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Effect of cryoprotectants for maintaining drug permeability barriers in porcine buccal mucosa

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ABSTRACT

Ex vivo drug permeation studies are useful for early screening of drug candidates for buccal delivery. However, it is not always feasible to obtain fresh tissue for each experiment. Therefore, a method for storing excised tissue for later use is needed. The purpose of this study was to determine if permeability barriers for small molecules (nicotine and diazepam) were maintained after freezing porcine buccal mucosa with cryoprotectants to −80 °C.

Combinations of dimethyl sulfoxide, bovine serum albumin, glycerol and sucrose were used as cryoprotectants. The permeability of nicotine and diazepam across fresh or frozen/thawed tissue was determined using modified Ussing chambers. Haematoxylin-eosin stained tissue sections for histology were prepared.

The permeability of nicotine across tissue frozen without cryoprotectants was significantly higher compared to tissue frozen with cryoprotectants or fresh tissue. Freezing with or without cryoprotectants did not significantly affect the flux of diazepam compared to fresh tissue. Only minor histological changes were seen in frozen/thawed porcine buccal mucosa compared to fresh tissue. In conclusion, permeability barriers for nicotine and diazepam were preserved after freezing with any of the combinations of cryoprotectants; however, the barrier may be damaged when freezing without cryoprotectants.

1. Introduction

Oral administration is often preferred by patients due to ease of administration. However, oromucosal drug delivery has attracted increased interest as a means to circumvent pre-systemic degradation and hepatic first pass metabolism because a drug enters the systemic circulation directly, *via* the jugular vein (Patel et al., 2011; Squier and Wertz, 1996). Furthermore, easy access to the oral cavity makes this administration site acceptable for patients and enables removal of the formulation in case of adverse events. The onset of action is fast since the formulation is directly in contact with the site of absorption. Despite the advantages of using oromucosal drug delivery, there are only few approved oromucosal preparations on the market. This may be due to a lack of standardized methods for *in vitro* evaluation of oromucosal delivery systems (Patel et al., 2012). It is important to have predictable *in vitro* models to enable early screening of possible drug candidates for oromucosal delivery. Previously, diffusion cells, such as modified Ussing chambers or Franz cells, have been used to assess the permeability of drugs across the buccal mucosa (Artusi et al., 2003; De Caro et al., 2012; Nicolazzo et al., 2005). Porcine buccal mucosa is considered the golden standard as a model for human buccal mucosa due to physiological similarities to the human tissue (Squier and Wertz, 1996). It can be difficult to obtain fresh tissue for each experiment, and when excess tissue is available it calls for proper storage at subzero temperatures to obey the principles of the three R's (Replacement, Reduction and Refinement), which guide to more ethical use of experimental animals (Russell and Burch, 1959). Not much is known about how the barrier properties of buccal mucosa are affected by freezing. Several studies have shown that freezing do not significantly affect the permeability of buccal mucosa (Caon and Simoes, 2011; Diaz

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; J_{SS}, steady state flux; Log P, logarithm of partition coefficient; MW, molecular weight; P_{app}, apparent permeability coefficient; PBS, phosphate buffered saline (adjusted to 290 mOsm with sodium chloride); SD, standard deviation.

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Del Consuelo et al., 2005; Nicolazzo et al., 2003), whereas others have found that freezing did have an effect on permeability (Lee et al., 2002; van Eyk and van der Biijl, 2006).

Ice crystal formation during freezing can cause cell damage due to cellular stress and deformation; furthermore, formation of ice crystal leads to an increase in solute concentration, which can damage the cell membrane and cause solute precipitation and changes in pH which can also lead to tissue damage (Brockbank and Taylor, 2006; Pegg et al., 1979). Several cryoprotectants are known; however, no structurally common features have been identified. Different mechanisms of actions for the cryoprotectants have been proposed; (I) interacting (hydrogen bonding) with polar molecules, *e.g.* water and (II) water replacement, both mechanisms lead to decreased ice crystal formation and thus decreased tissue damage, whereas the mechanism of dimethyl sulfoxide (DMSO) is (III) pore formation in the cell membranes (Brockbank and Taylor, 2006; Gurtovenko and Anwar, 2007). Cryoprotectants can be classified by their ability to penetrate the cell membrane or not, called intracelullar or extracellular, respectively. It can be beneficial to combine an intracellular and an extracellular cryoprotectant (Fahy and Wowk, 2015). Recently, DMSO and bovine serum albumin (BSA) have been used in combination as intra- and extracellular cryoprotectants, respectively, when freezing porcine buccal mucosa (Amores et al., 2014). Glycerol (intracellular) and sucrose (extracellular) are two other well-known cryoprotectants (Ashwood-Smith, 1987). Glycerol has low cell toxicity and can form hydrogen bonds to water molecules and thereby decrease the risk of ice crystal formation (Fuller, 2004). Sucrose has, besides hydrogen bonding with water, been shown to interact with phospholipids and to stabilize cell membranes during high solute concentrations due to ice crystal formation (Fuller, 2004). When reducing sugars are in contact with protein there is a risk that Maillard reactions will occur, where the carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid (Li et al., 1996). In order to decrease the risk of Maillard reactions, non-reducing sugars are preferred (such as sucrose rather than lactose or glucose). Three low molecular weight (MW) model drugs with different lipo- and/or hydrophilicity (log P values) were chosen: Diazepam (MW = 285 g/mol, log P = 2.86 (Macheras et al., 1990)), nicotine (MW = 162 g/mol, log P = 1.43 (Nielsen and Rassing, 2002)) and mannitol (MW = 182 g/mol, log P = −3.10 (Larhed et al., 1997)).

As mentioned above, freezing buccal mucosa with DMSO and BSA as cryoprotectants has previously been studied by Amores et al. (Amores et al., 2014). DMSO has exceptionally high solubility properties for both hydrophilic and hydrophobic compounds along with drug permeation enhancing properties. Thus, it is important to compare the use of DMSO to other known cryoprotectants when freezing porcine buccal mucosa for permeability studies. To the best of our knowledge, such studies have not been reported. Hence, the aim of this study was to examine and optimize the use of cryoprotectants for storage of porcine buccal mucosa for future experiments. The permeability of three model drugs across porcine buccal mucosa was evaluated after freezing and thawing of the tissue with and without a combination of cryoprotectants: DMSO, BSA, glycerol and sucrose. This was compared to the permeability of the drugs across fresh porcine buccal mucosa. Additionally, histomorphology of fresh and frozen tissue with and without cryoprotectants was compared.

2. Material and methods

2.1. Materials

DMSO, n-hexane >95%, ortho-phosphoric acid 85%, sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Merck KGaA (Darmstadt, Germany). Mannitol (Pearlitol® 200 SD) was obtained from Roquette Pharma (Lestrem, France). BSA, glycerol, sodium phosphate monobasic (NaH_2PO_4) and sucrose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nicotine bitartrate dihydrate was kindly donated by Fertin Pharma A/S (Vejle, Denmark). Diazepam was a kind gift from Takeda (Osaka, Japan). [^{3}H]-nicotine (specific activity 80.4 Ci/mmol), [^{3}H]-diazepam (specific activity 78.2 Ci/mmol), [^{14}C]-mannitol (specific activity 57.2 mCi/mmol) and Ultima Gold™ liquid scintillation fluid was purchased from Perkin Elmer, Inc. (Waltham, MA, USA). Deionized water was collected from Milli-Q water system, SG Ultra Clear 2002 from Evoqua Water Technologies LLC (Warrendale, PA, USA)

All compounds were of analytical or HPLC grade and no further purified. Compositions given throughout the manuscript in % are w/w unless otherwise stated.

2.2. Procedure for freezing and thawing

Tissue preparation: Cheeks used for experiments with nicotine were obtained from domestic pigs from abattoir Harald Hansens Efterfølger I/S, Denmark. Cheeks used for experiments with diazepam were obtained from healthy experimental control pigs (approx. 30 kg Danish Landrace/Yorkshire x Durox (D-LY)). After euthanizing, the cheeks were excised and transported to the laboratory and kept cold and moist with phosphate buffered saline adjusted to 290 mOsm with NaCl (PBS). The cheeks were trimmed with surgical scissors until a thickness of approximately 5 mm was obtained.

Freezing: The trimmed cheeks were directly frozen without medium (A) or submerged in 100 g freezing medium B, C or D (see Table 1), and placed on a shaking table (50 rpm, 20–25 °C) for 30 min to allow the cryoprotectants to penetrate the tissue. Tissue submersion in freezing medium B were performed in two steps; (I) the tissue was submerged in 50 g of 40% glycerol in PBS for 30 min, and (II) 50 g of 40% glycerol and 40% sucrose in PBS was added followed by an additional 30 min on shaking table. The sucrose in (II) was not completely dissolved until (I) and (II) were combined, giving 100 g of 40% glycerol and 20% sucrose in PBS (freezing medium B). While submerged in media the cheeks were placed in a −80 °C mechanical freezer in a polystyrene foam container to ensure slow and steady decrease in temperature. No more than 7 h had passed before placing the pig cheeks in the freezer after euthanizing the pigs. The cheeks were stored in the freezer for approximately 30 days.

Thawing and washing: The thawing medium was PBS (pH 6.8), stored at 5 °C. The frozen cheeks in freezing media were submerged in approximately 200 mL thawing medium on a shaking table (50 rpm). After 30 min, the cheeks were thawed and subsequently washed by submerging in 50 mL thawing medium for 10 min, still on a shaking table (50 rpm). The wash was repeated twice. The setup was kept cool during both thawing and washing (not exceeding 12 °C) by placing the thawing medium on an Icetainer® cooling tray during thawing and washing.

Table 1
Composition of the freezing media.

Components	Freezing medium			
	A	B	C	D
Glycerol (%)	–	40	40	–
Sucrose (%)	–	20	20	–
BSA (%)	–	–	–	4
DMSO (%)	–	–	2	10
0.1 M PBS pH 7.4 (%)	–	40	38	86