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ORIGINAL ARTICLE

First *ex vivo* cultivation of human Demodex mites and evaluation of different drugs on mite proliferation

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Abstract

Background *Demodex* spp. mites are the most complex resident of the human skin microbiome. Although they are considered commensals, they can be pathophysiologically relevant in inflammatory skin diseases like rosacea. Until now, there is no culture system available for these mites except for using live vertebrate hosts.

Objectives Our aim was to establish an *ex vivo* culture of human Demodex mites and to characterize the sebogenesis-dependent mite density.

Methods Demodex mites were cultivated in pilosebaceous units of human skin explants, called human organotypic skin explant culture (hOSEC). Furthermore, different sebogenesis-modifying additives were evaluated. Mites and mite survival were evaluated using light and fluorescence microscopy.

Results After 90 days of incubation, living Demodex mites – including eggs, larvae and nymphs – were detected in the dissected skin samples. Incubation for 30 days with anabolic steroids (testosterone and trenbolone) as well as retinol and retinoic acid (isotretinoin) yielded a reduced mite density.

Conclusions With this technique, mites can be cultivated *ex vivo* for the first time, thereby establishing new ways to investigate *Demodex* spp. The sebostatic effect of isotretinoin might explain the mechanism of action in the off-label treatment of rosacea. We anticipate our findings to be the basis of an accelerated research on our most complex commensal, its life, biology and physiology.

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Conflict of interest

The authors declare no conflicts of interest.

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Introduction

Demodex mites are the most complex residents of the human facial skin but also in many other mammals.[1] Excessive mite proliferation, being favoured by several factors, such as decreased host immunity, sebaceous hyperplasia and/or erythematotelangiectatic rosacea, can lead to pathophysiologically relevant skin conditions.[2] Although the mites were first described over 180 years ago,[3] little is known about their life, biology and physiology.

Many researchers studied their life cycle,[4] though in 1961 S. G. Spickett was the first to analyse it comprehensively and in very much detail, although the two co-existing human mite species (*Demodex folliculorum* and brevis) were not described yet and hence Spickett could not differentiate between them.[5] He extracted the mites at various life stages and investigated each stage individually. This led to the reconstruction of the whole life cycle of Demodex mites, which he estimated to be about 14.5 days in total. However, he failed to cultivate the mites for a prolonged time. Also Grosshans *et al.*[6] investigated Demodex mite cultivation without specifying their employed methods, but they were not successful. Another study investigated the effect of various media (paroleine, fat from pigs, human serum, 1640/seroculture solution, normal saline and blank) and temperatures (16–22°C, 25–26°C, 36–37°C) on mite survival; however, eventually all mites died.[7] Furthermore, in a study published by us in 2018, we introduced Leibovitz's L-15 medium to keep Demodex mites alive and record survival curves (to evaluate propidium iodide as a fluorescent indicator of death), which led to a survival up to 14 days – however, none of the mites reproduced, so eventually all mites died presumably of old age.[8]

Up to now, only Tani *et al.* and Caswell *et al.* published studies where canine Demodex mites supposedly reproduced in a non-host-bound *in vivo* set-up. In these studies, canine Demodex mites were transferred to skin explants of dogs and hamsters, which in turn were engrafted into severe combined immunodeficiency (SCID) mice. Mice were sacrificed 90 days after skin grafting and the xenografts were analysed histologically. Microscopic examination showed mite proliferation in both types of skin.[9,10]

These results indicate that Demodex mites presumably require their specific hosts pilosebaceous units (though some Demodex species might also accept other types of skin: e.g. a case report where canine Demodex mites caused demodicosis in a ferret[11]), but do not necessarily need host-specific components from their host's bloodstream.

As there are well-established *ex vivo* human skin explant techniques, we envisioned describing the first *ex vivo* Demodex mite cultivation model independent of vertebrate host animals, by using a human organotypic skin explant culture (hOSEC) system for mite proliferation.[12,13] Additionally, to substantiate the claim that sebum consistency might influence mite proliferation (authors' observation), skin samples were treated with sebogenesis-modifying drugs (anabolic steroids and vitamin A derivatives).

Materials and methods

Chemicals

All chemicals and buffers were purchased from Sigma Aldrich (Steinheim, Germany) except where noted otherwise.

Skin samples

The use of fully anonymized, excised excess facial skin from the operating room of the department of dermatology at the Ludwig-Maximilian-University in Munich, Germany, was approved by the local ethics committee (project 18-671 UE). The skin was kept in a humid environment and processed on the same day.

Nomenclature

In our study, we used a simplified nomenclature based on the nomenclature of Spickett for the purpose of faster handling and counting of the mites.[5] We identified six-legged immature forms (larva and protonymphs), which we call 'larva', and eight-legged immature forms (deutonymphs), which we call 'nymphs'.

Ex vivo cultivation

Facial skin explants were decontaminated with 70% ethanol on the epidermal side, excessive subcutaneous fat was removed and larger skin pieces were cut to discs with a 6 mm biopsy punch. The glass cylinders (height 10 mm) needed were prepared in advance by cutting four small notches into one side to enable contact between the surrounding cell culture media and the inside of the cylinder, then the cylinders were sterilized by steam autoclaving them. The resulting skin samples were put upside-down inside the small glass cylinder with the identical inner diameter (Fig. 1a). The cylinders were then turned upside-down, the epidermis glued to the glass border using ethyl 2-cyanoacrylate ('super glue') and then incubated in DMEM medium with 10% (v/v) foetal calf serum and antibiotics and antimycotic (100 µg/mL clindamycin, 500 µg/mL aztreonam and 100 U/mL nystatin, selected to match the endobacterial antibiotic susceptibility as published earlier[14]) in 12 well plates at 37°C with 5% CO_2. Care was taken to avoid trapped air bubbles below the skin explant impeding the nutrient supply. This set-up was chosen with the aim to ensure the most physiological environment for the samples by keeping the epidermis as dry as possible (Fig. 1b). The medium was changed every other day to prevent contamination. In case of contamination the glass cylinder was detached, the skin sample disinfected in 70% ethanol and a new glass cylinder was attached to prevent persisting contamination due to biofilm formation. A total of 36 samples were used. All of them were scanned for mites after incubation for

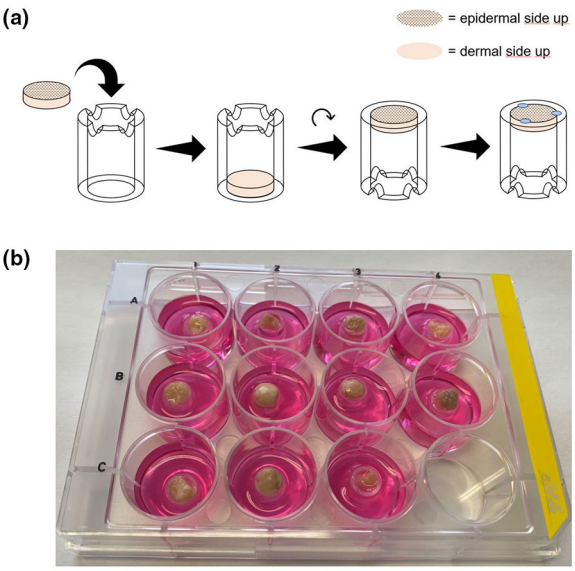

Figure 1 (a) Depiction of the mounting process of the skin samples (rosé-coloured disc) in the glass cylinder. The inverted skin sample (epidermal side down) is inserted to the inverted cylinder, then the cylinder is flipped and the skin sample is carefully attached to the cylinder with super glue (light blue ovals). (b) 12 well plate without lid showing 11 mounted skin samples in DMEM medium with additives (pink solution; freshly added, so not pH-balanced by atmospheric 5% CO_2). The one sample per well strategy is used to avoid cross-contamination.

90 days at 37°C. In three randomly selected, mite-harbouring samples the number of eggs, larvae, nymphs and adults was counted.

Sebogenesis-modifying treatment

To test the effect of sebogenesis-modifying drugs, the skin explants were incubated as described above for 30 days with the following drugs (concentration): testosterone (0.1 mg/mL), trenbolone (0.1 mg/mL), retinol (0.1 mg/mL) and isotretinoin (13-cis-retinoic acid, 0.1 mg/mL). All drugs were prepared as stock solutions in 100% ethanol and were freshly added at every change of media.

To ensure a similar starting mite density, all punch biopsies from a single experiment were taken from the same piece of skin. As a negative control, one sample was incubated without added drugs. The experiment was performed in triplicate, and resulting data were expressed in percent relative to the negative control (set to 100%) to enable comparison of the results.

Sample work-up

After 30 or 90 days of incubation (for drug testing or culture model establishment respectively) the samples were examined. The skin was detached from the glass cylinders and incubated in dispase (5000 caseinolytic units per 10 mL working concentration) solution at 4°C overnight. On the next day, the epidermis was carefully detached from the dermis using fine forceps. The epidermis was then transferred into a 10-mm tissue culture insert with a 8.0-µm pore polycarbonate membrane (Nunc, Roskilde, Denmark), carefully cut to smaller pieces and further digested with trypsin–EDTA-solution used for cell culture (0.5 g porcine trypsin and 0.2 g EDTA per litre of Hanks' Balanced Salt Solution) for 15–30 min at 37°C. To facilitate removal of smaller particles by capillary action, paper towels were pressed against the bottom of the insert. The remaining mites and larger debris particles were washed with phosphate buffered saline (PBS) and stored in 12 well plates in PBS until evaluation by microscopy.

Brightfield and fluorescence microscopy

Fluorescence microscopy was performed using an inverted Axio Observer 7 microscope (Carl Zeiss AG, Oberkochen, Germany) with 100–200× total magnification and the ZEN Imaging Software (Zeiss). Samples were imaged using either visible (VIS)- or UV-light with a blue (DAPI) (Filterset 02 or 49, Zeiss) or green (GFP) filter set (Filterset 38, Zeiss). Autofluorescence of the mites aided locating the mites among the debris of the digested samples in the transwell inserts. To determine viability of adult mites, movement of legs and chelicerae was evaluated. If an individual mite showed no movement after 1 min, it was considered dead. Eggs, larvae and nymphs were identified morphologically as they mostly show no movement.

Statistical analysis

For statistical analysis of the resulting data GraphPad Prism® 5.01 (GraphPad Software, Inc., La Jolla, CA, USA) was used. Statistical analysis was performed using the paired Student's *t*-test. A P-value < 0.05 was considered statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). All data are representative of at least three independent experiments.

Results

Ex vivo cultivation

After 90 days (representing approximately six generations) of incubation living Demodex mites were detected in 25 of 36 samples (69.4%). The 11 samples without mites did not have any mite exoskeletons, so presumably the skin did not contain any mites from the beginning of the experiment. Three samples with living adult mites were digested and all stages of development were counted. Eggs were found to occur sparsely (up to 6.3%), larvae were found up to 12.5%, nymphs up to 17.7% and mostly adult mites up to 76.5% (Table 1).

Sebogenesis-modifying treatment

Seborrhoeic drugs After 30 days (representing approximately two generations) of incubation with the seborrhoeic drugs testosterone and trenbolone (an anabolic steroid known to cause severe seborrhoea and acne in bodybuilders), the number of viable mites in three independent experiments decreased significantly compared to untreated samples (testosterone $P = 0.0286$, trenbolone $P = 0.0024$). The mean difference to the untreated control was 39.6% for testosterone and 55.7% for trenbolone (Fig. 2a).

Sebostatic drugs After 30 days (representing approximately two generations) of incubation with the sebostatic drugs retinol (precursor to retinoic acid) and isotretinoin (13-cis-retinoic acid), the number of viable mites in three independent experiments decreased significantly compared with untreated samples

Table 1 Three randomly selected, mite-harbouring samples were digested to count pre-adult and adult Demodex mites. The last column shows the sum of all three samples for each stage, the last line shows the total number of mites in each sample and the sum. Percentages are given in brackets in regard to the total number of mites in each sample (or the sum in the last column)

Stage	Sample 1	Sample 2	Sample 3	All samples
Egg	1 (6.3%)	1 (5.0%)	0 (0.0%)	2 (3.8%)
Larva	2 (12.5%)	1 (5.0%)	1 (5.9%)	4 (7.5%)
Nymph	1 (6.3%)	3 (15.0%)	3 (17.7%)	7 (13.2%)
Adult	12 (75.0%)	15 (75.0%)	13 (76.5%)	40 (75.5%)
Total per sample	**16**	**20**	**17**	**53**

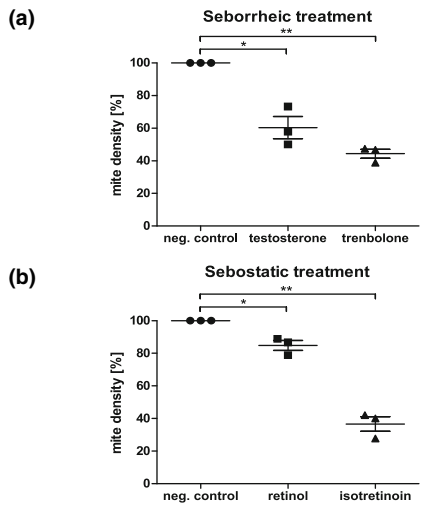

Figure 2 Graph showing the differences in mite density (mites per 0.283 cm^2 epidermis, equal to a circle with a diameter of 6 mm) in percent compared with a non-treated (negative) control. (a) With seborrheic treatment, testosterone and trenbolone led to a significantly decreased mite density ($P = 0.0286$ and $P = 0.0024$ respectively). The mean difference to the untreated control was 39.6% for testosterone and 55.7% for trenbolone. (b) With sebostatic treatment, retinol and isotretinoin led to a significantly decreased mite density ($P = 0.0378$ and $P = 0.0049$, respectively). The mean difference to the untreated control was 15.2% for retinol and 63.4% for isotretinoin, with isotretinoin having the strongest mite decreasing effect of all sebogenesis-modifying treatments.

(retinol $P = 0.0378$, isotretinoin $P = 0.0049$). The mean difference to the untreated control was 15.2% for retinol and 63.4% for isotretinoin, with isotretinoin having the strongest mite decreasing effect of all sebogenesis-modifying treatments (Fig. 2b).

Discussion

By being able to detect Demodex mites in the samples after 90 days of incubation, we were able to prove that Demodex mites can be cultivated in an *ex vivo* hOSEC. Andrade *et al.* describe hOSEC skin cells to actively divide up to 75 days in culture, as shown by immunohistochemical staining of Ki-67.[12,13] Assuming the lifespan of a Demodex mite is an estimated 14.5 days,[5] the mites found in the samples are therefore surviving and procreating in at least the sixth generation without a living vertebrate host. The 11 samples not containing *Demodex* spp. can possibly be assumed mite-free from the beginning.

Spickett described the life cycle of Demodex mites to start in the depth of the pilosebaceous unit. Here, the egg is laid by a female mite, which crawled deep into the follicle for oviposition. By moulding, the egg develops into a larva and subsequently a protonymph as it is extruded from the follicle with the flow of sebum. Then, the protonymph moulds to become a deutonymph. Both stages can be distinguished by the number of legs – 6 and 8 respectively – but only the latter displays leg movement and can actively move around. When the microanimal reached the follicle infundibulum, a final moulding step results in an adult, which is able to effectively yet slowly move around and conquer new habitats.[5]

Our experiments show the presumed manipulation of sebogenesis with different drugs can severely impair this intricate mechanism, a shortcoming of this study is however that we did not quantify the sebogenesis-modifying effect of the drugs. Seborrhoea is thought to lead to increased sebum flow, as the sebum is less viscous, this might lead to the mites getting flushed out of the pores faster, so they cannot develop and mould fast enough.

On the other side, sebostasis might slow down the flow of sebum and hinder the penetration of the pilosebaceous unit by the female mite for oviposition, as the pore contents are too firm. If the mite reaches sufficient depths of the pilosebaceous unit in this scenario; however, the lack of sebum flow would not inhibit mite development, as the resulting adult mite would be agile and strong enough to leave the pore (as was the parent mite to enter). Additionally, isotretinoin is known to atrophy sebaceous glands as well as the whole pilosebaceous follicle, which thereby could especially hinder *Demodex folliculorum* and brevis proliferation. This seems confirmed by our study which observed a reduced mite density with isotretinoin. Nevertheless, we did not study the potential atrophying action of isotretinoin on the hOSEC system, which would be interesting to investigate in future studies.

Both sebogenesis-modifying treatments led to decreased mite numbers; however, the only drug clinically employed for the treatment of rosacea (off-label) had the strongest effect, that is, the highest mean difference compared with the untreated control.

As Gao *et al.*[15] showed (and our observations confirmed) the short-term use of high-ethanol concentrations (70% used for disinfection of the skin explant) did not harm the mites in a substantial way. The drugs, dissolved in 100% ethanol, were added to the medium and thereby diluted.

One shortcoming of this study is the pronounced maceration of the epidermal layer towards the end the cultivation process, especially after 90 days at close to 100% air humidity. Future improvements might address this problem by exposing the epidermal side of the explant to a separate space with less air humidity. This in turn might lead to even higher mite survival and proliferation due to a more native setting.

Also, initially and occasionally (i.e. in certain time intervals) performed non-invasive imaging techniques could further improve and add insights to our culture model – for example, optical coherence tomography (OCT) or confocal laser scanning microscopy (CLSM). However, if these techniques are unavailable, even simpler techniques like UV-dermoscopy could reveal

some details, as published *in vivo* before.[16] Also, the lack of mite quantification in the beginning does not allow solid quantitative survival statistics; qualitatively however, mite survival was clearly shown.

When prolonged culture is desired, extraction of the mites and transferral to a new skin sample seems possible, as non-invasive imaging can be used to check for mites[17,18] and mites can efficiently be cryo-conserved.[19]

Being able to cultivate Demodex mites will establish a new way to investigate Demodex spp. It can accelerate new findings and ease future experiments by potentially losing the obstacle of having to have live vertebrate hosts to be able to do research on the most complex human commensal and its life, biology and physiology.

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Data availability statement

Data available on request from the authors.

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