGATIVE REPORT

Comparing the Effect of a Twice-weekly Tacrolimus and Beta-methasone Valerate Dose on the Subclinical Epidermal Barrier Defect in Atopic Dermatitis

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The proactive use of topical anti-inflammatory (TAI) therapy to address subclinical inflammation is an effective, contemporary clinical strategy for the management of atopic dermatitis (AD). The interaction of a proactive TAI dose with the subclinical epidermal barrier defect in AD is yet to be determined. A randomised, observer-blind, functional mechanistic study in 17 subjects with quiescent AD was performed to compare the effect of a twice-weekly dose of betamethasone valerate (0.1%) cream (BMVc), against tacrolimus (0.1%) ointment (TACo) on the biophysical and biological properties of the epidermal barrier. Application of BMVc preserved epidermal barrier function and stratum corneum (SC) integrity, but significantly elevated skin-surface pH with concomitant loss of SC cohesion. By contrast, TACo improved SC integrity, exerted an overall hydrating action, and significantly reduced caseinolytic and trypsin-like protease activity. The differential effects reported support the proactive use of TACo to promote reparation of the subclinical barrier defect in AD. Key words: topical corticosteroid; topical calcineurin inhibitor; epidermal barrier.

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The end-product of terminal keratinocyte differentiation, and a fundamental component of the epidermal barrier; the stratum corneum (SC) provides protection against multiple environmental insults including: chemical attack, allergen penetration, excessive water loss and microbial invasion (1). Contrary to the historical view that inflammatory skin diseases such as atopic dermatitis (AD) result from an acquired immunological disposition, current opinion attributes the breakdown of the epidermal barrier as the primary event or 'driver' of disease pathogenesis (2). Accordingly atopic skin is

dysfunctional, with increased permeability to water and environmental allergens a consequence of keratinocyte hyperproliferation, disturbed differentiation and elevated protease activity (3–5). Biophysical analysis of the SC reveals dryness and reduced integrity coupled with impaired permeability barrier function signaled by elevated transepidermal water loss (TEWL) that correlates with disease severity (4, 6, 7). Importantly, these barrier abnormalities persist sub-clinically, co-existing with Th1, Th2 and Th22 inflammatory mediators in normal-appearing, non-lesional atopic skin (4, 8, 9).

Topical corticosteroids (TCS) and more recently topical calcineurin inhibitors (TCI) provide efficacious first and second-line respective topical anti-inflammatory (TAI) therapies for both adults and children with acute flares of AD (10, 11). Once primary disease control (induction of remission) is established, current clinical practice advocates a twice-weekly TAI 'maintenance' dose in combination with a baseline daily emollient therapy as a preventative measure to suppress the repopulation of subclinical inflammatory infiltrate (12, 13). With clinical trials supporting the use of both TCS and TCI in this proactive manner, authors have questioned which class of TAI is clinically superior through careful evaluation of efficacy, safety, benefits and cost-effectiveness (14). Referring to epidermal barrier safety, compared to the damage associated with prolonged daily TCS use, the influence of TCI remains inconclusive (8, 15–17), with no studies to date reporting the interaction of a proactive TAI dose with the dysfunctional epidermal barrier.

To this end, a head-to-head, within-volunteer investigation was designed to compare the effect of a TCS, against a TCI dose on the biophysical and biological properties of the SC, when applied proactively (twice-weekly) in volunteers with quiescent AD. Not only do these subjects demonstrate a subclinical barrier defect (inhibited epidermal permeability barrier function, reduced SC hydration and elevated trypsin-like protease activity compared to healthy controls) (8), they are asymptomatic (minimum 6 months), allowing the interaction of the treatments with the defective epidermal barrier to be investigated, independent from their primary anti-inflammatory purpose of disease control.

METHODS

Subjects

A total of 23 volunteers with a self-reported recent history of AD (no symptoms/use of systemic, oral or topical anti-inflammatories in the past 6 months) were recruited for a randomized, observer-blind functional mechanistic study. Volunteers refrained from using emollients on the treatment sites for at least 7 days prior to participation and for the duration of the study. Basic exclusion criteria included pregnancy, breast-feeding, and being under the age of 18. Informed consent was obtained from each participant. The NHS Trent Multicentre Research Ethics Committee (MREC) approved the study under the project reference 04/MREC/70.

Treatment

Study sites (2 per-volunteer) were defined as the volar side of each forearm, 3 cm below the elbow flexure and 3 cm above the wrist. Volunteers were provided with a pre-weighed tube of BMVc (0.1% Betnovate® cream, GlaxoSmithKline, Uxbridge, UK) and TACo (0.1% Protopic® ointment, Astellas Pharma, Elisabethhof, Netherlands) in conjunction with a treatment diary, and instructed to evenly apply 2 fingertip units of the appropriate product to a randomly assigned treatment site, twice per week for 8 weeks. Treatment days were evenly spaced throughout the week. The aim of unsupervised self-treatment was to emulate normal clinical use by patients. Compliance to the regimen was assessed by verbal consultation and the satisfactory completion of the treatment diary. No more than 3 non-consecutive applications could be missed. Volunteers refrained from applying the treatments on the morning of the final measurement session.

Biophysical and biological assessments

A series of minimally-invasive, standardised assessments, including: TEWL combined with tape-stripping; infrared (IR) densitometry; measurement of skin-surface pH; capacitance; and assessment of SC protease activity were performed in climate-controlled conditions as previously reported by the research group (8). Volunteers acclimatised to the room conditions for 20 min prior to the assessments being made. All measurements were performed by the same, suitably trained technician.

Data analysis

Statistical significance ($p < 0.05$) was determined using Graphpad Prism v6.0b (Graphpad Software Inc., La Jolla, USA). All biophysical measurements followed a normal distribution, therefore group means were compared using a repeated measures 1 and 2-way analysis of variance. The Bonferroni test provided *post-hoc* analysis. A non-gaussian distribution was associated with protease activity, therefore the Friedman test combined with Dunn *post-hoc* analysis was used. Results are presented as the mean \pm the standard error of the mean (SEM).

RESULTS

Differential action of BMVc and TACo on the biophysical properties of the stratum corneum

A total of 17 volunteers with quiescent AD applied a mean of 19.39 (± 6.33 g) BMVc to one study site, and 17.11 (± 5.69 g) TACo to the opposing site over the

course of 16 applications (twice-per-week for 8 weeks total duration). A further 6 volunteers did not fully complete the treatment regimen and were therefore excluded from the analysis. To assess the SC in response to the study interventions, tape-stripping to experimentally damage the barrier combined with TEWL measurements every 5 discs was performed at baseline, and end of the maintenance regimen (Fig. 1a). At both sites, TEWL (disc 0) remained unchanged indicating preservation of epidermal permeability barrier function (inside-out) by both treatments. In contrast, by tape-stripping to 20 discs (TS20), a validated technique designed to non-invasively measure the structural integrity of the SC (8, 18), a significant difference between the sites was shown. By plotting TEWL against disc number and utilizing an "area under the curve" (AUC) analysis to account for the rate at which TEWL increases during tape stripping, it was demonstrated that application of TACo was associated with a significantly ($p < 0.01$) slower increase in TEWL (AUC $480.7 \pm 24.92 \text{ g/m}^2/\text{h.TS}_{20}$) compared to BMVc (AUC $598.5 \pm 36.22 \text{ g/m}^2/\text{h.TS}_{20}$). Of note, TEWL at disc 20 on the TACo site reached $45.54 \pm 3.94 \text{ g/m}^2/\text{h}$. This was significantly reduced compared to baseline (pre-treatment) measurements ($59.99 \pm 5.13 \text{ g/m}^2/\text{h}$), confirming the improvement of SC integrity by this agent ($p < 0.0001$). Interestingly BMVc had no detrimental effect on the integrity of the SC when applied twice-per-week for 8 weeks duration.

As a further assessment of the SC, infrared densitometry was employed as an indirect measure of protein mass (number of corneocytes) removed by tape-stripping, in response to the treatment interventions (Fig. 1b). By plotting the cumulative mass of protein removed every 5 discs, and conducting an AUC analysis on the resulting slope, it was shown that following application of BMVc, SC protein was removed at a significantly greater rate (AUC $2,064.8 \pm 75.24 \mu\text{g/cm}^2.\text{TS}_{20}$) compared to baseline (AUC $1,821.4 \pm 54.55 \mu\text{g/cm}^2.\text{TS}_{20}$) during tape-stripping to 20 discs ($p < 0.05$). At disc 5 significantly more SC protein ($p < 0.001$) was removed from the BMVc treated site ($171.3 \pm 8.60 \mu\text{g/cm}^2$) compared to baseline ($140.7 \pm 5.62 \mu\text{g/cm}^2$) confirming the damaging effect of this treatment on the cohesive properties of the SC.

To complete the biophysical assessments, SC capacitance as an indirect measure of hydration (Fig. 1c) and skin surface pH (Fig. 1d), was measured in response to the treatments. On the TACo site, capacitance (43.35 ± 1.45 RCU) was significantly elevated compared to BMVc (40.76 ± 1.47 RCU) indicating a superior beneficial effect of this treatment on the hydration state of the SC ($p < 0.05$). Furthermore TACo exerted a significant overall hydrating action in our quiescent AD cohort ($p < 0.01$) as evidenced by comparison to baseline measurements (37.29 ± 1.23 RCU). The greater effect of TACo compared to BMVc on SC capacitance could be due to the greater occlusivity of the ointment formulation compared to the cream

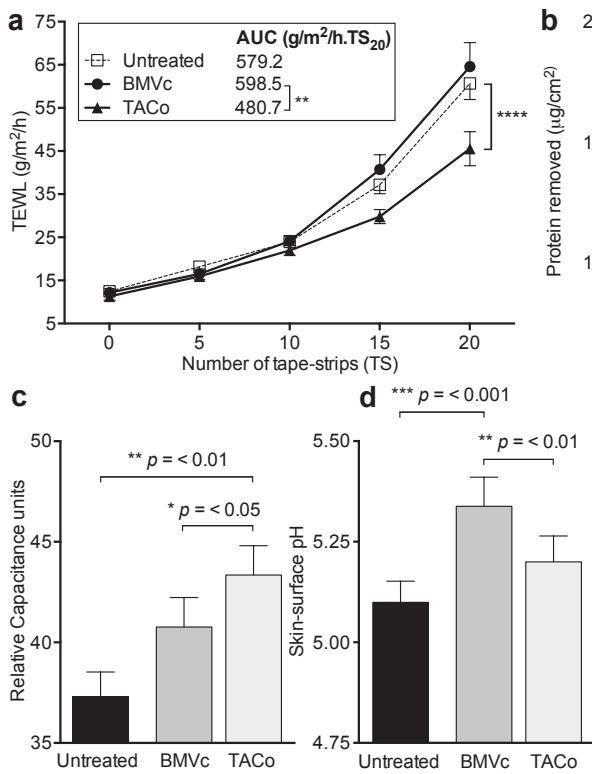


Fig. 1. Differential action of betamethasone valerate (0.1%) cream (BMVc) and tacrolimus (0.1%) ointment (TACo) on the biophysical properties of the epidermal barrier. (a) Stratum corneum (SC) integrity determined by transepidermal water loss (TEWL) measurements combined with tape-stripping to 20 discs before and after treatment. Area under the curve (AUC) analysis confirmed TEWL increased at a slower rate on the TACo site during tape stripping (***p* < 0.01). TEWL at disc 20 was significantly reduced on the TACo site compared to untreated skin (*****p* < 0.0001). (b) SC cohesion defined as the mass of protein (µg/cm²) removed during tape-stripping determined by infrared densitometry. AUC analysis confirmed increased SC removal on the BMVc site compared to untreated skin (**p* < 0.05). (c) Capacitance was significantly elevated by TACo compared to the BMVc (**p* < 0.05) and untreated skin (****p* < 0.01). BMVc was associated with a significant increase in skin-surface pH compared to TACo (***p* < 0.01) and untreated skin (****p* < 0.001). The graphs represent the mean of 17 volunteers with error bars indicating the standard error of the mean. Statistical significance was determined using a repeated measures 1 or 2-way analysis of variance with Bonferroni *post-hoc* analysis.

base. A trend for elevated skin-surface pH was observed at both treatment sites, albeit BMVc (5.34 ± 0.07 units) increased pH to a greater degree (*p* < 0.01) compared to TACo (5.20 ± 0.06 units). Of note the BMVc induced rise in skin-surface pH was also significant (*p* < 0.001) compared to baseline (5.10 ± 0.05 units).

Suppression of superficial stratum corneum protease activity by TACo

As an additional biological marker of epidermal barrier function, caseinolytic (broad-spectrum), chymotrypsin-like (Kallikrein-7), and trypsin-like protease activity (including Kallikrein-5 and -14) were quantified from discs 1-3 collected during tape-stripping (Fig. 2). An overall trend of suppressed protease activity by TACo was observed. Caseinolytic activity was statistically reduced (*p* < 0.05) on the TACo site (4.21 ± 0.27 nU/µg) compared to baseline measurements (4.73 ± 0.23 nU/µg). Quantification of trypsin-like proteases produced a similar result whereby TACo significantly (*p* < 0.05) reduced activity (1.20 ± 0.12 nU/µg) compared to baseline (1.66 ± 0.09 nU/µg). Thus TACo has the potential to reduce the aberrant protease activity associated with quiescent AD (8)

DISCUSSION

When performed consistently to defined experimental conditions, TEWL measurements combined with tape-

stripping offers a validated, minimally invasive method of assessing epidermal permeability barrier function, structural integrity and the cohesive properties of the SC (18–20). Using these techniques combined with a biological assessment of SC protease activity, a beneficial effect of TACo compared to BMVc on the epidermal barrier was demonstrated when applied proactively over an 8-week study duration. Most notably TACo significantly reduced protease activity and improved the integrity of the SC, coupled with an overall hydrating action. This suggests that a TACo formulation can promote repair of the subclinical barrier defect exhibited by subjects with quiescent AD (8) when applied at a twice-weekly frequency.

These new findings pertinent to a clinical maintenance regimen fit comfortably with the available literature reporting conventional daily TCI use, in that good efficacy is combined with repair of the defective epidermal barrier. For example both pimecrolimus cream (PIM), and TACo are highly effective at reducing disease severity, lowering TEWL and improving SC hydration in patients with mild-to-moderate AD (21, 22). With regards to PIM, the aforementioned beneficial actions were superior when directly compared to vehicle alone (21), suggesting the active ingredient itself (PIM) repairs the dysfunctional water-holding and barrier properties of the atopic SC. In support of this observation, mechanistically there is convincing evidence that TCI alter the defective structural lipid architecture of the epidermis. For example in lesional AD, investi-

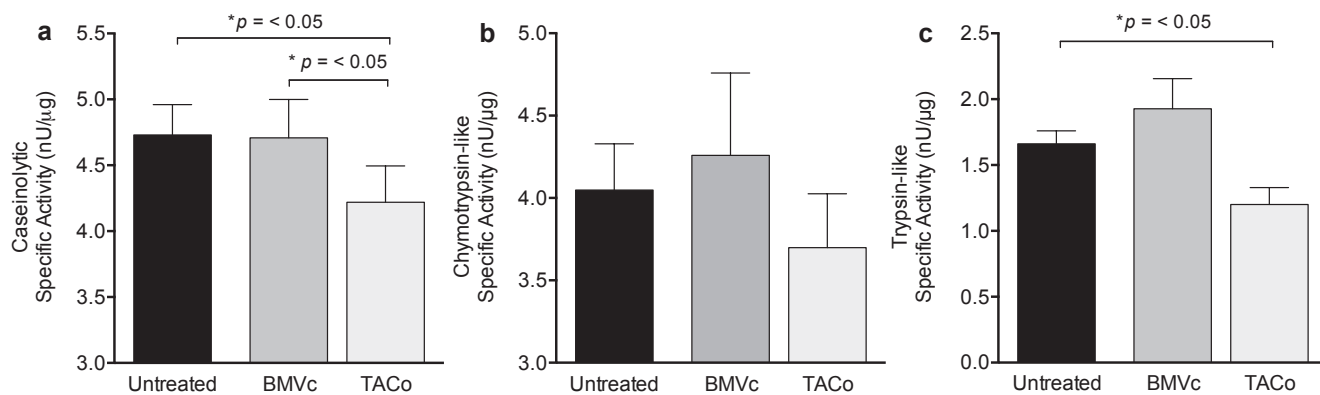


Fig. 2. Suppression of stratum corneum protease activity by tacrolimus (0.1%) ointment (TACo). Biological assessment of (a) Caseolytic, (b) chymotrypsin-like and (c) trypsin-like protease activity, quantified from discs 1–3 collected during tape-stripping. A significant differential effect of the 2 treatments was observed for caseolytic protease activity ($*p < 0.05$), contributed to by suppressed activity on the TACo site compared to untreated skin ($*p < 0.05$). TACo inhibited trypsin-like protease activity compared to untreated skin ($*p < 0.05$). The graphs represent the mean of 17 volunteers with error bars indicating the standard error of the mean. Statistical significance was determined using the non-parametric Friedman test with Dunn *post-hoc* analysis.

gators performing transmission electron microscopy confirmed a reordering of lipid bilayer architecture and restoration of disturbed lipid synthesis/trafficking following daily PIM and TACo use (15, 22, 23).

Given that a pathological consequence of the Th2-polarised immunological imbalance in AD is the negative impact on multiple components of epidermal barrier function (24), a plausible explanation for the concomitant rescue of the dysfunctional barrier by TCI use could be via the amelioration of inflammatory lesions. Alternatively here, by using a quiescent AD model that was visually flare-free for a minimum duration of 6 months, the results suggest a more direct action of TACo on the dysfunctional epidermal barrier, as opposed to a consequence of its primary anti-inflammatory and immunomodulating properties. This could be for example through the up-regulation of genes such as filaggrin and lorixin that are vital to skin hydration and the mechanical strength of the SC (3, 25, 26). A word of caution; the presence and modification of subclinical inflammation in these subjects was not experimentally determined throughout the duration of the study, therefore further work is required before drawing firm conclusions.

Due to technical limitations however, it was not feasible to differentiate the action of TACo from its vehicle (petrolatum base); an agent reported to improve hydration and promote barrier repair (27). Nevertheless a similar investigation identified on average a 3.4% rise in skin capacitance following intensive petrolatum treatment (8 applications spread over 2 days) on multiple anatomical sites in children with AD (28). By comparison we observed a much greater 14% rise in skin capacitance following a less intensive regimen, suggesting again that tacrolimus exerts an additive beneficial effect on the water-holding properties of the SC, supplementary to its formulated petrolatum base.

The tape-stripping procedure combined with IR densitometry to indirectly quantify the mass of protein

removed by each D-squame disc, provides a simple, minimally-invasive method for assessing the cohesive properties of the SC (18, 29). Using this methodology, the proactive use of BMVc was associated with a concomitant reduction in SC cohesion. This is in-line with previous studies identifying a similar effect by topical glucocorticoids applied daily over short and longer timeframes (8, 16). The negative impact of TCS on SC cohesion has been attributed to a diminution of corneodesmosome density (16). In addition to this change, a small but significant rise in SC pH associated with proactive BMVc use was shown. Deleterious barrier abnormalities such as reduced SC integrity/cohesion, delayed permeability barrier recovery, elevated serine protease activity and inhibited lipid synthesis are all reported consequences of a rise in skin pH (30). Although reduced SC cohesion combined with a trend for elevated desquamatory protease activity was noted by our study, no subsequent reduction in epidermal barrier function or integrity was observed following two days-per-week BMVc use. This is somewhat surprising given previous results (8), but could reflect the comparatively reduced exposure to this agent throughout the maintenance treatment regimen.

Through the degradation of corneodesmosomes, facilitating the shedding of terminally differentiated surface corneocytes, the SC undergoes a process of continual renewal to maintain the functional integrity of its barrier. Desquamatory proteases are fundamental to this process (31), but more recently, a broader role for these enzymes in the maintenance of barrier function has been intimated. For example Kallikrein (KLK)-5 activity has been linked with profilaggrin processing and KLK-7 with the generation of natural moisturising factor from filaggrin degradation (32, 33). But aberrant protease activity is linked to the onset of clinical AD (5, 8), evidenced by the overexpression of KLK-7 in transgenic mice promoting the development of chronic, inflammatory lesions (34).

Furthermore in skin diseases homologous to AD such as Netherton syndrome, elevated KLK-5 activity has been demonstrated to generate cutaneous inflammation independently from the adaptive immune system (35). Importantly here, reduced superficial caseinolytic and trypsin-like protease activity by TACo was demonstrated when applied proactively twice-per-week. Clarification is required to categorize the extent (depth) of inhibition, in addition to characterizing further the specific proteases suppressed, as the caseinolytic substrate encompasses a broad-spectrum of activity. Nevertheless the findings represent a novel beneficial action of this agent on the hyperactive desquamatory protease activity associated with quiescent AD.

In summary, when applied according to a proactive clinical treatment regimen for a duration of 8 weeks, BMVc compared to TACo elevated skin surface pH and reduced SC cohesion in subjects with quiescent AD. Although overall this effect was minimal, the twice-weekly use of BMVc appears unable to correct the barrier damage exerted throughout a daily TCS flare control regimen (8, 16). Alternatively, TACo was found to significantly improve SC integrity and hydration in conjunction with suppressed caseinolytic and trypsin-like protease activity compared to untreated skin. The differential effects observed suggest that TACo is more suitable for the proactive treatment of quiescent AD through a more reparative action on the epidermal barrier. Further studies are required to confirm whether this beneficial action remains over longer-term treatment durations. Of immediate interest, the complex relationship between reparation of the subclinical barrier defect, the degree of subclinical inflammation and the length of disease remission requires further investigation (1, 13). Treatment regimens focusing on maintaining or repairing the dysfunctional epidermal barrier, could have a significant influence on reducing the severity of AD in the long term and be disease modifying (36, 37).

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