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● *Original Contribution*

SPATIAL RESOLUTION OF TRANSDERMAL WATER MOBILITY USING NMR MICROSCOPY

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High resolution images were obtained using high-field nuclear magnetic resonance microscopy of in vitro preparations of hydrated hairless rat skin. The major anatomical features observed were comparable to those seen by electron microscopy and include the stratum corneum, the viable epidermis, sebaceous glands, the cell layers surrounding hair follicles (the outer and inner root sheaths), and regions of subcutaneous fatty deposits. Calculated diffusion maps demonstrated that signal intensity is sufficient to obtain quantitative water mobility data from the viable epidermis and the hair follicle/sebaceous gland regions. Images from skin immersed in D_2O clearly distinguish signal contributions that arise from fat from those which arise from water, and indicate that the calculated diffusion maps include only proton mobility from water in skin. These results may lead to further applications for using quantitative nuclear magnetic resonance microscopy for examining transdermal transport processes of in vitro skin preparations. © 1997 Elsevier Science Inc.

Keywords: **NMR microscopy; Transdermal water mobility; Skin structure.**

INTRODUCTION

Quantitative and qualitative analyses of the rates of molecular transport across skin are important for a number of biomedical engineering applications as well as for advancing our understanding of the barrier function and fundamental properties of skin. Commercial products are available for the transdermal delivery by passive diffusion of compounds such as scopolamine for motion sickness, nitroglycerin for heart disease, and clonidine for premenstrual syndrome (PMS).[1] The structure of mammalian skin is, however, exquisitely suited for restricting the transport of polar water soluble species and large macromolecules.[2] Mammalian skin is divided into two major domains: the epidermis and the dermis. The epidermis has been considered to be the primary barrier for restricting transport across the skin[3,4] and it consists of a series of layers of cells; the lower layer is an actively growing region, the viable epidermis, that produces cells which migrate to the upper region of the skin.[5–7] As these cells migrate to the upper region, termed the stratum corneum, they gradually lose their activity and become non-active anucleated cells that contain large proportions of the protein keratin and that are separated by regions containing highly lipophilic lipids.[8] The stratum corneum is approximately 10 to 20 microns thick. The nature of this structure will therefore serve to restrict the transport of water soluble species due to the highly hydrophobic interstitial layer of lipids between the corneocytes. Penetrating the stratum corneum (20 microns), viable epidermis (100 microns), and dermis (1 mm) are several structures, including hair follicles, sweat glands, and sebaceous glands, that have commonly been referred to in the literature as shunts because these structures may offer routes bypassing the lipophilic barrier of the stratum corneum.[4,9]

A wide range of methods have been pursued to facilitate the transport of targeted compounds across the skin. These methods may include the application of constant electrical fields (iontophoresis[10]), the application of

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pulsed electrical fields (electroporation[11,12]), the use of penetration enhancers (compounds such as dimethyl sulfoxide[13,14] which alter the lipid permeability of the skin), the use of ultrasonic pulses,[15,16] and the application of various metabolic approaches to alter skin structure.[17]

Analysis of the transport of various molecules across the skin has been limited to a few experimental techniques. The major experimental technique used for transdermal drug delivery research is the diffusion cell.[4,18] This system consists essentially of a two-compartment device where the two compartments, one the donor compartment and the other the receiver compartment, are separated by the skin sample. Overall flux measurements can be performed to measure macroscopic transport rates. No local information, however, on pathways through the skin, on local diffusion coefficients, or on the effects of the method of transport enhancement on the ultrastructure of the skin can be obtained. Fluorescent microscopy,[19] confocal microscopy,[20] and autoradiography[21] have also been adapted to consider the transport of molecules across the skin through visual observation of the location on the surface of the skin where the fluorescent molecules enter the skin. These methods give some information on the location of transport through the skin, but they do not give detailed information on local transport rates or of the pathways inside the skin not directly observable from the surface.

A number of issues concerning the transport of compounds across the skin have yet to be fully resolved due to the limitations of the above experimental techniques. The most important of these issues is the direct quantification of the relative rates of transport through the shunts, especially when applied electrical fields are used, by measurement of molecular mobility in the shunts directly. Another important issue is the direct measurement of the effects of the transport enhancement method on the structure of the skin. For example, the application of pulsed electrical fields are considered to reversibly alter the local structure of the stratum coroneum and recent evidence suggests that channels of large dimension, i.e., of order 50 microns, can open up in the skin upon application of high voltage pulses and are not associated with conventional shunts.[19]

Nuclear magnetic resonance (NMR) methods are ideally suited for answering some of the questions posed above. NMR methods have been investigated for in vivo skin studies.[22–24] Ablett et al.[22] performed high resolution magnetic resonance imaging (MRI) on the finger of a human subject and showed that the water content in the skin could be measured. Bittoun et al.[25] were able to differentiate epidermis from dermis and detect hair follicles in vivo using a whole body MRI system. MRI has also been used to examine relaxation properties of the dermis and epidermis.[26] Ono et al.[23] used NMR for in vivo skin tumor diagnostics. In vivo studies of drug delivery through skin may lead to the answers to some of the questions above. However, due to the limitations of resolution of current instrumentation, the difficulty of constructing devices that will allow simultaneous application of external electrical and magnetic fields on living organisms, and the desire to explore wider range of skin altering procedures, in vitro studies of transdermal transport using NMR are considered in the present work.

The major objectives of the present work are to quantify water mobility in hydrated skin from hairless laboratory rats, to determine spatially resolved diffusion maps so as to clearly distinguish possible pathways for transdermal drug delivery of water soluble species, and, in general, to explore the range of possible applicability of NMR microscopy for the study of transdermal drug delivery.

Hairless rats are a useful model for the study of certain aspects of transdermal transport since they do not contain sweat pores; this factor allows for direct quantification of the role of the hair follicle region and sebaceous glands on the transdermal transport of hydrophilic compounds. Future work will seek to compare the results obtained in the present study with results for transport through other skin types that have sweat pores.

METHODS

Male CD hairless rats (Charles River Laboratories, Wilmington, MA) were obtained between 21 to 42 days old and maintained until usage at $\leq$130 days old. Rats were euthanized with an overdose of sodium pentabarbital (60 mg/kg) immediately prior to skin isolation. Full thickness dorsal skin was tape stripped and shaved, cut into $\simeq 5 \times 5$-cm pieces, and placed on filter paper wetted with 0.9% NaCl and gentamicin (10 mg/L). The skin was sealed in sterile petri dishes and either stored at 4°C under maximal humidity for less than 2 weeks or frozen at −20°C.

NMR experiments were performed on a Bruker 600 MHz DMX widebore spectrometer (Bruker Instruments, Billerica, MA) complete with actively-shielded imaging gradients capable of reaching 960 mT/m along all axes. The bore size of the magnet is 89 mm, but with the gradient stack in place, the bore is reduced to 39 mm. A standard bore microimaging probe body was used in conjunction with a 10-mm birdcage radiofrequency coil insert that tuned to 600 MHz. Skin samples were cut in a circle to fit in the bottom of a flat-bottomed 10-mm NMR tube, and immersed in $\simeq$ 0.5 ml of 0.9% NaCl.

Two-dimensional spin-echo images were collected as 256×256 matrices with a 1.2 cm-square field of view. This yielded an in-plane resolution of 46.8×46.8 μm. Much higher resolution images were obtained (21.5

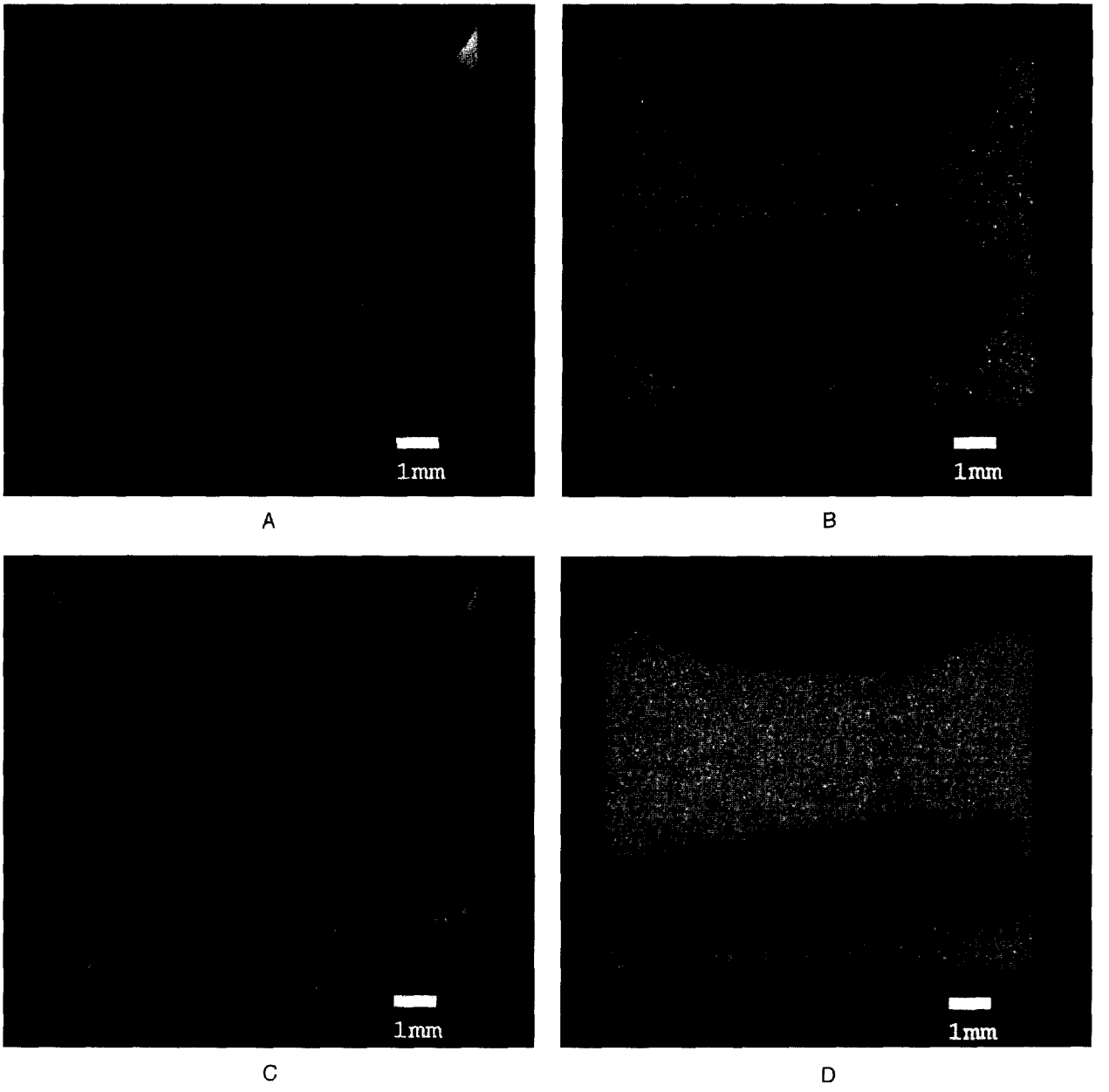
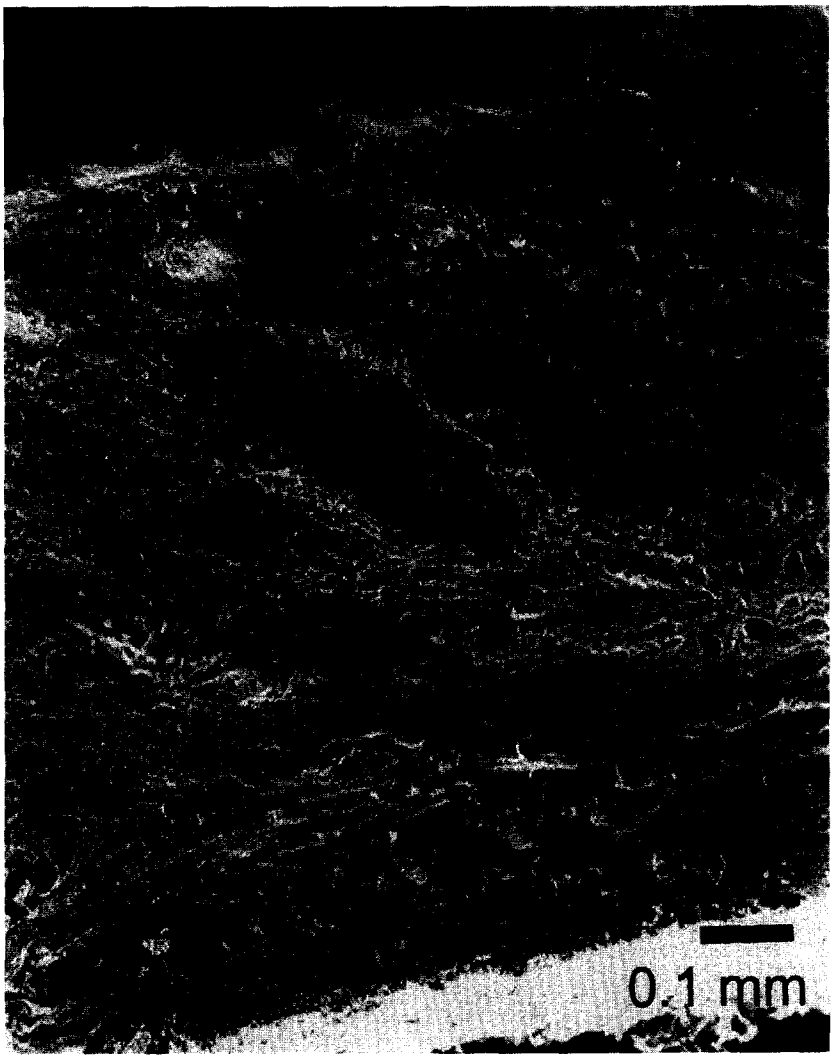


Fig. 1. Transverse slice across skin from a 58-day-old rat (A and B) and a 130-day-old rat (C and D). A and C are spin-echo images that are not diffusion weighted. The stratum corneum is the dark layer at the upper surface, below which lies a bright layer which is the viable epidermis. The hair follicles can be seen as regions of high signal crossing the skin, and fat can be seen at the base of the skin. B and D are calculated diffusion maps of the same slice from a series of diffusion-weighted spin echo images. D_{app} could be calculated for the viable epidermis and the hair follicle region.

μm $\times$ 21.5 μm $\times$ 100 μm) but did not improve the ability to resolve major anatomical structures, so routine images were collected with the coarser resolution to improve signal-to-noise ratio. Slice thickness was 200 μm and slice-selection was achieved with 1 ms, two-lobe sinc pulses for both the 90° and 180° pulses. The best images were obtained with the shortest echo-time possible, but for measuring diffusion coefficients, the diffusion time, Δ, must be long enough to allow sufficient diffusion-weighting. Therefore, images were collected with an echo time ranging from 16 to 21 ms, which corresponded to a Δ ranging from 10 to 15 ms. A series of four to five diffusion weighted images were collected from a single sample where the diffusion-sensitizing pulsed gradients were stepped through amplitudes ranging from 0 to 900 mT/m. In all cases,



A

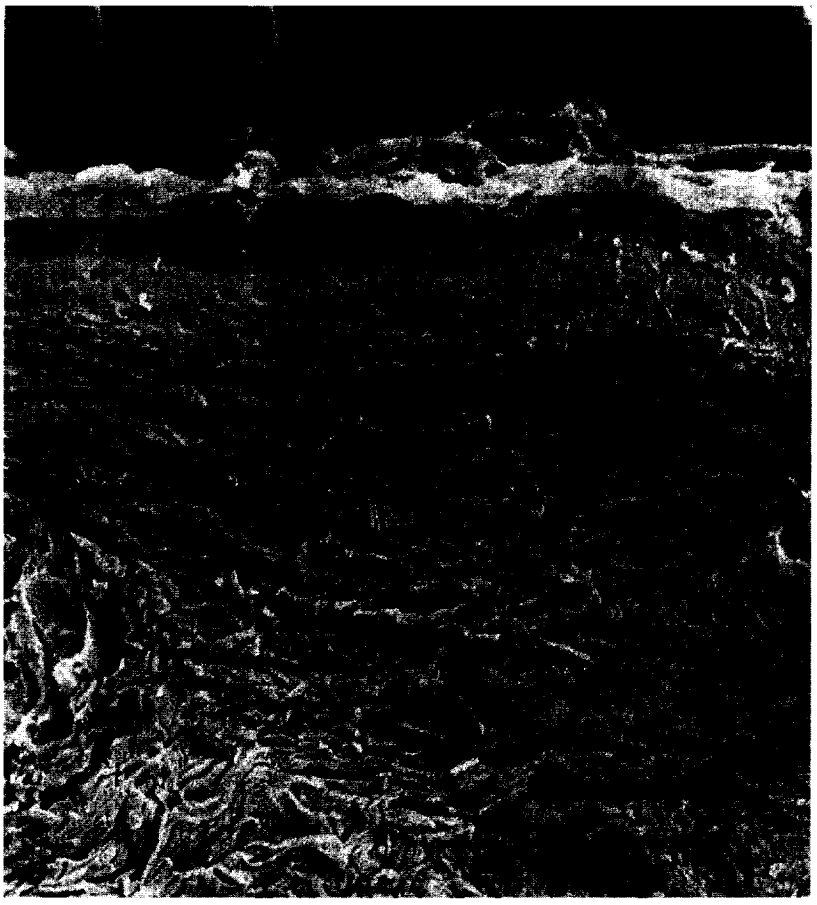
Fig. 2. SEM micrographs of rat skin showing the structures visible in the NMR images. (A) transverse cut comparable to the images in Fig. 1 showing the epidermis, dermis, and hair follicles (×75), (B) higher magnification view of the epidermal layers showing the stratum corneum and the viable epidermis (×200), and (C) cut in-plane with the skin comparable to the images in Fig. 3 showing hair follicles protruding through sebaceous glands (×35).

diffusion was measured across the skin (perpendicular to the skin surface). The image series was then used to calculate the spatially-resolved apparent diffusion coefficient of water, D_{app}, using a pixel by pixel two-parameter exponential fit to the Stejskal-Tanner diffusion equation. In all experiments 12 to 22 averages were collected, the recycle time was 1 s, and the diffusion gradient duration, δ, was 2 ms.

Samples to be used for scanning electron micrographs were cut into strips $\simeq 1 \times 20$ mm, pinned on dental wax, and immersed in cold 4% glutaraldehyde adjusted to pH 7.4 with 0.1 M sodium cacodylate buffer. After 1 h of fixation, the tissue was cut into 1 mm × 1 mm blocks and fixed for an additional 1.5 h on ice. After rinsing in three changes of buffer, the tissue was dehydrated in a graded ethanol series, critical point dried, mounted and sputter-coated, and viewed with a JEOL 850 (JEOL USA, Peabody, MA) scanning electron microscope.

RESULTS

Figures 1A and 1C illustrate the anatomical detail visible in cross sections of skin from 58- and 130-day-old hairless rats, respectively. The stratum corneum is visible as a very thin dark layer at the upper surface, below which lies the bright, somewhat thicker layer of the viable epidermis. The cell layers that surround the hair follicles (the outer and inner root sheaths) can be



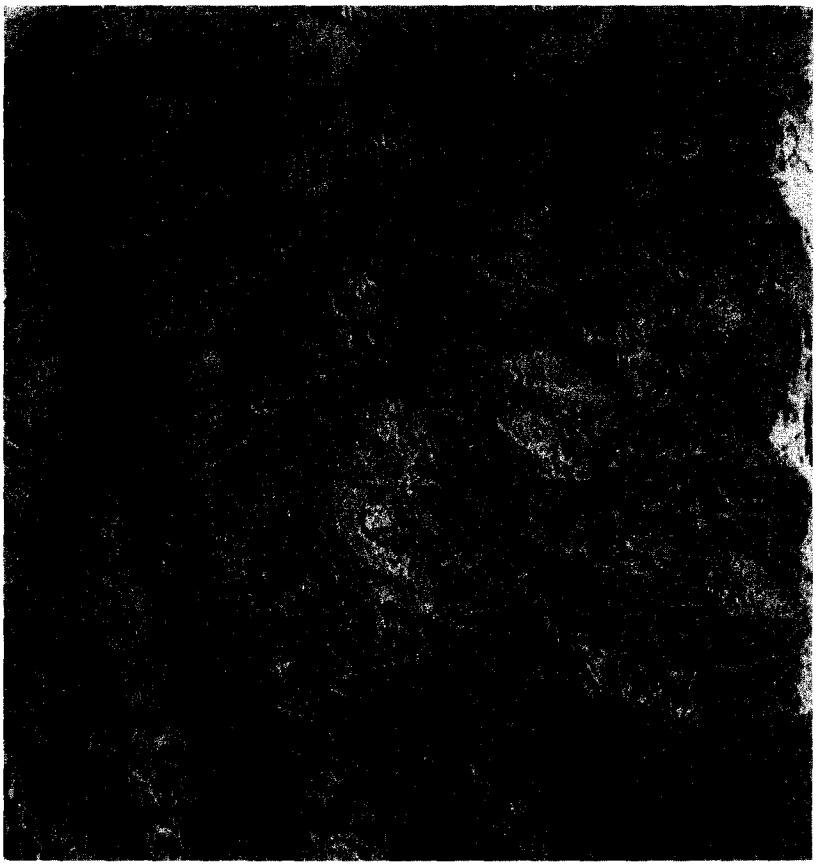
B

Fig. 2 continued.

seen as areas of relatively high signal that penetrate the dermis, which is largely devoid of signal. Sebaceous glands can also be seen associated with each hair follicle as regions where the follicle broadens at about 300 μm below the surface of the skin. Subcutaneous fat can be seen at the base of the dermis, and fatty projections are directed upwards into the dermis as regions of fairly faint signal (giving the false impression that the hair follicles protrude completely across the dermis—see D_2O results discussed below). The electron micrograph shown in Fig. 2A with magnification comparable to the NMR images shows similar features to those that are visible in the NMR images, except that the hair shafts are more easily observed. Fig. 2B is a higher magnification electron micrograph view showing the viable epidermis and the stratum corneum that can also be clearly seen in the NMR images.

Calculated diffusion maps of water mobility across the skin are shown in Figure 1B and 1D. Skin from both the younger and older rat had sufficient ^1H signal to measure D_{app} in the lower epidermal layer (viable epidermis) and along the cells surrounding the hair follicles, but the signal was too low to measure diffusion in the thin layer of the stratum corneum or in the dermis between hair follicles. The hair follicles are more obvious in the diffusion map of skin from the older rat (Figure 1D), primarily because the thicker, older skin generally had lower signal in the dermis than that of the younger skin (presumably because of lower water content in the older skin), and contrast was therefore better. The averages of D_{app} for the images in Figure 1 were $8.6 \pm 2.9 \times 10^{-6}$ and $8.2 \pm 2.6 \times 10^{-6}$ cm^2/s for the lower epidermis and hair follicles, respectively, in the younger skin, and $8.6 \pm 3.0 \times 10^{-6}$ and $7.6 \pm 2.3 \times 10^{-6}$ cm^2/s for the epidermis and hair follicles in the older skin.

Figures 3A and 3C show in-plane slices of the skin where the hair follicles and sebaceous glands are again clearly visible as oval regions of light signal. Figure 3A is a slice made just below the level of the sebaceous glands in skin from a 70-day-old rat. The hair



C

Fig. 2 continued.

follicles are plainly visible, and toward the top right and top left of the image some of the sebaceous glands included in the slice are visible as are a few hair shafts (pixels with no signal in sebaceous glands). Figure 3C is a slice that cuts through the sebaceous glands in skin from a 130-day-old rat. The anatomical detail again shows the same major structures present in a similar electron micrograph except for the clearly delineated hair shafts in the latter (Fig. 2C). Corresponding diffusion maps are shown in Figs. 3B and 3D and, as before, the contrast is better in the skin from the older rat. The average D_{app} of water in the younger rat in Fig. 3 was $7.6 \pm 1.3 \times 10^{-6}$ cm^2/s and $8.2 \pm 2.1 \times 10^{-6}$ cm^2/s, in the sebaceous glands and hair follicles, respectively, and D_{app} in the older rat was $8.3 \pm 1.3 \times 10^{-6}$ cm^2/s and $7.5 \pm 1.8 \times 10^{-6}$ cm^2/s, in the sebaceous glands and hair follicles, respectively.

To verify that the NMR signal in the diffusion maps is due to water, a skin sample from a 71-day-old rat was immersed for 20 h in a 0.9% NaCl solution in D_2O. The D_2O should exchange with the water in the skin, eliminating the water signal and leaving behind only the signal from fat. Figure 4 is an image of the D_2O prepared skin showing that all of the signal from the lower epidermis and hair follicles has disappeared. The fat signal clearly shows the basal fat deposits, as well as the line of sebaceous glands. A calculated diffusion map of this skin sample showed no signal; that is, the fat seen in Fig. 4 does not contribute to the diffusion maps shown earlier because of its low mobility. Therefore, our diffusion maps of skin are measurements of the water only.

DISCUSSION

In general the above data shows that NMR microscopy is useful for examining the detailed structure of in vitro samples of skin through quantitative measurements of spatially resolved water diffusion coefficients. Although previous work has applied NMR to in vivo skin studies, there are several advantages and applications for the use of in vitro preparations. These advantages include use of smaller samples thereby allowing for higher spatial resolution, the use of larger magnetic fields, also providing higher spatial resolution, and the application of a wider range of chemical and physical conditions that

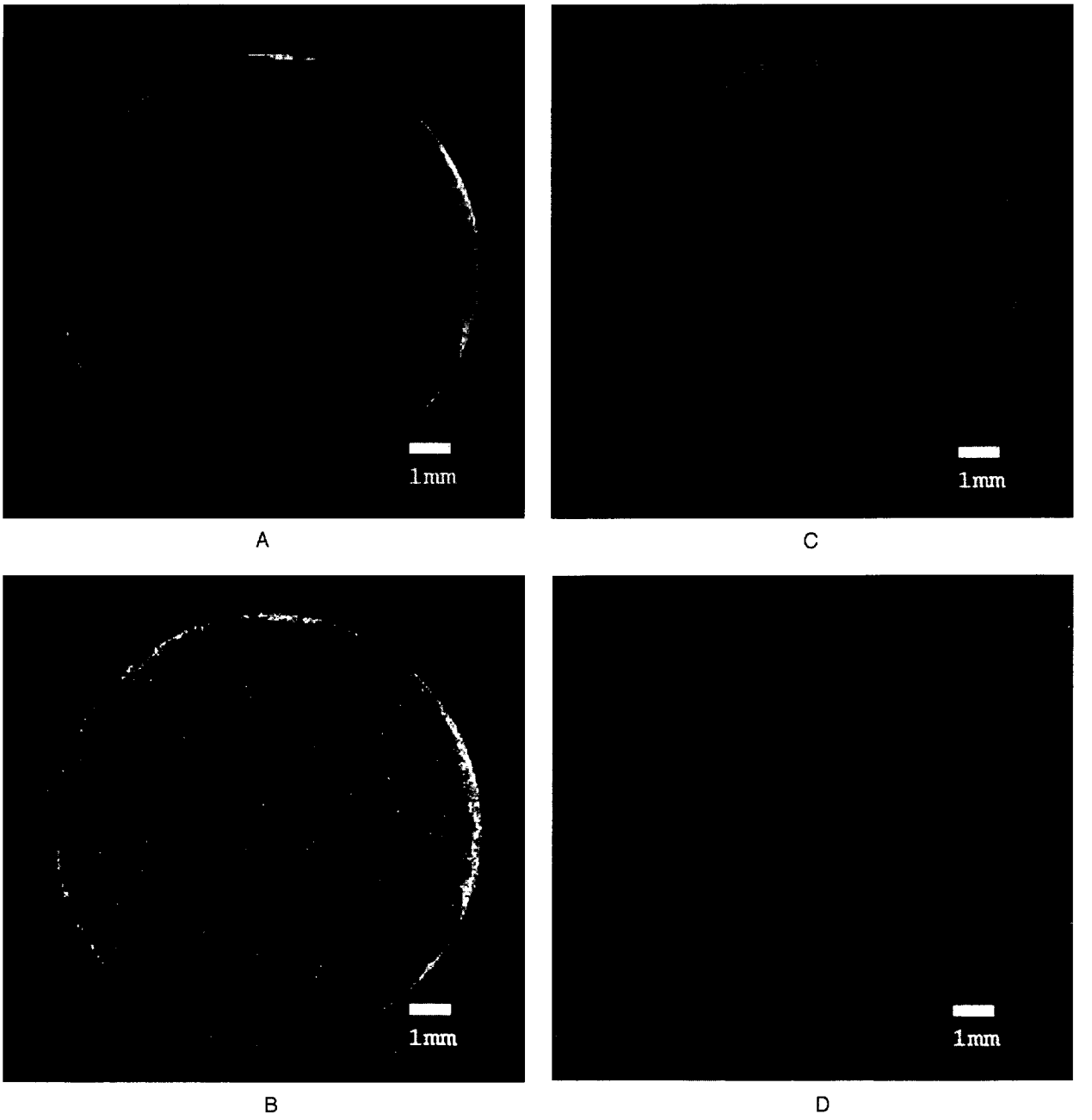


Fig. 3. Slice in-plane with the skin made just below the sebaceous glands in skin from a 70-day-old rat (A and B) and across the sebaceous glands in skin from a 130-day-old rat (C and D). A and C are spin-echo images that were not diffusion weighted and clearly show the cells around the hair follicles and the sebaceous glands. B and D are calculated diffusion maps of the same slice.

may alter skin structure. The present study has shown that high-field NMR microscopy allows visualization of gross anatomical detail, as observed in scanning electron micrographs of skin showing the major structures, including the stratum corneum, the hair follicles, the sebaceous glands, and the viable epidermis.

Quantitative determination of the rates of localized water mobility in the shunts, i.e., in the hair follicles and sebaceous glands, is expected to allow accurate assessment of various mathematical models that seek to quantify the relative rates and pathways for transdermal drug delivery. In addition, the capability to resolve water soluble and fat soluble regions will be valuable for qualitatively assessing transport pathways of lipophilic and hydrophilic compounds.

The D_{app} in the viable epidermis and hair follicles of

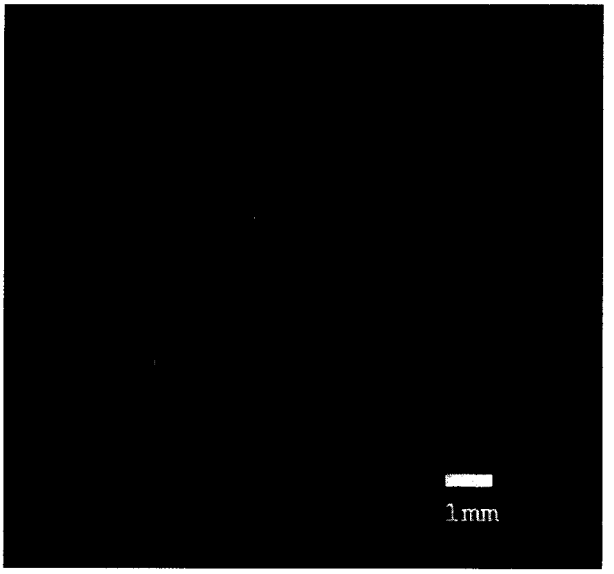


Fig. 4. Transverse slice comparable to Fig. 1A of skin from a 71-day-old rat that had been soaked for 20 h in D_2O. The D_2O replaced the H_2O fraction in the tissue and the resulting ^1H image contained only signal that originated from fat. The subcutaneous fat deposits and the line of sebaceous glands are clearly seen, but the hair follicles and viable epidermis seen in Fig. 1 are no longer visible. A calculated diffusion map of this tissue showed immeasurably low mobility for the fat signal indicating that our diffusion maps in Figs. 1 and 3 are showing mobility of the water soluble fraction only.

the skin from the young hairless rat shown in Figs. 1A and 1B were found not to differ significantly and to be about 30% of the value for free solution. The anatomical structures of the epidermis and hair follicle domain have been extensively studied by other methods.[6,27] It is clear from these previous studies that both of these regions contain actively growing cells that are tightly spaced. There are thus no direct open pathways for water and water soluble compounds to transport through these regions. Therefore, the predominate mode of transport for these types of species may be intracellular, although no definitive conclusion concerning this point can be made from the present studies. The diffusion coefficients in these regions are of similar magnitude to those found in other tissues and cells. Even though water has restricted transport rates of approximately 30% in these regions of the skin, this does not necessarily imply that larger species or more polar species will move at the same rates through these regions of the skin. Further work is necessary to measure the transport of larger species with different molecular properties and to distinguish between intercellular and intracellular pathways along the hair follicles.

Observation of water mobility in and/or around the sebaceous gland is of particular relevance in light of recent work[28,29] that indicates the importance of sebaceous glands in transdermal drug delivery. The sebaceous fluid consists of waxy-like substances, sebum[29,30] contained within a domain surrounded by actively growing cells. The sebum itself contains some water; however, the metabolic cells that line the outer walls of the sebaceous gland would be expected to contain more water. Transport of polar water soluble species through these cells may be similar to that through the cells surrounding the hair follicles and in the viable epidermis. Water mobility measurements, as observed in our experiments, would be expected to be similar in the cellular environments of the three regions.

No quantitative differences in water diffusion coefficients were therefore seen for rats of different ages; however, some qualitative differences with regard to the contrast of the hair follicle regions with respect to the dermis were observed. The older rats appeared to have less water in the dermis than the younger rats and this resulted in a higher contrast between the dermis and the hair follicles in the older rats.

CONCLUSIONS

The NMR methodology has been shown in the present study to be useful for examining water mobility in transdermal shunts (primarily hair follicles and sebaceous glands); this work is expected to find application in the analysis of drug delivery via either passive diffusion or transport facilitated by electric fields. The application to in vitro preparations provides for greater control of the experimental conditions and higher NMR resolution than in vivo preparations. The literature on transdermal transport of water soluble species indicates that the shunts, i.e., hair follicles and sweat pores, are the major pathways for the transport of hydrophilic species. The present work suggests that water mobility is high in the hair follicle region. It cannot, however, be concluded from the present work that the only pathway for the transport of water soluble species is in this region; further work with other skins is recommended. The water mobility in the viable epidermis and the hair follicles was about 30% of that in free solution. This reduction in water mobility represents an upper limit to the relative diffusion coefficients of larger water soluble species in these domains in the skin. Transdermal transport of other larger water soluble species is likely to be slower than that observed here for water.

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