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In Vivo Visualization of Hair Follicles by Ultrasound Biomicroscopy in Alopecia Areata and Its Correlation with Histopathology

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ABSTRACT Ultrasound biomicroscopy (UBM) is a non-invasive imaging technique used in examination of several skin diseases but never in imaging hair and scalp diseases. Main objective of this investigation was assessment of the efficacy of UBM for in vivo visualization of hair follicles in cases of alopecia areata (AA) and correlation of findings with histopathological findings. This study included 30 patients with AA. Two areas, one with AA and a control area, were marked, examined by UBM and then biopsied for histopathological examination. In patients with alopecia totalis (AT) or universalis (AU) only an AA area was examined. Non-echogenic conical shadows reaching the epidermal entrance echo (probably corresponding to the hair follicles) were seen and were wider and fewer in number in areas of AA than in normal control areas. No significant difference was found regarding number and width of hair follicles between UBM and histopathological examination. However, a significant increase in length of follicles in histopathology was detected, indicating that the UBM image was probably unable to reach the deepest part of the follicle. Main limitation of the study is small number of cases. No significant difference was found between UBM and histological measurements of hair follicle number and width in patients with AA, making UBM a useful tool for in vivo visualization of hair follicles.

KEY WORDS: alopecia areata, non-invasive imaging, in vivo visualization, histopathological examination, ultrasound biomicroscopy,

INTRODUCTION

Alopecia areata (AA) is an autoimmune, reversible, initially patchy hair loss most commonly involving the scalp (1). Histopathology is characterized by typical inflammatory lymphocytic infiltrates in the peribulbar region and increased numbers of hair follicles in a resting phase and catagen and telogen hairs (2).

Diagnosis and therapy of hair and scalp diseases were in the recent years subject to significant progress. One of the major developments was employing imaging techniques, including trichoscopy (3,4) and reflectance confocal laser scanning microscopy (5,6). Although ultrasound biomicroscopy (UBM) is one of the non-invasive imaging techniques used in examination of several skin diseases (7-15), no report of its role in hair and scalp diseases was found upon reviewing the literature.

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METHODS

The study was approved by the local ethical committee and included 30 adult patients with AA recruited from May until November 2011. Informed consent was obtained from all patients. All patients underwent: detailed history taking, clinical examination, and photography (Panasonic-Lumix DMB- FX07 digital camera). The extent of AA was determined using SALT score (16).

Examination of the scalp by UBM

A Paradigm Ultrasound Biomicroscope plus Model P45 UBM was used. It is a microprocessor-based digital instrument that uses very high frequency ultrasound (50 MHZ) to produce a two dimensional section view of the examined tissue in B-scan (brightness) mode for diagnostic evaluation, and A-scan that measures the axial length with depth of penetration up to 5 mm.

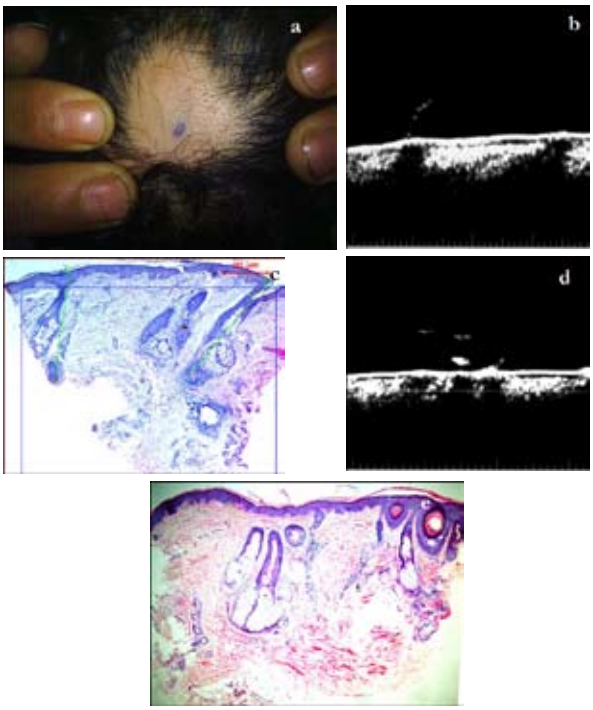

Figure 1. Alopecia areata (AA). A: Clinical appearance of the AA lesion with area to be examined by ultrasound biomicroscopy (UBM) and then biopsied for histopathological examination marked. B: UBM findings in AA area showing non echogenic conical shadows with the narrow end reaching the epidermal entrance echo corresponding to hair follicles. C: Histopathological findings of AA area. D: UBM findings of control area with more numerous thinner non-echogenic shadows and an echo-dense shadow above the epidermal entrance echo representing the hair itself. E: Histopathological findings of control area (hematoxylin and eosin ×40).

- Two areas were marked for UBM examination, one from an AA area, and the other from a normal hairy scalp as a control in cases of patchy AA (Figure 1). Only one area was examined in cases of alopecia universalis (AU) and totalis (AT) (Figure 2). Those areas would afterwards be taken for histopathological examination.
- Patients were examined in supine position or seated according to the site of the lesion, and a coupling agent (saline) was used to fill a reservoir created on the skin by a contact cup, which was firmly held on the surface of the skin to ensure no spilling of saline occurred.
- It was more difficult to apply the cup and probe to a hairy (control) area of the scalp but it was achieved by separating the hair around the selected site and applying the contact cup over the selected site with a few folded hair shafts over the scalp. This was preferred to cutting the hair short in that area, which would be cosmetically unacceptable to the patient.
- The tip of the scanning probe was immersed in the saline and moved parallel to the sagittal plane with the skin lesion placed in the focal plane to get the highest resolution.

UBM measurement

As the UBM cup measured 5 mm in diameter, sonographic findings in 4 mm of the field were taken to accurately reflect the 4 mm punch skin biopsy. In each field:

- The number of conical echo-poor areas denoting hair follicles was counted.
- The length of each follicle in mm from the epidermis to the lower end of the echo-poor area was measured, and an average length of all follicles per field was calculated for each patient.
- To calculate the width in mm, three measurements were taken: at the uppermost end just beneath the epidermis, at the middle, and at the lowermost part of the echo-poor area. An average width per follicle was calculated, and then an average of all follicles per field.

Histopathological examination

Skin biopsies were formalin fixed. Paraffin-embedded tissue blocks were prepared, and 5 micron thick sections with longitudinal cut were stained with hematoxylin-eosin (17) to show a longitudinal section of the hair follicles. Morphometric analysis was carried out using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England).

Table 1. The clinical data of the patient group

Variable	Cases
Type of alopecia:	
AT N (%)	1 (3.3)
AU N (%)	8 (26.7)
Ophiasis N (%)	1 (3.3)
Patchy N (%)	20 (66.7)
Duration of AA in years:	
Range	0.2-10
Mean±SD	2.32±2.82
Percentage of scalp affected by alopecia:	
Range	1-100
Mean±SD	41.53±43.84

AA: Alopecia areata, AT: alopecia totalis, AU: Alopecia universalis, SD: Standard deviation, N: Number

Histopathological measurements

The major axis (length) and the minor axis (width) of the hair follicle were measured on a real-time image in micrometers. In each microscopic field at ×40 power magnification.

- The number of follicles was counted.
- The length of each follicle was measured in mm from the epidermis to its lower end (Figure 2), and an average length of all follicles per field was calculated for each patient.
- To calculate the width of one follicle in mm, the average of three widths was taken per follicle (Figure 3), and then an average of all follicles per field was calculated for each patient.

STATISTICAL ANALYSIS

Data were described in terms of mean±standard deviation (SD), median and range, or frequencies and percentages when appropriate. Mann Whitney U-test was used for comparison between the study groups for independent samples. For group comparison, Wilcoxon signed rank test for paired (matched) samples was used. Correlation between variables was done using Spearman rank correlation equation for non-normal variables. A *P* value <0.05 was considered statically significant. All calculations were done using SPSS (SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

RESULTS

This study included 30 patients with AA aged 18-48 years (Mean±SD: 29.17±9.473) twenty (66.7%) men and ten (33.3%) women (Table 1).

UBM Examination

An epidermal entrance echo and a less echogenic dermal layer with non-echogenic conical shadows were seen (with the narrow end reaching the epidermal entrance echo), probably corresponding to the hair follicles. In areas of AA these shadows were wider and fewer in number than in normal control areas (Figure 1). In control areas, an occasional echo-dense shadow above the epidermal entrance echo representing the hair shaft (Figure 1) was seen. In some cases of AT and AU, non-echogenic globular shadows were present within the dermis but not reaching the epidermal entrance echo and probably corresponded to dilated sebaceous glands (Figure 3).

Histopathological Examination

In AA areas, peribulbar lymphocytic infiltrate with different densities and decreased number of terminal hair follicles was present (Figure 1). In some cases of AT and AU, marked hair follicles miniaturization was seen with a decreased anagen:telogen ratio and hypertrophy of sebaceous glands; the inflammatory infiltrate was sparse and involved the miniaturized hairs (Figure 3). In control areas the percentage of anagen follicles was greater than that in AA areas, and no inflammation was found (Figure 1).

Comparison of Histopathological and UBM findings

Number and width of hair follicles

No significant difference was found in number and width of hair follicles in all AA cases, AT& AU and patchy AA cases (Table 2).

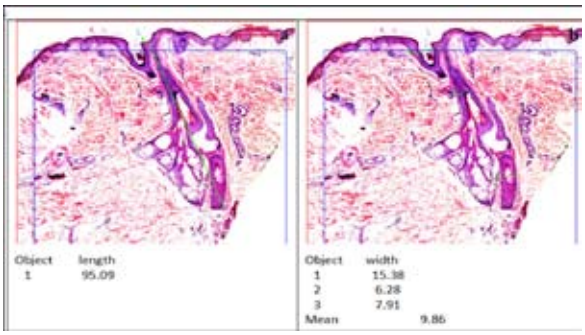

Figure 2. Alopecia areata. Calculation of length and width of the hair follicle in histopathological real time image. A) Number and length of follicles. B) Three width readings and their mean.

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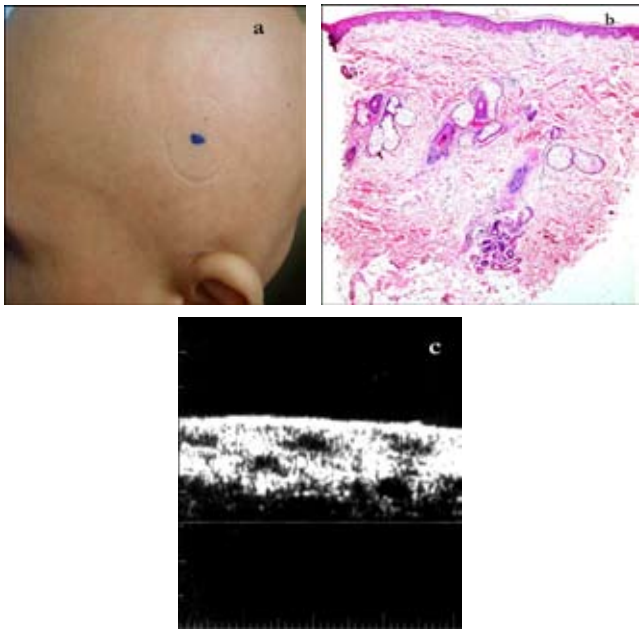

Figure 3. Alopecia universalis (AU). A: Side view of a case of long standing chronic AU with area to be examined by ultrasound biomicroscopy (UBM) and then biopsied for histopathological examination marked. B: UBM findings in the same patient showing non-echogenic globular shadows within the dermis not reaching the epidermal entrance echo, probably corresponding to the dilated sebaceous glands. C: Histopathological findings showing hypertrophy of sebaceous glands and sparse inflammatory infiltrate (hematoxylin and eosin ×40).

Negative correlation was found between number and width of follicles in UBM of all cases of AA and duration of illness (ρ: 0.05, r: -0.358 and ρ: 0.021, r:-0.428, respectively) (Supp Figure 4, 5). Negative correlation was also found between width of follicles in UBM and percentage surface area of scalp affected by alopecia (ρ:0.005, r: -0.503) (Supp Figure 6), reflecting disease severity.

In cases with patchy AA, a significant positive correlation was found between UBM and histopathology in the number of follicles in AA areas (ρ: < 0.001, r: 0.807) (Supp Figure 7).

Length of follicles

A significant increase in length of follicles in histopathology was detected in all cases with AA (*P*=0.008), AT and AU cases (*P*=0.017), and patchy AA cases (*P*<0.001) (Table 2).

Comparison of AA area versus control area revealed a significant difference in number of follicles in UBM (*P*<0.001) as well as histopathology (*P*=0.016) (Table 3).

DISCUSSION

Histopathologically, AA passes through several phases, including the acute stage (18), the subacute stage (19), and finally the chronic stage (20). In chronic stages with repeated episodes, the peribulbar lymphoid cell infiltrate also involves miniaturized hairs,

Table 2. Comparison of histopathological and ultrasound biomicroscopy (UBM) findings

Clinical group	UBM mean±SD	Histopathology mean±SD	*P*-value
All cases of AA			
No. of follicles	2.17±0.83	2.13±0.82	0.655
Width (in mm)	0.38±0.12	0.37±0.10	0.581
Length (in mm)	0.66±0.26	3.16±1.11	<0.001*
AT/AU cases			
No. of follicles	1.56±1.01	1.67±1.00	0.317
Width in mm	0.30±0.12	0.36±0.16	0.483
Length in mm	0.96±0.11	2.79±1.18	0.017*
Patchy AA cases AA area			
No. of follicles	2.43±0.60	2.33±0.66	0.317
Width in mm	0.41±0.11	0.37±0.06	0.281
Length in mm	0.54±0.19	3.31±1.08	<0.001*
Control area (in cases of patchy AA)			
No. of follicles	4.86±1.2	3.67±0.71	0.102
Width (in mm)	0.32±0.11	0.41±0.11	0.594
Length (in mm)	0.42±0.48	3.49±0.78	0.008*

AA: Alopecia areata, AT: Alopecia totalis, AU: Alopecia universalis, UBM: Ultrasound biomicroscopy, SD: Standard deviation, **P* value<0.05 was considered statistically significant

Table 3. Comparison of ultrasound biomicroscopy (UBM) and histopathological findings in patchy group of alopecia areata (AA) vs. control areas:

Findings in patchy group	AA area (mean±SD)	Control area (mean±SD)	*P* value
UBM findings			
No. of follicles	2.48 ± 0.60	4.86 ± 1.20	<0.001*
Width (in mm)	0.41 ± 0.11	0.32 ± 0.11	0.028*
Length (in mm)	0.54 ± 0.19	0.42 ± 0.48	0.130
Histopathological findings			
No. of follicles	2.33 ± 0.66	3.67 ± 0.71	0.016*
Width (in mm)	0.37 ± 0.06	0.41± 0.11	0.314
Length (in mm)	3.31 ± 1.08	3.50 ± 0.78	0.110

AA: Alopecia areata, UBM: Ultrasound biomicroscopy, SD: Standard deviation,
**P* value <0.05 was considered statistically significant

and in 10% of cases there is perifollicular fibrosis with follicular dropout (18). The total number of follicles is normal or decreased in AA compared to normal scalp (20).

In the acute stage of AA, the root sheaths and hair matrix are infiltrated by lymphocytes and there may be hair follicle pigment incontinence, keratinocyte necrosis, and vacuolar damage (21). An inflammatory infiltrate creates reduced echogenicity, which was previously reported in cases of chronic eczema and lichen planus (10). Pigmented lesions may also create hypoechogenic structures in UBM examination (22). In the present study, hairy areas of the scalp in cases of patchy AA showed the more numerous conical shadows of smaller width. An overlying echo-dense shadow of the hair was seen, confirming that these conical shadows were probably hair follicles.

In some cases of AT and AU, non-echogenic globular shadows present within the dermis, probably corresponding to the dilated sebaceous glands seen in histopathological examination. Fluids create areas of low echogenecity on UBM examination (13) as is the case in sebum filled sebaceous glands. Interestingly, fibrous bands did not create any visible UBM changes.

Trichoscopy allows in vivo visualization of hair and scalp structures at optical magnifications up to 70-fold and more. Characteristic trichoscopic features of AA are black dots, tapering hairs (exclamation mark hairs), broken hairs, yellow dots, and short vellus hairs (23). A disadvantage of trichoscopy is that structures of different histopathological nature may give an undistinguishable trichoscopy appearance (4). The yellow dots first reported by Ross *et al.* (3); considered to be a mixture of immature hair shaft and sebum (24), were also positively correlated with AA severity and had a tendency of positive correlation with AA activity (23). However, as a diagnostic marker, the yellow dots were sensitive but not specific, being frequently present in patients with female androgenic alopecia and may represent sebaceous glands or epidermal "sebum lakes" (25). Decreased total number of hair follicles in histopathological examination of AA is documented in the literature (20) and corresponds to USB findings in the present study. Yellow dots in trichoscopy may present dilated sebaceous glands, which might explain lack of reports of decreased hair follicle numbers in dermoscopic examination of patients with AA. Therefore, an advantage of in vivo UBM visualization over trichoscopy is the accurate counting of the number of follicles per UBM field.

In vivo confocal scanning laser microscopy may develop into a valuable tool in evaluation of hair shaft diseases (26,27). A major limitation of this technique has been the depth of imaging of about 200µm, confined to the upper dermis without the possibility of visualizing reticular dermis and hair follicles (5). An additional hurdle is the bright reflection of keratin, which decreases the perception of hair follicle details in the image (6).

Due to the limitations of available in vivo visualization tools for hair follicles, use of UBM as a non-invasive tool to monitor the progress of AA as reflected by the number and width of hair follicles (reflecting the density of inflammatory infiltrate) would be a great advantage. Identification of the small percentage of cases with perifollicular fibrosis and follicular drop-outs would also be of great value in avoiding unnecessary treatment protocols in this subset of cases. Another advantage would be the ability to easily examine different areas of the scalp, especially in cases of AT or AU, an option not available with skin biopsy.

Some neoplasms have been examined using high frequency ultrasonography, such as seborrheic keratosis (28) and melanomas (29), where physical measurements were found to agree reasonably well with histology. Analysis of data from all 30 cases of

AA as well as cases with patchy AA, AT, and AU separately showed no significant difference between UBM imaging and histopathology in the number of hair follicles, with a significant positive correlation indicating that the UBM findings sensitively reflect the histopathological changes in the scalp in the area imaged. A negative correlation was found between the number of follicles in UBM imaging of areas of AA and duration of illness, meaning that the longer the disease duration the fewer the number of hair follicles. The ability to determine the presence or absence of hair follicles can be particularly useful in identifying the 10% of cases with perifollicular fibrosis and follicular dropout (18), which will probably have limited response to therapy.

There was no significant difference between UBM imaging and histopathology in the width of the hair follicles. Korting *et al.* (30) observed increased dermal echogenicity after local treatment with corticosteroids due to decreased inflammatory infiltrate and suggested sonographic monitoring of the therapeutic effect of treatment. Similarly, decreased width of hair follicles might be expected in cases of AA with decreased density of inflammatory infiltrate in response to therapy. Indeed, healthy control areas of the scalp examined by UBM showed a significantly higher number of follicles with a significantly smaller width, supporting the above hypothesis. A negative correlation was also found between the width of the follicles in UBM imaging of AA areas and the duration of illness, which could be explained by the fact that two events occur in long-standing cases with slightly reduced inflammatory infiltration and miniaturization of hair follicles (19). Limitations of our study include increased width of the UBM follicle shadow due to dilated sebaceous glands and not inflammatory infiltration in some cases of AT and AU.

The significant difference between lengths of follicles in UBM imaging compared to histopathology indicates that the UBM image was unable to reach the deepest part of the follicle and cannot be used to measure the length of follicles.

CONCLUSION

Very good correlation was found in AA cases between UBM and histological measurements of hair follicle number and width in this study, indicating that UBM is a useful in vivo visualization tool of hair follicles. Further studies involving a larger number of cases and performing UBM examination of AA lesions before and after therapy would be needed to confirm our findings.

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