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

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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 h 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 h 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.

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

Abbreviations: CM, Cellophane membrane; CLSM, confocal laser scanning microscopy; DIRT, dynamic infrared thermography; FDC, Franz diffusion cells; HS, human skin; IR, infra red; IPHSF, isolated perfused human skin flap; KHb, modified Krebs-Henseleit buffer; PS, pig skin; PG, propylene glycol; SC, *stratum corneum*.

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forelimb (Wagner et al., 2003). 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, 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 et al. (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 h 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 et al. (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 (Fig. 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 (Fig. 1). The skin diffusion area (49 cm^2) exhibiting best perfusion (skin temperature of *ca.* 32 °C) 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 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.

Table 1
Characteristics of human skin flaps.

Flap number	Age (years)	Weight (g)	Thickness (cm)	Type of experiment
0a	51	–[a]	–[a]	Preliminary perfusion test
0b	42	1110	–[a]	Preliminary skin penetration experiment (calcein)
1	47	443	1.90	Skin penetration experiment (calcein)
2	41	526	1.63	Skin penetration experiment (calcein)
3	54	603	1.26	Skin penetration experiment (calcein)
4	40	1187	0.90	Skin penetration experiment (rhodamine)
5	57	1570	1.68	Skin penetration experiment (rhodamine)
6	66	525	0.93	Skin penetration experiment (rhodamine)
7	42	–	0.23[b]	*Ex vivo* skin penetration experiment in FDC (calcein)
8	42	–	0.17[b]	*Ex vivo* skin penetration experiment in FDC (rhodamine)

[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.