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Comparison of Reconstructed Human Skin Models with Human and Pig Skin in *in-vitro* Percutaneous Absorption

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ABSTRACT. This study was carried out in accordance with OECD guidelines on skin absorption test for the comparison of reconstructed human skin models, EFT-306™ and KeraSkinFT™ with human skin and pig skin. Three OECD standard compounds were used for penetration and absorption: Caffeine, benzoic acid and testosterone, all of which differ vastly in their lipophilicity. Reconstructed human skins were more permeable to low lipophilic compounds such as caffeine than human and pig skin. Differences became most obvious with KeraSkinFT™ showing a relatively weak penetration barrier on caffeine. EFT-306™ and KeraSkinFT™ both showed similar penetration patterns in the case of lipophilic compounds, benzoic acid and testosterone compared with human and pig skins. High correlation was observed on the penetration flux between the reconstructed human skin models and human and pig skin, even though different penetration rates existed. The reconstructed human skin models can be regarded as generally useful on lipophilic compounds for *in-vitro* penetration studies.

KEY WORDS: *In vitro* skin absorption, human skin, pig skin, reconstructed human skin model

Introduction

Skin absorption tests are essential for toxicological research of compounds commonly used in drugs and cosmetics. The most useful data on skin absorption are obtained from *in vivo* human volunteer studies and occupational bio-monitoring (Ross et al., 2005, Williams et al., 2006). However, these data are limited due to a lack of samples from volunteers. Consequently, most toxicological data on skin absorption have been derived from rodent models (Zendzian et al., 2000). However, it is well-documented that dermal absorption in rodents is greater than that in humans, because of compounds' rapid passage down the hair follicle and the relative thickness of the stratum corneum (SC) (Williams et al., 2006). As an alternative method, *in vitro* skin

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absorption techniques are used nowadays to investigate the degree and kinetics of chemical absorption into and through the skin, to give an overall estimate of the dose that reaches systemic circulation. For hazard analysis by skin absorption (Ponec et al., 2000), the OECD Test Guideline 428 describes *in vitro* methods for assessing absorption by using human and animal skin (Heylings et al., 2004). Moreover, animal experiments should be avoided whenever scientifically feasible, making the use of *in vitro* systems imperative. New *in vitro* methods are being developed as alternatives to animal protocols for generating data for consumer safety risk assessment.

Reconstructed human epidermis (RHE) models have been used to test the safety of applied drugs as a means of reducing the less ethical *in vitro* and *in vivo* methods. RHE models were introduced to study skin damage such as irritation, corrosion, genotoxicity, and sensitization (Coquette et al.,2003, Kidd et al., 2007, Tornier et al., 2010). A protocol of skin irritation testing using RHE was submitted to the European Centre for the Validation of Alternative Methods (ECVAM) Scientific Advisory Committee (Spielmann et al., 2007), and *in vitro* skin corrosion testing based on RHE has been approved in OECD Guideline 341 (Kandarova et al., 2006). In accordance with these international trends, many researchers are reporting that animal skin can be replaced *in vitro* by RHE models (Kandarova et al., 2006, Netzlaff et al., 2007, Schafer-Korting et al., 2008, Schmook et al., 2001).

In recent years, as a more advanced alternative skin model, reconstructed human full-tissue (FT) skin models were introduced to the market. FT models closely resemble human skin and represent all dermal multilayers, consisting of the SC, epidermis, basement membrane, and dermis (Ponec et al., 2000, Schafer-Korting et al., 2008). The KeraSkin FT™ FT skin model (Biosolutions Co., Seoul, Korea), with absorption characteristics closely resembling those of human skin, was introduced in Korea in 2008. The KeraSkin FT™ model exhibits *in vivo*-like morphological and growth characteristics, which are uniform and highly reproducible. KeraSkin FT™ consists of layers analogous to those found *in vivo*, and is metabolically active. Although there are multiple reports of skin irritation studies using FT skin models such as KeraSkin FT™ (Ahn et al., 2010, Choi et al., 2014), not much literature on KeraSkin FT™ absorption has been published. To date, Keraskin FT™ has only been tested for DNA plasmid penetration (Kang et al., 2004). For skin toxicity testing, Keraskin FT™ was validated to establish an *in vitro* method for skin irritation and corrosion in Korea, but has not been validated by the international community.

The purpose of this study was to validate the absorption characteristics of Keraskin FT™, in comparison with those of human skin, pig skin, and the well-characterized FT skin model EpiDermFT™, by using three compounds widely varying in physicochemical characteristics: testosterone, caffeine, and benzoic acid. The experimental conditions (quantity applied, exposure time, vehicle, receptor fluid, preparation of membranes, and analysis) were standardized according to a detailed protocol that adapted many of the guidelines proposed by the OECD. Our aim was to validate an alternative approach using commercially available models for the testing of new, or not yet fully characterised, chemical entities, such as pharmaceuticals and cosmetic ingredients.

Table 1. Test substances and their physieochemical properties

Test substances	CAS No.	MW	logPo/w
Testosterone	58-22	288.4	3.32
Benzoic acid	65-85-0	122.1	1.83
Caffeine	58-08-2	194.2	0.01

Materials and Methods

1. Test Substances

Caffeine, benzoic acid and testosterone were selected on the basis of their ranges of physicochemical properties (Table 1) according to

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recommendation of the OECD Guidance Document for the Conduct of Skin Absorption Studies (OECD, 428. Skin absorption: *in vitro* method, adopted 13, 4, 2004). Caffeine and benzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) and testosterone was purchased from Tokyo kasei kogyo Corporation, LTD. (Tokyo, Japan).

2. Test formulations

Donor solutions and receptor fluids were according to Schafer−Korting M et al., (2008). The donor solutions were prepared in ethanol/miglyol (E/M; 1:9, v/v) solution for benzoic acid and testosterone, while caffeine was dissolved in PBS. Concentration was 1% for each compound (Table 2). For experiments with caffeine and benzoic acid, PBS was used as the receptor fluid, while for experiments with testosterone, 5% bovine serum albumin (BSA, Sigma-Aldrich) was added to PBS to overcome solubility restrictions (Table 2) (Schafer−Korting et al., 2008). The PBS volume of the receptor fluid was 7 ml. By itself, testosterone has a very low level of permeability, the addition of 5% BSA to the receptor fluid was increased the flux of testosterone by protein binding and recommended by Schafer−Korting M et al., (2008) (Schafer− Korting et al., 2008).

3. Skin preparation

Human skin was purchased from HansBiomed Corporation (Seoul, Korea). Pig skin was obtained from the Garak slaughterhouse (Seoul, Korea). Human skin and pig skin were kept frozen at −20° C and used within 2 months. The range of skin thickness was 0.8-1.1 mm. Two reconstructed FT skin models were obtained from commercial sources. EpiDermFT™ (EFT−306) was purchased from MatTek Corporation (Ashland, MA, USA), and KeraSkin FT™ from Modern Cell & Tissue Technologies (Seoul, Korea). The reconstructed FT skin models were incubated overnight in growth medium at 37° C, with 5% CO_2 and saturated humidity as suggested by the manufacturers.

4. Experimental design

This study was performed according to OECD Test Guideline 428 for doses of test substances, vehicles, duration of the experiment, skin thickness, receptor fluid type, and temperature. First, skin samples were thawed at room temperature and mounted onto static Franz−type diffusion cells. Each skin sample (exposed surface area 2 cm^2) within each diffusion cell, along with the receptor fluid, was maintained at a constant temperature close to normal skin temperature of 32 ± 1° C using a water circulation system (OECD, 2004b) (Heylings et al., 2004). The receptor fluid was stirred by a magnetic bar at 250 rpm. Next, 250 $\mu L/cm^2$ of 1% donor solution of caffeine, benzoic acid, or testosterone was applied to the skin surface. At defined time intervals (1, 2, 4, 6, 8, 10, and 24 h), 1 mL receptor fluid aliquots were collected and replaced.

5. High-performance liquid chromatography (HPLC)

UltiMate 3000 (Dionex, Sunnyvale, CA, USA) HPLC for isocratic chromatography, ZORBAX SB−C18 column for benzoic acid and testosterone (Agilent Technologies, Santa Clara, CA, USA), and Symmetry C18 columns for caffeine (Waters Corporation, Milford, MA, USA) were used at 70° C

Table 2. Test formulations

	Testosterone	Benzoic acid	Caffeine
Donor	E/M(10:90 v/v)	PBS	E/M(10:90 v/v)
Receptor fluid	PBS	PBS	PBS + 5% BSA
Concentration	1%	1%	1%
Dose (μl/cm^2)	250	250	250

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Table 3. Permeation rate (flux) and lag time of different skin types obtained from 24 h permeation experiments. Each compound was tested in 3 independent skins in triplicate. (Mean values ± SD)

		Pig skin	Human skin	KeraSkinFT™	EFT-306™
Testosterone	Lag time (h)	5.48 ± 1.50	4.28 ± 0.98	4.62 ± 0.34	4.40 ± 0.26
	Flux (μg/cm2/h)	0.09 ± 0.06	0.46 ± 0.03	0.28 ± 0.03	0.88 ± 0.67
Benzoic acid	Lag time (h)	1.66 ± 1.41	1.68 ± 1.89	1.32 ± 0.45	0.43 ± 0.73
	Flux (μg/cm2/h)	40.85 ± 13.85	79.56 ± 0.32	219.68 ± 12.79	155.54 ± 29.25
Caffeine	Lag time (h)	2.21 ± 2.16	2.40 ± 0.49	0.15 ± 1.06	1.50 ± 0.46
	Flux (μg/cm2/h)	3.68 ± 0.49	17.77 ± 23.19	236.04 ± 22.17	10.61 ± 2.33

(flow rate: 2.0 mL/min). The protocol of van de Sandt *et al.* (2004) was used for HPLC-UV analysis of benzoic acid and caffeine. Testosterone was analyzed according to Bogaards et al. (1995) (Bogaards et al., 1995).

6. Histological investigation

Tissue samples were fixed in 4% neutral-buffered formalin for varying lengths of time and were embedded in paraffin. The formalin-fixed, paraffin-embedded 4-6 μm tissue sections were deparaffinized, dehydrated in graded alcohol, and stained with hematoxylin and eosin (H&E).

7. Statistical analysis

Statistical analysis was performed by ANOVA. All data are expressed as means ± SEM. Student's *t*-test was used for unpaired comparisons; *p*-values <0.05 were considered statistically significant. All experiments were conducted in triplicate.

Results

1. Percutaneous Absorption

The permeability of the Keraskin FT™ model was compared to human skin, pig skin and commercially available, reconstructed full thickness skin, EFT-306™ (Fig 1). Testosterone displayed only a low degree of penetration after a 24-h exposure (Fig. 1A). It did not penetrate pig skin until after 6 h of exposure. In the FT models (Keraskin FT™ and EFT-306™) and human skin, however, testosterone penetrated at 2 h. The flux of testosterone through the four skin

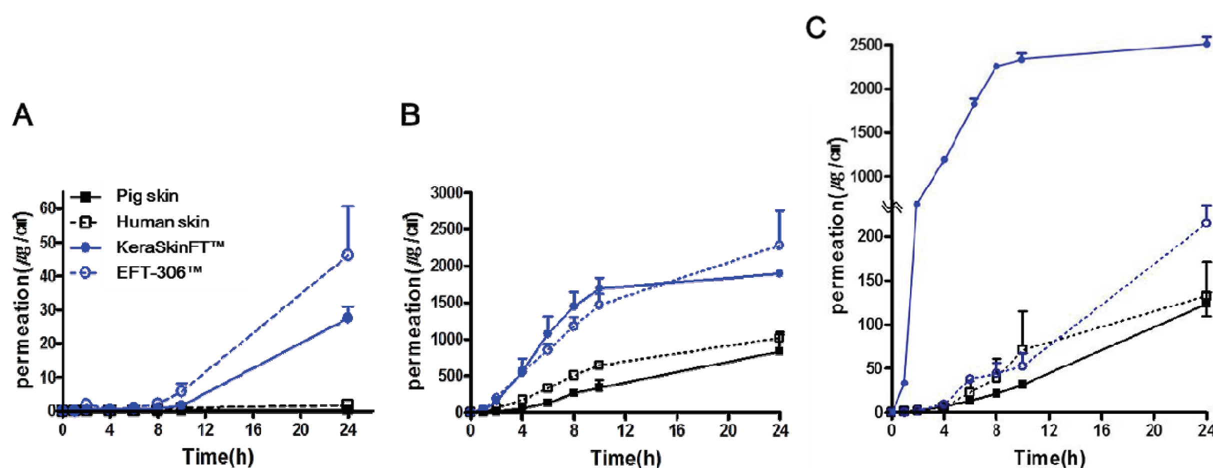

Fig. 1. Permeation of 1 % dornor solution (250 μl/cm2) across excised pig skin (■), human skin (□), KeraSkinFT™ (●), EFT-306™ (○) following a 6h exposure. Results are means ± sem for triplicate for each model. A. Testosterone, B. Benzoic acid, C. Caffeine.

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substrates tested and their corresponding lag times were as follows: human skin (0.46 ± 0.03 $\mu g/cm^2/h$, 4.28 ± 0.98 h), pig skin (0.09 ± 0.06 $\mu g/cm^2/h$, 5.47 ± 1.50 h), EFT-306™ (0.88 ± 0.67 $\mu g/cm^2/h$, 4.40 ± 0.26 h), and KeraSkin FT™ (0.28 ± 0.03 $\mu g/cm^2/h$, 4.62 ± 0.34 h). The flux of testosterone in EFT-306™ was substantially higher than in the other substrates. The lag time in human skin, however, was similar to that in KeraSkin FT™ and EFT-306™ (Table 3). For benzoic acid, the rates of flux in KeraSkin FT™ and EFT-306™ were 2- to 5-fold higher compared with flux rates across human skin and pig skin (Fig. 1B). The flux rate of benzoic acid was the highest in KeraSkin FT™. The rate of final penetration in EFT-306™ was higher than in KeraSkin FT™ at 24 h. Lag time in EFT-306™ was the lowest. Overall, the lag time in FT models was lower than in human skin and pig skin (Table 3). Lag times of testosterone and benzoic acid were well in accordance with those reported by Ackermann et al. (2010) (Ackermann et al., 2010). For caffeine, the flux through human skin was 17.77 ± 23.19 $\mu g/cm^2/h$, and lag time was 2.40 ± 0.49 h (Table 3, Fig. 1C). The penetration in pig skin was 3.68 ± 0.49 $\mu g/cm^2/h$. The lag time in pig skin (2.21 ± 2.16 h) was similar to the lag time in human skin. The rates of penetration in KeraSkin FT™ and EFT-306™ were at least

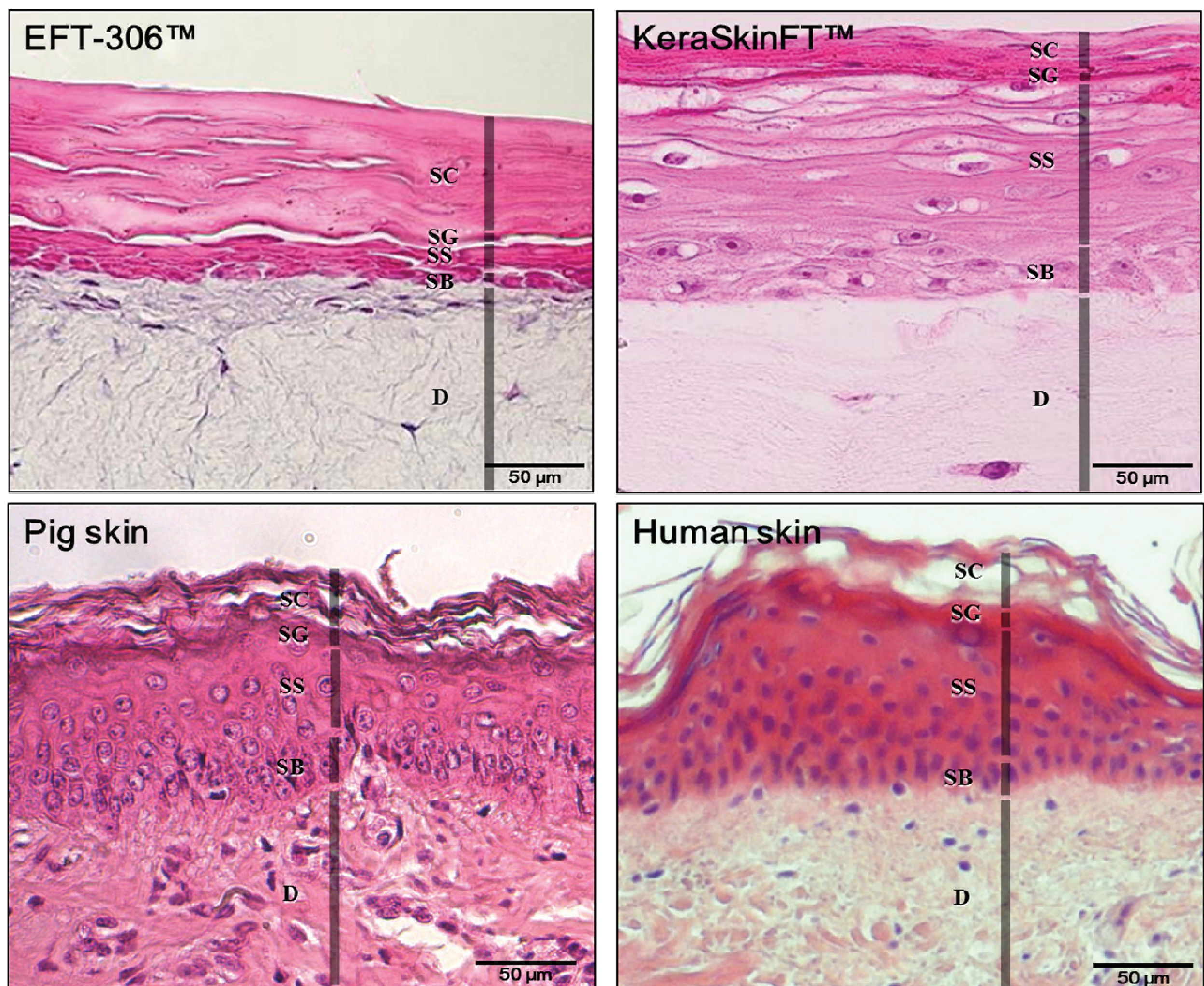

Fig. 2. Histological appearance of skin models. Hematoxylin and eosin stained paraffin sections of skin models (SC; Stratum Corneum, SG; stratum granulosum, SS; stratum spinosum, SB; stratum basale, D; dermis). Scale bar: 50 μm.

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17.8-fold and 2.3-fold higher than the rate of penetration in human skin, respectively. Lag time of caffeine penetration in KeraSkin FT™ samples was at least 1-fold higher than in human skin, pig skin, and EFT-306™. Moreover, between the two reconstructed FT skin models, KeraSkin FT™ was more penetrable than EFT-306™. All tested chemicals penetrated the FT models earlier (shorter lag time) and more rapidly (flux) than human skin and pig skin, especially KeraSkin FT™.

2. Histological investigation

The FT model was examined histologically using vertical sections with light microscopy (Fig. 2). The FT model presented a characteristic tissue architecture and morphology. Some differences between KeraSkin FT™ and EFT-306™ were observed in the four layers of epithelial cells in the epidermis. The thickness of the SC in the EFT-306™ RHE was up to 4.5-fold higher than that of KeraSkin FT™. The stratum granulosum was distinctly observed in KeraSkin FT™, but rarely in EFT-306™. The stratum spinosum was thicker in KeraSkin FT™ than in EFT-306™. Compared with human skin and pig skin, KeraSkin FT™ exhibited a more similar pattern of epidermal layers than EFT-306™.

Discussion

Reconstructed human FT skin models consist of normal human epidermal keratinocytes cultured to form all layers of skin, closely resembling the characteristics of human skin. Efforts to reconstruct human skin *in vitro* are reflected in numerous reports on the development of different culture systems and their assessment as penetration models. KeraSkin FT™ and EFT-306™ are composed of all epidermal layers. Skin layers in all tested substrates were taken into consideration to replicate a scenario similar to *in vivo* conditions (Jakasa et al., 2008).

The barrier property of new FT human skin models was studied by penetration of three standard compounds. We found that the FT models more efficiently retarded the permeation of benzoic acid and testosterone. These results suggest that the barrier properties of reconstructed FT models closely mirror penetration by lipophilic compounds across human skin. However, permeability of a hydrophilic compound, caffeine, was higher in the KeraSkin FT™ and EFT-306™ models than in human skin for all test compounds. Interestingly, the same was observed by Schäfer-Korting et al. (2006) (Schafer-Korting et al., 2006), who found an increase in permeability of hydrophilic compounds across bovine skin compared to human and pig skin, and attributed it to different lipid composition. These data imply that the FT models may also contain a lipid composition, which differs from the pattern in human skin (Ackermann et al., 2010, Tornier et al., 2010).

Under histological examination, a difference in layer structure was observed in KeraSkin FT™ compared with EFT-306™, pig skin and human skin. Although the skin layer composition was similar, KeraSkin FT™ exhibited a different cell density in the SS and SB. We suggest that the different cell density contributed to an ineffective barrier against penetration by hydrophilic caffeine.

In the present study, KeraSkin FT™ provided a sufficient barrier to testosterone and benzoic acid, but was permeable for the more hydrophilic caffeine. Although the FT models and native skins exhibited differing rates of penetration by our test compounds, the same trends of testosterone and benzoic acid permeability were observed in all skins. KeraSkin FT™, although in need of improvement in the barrier function against hydrophilic compounds, can be leveraged as a better substitute for *in vitro* native skin.

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Disclosure

The authors declare no conflict of interest.

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