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Research paper

# Differential stripping demonstrates a significant reduction of the hair follicle reservoir in vitro compared to in vivo

Alexa Patzelt [a,*], Heike Richter [a], Rolf Buettemeyer [b], Hans Joachim Roewert Huber [a], Ulrike Blume-Peytavi [a], Wolfram Sterry [a], Juergen Lademann [a]

[a] Department of Dermatology, Charité – Universitätsmedizin Berlin, Berlin, Germany
[b] Department of Surgery, Charité – Universitätsmedizin Berlin, Berlin, Germany

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**Abstract**

Penetration studies are commonly performed on in vitro models, presumably due to a lack of non-invasive in vivo methods. To date, it is not clear whether in vitro models are suitable to reflect the in vivo conditions for percutaneous penetration. Apart from inter and intraspecies skin differences, the excision of a skin sample may influence the penetration rate inter alia as a result of the contraction of the elastic fibres in the skin during excision. Therefore, the aim of the present study was to investigate the follicular reservoir of the hair follicles of human skin in vivo and in vitro utilizing the method of differential stripping. The results obtained revealed a significantly reduced follicular reservoir in vitro, which was only $9.5 \pm 10.6\%$ of the in vivo reservoir. These results are important for the interpretation of earlier *and* future penetration investigations. It can thus be assumed that excised skin models are suitable for penetration studies only to a limited extent, as follicular penetration is greatly diminished due to the contraction of the elastic fibres of the skin.


*Keywords:* Follicle penetration; In vivo; In vitro skin model; Hair follicle reservoir; Elastic fibres

## 1. Introduction

In recent years, hair follicles have attracted much attention as it has been shown that they are important for percutaneous penetration of topically applied substances and for drug delivery [1–4].

The former assumption that hair follicles are not relevant for penetration as they cover only 0.1% [5] of the skin surface has been abandoned. Instead, investigations have shown that the hair follicle reservoir greatly varies on different body sites and is comparable to the reservoir of the stratum corneum (SC) in some body areas [6]. In addition, the hair follicles are currently esteemed to represent an important target for drug delivery as they are surrounded by a close network of blood capillaries and dendritic cells [7]. The relevance of the hair follicles for the percutaneous penetration process has been identified in several investigations utilizing a multitude of in vivo and in vitro models [1,2,4,8–12].

Generally, apart from the tape stripping procedure and the determination of blood and urine levels, non-invasive in vivo methods are seldom available for penetration studies [13]. Therefore, in vitro investigations are usually performed for ethical reasons. Common in vitro models are, for example, the Franz diffusion cell utilized with split or full skin, the Saarbruecken penetration model, the porcine ear skin model, and other animal models [14]. Recently, two in vivo methods have been introduced to investigate follicular penetration: the selective blockage of hair follicles [15] and the differential stripping procedure [11].

Nevertheless, the question remains whether in vitro models are able to reflect in vivo conditions. Rare inves-

* Corresponding author. Center of Experimental and Applied Cutaneous Physiology, Department of Dermatology, Charité – Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany. Tel.: +49 30 450 518 106; fax: +49 30 450 518 918.
*E-mail address:* alexa.patzelt@charite.de (A. Patzelt).

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tigative approaches correlating in vivo and in vitro data revealed in vitro penetration values, which were twice the amount of the in vivo values [14]. Differences in size, type and density of the follicles, lipid composition and stratum corneum thickness have been used to explain this discrepancy [14]. In order to obtain a representative comparison of in vivo and in vitro data, skin of the same species and body area should be used, ideally from the same donor. Irrespective of inter-and intraindividual skin differences, the excision of the skin may also have an influence on the penetration rate. After cutting a piece of skin *and* removing the subcutaneous fat tissue, the skin contracts to a certain degree. This may be due to several physiological factors such as water loss and the sudden absence of blood flow. Additionally, the elastic fibres which endow the skin with resilience [16] presumably contract after being cut, acting as a severed rubber band. On the one hand, this means that the hair follicle density per $cm^2$ increases, possibly influencing the follicular penetration rate. This could, however, be easily avoided by stretching the skin to the initial size before investigation. On the other hand, when considering the distribution of the elastic fibres which closely surround the hair follicles [17], it is highly probable that after cutting the skin, the hair follicles are constricted by the contracting elastic fibres and, therefore, are significantly less receptive for the penetration process. Because of the close network of the elastic fibres, stretching of the skin would presumably not reopen the hair follicles.

The aim of the present study was to verify the assumption that in excised skin, the hair follicle reservoir is significantly reduced. Therefore, we utilized the recently introduced method of differential stripping [11] to compare the follicular reservoir of human skin, in vivo and in vitro, on the same donor and body site. Furthermore, histological investigations were performed to show the distribution of elastic fibres in human skin.

## 2. Materials and methods

In the present study, the reservoir of the hair follicles was investigated in vivo and in vitro. Therefore, the skin of the same patients and the same skin sites was utilized both for the in vivo and the in vitro investigations. Two study designs were applied and histological investigations followed subsequently.

*Study design A:* In vivo investigation of the hair follicle reservoir of humans in the region of the abdomen or the inside of the thigh by means of differential stripping.
*Study design B:* In vitro investigation of the hair follicle reservoir of the corresponding contralateral excised skin sample of the same volunteers by means of differential stripping.
*Study design C:* Histological investigation of the fibre distribution around and between the hair follicles.

### 2.1. Volunteers/excised skin

The study was performed in vivo on six female volunteers, mean age $43.5 \pm 11.3$ years. The volunteers were all patients at the Department of Surgery, Charité – Universitätsmedizin Berlin, suffering from adiposis, and planned to receive a reduction of their adipose tissue in the region of the abdomen (5 volunteers) or the inside of the thigh (1 volunteer).

One day prior to the surgical intervention, study design A was applied in vivo on one body side. After surgery, the excised skin of the corresponding contralateral body area was utilized for performing the in vitro investigations (study design B). The excised skin was analyzed immediately after removal and, therefore, there was no necessity for fixation or inlaying.

Approval for these investigations had been obtained from the Ethics Committee of the Charité. The study was conducted in compliance with the ethical rules stated in the Declaration of Helsinki Principles. The volunteers participating in the study had given their informed and written consent.

### 2.2. Topically applied substance

For investigation of the follicular reservoir, an o/w formulation containing nanoparticles of 100 nm was applied. The formulation contained 0.12% of the lipophilic dye curcumin. A particle containing substance was chosen because of its preferential penetration into the hair follicles [18].

### 2.3. Study design A, in vivo, preoperative

#### 2.3.1. Application of the topically applied substance

A skin area of $4 \times 4$ cm was marked in the region of the abdomen or the inside of the thigh using a permanent marker. Subsequently, 2 $mg/cm^2$ of the emulsion was applied and distributed homogeneously within the demarcated skin area utilizing a saturated glove finger.

#### 2.3.2. Differential stripping

After a penetration time of 30 min, differential stripping was applied to determine the follicular reservoir as described previously [11]. Differential stripping is a combined method of tape stripping and cyanoacrylate skin surface biopsies. The tape stripping procedure was performed as described by Weigmann et al. [19]. Adhesive tape strips (*tesa* No. 5529, Beiersdorf, Germany) were pressed onto the skin using a roller, which stretches the skin surface hereby *avoiding* the influence of furrows and wrinkles [20]. The tape strips were then removed with a quick movement. This process was repeated until the topically applied substance was completely removed from the stratum corneum (after an average of 10 tape strips). This could be detected visually by a disappearing fluorescent signal, using laser scanning microscopy (LSM 2000, Carl Zeiss, Jena, Germany). Subsequent to the SC removal, a drop of cyanoacrylate superglue (UHU

GmbH, Brühl, Germany) was applied onto the stripped skin area and was covered with a further tape strip using light pressure. After polymerization of the superglue (approximately after 5 min), the tape strip was removed and a cyanoacrylate skin surface biopsy was obtained containing the follicular casts and corneocytes.

#### 2.3.3. Quantitative analysis of the cyanoacrylate skin surface biopsies

After removal, the cyanoacrylate skin surface biopsies were punched to a constant size of 15 mm diameter and extracted in ethanol (Uvasol, Merck, Darmstadt, Germany) using ultrasound (Sonorex Super RK102H, Bandelin Electronic, Berlin, Germany) and centrifugation (at rcf = 1487, 36*g* fr 10 min at 20 °C, Centrifuge MR1812, Jouan GmbH, Unterhaching, Germany). This was followed by the determination of the relative concentration in arbitrary units of the curcumin using fluorescent measurements (Luminescent LS 50B, PerkinElmer, Überlingen, Germany). The maximum fluorescence intensity was detected at a wavelength of 510 nm.

### 2.4. Study design B, in vitro, postoperative

#### 2.4.1. Excision of skin

After anaesthesia of the patient, but before excision of the skin, a jig of 4 × 4 cm was applied onto the corresponding contralateral skin region to that used for the in vivo experiments, and the contour of the jig was carved into the skin. The skin was then excised during surgery.

#### 2.4.2. Preparation of excised skin

After excision of the skin, the majority of the adipose tissue was removed apart from the upper 1 cm. As the skin contracts after excision, in order to receive the original skin size, the jig of 4 × 4 cm was applied again and the skin was stretched until the carved skin area was of identical size with that of the jig. The excised skin was fixed in this position.

#### 2.4.3. Application of the topically applied substance

Within the carved skin area of 4 × 4 cm, 2 mg/cm$^2$ of the formulation was distributed on the skin surface utilizing a saturated glove finger. In contrast to the in vivo experiments, the formulation was applied by means of a massage appliance, as it has been shown that massage substitutes the physiological movement of the hairs in the follicles, which is essential for an optimal penetration of particles into the hair follicles [18].

Then, differential stripping was performed analogously to study design A.

### 2.5. Study design C

Biopsies of 8 mm in diameter were sampled from human skin in vivo under local anaesthesia. The samples were fixed in a formaldehyde solution and embedded in paraffin. Sections were cut vertically to the skin surface and stained with Elastica-van-Gieson-staining, conforming to histological standard procedures.

### 2.6. Data analysis

For a better comparison of the obtained data, the relative preoperative concentrations of *curcumin* were set at 100% and compared with the postoperative values.

For statistical analysis, we utilized the Kolmogorov–Smirnov test (SPSS 11.0) to demonstrate that the obtained data were not not-normal distributed ($p > 0.05$). We then calculated the mean values and standard deviations of the recovered concentrations of the curcumin. Furthermore, we utilized the Wilcoxon test to show significant differences between pre-and postoperative values ($p < 0.05$).

## 3. Results

The aim of the present investigation was to demonstrate differences between the follicular reservoir in vivo and in vitro by means of differential stripping. Furthermore, histological analysis was performed to characterize the fibre distribution between and around the hair follicles.

The comparison of the follicular reservoir of in vivo and in vitro skin revealed that the follicular reservoir of excised in vitro skin is only 9.5 ± 10.6% of the follicular reservoir in vivo. These results are presented graphically in Fig. 1. Statistical evaluation of the data exposed a significantly decreased follicular reservoir in vitro ($p < 0.05$).

The evaluation of histological sections (see Fig. 2) revealed that the elastic fibres are circular in the region of the hair follicles (see white arrow), while they are straighter and more parallel in the interfollicular region of the skin (see black arrows).

## 4. Discussion

The present investigation of the follicular reservoir using in vivo and in vitro methods confirmed the assumption that

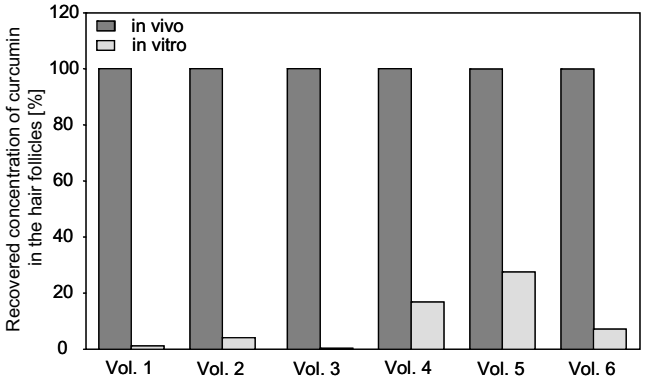


Fig. 1. Comparison of the recovered concentrations of curcumin in vivo and in vitro.

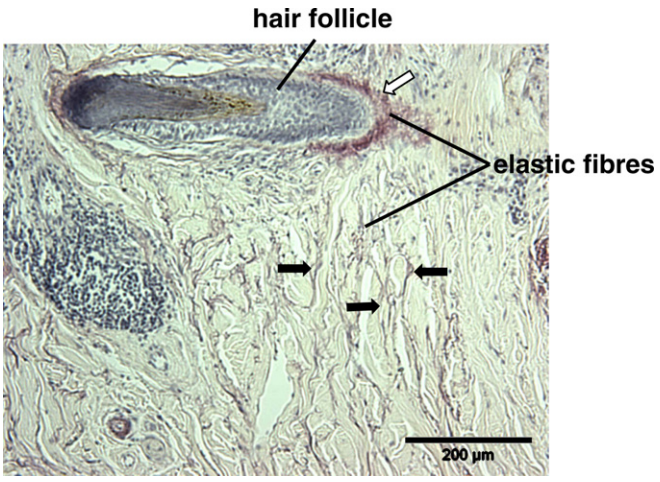


Fig. 2. Histological section of human skin in Elastica-van-Gieson-staining. The distribution of elastic fibres is rather circular in the region of the hair follicles (see white arrow) while they are straighter and more parallel in the interfollicular region (see black arrows).

the follicular reservoir is significantly reduced in the in vitro skin model. Histological investigations gave implications for a possible explanation of this phenomenon. The section of the hair follicles (Fig. 2) shows that the hair follicle is surrounded by a relatively close network of elastic fibres, whereas the elastic fibres in between the hair follicles are straighter and more parallel. This observation is in concordance with investigations of Starcher et al. [17]. They investigated the distribution of elastic fibres in avian and mammalian skin including human skin. Avian skin is an exaggerated example of how elastic fibres in the skin possess a primary role in the movement of skin covering. Elastic fibres surround the feather follicles and connect them to large smooth muscle bundles with an elastic fibre linkage. The follicles are also connected to each other with elastic fibres. No additional elastic fibres throughout the skin were found which might contribute to skin elasticity.

The elastic fibres in mammalian skin essentially perform the same function as the elastic fibres in avian skin. The muscle M. arrector pili is more slender in mammals, however, possibly due to the fact that the forces required to raise a hair are lower than those needed to move and keep a feather in place during flight. The hair follicles of most animals are surrounded by an elastic fibre network, which connects the follicles to each other. This is also the case in humans and pigs but to a lesser degree, as the fibres not only surround the hair follicles but also lie as parallel fibres throughout the entire dermis. This different pattern of elastic fibres suggests an important role in the elasticity of skin [17].

The present investigation and the observations by Starcher et al. [17] emphasize the assumption that the elastic fibres surrounding the hair follicles contract if the skin is excised, possibly leading to a significant reduction of the follicular penetration pathway. However, other factors concerning the reduced follicular penetration must also be taken into consideration, such as loss of humidity and the absence of blood flow in excised skin. These aspects presumably additionally contribute to the differences in the penetration pathways to a certain extent.

Although the mechanisms leading to the reduced follicular reservoir have not been fully clarified, the reality of the topic cannot be disregarded. Nevertheless, several consequences for in vitro investigations should be derived from the observations of the present investigation. For penetration studies, excised human skin is suitable only to a limited extent as the follicular reservoir for penetration is significantly reduced. This is especially valid in regard to follicular penetration studies, or if substances are applied which preferentially penetrate into the hair follicles, such as particulate substances [18,21]; or caffeine [17,22], which utilizes the hair follicles as a fast penetration pathway into the blood.

Generally, porcine skin is a frequently used model for human skin [23], showing a similar composition and penetration behaviour for topically applied substances [24] as well as a comparable arrangement of elastic fibres [17]. Nevertheless, it must be admitted that stratum corneum thickness, follicular density and size are increased in porcine skin [14]. Such variations can, however, also be observed in the different body regions of humans [6]. In this context, Feldmann et al. [25] for example observed that the absorption of hydrocortisone is increased in regions with large or numerous hair follicles.

In regard to the difficulties concerning elastic fibre contraction, the porcine ear skin model – if not utilized in Franz diffusion cells – is probably the most advantageous, as the skin is not excised but, rather more, fixed on the cartilage which inhibits contraction. For other in vitro animal models, the contracting phenomenon of the elastic fibres may even be increased as studies have reported the concentration of elastic fibres mainly around the hair follicles [17]. In general, the present investigation revealed that results of in vitro penetration studies utilizing excised skin such as Franz diffusion cell experiments must be regarded critically.

In conclusion, the results of this investigation revealed that excised human skin is suitable only to a limited extent for penetration investigations, as the elastic fibres, which are cut during excision, seem to constrict the follicular openings and thus reduce the follicular reservoir and penetration pathway. To overcome this problem, the development of non-invasive in vivo methods should be emphasized. If the application of in vivo models is not feasible, the utilization of the skin of a complete porcine ear seems to represent an appropriate alternative. However, a reduction in the follicular reservoir is highly presumable for Franz diffusion cells requiring the removal of the skin from the cartilage, as well as in excised skin.

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