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Effect of Frozen Human Epidermis Storage Duration and Cryoprotectant on Barrier Function Using Two Model Compounds

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Key Words

Percutaneous penetration · Skin absorption · Human skin · Freezing · Storage conditions · Skin barrier function · Steady-state flux · Lag time · Diethyl phthalate · Caffeine

Abstract

Skin is commonly stored frozen and then thawed prior to use for in vitro permeation experiments. Does frozen storage of skin alter its barrier property? Numerous studies have found contradictory answers to this question. In this study, the steady-state flux and lag time of diethyl phthalate (DEP) were measured for fresh human skin and skin frozen at –85°C for 1, 2, 3, 6, 9, 12, and 18 months with 10% glycerol as a cryoprotective agent. No significant differences in steady-state flux were found between fresh and previously frozen samples ($p = 0.6$). For lag time, a significant ($p = 0.002$) difference was found among all groups, but comparisons with fresh skin were not significant. Does glycerol have a cryoprotective effect? The steady-state flux and lag time of DEP and caffeine were measured through human skin stored at –85°C for up to 12 months with and without 10% glycerol. No significant differences in steady-state flux or lag time were found between samples stored with or without glycerol for either DEP or caffeine ($p \geq 0.17$). These findings support the use of frozen skin to measure the passive permeation of chemicals in studies unconcerned with viability and metabolism.

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Introduction

Previously frozen excised human skin is normally used in permeability studies because fresh skin is rarely available at the exact time in vitro permeation experiments are conducted. Numerous studies have addressed the effect of storage conditions on skin permeability, but with imprecise or contradictory results. It has been shown that storage diminishes the metabolic activity of the skin [1], but its effect on the physical barrier properties of the skin was not clear from these results. Studies on the subject have employed diverse objectives and methods, rendering their findings difficult to compare. Furthermore, there is an inherent variability associated with percutaneous absorption, raising the question of the exact variability that conclusively demonstrates frozen storage has a significant effect. For example, caffeine permeation has a higher variability in comparison to more lipophilic compounds [2]. Hydrophilic compounds may follow differ-

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Table 1. Studies investigating the effect of frozen storage on human skin permeation

Ref.	Test chemical	Temp., °C	Max. storage, days	Species	Anatomic site	Skin prep. method	Cryo	Freezing effect
8	Water	Icebox	27	Human	Epigastrium	FT	No	No
9	NR	NR	90	Human	Abdomen	FT	No	No
10	Water	–20	180	Human	Thighs	Derm	No	No
11	Water	–20	360	Human cadaver	Abdomen	Derm	No	No
12	Water	–20	466	Human cadaver	Abdomen	Derm	No	No
13	Water, 17β-estradiol, dextrans	–85	300	Human	Buccal and vaginal mucosa	FT	No	No
14	Chloroform, trichloroethylene, tetrachloroethylene	–20	NA	Human	Breast and abdomen	Derm	No	No
15	Benzo[a]pyrene, ethylene glycol, methyl parathion, naphthalene, nonylphenol, toluene	–19	60	Human	Breast	Derm	No	No (when including skin depot)
16	Anisole, cyclohexanone, 1,4-dioxane	–20	30	Human	Abdomen	Derm	No	No
17	Caffeine	–20	21	Human	Abdomen	FT	No	No
17	Caffeine						Gly	No
18	Chromone acid (FPL 57787)	–17	2.5	Human	Thighs	HEM	No	No (for skin stored dry)
18	Chromone acid (FPL 57787)	–17	2.5	Human	Thighs	HE	No	Yes
19	T-2 toxin (trichothecene mycotoxin)	–60	10	Human cadaver	Abdomen	FT	No	Yes
17	Caffeine	–80	21	Human	Abdomen	FT	No	Yes

Cryo = Cryoprotectant; Derm = dermatomed; FT = full thickness; Gly = glycerol; NR = not reported; Ref. = reference number; Temp. = temperature.

ent pathways and may also bind to polar groups as well as keratin or cornified envelopes [2].

In vitro permeation experiments may use epidermal membranes, dermatomed skin, or full-thickness skin. The use of epidermis only is the most physiologically defensible, as it more closely mimics the in vivo situation where continuous blood flow within watery dermis acts as a sink in much the same way as the receptor compartment of an in vitro setup. Watery dermis in dermatomed and especially in full-thickness skin acts as an additional barrier, which may be substantial, that does not exist in the in vivo setting. On the negative side, epidermal membranes are obtained by heat separation (60°C for 1–2 min followed by peeling from dermis), which destroys metabolic activity. They are fragile and can be prone to leaks if hair follicles are damaged in the separation process. Nevertheless, their use is broadly accepted by advisory and regulatory agencies [3].

Some international advisory and regulatory agencies offer recommendations regarding the use of previously frozen skin for in vitro dermal absorption testing. The Organization for Economic Cooperation and Development (OECD) guidance allows its use for all compounds that are not dermally metabolized [3]. The International Programme on Chemical Safety in their Environmental Health Criteria on dermal absorption state that human skin can be stored at –20°C for up to 1 year [4]. The EU Scientific Committee on Consumer Products recommends storage at –20°C or lower, with no specification on duration [5]. The US Environmental Protection Agency (EPA) permits 'frozen (–20°C)' storage for up to 3 months, provided that barrier properties of the samples can be confirmed [6]. These conditions echoed the European Cosmetic Toiletry and Perfumery Association's (COLIPA) guidelines for dermal rate testing of cosmetic ingredients [7].

A number of studies have been published that address the issue of frozen skin storage and potential deleterious effects on barrier function as measured by skin permeability to various compounds. Previous studies are sum-

Table 2. Studies investigating the effect of frozen storage on animal skin permeation

Ref.	Test chemical	Temp., °C	Max. storage, days	Species	Anatomic site	Skin prep. method	Cryo	Freezing effect
20	Levamisole	–30	7	Sheep and calf	Dorsal	Derm	No	No
21	Abamectin	–20 and –70	180	Cattle	NR	Derm	No	No
22	Diclofenac	–20	14	Rat	Abdomen	FT	No	No
23	Carbamazepine, triamcinolone-acetonide	–80	30	Pig	Buccal, esophaegal	FT	No	No
24	Nicorandil, isosorbide dinitrate, flurbiprofen	–80	30	Rat	Dorsal	FT	No	No
25	Caffeine	–20	7	Rabbit	Ear	FT	No	No
26	N,N-diethyl-m-toluamide (m-DEET)	–80	42	Pig	Back	Derm	No	Yes
27	Salicylic acid	–20	63	Hairless rat	Dorsal	FT	No	Yes
21	Hydrocortisone, water, uracil, ivermectin	–20 and –70	180	Cattle	NR	Derm	No	Yes
28	Melatonin	–22	180	Hairless rat	Dorsal	FT	No	Yes
							Gly	Yes
28	Nimesulide	–22	180	Hairless rat	Dorsal	FT	No	Yes
							Gly	Yes
22	Diclofenac	–20	14	Pig	Ear	FT	No	Yes
22	Diclofenac	–20	14	HGP	Abdomen	FT	No	Yes
29	Hydrocortisone	–20	360	Canine	Various	FT	No	Yes
30	Estradiol patch	–20	30	Rabbit	Ear	FT	No	Yes[1]
25	Caffeine	–20	7	Pig	Ear	FT	No	Yes
25	Caffeine	–20	7	Rat	NR	FT	No	Yes[1]
19	T-2 toxin	–60	10	Monkey	Abdomen	FT	No	Yes

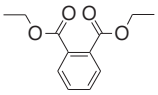
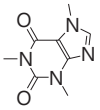
Cryo = Cryoprotectant; Derm = dermatomed; FT = full thickness; Gly = glycerol; HGP = hairless guinea pig; NR = not reported; Ref. = reference number; Temp. = temperature. [1] Enhanced barrier function with frozen storage.

marized in tables 1 and 2. Table 1 describes results on human skin and table 2 presents results for animal skin. From these data, the overall picture remains one of uncertainty: some investigators have found no evidence of an effect of freezing on barrier function, while others have found significant effects. Notably, one study [26] found adverse effects of storage at –80°C on dermatomed pig skin. This single study prompted the OECD [3] to proclaim, 'Skin should not be stored at very low temperatures since it has been shown that the storage of skin at –80°C can enhance permeability.'

Several techniques, other than permeability, have been used to assess the skin barrier, including transepidermal water loss, resistance or impedance [31], and imaging techniques. Multiphoton excitation fluorescence microscopy found tissue structural damage due to frozen storage correlated to increasing permeation of caffeine, with damage most pronounced in skin stored at –80°C [17]. Regardless of any effects on barrier function, heat separation and skin freezing ought not to be used in permeation studies requiring skin viability and metabolism [32].

The present work was prompted by anecdotal evidence in our laboratory which suggested that skin barrier function is maintained for well over a year following our protocol [heat-separated human epidermal membranes (HEMs) saturated in buffer with 10% glycerol as cryoprotectant and stored at –85°C]. The present work was designed to quantitatively assess this observation and also to address the OECD prohibition against low-temperature skin storage [3]. The permeation of diethyl phthalate (DEP) through HEMs from a single skin donor, fresh and stored up to 18 months at –85°C with 10% glycerol, is presented here. Also studied was the effect of frozen storage with and without glycerol on the skin permeability of two model permeants, DEP and the hydrophilic compound caffeine. Multiple skin donors were used in this second part of the study. During the course of the

Table 3. Properties of DEP and caffeine

Name/formula/CAS	Structure	MW, g/mol	log K_{ow}	MP, °C	BP, °C	S_w, mg/ml, 25°C
DEP $C_{12}H_{14}O_4$ 84-66-2		222.2	2.47	–41	295	1.08
Caffeine $C_8H_{10}N_4O_2$ 58-08-2		194.2	–0.07	236	352	21.6

CAS = Chemical Abstracts Service No.; MP = melting point; MW = molecular weight; BP = boiling point; S_w = water solubility. Source: PubChem Open Chemistry Database (http://pubchem.ncbi.nlm.nih.gov/).

study, a freezer failure went undiscovered for at least 2 days, which permitted the study of the effect of thawing and refreezing on barrier function.

Methods

Chemicals and Solutions

DEP of 99% purity and caffeine of 99.0% purity (Fluka brand) were both obtained from Sigma-Aldrich (St. Louis, Mo., USA). Table 3 presents some physical properties of these compounds.

The buffer used was Hanks' Balanced Salt Solution (Gibco, Invitrogen, Carlsbad, Calif., USA), with 50 mg/l of gentamicin sulfate, 0.32 g/l of sodium bicarbonate, and 5.96 g/l of HEPES (Sigma-Aldrich). The pH of the buffer was adjusted to 7.4 at 37°C. The buffer was filtered (pore sizes: 0.2 μm, SFCA; Nalgene, Thermo Fisher Scientific, Rochester, N.Y., USA) and degassed prior to use by warming to 40°C and stirring under a laboratory vacuum.

Donor formulations consisted of water saturated with excess DEP or 20 mg/ml caffeine in water.

Skin Sample Preparation and Storage

Human skin samples were used for these studies. Skin was obtained from breast reduction surgeries from 4 Caucasian females, aged 38–62 years, from the West Virginia University Skin Bank. Skin was obtained fresh on the day of surgery and HEMs were prepared by submersing the skin in buffer at 60°C for 60 s. Epidermis was separated from remaining dermis using cotton swabs. A pool of buffer, with or without 10% (v/v) glycerol, was placed on a cutting board. The epidermal membrane was floated onto the buffer and epidermal discs were cut using a stainless steel punch (1.59-cm diameter). Discs were floated onto a pool of buffer with or without glycerol, covered with gauze, and wrapped in foil. The discs were either used fresh or stored in a freezer at –85°C. Fresh (unfrozen) epidermal discs served as the control; these were never in contact with glycerol. Fresh discs were placed in a refrigerator (4°C) until use within 2 days of harvesting. The freezer temperature was registered on a graphic chart changed weekly.

A freezer malfunction after ~9 months of storage led to thawing of some skin samples to room temperature for 2–4 days, after which the skin was refrozen. This incident motivated a study of the effect of thawing and refreezing on barrier function after a total of 12 months of storage.

Sample Size

A sample size of six discs was selected for each treatment. Based on an expected coefficient of variance (CV) in permeability of about 30%, it is possible to find a significant difference if two groups exhibit about a 1.75-fold or greater difference, with 95% confidence [33]. This statistical power was deemed reasonable, as differences in means exceeding 2-fold are commonly reported in studies of in vitro dermal absorption [34]. It may be important to point out that studies of this nature cannot be used to conclude that there is 'no difference' among treatment groups: insignificant differences in mean values provide, at best, weak support for the null hypothesis.

For the DEP permeation studies with glycerol as a cryoprotectant, HEMs from a single individual human donor were used to eliminate the effect of interindividual variability. This benefit is offset by the question of generalizability across other donors; however, the permeability results for this donor agree with previously published data (described in Results). For other studies, HEMs from 1 or 2 donors were used.

Permeation Studies

HEMs were thawed at room temperature and then floated on a pool of buffer for ~20 min. They were mounted on Franz-type (static) diffusion cells (1.6 cm^2 diffusion area and 5-ml volume; PermeGear Inc., Hellertown, Pa., USA) with dialysis tubing (MWCO 12–14,000; Spectrum Laboratories Inc., Rancho Dominguez, Calif., USA) used as a membrane support. After mounting, the skin was rinsed 3 times with water. Skin discs were equilibrated overnight with pH 7.4 buffer in receptor compartments. The skin surface was maintained at 32°C by recirculating water at 37°C through the diffusion cell jackets. Each set of experiments was performed with six diffusion cells, with all sets from the same skin donor. Prior to the dosage, transepidermal water loss measurements were performed. For DEP, 500 μl of donor solution was

placed in the donor compartments and samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, and 8 h, replenishing with fresh buffer after each sampling. For studies at 12 and 18 months of storage, the exposure duration was extended to 24 h. Donor solution was replaced every 2–4 h to assure 'infinite dose' conditions. For caffeine, the same conditions were used, but sampling was done at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 22, 23, and 24 h. Donor solution was replaced at 4, 8, 12, and 22 h.

HPLC Analysis

Aliquots of 20 µl from each sample were analyzed in an Agilent 1100 HPLC system. For DEP, an Onyx Monolithic C18 column, 3 × 100 mm (Phenomenex, Torrance, Calif., USA), was used and maintained at 22°C. The isocratic mobile phase consisted of acetonitrile-water (40:60) at a flow rate of 1 ml/min. DEP was detected at a wavelength of 232 nm with a retention time of 2.7 min. Calibrations were performed over a range of 0.01–25 µg/ml prior to each experiment, and they were linear (r^2 = 0.999). The limit of detection was 0.01 µg/ml and the limit of quantification was 0.025 µg/ml.

For caffeine, a Kinetex C18 column, 4.6 × 100 mm and 2.6-µm particle size (Phenomenex), was used and maintained at 22°C. The isocratic mobile phase was acetonitrile-water (10:90) at a flow rate of 0.6 ml/min. Caffeine was detected at 272 nm with a retention time of 6.4 min. Linear (r^2 = 0.999) calibrations over a range of 0.01–50 µg/ml were performed prior to each experiment. The limit of detection was 0.01 µg/ml and the limit of quantification was 0.03 µg/ml.

Calculation of in vitro Permeability Parameters

The sampled concentrations were corrected to account for the amount of test substance that had been removed from the receptor at each sampling time. The total amount of chemical that had penetrated the skin into the receptor fluid, normalized by the area of exposed skin [$m(t)$] was calculated from the measured receptor concentrations at each sample time point, taking into account the amount removed with each sample. Steady-state flux (J_{ss}) and lag time (t_{lag}) were calculated by nonlinear regression to the experimental data, as described previously [35]. Briefly, for each diffusion cell, the best fit of the data to the first 7 terms of the diffusion equation for a homogeneous membrane [36] was determined using SigmaPlot 12.5 (Systat Software):

$$m(t) = J_{SS}t - J_{SS}t_{lag} - 12\frac{J_{SS}t_{lag}}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(-n^2\pi^2\frac{t}{6t_{lag}}\right). \quad (1)$$

The analysis yields estimates for the 2 variables J_{ss} and t_{lag}. The quality of the regressions was excellent, with an average r^2 of 0.999 and a minimum of 0.979. The permeability coefficient, k_p, was calculated as

$$k_p = \frac{J_{SS}}{C_d}, \quad (2)$$

where C_d is the donor concentration (here, the average measured quantities).

Statistical Analyses

Steady-state flux and lag time of DEP permeation through skin stored 0, 1, 2, 3, 6, 9, 12, and 18 months were analyzed with one-way analysis of variance (ANOVA) for multigroups using SigmaPlot 12.5, which executes the Shapiro-Wilk normality test and the equal variance pairwise multiple comparison procedures. When a significant difference of $p \leq 0.05$ emerged, an additional method, Holm-Sidak, was run to compare all groups to the fresh skin control. Linear regressions were done using SigmaPlot. The statistical analysis of the effect of glycerol was done with a t test, also using SigmaPlot. For each storage duration studied, the comparison was made between skin stored with and without glycerol.

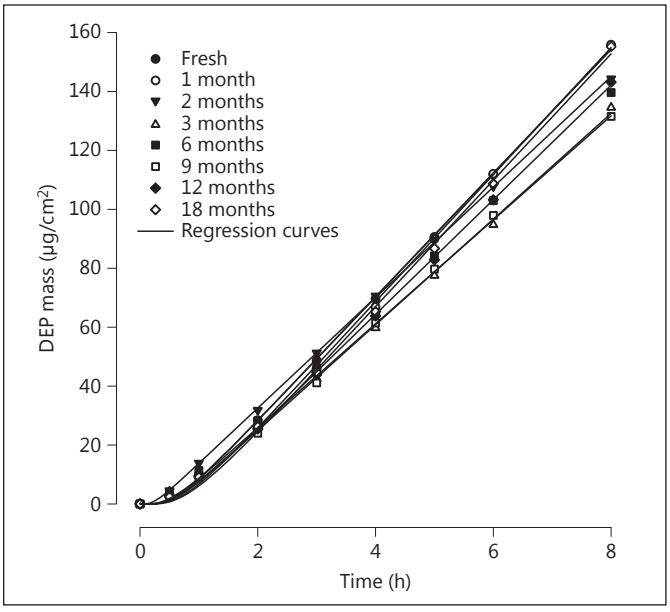


Fig. 1. DEP permeation through heat-separated human epidermis. Mass accumulation in receptor compartments through skin stored up to 18 months at –85°C. Means, n = 6 per group; all samples are derived from a single human donor. Solid lines represent nonlinear regression of data with the diffusion equation (equation 1).

Results

Effect of Frozen Storage with Glycerol on DEP Permeation

The cumulative penetration of DEP through fresh skin and skin stored at –85°C with 10% glycerol up to 18 months is displayed in figure 1 and the results are tabulated in table 4. The average donor concentration was 1,020 ± 199 µg/ml. The average values for the six discs are displayed for each storage duration. All data were included in the analysis; no data were rejected as outliers. As shown in figure 2 and table 4, the DEP steady-state flux ranged from 21.6 µg/(cm² h), its highest value after 1 month of storage, to 17.8 µg/(cm² h) at 3 months of storage, a 1.22-fold decrease. The overall average was 19.6 ±

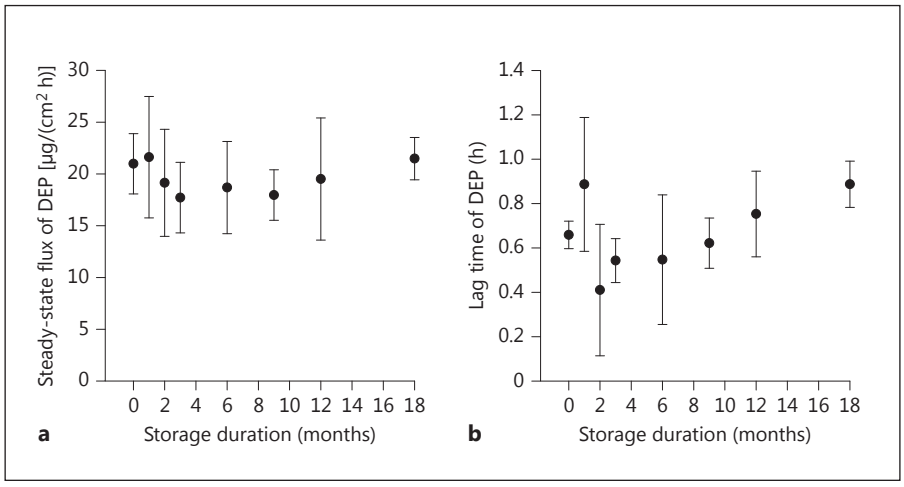


Fig. 2. Steady-state flux (**a**) and lag time (**b**) of DEP through heat-separated human epidermis stored up to 18 months at –85°C. Means ± SD, n = 6 per group from a single donor.

Table 4. Effect of –85° C storage with glycerol on DEP permeation

	Storage duration							
	0 months	1 months	2 months	3 months	6 months	9 months	12 months	18 months
J_{ss}, μg/(cm² h)	21.0±2.9	21.6±5.9	19.1±5.2	17.7±3.4	18.7±4.4	18.0±2.4	19.5±5.9 20.0±5.1	21.5±2.0 21.7±1.1
k_p, 10^{-3} cm/h	20.6±2.8	21.2±5.8	18.7±5.1	17.4±3.2	18.3±4.3	17.6±2.4	19.1±5.8 19.6±5.0	21.1±2.0 2.13±1.1
t_{lag}, h	0.66±0.06	0.89±0.30	0.41±0.30	0.54±0.10	0.55±0.29	0.62±0.11	0.75±0.19 0.88±0.35	0.89±0.10 0.94±0.34

Values represent means ± SD, n = 6. Data in the second row at 12 and 18 months are calculated from 24 h of exposure.

4.2 μg/(cm² h) (CV: 21.4%). ANOVA found that the differences in the mean values among the treatment groups were not great enough to exclude the possibility that the difference was due to random sampling variability; there was not a statistically significant difference (p = 0.6).

The DEP lag time, also plotted in figure 2, varied from its lowest value of 0.41 h at 2 months of storage to 0.94 h at 18 months, a 2.3-fold increase. The average lag time was 0.66 h and the CV was 37.5% overall. The differences in the mean values among the treatment groups were greater than would be expected by chance; there was a statistically significant difference (p = 0.002). However, multiple comparisons with the control group (fresh skin) showed no significant differences.

To observe if extended frozen storage led to an exposure duration-dependent deteriorating effect on skin barrier, the chemical exposure was extended to 24 h for both the 12- and 18-month storage studies. The steady-state fluxes from the longer exposures were compared to the ones obtained using only the initial 8 h of exposure data (table 4). The differences in the means of steady-state fluxes and lag times were not great enough to reject the possibility that the difference was due to random sampling variability (flux: p > 0.8; lag time: p > 0.4).

Effect of Frozen Storage with and without Glycerol on DEP Permeation

To evaluate the cryoprotective effect of glycerol, HEM samples were stored with and without glycerol for 3, 6, and 12 months at –85°C prior to DEP permeation testing. The steady-state fluxes and lag times of DEP through these skin samples are compared in figure 3 and table 5. No significant differences were found (flux: p ≥ 0.4; lag time: p ≥ 0.15).

Effect of Frozen Storage and Thawing with and without Glycerol on DEP Permeation

Two sets of six discs, one with glycerol and the other without, were stored for a total time of 12 months, but thawed 9 months into storage time. Data are listed in table 5. The steady-state flux values were 28.3 ± 3.8 and 30.4 ± 7.9 μg/(cm^2 h) and the lag time values were 0.74 ± 0.15 and 0.53 ± 0.28 h, respectively. These values were not significant. There were significant differences between these values and corresponding values at 12 months without thawing. However, these two skin sets were derived from 2 different individuals, and thus intraindividual variance was a confounding influence.

Effect of Frozen Storage with and without Glycerol on Caffeine Permeation

To evaluate the cryoprotective effect of glycerol on skin permeation to a hydrophilic chemical, caffeine, HEM samples with and without glycerol were stored for 4, 8, and 12 months at –85°C. The average measured donor concentration was 19,465 ± 1,272 μg/ml (CV: 6.5%). The caffeine steady-state fluxes and lag times through fresh skin and skin stored for 4, 8, and 12 months are presented in figure 4 and table 6. The difference in the mean values of steady-state flux and lag time with and without glycerol was not great enough to reject the possibility that the difference is due to random sampling variability (all p ≥ 0.34).

Discussion

The present study was designed to evaluate the change in barrier function of heat-separated human epidermis with storage duration at –85°C. Results indicate that barrier function is preserved with storage up to 18 months with glycerol as a cryoprotectant. The average DEP permeability through fresh and frozen skin stored up to 18 months with glycerol was (1.98 ± 0.52) × 10^{-2} cm/h, the steady-state flux was 19.64 ± 4.20 μg/(cm^2 h), and the lag time was 0.67 ± 0.25 h, similar to the results from our previous study [37]. The overall CV for steady-state flux of DEP was 21%, similar to intradonor permeability CV on previous studies [2, 38]. The differences in the mean values of lag time for DEP among the treatment groups were greater than would be expected by chance (p = 0.002). However, multiple comparisons versus the control of fresh skin by the Holm-Sidak method showed no significant differences. The regression indicated a slight increase in lag time with storage time, although others reported a decrease in lag time [17, 29].

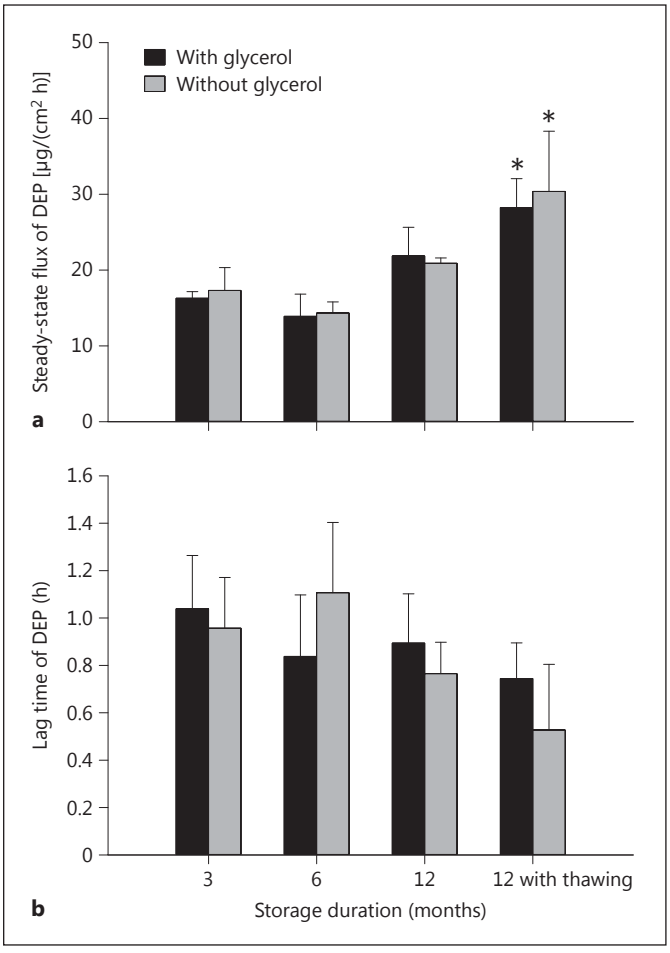


Fig. 3. Effect of glycerol on DEP steady-state flux (**a**) and lag time (**b**) on human epidermis stored at –85°C. Mean ± SD, n = 6 per group. * Significant difference (p < 0.02) between corresponding values at 12 months without thawing.

Table 5. Effect of –85° C storage with (+) or without (–) glycerol on DEP permeation

		Storage duration			
		3 months	6 months	12 months	12 months with thawing
J_{ss}, μg/(cm^2 h)	+	16.3±0.9	13.9±2.9	21.9±3.8	28.3±3.8
	–	17.3±3.0	14.3±1.5	20.9±0.7	30.4±7.9
k_p, 10^{-3} cm/h	+	16.0±0.9	13.6±2.8	21.5±3.7	27.7±3.7
	–	17.0±2.9	14.0±1.5	20.5±0.7	29.8±7.7
t_{lag}, h	+	1.04±0.22	0.84±0.26	0.89±0.21	0.74±0.15
	–	0.96±0.21	1.11±0.30	0.77±0.13	0.53±0.28

Values represent means ± SD, n = 6.

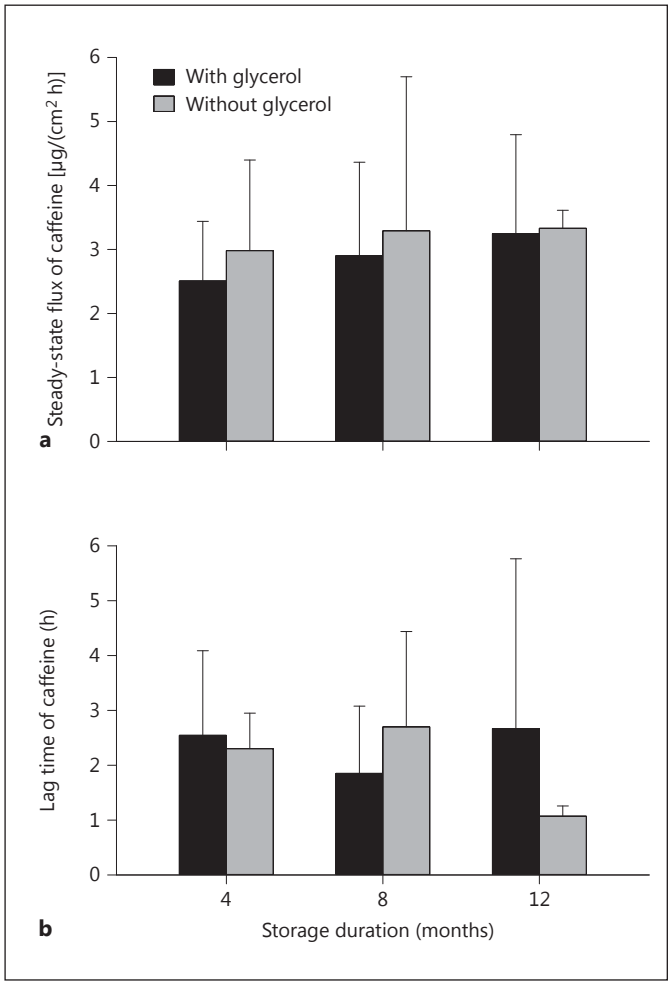


Fig. 4. Effect of glycerol on caffeine steady-state flux (top) and lag time (bottom) for skin stored at –85°C up to 12 months. Mean ± SD, n = 6 per group.

Table 6. Effect of storage at –85°C with (+) or without (–) glycerol on caffeine permeation

		Storage duration		
		4 months	8 months	12 months
J_{ss}, µg/(cm² h)	+	2.51±0.93	2.91±1.46	3.25±1.55
	–	2.98±1.42	3.29±2.41	3.33±0.28
k_p, 10^{-4} cm/h	+	1.29±0.47	1.49±0.75	1.67±0.80
	–	1.53±0.73	1.69±1.24	1.71±0.14
t_{lag}, h	+	2.55±1.54	1.85±1.23	2.67±3.09
	–	2.30±0.65	2.70±1.74	1.07±0.19

Values represent means ± SD for n = 6.

A hydrophilic chemical, caffeine, was the second model chemical tested, and it was used to investigate the cryoprotective effect of glycerol. Limited skin availability permitted the study only up to 12 months of storage. The overall average permeability of caffeine through human fresh skin and skin stored up to 12 months with and without glycerol was $(1.5 \pm 0.79) \times 10^{-4}$ cm/h, the steady-state flux was 2.95 ± 1.5 µg/(cm² h), and the lag time was 2.3 ± 1.5 h. These results are comparable to other studies [2, 34, 39], although one study reported permeability 10 times higher [17]. The CV for steady-state flux was 44.5% for the samples with glycerol and 51% for the ones without glycerol and 49.8% overall. The CV was higher than DEP, perhaps due to the hydrophilic nature of caffeine and the interdonor skin variation.

The addition of 10% glycerol made no statistically significant difference in DEP or caffeine steady-state flux for samples stored up to 12 months. For caffeine, the CV of steady-state flux was slightly smaller with glycerol (44.5%) than without glycerol (51%), but for DEP the CV was 25.4% with glycerol and 18.7% without. Also there was not a significant trend regarding the CV versus length of storage for either DEP or caffeine. Several authors recommend the use of glycerol [40, 41] since it is a known cryoprotective agent. Our original hypothesis was that storage with glycerol maintained barrier function compared with storage without glycerol; however, our results do not support that premise.

Previous works analyzing the effect of frozen storage on skin permeation are listed in tables 1 and 2. In summary, permeation in general increased with frozen storage in the range of 2–3 times for about half of all the studies, but most of these were nonhuman studies. Some of the studies that found a significant effect from frozen storage are discussed below. It is interesting to note that several of those studies with human skin [17, 19] only observed storage time up to 6 weeks. Nielsen et al. [17] concluded that tissue structural damage due to storage correlated to increasing permeation of caffeine, with effects most pronounced with storage at –80°C for 3 weeks, based on very few samples: only two donors with a total of 3 samples (one diffusion cell for donor No. 1 and two diffusion cells for donor No. 2), resulting in a 4-fold increase in permeability for donor 1 and a 1.7-fold increase for donor 2. Donor 1 is disproportionately responsible for the overall results, and the low sample number renders their conclusion statistically uncertain. On the other hand, their results were supported by tissue structural changes that were observed with deteriorating barrier function. Kemppainen et al. [19] found a 2.4-fold increase

in the rate of permeation of skin stored at –60°C for 10 days versus fresh skin; they included 83 samples from multiple donors. Ahlstrom et al. [29] investigated the effect of freezing on the permeation of hydrocortisone (log K_{ow} 1.43) through canine skin stored at –20°C for 1, 4, 8, and 12 months. They found that the pseudo-steady-state flux of hydrocortisone through skin frozen for 1, 4, 8, and 12 months was 1.27, 1.34, 1.90, and 1.85 times greater than through fresh skin, respectively. This represents a clear increase with storage time.

Babu et al. [28] found a 2.2-fold increase in flux of melatonin (log K_{ow} 1.2) with 10% glycerol and a 5-fold increase without glycerol through hairless rat skin stored for 6 months. For nimesulide (log K_{ow} 2.6), the increase in flux was 2- and 2.5-fold with and without glycerol. Their sample size was 9 for all experiments. For nimesulide, a large increase in flux occurred in the first 4 days and then decreased to about 2-fold and stayed constant from then on. Their study clearly shows an effect of frozen storage on permeability of those two compounds on rat skin as well as the benefit of glycerol for melatonin permeation. Their results cover a reasonable period of storage as well as an appropriate number of samples.

It is unclear why the results from the present study fail to observe an adverse effect on barrier function with frozen storage duration. Perhaps the results depend on the permeating chemical; however, our results with caffeine contradict the findings of Nielsen et al. [17]. Furthermore, from other data generated in our lab using different donor formulations, there have not appeared to be any significant differences in permeability over storage duration time. It therefore seems prudent for individual labs to test their own results using the particular storage conditions within their lab.

Some potential limitations of this study have been identified. First, only breast tissue was used here. It is known that skin from different body parts exhibits different permeation properties for a given chemical. Breast and abdominal skin are most commonly used in absorption studies through human skin [3]. It may be conceivable that skin other than breast skin exhibits different effects from freezing.

Second, skin from only one human donor was used in the DEP permeation study on the effect of freezing duration with glycerol (fig. 1, 2; table 3). The motivation was to eliminate intraindividual variability. A limitation of this approach is that only one donor was studied, which may not be representative of other human donors. However, as pointed out in the Results section, the permeability results for this donor are well within the data range of previously published results.

The model compounds studied here represent a small range of molecular weight (194.2 to 222.2) and a moderate range of lipophilicity (log K_{ow} –0.07 to 2.47). Results presented here for caffeine and DEP may not be applicable to other chemicals of differing properties.

Finally, results may have been affected by the type of buffer. A balanced salt solution such as the HEPES-buffered HBSS used here for both frozen storage and as a receptor fluid is a solution made to a physiological pH and salt concentration. Balanced salt solutions provide cells with water and inorganic ions while maintaining a physiological pH and osmotic pressure. It is possible that the use of simpler saline solutions (e.g. phosphate-buffered saline) may lead to different results.

Conclusions

The results from this study of DEP permeation through human epidermal membranes fresh and previously frozen at –85°C for 1, 2, 3, 6, 9, 12, and 18 months showed no significant effect from frozen storage. Furthermore, skin seems to conserve its permeability after 24 h of chemical exposure under the conditions described here. As others have demonstrated, there is no doubt that freezing affects the structure of skin, but the present study suggests that barrier function is maintained. The addition of 10% glycerol made no statistically significant difference in DEP or caffeine permeability for samples stored up to 12 months. The barrier property of epidermal membranes appears to be robust. These results support the use of skin frozen at –85°C for up to 12 months or more for studies on barrier function. These conclusions may not apply to frozen storage at higher temperatures and should be considered in light of the possible limitations described previously.

Disclosure Statement

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