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Research Paper

Effects of Growth Conditions on the Barrier Properties of a Human Skin Equivalent

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Purpose. Development of transdermal and topical formulations requires extensive skin permeation testing and the availability of reproducible test models. We have worked on development of a Human Skin Equivalent (HSE) by culture with a combination of additives, including the PPAR-α agonist clofibrate, in order to simulate the cutaneous barrier of human skin.
Methods. HSEs were constructed by culturing human keratinocytes on dermal matrices consisting of human fibroblasts and collagen and cultured in specific growth conditions (combination of clofibrate, ascorbic acid and fatty acids). The resulting HSEs were characterized for their morphology, lipid composition and permeability profile and compared to human skin and EpidermFT®.
Results. The unique media growth additives combination normalized the lipid profile and significantly increased the permeability barrier of the HSEs to caffeine and hydrocortisone ($p<0.05$). The HSEs overestimated the permeation of most compounds by 2–7 fold as compared to human skin. The permeability profiles obtained though were very similar and not significantly different ($p<0.05$) from those of EpidermFT®.
Conclusions. Culture with the growth media additives combination produced a pronounced effect on the permeability barrier of the HSEs. Further validation of permeability with additional agents could comprise the first step toward their use in skin permeability screening.

KEY WORDS: clofibrate; permeability; reconstructed skin; skin equivalents; transdermal.

INTRODUCTION

Transdermal drug delivery systems (TDDS) have offered several clinical advantages in drug delivery since their inception more than 20 years ago, and have been gaining momentum in the past decade due to their ability to provide controlled release of molecules, avoidance of first pass metabolism, reduced side effects and increased patient compliance. This has led to the development of several novel delivery devices that utilize the skin as a port for systemic delivery of agents. Also, skin is the site of application of various topical products that aim for localized delivery to its various layers. One of the primary factors in development of transdermal and topical drug delivery systems is the assessment of the percutaneous absorption and skin deposition of the agents from these formulations/devices. Traditionally, excised human cadaver or animal skin from rat, hairless mouse, guinea pig, snake and others has been used extensively for permeability testing of formulations (1). Though the data collected from human skin studies would most reflect *in vivo* performance, its advantages are offset by high data variability between skin tissues obtained from different donors or different sites of the body, increasing difficulties in procurement and high cost. Animal skin is easily procured as compared to human skin, but differs from human skin in terms of the morphological characteristics, water content and the lipid composition of the stratum corneum, which is essential to the maintenance of the skin's barrier properties (2). The need for a suitable model for permeability testing has necessitated the development and evaluation of several tissue-cultured *in vitro* skin models, frequently known as bioengineered skin substitutes or skin equivalents (SEs). Skin equivalents have been developed and researched over the past years for various applications, mainly as skin replacements in burns or wounds (3,4), for skin biology research (5), for cutaneous irritation and toxicity testing (6,7) and as models for permeability testing of agents and formulations (8,9). These skin substitutes are cultured in a controlled environment, thus increasing reproducibility and

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NOTATIONS: ALI, Air–liquid interface; DEET, *N*,*N*-diethyl-*m*-toluamide; HSE, Human skin equivalent; HSE-OP, Human skin equivalents with the optimal concentrations of the growth additives (clofibrate, ascorbic acid and lipids); HSE-C, Human skin equivalent control without clofibrate, ascorbic acid and lipids; HPLC, High-performance liquid chromatography; PPAR, Peroxisome proliferator activated receptor; SE, Skin equivalent; TDDS, Transdermal drug delivery systems; TLC, Thin layer chromatography.

reducing scope for larger standard deviations normally encountered with human skin data. Today, reconstructed models are commercially available and have a well defined architecture and lipid composition but are more frequently being used for irritation and toxicity testing (10) and to a lesser extent for evaluation of cutaneous absorption. This application is nevertheless being increasingly evaluated in recent years and has received a boost due to the acceptance of SE models by the OECD guidelines 431 for corrosivity testing (11), while the test procedure for cutaneous absorption based on reconstructed models is still open for discussion and subject to the equivalence of the permeability data obtained from the SEs to that obtained with human skin.

The skin permeability barrier is endowed with a unique combination of skin lipids, namely ceramides, cholesterol, cholesterol esters and free fatty acids, which accumulate during the keratinocyte differentiation process and are packed in lamellar granules, the content of which is extruded in the intercorneocyte space forming multilamellar sheets (12). Thus, normalization of the epidermal differentiation process and the subsequent accumulation of lipids are crucial to the formation of permeability barrier in the reconstructed skin models. Though the synthesis of intercellular lipids *in vitro* and their subsequent organization into lipid lamellae have been established in the skin models, and the intercorneocyte route has been confirmed as the main route of permeation (13), the lipid content and profile of SEs differ from that of human SC and has been suggested as the cause of the weaker permeability barrier (14).

The permeability of tested SEs in literature has been found to be several fold higher to various tested compounds when compared to human skin (15, 16). However, alterations of culture conditions of the SE, especially the culture media constitution can lead to alterations in the differentiation process and the lipid profiles of the SEs.

We have developed a human skin equivalent (HSE) that serves as a bioengineered three-dimensional skin model possessing both epidermal and dermal components (17). Many skin equivalent models that are commercially available (Epiderm® (MatTek, USA) and SkinEthic® (Nice, France) consist of only an epidermal layer seated on various inert membranes. Some others, such as Episkin® (L'Oreal, France) consist of an epidermal layer housed on a type I collagen base. Also, limited literature is available on the permeability characteristics of full thickness skin equivalents such as EpidermFT® (MatTek, USA). Our HSE is a full thickness skin equivalent, where the epidermal and the dermal cells are primary cells derived from humans, which in combination with the Type I collagen matrix form a system that can be better compared to human skin *in vivo*. Since our primary objective was to create a HSE model that possesses suitable barrier properties for permeability testing, we have grown the HSE under specific culture conditions, a combination of ascorbic acid, external fatty acids and a PPAR-α agonist and evaluated its morphology, lipid composition and the permeability profiles. We propose that the combination of the above factors will further enhance the HSE permeability barrier as compared to SE models that have utilized each of these culture media components alone. Our HSE has demonstrated lipid and permeability profiles that are significantly closer to human skin than that of the controls (without growth additives combination) and similar to the full thickness EpidermFT®.

MATERIALS AND METHODS

Cell Culture

Human dermal fibroblasts and human keratinocytes were obtained from Invitrogen Corp., (Carlsbad, CA) or were isolated from the epidermal and dermal fragments of neonatal foreskins obtained during routine circumcisions from the Department of Pediatrics, University Hospital, University of Medicine and Dentistry, Newark, NJ. Secondary cultures of fibroblasts were established with media containing Dulbecco's Modified Eagles Medium (DMEM) (Gibco-Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). Secondary cultures of keratinocytes were established with supplemented Epilife® medium (Invitrogen Corp., Carlsbad, CA) containing 0.06 mM calcium chloride, bovine pituitary extract (0.2% *v/v*), bovine insulin (5 μg/ml), hydrocortisone (0.18 μg/ml), bovine transferrin (5 μg/ml), and human epidermal growth factor (0.2 ng/ml).

Preparation of the Dermal Layer

The human skin equivalents were made in two stages, the preparation of the dermal layer followed by seeding of the keratinocytes on the dermal layer and their subsequent proliferation and differentiation (Fig. 1). The dermal layer was made by mixing in a 60 mm petri dish an aliquot of 2X DMEM (1.6 ml), FBS (1.6 ml), 0.1 N NaOH (0.2 ml), bovine type I collagen (2.0 ml) (Inamed, Santa Barbara, CA), and 220,000 fibroblasts in 1.0 ml of DMEM. The mixture was incubated for 7 days in 10% CO_2 at 37°C in a humidified incubator, to allow for contraction of this matrix to a tissue-like scaffold. This contracted collagen scaffold (~1 cm diameter) was transferred to a Transwell® insert (12 mm diameter, pore size=0.4 μm, Corning Costar Corporation, Cambridge, MA) placed in 12 well plates (Corning Costar Corporation, Cambridge, MA).

Preparation of the Full Thickness HSE Model

The surface of the dermal layer was coated with a mixture of human type I and type IV collagens (Invitrogen Corp., Carlsbad, CA) just before seeding of the keratinocytes in order to optimize the extracellular matrix for attachment of keratinocytes to the dermal layer. Seven hundred thousand keratinocytes (passage 2–3) were seeded on the top of the dermal layers in 75 μl of the media and allowed to attach for 1.5 h in a laminar hood. The attachment media consisted of supplemented Epilife® with 1.2 mM calcium chloride to enhance the attachment of keratinocytes to the dermal scaffold. DMEM (without serum) was then added to the wells from the bottom and the skin cultures were submerged in Epilife® and cultured submerged for 6 days (0.06 mM calcium) at 37°C, 10% CO_2 and 75% RH. During the last 4 days of the submerged culture, the feeding media was

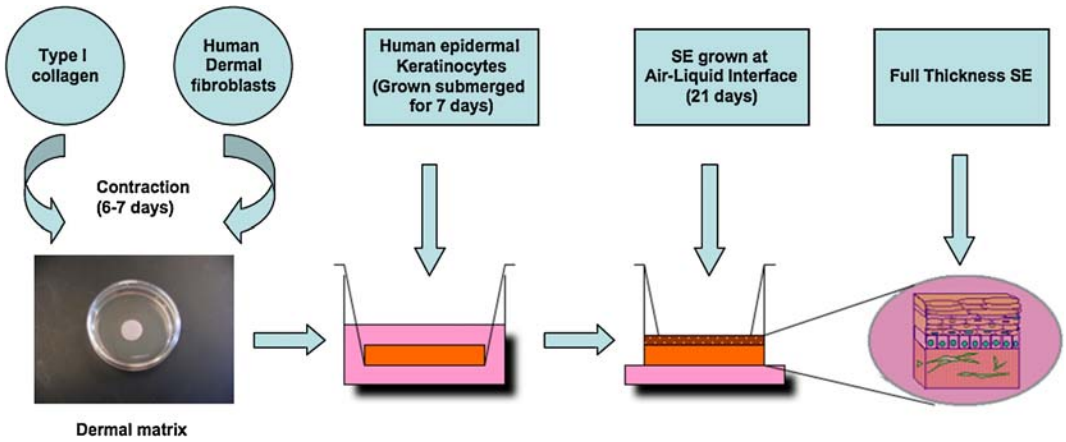

Fig. 1. Preparation of the human skin equivalent. The dermal matrix was made by contraction of bovine Type I collagen by dermal fibroblasts. Keratinocytes (Passage 2–3) were seeded onto the dermis, where they proliferate and differentiate (when exposed to the ALI) leading to formation of a multilayered epidermis. *SE* skin equivalent.

supplemented with an external free fatty acids/lipids mixture (25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid, 7 μM arachidonic acid, and 100 μM bovine serum albumin) (18). Thereafter, the skin cultures were only fed from the bottom and the surface was exposed to air to promote the differentiation of the epidermis *in vitro* (19,20). The skin equivalents were cultured at the Air–Liquid Interface (ALI) for 21 days to allow full differentiation of keratinocytes and fed two or three times a week. The feeding media at this stage was supplemented with lipids (25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid, 7 μM arachidonic acid, and 100 μM bovine serum albumin) and different concentrations of ascorbic acid (0, 50, 100, 150 μg/ml). All lipids and ascorbic acid were purchased from Sigma, St. Louis, MA.

Effect of a PPAR-α Agonist, Clofibrate on Formation of the Skin Permeability Barrier in Conjunction with Ascorbic Acid and Fatty Acids

Clofibrate (Calbiochem, San Diego, CA) in concentrations ranging from 100 to 400 μM was added to the culture media throughout culture of the equivalents at the ALI alone and in combination with optimized concentrations of ascorbic acid and lipids. Full thickness skin equivalents with the optimal concentrations of the growth additives (HSE-OP) were then characterized by light microscopy, lipid analysis and permeability barrier evaluation as per methods described below, and were compared to a control (HSE-C: without clofibrate, ascorbic acid and lipids) and to human cadaver skin. Skin thickness of the HSE post 21 days of ALI culture was measured with the aid of a micrometer (Mitutoyo, Japan) and reconfirmed by microscopy.

Visualization of the Cell Dynamics in the Skin Equivalents

In order to visualize the cell dynamics occurring within the collagen based scaffolds, rat neonatal GFP-expressing fibroblasts were used to make the scaffold instead of the regular human dermal fibroblasts. The dermal matrices were made as described under "Preparation of the dermal layer" and the layers were observed under a fluorescent microscope (Nikon Eclipse TE2000-E) during the 1st, 4th and 6th day of contraction. To visualize the attachment and distribution of keratinocytes, the nuclei of the human neonatal keratinocytes in suspension were labeled with Hoechst 33342 dye (component B of Image-iT Live Plasma Membrane and Nuclear Labeling Kit, Molecular Probes, Invitrogen Detection Technologies, Eugene, OR). All labeling procedures were conducted as per manufacturer's instructions. The labeled keratinocytes in suspension were then seeded on top of dermal layers prepared from unlabeled human dermal fibroblasts and bovine collagen as per the procedure described in "Preparation of the dermal layer". The dermal layers were fed with media, and stored in the humidified incubator at 37°C and 10% CO_2. On the subsequent day and on the 4th day after seeding, the layers were observed under a fluorescence microscope (350/461 nm) for attachment of the keratinocytes to the dermal layers and their distribution.

In Vitro Permeability Studies

The cultured HSEs (HSE-OP) were tested for their barrier properties, evaluated by comparison of their permeability to model agents as compared to split thickness human cadaver skin (obtained dermatomed to ~500 μm, Allosource, Cincinnati, OH), the control HSEs (HSE-C), and a commercially available model EpidermFT® (MatTek Corporation, Ashland, MA). EpidermFT® is a full thickness skin model developed and marketed by MatTek Corp. and is shipped on medium supplemented agarose gels at 4°C. On receipt, the tissue wells were supplemented with the provided medium and equilibrated at 37°C, 5% CO_2 overnight. All tissues were used within 1–2 days of the equilibration for permeability studies. Human skin equivalents cultured in house for 21 days at the ALI were used for the permeability studies. The skin equivalents (HSE or EpidermFT®) were transferred to

jacketed vertical Franz diffusion cells (volume 4.1 ml, diffusion area 0.196 cm^2) (PermeGear Inc., Bethlehem, PA) containing phosphate buffered solution (PBS, pH 7.4) as the receptor solution being continuously stirred at 600 rpm. Smaller size diffusion cells were used to accommodate the smaller diameters (~1.2 cm) of the skin equivalents. The diffusion cells were maintained at 37°C with the help of a thermostatic water pump (Haake DC10, Karlsruhe, Germany) leading to skin temperatures of 32±0.5°C. Saturated suspensions of drug agents in propylene glycol (5 µl) were added to the donor compartments, which was covered with Parafilm®. Samples (300 µl) were withdrawn from the receptor over 24 h and replaced with an equivalent volume of the buffer. Results were compared to data obtained from similar experiments with human skin.

HPLC Methods and Data Analysis

Analysis for drug content was conducted on an Agilent 1100 HPLC system (Agilent Technologies, USA) with an Agilent C_{18} RP column (Eclipse XDB-C18, 4.6×150 mm, 5 µm) (Agilent Technologies, USA). All methods were validated for linearity of the calibration curves, limits of detection and intra and inter day variability. All parameters are reported in Table I.

For evaluation of permeability parameters, the cumulative amounts of drug per area (µg/cm^2) were plotted against time, and the steady state flux (J_{ss}, µg/cm^2/h) was calculated as the slope of linear portion of the plot using linear regression (Microsoft Excel). Lag time (h) was obtained as the *x*-axis intercept of this linear part of the plot. Thereafter, the permeability coefficient (K_p, cm/h) was calculated as the ratio of the steady state flux to the concentration of the drug in the vehicle/formulation (Co, mg/ml).

Histology

Cultures were fixed in 4% buffered formalin overnight and then processed for embedding in paraffin. Sections (6 µm) were obtained and stained with hematoxylin and eosin. Stained samples were analyzed by light microscopy.

Lipid Analysis

The epidermal lipids were quantitatively determined using Thin Layer Chromatography (TLC) (21) following their extraction from cultures or from human cadaver skin (used as controls). Following lyophilization, lipids were extracted from the dried tissue successively at 2-hour intervals with chloroform: methanol mixture (2:1, 1:1 and 1:2). Combined extracts from each set of cultures were dried under a gentle stream of nitrogen, redissolved in 100 µl of chloroform: methanol (2:1) and the dissolved lipids were separated using TLC. 20×20 cm glass plates coated with 0.25-mm-thick silica gel G (Adsorbosil-plus-1; Alltech Associates; Deerfield, IL) were washed with chloroform: methanol, 2:1, activated in a 110°C oven, and the adsorbent was scored into 6-mm-wide lanes. Samples (5 or 10 µl) were applied 2–3 cm from the bottom edge of the plate, and the chromatogram was developed. Two development regimens were used. To resolve ceramides, glucosylceramides and phospholipids, chromatograms were developed with chloroform:methanol:water, 40:10:1, to 6 cm; followed by chloroform:methanol:acetic acid, 190:9:1, to 20 cm; followed by hexane:ethyl ether:acetic acid, 70:30:1 to 20 cm. To resolve cholesterol, fatty acids, triglycerides and cholesterol esters, chromatograms were developed with hexane to 20 cm; followed by toluene to 20 cm; followed by hexane:ethyl ether:acetic acid, 70:30:1, to 12 cm. For quantification, the developed chromatograms were air dried, sprayed with 50% sulfuric acid and charred by slowly heating to 220°C. The charred chromatograms were scanned with a photodensitometer.

Statistical Analysis

All data was analyzed with the aid of Microsoft Excel and Kaleida Graphs 4.02, (Synergy Software, Reading, PA). ANOVA ($p<0.05$) was used to determine statistical significance of data.

Table I. Development and Validation of HPLC Methods

Drug	Mobile phase	Flow rate (ml/min)	Detection wavelength (nm), retention time (min)	Intra and inter-day variability, limit of detection
Caffeine	Acetonitrile: water:methanol (10:70:20 *v/v/v*)	1	270, 3.2	0.06, 0.81, 0.5 µg/ml
Hydrocortisone	Acetonitrile: water (40:60 *v/v*)	1	242, 2.90	0.23, 2.78, 0.5 µg/ml
Ketoprofen	Acetonitrile:water (0.1% TFA) (80:20 *v/v*)	1	264, 1.82	0.08, 1.31, 0.05 µg/ml
N,*N*-Diethyl-*m*-toluamide (DEET)	Methanol:water (80:20 *v/v*)	0.7	240, 3.08	0.45, 0.48, 0.5 µg/ml
Paraoxon	THF:acetonitrile:sodium phosphate buffer (pH 7.4) (12:25:63 *v/v/v*)	1	280, 7.2	0.07, 1.67, 0.5 µg/ml
Malathion	Acetonitrile:water (70:30)	1.25	215, 3.1	0.23, 1.78, 0.1 µg/ml

The correlation coefficient of linearity for all methods was >0.99

RESULTS

Macroscopic, Histological and Ultrastructural Features of Skin Equivalents

The dermal layers made from collagen and fibroblasts contracted gradually over 6 days to a tissue about 1 to 1.2 cm in diameter, after which they were seeded with keratinocytes from the top. Macroscopic observation of the surface of the skin equivalents post exposure to the ALI showed a transformation from wet and shiny surfaces to dry and matte yellowish surfaces 2–3 days post exposure to air, indicating the formation and differentiation of an epidermal layer. The HSE cultures were ~1,000 μm thick, and histological analysis after 21 days showed an epithelial architecture resembling native epidermis with formation of a multilayered and differentiated epidermis, including the basal, spinous, and granular layers and a flattened stratum corneum (Fig. 2). Epidermal markers of differentiation such as keratohyalin granules were present in cultures, and were significantly increased when cultures were grown in the presence of clofibrate and ascorbic acid (Fig. 2b, c, d).

Distribution and Attachment of Cells in the Skin Equivalent Matrix

In order to visualize the distribution and viability of fibroblasts in the skin equivalent matrix, we used rat neonatal GFP-expressing fibroblasts to make the dermal equivalents and observed the matrices over the 6 days of contraction under a fluorescent microscope. On the 1st day, the fluorescent cells were found to be uniformly dispersed throughout the matrix and exhibited a circular shape with a circularity index of 0.86 (SD: 0.04) (Fig. 3a). On day 4, and more so on day 6, the fibroblasts looked increasingly fibrous, displayed a uniform dispersion throughout the matrix and appeared to be growing well and proliferating in the collagen matrix (Day 4 circularity index: 0.43 (SD: 0.12) (Fig. 3b), day 6 circularity index: 0.22 (SD: 0.07) (Fig. 3c). The circularity indices obtained for cells on day 4 and 6 were significantly different from that of day 1 ($p<0.05$). The health and homogeneity of the cells corresponded to the gradual contraction of the collagen matrix over 6 days to a dermal tissue.

In order to determine the attachment of keratinocytes on the surface of the collagen matrix and their homogeneity when attached, we labeled the nucleus of keratinocytes in suspension with Hoechst 33342 dye and seeded them on dermal matrices made with type I collagen and unlabeled dermal fibroblasts. When viewed under a microscope 1 day after seeding, the keratinocytes had attached to the surface of the matrix and appeared to be uniformly distributed throughout the surface of the dermal layer (Fig. 4).

Effect of the PPAR-α Agonist Clofibrate Alone and in Combination with Ascorbic Acid and Lipids on the Morphology and the Lipid Profile of the HSE

Culture of the skin equivalents in the presence of different concentrations of clofibrate (100, 200, 300,

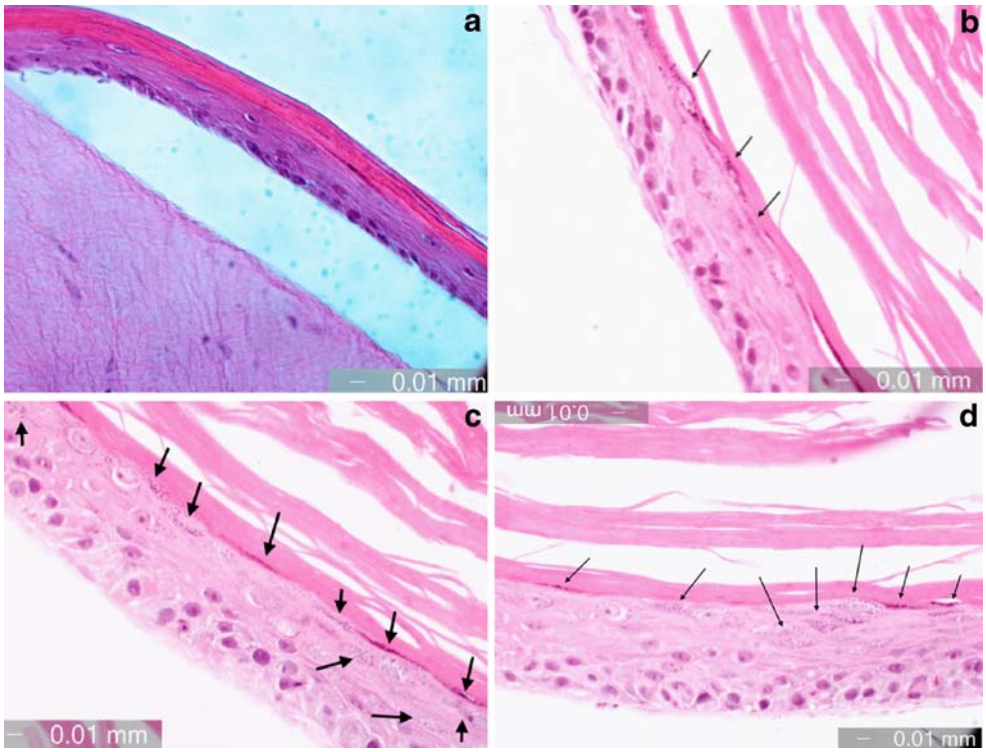

Fig. 2. Morphology of HSE (**a**) in the absence of fatty acids, ascorbic acid and clofibrate (HSE control), (**b**) in the presence of ascorbic acid (100 μg/ml), (**c**) in the presence of clofibrate 300 μM, (**d**) in the presence of fatty acids, ascorbic acid (100 μg/ml) and clofibrate (300 μM). Paraffin embedded sections were stained with Hematoxylin and Eosin. *Arrows* indicate the presence of keratohyalin granules.

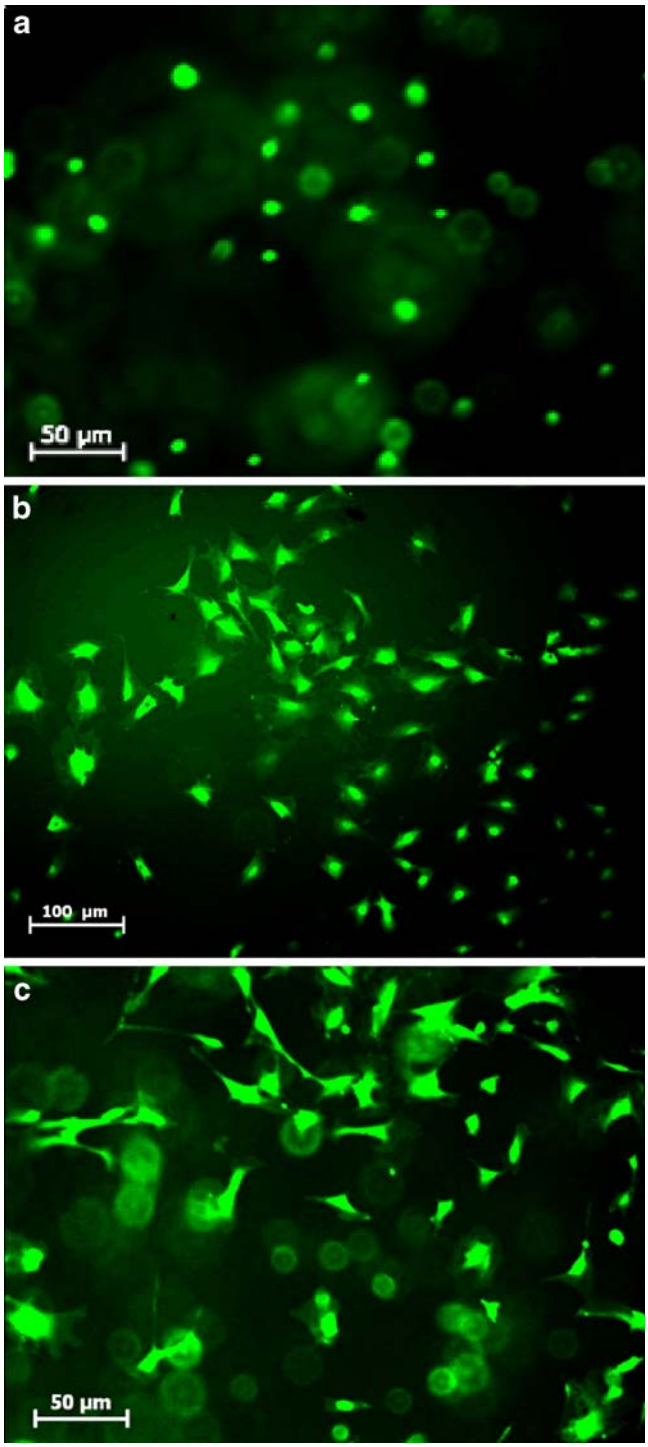

Fig. 3. Dermal layers made with GFP expressing fibroblasts. On day 1 (**a**), cells appear circular and homogenously distributed in the collagen matrix. Circularity index: 0.86 (SD: 0.04) Image J software, NIH, USA. Cells are growing well and are fibrous on day 4 (**b**), Circularity index: 0.43 (SD: 0.12) and on day 6 (**c**), Circularity index: 0.22 (SD: 0.07) and appear to be proliferating in the matrix.

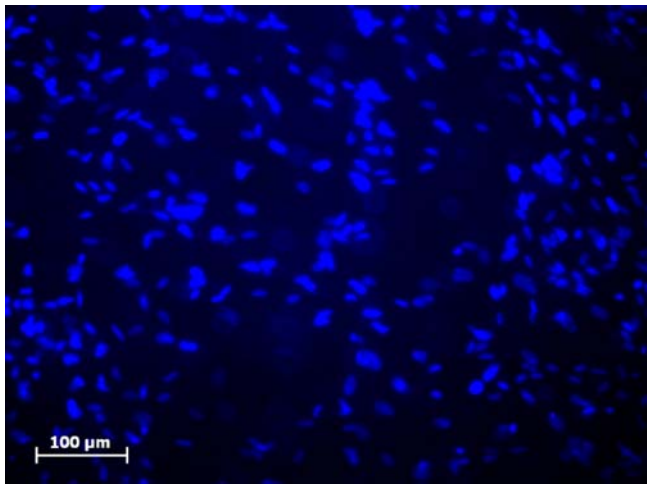

Fig. 4. Nuclei of keratinocytes labeled with Hoechst 33342 dye seen attached on the surface of the fibroblast–collagen matrix. The attachment of keratinocytes is homogenous.

400 µM) alone and in conjunction with external lipids and ascorbic acid (HSE-OP) demonstrated significant changes in the ultrastructural features of the HSE (Fig. 2c, d) with an increase in number of viable epidermal layers and the thickness of the stratum corneum. Significant increase in the amount of keratohyalin granules, which are markers of the keratinocyte differentiation process, was also observed indicating normalization in the differentiation process and the subsequent lipid metabolism in the epidermis (Fig. 2d).

Based on previous studies that have reported the deleterious effect of serum on the morphogenesis, differentiation and lipogenesis of the epidermis grown at the ALI (22, 23), we used a serum free culture for culturing the HSEs. Analysis of the lipid composition of the HSE cultures by Thin Layer Chromatography allowed good separation of ceramides, cholesterol, cholesterol esters and free fatty acids, a unique combination of lipids found in upper layers of skin.

We observed a significant difference in the total lipid and ceramide composition in the HSE cultures grown with or without ascorbic acid, fatty acids and clofibrate. As shown in Table II, the lipid components of the control HSE without any added ascorbic acid, fatty acids and clofibrate (HSE-Control) differed from that of native skin. The content of glucosylceramides, acylglucosylceramides, ceramides and fatty acids in the control HSE were much lower than that of human skin, while the content of triglycerides was 3.6 fold higher than native tissue. Also, the ceramide profile also deviates from that of native skin: the content of ceramides 5, 6 and 7 was very low while that of ceramide 2 was higher than human skin (Table III). Cultures supplemented with clofibrate alone showed an increase in the total ceramide content as clofibrate concentration increased from 100 to 300 µM as compared to the control, where the highest total ceramide content was obtained with addition of 300 µM of clofibrate (61.6% increase as compared to control) (Table II). While the amount of ceramides 4, 5, 6 and 7 increased 2.2, 3.4, 1.7 and 12 fold respectively, there was a 33.6% decrease in the amount of ceramide 3 (Table III). Skin equivalents cultured at the ALI with this optimal concentration of clofibrate (300 µM) in conjunction with external lipids and ascorbic acid (100 µg/ml) (HSE-OP) demonstrated an increase in the content of glucosylceramides and acylglucosylceramides and a decrease in triglycerides as compared to control (Table IV). Further increase in ceramides 5, 6 and 7 and a decrease in the

Table II. Lipid Composition of HSE Treated with Different Concentrations of Clofibrate Only (Without Ascorbic Acid and Lipids)

Lipid class	Control HSE	HSE cultured with different clofibrate concentrations				Human cadaver skin
		100 μM	200 μM	300 μM	400 μM	
Phospholipids	19.5±3.8	23.6±2.3	19.6±2.1	21.4±2.7	24.8±2.4	39.2±2.1
Glucosylceramides	1.0±0.3	0.8±0.2	0.6±0.1	0.7±0.1	0.3±0.1	4.8±0.7
Acylglucosylceramides	0.2±0.1	0.5±0.2	0.5±0.1	0.3±0.1	0.2±0.0	Trace
Ceramides	8.6±1.4	9.6±1.1	11.8±1.0	13.9±0.9	7.6±0.4	12.2±2.2
Cholesterol	25.9±3.6	23.8±2.1	21.4±2.1	18.3±1.6	16.1±1.3	19.4±2.9
Fatty acids	6.7±0.6	9.1±1.5	11.2±2.1	8.2±1.1	8.9±1.2	8.1±0.6
Triglycerides	33.5±3.1	28.5±2.6	29.6±2.4	33.2±4.1	34.6±3.5	9.2±0.9
Cholesterol esters	4.6±0.8	4.1±0.9	5.3±0.8	5.0±1.1	5.9±1.2	7.1±0.5

Control HSE was cultured with no added clofibrate

content of ceramide 2 was observed with this combination of a PPAR-α agonist, ascorbic acid and lipids (Table V). It can be stated that a large contribution to the increase in glucosylceramide and ceramide content in the cultures was due to the presence of ascorbic acid as observed from cultures grown only in the presence of ascorbic acid (data not shown). This is consistent with previous studies that have established the role of ascorbic acid in the normalization of the ceramide profile in skin equivalents (24).

Permeability of the HSE to Model Drugs

The culture of the skin equivalents with clofibrate in combination with ascorbic acid and lipids resulted in a statistically significant decrease ($p<0.05$) in their permeability to model drugs caffeine and hydrocortisone as compared to the control HSE. (Fig. 5a, b; Tables VI and VII). While the permeability of HSE to caffeine decreased by about 67% with the addition of clofibrate alone and by 76% by the addition of clofibrate, ascorbic acid and lipids, the permeability of the HSE to hydrocortisone decreased by 58% with the addition of clofibrate and by 79% with the addition of clofibrate, ascorbic acid and lipids (Fig. 5c, d). Also, the combination of all three constituents increased the lag time of the HSE cultures, indicating a higher penetration barrier (Tables VI and VII). The decrease in skin permeability of the optimized cultures can be correlated to the improvement in barrier properties as a result of improved keratinocyte differentiation and lipid metabolism. We also compared the permeability profiles of the HSE-OP with a commercially available model, EpidermFT® using similar experimental conditions (Table VIII). Both HSE-OP and EpidermFT® demonstrated a lower permeability barrier to all drugs as compared to human skin (from ~2 to 25 fold higher permeability than human skin for different agents tested). However, their permeability profiles for most drugs was similar to each other, albeit a few deviations (Fig. 6). A significant difference ($p<0.05$) in permeability between the HSE and EpidermFT® was observed for the model drug caffeine, where the HSE estimated lower permeability than EpidermFT® (Fig. 6a). However, for all other agents, the permeability profiles were very close, such that no significant difference was observed.

DISCUSSION

The enormous potential and growth of transdermal and topical delivery systems necessitates the use of reliable models that aid in the evaluation of skin permeation of compounds and their subsequent screening and formulation development. In order to obtain reliable permeability data, the skin models should possess a permeability barrier that reflects the barrier provided by human skin. We have cultured a HSE with a media formulated with a combination of external lipids, ascorbic acid and the PPAR-α agonist clofibrate. Media supplemented with external lipids has been shown to induce the synthesis of lipid precursors and formation of lamellar bodies in serum free SE culture (18). Also, the presence of ascorbic acid in cultures has been shown to markedly improve the lipid profile and the barrier formation in reconstructed skin (25). Pasonen-Seppanen *et al.* have shown that culture of an organotypic keratinocyte culture with 40 μg/ml of ascorbic acid for 3 weeks reduced the

Table III. Ceramides Composition of HSE Treated with Different Concentrations of Clofibrate Only (Without Ascorbic Acid and Lipids)

Ceramides	HSE control	HSE cultured with different clofibrate concentrations				Human cadaver skin
		100 μM	200 μM	300 μM	400 μM	
1	16.9±2.3	13.2±1.1	14.3±2.2	13.4±1.4	16.5±1.3	10.2±1.8
2	49.8±2.9	53.6±3.1	49.2±3.3	48.1±3.6	47.3±2.9	22.1±0.2
3	23.2±3.1	19.5±1.8	18.7±2.1	15.4±1.1	16.7±2.4	21.9±1.6
4	5.2±1.0	7.1±0.9	7.5±1.1	11.6±1.6	10.2±1.2	6.9±1.3
5	3.7±0.8	3.4±0.7	5.7±1.0	6.5±1.0	5.4±1.0	16.7±1.6
6	1.1±0.0	2.1±0.4	3.3±0.8	3.8±0.6	2.3±0.1	7.5±1.5
7	0.1±0.0	1.1±0.2	1.3±0.1	1.2±0.5	1.6±0.1	14.7±0.8

Control HSE was cultured with no added clofibrate

Table IV. Lipid Composition of HSE Treated with Ascorbic Acid, Lipids and Clofibrate

Lipid class	HSE control	Ascorbic acid (100 μg/ml) and external lipids	Clofibrate 300 μM, ascorbic acid (100 μg/ml) and external lipids	Human cadaver skin
Phospholipids	19.5±3.8	24.5±3.1	23.8±2.1	39.2±2.1
Glucosylceramides	1.0±0.3	5.1±0.3	5.3±0.3	4.8±0.7
Acylglucosylceramides	0.2±0.1	0.7±0.0	0.6±0.1	Trace
Ceramides	8.6±1.4	13.4±1.0	14.1±0.5	12.2±2.2
Cholesterol	25.9±3.6	28.5±2.8	25.3±3.1	19.4±2.9
Fatty acids	6.7±0.6	9.5±0.6	10.1±0.5	8.1±0.6
Triglycerides	33.5±3.1	13.5±1.0	15.5±1.6	9.2±0.9
Cholesterol esters	4.6±0.8	4.8±1.0	5.3±1.2	7.1±0.5

The external lipid mixture consisted of a combination of 25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid and 7 μM arachidonic acid. Control HSE was cultured with no added clofibrate, ascorbic acid and external lipids

TEWL (TransEpidermal Water Loss) by approximately 50% (26). The effect of PPAR agonists on skin homeostasis has been studied by some groups since the 1990s. The PPAR receptor subtypes (α, β, γ) are expressed in man and have shown to heterodimerize with Retinoid X Receptors (RXRs) and play an important role in lipid metabolism. All three PPAR subtypes are expressed in human epidermal keratinocytes and the expression of PPAR (α, and γ) has been found to increase during the course of keratinocyte differentiation (27). PPAR-α agonists such as clofibrate have also been shown to play a role in the stimulation of epidermal lipid synthesis, increase in lamellar body secretion, acceleration of extracellular lipid processing, all eventually leading to improvement in epidermal permeability barrier homeostasis (28).

We cultured HSEs in serum-free conditions in the presence of external fatty acids/lipids (25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid, 7 μM arachidonic acid) used during the last 4 days of submerged culture and a combination of ascorbic acid (100 μg/ml), a PPAR-α agonist clofibrate (300 μM) and the external fatty acids during the ALI culture (HSE-OP). The addition of clofibrate alone and in combination with ascorbic acid and free fatty acids produced a significant difference not only in the morphological aspects of the HSE, but also in the lipid profile, epidermal homeostasis and the permeability barrier. Activation of PPAR-α receptors by clofibrate normalized the ceramide profile of the HSE and resulted in an increase in the total amount of ceramides and also in the amounts of ceramides 4, 5, 6 and 7, as compared to the control. The addition of naturally occurring fatty acids, which are also known potent activators of PPAR-α (29) and ascorbic acid to the culture in addition to clofibrate led to further normalization of the lipid profile of the HSE resulting in an array that is closer to human skin (Table IV). While the content of ceramide 2 was decreased, the amount of selected ceramides and glucosylceramides was increased. The amount of ceramide 4, 5, 6 and 7, all polar α- and ω-hydroxylated species were increased significantly (Table V), probably in part due to the ascorbic acid led stimulation in the activity of ceramide synthase, a key enzyme in epidermal sphingolipid metabolism and generation of hydroxylated and non-hydroxylated ceramides (24).

The normalization of the lipid profile in the HSE-OP led to a decrease in the permeability of the HSE to model agents as compared to the control, due to the increase in the permeability barrier residing in the epidermis. For evaluation of the permeability barrier of the HSE, we chose compounds differing in their physico-chemical properties and their therapeutic potential. Caffeine (stimulant) (log P: −0.13± 0.37, MW: 194.12), hydrocortisone (steroid hormone) (log P: 1.42±0.47, MW: 362.47), DEET (insect repellant) (log P: 1.95 ±0.23, MW: 191.27), malathion (organophosphate insecticide) (log P: 2.92±0.34, MW: 330.4), paraoxon (organophosphate insecticide) (log P: 2.30±0.30, MW: 275.22), and ketoprofen (non-steroidal anti-inflammatory drug) (log P: 2.81±0.32, MW: 254.3) were all evaluated for their permeability through HSE, EpidermFT® and human cadaver skin. In addition, we also used caffeine and hydrocortisone to evaluate the changes in the epidermal barrier by supplementation with free fatty acids, ascorbic acid and clofibrate. The HSE-OP demonstrated significantly reduced permeability to both

Table V. Ceramides Composition of HSE Treated with 300 μM of Clofibrate in Conjunction with Ascorbic Acid (100 μg/ml) and External Lipids

Ceramides	HSE control	Clofibrate 300 μM	Clofibrate 300 μM, ascorbic acid (100 μg/ml) and lipids	Human cadaver skin
1	16.9±2.3	13.4±1.4	14.8±0.9	10.2±1.8
2	49.8±2.9	48.1±3.6	26.2±2.3	22.1±0.2
3	23.2±3.1	15.4±1.1	19.6±2.2	21.9±1.6
4	5.2±1.0	11.6±1.6	10.7±1.8	6.9±1.3
5	3.7±0.8	6.5±1.0	15.8±1.3	16.7±1.6
6	1.1±0.0	3.8±0.6	5.4±0.7	7.5±1.5
7	0.1±0.0	1.2±0.5	7.5±1.7	14.7±0.8

The external lipid mixture consisted of a combination of 25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid and 7 μM arachidonic acid. Control HSE was cultured with no added clofibrate, ascorbic acid and external lipids

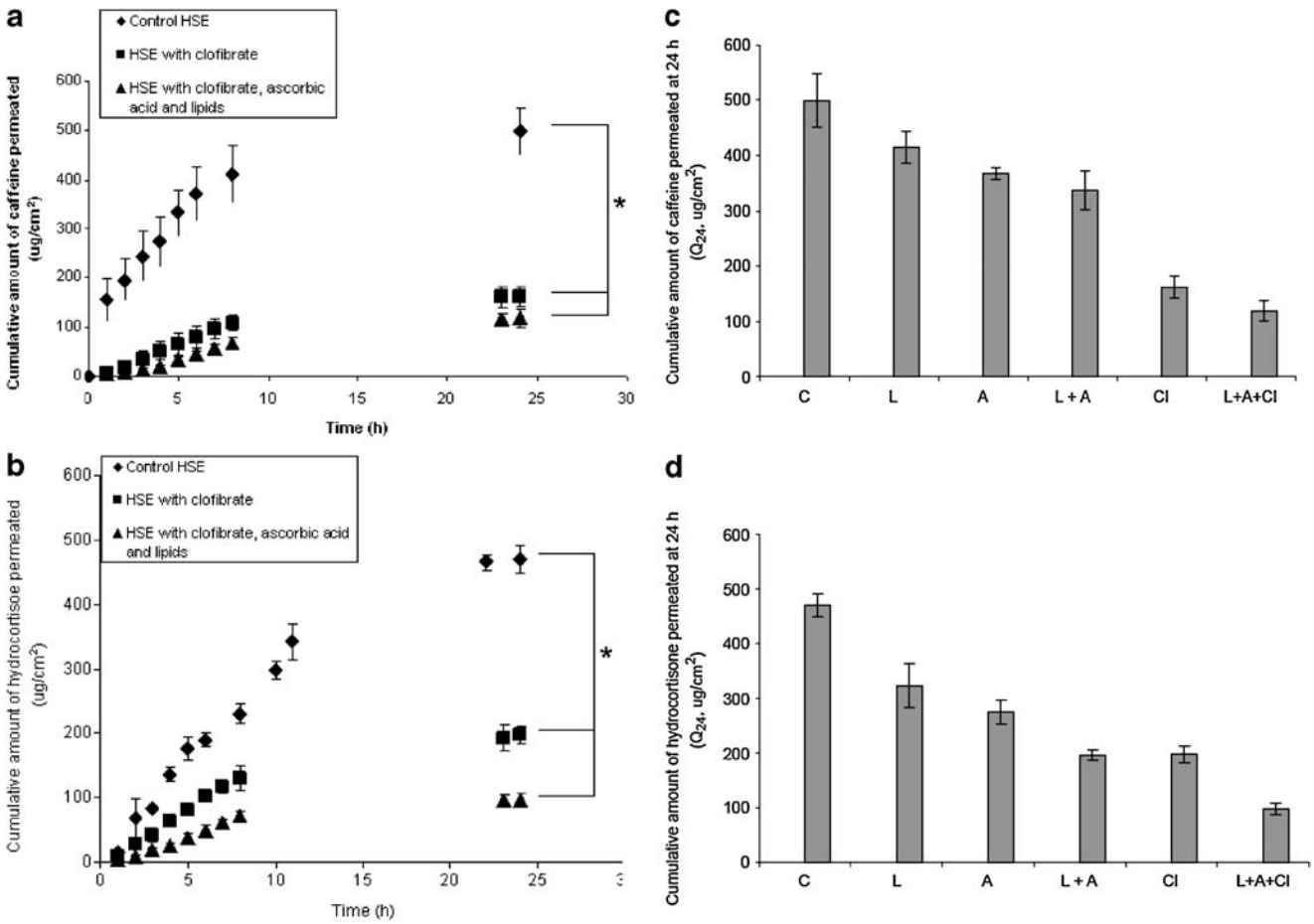

Fig. 5. Cumulative amount of caffeine (**a**) and hydrocortisone (**b**) permeated through control and optimized HSEs over 24 h. Culture of the HSE with specific combination of growth additives (external lipids, ascorbic acid (100 μg/ml) and clofibrate (300 μM)) significantly decreased the permeability of the HSE ($p<0.05$) to caffeine (76% decrease) and hydrocortisone (79% decrease). (**c**) and (**d**) represent the decrease in permeability of the HSE to caffeine and hydrocortisone after culture with each of the external additives added to culture media. *c* control, *L* external lipids, *A* ascorbic acid, *Cl* clofibrate. Note: all treatments, individual and in combination were found to be significantly different than the control ($p<0.05$).

caffeine and hydrocortisone as compared to the control HSE, although permeability was still 2–4 fold higher than native tissue (Figs. 5 and 6, Tables VI and VII). However, the HSE-OP demonstrated very similar permeation profiles for most agents when compared to the commercially available full-thickness skin equivalent model EpidermFT® (Fig. 6). However, a significant difference was observed between HSE-OP and EpidermFT® for caffeine permeation, where the permeation parameters for HSE-OP (Q_{24}: 118.56 μg/cm^2, *J*: 9.46 μg/cm^2/h) were significantly lower than EpidermFT® (Q_{24}: 504.56 μg/cm^2, *J*: 38.53 μg/cm^2/h) ($p<0.05$). The lower permeability of the HSE-OP to the hydrophilic low

Table VI. Permeation of Caffeine through HSE Treated with Clofibrate (300 μM) with/without Ascorbic Acid (100 μg/ml) and External Lipid Supplementation

Parameter	HSE control	Clofibrate only	Clofibrate with ascorbic acid and lipids
Q_{24} (μg/cm^2)	498.4±48.9	**161.8±19.8**	**118.5±18.6**
Flux (μg/cm^2/h)	36.4±2.1	**14.7±1.3**	**9.4±1.2**
Lag time (h)	0	0.8±0.4	1.1±0.1

The external lipid mixture consisted of a combination of 25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid and 7 μM arachidonic acid. Control HSE was cultured with no added clofibrate, ascorbic acid and external lipids. Statistically significant differences from control indicated by bold font ($p<0.05$)

Table VII. Permeation of Hydrocortisone through HSE Treated with Clofibrate (300 μM) with/without Ascorbic Acid (100 μg/ml) and External Lipid Supplementation

Parameter	Control	Clofibrate only	Clofibrate with ascorbic acid and lipids
Q_{24} (μg/cm^2)	469.6±20.8	**196.9±14.6**	**96.7±10.1**
Flux (μg/cm^2/h)	30.5±1.6	**16.6±1.4**	**7.9±0.4**
Lag time (h)	0	0	1.0±0.2

The external lipid mixture consisted of a combination of 25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid and 7 μM arachidonic acid. Control HSE was cultured with no added clofibrate, ascorbic acid and external lipids. Statistically significant differences from control indicated by bold font ($p<0.05$)

Table VIII. Permeation Parameters for Six Agents after 24 h *in vitro* Permeation Experiments with Three Skin Models: HSE, EpidermFT® and Human Skin

Agent	Co (mg/ml)	HSE			EpidermFT®			Human skin		
		Q_{24} (μg/cm²)	J_{ss} (μg/cm²/h)	$K_p \times 10^{-5}$ (cm/h)	Q_{24} (μg/cm²)	J_{ss} (μg/cm²/h)	$K_p \times 10^{-5}$ (cm/h)	Q_{24} (μg/cm²)	J_{ss} (μg/cm²/h)	$K_p \times 10^{-5}$ (cm/h)
Caffeine	12.87	118.5±18.6	9.4±1.2	73.5±9.5	504.5±47.3	38.5±10.3	299.3±80.5	65.6±48.8	2.2±1.5	17.4±11.7
Hydrocortisone	19.64	96.7±10.1	7.9±0.4	40.5±2.1	111.8±29.7	6.4±1.9	32.9±9.8	28.6±31.7	2.0±2	10.3±11
Ketoprofen	54.32	2,432.5±1153	140.5±41.5	258.7±76.4	3051.9±1185	166.4±40.8	306.4±75.2	561.7±376	19.3±17.5	35.5±32.2
DEET	295.26	15,030.5±1253	837.9±57.3	283.7±19.4	15,088.8±355.12	942.5±54.3	319.2±18.4	2,076.5±458.4	100.7±33.9	34.1±11.5
Malathion	145.60	195.6±34.2	8.4±1.2	5.7±0.8	200±8.2	11.6±3	7.5±2.1	76.2±23.5	5.5±1.3	3.8±0.9
Paraoxon	25.39	2,811.4±45.6	115.9±2.5	456.8±9.8	2,964.9±691	123.6±18.7	487.1±123	94.4±4.54±0.1	4.54±0.1	17.9±0.2

Cumulative amounts of drug per square centimeter of skin area was plotted against time, and the steady state flux (J_{ss}) was calculated as the slope of linear portion of the plot. The permeability coefficient (K_p) was obtained as the ratio of the steady state flux and the solute concentration in the donor solution
Co concentration of the solute in the donor solution (mg/ml); Q_{24} cumulative amount of solute permeated after 24 h (μg/cm²); J_{ss} steady state flux (μg/cm²/h); K_p permeability coefficient (cm/h)

molecular weight drug caffeine (MW: 194.19) may indicate intact structures of the lipid bilayers and the corneocytes in the epidermis of the HSE-OP which may provide a high barrier to diffusion for hydrophilic compounds. However, as no difference between HSE-OP and EpidermFT® was observed with other compounds of varying lipophilicities, further studies on the permeation pathways in reconstructed skin models and penetration profiles of other low molecular weight hydrophilic solutes will have to be intensively evaluated in order to derive significant conclusions. Overall, both reconstructed models depicted significantly higher permeability to all agents as compared to human skin. While permeabilities of caffeine, malathion and hydrocortisone were approximately 2, 3, 4 fold respectively of human skin, permeability of ketoprofen and DEET with HSE-OP was ~5 and 7 times that of human skin. The overestimation of permeability by reconstructed skin models has been observed by several groups in literature (8, 15, 30–32). In a skin absorption German prevalidation study, Schäfer-Korting *et al.* found that Epiderm®, Episkin® and SkinEthic® were 4.0, 46.0 and 60.0 fold permeable to caffeine as compared to human epidermis (30).

A high difference in the permeability of HSE to paraoxon (MW: 275.19) was observed (~25 fold higher permeability with HSE-OP as compared to human skin), with EpidermFT® mirroring the same permeability trend. The high permeability obtained for paraoxon with the skin equivalents could be a result of an underestimation of paraoxon permeability through human skin due to its decomposition during the extensive epidermal passage of native human skin (33). Also, the human dermis has been postulated to exhibit a depot for moderately lipophilic compounds *in vitro* (34) and the dermatomed split thickness skin used in the experiments could have lead to increased diffusion and retention of this agent in the deeper skin layers thereby decreasing the transdermal permeation.

In addition to the permeability profiles, we have also evaluated the mechanical strength of HSE (data not shown). Overall, the mechanical constitution of the HSE was fragile, just like all available collagen-matrix based models. Our laboratory is currently investigating the integration of unique polymer scaffolds into this collagen model, which will enhance the mechanical strength, handling properties and the long term stability of the HSE.

CONCLUSION

A HSE was developed by culture in unique conditions with a combination of external free fatty acid mixtures, ascorbic acid and the PPAR-α agonist clofibrate. The resulting HSE demonstrated all morphological characteristics of native epidermis and demonstrated the presence of important markers of the epidermal differentiation process. Culture with the combination of free fatty acid mixtures, ascorbic acid and clofibrate led to development of reconstructed skin with lipid profile, epidermal homeostasis and permeability barrier closer to human skin than the control HSE. Barring the case of paraoxon, the HSE-OP overestimated the permeability of agents by about 2 to 7 fold, making it a suitable model for screening of skin absorption of various compounds. However, the behavior of skin models to permeability of compounds with various physico-chemical properties can only be validated with a large library of

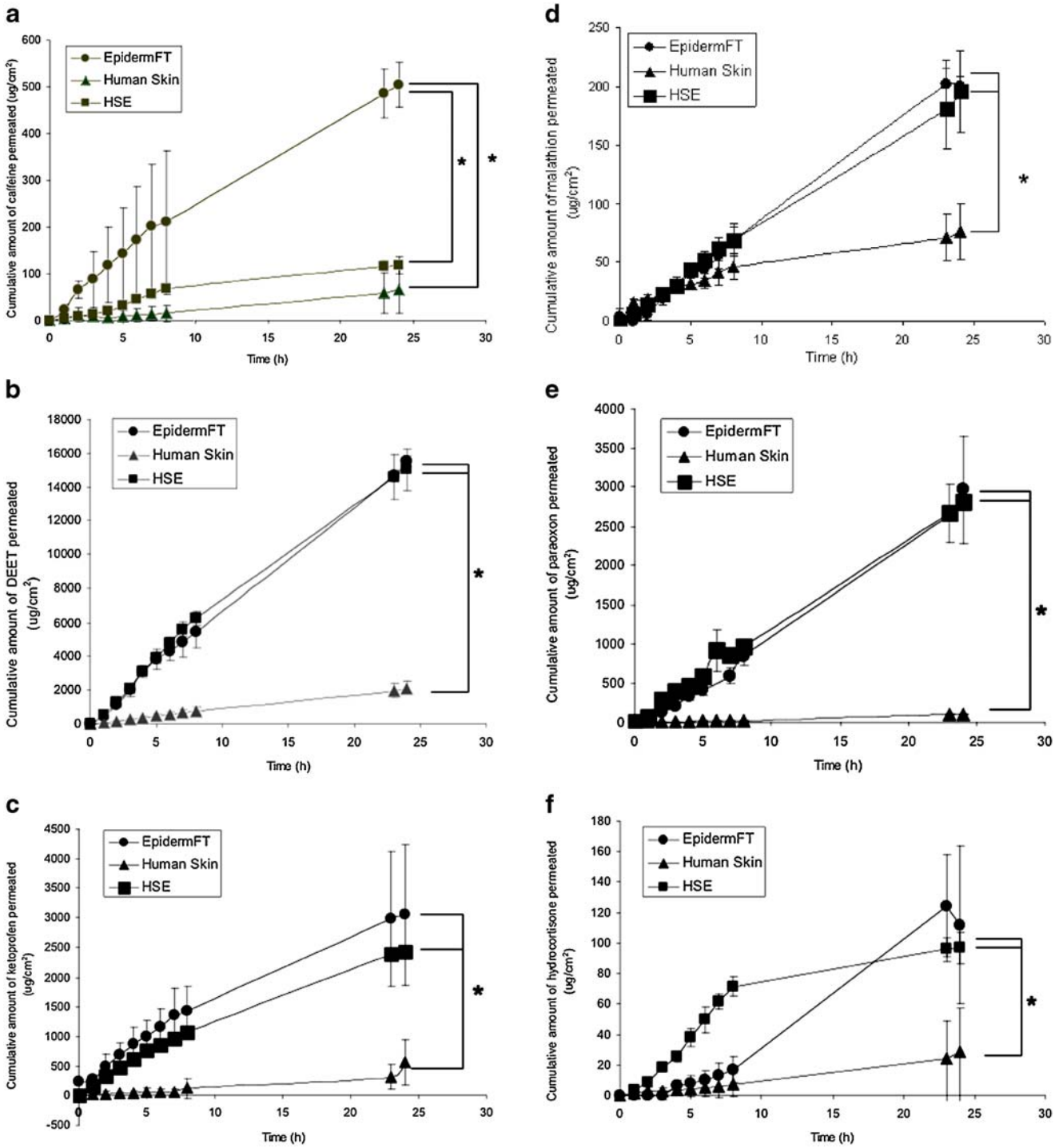

Fig. 6. Permeation of model compounds through HSE-OP, EpidermFT® and human skin. (**a**) Caffeine; (**b**) DEET; (**c**) ketoprofen; (**d**) malathion; (**e**) paraoxon; (**f**) hydrocortisone. All permeation studies were conducted over 24 h in static Franz Diffusion cells. Each data point represents the mean±S.D. (n=3–4 for all skin equivalents, n=6–8 for human skin). The HSE-OP overestimated the permeability of agents by about 2 to 7 fold of human skin, except for paraoxon (25 fold). The permeability profile of the HSE-OP was significantly different than human skin ($p<0.05$), but not significantly different than that of EpidermFT®, except in the case of caffeine.

compounds with differing properties. We are however, in the process of investigating the reproducibility of the HSE-OP to the agents used and the contribution of intra- and inter-batch variability to the total variation. We conclude that the development and testing of the HSE-OP, along with its future validation represents an important step toward the use of these skin equivalents as reproducible and accessible skin models for permeation testing. The primary drawback of the HSE-OP, its overestimation of permeability, will be the focus of further research and attempts to further improve the lipid profile in the epidermis could result in their universal use for formulation testing and also in drug delivery.

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