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Cumulative evidence of the low reliability of frozen/thawed pig skin as a model for *in vitro* percutaneous permeation testing

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1. Introduction

The *in vitro* permeation tests using a Franz diffusion cell system have become an eminent tool in the field of percutaneous absorption and topical drug research. It has also been well-established that the *in vitro* percutaneous studies using *ex-vivo* skin can accurately assess bioavailability and bioequivalence throughout all phases of the topical and transdermal drug development. Due to the limited bioavailability of good-quality human skin specimens, porcine skin has frequently been used as an *in vitro* model that simulates penetration to and through human skin (Simon and Maibach, 2000; Schmook et al., 2001). Porcine skin is considered as an excellent alternative and a model for human skin because the stratum corneum lipids are generally similar to human lipids (Gray and Yardley, 1975; Wertz, 2013), the hair density is similar (Jacobi et al., 2005), and there is a similarity in skin permeability (Andega et al., 2001). Nevertheless, several researchers have noticed that freezing and then thawing the tissue result in enhancement of drug permeation. Kasting and Bowman (1990a, 1990b) have found that the electrical resistance of frozen skin was lower than that reported for skin *in vivo*, and fresh skin was less permeable to sodium ions during passive diffusion and less conductive than frozen skin at low current. Bosman et al. (1998) reported that pig skin, which had been frozen for 2 months at −80 °C, resulted in a higher permeability of the anticholinergic, dexetimide, without any lag time. A later study by Jacobi et al. (2005), who compared the penetration of flufenamic acid in porcine and human skin, has shown that the drug concentrations detected in human skin layers *in vitro* and *in vivo* were much smaller than in a refrigerated porcine tissue. The present commentary article is aimed at explicitly demonstrating by using more evidence that the convenient protocol of freezing pig skin for *in vitro* percutaneous studies results in low-reliable penetration data and should therefore be avoided.

2. Material and Methods

2.1. In Vitro Skin Penetration Studies

2.1.1. Diffusion Tests

The permeability of the drugs through animal skin was determined *in vitro* using a Franz diffusion cell system (Permegear, Inc., Bethlehem, PA). The diffusion area was 1.767 cm^2 (15 mm diameter orifice), and the receptor compartment volumes varied from 11 to 12 ml. The solutions in the water-jacketed cells were thermostated at 37 °C and stirred by externally driven, Teflon-coated magnetic bars. Each set of experiments was performed with at least four diffusion cells ($n \geq 4$), each containing skin from pig ears. Full-thickness porcine skin was excised from fresh ears of slaughtered white pigs (breeding of Landres and Large White). After subcutaneous fat was removed with a scalpel, the skin (1.0–1.5 mm thickness) was freshly used (no >5 h after slaughtering) or kept at −20 °C until used within two weeks. All skin sections were measured for transepidermal water loss (TEWL) before mounted in the diffusion cells or stored at lower temperatures until used. TEWL examinations were performed on skin pieces using Dermalab® Cortex Technology instrument, (Hadsund, Denemark) and only those pieces that the TEWL levels were <15 $g/m^2/h$ were introduced for testing. The skin was placed on the receiver chambers with the stratum corneum facing upwards, and the donor chambers were then clamped in place. The excess skin was trimmed off, and the receiver chamber, defined as the side facing the dermis, was filled with phosphate buffered saline (PBS, containing 10 mM PO_4^{-3}, 137 mM NaCl, and 2.7 mM KCl, pH 7.4). After 15 min of skin washing at 37 °C, the buffer was removed from the cells. Aliquots (0.5 ml) of aqueous solutions of diclofenac sodium or caffeine (1.0%w/w), or a micellar system containing 1.8% L-DOPA, were applied on the skin (an infinite dose application), and the receiver chambers were refilled with a fresh phosphate buffered saline (PBS, pH = 7.4). Samples (2 ml) were withdrawn from the receiver solution at predetermined time intervals, and the cells were replenished up to their marked volumes with fresh buffer solution each time. Addition of PBS to the receiver compartment was performed with great care to avoid trapping air beneath the dermis. The receiver samples were taken into 1.5-ml vials and kept at −20 °C until analyzed.

2.1.2. Drug Analysis

Diclofenac (Sintov and Botner, 2006) – Aliquots of 20 μl from each sample were injected into a HPLC system, equipped with a prepacked column (Betasil C_{18}, 5 μm, 250 × 4.6 mm, Thermo electron corporation, UK). The HPLC system (Shimadzu VP series) consisted of an autosampler and a diode array detector. The quantification of diclofenac was carried out at 280 nm. The samples were chromatographed using an isocratic mobile phase consisting of acetonitrile – sodium acetate buffer, pH 6.3 (40:60) at a flow rate of 1 ml/min. *Caffeine* (Sintov and Greenberg, 2014) - Aliquots of 20 μl from each sample were injected into a HPLC system (Shimadzu VP series), equipped with a prepacked column (ReproSil-Pur 300 ODS-3, 5 μm, 250 mm × 4.6 mm, Dr. Maisch, Germany), which was constantly maintained at 30 °C. The quantification of caffeine was carried out at 273 nm. The samples were chromatographed using an isocratic mobile phase consisting of methanol – 1% acetic acid solution (50:50) at a flow rate of 1 ml/min. L-DOPA: Samples were analyzed using liquid chromatography – tandem mass spectrometer - LC/MS (API 2000 LC/MS/MS, Applied Biosystems/MDS SCIEX, Ontario, Canada, combined with a Liquid Chromatograph model LC-20AD, Shimadzu USA Manufacturing, Inc., OR, USA). LCMS analysis was performed using a Reprosil C18-AQ column (5 μm, 100

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× 2mm, Dr. Maisch, Germany) preceded by a guard column packed with the same material. A mobile phase consisting of a 95:5 mixture of 0.5% acetic acid in water (eluent A) and 0.5% acetic acid in acetonitrile (eluent B). The HPLC isocratic flow rate was 0.2 ml/min. Positive ion mode was used, and selected-ion monitoring was accomplished at *m/z* 198 (152).

2.2. Calculation of Drug Permeation

As a result of the sampling of large volumes from the receiver solution (and their replacement with equal volumes of buffer), the receiver solution was constantly being diluted. Taking this process into account, the cumulative drug that permeated out into the receiver ($Q_{out}(t_n)$) at the end of the *n*th sampling time (n ≥ 0) was calculated according to the following equation:

$$Q_{out}(t_0) = C_{out}(t_0) = 0;\ t_0^- = t_0 = 0$$

$$Q_{out}(t) = VrC_{out}(t_n^-) + \sum_{i=0}^{n-1} VsC_{out}(t_n^-) \qquad n \geq 1$$

$$C_{out}(t_n) = \left[C_{out}(t_n^-) \cdot (Vr - Vs)\right]/Vr$$

where $C_{out}(t_n)$ is the drug concentration in the receiver at sampling time t_n, expressed by a running number ($t = 1, 2, 3\ldots t_n$). *Vr* and *Vs* are the constant volumes of the receiver and the sample solutions, respectively. Data was expressed as the cumulative drug permeation per unit of membrane surface area, $Q_{out}(t_n) / S$ ($S = 1.767\ cm^2$). The steady state fluxes (J_{ss}) were calculated by linear regression interpolation of the experimental data a steady state:

$$J_{ss} = \Delta Q_{out}(t_n)/(\Delta t \cdot S)$$

3. Results and Discussion

We have accomplished several studies concerning the difference between frozen and fresh skin (Sintov and Botner, 2006; Sintov and Greenberg, 2014), and we wish to further clarify that issue for researchers in dermatology to pay attention to the low-reliable use of the convenient protocol of storing pig skin at −20 °C. Three model drugs have been investigated with different lipophilic-hydrophilic properties, diclofenac (logP = 4.2), caffeine (logP = −0.07), and L-DOPA (logP = −2.4). It has been clearly shown (Sintov and Botner, 2006) that frozen/thawed pig skin had a higher permeability to diclofenac compared to that obtained by using fresh skin. The results have further demonstrated that the abnormal permeability through frozen pig skin is independent of the effect of different formulations, placing a question mark on the validity of the *not* freshly-used porcine skin for *in vitro* penetration studies. In the other study (Sintov and Greenberg, 2014), we explored the percutaneous permeability of caffeine across several commonly used skin models. Whereas caffeine was transported at similar rates across rat and rabbit skin (fresh or frozen), differences were noted between caffeine transport rates across fresh and frozen/thawed pig skin. It was also evident that the permeability of caffeine through frozen/thawed pig skin was abnormally high and independent of the vehicle properties, *i.e.*, its hydrophilic or lipophilic nature. Fig. 1 presents the said difference in the permeability of diclofenac and caffeine between fresh and frozen-thawed pig skin. In our recent study (unpublished), we have also shown that frozen-thawed pig skin lost its barrier and turned to be significantly more permeable to L-DOPA than a freshly excised skin. Table 1 summarizes the permeation rates (at steady state) of diclofenac, caffeine, and L-DOPA through fresh and frozen pig skin, demonstrating an increase of 5.2-, 8.6-, and 3.8-fold, respectively, when drug was applied on the frozen/thawed skin. As the drug permeation fluxes through frozen rat and rabbit skin were similar or even lower than the fluxes through fresh skin sections (Sintov and Greenberg, 2014; Nicoli and Santi, 2007), it is reasonably postulated that the relatively high ceramide and the low cholesterol levels in porcine stratum corneum (while the opposite was shown in rat and rabbit skin) (Gray and Yardley, 1975; Nicoli et al., 2008) may be ascribed to the

Table 1
Permeation fluxes of various drugs across fresh and frozen-thawed pig skin.

Drug	Permeation flux (J_{ss}), $\mu g \cdot cm^{-2} \cdot h^{-1}$		
	Fresh skin	Frozen–thawed skin	J_{ss} increase
Diclofenac sodium[a]	1.12 (±0.45)	5.80 (±2.38)*	×5.2
Caffeine[b]	3.77 (±1.38)	32.37 (±9.69)*	×8.6
L-DOPA[c]	3.67 (±1.12)	14.07 (±1.66)*	×3.8

[a] Data obtained from Sintov and Botner (2006).
[b] Data obtained from Sintov and Greenberg (2014).
[c] 1.8% in a micellar system; unpublished data.
* Significantly different than the corresponding flux obtained through fresh skin (ANOVA, $p < 0.05$).

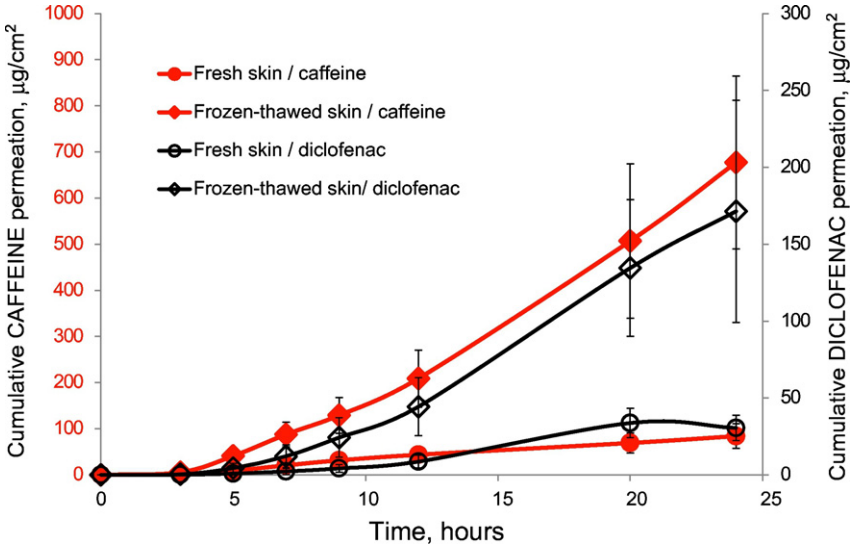

Fig. 1. Permeation of caffeine (red, closed symbols) and diclofenac sodium (black, open symbols) through fresh pig skin (circles) and frozen-thawed pig skin (diamonds).

abnormal increased permeability across frozen/thawed pig skin. A relatively high *ceramide-to-cholesterols* ratio in the stratum corneum's extracellular lamellar membranes implies that it can be more easily disrupted by internal ice crystalline formation, leading to barrier abrogation. As a high *ceramide-to-cholesterols* ratio indicates a consequently high resilient barrier integrity (as evidenced by the relatively low drug permeability of pig skin), it also indicates according to our findings that the lipid layer structure possesses a plastic-type compressibility property and is prone to irreversible creation of pores by intercalating tiny solid particles such as formed ice. The scarcity of nonpolar (neutral) lipids such as cholesterol and especially cholesteryl esters in porcine stratum corneum may result in a less isolation of the water phase in the lipid bilayer thus increasing its transition to ice. It has been hypothesized therefore that the reason for the increased permeation of drugs through frozen/thawed pig skin is not related to inactivation of metabolic enzymes (as some researchers has pointed out), but to the fact that porcine skin has a dissimilar *ceramide-to-cholesterol* ratio compared to rat and rabbit skin.

Conflict of Interest

The author states no conflict of interest

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