The isolated perfused human skin flap model: A missing link in skin penetration studies?

Selenia Ternullo[a], Louis de Weerd[b], Gøril Eide Flaten[a], Ann Mari Holsæter[a], Nataša Škalko-Basnet[a,*]

[a]Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø The Arctic University of Norway, Universitetsveien 57, 9037 Tromsø, Norway; Email: selenia.ternullo@uit.no (S.T.); goril.flaten@uit.no (G.E.F.); ann-mari.holsater@uit.no (A-M.H.)

[b]Department of Plastic and Reconstructive Surgery, University Hospital of North Norway, Sykehusvegen 38, 9019 Tromsø and Department of Clinical Medicine, University of Tromsø The Arctic University of Norway, Universitetsveien 57, 9037 Tromsø, Norway; Email: Louis.De.Weerd@unn.no

*Corresponding author: Nataša Škalko-Basnet, Drug Transport and Delivery Research Group, Department of Pharmacy, University of Tromsø The Arctic University of Norway, Universitetsveien 57, 9037 Tromsø, Norway; Tel: +47-776-46640; Fax: +47-776-46151; Email: natasa.skalko-basnet@uit.no

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Abstract

Development of effective (trans)dermal drug delivery systems requires reliable skin models to evaluate skin drug penetration. The isolated perfused human skin flap remains metabolically active tissue for up to 6 hours during *in vitro* perfusion. We introduce the isolated perfused human skin flap as a close-to-in vivo skin penetration model. To validate the model's ability to evaluate skin drug penetration the solutions of a hydrophilic (calcein) and a lipophilic (rhodamine) fluorescence marker were applied. The skin flaps were perfused with modified Krebs-Henseleit buffer (pH 7.4). Infrared technology was used to monitor perfusion and to select a well-perfused skin area for administration of the markers. Flap perfusion and physiological parameters were maintained constant during the 6 hours experiments and the amount of markers in the perfusate was determined. Calcein was detected in the perfusate, whereas rhodamine was not detectable. Confocal images of skin cross-sections shoved that calcein was uniformly distributed through the skin, whereas rhodamine accumulated in the *stratum corneum*. For comparison, the penetration of both markers was evaluated on *ex vivo* human skin, pig skin and cellophane membrane. The proposed perfused flap model enabled us to distinguish between the penetrations of the two markers and could be a promising close-to-in vivo tool in skin penetration studies and optimization of formulations destined for skin administration.

Key words: human skin; skin therapy; skin models; skin penetration; isolated perfused human flap

Abbreviations: Cellophane membrane (CM); confocal laser scanning microscopy (CLSM); dynamic infrared thermography (DIRT); Franz diffusion cells (FDC);

human skin (HS); infra red (IR); isolated perfused human skin flap (IPHSF); modified Krebs-Henseleit buffer (KHb); pig skin (PS); propylene glycol (PG); ~~pig skin (PS);~~ *stratum corneum* (SC)

1. Introduction

(Trans)dermal delivery of drugs and cosmeceuticals has gained increasing interest in pharmaceutical and cosmetic fields. The skin represents an attractive route of drug delivery for both local and systemic effects. In this context, investigating the drug penetration into/through the skin is of fundamental importance regarding both the desired drug's therapeutic efficacy and its potential toxicity (DeLouise, 2012; Prow et al., 2011). Skin, especially the *stratum corneum* (SC) layer, exhibits very efficient barrier properties which limit drug penetration into/through the skin and needs to be overcome for successful trans(dermal) delivery (Bouwstra et al., 2003; Lane, 2013). Therefore, reliable skin models able to predict and evaluate the (desired or undesired) penetration of molecules/nanosystems *in vivo* and serve as a tool in optimization of topical formulations are required (Flaten et al., 2015). *In vivo* studies, especially in humans, are the golden standard tool in skin penetration studies. However, in the early stages of drug development, *in vivo* studies are restricted due to ethical and economical concerns (Parra et al., 2016). Moreover, new regulations limit the use of animals for *in vivo* studies in the initial stages of product development (Flaten et al., 2015). Therefore, *in vitro* and *ex vivo* techniques are gaining more interest as tools to study skin penetration (Patel et al., 2016).

The skin perfusion models comprise a surgically prepared portion of skin (flap) including subcutaneous fatty tissue with assured continuous vascular circulation. These models offer the benefits of living metabolically-active tissue and are considered the missing link between *in vitro* and *in vivo* methods (Schaefer et al., 2008). These models overcome some of the limitations of the *in vitro* studies using human or animal skin, such as the use of only epidermis and the upper part of the dermis and the lack of a dermal vascular system (de Lange et al., 1992; Patel et al.,

2016). Several animal specimens have been used for the skin perfusion model, such as the isolated perfused pig skin flap (Riviere et al., 1986), the isolated blood-perfused pig ear (de Lange et al., 1992), the isolated perfused bovine udder (Kietzmann et al., 1993) and the pig forelimb (Wagner et al., 2003). ~~Several animal skin perfusion models are in use (Riviere et al., 1986; de Lange et al., 1992; Kietzmann et al., 1993; Wagner et al., 2003); however, their use retains the limitations of correlations between animal and human skin.~~ Pig skin is considered the most suitable animal model to mimic human skin. Therefore, pig skin flap has been widely studied as skin perfusion model and skin penetration of different substances has been investigated using the isolated perfused pig skin flap model (Carver et al., 1989; Carver et al., 1990; Williams et al., 1990; Wester et al., 1998; Inman et al., 2003).

However, use of animal skin retains the limitations of correlations between animal and human skin. Kreidstein et al. (1991) designed the isolated perfused human skin flap model using transverse paraumbilical skin flap. This tissue is normally discarded in abdominal dermolipectomy (Kreidstein et al., 1991). Several techniques have confirmed the perfusion of the flap (Black et al., 2001; Kreidstein et al., 1995; Lipa et al., 1999; Miland et al., 2008). Miland and colleagues (2008) confirmed the suitability of the dynamic infrared thermography (DIRT) to monitor skin flap perfusion and to differentiate between well and less perfused areas.

To the best of our knowledge, the isolated perfused human skin flap (IPHSF) has not been used to study (trans)dermal penetration. Such a model could be a valuable tool in skin penetration studies and in optimization of dosage forms/delivery systems for skin therapy.

This study evaluated the feasibility of the IPHSF as a skin penetration model. To validate the IPHSF model, two fluorescent markers, a hydrophilic (calcein) and a

lipophilic (rhodamine), were used and their penetration investigated over a 6 hours period. Confocal laser scanner microscopy (CLSM) technique was used to follow the fluorescent markers penetration through the IPHSF. These data were compared with the *ex vivo* (human and pig skin) and *in vitro* (cellophane membrane) penetration studies in Franz diffusion cells (FDC).

2. Material and methods

2.1. Material

Calcein, rhodamine B, sodium chloride, potassium chloride, magnesium sulfate, sodium bicarbonate, trichloroacetic acid (≥99.0 %), ethanol (96 %, v/v) and Triton™ X-100 were from Sigma-Aldrich Chemie (Steinheim, Germany); human serum albumin (30 mg/mL) from Octapharma AG (Lachen, Switzerland); propylene glycol (PG) from NMD – Norwegian Medical Depot AS (Oslo, Norway) and glucose, calcium chloride and potassium dihydrogen phosphate from Merck KGaA (Darmstadt, Germany). Sucrose was product of VWR International bvba/sprl (Leuven, Belgium). Pig ears were purchased from Nurtura AS (Bardufoss, Norway).

2.2. Human skin flap

Eight human skin flaps were used in this study and were obtained from the abdomen of female patients (mean age 49.5 years, range 40-66 years) who underwent abdominoplasty (Table 1). All patients gave their written consent prior to the surgery and the experiments were performed according to the Declaration of Helsinki Principles. Since these skin panni are normally disposed of by incineration, no ethical approval for their use was required according to Norwegian Ethical Committee. The

procurement and disposal of human skin flaps were in accordance with the policy of the University Hospital of North Norway, Tromsø.

2.3. Preliminary perfusion experiment

A modification of the perfusion design of the model described by Miland and co-workers (2008) was used. The human skin flap, after its excision, was wrapped in gauze soaked with physiological solution and placed in a sealed plastic box to maintain it at room temperature until it was transferred in the laboratory where the flap experiments were performed. To perfuse the human skin flap, it was placed on a metal grid and one vessel was selected and cannulated with an arteriotomy cannula (diameter 1 mm; DLP® Metronic Inc, Minneapolis, USA), which was then connected to the perfusion apparatus (Figure 1). The cannulation was performed at room temperature. The perfusate was modified Krebs-Henseleit buffer (KHb) comprising (in mM): 110 NaCl, 3.8 KCl, 1.4 KH2PO4, 1.2 MgSO4, 31 NaHCO3, 2.5 CaCl2, 11 glucose and 10 sucrose. Human serum albumin (30 mg/ml) was added to the perfusate. The perfusate had a pH of 7.4 and an osmolarity of 290 mOsm mimicking the physiological conditions.

The perfusion flow rate (6-8 mL/min) was monitored using a drop counter and the pressure by inline pressure transducer (Transpac® IV; Abbott Laboratories, North Chicago, IL, USA).

All perfusions were initiated within 90 min after the excision of skin panni (Miland et al., 2008).

2.4. Validation of the IPHSF model

Preliminary calcein skin penetration experiments through the IPHSF were performed to validate the experimental setups and the analytical method of the quantification of penetrated marker in the perfusate. Calcein solution (10 mM) in KHb was applied onto the perfused skin flap (Figure 1). The skin diffusion area (49 cm^2) exhibiting best perfusion (skin temperature of ca. 32 ºC) was measured with an IR camera (FLIR ThermaCAM S65 HS, FLIR Systems). An adhesive patch constituted the donor chamber and calcein solution (7 mL) was applied onto the selected well-perfused skin area using a syringe. The experiment was carried out for 6 h when no leakage of the solution was observed. The weight of the flap was determined before and after the perfusion period. The penetrated calcein was assessed spectrofluorometrically ~~spectrophotometrically~~ in the perfusate collected after 6 h from a metal container placed under the flap. To assure that no inherent fluorescent skin constituents were detected, the perfusate was also collected when calcein solution was applied (time 0). Moreover, the non-penetrated calcein (retained on the flap surface) was swept and quantified at the end of experiment.

Figure 1.

2.4.1. Quantification of calcein in the perfusate

The analytical method used to detect and quantify calcein in the perfusate was based on Bahia and co-workers (2010) with modifications. The collected perfusate was centrifuged to remove blood cells (1914 *g*, 20 min). To assure that no calcein precipitated with the blood cells the pellets were firstly washed with KHb (10 mL) and secondly dissolved in Triton solution 5% (w/v; 10 mL). Trichloroacetic acid (58.82%, w/v) was used to precipitate plasma protein. Calcein concentrations in the

supernatants, pellets washed after blood cells precipitation and pellets dissolved in Triton solution were determined spectrofluorometrically ~~spectrophotometrically~~ (excitation and emission wavelengths at 485 and 520 nm, respectively) on a Polarstar fluorimeter (Fluostar; BMG Technologies, Offenburg, Germany) using a multiplate reader (COSTAR 96). Three parallels were determined for each sample. Equation 1 (derived from the Fick's first law) was used to calculate the apparent permeability coefficient (P_{app}) where J is the observed flux rate (μg/cm^2/s) and C_d is the concentration of calcein solution in the patch (μg/mL). The flux was calculated from the linear part of the curve, representing the steady-state condition.

$$P_{app}\,(\text{cm/s}) = J / C_d \qquad (1)$$

2.5. Skin penetration experiments on IPHSF model

Calcein (10 mM) in KHb and rhodamine (10 mM) in KHb/PG (0.5%, v/v) were applied onto the ~~perfused~~ skin flaps perfused with KHb (Figure 1). The weight and thickness of each flap were determined before and at the end of the experiment. The perfusate was collected at time 0 and every hour for a period of 6 h and sink conditions were maintained. The penetrated and non-penetrated markers were quantified as described in the methods for validation of the IPHSF model.

2.6. Confocal laser scanning microscopy (CLSM)

CLSM analysis was performed on a Leica TCS SP5 microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) equipped with an Argon laser. The samples were prepared using the imprint method (Brommeland et al., 2003). A cross-sectioned slide

of the flap was cut by a scalpel and attached onto a microscope slide. The cells remaining on the slide were fixed in formalin (SPRAYFIX®, Histolab Products AB, Gothenburg, Sweden). The flap areas used in CLSM are shown in Figure 2a. Calcein and rhodamine were excited using 488 and 568 nm laser lines, respectively. Fluorescence was detected using the following spectral range: 505~~0~~-550 nm for calcein, 570-610 nm for rhodamine. When required, images were acquired in Z-sections of 1 to 100 µm thickness and were superimposed.

2.7. Ex vivo and in vitro skin penetration experiments

Ex vivo and *in vitro* skin penetration experiments were carried out on Franz diffusion cells (FDC; PermeGear, Bethlehem, USA) of 1.77 cm^2 diffusion area and with 12 mL receiver volume (Hurler et al., 2012). *Ex vivo* skin penetration experiments were performed using human skin (HS) and pig ear skin (PS). Human skin (HS) was obtained from human skin flaps, which were not the same as the flaps used in skin penetration experiments on IPHSF model (Table 1). ~~and pig ear skin (PS) were used in *ex vivo* skin penetration experiments, while~~ C~~c~~ellophane membrane (CM; Max Bringmann KG, Wendelstein, Germany) was employed in *in vitro* experiments. The same solutions of the fluorescent markers tested in the IPHSF were added into the donor chamber (600 µL). The receiver chamber was filled with KHb. Samples (500 µL) were collected at 1, 2, 4 and 6 h and replaced by fresh acceptor medium to maintain sink conditions. All experiments were carried out at 32 °C. Calcein and rhodamine concentrations were determined spectrofluorometrically~~spectrophotometrically~~. Equation 1 was used to calculate the P_{app}.

2.8. Statistical evaluation

The Student's *t*-test was used for the comparison of two means. A significance level of $p < 0.05$ was considered significant.

3. Results

Any model destined for application in the development and optimization of skin formulations needs to be validated to confirm its robustness and wider applicability.

3.1. Validation of IPHSF model

Table 1 provides an overview of the characteristics of the flaps used in experiments. The perfusion experimental set up of Miland et al. (2008) was modified to identify the areas of the flap with skin temperature of at least 32 ºC corresponding to adequate perfusion (Figure 1). This enabled us to establish the area (49 cm^2) of well-perfused skin surface suitable to apply fluorescent markers. Once the area was selected, we focused on optimizing the volume (mL) of the marker solution to be applied. ~~A fluorescent marker c~~Calcein solution (7 mL) was injected into an adhesive patch (donor chamber) attached to the selected well-perfused skin area and penetration studies performed to further validate the model and confirmed its applicability.

The IPHSF was further validated in respect to possible oedema formation and the weight changes. Variation of less than 10 % in the weight observed during 6 h of perfusion was found acceptable (data not shown).

Calcein was detected in the perfusate. Moreover, in the perfusate collected at time 0 no fluorescence was detected, indicating that no inherent fluorescent skin constituents were present in the perfusate and could be detected spectrofluorometrically.

3.2. Assuring perfusion during the skin penetration studies

Physiological parameters were monitored throughout the experiment. The flow, perfusion pressure and inlet temperature were kept constant during 6 h (Table 2).

Table 2.

Figure 2.

All areas where the markers were applied were well perfused throughout the experiment and no leakage of markers from the donor chambers were observed (Figure 2a). DIRT images, taken 1 min after the start of the perfusion, showed a hot spot indicating where the skin perfusion started (Figure 2b). A distinct area of rewarming appeared corresponding to the perfusion area of the cannulated vessel. DIRT images were also taken before and after the application of markers (Figure 2c) and confirmed well-perfused skin in areas where the markers were applied throughout the experiment (Figure 2d, e).

3.3. Penetration of fluorescent markers in IPHSF model

DIRT images of flap areas where markers were applied showed that the area was still perfused after 6 h. Interestingly, DIRT images of flap areas next to where rhodamine was applied showed also the less well-perfused area (skin surface temperature lower than 32 ºC) (Figure 2e). To further investigate this area, a cross-section of the area was analyzed by CLSM. CLSM images of the marker-free skin area showed no presence of both markers, as expected (Figure 3a). CLSM images of calcein (green) in the perfused-treated area indicated a uniformly distributed fluorescence through the skin flap, while rhodamine (red) exhibited a bright fluorescence in the SC but decreasing fluorescence in the viable epidermis and subcutaneous fat tissue (Figure 3b). CLSM images of both markers in the well-perfused-untreated area indicated a weaker fluorescence as compared to the treated area (Figure 3c). In the less well-perfused areas we could only detect a weak fluorescence of rhodamine (Figure 3d).

Figure 3.

CLSM images confirmed the skin penetration profiles of the two markers in the IPHSF model.

3.4. Correlation between the skin penetration in the IPHSF model and ex vivo and in vitro studies

Penetration of two markers through IPHSF model is presented in Figure 4a. No fluorescence was detected in the perfusate at time 0, assuring that no inherent skin constituents were detected with our analytical method employed to quantify the penetrated markers in the perfusate. Calcein was detected in the perfusate, whereas rhodamine was not detectable. *Ex vivo* skin penetration studies using human skin revealed lower calcein penetration as compared to IPHSF ($p<0.05$) (Figure 4b). The

same penetration profiles of rhodamine were observed both in the IPHSF and in *ex vivo* studies on human skin (Figure 4a, b). More calcein and rhodamine penetrated through the pig skin and cellophane membrane compared to the *ex vivo* human skin and the IPHSF (Figure 4c, d). Calcein exhibited higher flux and P_{app} than rhodamine; whereas the non-penetrated rhodamine was recovered to higher extent (Table 3).

Figure 4.

Table 3.

4. Discussion

The organization of the lipid domains in the SC is considered the main contributor to the barrier property of the skin (Bouwstra et al., 2003; Schmieder et al., 2015). This barrier property of the skin can be seen as a synergy between the cooperation and interactions between SC macro- and micro-structure, bi- and three-dimensional supramolecular organization of the lipid matrix and composition of the SC (Baroli, 2010). However, dermal circulation is responsible for the clearance of the drug from the skin and should not be neglected (Lane, 2013). The introduction of nanocarriers has opened a means to improve penetration of drug into/through the skin. Nanoparticles are expected to enhance or limit the ingress and diffusion of drugs into/through the skin depending on their physicochemical properties, particularly size (Vanić et al., 2015). ~~Penetration of molecules and particles in and through skin is gaining increased attention due to increased focus on the transdermal therapies, safety of cosmetic products, possible penetration of environmental compounds and skin decontamination (Bolzinger et al., 2012).~~

Consequently, there is an increasing need to understand the penetration into/through the skin and the interaction between the carrier, drug and the skin not only to optimize the therapeutic applications, but also to minimize potential side effects (Bolzinger et al., 2012; DeLouise, 2012). However, reliable skin models able to predict and investigate desired or undesired penetration through the skin remain challenging. Human skin perfusion models offer the benefits of living tissue with active microcirculation, mimicking the application in human to a greater extent (Schaefer et al., 2008; Patel et al., 2016). Moreover, they provide a mean to study the effects of dermal vasculature on the systemic absorption. However, no human skin perfusion model has been used to assess the penetration of drugs/markers into the skin and as a tool in dermatological product development. Kreidstein and co-workers (1991) have shown that the human skin flap is metabolically and physiologically stable for at least 5 h of *in vitro* perfusion. We used DIRT to measure skin perfusion. A number of studies have shown a good correlation between skin temperature and skin perfusion. As such DIRT can provide us indirectly with information on skin perfusion and its dynamics. (de Weerd et al., 2006; de Weerd et al., 2009).

The perfusion of IPHSF was performed using the established method by Miland et al. (2008) with modifications. To validate the model, preliminary skin penetration studies were performed on perfused skin flaps in order to establish the experimental design of skin penetration studies on IPHSF. In both preliminary and skin penetration studies, In all flaps we observed the same dynamics of perfusion. This confirmed that experimental conditions remained constant throughout the experiments. Moreover, little variation (less than 10 %) in the weight and thickness of all flaps during 6 h perfusion excluded oedema formation and assured a good tissue perfusion as reported earlier (Black et al., 2001; de Lange et al., 1992; Lipa et al., 1999). Similar to Miland

et al. (2008) a perfusion flow rate between 6 and 8 ml/min was maintained during the experiments. The perfusion pressure was higher than 50 mmHg, which we attribute to the smaller cannula diameter and the different software used to monitor the baseline perfusion in our experiments as compared to Miland et al. (2008).

The IPHSF model was able to distinguish between the penetrations of two markers (0.7% calcein and 0% rhodamine). Calcein penetrated to a lower extent, as expected, since it is a hydrophilic marker with low log*P* (-5.02) (Gillet et al., 2011) with limited skin penetration potential (Bolzinger et al., 2012). Interestingly, calcein was detected in the perfusate, which would suggest that the circulation enabled its penetration. Rhodamine has a higher log*P* (1.95) (Anissimov et al., 2012) and due to its lipophilicity was prepared in solution containing PG (0.5 %; v/v) as a solubilizing agent. Therefore, we expected that PG would act as a penetration enhancer (Lane, 2013). However, the concentration of PG used in our study was relatively low compared to the PG concentration used in other studies suggesting its role as penetration enhancer (Trottet et al., 2004; Watkinson et al., 2009). Similar penetration results were reported by Wester and co-workers (1998) and their studies on the isolated perfused pig skin flap model. Tested compounds with lower log*P* were detected in higher percentage in the perfusate compared to compounds with higher log*P*. Although we could not detect rhodamine in the perfusate, the amount of rhodamine retained at the administration site on the flap was less than calcein. This suggests that rhodamine remained within SC rather than penetrating deeper into epidermis, as expected (Bolzinger et al., 2012). ~~The lipids in human skin barrier are organized as stacked bilayers of fully extended ceramides with cholesterol molecules associated with the ceramide sphingoid moiety, providing barrier towards both~~

hydrophilic and lipophilic molecules (Iwai et al., 2012). The extracellular space in SC and stratum granulosum exhibits hydrophobic properties due to its lipid-rich composition, whereas the layers below the stratum granulosum represent hydrophilic environment due to desmosome-rich composition (Iwai et al., 2012; Schmieder et al., 2015). The IPHSF results were supported by the CLSM images of the cross-section of skin flaps. While calcein was found uniformly distributed throughout the flap, rhodamine exhibited a bright fluorescence in the SC but weaker fluorescence in the viable epidermis and subcutaneous fat tissue. Gillet and co-workers (2011) reported similar accumulation of rhodamine in the SC and negligent amount within the epidermis. The same penetration profile of rhodamine observed in the IPHSF model was seen in full human skin in FDC. Water present on the surface of skin might cause swelling of the polar head-group regions in the lipid bilayers, and hence disruption of the lipid domains, which could explain the higher fluxes of hydrophilic molecules such as calcein (Bolzinger et al., 2012). However, the percentage of penetrated calcein through full human skin in FDC was significantly lower ($p<0.05$) than through the IPHSF model indicating that the perfusion might play an important role in the penetration of compounds through the skin, as recently suggested by Patel et al. (2016). For the hydrophilic marker calcein we observed a positive effect of perfusion on its skin penetration, whereas for the lipophilic marker rhodamine the penetration profile was not influenced by the perfusion to a greater extent.

These results support considerations that *ex vivo* skin studies do not resemble the *in vivo* situations to a satisfactory extent. This is a clear advantage of the skin perfusion models, since these models investigate the drug penetration considering also the dermal perfusion (Schaefer et al., 2008). Wagner and co-workers (2002) also reported that hydrophobic flufenamic acid penetrated less through the human skin in FDC

system than in *in vivo* studies. We confirmed that the perfusion played a role in the penetration of both markers by analyzing cross-section of perfused skin flap areas where the markers were not applied (as visible by DIRT images). CLSM images of these untreated perfused areas showed the presence of both markers in the skin and subcutaneous tissue of untreated areas confirming that perfusion affected the penetration. Such findings stress the importance of perfusion in a skin penetration model.

The results obtained with the IPHSF model were compared with the results obtained on the established skin models. Both penetration studies through the pig skin and cellophane membrane showed a higher penetration of the markers compared to their penetration through the IPHSF model and human skin in FDC system, strongly indicating the importance of having a model that contains human skin. Interestingly, calcein penetrated through pig skin more than rhodamine. Observed P_{app} of calcein across pig skin was similar as in an established *in vitro* model mimicking human SC lipid composition (Engesland et al., 2013). Rhodamine penetration through the pig skin, IPHSF model and human skin on FDC system was similar and as expected since it is known that pig skin mimics human skin better for the lipophilic penetrants (Dick and Scott, 1992). Penetration of both markers through cellophane membrane was significantly higher than in other models used in this study ($p<0.05$); calcein penetrated more than rhodamine in agreement with Ansari and co-workers (2006) who tested the penetration of three drugs with different lipophilicity through human skin, cellophane and natural membranes. They found that penetration of water soluble diclofenac sodium through cellophane was higher than through all other membranes, while penetration of lipophilic erythromycin through cellophane was lower (Ansari et al., 2006).

Although different *in vitro* and *ex vivo* animal skin models help us to simplify the drug penetration, the results obtained from those models differ from the results obtained with IPHSF model suggesting the importance of this missing link in reaching the close-to-in vivo situation.

The limitation of this study is the relatively small number of experiments; therefore, the results should be interpreted within the context of this limitation. Moreover, the IPHSF model is metabolically active for 6 h and skin drug penetration studies on IPHSF model have to be performed within this time limit. Specific equipment, such as infrared camera, is needed to perform skin flap experiments as well as the help of selected professionals to cannulate the skin flap. Therefore, we are aware that the IPHSF model cannot be used as skin perfusion model in the early stages of drug development. However, the model might be used, after the fully validated methodologies have been employed, to study skin drug penetration in conditions closer to the *in vivo* human ones since ~~However,~~ the importance of perfusion and the use of human skin in skin penetration studies is clear.

The next step in full utilization of the proposed model is to evaluate the penetration of nanoparticles and nanoparticle-associated drugs.

5. Conclusions

We demonstrated that the isolated perfused human skin flap model can be used in the skin penetration studies since it provides constant experimental conditions and perfusion, assuring reproducible results. Moreover, the model proves the benefits of working with living tissue using a byproduct of surgery, and avoiding animal use. The

model was able to distinguish between the penetrations of two markers and is a promising tool in optimization of formulations destined for skin administration.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Figures and figure legends:

Figure 1. Flap perfusion design

The perfusate was filtered and gassed with 95% O_2/5% CO_2 using a rotating surface oxygenator. The perfusion flow rate (6-8 mL/min) was monitored using a drop counter and the perfusion pressure measured with an inline pressure transducer. A heating circulator was set at 38 ºC and a thermocouple inserted in the cannula to monitor the temperature of the perfusate entering the flap. The IR camera monitored the skin surface temperature throughout the perfusion period. The flow, pressure and temperature of the perfusate were monitored on-line using PhysAcq software. The perfusate from IPHSF was collected using a metal container.

Figure 2. Photographs and DIRT images of representative flaps in skin penetration experiments

The **a** represents the photographs of flaps. The **b** are DIRT images recorded 1 min after the start of the perfusion (skin temperature below 32 ºC). The **c**, **d** and **e** are DIRT images recorded at time 0, 3 and 6 hours after the application of the markers, respectively (skin temperature at ca. 32 ºC).

Figure 3. Representative CLSM images of cross-sections of IPHSF at the end of skin penetration experiments

CLSM images of calcein (green fluorescence) in IPHSF are shown in the left column, while CLSM images of rhodamine (red fluorescence) are shown in the right column. The **a** represents the intact flap cut before the perfusion (control). The **b** is treated

area; **c** untreated area. The **d** is a non-perfused area observed by DIRT in flap where rhodamine was applied.

Cross-sectioned slides of IPHSF were cut starting from the skin surface (S; left side) to the subcutaneous fat (SF; right side). Scale bar represents 500 µm.

Figure 4. Penetration profiles of calcein and rhodamine through IPHSF (a), HS (b), PS (c) and CM (d).

The concentration of calcein in KHb and rhodamine in KHb/PG (0.5%, v/v) solutions was 10 mM. The penetration of both markers was investigated for a period of 6 h. All experiments were conducted in triplicates and the results are presented as mean ± SD. * $p<0.05$.

Tables and table legends:

Table 1. Characteristics of human skin flaps

[a] Weight and thickness of the flaps were not determined in the preliminary study used to validate the experimental design of the perfusion technique and experimental set up.

[b] Thickness (cm) refers to the full skin obtained from the human skin flap. Weight was not determined since not relevant for *ex vivo* study.

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Table 2. Characteristics and physiological parameters of IPHSF recorded during 6 h of experiment (mean ± SD)

[a] Variation (%) calculated from weight and thickness measured before and after perfusion of flaps.

Table 3. *In vitro* and *ex vivo* penetration of calcein and rhodamine (mean ± SD)

[a] The amount retained at skin surface.

[b] nd, flux and Papp were not determined because no penetrated rhodamine was detected.