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Porphyrin Distribution After Topical Aminolevulinic Acid in a Novel Porcine Model of Sebaceous Skin

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Background and Objective: Aminolevulinic acid photodynamic therapy (ALA-PDT) depends on drug metabolism into porphyrins. Clinically, ALA-PDT has been used with a wide range of protocols for treating both epidermal and dermal targets, despite limited understanding of porphyrin biodistribution over time. We studied porphyrin accumulation after topical application of ALA in vivo, and also describe the porcine ear as a new animal model to study adnexal glands.

Study Design/Materials and Methods: The microanatomy of anterior ear skin of swine was measured. Topical 20% ALA in water/ethanol was applied under occlusion. Biopsies taken after 5, 10, 15, and then every 15 minutes for a total of 3 hours were examined by fluorescence microscopy of frozen sections to assess accumulation and distribution of porphyrins.

Results: Porphyrin fluorescence of digital photomicrograph images was not visually apparent until 30–45 minutes after application, although quantitative pixel analysis showed a statistically significant increase in epidermal fluorescence only 15 minutes after ALA application. From 30 to 120 minutes, epidermis, hair follicles (HF), and sebaceous glands (SG) became progressively more fluorescent. Eccrine gland fluorescence began to be detected after 30 minutes; SG showed fluorescence starting at 45–75 minutes. Fluorescence in all sites reached maximum intensity from 75 to 180 minutes of incubation. There was a trend for HF and SG to express stronger fluorescence compared with epidermis and eccrine glands.

Conclusion: Anterior pig ear skin is microanatomically similar to human sebaceous skin. The time-dependent accumulation of porphyrins in pilosebaceous units and eccrine glands in this model suggests other routes of uptake of topical ALA in addition to the trans-epidermal route. Apparently, time interval between ALA application and light exposure could be optimized for different uses of ALA-PDT.
usually located at the site where the PS is accumulated, such that selective targeting of microscopic tissue structures is possible if the PS is localized. 5-Aminolevulinic acid (ALA) is a natural PS precursor, found in all human cells as part of the heme synthesis pathway [5]. When exogenously applied, ALA overdrives the pathway, producing porphyrins, the active PS. Because aminolevulinic acid photodynamic therapy (ALA-PDT) depends on drug delivery followed by metabolism into porphyrins, the distribution of PS within tissue varies with time after ALA administration, route of administration, and any factor that affects ALA metabolism.

ALA-PDT has been described for the treatment of various skin problems, including actinic keratoses [6], superficial skin cancers [7], psoriasis [8], warts [9], cutaneous T-cell lymphoma [10], acne [4], and cosmetic rejuvenation [11]. Topical 20% ALA (water/alcohol) solution for 12 hours followed by blue light exposure is currently FDA approved and commercially available in the US as a treatment for actinic keratosis, but in practice a wide range of application times and light sources are used. Methyl-ester of ALA is also used in Europe in similar fashion. For acne treatment, different protocols have been reported with variable clinical responses to ALA-PDT. A wide variety of blue, yellow or red lamps, LED sources and lasers have been used for ALA-PDT of acne. The time of incubation, measured from when the drug is applied until the activating light is given, has varied from 15 minutes [12] to 4 hours [13]. Despite clinical exploration of topical ALA-PDT for acne treatment under a wide variety of conditions, little is known about the kinetics and distribution of PS induced in sebaceous skin after topical ALA. We decided to study this in porcine sebaceous skin.

There is no ideal animal model for the study of acne. Animal models include the rabbit ear [14–16], Mexican hairless dog [17], rhino mouse [18], rat ear [18,19], and Syrian hamster flank organ [20], none of which simulate exactly the anatomical and physiological conditions involved in human acne. For studies of a topical agent such as ALA, penetration of the agent is affected by the stratum corneum and by density of hair follicles (HF). Rodent and furred animal models do not mimic human skin well in these regards. Pigs are well known to be a good cutaneous animal model, as they are non-furred, non-rodent animals with skin that resembles non-sebaceous human skin anatomically and physiologically [21–23]. Porcine skin has already been shown to be a good animal model for ALA-PDT studies in vivo [24]. Similar to humans, pigs are proven to metabolize ALA into porphyrins that can be directly measured (by porphyrin extraction) and/ or indirectly analyzed (e.g., by fluorescence photography or spectrofluorimeter measurements). However, the very small SG of swine skin are a major limitation to the use of this model for photodynamic studies related to sebaceous follicles. SG secrete wax esters, and tend to be prominent in mammalian ear skin. We therefore examined pig ears, found an abundance of large SG similar to those in human sebaceous skin, and performed a study of the kinetics and distribution of porphyrins synthesized from topical ALA.

### MATERIALS AND METHODS

#### Defining an Animal Model to Study Adnexal Glands and Porphyrin Metabolism

The anterior (inner) ear skin of two female adult Yucatan mini-pigs (retired breeders) (about 120 kg) and of three young female Yorkshire pigs (about 30–50 kg) were harvested for microanatomy analysis. Different types of pigs were used to verify the existence of same skin structures in these different breeds. Six random biopsies (4-mm punch) from the anterior of each animal’s ear (helix and lobule) were examined after H&E staining under light microscopy. The thickness of epidermis, size, and depth of HF, SG, and eccrine/apocrine glands (EG) were measured. Depth of the follicles and glands was measured relative to the bottom of the epidermis. For epidermis, relative maximum and minimum thickness were measured. For HF, SG, and EG the two maximum axial diameters were measured, and averaged. For comparison with human facial skin, three discarded preauricular skin samples from face lifts in adult females were processed and analyzed similarly, in addition to comparison with published microanatomy studies of human skin. These non-identifiable skin specimens had MGH Institutional Review Board approval to be used in this research.

#### Porphyrin Production After Topical Application of 20% ALA In Vivo

**Animals.** Three female domestic Yorkshire swine weighing 30–50 kg were used in the study, which was approved by the MGH Institutional Subcommittee for Research Animal Care. Animals were anesthetized with intramuscular (IM) Telazol/Xylazine (4.4 and 2.2 mg/kg) and inhaled Isoflurane 2.0% with 3.0 L/min in oxygen after fasting overnight. Temperature-controlled blankets were used under the animals to maintain core temperature while under anesthesia. Euthanasia was performed with pentobarbital 100 mg/kg IV while under anesthesia.

**ALA solutions.** 5-ALA hydrochloride from Biosynth International, Inc. (Naperville, IL) was prepared as a 20% solution in equal parts of ethanol/water for topical application of 0.5 cm² per 10 cm² skin area. After ALA administration, the skin was occluded with plastic sheeting (Saran wrap) and covered with aluminum foil, to avoid ambient light exposure during the experiment.

**Study design.** Each animal’s inner ears were cleansed with isopropanol, rinsed with water, clipped of hair, cleansed and rinsed again, dried with a towel. The drug was homogeneously applied onto the skin test sites (both ears of each animal), then occluded with Saran plastic wrap. Control sites were also occluded, but received no topical application. Biopsies for fluorescence microscopy were taken at baseline and after 5, 10, and 15 minutes of drug application; and then every 15 minutes for a total of 3 hours after ALA application. Four millimeter punch biopsies were separated from each other by at least 0.5 cm. Each biopsy site was kept occluded until immediately
before the biopsy, which took less than 1 minute, and covered immediately after the procedure.

**Fluorescence microscopy.** For each time after ALA application, two biopsies were taken and prepared as a fresh-frozen specimen for analysis by fluorescence microscopy. Biopsies were also taken and processed from the control sites. Frozen sections (15 μm thick) were made using a cryostat (Leica CM 1510S-3, Leica Microsystems Nusslock GmbH, Nussloch, Germany). Digital fluorescence photo-microscopy was performed using a Nikon Eclipse TE 2000-S (Tokyo, Japan) at 10× magnification, with 415 nm excitation and 635 nm emission band-pass filters. Images were captured using a light CCD camera (RT3® slider,Diagnostic Instruments, Inc., Sterling Heights, MI) without automatic gain control or other signal processing, and analyzed using software (Spot® software, Diagnostic Instruments, Inc.). For each biopsy, a series of two to four representative frozen sections containing at least one skin structure (epidermis, HF, SG, and sweat glands) was analyzed, including at least two slides containing each skin structure for each time of sampling. The photomicrography settings during fluorescence microscopy were manually set and kept the same for all samples (10 seconds of integration time), which were analyzed under low light conditions to minimize porphyrin photobleaching, and as a batch to minimize long-term instability of the microscopy source. For each batch, the excitation light was stable during the photograph acquisition, at an intensity of 6.5 mW/cm².

Direct fluorescence assessment through the microscope was avoided to minimize porphyrins photobleaching during the light excitation; therefore, the analysis of fluorescence was performed using the digitally acquired images only.

**Image analysis.** Experiment and control frozen section slides were transformed into microscopic fluorescent photographs at 10×. In each of those photographs, four 50×50 pixel square regions of interest (ROI) corresponding to 100 μm×100 μm of tissue, were extracted from epidermis, HF epithelium, SG, EG, dermis, and background (no tissue) for analysis. The ROI of each skin structure were randomly chosen sites that included as fully as possible the structure of interest. For HF, SG and epidermis, hair shafts, and stratum corneum were excluded from ROI to minimize errors due to the noted autofluorescence. Size of the ROI was chosen as a compromise between the goals of averaging over a sufficiently large region of the structures, and excluding adjacent or surrounding tissue from the ROI. Every ROI was analyzed using WCIF—Image J 1.39u software—sWayne Rasband National Institutes of Health, USA. This software transformed each ROI into numeric values of fluorescence intensity per pixel. Statistical analysis was performed using SPSS Version 15. (SPSS, Inc., Chicago, IL), Microsoft® Excel 2000, Microcal® Origin® software. Tests of normality were performed to determine the choice of statistical methods. From each structure ROI a correspondent background ROI value was subtracted to minimize potential errors due to environment light effects.

### RESULTS

#### Pig Ears Microanatomy

The results of the measurements of skin structures’ sizes and location are summarized and compared to human facial skin in Figure 1. In an area of 5 mm² the average number of HF, SG, and sweat glands were respectively about 40, 2, and 227 in young Yorkshire pig ears; and 30, 20, and 624 in adult Yucatan pig ears. Both adult and young pigs showed similar morphological structures compared to human facial skin (Fig. 2a,b), but young pigs had somewhat slightly smaller adnexal glands compared to adult pig and human skin.

**Fluorescence Microscopy Analysis**

Fluorescence was assessed subjectively as “present” or “not present” while analyzing the digitally acquired photomicrographs. Baseline and control (no ALA) samples showed weak autofluorescence of HF and stratum corneum only, attributed mainly to keratins but potentially including weak fluorescence from endogenous porphyrins. Porphyrin fluorescence was not visually apparent until 30–45 minutes after drug application and then increased with longer periods of incubation (Fig. 3).
Hair follicle and eccrine gland fluorescence began to be visually apparent after 30 minutes, while SG fluorescence was visually apparent beginning at 45 minutes. Epidermal fluorescence was visually greater than the baseline epidermal autofluorescence from 30 to 45 minutes of incubation, and was strongly visually apparent starting at 60–90 minutes. Visually apparent fluorescence was greatest at the longest incubation times of 135–180 minutes after ALA application at which time HF and SG fluorescence was apparently brighter than epidermal fluorescence. Quantitative analysis of porphyrin fluorescence allowed more accurate assessment of distribution and kinetics after ALA administration.

To confirm the assumption of normality of the results taken from the pixel analysis of fluorescence micrographs, we used the Kolmogorov–Smirnov (KS) test. Results indicated no significant departures from a normal (Gaussian shaped) distribution at any time point for sweat glands, SG, HF, epidermis, and dermis (all $P > 0.05$), with the exception of epidermis at 30 minutes which showed some evidence of skewness ($Z = 1.953$, $P = 0.001$). Even considering this exception, a parametric strategy was warranted and more appropriate in this context, particularly since the vast majority of the KS tests confirmed a normal distribution and revealed little evidence of serious outliers or extreme values. Therefore, parametric methods were applied, including $t$-tests and ANOVA (as well as regression models for assessing time related changes in fluorescence microscopy over time for each skin structure) (Fig. 4).
Table 1 indicates significant increases in fluorescence with incubation time (i.e., time since ALA application) for all five skin structures, with fastest time-related changes (i.e., steepest slopes) for SG and HF followed by epidermis. Lowest correlations (i.e., significantly less time-related changes) were observed for the dermis and sweat glands. For all five structures, statistical analysis indicated reasonably good linear fit (all Pearson r values are significant, ranging from $r=0.36–0.90$). Goodness-of-fit $F$-tests all showed significant linear fit (they are all $P<0.01$).

At 30 minutes, EG began to show statistically significant increase in fluorescence ($P<0.00001$) compared to control, 5, 10, and 15 minutes of incubation. SG showed significant fluorescence compared to baseline starting at 60–75 minutes of incubation ($P<0.00001$). In HF, porphyrin fluorescence became significantly greater than the baseline autofluorescence at 45 minutes after ALA application ($P<0.000001$). Even though subjectively undetectable while analyzing the digital images, quantitative pixel analysis of the digital photomicrographs from epidermis showed very early porphyrin accumulation after ALA. Within 15 minutes after drug application, a statistically significant increase in epidermal fluorescence was measured over the baseline autofluorescence ($P = 0.04$). Epidermal fluorescence then increased over time. Dermis showed weak fluorescence that increased slightly with time after ALA application. As shown in Figure 4, porphyrin fluorescence generally increased during the entire 180 minute period of observations in this study.

**DISCUSSION**

The major findings of this study are: (1) the anterior skin of porcine ears appears to be microanatomically similar to human sebaceous facial skin, (2) porphyrins accumulate in different skin structures at different rates after topical application of ALA, according to fluorescence microscopy measurements, and (3) eccrine and apocrine glands accumulate statistically significant levels of porphyrins after topical ALA application compared to baseline.

The first finding suggests that pig ear might be developed as a model for sebaceous skin. Lack of an animal model similar to human sebaceous skin encouraged us to closely examine pig ear skin. Porceine skin is generally similar to human skin [21–23], and similarly metabolizes ALA [24]. However, pigs have few and small SG over most of their body surface. We found that both young and adult porcine ears have skin structure, microanatomy, and morphology similar to human facial skin. As in humans, the SG in young pigs are smaller than those in the adults. We also found eccrine and apocrine glands in pig ear skin. Unlike humans, where apocrine gland lumens are usually 10 times larger than eccrine glands [25], pig ears have apocrine glands somewhat smaller than the eccrine glands. In addition, human facial skin rarely contains apocrine glands [25]. Both types of sweat glands are numerous in pig ears, and located close to HF. The anterior (inner) surface of adult pig ears appears to be a good animal model for adnexal glands.

The second finding suggests that different skin structures may be preferentially affected by ALA-PDT depending on the time between ALA application and light exposure. Specifically, the earliest porphyrin accumulation is in epidermis, followed by eccrine and apocrine glands, then HF and SG, until maximum levels are reached in these structures over a 3-hour period. Three hours was the longest time period of application studied. Light exposure 3 hours after application of ALA under occlusion is likely to affect all of these structures. For the purpose of ALA-PDT to target SG, this study suggests that a long incubation time is needed, after which epidermis and other glands will also be affected.

It is possible that porphyrins levels might accumulate further after longer application times since according to Figure 4 there is no evidence of fluorescence levels reaching a plateau. Since Kennedy et al.’s initial report [5], ALA-PDT has become a popular therapeutic method in dermatology. Various indications and different protocols for treatments have been reported. To date, there is no real consensus about the incubation time after ALA application needed to allow accumulation of sufficient PS before light irradiation. Following Hongcharu et al.’s initial report [4] of
long-lasting efficacy for acne treatment in a prospective, controlled study using high-fluence red light given 3 hours after ALA application under occlusion, several studies were reported using different incubation times from 15 minutes [12] to 4 hours [13,26] after ALA application. A wide variety of blue, yellow, and red light sources and treatment fluences have also been used. Another confounding factor is that unintentional exposure to environmental light sources such as sunlight, may in practice play a therapeutic role [27]. Methyl-ALA, which is rapidly converted to ALA after uptake into skin, has been developed and studied as an alternative to topical ALA. The efficacy of topical methyl-ALA for acne therapy is apparently similar to that of topical ALA, when used with long application time and high fluences of red light.

This study has several limitations. It is difficult to absolutely quantify porphyrins by fluorescence microscopy, due to variations in frozen section thickness, porphyrin photobleaching during sample processing and imaging, and in theory, site-dependent quenching of porphyrin fluorescence due to various microenvironments in the tissue. We compensated for baseline tissue autofluorescence and for background light levels in the image analysis. However, other sources of variability exist that were not compensated. These include random variations in frozen section thickness (nominally 15 μm), random variations in the amount of porphyrin photobleaching during tissue handling and microscopy, potential diffusion of porphyrins between structures in the sections, and optical scattering of fluorescence emission during imaging. Dermal fluorescence in many individual tissue sections was noted to correspond with the presence of brightly fluorescent follicles, glands or epidermis in the particular sample. This suggests that at least part of the weak dermal fluorescence noted in our study is an artifact, for example, due to porphyrin leakage from adjacent structures in the section, and/or due to optical scattering within the sample. Several different fluorescent porphyrin species may also be present, and we did not attempt to isolate them. For these reasons, the study provides semi-quantitative relative values rather than an absolute analysis of porphyrin concentration among the various skin structures.

Sampling errors can also occur. Due to the smaller size of SG in the young pigs, detection of SG with weak fluorescence after short periods of incubation was more difficult and could have created a negative bias in our analysis. Another potential source for error in this study was difficulty identifying non-fluorescent structures at the early times after ALA application. While epidermis and HF are readily seen in the absence of any tissue stain, it can be difficult to find glands. A potential bias therefore exists by omitting individual non-fluorescent structures from the analysis, simply by failing to find them. Finally, selective targeting of skin structures depends on other variables other than the time of incubation. Statistically significant fluorescence does not necessarily equate to adequate porphyrin levels for a photodynamic response, but lack of fluorescence during early periods after ALA application strongly suggests that early periods may not produce sufficient porphyrin levels. The third finding suggests that some eccrine and/or apocrine gland disorders may be treatable with ALA-PDT. For example, hydradenitis suppurativa is a painful, inflammatory and scarring condition of the groin and/or axillae that is associated with both sebaceous and apocrine gland occlusion. Cases of this condition have been anecdotally reported to respond to ALA-PDT [28]. To the best of our knowledge, eccrine or apocrine gland-targeted PDT is a novel observation for ALA or any other PS. While eccrine gland function for thermoregulation is well known, the function(s) of apocrine glands is unclear and may be related to scent. In humans, bacterial metabolism of apocrine sweat is largely responsible for axillary odor. Apocrine sweat is formed by “decapitation” of cellular cytoplasm, when the apical portion of the secretory cell cytoplasm is eliminated into the lumen of the gland as shown in Figure 2h, producing a proteinaceous secretion (mostly sialomucin) [25]. In contrast, SG shed cells that have synthesized a mixture of lipids and wax esters, to produce sebum. The pathway(s) by which ALA reaches these three secretory glands in skin following topical application have not been determined. The secretory coils of eccrine and apocrine glands are very deep structures.

The rapid appearance of porphyrin fluorescence suggests that direct uptake of ALA may be occurring through the ducts that communicate with the skin surface. This hypothesis deserves further testing; manipulation of the ALA solution vehicle could potentially optimize such uptake. In this limited study, only one ALA vehicle (ethanol/water) was tested. After penetration by whatever route(s), ALA is taken up by cells and metabolized to the coproporphyrinogen, a precursor of heme, which is then taken up by mitochondria via peripheral benzodiazepine receptors [29].

In summary, it is clear that topical 20% ALA induces early epidermal porphyrin synthesis, followed by progressive time-dependent and strong fluorescent porphyrin accumulations in epidermis, eccrine and apocrine glands, HF epithelium, and SG. There is weak fluorescent porphyrin accumulation in the dermis, and none was noted in subcutaneous fat following topical ALA. These findings suggest that topical ALA-PDT of epidermal targets may require relatively short application times, for example, 15–30 minutes. For SG targeting in this animal model, an application time of at least 45–60 minutes appears to be needed. In this study, we show that pig ear skin is largely anatomically similar to human sebaceous skin, but we did not study functional aspects such as sebum output, response to hormones, secretory stimuli, or follicular occlusion, for example. Development of pig ear as a functional model deserves further study. We aim to develop and utilize this model for studying ALA-PDT of sebaceous, eccrine and apocrine structures, and for further comparison with human skin.

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