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Research paper

Comparison of two in vitro models for the analysis of follicular penetration and its prevention by barrier emulsions

Jürgen Lademann [a,*], Alexa Patzelt [a], Heike Richter [a], Sabine Schanzer [a], Wolfram Sterry [a], Alexander Filbry [b], Kerstin Bohnsack [b], Frank Rippke [b], Martina Meinke [a]

[a] Department of Dermatology, Charité-Universitätsmedizin Berlin, Berlin, Germany
[b] Beiersdorf AG, Hamburg, Germany

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ABSTRACT

The penetration of topically applied substances in and through the human skin is of special interest for the development and optimization of topically applied drugs and cosmetic products. In the present study, the efficacy of barrier emulsions in the prevention of the penetration of pollen allergens into the hair follicles was investigated. Because of the sensitising potential of the used pollen allergens, the study was carried out under in vitro conditions. Therefore, excised human skin and porcine ear skin were used as tissue models. Applying laser-scanning microscopy and fluorescent-labeled grass pollen allergens, we found that the preventive efficacy of the barrier emulsions could be significantly better investigated on porcine ear skin than on excised human skin. This might be due to the contraction of the elastic fibres around the hair follicles in excised human skin after its removal. In contrast to the excised human skin, the porcine ear skin remains on the cartilage during the experiment. Therefore, contraction of the tissue can be avoided. The results give further indication that in vitro studies based on membranes of excised skin are not suitable for the investigation of the follicular penetration pathway of topically applied substances.

1. Introduction

Recently, numerous publications have demonstrated that hair follicles represent efficient long-term reservoirs for topically applied substances [1–3]. Moreover, hair follicles are considered to be important targets for drug delivery, as they are surrounded by a close network of blood capillaries [4,5], and the host of the stem and dendritic cells, which are important for regenerative medicine and immunology [6,7].

On the contrary, the hair follicles display interruptions in the skin barrier. Therefore, strategies are required to prevent the penetration of hazardous substances, such as allergens, into the hair follicles [8,9].

The selective investigation of follicular penetration, both in vivo and in vitro, poses a challenge. Previously, the main problem was the lack of quantitative model systems that are truly follicle free but retain the structural, biochemical and barrier properties of normal skin [10].

At the present time, few methods are available to investigate follicular penetration. Laser-scanning microscopy (LSM), e.g., allows the non-quantitative visualisation of follicular penetration of fluorescent or fluorescent-labeled substances [11–13]. Artificial closing of the hair follicles by a special lacquer-wax mixture [14–16] and the method of differential stripping, which is a combination of tape stripping and cyanoacrylate surface biopsies [17], offer further possibilities to investigate follicular penetration in vivo selectively and quantitatively.

In the case of harmful substances, in vivo investigations are not feasible. Consequently, skin models have to be constituted. Usually, excised human skin or porcine ear skin is applied in these cases. In order to investigate follicular penetration in vitro, cryo sections can be prepared from tissue biopsies and analyzed after the treatment of the skin with the topically applied formulation [18,19]. Using microscopic techniques, such as fluorescence, Raman or electron microscopy, the distribution of topically applied substances in the hair follicles can be analyzed [20,21].

Excised human skin has the disadvantage of contracting after removal and has to be re-stretched to its original size for the penetration experiments. Nevertheless, it is still unclear, whether contracted and re-stretched skin still offers a valid model to investigate follicular penetration. In this context, Patzelt et al. [22] proposed that during the re-stretching process, the dense circular fibre network around the hair follicles remains contracted, whereas the parallel interfollicular elastic fibres are able to be re-stretched resulting in a reduced follicular reservoir. Therefore,

* Corresponding author. Center of Experimental and Applied Cutaneous Physiology, Department of Dermatology, Charité-Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany. Tel.: +49 30 450 518 100; fax: +49 30 450 518 918.
E-mail address: juergen.lademann@charite.de (J. Lademann).

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porcine ear skin was proposed to represent a more suitable model as the ear skin remains fixed to the ear cartilage during the experiments, which inhibits any contraction. On the other hand, porcine hair follicles are per se larger than the human hair follicles. This may lead to an over-estimation of the follicular penetration process.

In the present study, the penetration of pollen allergens into the hair follicles of excised human and porcine ear skin was studied and compared. Additionally, the penetration of the pollen allergens into the hair follicles was investigated with and without pre-application of two barrier enhancing formulations, lotion 1 and lotion 2.

2. Materials and methods

2.1. Tissue samples

The study was carried out on both porcine ear skin and excised human skin.

The porcine ears were obtained from freshly slaughtered pigs. Pre-treatment of the porcine ears consisted only of washing with cold water and drying with paper towels. Approval for these experiments had been obtained from the Government Office of Veterinary Medicine in Berlin-Treptow, Germany.

The human facial skin samples from the retro auricular region were obtained during surgical interventions. This skin area was chosen because it offered terminal hair follicles, as well as vellus hair follicles. The sizes of the excised skin samples were measured before and after removal of the skin. As the samples contracted after removal, they were re-stretched to their original size before treatment. Approval of the experiments had been obtained from the Ethics Committee of the Charité Berlin, Germany.

2.2. Formulations

Two different formulations from Beiersdorf, Hamburg, Germany were applied: Oil in water emulsion Eucerin® pH5 Lotion (lotion 1) and water in oil emulsion Eucerin® pH5 Lotion F (lotion 2).

The ingredients of the formulations are listed below:

pH5 Lotion (lotion 1): aqua, alycerin, cetyl palmitate, paraffinum liquidum, panthenol, cyclomethicone, cetyl alcohol, sorbitan stearate, aluminum starch octenylsuccinate, phenoxyethanol, tocopheryl acetate, methylparaben, carbomer, sodium citrate, propylparaben, citric acid, perfume, linalool, hydroxyisohxyl 3-cyclohexene carboxaldehyde, hexyl cinnamal, benzyl salicylate, alpha-isomethyl ionone, butylphenyl methylpropional and limonene.

pH5 Lotion F (lotion 2): aqua, isopropyl stearate, paraffinum liquidum, glycerin, panthenol, polyglyceryl-2 dipolyhydroxystearate, polyglyceryl-3 diisostearate, tocopheryl acetate, phenoxyethanol, magnesium sulfate, methylparaben, sodium citrate, propylparaben, citric acid, perfume, linalool, hydroxyisohxyl 3-cyclohexene carboxaldehyde, hexyl cinnamal, benzyl salicylate, alpha-isomethyl ionone, butylphenyl bethylpropional, limonene trisodium EDTA, silica, acrylates/C10–30 alkyl acrylate crosspolymer, propylparaben, BHT and perfume.

2.3. Labeling of the pollen allergens

The fluorescent labeling of the pollen allergens was undertaken in accordance with Jacobi et al. [9]. Two ampoules with 1.26 mg extract of grass pollen (Gräsermischung, 450,000 SQ U, ALK Scherax, Hamburg, Germany) were solved in 1 ml PBS buffer (Sigma–Aldrich Chemie GmbH, Deisenhofen, Germany). The extract contained purified allergens with protein sizes from 10 to 100 kDa. A 10 mM solution (2 mg in 1 ml PBS buffer) of fluorescein isothiocyanate FITC (Sigma–Aldrich Chemie GmbH, Deisenhofen, Germany) was added to the solution of the pollen extract. This mixture was incubated for 2 h at 4 °C.

The purification of the FITC-labeled pollen proteins was performed using membrane dialysis (Spectra/Por®, MWCO 6-8000, Theodor Karow GmbH, Berlin, Germany). The dialysis was carried out in 50 ml PBS buffer in the absence of light at 4 °C (ice cooled). The PBS buffer was stirred and renewed hourly for a total of 32 times. Afterwards, it was renewed daily for the next 8 days. The fluorescence of the removed PBS buffer was measured regularly. The dialysis was finished, if the fluorescence measured was constant.

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 non-bonded FITC. Both the solutions were incubated for 2 h at 4 °C.

Aliquots were dried at 30 °C using a lyophilisator (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 to ensure that the measured fluorescence of the test areas derived only from the FITC-labeled allergens and not from free fluorescein isothiocyanate.

For application on pig ears, one aliquot of the lyophilisates (450,000 SQ U) was dissolved in 200 μl (2250 SQ U/μl).

2.4. Treatment of the skin

In total, four porcine ears were investigated. Three skin areas of 3 cm × 2 cm were separated on each skin ear using a permanent marker (edding 140 S, OHP-marker permanent, edding, Ahrensburg, Germany). Subsequently, a silicon barrier was applied around the skin areas in order to avoid lateral spreading of the consecutively applied substances. The first area remained without pre-treatment. The second and third skin areas were pre-treated with 2 mg/cm^2 of the barrier enhancing formulations lotion 1 and lotion 2, respectively. The formulations were applied by means of massage appliance (for 3 min) using a hand-held device (Massagegerät PC 60, Petra-electric Elektrogeräte Fabrik Burgau, Germany).

After a 30-min penetration time, 5 μL/cm^2 of the FITC-labeled allergen solution of 2250 SQ U/μL was carefully applied onto all the three areas using a pipette (10–100 μl, Eppendorf, Hamburg, Germany) and a spatula spoon. For each new production of labeled pollen allergens, 5 μL/cm^2 of the control solution was applied once on a separate area and treated as the area without pre-treatment.

After further 60 min at room temperature, possible supernatant was carefully removed. From each application area, several biopsies of 0.5 cm × 0.5 cm were removed and shock frozen in liquid nitrogen.

The same procedure was performed on four excised human skin samples with the exception that due to the small size of the human facial skin samples, the size of the three skin areas had to be decreased to 1 cm × 1 cm. On account of the reduced amount of emulsion necessary to pre-treat the skin areas, an error of 10% had to be accepted for the amount of topically applied substances.

2.5. Biopsies/laser-scanning microscopy (LSM)

After the removal of the biopsies from the porcine ear skin and from the excised human facial skin, each frozen biopsy was cut into 10 μm thin slices. The cryo sectioning was carried out vertically from right to left in order to avoid contamination with the labeled allergens from the surfaces.

Sections containing follicles were marked and investigated using in vitro laser-scanning microscopy (LSM 410 invert Zeiss, 488 nm). The auto fluorescence of the pig ear was blocked using filters. The penetration depth of the FITC-labeled allergen was mea-

sured on each LSM picture and selected pictures were stored. In preparation experiments, it was ensured that without the application of the labeled allergens, no fluorescent signal could be obtained from the histological sections.

The penetration depth could be estimated quantitatively given in μm, and the fluorescence intensity could be estimated qualitatively (strong, weak or no fluorescence). The fluorescence of the control solution was considered in the interpretation.

In the case of pig ear skin, 70 hair follicles were investigated for the non-pre-treated area, 91 for skin area pre-treated with lotion 1 and 69 for skin area pre-treated with lotion 2.

For human skin, six vellus hair follicles and 12 terminal hair follicles were investigated for the non-pre-treated area, 10 vellus hair follicles and 13 terminal hair follicles for the skin areas pre-treated with lotion 2 and 5 vellus hair follicles and 14 terminal hair follicle for the skin areas pre-treated with lotion 1.

2.6. Statistical analysis

For the statistical analysis, the Wilcoxon test was used (SPSS 16.0).

3. Results

In Fig. 1, typical transmission and fluorescence images of a histological section are presented, deriving from a non-pre-treated porcine ear skin area, 60 min after application of the allergens. The histological section contains a hair follicle. The distribution of the fluorescent-labeled allergens in the hair follicle can be well recognized in the fluorescent mode as light areas. The control solution from the last dialysis showed no significantly enhanced fluorescent signal compared to the completely untreated skin.

Analyzing 70 hair follicles of the non-pre-treated skin areas revealed that in 98% ± 4% of the hair follicles pollen allergens could be detected.

In the case of skin pre-treatment, different results were obtained. After pre-treatment of the skin areas with lotion 1 or lotion 2, 71% ± 9% and 39% ± 4%, respectively, of the hair follicles showed fluorescence. The mean values are presented in Fig. 2. The significance of the differences was shown by the Wilcoxon test ($p < 0.01$). The intensity of fluorescent signal detected in the hair follicles was weak for parts of the hair follicles in the case of pre-treatment with lotion 1 and lotion 2. In this case, the penetration depth was reduced by 52% ± 15% from the mean value 724 μm ± 89 μm to 373 μm ± 70 μm.

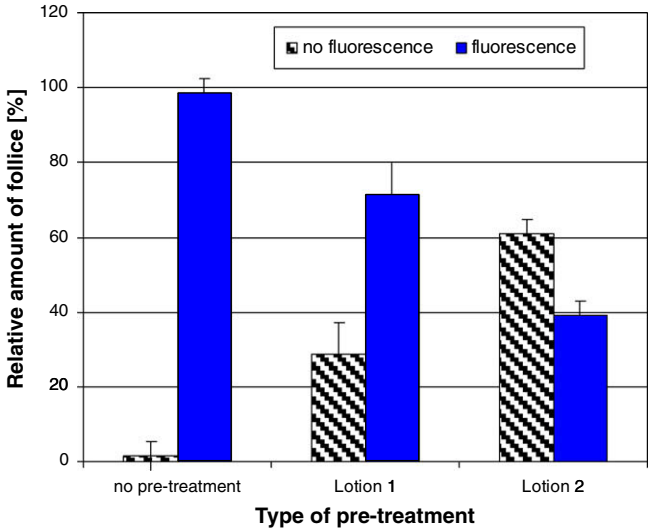

Fig. 2. Relative amount of follicles with or without fluorescence of pollen allergens in relation to all investigated follicles in the respective area. The results are shown for non-pre-treated, lotion 1 and lotion 2 pre-treated porcine skin. Both the lotions reduce significantly the amount of follicles with pollen allergens but to a different degree.

The results obtained from excised human facial skin considered data both from terminal hair follicles as well as from vellus hair follicles. For the non-pre-treated human facial skin, similar results were obtained as in the case of the experiments on porcine ear skin. All hair follicles contained a fluorescent signal, the intensity of these fluorescent signals was lower, in comparison to the fluorescent signals obtained from porcine hair follicles without pre-treatment (Fig. 3). The fluorescence was comparable to the weak fluorescence signal obtained from porcine hair follicles with pre-treatment using lotion 2. The mean penetration depth in terminal hair follicle without pre-treatment was 650 μm with a relative SD of 17%, and for vellus hair follicle a penetration depth of 350 μm with 38% SD was measured.

In the case of the pre-treatment of the human skin with the barrier emulsions, almost no fluorescent signal could be detected in the hair follicles, as shown in Fig. 4. In each area, only one follicle with a weak fluorescence was found. Consequently, a distinction between the barrier emulsions lotion 1 and lotion 2 was not possible and no differences could be observed between vellus and terminal hair follicles.

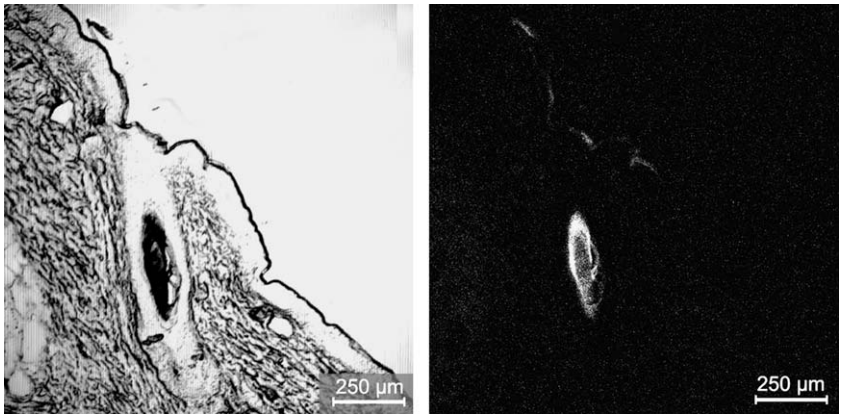

Fig. 1. Native (left) and fluorescent (right) figure of a histological 10 μm section of porcine ear skin with a strong fluorescence as found for non-pre-treated skin after application of fluorescent-labeled pollen allergens

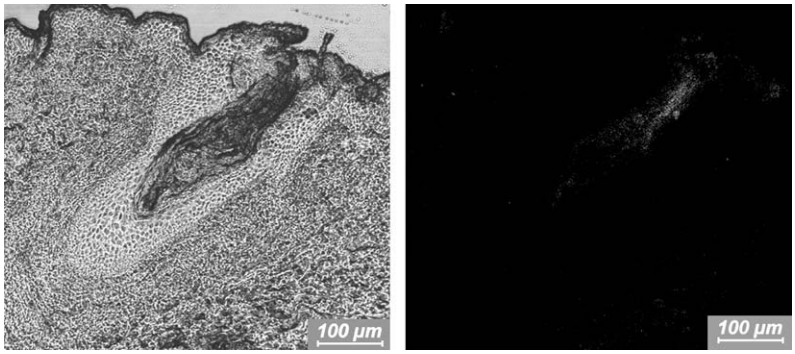

Fig. 3. Native (left) and fluorescent (right) figure of a histological 10 µm section of human excised skin with a weak fluorescence signal as found for non-pre-treated human skin after application of fluorescent-labeled pollen allergens.

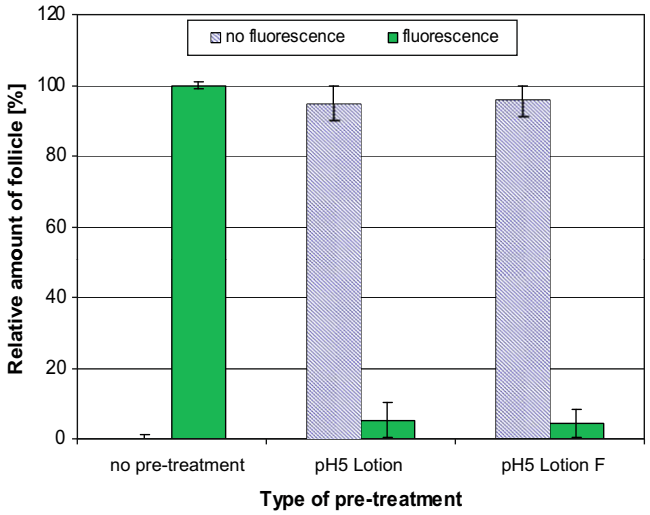

Fig. 4. Relative amount of follicles with or without fluorescence of pollen allergens in relation to all investigated vellus and terminal human hair follicles in the respective area. The results are shown for non-pre-treated, lotion 1 and lotion 2 pre-treated human skin. For both the lotions, no differences were found.

4. Discussion

Excised human skin can be only doubtfully considered as a suitable model for follicular skin penetration. Excision of skin leads to skin damage due to dehydration and contraction amongst others. The results of the present investigation may be able to emphasize the suggestion that during re-stretching only the interfollicular elastic fibres will be decompressed, whereas the circular elastic fibres around the hair follicles remain contracted [22]. When comparing the results obtained from the investigation performed on human facial skin and on porcine skin, it becomes conspicuous that the fluorescent intensity in the non-pre-treated areas of human skin was as weak as in the case of the weak signal of the pre-treated porcine skin.

Differences concerning the efficacy of the two different barrier formulations, lotion 1 and lotion 2, could only be detected on porcine skin. In the case of the non-pre-treated human facial skin, only a weak fluorescence signal was detected, possibly on account of the contraction of the hair follicles.

In the case of the pre-treated human facial skin, no fluorescent signal was detectable in the hair follicles. This may be due to contraction of the hair follicles on the one hand and to the barrier emulsions on the other hand, which are able to close the hair follicle orifices and prevent penetration. For the weak fluorescence signal, the sensitivity of the LSM is close to the detection limit, further differentiation was not possible.

These results must be considered as first indications that porcine ear skin might be a better model for the analysis of follicular penetration than the excised human skin.

In the case of the porcine ear skin, the physiological conditions of the tissue can be kept almost constant even after removal of the ears. The skin barrier is not interrupted, dehydration is decelerated, and contraction does not take place [23]. Because of the larger follicular size on porcine ear skin, an over-estimation of the penetration of the pollen allergens into the hair follicles can be expected when interpreting the results, whereas interpretation of the results obtained from excised human facial skin will lead to under-estimation of the potential risks. For the development and evaluation of prevention strategies and for risk assessment, an in vitro over-estimation is safer than under-estimation, so that under in vivo conditions on human skin, even better results can be expected than on porcine ear skin.

A comparison of the penetration depths (Fig. 5) shows that the penetration depth of the allergens into the terminal hair follicle is comparable to the one of the porcine hair follicle, whereas the pollen allergens inside the vellus hair follicle shows only half of the penetration depth. The obtained penetration depths of the pollen allergens in the vellus and terminal hair follicles correlate with the total lengths of the infundibulum of the vellus and terminal hair follicles at this body area of 225 ± 34 µm and 646 ± 140 µm, respectively [24]. The length of the infundibulum of the porcine hair follicle was determined by Jacobi et al. [23] to be between 500 and 659 µm, and is therefore comparable to that of the terminal hair follicle. Also, the penetration depths into human terminal

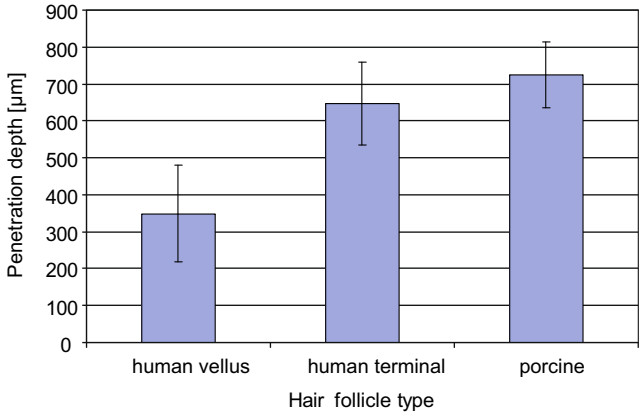

Fig. 5. Comparison of the penetration depths of the different hair follicle types: human vellus and terminal hair and porcine hair follicles. The penetration depths of the allergens into human terminal and porcine skin hair follicles were comparable.

hair follicle and porcine hair follicle were shown not to be significantly different (Wilcoxon test, $p = 0.328$). The measured marginal deeper penetration depth of the pollen allergens into the porcine hair follicle of 724 μm ± 89 μm compared to the total length of the infundibulum determined by Jacobi could be due to the strong fluorescence signal. Furthermore, the diameter of the infundibulum is comparable for porcine skin (173–230 μm) [23] and for human terminal hair follicles (172 ± 70 μm) [24]. Therefore, porcine skin should be a very good model for human skin with terminal hair follicles.

The results obtained in the present study are relevant for the in vitro analysis of dermatopharmacokinetics for topically applied substances. Such experiments are frequently carried out using diffusion cells [25–29]. This involves the topical application of substances to human skin membranes, several hundred micrometers thick, to investigate their penetration and storage characteristics. The follicular penetration pathway, which is an efficient route for a number of substances [14], was not investigated or considered in these experiments. In many cases, it is difficult to draw any conclusions from the results obtained under in vitro conditions and relate them to the in vivo situation.

Moreover, assuming that the contraction of the elastic fibres around the hair follicles actually takes place, this might be an explanation why diffusion cell experiments may work concerning intercellular penetration investigations. The length of a terminal hair follicle can be up to 3900 μm [24], whereas split skin samples utilized for diffusion cell experiments are usually not thicker than 1000 μm. This means that the hair follicles have to be considered as straight tunnels leading through the skin sample directly into the receptor medium. Only the contraction of the elastic fibres around the hair follicles can prevent an immense and straight transfollicular penetration.

5. Conclusion

In contrast to the results obtained from porcine ear skin, no differences were found between the barrier emulsions in the prevention of follicular penetration of pollen allergens using human excised skin. On the other hand, the penetration depths of the pollen allergens show comparable results for human terminal and porcine hair follicles. Summarizing the results, it can be concluded that for the analysis of the follicular penetration porcine ear skin is a more suitable in vitro model than the excised human skin.

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