

Prevention of Follicular Penetration: Barrier-Enhancing Formulations against the Penetration of Pollen Allergens into Hair Follicles

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Key Words

Barrier-enhancing emulsions • Skin protection • Pollen allergens, penetration

Abstract

The hair follicles could be a reservoir for topically applied substances. They are not only surrounded by a close network of blood capillaries, which makes them interesting for drug delivery, but they are also the host of dendritic cells, which are important for immunomodulation. Previously, pollen allergens were shown to penetrate into the hair follicles. The application of barrier-enhancing formulations might represent an effective strategy to prevent pollen protein penetration into the hair follicle. In the present study, porcine skin areas were pretreated with 4 barrier-enhancing emulsions. One skin area served as control and remained without pretreatment. Afterwards, fluorescein-isothiocyanate-labeled grass pollen proteins were applied to the porcine skin samples, and their penetration was investigated via fluorescent laser scanning microscopy. It was shown that the barrier-enhancing formulations were able to significantly reduce the penetration of exogenous proteins into the hair follicles, the extent of such reduction depending on the formulation.

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Introduction

A broad knowledge of the penetration pathways of substances deliberately or unintentionally applied topically is essential for the stimulation or prevention of the penetration of these substances through our skin barrier. In the past, it was assumed that the intercellular penetration route inside the lipid layers around the corneocytes is the only penetration pathway of importance for topically applied substances [1–3]. Recently, it was shown that also the hair follicles represent a fast penetration pathway through the skin barrier for substances which can pass the stratum corneum (SC) [4–6]. Additionally, it was shown that the reservoir of the hair follicles can be equivalent to the reservoir of the SC in some body sites [7]. Furthermore, substances, which penetrate into the hair follicles and cannot pass the barrier into the viable dermis or epidermis such as particles, are stored for up to 10 times longer than in the SC [8, 9].

Recently, the hair follicles have been attracting attention, particularly in drug delivery and prevention strategies. On the one hand, the hair follicles represent disruptive shunts of the skin barrier and are easily accessible; on the other hand, they are the host of stem cells and are surrounded by a close network of blood capillaries and den-

dritic cells, which makes them interesting for immunological processes. Lately, the hair follicles were selected as a promising target for topical vaccination [10]. Other investigations disclosed that also pollen proteins are able to enter the organism via the hair follicles. Jacobi et al. [11] applied fluorescein-isothiocyanate (FITC)-labeled pollen proteins on test skin and observed a penetration into the SC and into the hair follicles. Additionally, the pollen proteins were located in the perifollicular region of the dermis. Jacobi et al. concluded that the penetration into the dermis must have proceeded via the follicular pathway. Also, Jacobi et al. [12] have shown that pollens disintegrated after coming into contact with sweat, and pollen proteins were released. Gunawan et al. [13] have shown that the pollens tested in their study could release serine and/or cysteine endopeptidases when contacting saline solution.

It is generally accepted that also for atopic dermatitis (AD), e.g. type I allergens play an important pathophysiological role and might even increase the risk factors for AD and for AD severity not only by means of inhalation, but also by direct contact with the skin [11, 14–16]. Atopy patch testing with aeroallergens in patients with AD and controls showed significant differences (more positive reactions for AD) between the two groups [17–19]. Atopic dermatitis goes along with an impairment of the barrier function due to decreased ceramide levels. Recently, mutations of the filaggrin structure protein have been described in AD patients, which may contribute to a disrupted skin barrier function [20]. Recently published data suggest that additional AD risk factors exist in the epidermal differentiation complex apart from filaggrin mutations [21]. These epidermal changes contribute to an increased antigen absorption into the skin which results, in comparison to systemic or airway allergic disorders, in higher levels of Th2 responses [22] or can lead to primary systemic sensitization. Epidermal exposure to food antigens, e.g. can prevent the normal induction of oral tolerance and also lead to airway eosinophilia following inhalation [23]. Eczematous reactions to type I allergy-inducing antigens are documented in a subgroup of patients with atopic eczema. It was demonstrated that pollen grains induce an inflammatory reaction in susceptible individuals with atopic eczema [24]. Ongoing investigations in Kiel and Hanover have shown that the atopic eczema is correlated with the pollution by birch pollen, with the symptoms becoming worse on those parts of the body which are not protected by textiles [25]. This indicates that prevention is important for disrupted barriers. Although it is not clear whether pollen allergens can enter the intact

barrier, it is interesting to know whether the possible follicular pathway can be inhibited by barrier-enhancing formulations. The results could be of importance to other particles, exogenous proteins or allergens which should be prevented from entering the hair follicles.

Therefore, in the present study, 4 barrier-enhancing formulations were investigated for their protection properties, especially in relation to the hair follicles. For the investigations, grass pollen allergens were labeled with a fluorescent dye [11] and applied on porcine ear skin, which is a suitable model for human skin [26, 27]. Biopsies were taken from the skin samples after application and penetration of the pollen allergens. Laser scanning microscopy [28–32] was used to analyze the distribution of the allergens on the histological sections. The experiments had to be carried out *in vitro* because of the high number of biopsies to be taken from the tissue. Excised human skin is less suited for the investigation of follicular penetration than porcine ear skin, because the skin contracts after removal, which can lead to a reduction of the follicular reservoir up to 90% [33]. The use of pig ear skin represents a worst-case scenario because the orifices are bigger. If the application of skin care products results in a reduced penetration into porcine skin, this effect is expected to be higher in human skin [34].

Materials and Methods

Labeling of the Pollen Allergens

Pollen allergens were labeled with a fluorescent dye as described in detail by Jacobi et al. [11]. The used extract contains purified allergens with protein sizes from 10 to 100 kDa. Two ampoules with 1.26 mg extract of grass pollen [Gräsermischung, 450,000 standardized quality units (SQU), ALK Scherax, Hamburg, Germany] were dissolved in 1 ml PBS buffer (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). A 10-mM solution (2 mg in 1 ml PBS buffer) of FITC (Sigma-Aldrich Chemie) was added to the solution of the pollen extract. This mixture was incubated in the dark for 2 h at 4°C.

The FITC-labeled pollen proteins were purified using membrane dialysis (Spectra/Por®, molecular-weight cutoff 6–8,000, Theodor Karow GmbH, Berlin, Germany).

An excess of L-alanyl-L-glutamine (Dipeptamin®, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) was added to the FITC-labeled proteins and the dialysate in order to inactivate nonbonded FITC. Both solutions were incubated for 2 h at 4°C.

Aliquots were dried at 30°C using a lyophilizer (Concentrator 5301, Eppendorf, Hamburg, Germany). The dry lyophilisates were stored at –20°C in the absence of light. The lyophilisates of the dialysate were used as controls in the experiments.

For application on pig ears, one aliquot of the lyophilisates (450,000 SQU) was dissolved in 200 µl phosphate buffer saline (2,250 SQU/µl).

Formulations

Four different formulations were applied:

- Eucerin® pH 5 lotion (o/w) = lotion 1,
- Eucerin® pH 5 lotion F (w/o) = lotion 2,
- Eucerin® pH 5 cream (o/w) = cream 1,
- Eucerin® pH 5 cream F (o/w) = cream 2.

These formulations were evaluated because they contain a specific combination of active agents, pH 5 citrate buffer, glycerol and dexpanthenol, which significantly enhances SC functions, as described earlier [35]. All formulations had been tested in a study on patients with sensitive skin associated with allergic respiratory diseases. The results of the study confirmed the suitability of the products in the specific, barrier-invigorating skin care and therapy of skin sensitivity for patients with allergic airway disease [36].

Tissue Samples and Skin Treatment

The experiments were carried out on ears of freshly slaughtered domestic pigs (German domestic pigs, 6 months old). Each formulation was investigated at least on 5 pig ears. Permission for the experiments had been obtained from the Veterinary Governmental Office in Berlin-Treptow.

Before commencement of the experiments, the porcine ears were cleaned with cold water and dried with paper tissues. On each ear, 5 skin areas of $3 \times 2 \text{ cm}^2$ in size each, were marked for investigation (edding 140 S, OHP-marker permanent, Edding, Ahrensburg, Germany). The skin areas 2–5 were pretreated applying 2 mg/cm^2 of each formulation (all Beiersdorf AG, Hamburg, Germany) onto the skin areas for 3 min using a hand-held massage device (Massagegerät PC 60, Petra-electric Elektrogeräte Fabrik Burgau). Area 1 was left untreated.

Skin area 1: control skin area (S_{CO}), no pretreatment

Skin area 2: pretreatment with lotion 1 (S_{L1})

Skin area 3: pretreatment with lotion 2 (S_{L2})

Skin area 4: pretreatment with cream 1 (S_{C1})

Skin area 5: pretreatment with cream 2 (S_{C2})

Thirty minutes after pretreatment, $5 \mu\text{l/cm}^2$ of the FITC-labeled allergen solution of 2,250 SQU/ μl were carefully applied to all 5 areas. After a penetration time of 1 h, biopsies were taken from all skin areas.

Histological Section

Two biopsies of $0.5 \times 0.5 \text{ cm}^2$ each were taken from the skin areas of every porcine ear. The biopsies were shock frozen in liquid nitrogen and stored at -30°C . The frozen punch was cut vertically to the skin surface in sections of $10 \mu\text{m}$ thickness using a cryotome (Kryostat MT Slee-Technik, Typ HS, Mainz, Germany) in order to avoid contamination of the labeled allergens from the surfaces.

Laser Scanning Microscopy

Histological sections obtained from biopsies were analyzed by laser scanning microscopy (LSM 410 invert, Karl Zeiss, Jena, Germany), both in the transmission and fluorescent modes [37, 38]. A wavelength of 488 nm was used for fluorescence excitation, and the emission was measured from 505 to 600 nm.

The penetration depth of the pollen allergens in the hair follicles was determined using the software program of the LSM for distance measurements.

In total, 127 follicles were investigated for the control area (S_{CO}), 91 for lotion 1 (S_{L1}), 69 for lotion 2 (S_{L2}), 56 for cream 1 (S_{C1}) and 77 for cream 2 (S_{C2}).

Statistical Analysis

A descriptive explorative data analysis was performed using PASW (SPSS Inc., Chicago, Ill., USA). Because of the limited number of data, parametric tests were performed. The data were analyzed using related sample Friedmann's two-way analysis of variance by ranks. Differences among the means were analyzed by the Wilcoxon signed-rank test. Differences were considered significant at $p < 0.05$.

Results

In figure 1, a transmission and fluorescence image of a histological section is shown, which contains a hair follicle. The histological section was obtained from a biopsy removed from the control area (S_{CO}) treated with pollen allergens. Detection of fluorescence was equalized to penetration of allergens, as previous investigations have shown that untreated skin reveals no fluorescence. As can be seen from figure 1, the allergens are almost completely located in the hair follicles, only a very weak fluorescent signal can be detected on the skin surface. The penetration depth of the fluorescent signal into the hair follicle was determined by using the software program of the laser scanning microscope, as shown in figure 2.

The intensity of the fluorescent signal was subdivided by visual assessment into 3 groups: A = strong fluorescent signal, B = weak fluorescent signal or C = no fluorescent signal. Examples for this classification are given in figure 3.

In figures 4 and 5, the percentages of the hair follicles exhibiting fluorescent signals are demonstrated for each pretreatment.

In the control skin area (S_{CO}), almost all of the hair follicles contained pollen allergens, most of which showed a strong fluorescent signal whereas only about 10% showed a weak fluorescent signal. After pretreatment with lotion 1 (S_{L1}), fewer hair follicles displayed a fluorescence at all and more than half of them showed a weak fluorescent signal. The pretreatment with lotion 2 (S_{L2}) reduced the penetration into the hair follicle to below 60%, and the strong fluorescent signal was found in less than 10%. Both creams caused the strong fluorescence signal to drop to almost zero, whereas in almost 80%, the penetration of pollen allergens into the hair follicle was prevented completely by cream 2.

Comparing the various formulations, the reductions in penetration of pollen allergens were significantly different ($p < 0.05$) with the exception of cream 1 and lotion 2, and were ranked $S_{C2} > S_{C1} > S_{L2} > S_L$.

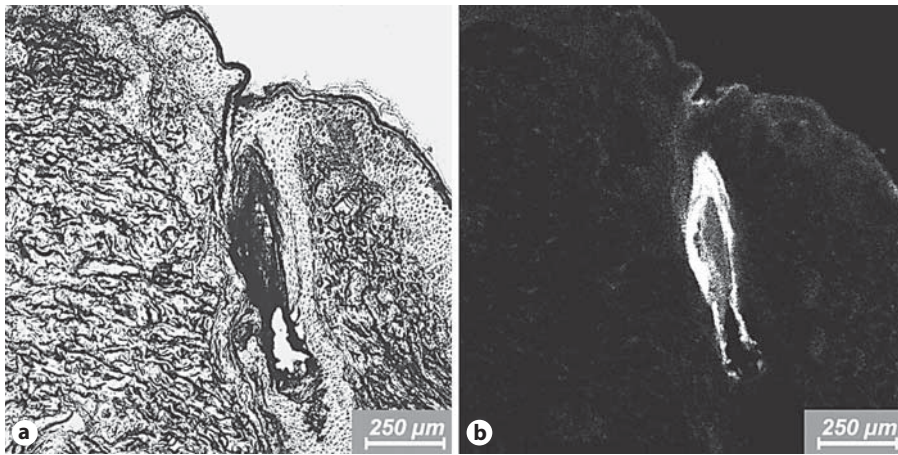


Fig. 1. Laser scanning transmission (a) and fluorescence (b) images of histological section which contains hair follicles.

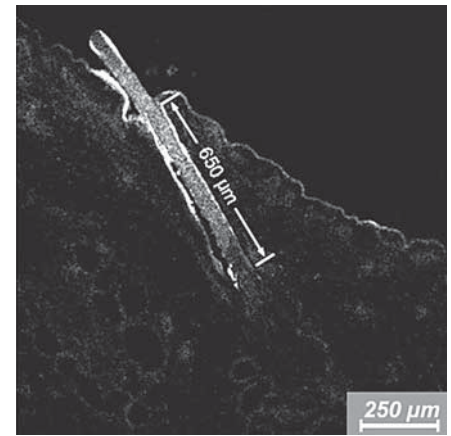


Fig. 2. Determination of the penetration depth of the fluorescent signal into the hair follicle using the laser scanning microscope software program.

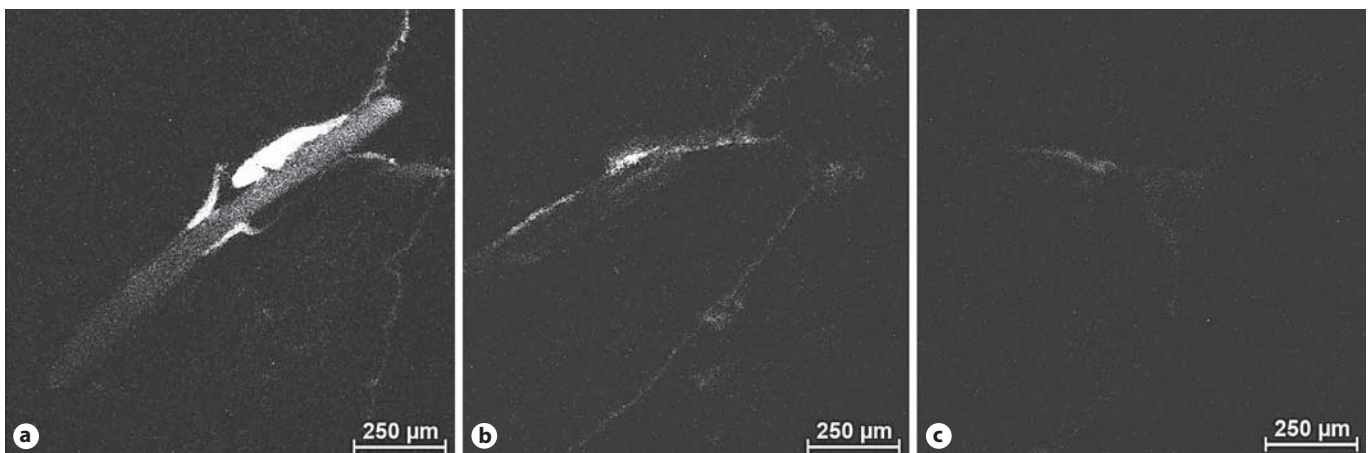


Fig. 3. Laser scanning fluorescence images of strong fluorescent signal (a), weak fluorescent signal (b) and no fluorescent signal (c).

A weak fluorescent signal always corresponded with a lower penetration depth (fig. 6). The mean penetration depth of the strong fluorescent signal was $723 \pm 118 \mu\text{m}$ and the weak fluorescence shows a significantly lower mean penetration depth to $377 \pm 89 \mu\text{m}$ ($p < 0.001$). The penetration depths for weak and strong fluorescent signals were nearly comparable for all 5 skin areas.

Discussion

When applied to the control skin area (without pretreatment), the pollen allergens penetrated in almost all cases deeply into the hair follicles, as strong fluorescent signals were observed. In contrast, the fluorescent signal on the skin surface was constantly significantly weaker suggesting that in particular the hair follicles represent an efficient reservoir and penetration pathway for these topically applied allergens.

Fig. 4. Relative amount of follicles with and without fluorescence of pollen allergens for different pretreatments. All formulation-treated areas showed significantly lower allergen penetration compared to the untreated one ($p < 0.05$). * $p < 0.05$: significant changes between the neighbors.

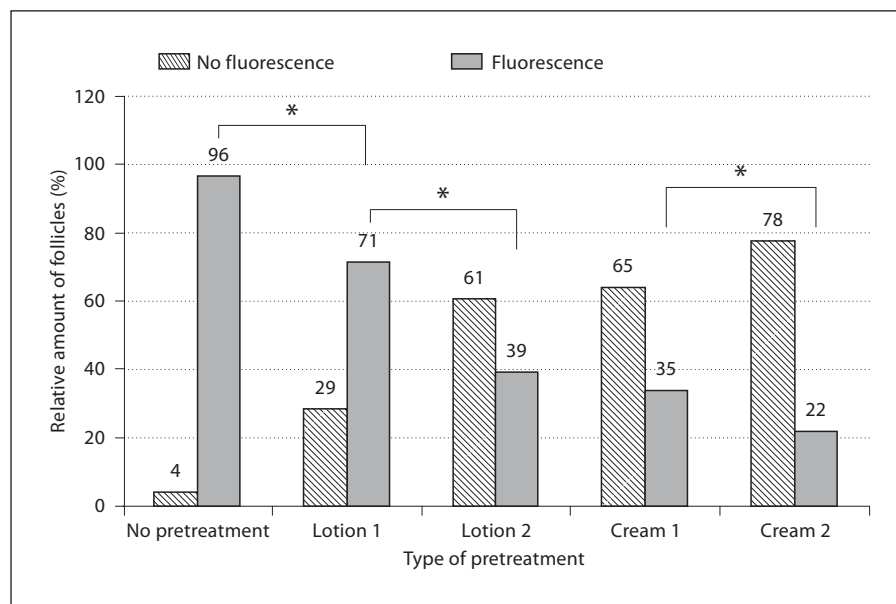
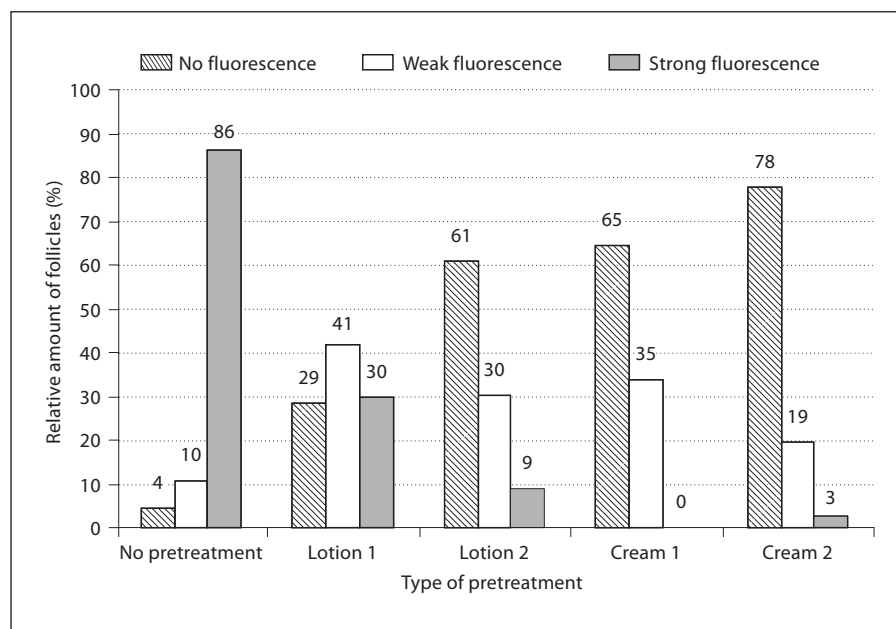


Fig. 5. Relative amount of follicles with strong, weak and without fluorescence of pollen allergens for different pretreatments.



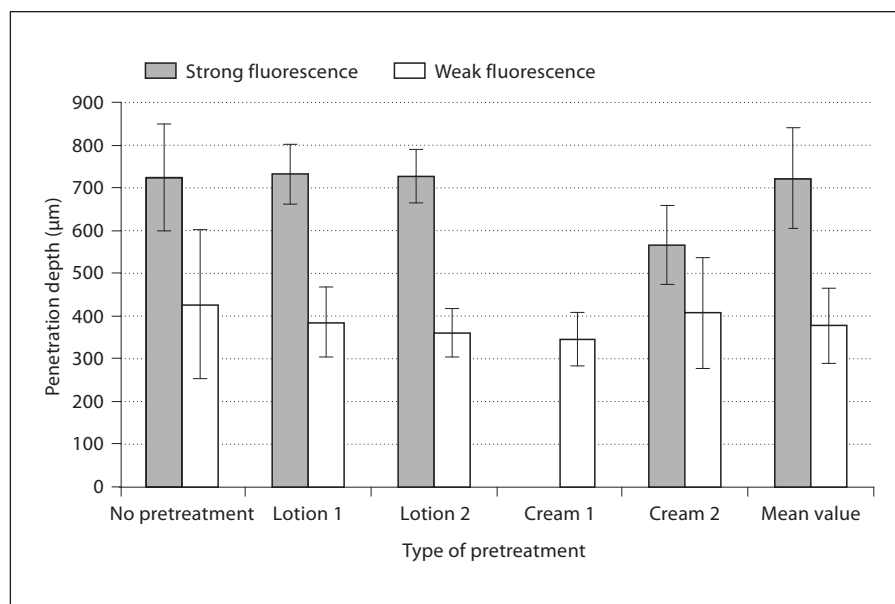
All pretreatments of the skin reduced significantly the percentage of follicles containing any pollen allergens (increased absence of fluorescent signals), the concentration of penetrated allergens (increase in weak fluorescent signals) and the penetration depth of the allergens into the hair follicles.

For cream 2 (S_{C2}), the best results were obtained, whereas lotion 2 (S_{L2}) and cream 1 (S_{C1}) were nearly identical but hardly less effective, which might be due to the higher consistency (higher viscosity) of both creams compared to the

lotions. Regarding the lotions, the w/o lotion 2 shows significantly better results than the o/w lotion 1 ($p < 0.05$).

In principle, barrier-enhancing emulsions may act on 3 different levels of the epidermis. An immediate effect is provoked by adding a lipid mixture to the skin surface (occlusion), an intermediate effect by adding a lipid mixture to the intercellular spaces and a delayed effect by providing lipids to the epidermal cells, which should restore the natural barrier function of the skin [39]. As in the present study the formulations were applied only 30

Fig. 6. Penetration depths of pollen allergens with strong and weak fluorescence in pretreated and not pretreated follicles compared to the mean value of all groups.



min before allergen contact, the immediate effect of occlusion has to be assumed. In the case of long-term use, also the other effects might become relevant. The test formulations contain a combination of active agents, pH 5 citrate buffer, glycerol and dexpanthenol, which significantly enhances SC enzyme function, as described earlier [35]. This is essential for the maintenance and re-establishment of the skin's own defensive functions [40]. This combination together with the type of emulsion seems to influence the degree of efficacy.

In summary, the application of the investigated formulations reduced the penetration of pollen proteins into the hair follicles significantly, and therefore the results achieved might be interesting for endangered persons and motivate them to reduce their cutaneous exposure to harmful allergens or other exogenous proteins. The formulations should be applied each time before an expected skin exposure to allergens or other exogenous proteins.

Nevertheless, clinical studies have to be carried out to investigate the clinical relevance of these findings.

Conclusion

The treatment of the porcine ear skin with 4 medical skin care products significantly reduced the penetration of pollen allergens into the hair follicles. The reduction in penetration ranged from 29% using o/w lotion 1 to 78% using w/o cream 2 with the measured fluorescent signals

in the hair follicles being predominantly weak. A weak fluorescent signal always corresponded to a lower penetration depth of $377 \pm 89 \mu\text{m}$ instead of $723 \pm 118 \mu\text{m}$ which was determined for strong fluorescence.

Acknowledgments

The project was partly supported by Beiersdorf AG and by the 'Skin Physiology' Foundation of the Donor Association for German Science and Humanities.

Disclosure Statement

All authors have no conflicts of interest.

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