Journal of Controlled Release 226 (2016) 66–76

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

The application of local hypobaric pressure — A novel means to enhance macromolecule entry into the skin

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ARTICLE INFO

Article history:
Received 27 December 2015
Received in revised form 25 January 2016
Accepted 27 January 2016
Available online 29 January 2016

Keywords:
Hypobaric pressure
Macromolecule
Skin
Follicle
In vivo
Blood flow
Bioavailability
Drug delivery
Penetration enhancement

ABSTRACT

The local application of controlled hypobaric stress represents a novel means to facilitate drug delivery into the skin. The aims of this work were to understand how hypobaric stress modified the properties of the skin and assess if this penetration enhancement strategy could improve the percutaneous penetration of a macromolecule. Measurements of skin thickness demonstrated that the topical application of hypobaric stress thinned the tissue ($p < 0.05$), atomic force microscopy showed that it shrunk the corneocytes in the *stratum corneum* ($p < 0.001$) and the imaging of the skin hair follicles using multiphoton microscopy showed that it opened the follicular infundibula ($p < 0.001$). Together, these changes contributed to a 19.6-fold increase in *in vitro* percutaneous penetration of a 10,000 molecular weight dextran molecule, which was shown using fluorescence microscopy to be localized around the hair follicles, when applied to the skin using hypobaric stress. *In vivo*, in the rat, a local hemodynamic response (*i.e.* a significant increase in blood flow, $p < 0.001$) was shown to contribute to the increase in follicular transport of the dextran to produce a systemic absorption of 7.2 ± 2.81 fg·mL^{-1}. When hypobaric stress was not applied to the rat there was no detectable absorption of dextran and this provided evidence that this novel penetration enhancement technique can improve the percutaneous penetration of macromolecules after topical application to the skin.

1. Introduction

The delivery of macromolecules such as peptides, proteins and antibodies *via* the skin is an attractive proposal when considering the treatment and prevention of diseases. However, their administration *via* this route is a challenge due to the skin's highly stratified structure [1,2]. The *stratum corneum* and the multiple underlying layers, which constitute the cutaneous tissue, exhibit highly selective permeability that only allows the rapid absorption of relatively small, lipophilic compounds to enter the body after topical administration (<500 Da, Log P 0.8–3) [3,4]. Therefore, the only means by which macromolecules can enter the skin tissue at a rate that is suitable for therapeutic applications is by the use of a penetration enhancement strategy [5–9]. While many of the developed approaches to allow macromolecules into the skin are effective they each have associated cost, compliance and safety issues that make their use limited in clinical practice [10]. There therefore remains a need to develop new technologies to facilitate the passage of high molecular weight therapeutic agents into the skin.

The few documented reports that have assessed the capability of applying local hypobaric stress to alter the properties of the skin suggest that it could represent a novel means to enhance drug percutaneous penetration. For example, the application of local hypobaric stress has been documented to significantly increased transepidermal water loss (TEWL) and decrease the *stratum corneum*'s water content [11]. These effects could potentially thin the skin and facilitate drug entry. In addition, the hypobaric stress induced stretching of the skin has been shown to cause disorganization of the intercellular lipid bilayers and rupture of the skin's corneosomes [12,13]. Increasing the lipid fluidity is a mechanism that chemical penetration enhancers use to increase percutaneous penetration and therefore this could be an important means by which hypobaric stress could act to enhance percutaneous penetration. Furthermore, the continuous application of localized sub-atmospheric pressures has been reported to significantly increase cutaneous blood flow [14,15]. Increasing the blood flow under local hypobaric stress conditions may increase the systemic absorption of topically applied agents due to the dilation of the dermal blood vessels and their displacement towards the skin surface [16]. However, how these effects of hypobaric stress influence skin penetration of macromolecules is currently unknown.

The purpose of the present study was to assess the influence of locally applied hypobaric stress upon the mechanical and physiological properties of cutaneous tissue and then using this knowledge test if hypobaric stress could enhance the percutaneous penetration of macromolecules. To apply controlled hypobaric stress to the skin one of the

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http://dx.doi.org/10.1016/j.jconrel.2016.01.052

most widely used test systems for studying skin permeability, the Franz diffusion cell, was adapted to operate at a sub-atmospheric pressure of 500 mBar. A series of fluorescein isothiocyanate (FITC)-labeled dextrans was used as the model macromolecules for this work because of their molecular homogeneity, their excellent chemical and physical stability, their commercial availability and their ability to be tracked *in vitro* and *in vivo* [17]. *In vitro* permeation studies were performed using rat skin. It is accepted that rat skin is more permeable compared to human skin, but this membrane was selected primarily because this allowed similar studies to be performed *in vitro and in vivo*. In addition, the rat was easily amenable to the measurement of blood flow using full-field laser perfusion imaging (FLPI) and systemic bioavailability using standard blood sampling protocols [18–20]. Porcine skin was also utilized as a permeation barrier to explore the transdermal transport mechanism of the macromolecules because it is the most relevant animal model for human skin [18]. In addition, this barrier is especially suitable for studying follicular structures as the ear cartilage prevents contraction of tensile fibres and closure of follicles upon sample preparation, which can occur with human and rat skin [21]. The changes in the skin caused by the application of hypobaric stress were assessed using multiphoton microscopy, light microscopy and atomic force microscopic (AFM) imaging. In addition, the entry of the dextran into the cutaneous tissue was followed using fluorescence microscopy to try and elucidate the route of entry of this molecule into the skin.

2. Materials and methods

2.1. Materials

Acetonitrile and methanol both HPLC grade, DPX mounting medium, xylene and Optiphase scintisafe gel were purchased from Fischer Scientific (Leicester, UK). Tetracaine base BP grade (99.9%), formalin solution neutral buffer 10%, DAPI medium, ethanol, heparin sodium salt (I-A), urethane, 0.7% glacial acetic acid, isopropanol and FTIC-dextran with average molecular weight (Mw) of 4 kDa (FD-4) and 10 kDa (FD-10S) used without any further purification steps were supplied by Sigma Aldrich (Dorset, UK). Concentrated hydrochloric acid and sodium hydroxide was from Fluka (Dorset, UK). Sodium acetate was provided by Alfa Aesar (Heysham, UK). Silicone membranes with a thickness of 0.25 mm were purchased from GBUK Healthcare (Selby, UK). Phosphate buffered saline (Dulbecco A) tablets were obtained from Oxiod Limited (Hampshire, England). The Tissue-Tek® O.C.T™ compound, scintillation vials and hydrogen peroxide 30% were obtained from VWR International (Leicestershire, UK). Dextran (carboxyl-^{14}C) with an average M.W. of 10 kDa and specific activity of 0.00006 Ci/mmol was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, USA). Soluene® 350 was provided by Perkin Elmer (Bucks, UK). Isoflurane 100% (w/v) inhalation vapour liquid was obtained from Animal Care Ltd. (York, UK).

2.2. Animals

All procedures were conducted in accordance with the UK Animal Scientific Procedures Act (1986) and Amendments Regulations (2012) and approved by the King's College London Animal Welfare and Ethical Review Body. Sprague Dawley male rats (6–9 weeks old, *ca.* 220–250 g; Charles River, Kent, UK) were caged in groups of 4 with free access to water and food. A temperature of 19–22 °C was maintained, with a relative humidity of 45–65%, and a 12 h light/dark cycle. Animals were acclimatized for 7 days before each experiment.

2.3. In vitro dextran permeation studies

Rats were killed by intraperitoneal injection of sodium pentobarbital. The dorsal hair was removed using an animal hair clipper and full thickness skin was excised. The excess fat adhering to the dermis side was removed carefully with a scalpel. A standard Franz diffusion cell was attached to an in-house designed aluminium support frame (Supplementary data Fig. S1a, b and c) that was able to pressure seal the donor compartment (Supplementary data Fig. S1d). To develop a sound experimental protocol using the assembled pressure cell set up, a series of transport experiments with porcine skin were conducted. These preliminary studies indicated that the receiver chamber of the Franz cell could not be filled with a liquid because the hypobaric pressure caused suction of the liquid from the donor phase into the receiver compartment. As a consequence, the traditional Franz cell was adapted to use a sponge in order to collect the drug exiting the dermal side of the skin. The sponge was shown to remain in contact with the skin through the experiments and it allowed the complete recovery of the drug that had passed through the skin (Supplementary data Fig. S2). For the *in vitro* dextran permeation studies (note: fluorescein isothiocyanate (FITC)-dextran was used throughout the *in vitro* section of work), rat skin was employed using the permeation methodology developed with the porcine skin. The depilated rat skin harvested from the animals was cut into pieces of a suitable size and mounted with the *stratum corneum* facing the donor compartment in the Franz diffusion cell (University of Southampton, UK). The pressure cell sealed donor compartment was attached to the Franz cell base and each diffusion cell was placed on a submersible stirring plate in a pre-heated water bath (Grant Instruments, Cambridge, UK) set at 37 °C, to obtain a temperature of 32 °C at the membrane surface [22]. The sealing of the cells was evaluated by their resistance to air removal and the absence of significant solvent back diffusion into the donor compartment prior to the application of hypobaric pressure. Any leaking cells were not used in the experiments and hence all the data was used in the data analysis. The experiments were initiated by the application of 1 mL of a donor solution (containing either 125 μM of FD-4 or FD-10S in phosphate buffer solution at pH 7.4) to the apical surface of the skin. Permeation experiments were conducted under atmospheric (1010 mBar) and hypobaric pressure (500 mBar) for 1 h using the pressure cell assembly. The hypobaric conditions had previously been tested in a pilot study and it appeared to cause no skin blistering (data not shown), but this was investigated further using skin histology (detailed in a subsequent section). The hypobaric pressure was applied to the skin immediately after the application of the donor solution by the removal of air from the Franz cell donor compartment, which was sealed onto the skin and thus acted as a vacuum chamber. A manometer was mounted on the donor compartment in order to record the pressure applied. Hypobaric stress was applied for 1 h and any small loss in pressure was corrected by extracting further air from the Franz cell. After the 1 h time period of hypobaric stress the cells were allowed to equilibrate at atmospheric pressure and the permeation experiments were continued for a total of 20 h to allow suitable drug levels to accumulate in the skin to facilitate accurate analytical quantification. At the end of the transport studies the *stratum corneum* was removed by tape stripping (*ca.* 20 strips until the skin was translucent) using adhesive tape (Scotch 845 book tape, 3 M, Bracknell, UK) and the epidermis was separated from the dermis as previously described [23]. Dextran was extracted from the receptor compartment, adhesive tape and skin using a phosphate buffer saline (pH 7.4) extraction solution. The dextran was quantified using a stand-alone fluorescence spectrometer (Varian Cary Eclipse fluorescence spectrophotometer, Agilent, Cheadle, UK) at an excitation wavelength of 495 nm and fluorescent emission wavelength of 515 nm. The assay was verified as 'fit for purpose' by determination of linearity, precision and sensitivity (data not shown). Drug extraction was within the 100 ± 15% recovery rates published in guidelines [24]. The effect of local hypobaric stress upon dextran cutaneous bioavailability was represented as an enhancement ratio (*ER*), which was calculated according to Eq. (1) where C_P and C_{AT} were the amount of dextran (μg) per cm^2 of skin under hypobaric and atmospheric pressure conditions, respectively.

$$ER = \frac{C_P}{C_{AT}}. \quad (1)$$