A BIOENGINEERED HUMAN SKIN EQUIVALENT (HSE) FOR THE EVALUATION OF PROTECTANTS.

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ABSTRACT

Estimation of permeation of compounds such as chemical warfare agents (CWAs) alone or through barrier creams such as SERPACWA (Skin Exposure Reduction Paste Against Chemical Warfare Agents) requires animal experiments or *in vitro* testing using animal or human skin. While animal skin does not reflect the same barrier properties as human skin, human skin poses problems of procurement, cost and large variations from donor to donor. Skin equivalents, such as the Human Skin Equivalent (HSE) developed in our laboratory, are *in vitro* cultured skin models that can be used for permeation/toxicity testing of compounds. The HSE is a full thickness skin equivalent that has been optimized by addition of various growth factors, such as ascorbic acid, lipids and a PPAR-α agonist. It has been characterized for morphology, lipid composition and barrier properties and compared to the commercially available skin equivalents. Compared to these, the HSE possesses closer lipid composition and barrier properties to human skin. The morphology shows a highly differentiated epidermis that provides a good barrier to the permeation of tested model agents such as caffeine. The HSE also provides lower variation in permeability data as compared to human cadaver skin and can be a consistent and reproducible model for permeability testing of agents.

1. INTRODUCTION

The skin serves as a convenient route for the potential delivery of agents such as drugs and cosmetics. As a model, it is often used for product safety/toxicity testing of compounds. Various agents can have their site of action in different layers of the skin such as the stratum corneum, epidermis, dermis, various appendages or can be targeted for transdermal delivery into the body. Permeation or toxicity testing of agents has in the past been carried out on animal models. Animal skin however, does not possess the same structure and properties of human skin. Moreover, more stringent regulations have been recently been imposed on the safety aspects of conducting such tests on animals. Also, human testing of toxic compounds such as pesticides and Chemical Warfare Agents (CWAs) is not possible. Human cadaver skin has been extensively used for permeability testing of various agents, but poses difficulties of expense, procurement, especially when required in large numbers, and high variability between different donors and different sites of the body, leading to high standard deviations in data.

The current study addresses the significant need of a reliable, viable full-thickness human skin model which can be used for rapid screening of topical formulations and for testing the permeability of environmentally encountered agents or drugs. Skin Equivalents are *in vitro* cultured skin models that are designed to meet the research and testing needs of the pharmaceutical and cosmetic industry. They can be used to study the molecular biology of the skin, for permeation and toxicity studies, in treatment of wounds and for skin replacement in burns (Michel *et al.* 1993: Ponec; Kempenaar 1995: Slivka *et al.* 1993). Various types of skin equivalents have been researched and generated by different groups (Bell *et al.* 1981) and some of these are available commercially such as Skinethic™ (Skinethic, Nice, France), Episkin™ (L'Oreal, France), and Epiderm™ (MatTek Corporation, Ashland MA, USA), and are well documented in the literature (Bernard *et al.* 2000) (Boelsma *et al.* 2000). All three of the marketed models are epidermis - only models which means that the thickest skin layer, the dermis, is absent. Currently, Epiderm FT™, a full thickness model is the main model in the U.S. specifically marketed for permeability studies. These models though consistent in permeability and responsiveness, have a limitation of possessing weak barrier function. Several possible reasons for this have been suggested, including impaired desquamation (Vicanova *et al.* 1996), abnormal skin lipid profiles, and the presence of unkeratinized microscopic foci (Mak *et al.* 1991).

Our group has developed and optimized a full-thickness Human Skin Equivalent (HSE) model that is essentially living skin that is grown *in vitro* (Song *et al.* 2003) (Asbill *et al.* 2000) (Godwin *et al.* 1997). The full thickness model has a fully differentiated epidermis (including a stratum corneum) grown on a dermal base made from collagen and fibroblasts. The HSE has been optimized in terms of the lipid composition, morphology and the barrier properties by modulating the growth media

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used to grow the cultures. We have further tested the permeability of various model agents through the HSE and have conducted comparative studies for some through the commercially available Epiderm FT™. The optimized HSE can serve as a reproducible *in vitro* model for testing of chemical warfare agents as well as the protectant properties of barrier creams such as SERPACWA (Skin Exposure Reduction Paste against Chemical Warfare Agents).

2. MATERIALS AND METHODS

2.1 Establishment of Cell Lines

Keratinocytes and dermal fibroblasts were obtained from human foreskins obtained from the Department of Pediatrics, University Hospital (Newark, NJ) as well as from Cascade Biologicals (Portland, OR). The fibroblasts were grown in Dulbecco's Modified Eagles Medium (DMEM) (Gibco-Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), and passages 3 to 10 were used for the skin equivalent preparation. Keratinocytes were cultured in supplemented Epilife (Cascade Biologicals, Portland, OR). (Supplemented Epilife contains 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). Keratinocytes passages 2 or 3 were used for the preparation of the skin equivalents.

2.2 Preparation of the Dermal Matrix

The dermal matrix of the skin equivalent was prepared by mixing 2X DMEM (Invitrogen Corp., Carlsbad, CA), FBS (Atlanta Biologicals, Lawrenceville, GA), 0.1 N NaOH (Sigma, St. Louis, MO), bovine type I collagen (Inamed, Santa Barbara, CA), and 1 ml of a suspension of fibroblasts at a concentration of 150,000 cells/ml in a 60 mm petri dish. The petri dish was then incubated for 7 days at 10% CO_2, 37 °C in a humidified incubator, during which time the fibroblasts contract the hydrated collagen into a tissue like structure. This contracted collagen gel (about 1cm diameter) was transferred to a Snapwell® insert system (Corning Costar Corporation, Cambridge, MA).

2.3 Preparation of Full Thickness Skin Equivalents

The surface of dermal layers placed in the Snapwell® inserts was coated with a mixture of human type I and type IV collagens (Cascade Biologics, Portland, OR) just before seeding of keratinocytes to optimize the extracellular matrix for attachment of keratinocytes to the dermal layer. 700,000 keratinocytes were seeded directly on the top of each dermal layer. Then the skin equivalents were fed from the top with Epilife® and the bottom with DMEM (no serum) and cultured submerged for 7 days at 37 °C, 10% CO_2, 75% Relative Humidity (RH) to allow proliferation of keratinocytes and the development of the basal layer of epidermis. On the 7th day, the cultures were lifted to the Air-Liquid interface (ALI) and fed only from the bottom. Culture at the ALI for 25-30 days allowed full differentiation of keratinocytes leading to formation of a multilayered, highly differentiated, full thickness skin model equivalent. The preparation of the skin equivalent is further described in Fig 1.

2.4 Optimization of the HSE by Addition of Growth Factors

In order to obtain a fully differentiated skin model that depicts the biochemical properties of human skin, the growth media can be supplemented with various growth factors. Some of these factors have been used by several groups to develop their skin equivalents, such as lactic acid (Rawlings *et al.* 1996), retinoic acid (the biologically active metabolite of vitamin A) (Rittie *et al.* 2006), pH, cholesterol sulfate, fatty acids, calcium (Bouwstra *et al.* 1998) (Vicanova *et al.* 1998), and several others. We have optimized the HSE by addition of fatty acids, cholesterol, ascorbic acid and a Peroxisome Proliferator Activated Receptor (PPAR) agonist clofibrate to the growth media.

Medium supplemented with 25 μM palmitic acid, 15 μM linoleic acid, 25 μM oleic acid, 7 μM arachidonic acid, 0.25% cholesterol, and 100 μM bovine serum albumin with 0.1% DMSO (as vehicle) were used for the last 4 days of submerged conditions and for the whole exposed stage. All the above agents were purchased from Sigma, St. Louis, MO. In addition, a varying range of concentrations of ascorbic acid (50, 100, 150 μg/ml) (tissue culture tested, Sigma, St. Louis, MO) and clofibrate (100, 200, 300, 400 μM) with 0.1% DMSO (as vehicle) (Calbiochem, San Diego, CA) were used when the cultures were exposed to the air-liquid interface.

2.5 Morphology, Lipid Composition and Analysis of Expression of Differentiation Markers

The HSE cultures, post fixation and sectioning, were stained with Hematoxylin and Eosin (H & E) and the structure was analyzed by light microscopy. The lipid composition was determined by extraction with solvents and subsequent thin layer chromatography. The expression of involucrin was used as a marker for keratinocyte

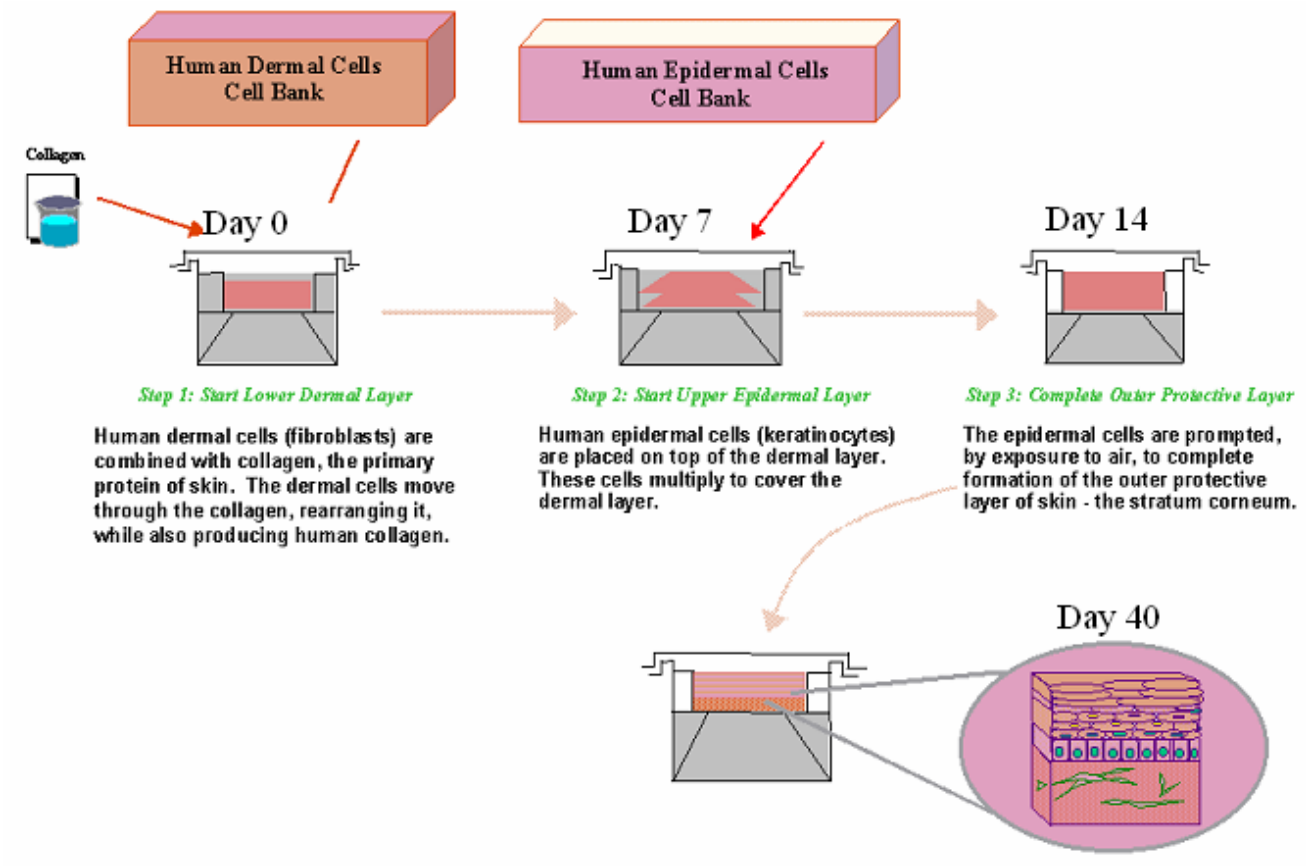

Fig. 1: Formation of the Human Skin Equivalent (www.organogenesis.com 1998)

differentiation and was detected with the help of an involucrin immuno-kit (Biomedical Technologies, Inc., Stoughton, MA).

2.6 Validation of the HSE and Permeability Studies

Validation of the HSE was conducted by determining its permeability characteristics with the help of the model drug caffeine. In addition the permeability profiles of Epiderm FT™ (MatTek Corp, Ashland, MA) and human cadaver skin with the model drug caffeine were determined. Other model agents such as hydrocortisone, ketoprofen, the insect repellant DEET and mimics of the warfare agent VX (malathion and paraoxon) were also assayed through Epiderm FT™ and human cadaver skin and their permeability profiles determined. Current studies in the lab will analyze the permeability profiles of these drugs through developing HSE cultures. All the above chemicals were purchased from Sigma, St. Louis, MO, and HPLC methods were developed using an Agilent HP1100 system and an Eclipse XDB-C_{18} 5 µm column (Agilent Technologies).

The permeability studies were conducted with the aid of Franz diffusion cells (Permegear, Inc., Bethlehem, PA). The receptor compartment of the diffusion cells was filled with isotonic phosphate buffer (pH 7.4) and was maintained at 37 ± 0.5 °C with constant stirring at 600 rpm. 5.0 µl of a saturated suspension/neat solution of the respective agents were applied to each cultured HSE or Epiderm FT™. In case of human cadaver skin, 100 µl of the saturated suspension/neat solution of the respective agents was applied to each piece of skin, as the human cadaver skin experiments were carried out in larger Franz cells (receptor volume 5.1 ml, diffusion surface area 0.64 cm^2) as compared to smaller cells for HSE and Epiderm FT™ (receptor volume 4.1 ml, diffusion surface area 0.196 cm^2). All cell donors were occluded with Parafilm® to prevent evaporation. At predetermined time points, 300 µl samples were taken from the receptor compartment over 24 hours and were immediately replaced by the same volume of a fresh buffer solution. The samples were kept frozen at –20 °C prior to HPLC analysis. The amount of drug/agent withdrawn was corrected in the subsequent calculations of cumulative amount penetrated. For all experiments, n was equal to at least 3.

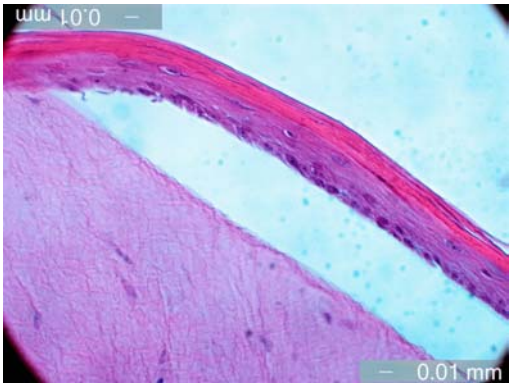

Fig. 2: A cross section of a full thickness HSE with no additional growth factors (Control). Seen above is the epidermis with the stratum corneum separated from the dermal component.

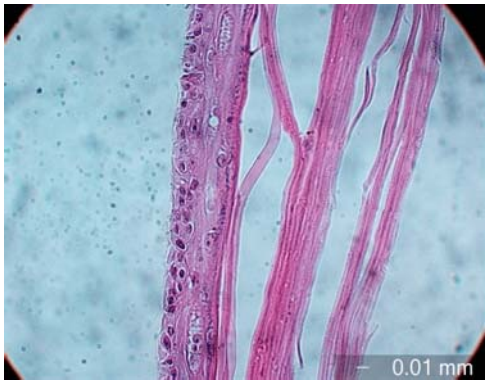

Fig. 3: A cross section of the HSE cultured with lipid supplementation. The differentiated epidermis shows a multilayered stratum corneum.

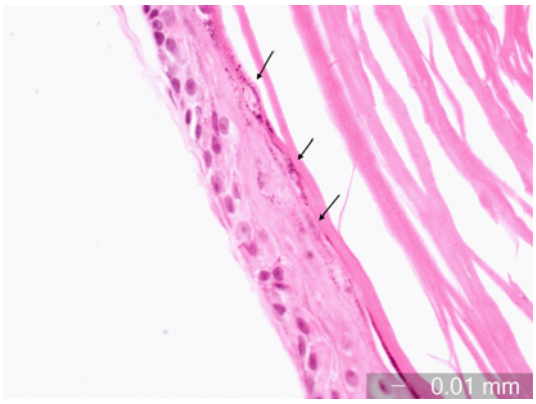

Fig. 4: A cross section of the HSE with ascorbic acid supplementation (100 µg/ml). The well differentiated epidermis shows the presence of keratohyalin granules (indicated by arrows).

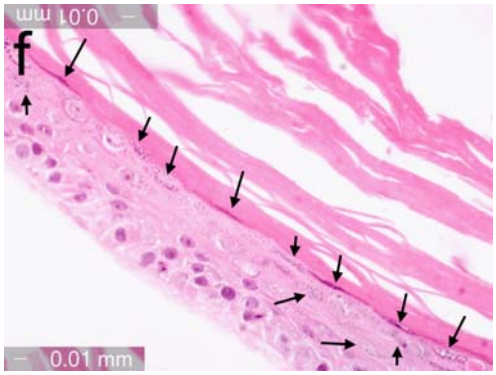

Fig. 5: A cross section of the HSE with clofibrate supplementation (300 µm). (Arrows indicate keratohyalin granules)

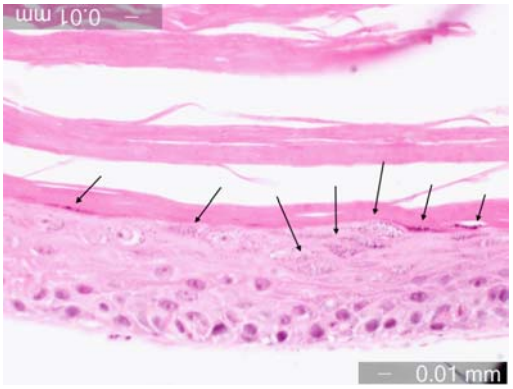

Fig. 6: A cross section of the HSE with lipids, ascorbic acid and clofibrate supplementation. The combination of these three factors showed a relatively thicker epidermis and stratum corneum (Arrows indicate keratohyalin granules)

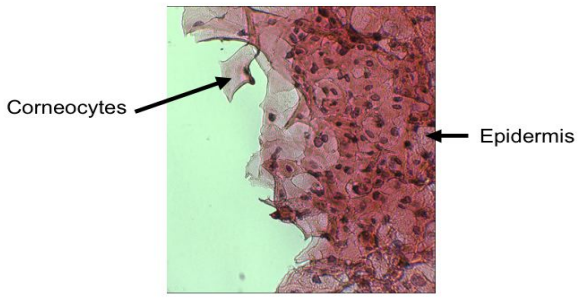

Fig. 7: A horizontal section of the epidermis (bottom up) from the human skin equivalent (HSE) showing the flattened corneocytes forming the stratum corneum, situated above the differentiating epidermis. (Magnification: 20 X).

Table 1: Lipid Profile of HSE Cultures Without Any Additional Growth Factors (Control), With Clofibrate Only, With Clofibrate, Ascorbic Acid and Lipids Compared With the Lipid Profile of Native Human Skin.

Lipid class	Control	Clofibrate 300 μM		Human cadaver skin
		No ascorbic acid or lipids	Ascorbic acid and lipids	
Phospholipids	19.5 ± 3.8	21.4 ± 2.7	23.8 ± 2.1	39.2 ± 2.1
Glucosylceramides	1.0 ± 0.3	0.7 ± 0.1	5.3 ± 0.3	4.8 ± 0.7
Acylglucosylceramides	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	Trace
Ceramides	8.6 ± 1.4	13.9 ± 0.9	14.1 ± 0.5	12.2 ± 2.2
Cholesterol	25.9 ± 3.6	18.3 ± 1.6	25.3 ± 3.1	19.4 ± 2.9
Fatty acids	6.7 ± 0.6	8.2 ± 1.1	10.1 ± 0.5	8.1 ± 0.6
Triglycerides	33.5 ± 3.1	33.2 ± 4.1	15.5 ± 1.6	9.2 ± 0.9
Cholesterol esters	4.6 ± 0.8	5.0 ± 1.1	5.3 ± 1.2	7.1 ± 0.5

3. RESULTS AND DISCUSSION

3.1 Morphology, Lipid Composition and Analysis of Expression of Differentiation Markers

When cultured without any additional growth factors (lipids, ascorbic acid and clofibrate), a full thickness HSE was obtained with a differentiated epidermis (Fig 2). Lipid supplemented medium (described in Materials and Methods) was used in cultures because external lipids have been reported to induce formation of lamellar bodies and serve as precursors of barrier lipids in human skin equivalent cultures (Boyce; Williams 1993). The addition of lipids to the HSE growth media increased the thickness of viable epidermis and stratum corneum in the skin culture, which displayed the artifactual basketweave separation of corneocytes like human skin (Fig 3). Ascorbic acid was added as a growth factor based on its ability to catalyze hydroxylation reactions that are required for the formation of certain essential lipids and ceramides in skin. With the addition of ascorbic acid (optimal concentration found to be 100μg/ml) the HSE displayed a thicker stratum corneum than control (Fig 4). Also, the histological images showed the presence of keratohyalin granules. Similar results were obtained with HSEs cultured with clofibrate (Fig 5). Clofibrate is a PPAR-α receptor agonist, and these receptors in the skin are involved in lipid metabolism and homeostasis. Activators of PPAR-α have been shown to accelerate epidermal barrier maturation in fetal rat skin both *in vivo* and *in vitro* (Hanley *et al.* 1999: Komuves *et al.* 1998).

When the skin equivalents were cultured with lipids, ascorbic acid (100μg/ml) and clofibrate (optimal concentration determined to be 300 μm), the HSE exhibited a relatively thicker viable epidermis and stratum corneum as compared to the control as well as all other treatments mentioned above. The epidermis obtained with the combination of all three factors was similar to native human skin (Fig 6). Similarly, the HSE cultured with all three factors together showed the most optimized lipid profile, one that closely resembled that of native human skin. Since the unique composition and organization of lipids in skin play a major role in skin permeability, a lipid composition as close as possible to that of the native tissue is essential to the barrier properties of skin equivalents. The lipid profile obtained with the combination of all three growth factors (optimized HSE) was found to be better than that obtained with control (no additional growth factors) with the optimized model depicting higher content of glucosylceramides and acylglucosylceramides and lower amount of triglycerides as compared to control. The lipid profile of the optimized model was also better than the HSE with only lipids (data not shown), only ascorbic acid (data not shown) and only clofibrate (Table 1).

The immunohistochemistry studies conducted for the expression of involucrin revealed positive involucrin expression in cultures treated with clofibrate as early as the first week of skin culture development. In the skin culture without clofibrate treatment, no expression of involucrin was found during the same time frame. Clofibrate thus seems to regulate the differentiation of keratinocytes in the HSE.

3.2 Permeability Studies

The optimized HSE with all three growth factors (lipids, ascorbic acid and clofibrate) showed better barrier

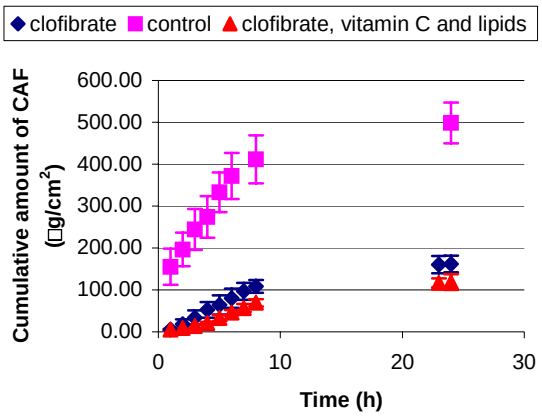

Fig. 8: Cumulative amount of caffeine permeated through HSE over 24 hours with treatment of 300 µM clofibrate with and without ascorbic acid and lipid supplementation.

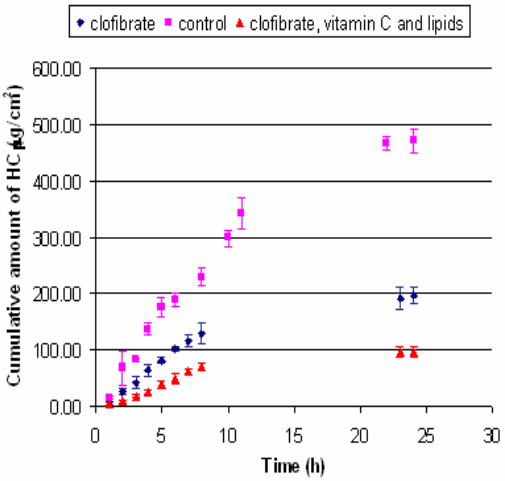

Fig. 9: Cumulative amount of hydrocortisone permeated through HSE over 24 hours with treatment of 300 µM clofibrate with and without ascorbic acid and lipid supplementation.

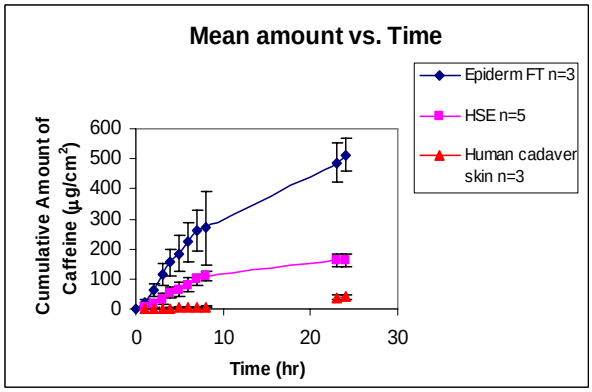

Fig. 10: Cumulative amount of caffeine permeated through optimized HSE, Epiderm FT™ and human cadaver skin over 24 hours.

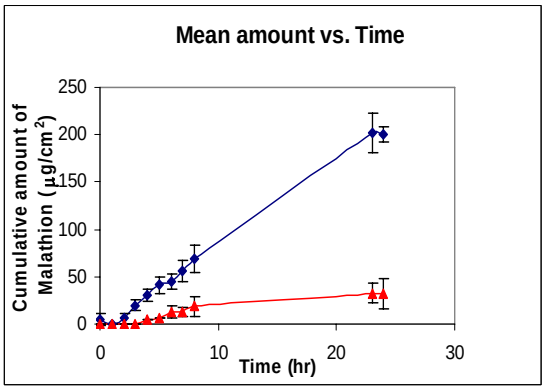

Fig. 11: Cumulative amount of malathion permeated through Epiderm FT™ and human cadaver skin over 24 hours.

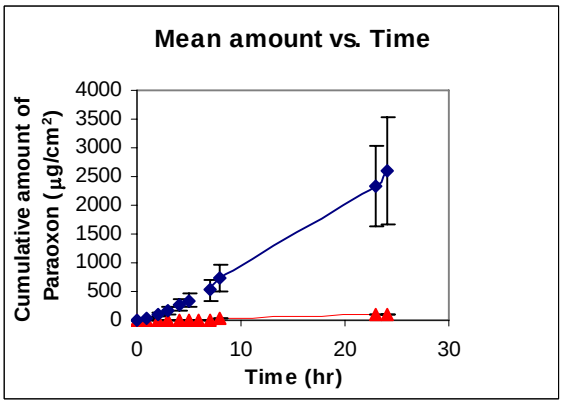

Fig. 12: Cumulative amount of paraoxon permeated through Epiderm FT™ and human cadaver skin over 24 hours.

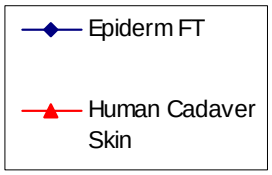

Table 2: Comparison of Permeability Parameters of the Skin Equivalents and Human Skin

	HSE optimized (with lipids, ascorbic acid and clofibrate)	**HSE control**	**Epiderm FT™**	**Human skin**
Hydrocortisone				
Q_{24} (μg/cm^2)	96.7 ± 10.1	469.6 ± 20.8	111.87 ± 29.72	28.65± 51.7
J (μg/cm$^{2/}$hr)	7.9 ± 0.4	30.5 ± 1.6	6.47±1.94	2.03±2.18
Caffeine				
Q_{24} (μg/cm^2)	118.5 ± 18.6	498.4 ± 48.9	513.86 ± 53.32	65.61± 48.81
J (μg/cm$^{2/}$hr)	9.4 ± 1.2	36.4 ± 2.1	38.53 ± 10.36	2.24±1.51

properties than control (no additional growth factors) (Fig 8), HSE with lipids only (data not shown), HSE with ascorbic acid only (data not shown) and clofibrate only (Fig 8 and Fig 9). The permeability parameters Q_{24} - amount of drug permeated per square cm of membrane in 24 hr (μg/cm^2) and flux (J) in (μg/cm^2/hr) are reported in Table 2. The permeability profiles of the optimized HSE were closer to those obtained from human cadaver skin as compared to the permeability profiles obtained from Epiderm FT™ (Fig. 10). Permeability studies conducted with mimics of the CWA VX (malathion and paraoxon) and the insect repellant DEET showed much higher permeability and lower barrier functions for Epiderm FT™ as compared to human cadaver skin (Fig. 11, Fig. 12). Permeability studies of these agents on the optimized HSE are to be conducted in the future. From the results obtained to date, the optimized HSE has shown better permeability profiles as compared to Epiderm FT™ though the standard deviations obtained with both the *in vitro* bioengineered cultures HSE and Epiderm FT™ were much smaller than those obtained with human cadaver skin.

4. CONCLUSION

The skin serves as both an absorptive organ and a barrier against the transport of toxic compounds. Therefore, the extent to which the skin will function in these capacities will depend on both the physicochemical nature of the agent in question as well as on the physiology of the skin. The development of a reproducible *in vitro* skin equivalent model is of great benefit to the military defense organizations for testing the permeation of toxic compounds, CWAs as well as for evaluating the barrier properties of protectant creams such as SERPACWA.

The barrier function of skin is located mainly at the stratum corneum and thus the formation of a fully differentiated epidermis with the optimal lipid profile is key for developing good barrier properties. The addition of lipids, clofibrate and ascorbic acid to the growth media of the HSE optimized the skin culture and demonstrated a highly differentiated epidermis with the stratum corneum.

The lipid profiles of the optimized HSE were close to that of human skin with higher content of glucosylceramides and acylglucosylceramides and lower amount of triglycerides as compared to control. Also, the barrier functions of the HSE were better than those of the commercially available full thickness model Epiderm FT™. The promotion of barrier function with lipid supplementation and ascorbic acid treatment were mainly due to their effect on lipid metabolism, where the lipids supplementation provided the precursors for lipid synthesis and ascorbic acid facilitated synthesis of critical and unique lipids present in the stratum corneum. In addition, ascorbic acid may also have shown some effects in regulation of keratinocyte differentiation. The effects of clofibrate could be ascribed to the promotion of differentiation of keratinocytes and lipid metabolism. The combination of these three factors led to the production of a highly differentiated skin model.

From our optimization results and the data from the permeability studies of caffeine on HSE, Epiderm FT™ and human cadaver skin, we predict that our HSE will depict better barrier properties to various other agents just as it did for caffeine, and thus will demonstrate lower permeability. It is also to be noted that the HSE showed much lower standard deviations in the results as compared to human cadaver skin underscoring its ability to act as a reproducible and consistent model for permeability testing.

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