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Use of the skin sandwich technique to probe the role of the hair follicles in sonophoresis

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ABSTRACT

The human skin sandwich technique was used to explore the effect of brief ultrasound exposure on the transfollicular pathway of absorption. Hydrocortisone was used as a model drug. In order to calculate the permeability coefficient of hydrocortisone, its concentration at saturation in the PBS donor solution was determined. Skin samples were prepared by sandwich technique with total hydration of the epidermal and sandwich membranes. The skin was sonicated for 0 s (control), 30 s or 45 s using a pulsed mode (10% duty cycle) with the spatial and temporal average intensity (SATA) of 3.7 W/cm^2. The transducer was then removed and permeation was allowed to proceed for 52 h. Then the percentage follicular contribution was determined. It was determined that without ultrasound, drug entry into follicles accounted for 46% of total penetration. As the duration of sonication increased, the follicular contribution fell to zero even though total transepidermal flux dramatically increased. This is explained by ultrasound exposure causing sloughing off of the uppermost stratum corneum. This permeabilises the continuous surface but at the same time the disturbed cornceocytes will plug hair follicle orifices.

1. Introduction

For many years, researchers investigating topical and transdermal drug delivery have questioned the relative importance of drug absorption through the continuous stratum corneum versus permeation through the hair follicles. Early work indicated that follicular transport was significant only during the initial stage of drug absorption but made only a very small contribution to the flux during the steady-state period (Scheuplein, 1967; Scheuplein et al., 1969). This was believed to be due to follicular openings only making up ~0.1% of the total skin surface area. However, more recent research, using many different approaches and methods has indicated that hair follicles may have a greater role than previously believed (Ogiso et al., 2002; Dokka et al., 2005; Grams et al., 2005; Teichmann et al., 2005, 2006). This new thinking has been associated with the realisation that the follicles really represent invaginated or inward folded areas of the stratum corneum. So the actual surface area for possible absorption is a lot greater than 0.1% (Meidan et al., 2005).

Several animal models have been used over the years to investigate the role of the hair follicles. One simple approach is to simply compare the penetration of a drug through a hairy and hairless rodent species. For example, Hisoire and Bucks (1997) deduced the delivery of retinoic acid to hair follicles by comparing the acid's percutaneous penetration through both hairy and hairless guinea pigs in vitro. A problem with this method is that hairless species of rodents do actually have skin containing underdeveloped follicles and are so not really follicle-free (Meidan, 2010).

Other animal models have included the use of the macaque monkey, the Syrian hamster as well as comparisons between hairy and hairless sites of the guinea pig ear. However, a general problem with all animal models is that it is difficult to show that the barrier properties of any follicle-containing continuous skin membrane are structurally comparable to those of the "control" follicle-free continuous membrane. The problem was addressed by Barry et al. who devised a novel in vitro technique, called the 'skin sandwich' approach (El Maghraby et al., 2001; Barry, 2002; Essa et al., 2002).

In the skin sandwich system, the role of shunts in total percutaneous absorption is determined by comparing drug flux across hydrated epidermal membrane with that through a hydrated 'sandwich' of epidermal membrane with an extra stratum corneum on top. Fig. 1 illustrates such a sandwich. Remembering that follicles typically represent only about 0.1% of the total skin surface area, it follows that there is a negligible probability that hair follicle openings in the two stratum corneum membranes will superimpose. So the additional stratum corneum blocks virtually all available follicular routes. If the shunts route plays no part in the permeation

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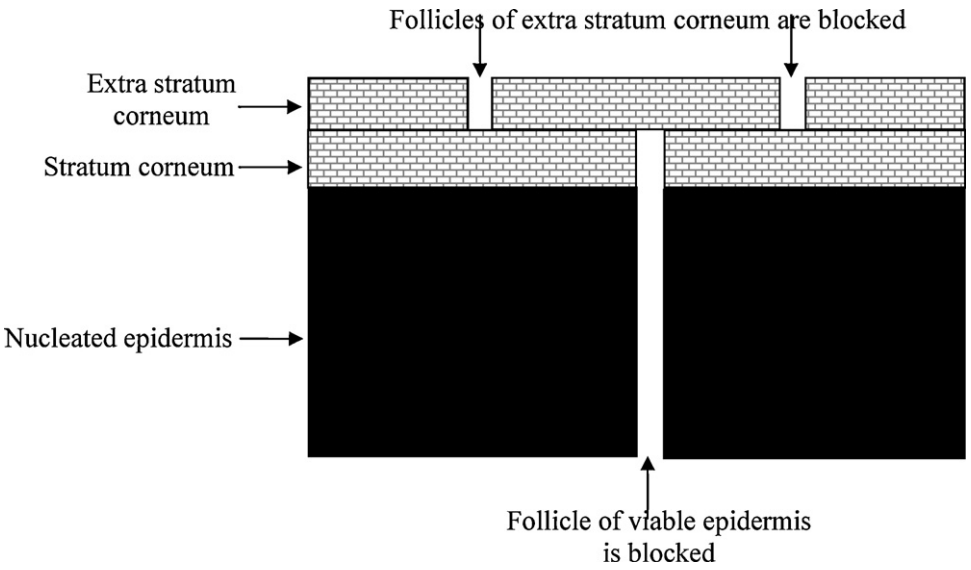

Fig. 1. Scheme demonstrating the basis of the skin sandwich approach. The sandwich consists of an epidermal membrane with an overlying extra stratum corneum. The arrangement effectively blocks the follicular passage of applied solutes.

process then steady state flux through the sandwich is half that of the single membrane. If shunts are responsible for all drug transport then flux through the 'sandwich' is zero. Hence, the magnitude of flux decrease allows quantifiable identification of shunt contribution to total absorption. The shunts are believed to correspond to the hair follicles since the much smaller sweat duct openings close over when the tissue is fully hydrated.

Two further basic points should be considered. Firstly, it has long been established that as long as relatively non-lipophilic drugs ($\log K_o/w < 2.7$) are used, the nucleated epidermis does not contribute significantly to skin barrier properties. As a result, the presence of the nucleated epidermis in the sandwich membrane can be neglected in permeability calculations and it just acts as a mechanical support. Hence, for the purposes of calculations, the thickness of the sandwich is considered to be double the thickness of the single stratum corneum (with the nucleated epidermal membrane). Another important point is that when the skin sandwich system is used, the extra stratum corneum and the lower stratum corneum are extracted from adjacent skin areas on the same donor, thus reducing as far as possible, natural variations in skin barrier properties.

The objective of present study was to use the skin sandwich technique to explore the effects of ultrasound on the transfollicular route of absorption. Hydrocortisone was employed as a model drug. Since initial pilot studies have indicated that epidermis and stratum corneum isolation was very difficult to achieve with pig skin, human skin was used in these studies.

2. Materials and methods

2.1. Materials

Hydrocortisone, bovine pancreatic trypsin (T-4665) and phosphate buffer saline (PBS) tablets (pH 7.4) were all purchased from Sigma–Aldrich (Poole, UK). Tritiated [1,2,6,7-3H]-hydrocortisone (74 Ci/mmol) was obtained from Amersham Biosciences (Little Chalfont, UK). Sodium azide was supplied by Acros Organics (Geel, Belgium). Scintillation fluid (Optiphase HiSafe 3) was supplied by Fisher Scientific (Loughborough, UK). Scintillation vials were purchased from Fisher Packard Instrument Co. (Meriden, CT). Double distilled, de-ionised water was employed throughout.

2.2. Preparation of the hydrocortisone-containing donor solution

The donor solution consisted of hydrocortisone present as a saturated solution in phosphate buffer saline (PBS, pH 7.4). This was made by adding excess 'cold' hydrocortisone to PBS (pH 7.4) and stirring for 48 h at 32 °C. The solutions were subsequently filtered through a 6 μm pore cellulose membrane filter (Whatman Ltd., Brentford, UK). Finally, the 'cold drug' saturated solution was spiked with a tiny amount of tritiated hydrocortisone solution and mixed thoroughly such that each donor solution exhibited an activity of 5 μCi/ml. Although addition of this small volume was technically moving the solute concentration away from saturation, the change was negligibly small given the tiny aliquot volume.

2.3. Determination of hydrocortisone solubility in PBS

In order to be able to calculate the permeability coefficient of hydrocortisone, it is necessary to know its concentration at saturation in the PBS donor solution. The value was determined by using a radioactivity-based method. An aliquot of tritiated hydrocortisone (20 μCi) was added to 10 mg of 'cold' hydrocortisone. This was allowed to evaporate to dryness overnight. Subsequently, 20 ml of ethanol was added to hydrocortisone and thoroughly mixed to provide radiolabelled hydrocortisone solution. Aliquots of increasing volume (representing increasing mass of hydrocortisone) were added to separate scintillation vials, allowed to evaporate overnight, and reconstituted with 100 μl of PBS (pH 7.4). Liquid scintillation counting of this solution yielded standard curves relating disintegrations per minute to total drug mass. To determine saturation solubilities in PBS, remaining solution of hydrocortisone was separately evaporated to dryness overnight and 10 ml PBS (pH 7.4) was added. The solution was stirred and filtered as described above to prepare radiolabelled saturated solution of hydrocortisone. Each saturated solution was then assayed by liquid scintillation counting. Each experiment was performed in triplicate.

2.4. Sourcing of human skin

Full-thickness cadaveric human skin samples were obtained from the National Disease Research Interchange (Philadelphia, PA). The skins were derived from the abdominal sites of elderly

Caucasian females. All the tissues were previously sero-tested for HIV and hepatitis B and showed to be clear of these viruses. Furthermore, full ethical approval was obtained for both sourcing and use of these skins. The supplied skin sections were stored at −20 °C for a period of up to 6 months.

2.5. Preparation of human skin epidermal and sandwich membranes

Just before each permeation study, the skins were thawed at room temperature and epidermal membranes were prepared using the established heat separation method (Kligman and Christophers, 1963). Basically, surplus fat and adipose tissues were removed from the skin sections. These were then immersed for 45 s in a water bath maintained at 60 °C. The epidermis was then gently teased off the lower dermis and floated on an aqueous solution containing 0.002% (w/v) sodium azide. Stratum corneum (SC) membranes were prepared according to the methodology developed by Essa et al. (2002) with very slight modifications. This was performed by having the epidermal membranes floating with the epidermal side down on an aqueous solution containing 0.0001% trypsin and 0.5% (w/v) sodium hydrogen carbonate maintained at 37 °C. These conditions allow the enzyme to fully digest the viable tissue. After 12 h, the membranes were picked up on filter paper and any digested cells were washed off with distilled water. The SC membranes were then floated on water for 2 h to remove any remaining digested cells and trypsin. In order to hydrate both the epidermal and SC membranes, these were floated with the SC side uppermost on 0.002% sodium azide solution for at least 24 h. Subsequently, SC membranes were placed upon epidermal membranes derived from adjacent skin regions in order to produce SC/epidermal sandwiches.

2.6. Total hydration of the epidermal and sandwich membranes

Successful application of the skin sandwich approach involves the use of fully hydrated single epidermal and sandwich membranes. This is because the tissues will absorb water over time and thus swell. This swelling changes the dimensions of the membranes with time and thus the basic concept of the technique is undermined. Hence, studies have shown that it is best to use single membranes and sandwiches that are totally hydrated and thus do not enlarge further upon exposure to water (Frum et al., 2008). In addition, it is essential to ensure that sweat gland openings are sealed shut by prolonged hydration. The static Franz diffusion cells were used in this work to fully occlude and hydrate the membranes.

An aqueous solution containing 0.002% (w/v) sodium azide was degassed by ultrasonication (Camlab Transsonic T310, Cambridge, UK) for 15 min. The receiver compartments were then filled with this solution while epidermal or sandwich membranes were inserted as barrier membranes with the stratum corneum side uppermost. The donor compartments were filled with 0.002% (w/v) sodium azide solution and subsequently sealed with Parafilm®. The Franz cells were left for 24 h, thus facilitating virtually full hydration of the membranes. Subsequently, the preservative solution in the donor compartment was removed. Each cell was then carefully inverted to allow any entrapped air bubbles to escape from the solution in the side-arm. Hydrocortisone skin sandwich studies could then be started.

2.7. Permeation studies

A 200 μl volume of saturated hydrocortisone solution in PBS was added on to each hydrated epidermal or hydrated sandwich membranes mounted in the Franz cell. Ultrasonication at 20 kHz was then undertaken. This was achieved by placing the ultrasound transducer probe inside the donor compartment with its active tip face located 5 mm above the skin membrane surface. The skin was sonicated for 0 s (control), 30 s or 45 s using a pulsed mode (10% duty cycle) with the SATA intensity of 3.7 W/cm^2 (Sarheed and Abdul Rasool, 2011). The transducer was then removed and permeation was allowed to proceed for 52 h. At selected time points, a 100 μl aliquot of receiver solution was withdrawn from each receiver cell and replaced with the same volume of blank receiver solution. Each 100 μl aliquot was vortexed with 3 ml of scintillation fluid using an IKA MS2 mini-shaker (Staufen, Germany). Each transport experiment was performed with at least 6 replicates runs.

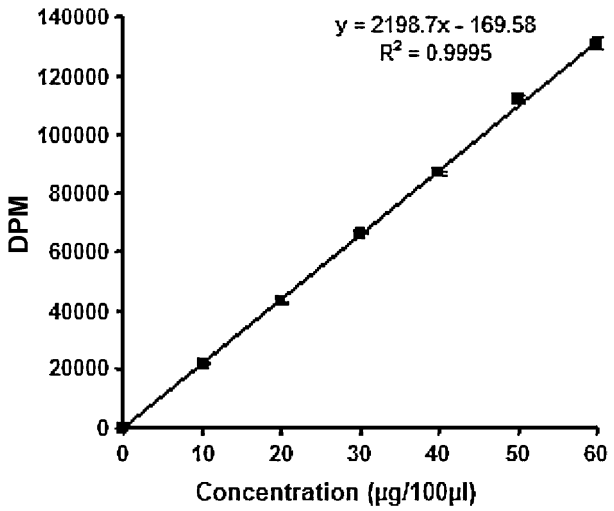

Fig. 2. Calibration curve for hydrocortisone in PBS (pH 7.4), as determined by liquid scintillation counting. Error bars represent standard deviation values, where $n = 3$.

Hydrocortisone permeation into the receiver solution was determined from liquid scintillation counter (Packard, TriCarbTM 1600TR) measurements and mathematical corrections were undertaken for the progressive dilutions. Linear regression analysis was used to determine the gradient of the steady-state segment of each hydrocortisone permeation experiment. The percentage follicular contribution was determined according to the equation below:

$$\%FC = \left[1 - 2 \times \frac{J_{Sand}}{J_{Ep}}\right] \times 100$$

where %FC is the percentage follicular contribution to total percutaneous absorption, J_{Sand} and J_{Ep} are the mean steady-state flux values for sandwich and single epidermal membranes, respectively.

3. Results

3.1. Hydrocortisone concentration in the donor solution

As described above, a radioactivity-based method was used to determine the concentration of hydrocortisone when it was at saturation in the PBS donor solution. These calibration curves related disintegrations per minute to total drug mass (see Fig. 2). The saturation solubility of hydrocortisone in PBS was found to be 0.29 mg/ml.

3.2. The influence of ultrasound on hydrocortisone permeation

Fig. 3 shows the hydrocortisone permeation plot under passive conditions (i.e. no ultrasound). Drug flux through single epidermal as well as sandwich membranes is shown. Steady-state drug flux across human epidermis was 34.09 ± 8.91 ng cm^{-2} h^{-1} and this

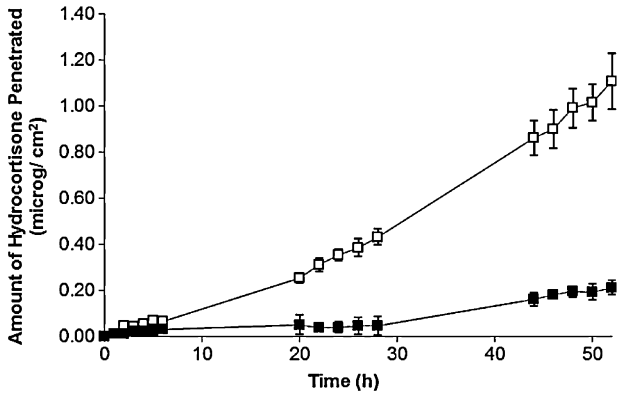

Fig. 3. Hydrocortisone permeation through single epidermal and sandwich membranes under passive conditions. (■) Represents sandwich data and (□) represents single epidermal data. Error bars represent standard error of the mean ($n \geq 6$).

is equivalent to a mean k_p of 1.16×10^{-4} cm h^{-1}. It is noteworthy that others have published similar mean k_p values for hydrocortisone flux through human epidermis such as 1.2×10^{-4} cm h^{-1} (Hadgraft and Ridout, 1987) and 2.3×10^{-4} cm h^{-1} (Johnson et al., 1995). Variations in skin sites used, preparation conditions and experimental conditions explain the value discrepancies between studies. For the sandwich, steady-state hydrocortisone flux was 9.19 ± 2.82 ng cm^{-2} h^{-1}. This means that the J_{Sand}/J_{Ep} value was 0.27 and this corresponds to a follicular contribution of 46%.

Fig. 4 presents the cumulative hydrocortisone permeation as a function of time following 30 s of pulsed ultrasound application. The permeation data for both single membranes and sandwiches is shown. Mean steady-state hydrocortisone flux across single and double membranes was 25.48 ng cm^{-2} h^{-1} and 9.8 ng cm^{-2} h^{-1} respectively. This is equal to a J_{Sand}/J_{Ep} value of about 0.38, which is equivalent to a follicular contribution to flux of 24%.

As Fig. 5 shows, when human skin was treated with ultrasound for a longer duration of 45 s, mean steady-state hydrocortisone flux across single epidermal samples was much higher at 298.39 ± 56.15 ng cm^{-2} h^{-1}. This is equivalent to a permeability coefficient of 29.84 ng/cm^2. For sandwich experiments, the measured steady-state flux was 150.17 ± 54.07 ng cm^{-2} h^{-1}. This calculates as a J_{Sand}/J_{Ep} value of 0.5, which means the follicular component was zero. Table 1 shows all the measured and calculated parameters for this study.

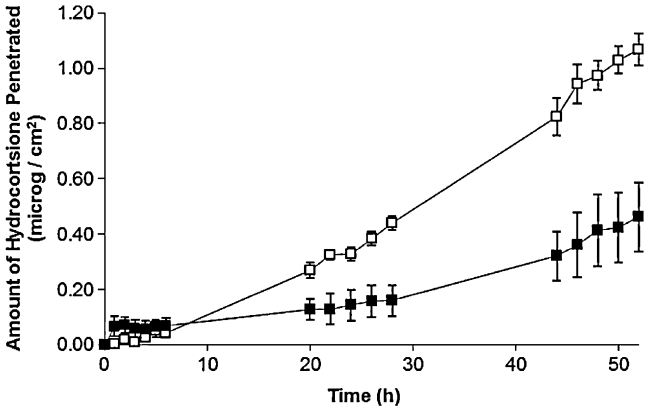

Fig. 4. Hydrocortisone permeation through single epidermal and through sandwich membranes following a 30 s application of 20 kHz ultrasound. (■) Represents sandwich data and (□) represents single epidermal data. Error bars represent standard error of the mean ($n \geq 6$).

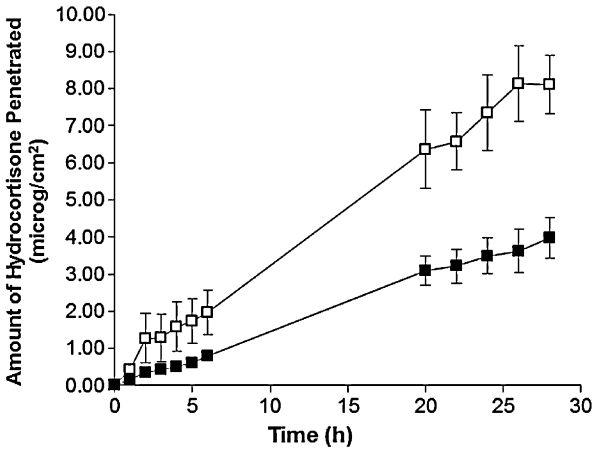

Fig. 5. Hydrocortisone permeation through single epidermal and through sandwich membranes following a 45 s application of 20 kHz ultrasound. (■) Represents sandwich data and (□) represents single epidermal data. Error bars represent standard error of the mean ($n \geq 6$).

4. Discussion

4.1. The nature of the information gained from the skin sandwich system

Before interpreting the results of the skin sandwich experiments, it is useful to consider the limitations of the information yielded by this methodology. The skin sandwich provides information on how much drug enters the follicular orifice as opposed to penetrating through the continuous stratum corneum surface. Hence, the %follicular contribution refers to the flux of drug entering the follicular opening as a fraction of total drug flux into the skin. Importantly, the skin sandwich system does not provide any information with regards to drug fate inside the follicle.

Another issue is the integrity of the stratum corneum. Relatively large tears or holes in stratum corneum samples would cause unusual penetration–time plots in both single membranes and double membranes. Such plots were not observed which indicates that our skin samples were mostly free from such tears. Yet the presence of smaller follicle-size holes in some of our stratum corneum samples is maybe possible. These would cause our follicular component values to be higher than the actual values.

In the skin sandwich system, the stratum corneum is highly hydrated. This would be expected to increase hydrocortisone flux through the continuous skin above the levels expected in the clinical situation. On the other hand, it is believed that hydration causes swelling of the cells in the stratum corneum, causing partial closure of the follicular orifices (El Maghraby et al., 2001). This effect would cause follicular absorption to be underestimated.

An important point is that in the skin sandwich system, sebum and hair shafts are absent from all membranes. Some research has indicated that the outward sebum flow in vivo can reduce the drug absorption into the pilosebaceous units (Meidan et al., 2005; Singh et al., 2000). However, Lademann et al. (2001) used stripping, staining and laser scanning microscopy to show that human hair follicles were either active or inactive. Active follicles were characterised by sebum generation and/or hair growth while inactive follicles did not do either. Surprisingly, curcumin penetration into the hair follicles of volunteers only occurred when there was sebum production and/or hair growth. It was proposed that a 'pumping' process by ridges on the growing hair itself was necessary for permeation to occur. This surprising concept was later demonstrated in other studies by the same team (Ossadnik et al., 2007; Lademann et al., 2007). On the other hand, the presence of a hair will reduce the follicle orifice surface area (Ferry et al., 1995) as well as its

Table 1
Data derived from skin sandwich studies.

Sonication period	J_{Ep} (ng cm^{-2} h^{-1}) Mean ± s.e.m.	Mean epidermal k_p (cm h^{-1} × 10^{-4})	J_{Sand} (ng cm^{-2} h^{-1}) Mean ± s.e.m.	J_{Sand}/J_{Ep}	Follicular contribution (%)
0 s	34.09 ± 3.98	1.16	9.19 ± 1.63	0.27	46
30 s	25.48 ± 3.49	0.87	9.80 ± 2.72	0.38	24
45 s	298.39 ± 22.92	10.15	150.17 ± 22.07	0.50	0

volume (Otberg et al., 2004). In summary, it is still uncertain how the absence of hair and sebum in the skin sandwich system affects the accuracy of follicular contribution measurements.

4.2. Sonophoresis and the hair follicles

Despite the limitations and questions mentioned above, the skin sandwich system can still provide important information on the drug's preference for the follicular orifice over the continuous stratum corneum in vitro. The results of our study indicated that for passive hydrocortisone absorption, 46% of absorption involved the drug penetrating into the follicles. This compares quite well with results obtained from use of the human follicle-free scar skin system where it was found that 58% of hydrocortisone flux was mediated via follicles (Hueber et al., 1994).

As Table 1 shows, application of ultrasound for 30 s only slightly affected hydrocortisone permeation through both single membranes and sandwiches. The calculated follicular contribution fell to 24%, indicating that 30 s of ultrasound permeabilised the continuous stratum corneum at the expense of the follicular route. One possible explanation is that the ultrasound dislocates and sloughs off the very top corneocytes in the stratum corneum, reducing the barrier function of the continuous surface but causing partial plugging of the follicular orifice. Since the follicular route of absorption is thus reduced, this could possibly explain the slightly reduced J_{Ep} value compared to the passive control. Sonication for 45 s caused much larger changes. There was an almost 9-fold increase in average flux through single epidermal membranes when compared to passive hydrocortisone flux. Also, there was a more than 16-fold increase in mean hydrocortisone flux through sandwich membranes. Under these conditions, the follicular contribution to total percutaneous absorption was zero. This indicates that 45 s of pulsed 20 kHz greatly permeabilised the continuous stratum corneum such that virtually all the hydrocortisone was entering the skin through this route. Again, this is explained by further sloughing off of the uppermost stratum corneum causing opening up of the continuous surface together with virtually total blockage of the hair follicle orifices. This hypothesis makes sense remembering ultrasonic changes to the corneocyte layers in full-thickness pig skin as visualised by scanning electron microscopy in our previous study (Sarheed and Abdul Rasool, 2011). Also, the follicles constitute a very tiny fraction (≤0.1% approximately) of the total skin surface area so there will be a large number of detached corneocytes for every hair follicle opening.

5. Conclusions

The human skin sandwich technique was used to explore the effect of brief ultrasound exposure on the transfollicular pathway of absorption. Hydrocortisone was used as a model drug. It was determined that without ultrasound, entry into follicles accounted for 46% of total penetration. As the duration of sonication increased, the follicular contribution fell to zero even though total transepidermal flux dramatically increased. This is explained by ultrasound exposure causing sloughing off of the uppermost stratum corneum. This permeabilises the continuous surface but at the same time the disturbed cornceocytes will plug hair follicle orifices.

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