UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

LUCIANO SERPE

PADRONIZAÇÃO DE MODELOS DE BARREIRAS DE MUCOSA, CONDIÇÕES EXPERIMENTAIS E USO DE MICROAGULHAS COMO PROMOTOR DE ABSORÇÃO EM ESTUDOS DE PERMEAÇÃO *IN VITRO*

STANDARDIZATION OF ORAL MUCOSA BARRIER MODELS, EXPERIMENTAL CONDITIONS AND USE OF MICRONEEDLES AS PERMEATION ENHANCER FOR *IN VITRO* PERMEATION STUDIES.

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Odontologia, na Área de Farmacologia, Anestesiologia e Terapêutica

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dentistry, in Pharmacology, Anesthesiology and Therapeutics Area.

Orientador: Profa. Dra. Michelle Franz Montan Braga Leite
Coorientador: Prof. Dr. Francisco Carlos Groppo

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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RESUMO

O uso de formulações tópicas na cavidade oral apresenta baixa eficácia devido à complexidade e variabilidade da mucosa de revestimento e difícil acessibilidade aos tecidos profundos, como a polpa dental. Microagulhas têm sido relatadas como um eficiente e indolor sistema transdérmico e transmucosa de fármacos para ação local e/ou sistêmica que poderia melhorar ação de fármacos tópicos em Odontologia. Visando a melhora da biodisponibilidade de formulações tópicas, estudos de permeação *in vitro* são muito importantes pois podem permitir a previsão do comportamento destas frente à barreira utilizada. No entanto, não existe um modelo de barreira que mimetize as condições reais da cavidade oral. Nesse contexto, este estudo teve como objetivos padronizar barreiras de tecidos espessos da cavidade oral de suínos, adaptar células de difusão e condições experimentais para realização de estudos de permeação *in vitro* com essas barreiras, visando o desenvolvimento de produtos tópicos em Odontologia. Além disso, avaliar a eficiência e aplicabilidade de microagulhas revestidas ou não como promotores de absorção *in vitro*, na metodologia desenvolvida. **Artigo 1:** foram padronizados os tecidos espessos de palato de porco (com e sem osso) como barreiras e condições experimentais para realização de ensaios de permeação *in vitro*. Em seguida, o sistema de microagulhas Dermaroller® (0,2, 0,5 e 1 mm de comprimento) foi aplicado naqueles tecidos, os quais foram montados em células de difusão vertical (Franz) adaptadas. Foram avaliados o fluxo, quantidade total permeada e o fator de promoção de permeação (EF) para os anestésicos locais lidocaína e prilocaína. Os tecidos foram padronizados com sucesso e a célula de Franz adaptada permitiu manter as condições ao longo das 12h dos experimentos de permeação. A permeação dos fármacos analisados ocorreu eventualmente, demonstrando a resistência das barreiras à difusão de fármacos. A aplicação prévia das microagulhas nos tecidos aumentou a permeação das drogas-teste em relação aos grupos-controle. **Artigo 2**: microagulhas de 0,7 mm foram revestidas com a droga Sulforrodamina B, aplicadas na superfície de mucosa jugal de suínos, seguida de ensaios de permeação (24h). Dois tipos de fluxo salivar (estático e dinâmico) foram simulados no compartimento doador da célula e comparados com a presença de umidade como controle negativo. Foram avaliados o tempo para início da permeação, fluxo e o EF. Ambos os tipos de fluxos salivares alteraram o perfil de permeação da droga modelo, aumentando a quantidade de droga permeada, em comparação com o controle, demonstrando a importância da saliva na realização de testes de permeação *in vitro*. **Conclusões gerais:** os tecidos foram padronizados e permitiram realizar ensaios de permeação nas células de Franz adaptadas. O uso de microagulhas foi eficaz em aumentar a permeação de fármacos nas condições avaliadas. A presença de fluxo salivar

demonstrou ser importante para simular as condições reais da cavidade oral em permeação *in vitro*, pois pode ser fundamental na dinâmica da permeação através do tecido. O presente estudo representa um aprimoramento na realização de experimentos de permeação *in vitro* visando a melhora de formulações tópicas e promotores de absorção para uso em Odontologia.

Palavras-chave: Permeação. Microagulhas. Mucosa Oral. Técnicas *In Vitro*.

ABSTRACT

The use of topical formulations in the oral cavity presents low efficiency because of the complexity and variability of the mucosal lining and hard accessibility of deep tissues, as dental pulp. Microneedles have been reported as an efficient and painless transdermal and transmucosal drug systems for local and/or systemic effect that could improve action of topical drugs in Dentistry. Aiming at improving the bioavailability of topical formulations, *in vitro* permeation studies are very important because it may allow predicting the behavior of these formulations in front of the barrier used. However, there is no barrier model that mimics the actual conditions of the oral cavity. In this context, this study aimed to standardize thick tissue barriers of the oral cavity from pigs, adapt diffusion cells and experimental conditions for performing *in vitro* permeation studies with these barriers, in order to develop topical products in Dentistry. Moreover, to evaluate the efficiency and applicability of microneedles coated or not as *in vitro* absorption enhancers in this methodology. **Article 1**: pig thick palate tissues (with and without bone) and experimental conditions were standardized to carry out *in vitro* permeation experiment. The microneedle device Dermaroller® (0.2, 0.5 and 1 mm length) was applied in those tissues, which were mounted in adapted Franz-type vertical diffusion cells. The Flux and the permeation enhancement factor (EF) of the local anesthetics lidocaine and prilocaine were evaluated. Tissues were successfully standardized and the adapted Franz cell allowed to maintain the experimental conditions through the 12h of permeation assay. The permeation of the analyzed drugs has occurred eventually, demonstrating the effectiveness of the barriers. The microneedles pretreatment on tissues increased the permeation flux of the tested drugs, in comparison to control groups. **Article 2**: microneedles of 0.7 mm were coated with the drug Sulforhodamine B, and applied on the porcine buccal mucosa surface, followed by permeation tests (24h). Two types of salivary flow (static and dynamic) were simulated inside the donor chamber and were compared with a moistened gauze as a negative control. We evaluated the onset time to permeation (Lag Time), flux and EF. Either of the simulated salivary flux affected the permeation profile of the model drug, by means of increasing drug permeation, as compared to the negative control, demonstrating the importance of saliva during *in vitro* permeation studies. **General conclusions:** tissues were standardized and allowed to perform permeation assays with adapted Franz cells. The use of microneedles in those barriers was effective to increase the permeation of drugs under the conditions evaluated. The presence of salivary flow has proved to be important to simulate the real conditions of the oral cavity in *in vitro* permeation, which

might have an essential role on permeation dynamics across the tissue. This study represents an advancement to perform *in vitro* permeation assays, aiming at the improvement of topical formulations and absorption enhancers for use in Dentistry.

Key words: Permeation. Microneedles. Oral mucosa. *In vitro* techniques.

SUMÁRIO

1 INTRODUÇÃO

A busca por sistemas que sejam capazes de melhorar a absorção de fármacos topicamente aplicados nos tecidos como pele e mucosas, de forma indolor e de baixo custo é um constante desafio na área da saúde. Para isso, diversos sistemas de liberação e promotores de absorção vêm sedo pesquisados. Em Odontologia, pouco avanço tem sido relatado, e poucas formulações eficazes para aplicação tópica em mucosa oral são comercialmente disponíveis.

Diversas condições que afetam a mucosa oral como mucosite, periodontite, estomatite aftosa recorrente, úlceras, líquen plano, herpes simples, câncer, doenças vesicolo-bolhosas, disfunções salivares e candidíase poderiam ter o tratamento beneficiado através de uma formulação tópica, a qual apresenta uma abordagem atraente (Paderni et al., 2012, Sankar et al., 2011).

Além dessas doenças, a ansiedade gerada pelo medo de sentir dor ainda é uma barreira para o atendimento odontológico (Nuttall et al., 2001). A anestesia local elimina a dor durante os procedimentos em odontologia, no entanto, esta etapa é um dos mais poderosos agentes indutores de estresse e ansiedade (Meechan, 2002). Um anestésico tópico capaz de eliminar a dor durante a punção e a injeção de uma solução anestésica nos tecidos orais seria um grande benefício à Odontologia. As formulações tópicas atualmente disponíveis não garantem uma eficácia de 100%, especialmente em mucosa palatina (Franz-Montan et al., 2012b, Meechan, 2002, Meechan et al., 2005).

De maneira semelhante à pele, a mucosa oral é uma eficiente barreira às camadas profundas, o que limita a penetração de substâncias do meio externo para o meio interno (Squier and Hopps, 1976, Lesch et al., 1989). Apesar da permeabilidade da mucosa oral ser em torno de 4000 vezes maior que à da pele (Galey et al., 1976, Squier and Hall, 1985), o conceito de que a mucosa é altamente permeável é errôneo. Pelo contrário, a mucosa oral representa uma eficiente barreira e a aplicação transmucosal de fármacos ainda é um grande desafio em *drug delivery*. Outras limitações mecânicas na aplicação tópica na cavidade oral consistem na presença de saliva, a qual continuamente lava todas as superfícies da mucosa, deglutição, fala e mastigação (Paderni et al., 2012, Chinna Reddy et al., 2011).

A mucosa oral é revestida por um epitélio escamoso estratificado, chamado de epitélio oral, e uma camada de tecido subjacente, chamada de lâmina própria. Essa é composta por fibras de colágeno (camada de suporte ao tecido conjuntivo), capilares

sanguíneos, terminações nervosas e músculo liso (Wertz and Squier, 1991). As principais funções da mucosa oral são proteção, secreção e sensorial (Squier and Brogden, 2011). A espessura total desta mucosa é estimada ser em torno de 500 a 800 µm, dependendo da região. O epitélio oral, que consiste na primeira barreira entre o ambiente intraoral e os tecidos mais profundos, é composto por células bem aderidas umas às outras, e dispostas em números distintos de camadas - 40 a 50 camadas de células, ligadas ao tecido conjuntivo (Harris and Robinson, 1992, Squier and Brogden, 2011). Esse epitélio pode ser dividido em dois tipos, o queratinizado, cobrindo áreas da mucosa mastigatória como palato duro e gengiva, e o não-queratinizado, cobrindo regiões como bochechas, palato mole e assoalho da boca (Squier and Brogden, 2011).

A mucosa oral de suíno é um modelo bastante utilizado para testes *in vitro*, devido à sua similaridade com a mucosa oral de humanos em termos de organização, composição lipídica, histologia e permeabilidade (Lesch et al., 1989, Wertz and Squier, 1991, de Vries et al., 1991a).

Inúmeros esforços tem sido relatados em busca do desenvolvimento de novas tecnologias para *drug delivery* em aplicação tópica em mucosa oral, como pastilhas, filmes bioadesivos, spray, enxaguatórios bucais, géis, pastas (Hearnden et al., 2012, Paderni et al., 2012). Mais recentemente, microagulhas tem sido demonstradas como um sistema efetivo e indolor em aplicação tópica transdérmica (Gill et al., 2008). E mais recentemente, foi demonstrado ser um sistema promissor em aplicação tópica na mucosa oral (Wang and Wang, 2015, Wang et al., 2015, Ma et al., 2014, Ma et al., 2015).

Microagulhas representam uma nova abordagem na aplicação tópica dermatológica de fármacos, com tamanhos variados, normalmente não passando de alguns milímetros. A literatura relata diversos tipos de sistemas de microagulhas (Bariya et al., 2012). O sistema mais simples é aquele no qual a microagulha é utilizada para perfurar as estruturas superficiais do tecido previamente à aplicação tópica de formulações. Essas microagulhas são normalmente usadas em dermatologia para romper a barreira do estrato córneo, camada que dificulta a penetração de fármacos (Qiu et al., 2008, Li et al., 2010, Coulman et al., 2009, Badran et al., 2009, Duan et al., 2011). Nesse sentido, essas microagulhas já foram testadas para aplicação de vacinas contra o vírus H_1N_1 (Kim et al., 2010), difteria (Ding et al., 2011), anestésicos locais (Li et al., 2010, Zhang et al., 2012), insulina (Martanto et al., 2004) e naltroxeno (Banks et al., 2011).

Microagulhas revestidas com fármacos, representam uma abordagem interessante, uma vez que o fármaco do revestimento fica retido no tecido após sua aplicação (Ma and Gill, 2014, Ma et al., 2014, Ma et al., 2015). No entanto, uma limitação desse sistema é a quantidade máxima de fármaco que pode ser colocada no revestimento, não ultrapassando 1 mg. Esse sistema já está comercialmente disponível e aprovado para uso nos Estados Unidos (Solid Microneedles System®, 3M Drug Delivery Systems®), sendo composto de 300-1500 microagulhas sólidas medindo 250 a 700 µm, revestidas com o fármaco como proteínas altamente potentes e vacinas.

Ainda, outra abordagem relatada é o uso de microagulhas ocas, contendo o fármaco em seu interior, permitindo a aplicação total de até 1,5 mL de formulação na forma líquida (Gupta et al., 2012, Gupta et al., 2011b). Este sistema também já está disponível comercialmente nos Estados Unidos (Hollow Microneedles System®, 3M Drug Delivery Systems®). O sistema consiste em um arranjo de 18 microagulhas ocas por cm^2, cada uma medindo 900 µm.

Outro sistema mais moderno utiliza microagulhas feitas de polímeros e polissacarídeos contendo o fármaco em sua estrutura (Wang et al., 2015, Caffarel-Salvador et al., 2015, Lu et al., 2015). Essas microagulhas ficam retidas no local após a aplicação, degradando-se e liberando o fármaco no interior do sítio de aplicação.

Existem vários sistemas de microagulhas já disponíveis no mercado mundial, principalmente nos Estados Unidos (Bariya et al., 2012). No Brasil, atualmente, existem três marcas de microagulhas todas do tipo *roller* registradas na ANVISA (Agência Nacional de Vigilância Sanitária): microagulhas Dermaroller® (Dermaroller®, Dermaroller Deutchland S.A.R.L. Alemanha), alvo de um dos artigos da presente tese; Ogival (W.T.F. Trovo Importação & Exportação – EPP, BRASIL; e Dr. Roller (Moohan Enterpreise CO., LTD., Coréia do Sul).

O Dermaroller® (Figura 1) apresenta um sistema de rolagem, no qual as microagulhas ficam inseridas, podendo estar dispostas em 4 ou 8 fileiras. O dispositivo composto de 4 fileiras é comercializado com microagulhas nos tamanhos de 0,5; 1; 1,5; 2,0 ou 2,5 mm. Já o dispositivo com 8 fileiras, está disponível nos tamanhos de 0,2; 0,5; 1; 1,5; 2,0 ou 2,5 mm (ANVISA, 2015). No presente trabalho, os dispositivos com 8 fileiras com microagulhas de 0,2; 0,5 e 1 mm foram utilizados.

Figura 1. Imagem de um aparelho Dermaroller® com microagulhas no tamanho de 1mm (Aumento de 1x).

Apesar desse sistema comercialmente disponível já ter sido avaliado extensamente para tratamentos estéticos na área de dermatologia, até o momento ainda não foi avaliada a aplicabilidade desse sistema em mucosa oral.

Estudos de permeação são fundamentais na fase pré-clínica de desenvolvimento de medicamentos. Estes ensaios permitem a previsão do comportamento desta formulação, frente à barreira utilizada, i.e., pele ou mucosa. Esses estudos são principalmente realizados em células de difusão vertical (Chinna Reddy et al., 2011, Nair et al., 2013, Squier, 1991), sendo a célula descrita por Franz na década de 70 (Franz, 1975) o modelo mais utilizado até os dias de hoje.

Quando uma formulação está sendo avaliada para aplicação tópica em mucosa oral, o epitélio de mucosa bucal ou de esôfago são os modelos de barreira não queratinizada mais utilizados (Diaz Del Consuelo et al., 2005, Diaz-Del Consuelo et al., 2005, Kulkarni et al., 2009, Kulkarni et al., 2010, Kulkarni et al., 2011). Uma das maiores vantagens da utilização dessas barreiras é a similaridade com a mucosa oral de humanos em termos de organização, composição lipídica, histologia e permeabilidade (Lesch et al., 1989, Wertz and Squier, 1991, de Vries et al., 1991a). No entanto, quando o alvo do fármaco é abaixo do osso, como por exemplo o tecido pulpar, este modelo de barreira não representa uma condição real e não pode ser considerado um método viável para avaliação.

Nesse contexto, os objetivos do presente estudo foram padronizar o preparo de barreiras de tecidos espessos da cavidade oral de suínos (mucosa palatina com e sem osso); adaptar células de difusão e condições experimentais para realização de estudos de permeação

in vitro com essas barreiras; e avaliar a eficiência e aplicabilidade de microagulhas revestidas ou não como promotores de absorção *in vitro*, na metodologia desenvolvida.

Para atingir esses objetivos, a presente tese será apresentada no formato alternativo*, e será composta de 2 artigos científicos, que se encontram em fase de submissão para revistas científicas.

Para uma melhor compreensão desta tese, a Figura 2 ilustra um fluxograma com o resumo dos artigos que serão apresentados.

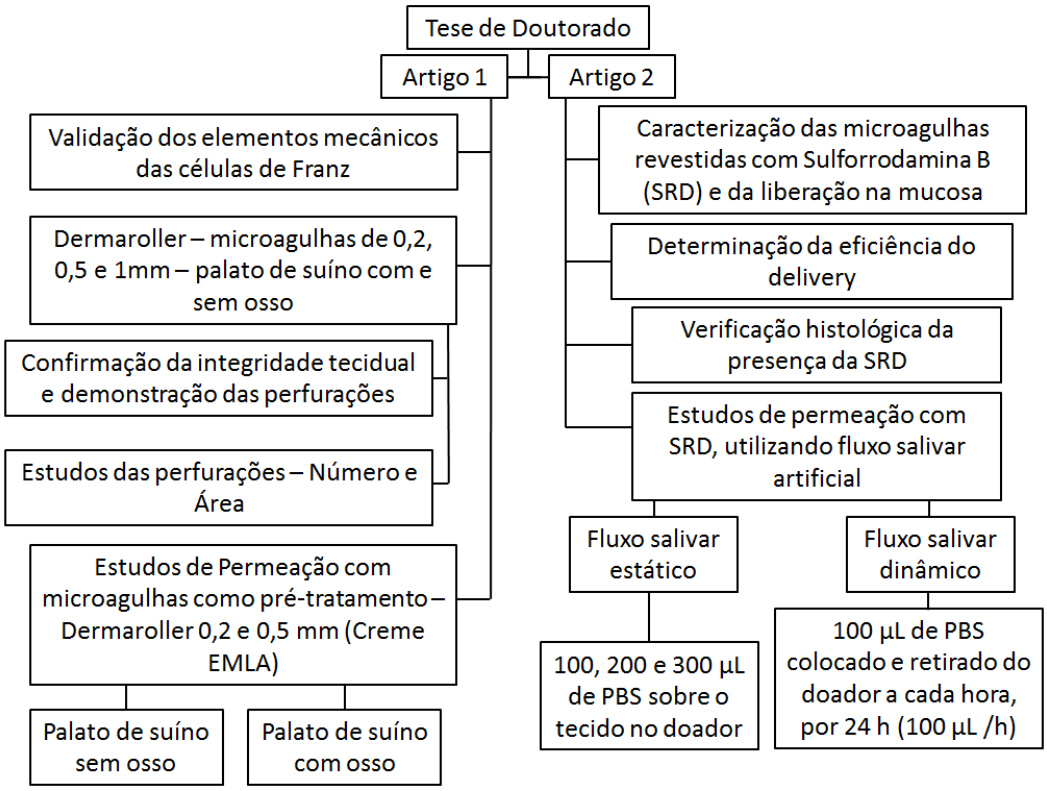

Figura 2 – Fluxograma com o resumo dos artigos que compõe a tese de doutorado.

* De acordo com as normas estabelecidas pela deliberação 001/2015 da Comissão Central de Pós-Graduação da Universidade Estadual de Campinas.

2 ARTIGOS

2.1 Artigo 1: Evaluation of full thickness oral mucosa barrier models for drug permeation studies.

Artigo será submetido ao periódico *European Journal of Pharmaceutical Sciences*

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ABSTRACT

Permeation studies are essential during formulation development. There is a lack in the literature of permeation methods description using oral tissues that represents a challenging barrier to permeation. Therefore the objective of this study was to present a new model of permeation using a full thickness porcine palate mucosa with and without the subjacent cortical bone. In addition, ideal experimental conditions to perform *in vitro* permeation studies in adapted Franz-type vertical diffusion cells are presented. Pieces of fresh porcine palatal mucosa with and without bone were cut in circular shape by using surgical instruments and high-speed air turbine drills. Adapted Franz cells were validated in terms of cells dimension and volume, sealing ability, stirring and dissolution efficiency, temperature control during permeation experiment, and confirmation of uniaxional flux. In order to test the effectiveness of barriers and to evaluate permeation conditions, commercially available handheld devices containing microneedles (0.2, 0.5 or 1.0 mm in length) were applied on palatal mucosa prior to the permeation studies, acting as permeation enhancers. Imaging studies (optical and fluorescence microscopy) of palatal mucosa were performed to verify tissue integrity after microneedles use and to characterize the micro perforations created (number and area). *In vitro* permeation studies were conducted across palatal barriers with lidocaine and prilocaine (lidocaine hydrochloride solution, and a eutectic mixture of lidocaine and prilocaine). The barriers were standardized and permeation studies were successfully conducted. The proposed method was able to maintain the experimental conditions throughout 12 h of permeation. The microneedles effectively created micro pores in the palatal mucosa. As expected, the *in vitro* permeation assays demonstrated that the palatal mucosa with bone was more effective as a barrier. Microneedles were able to enhance the permeation in the *in vitro* assays when palatal mucosa without bone was used as a barrier. The present study represents a step forward in methods to perform *in vitro* permeation studies

aiming to evaluate new topical formulations and permeation enhancers focusing on deep tissues of oral cavity.

Keywords: Oral mucosa, drug delivery, Permeability, Diffusion

1. INTRODUCTION

The oral cavity is an attractive site for topical formulations aiming systemic or local delivery. However, due to the complexity and reduced permeability of oral mucosa, few formulations are available for this purpose (Paderni et al., 2012, Sankar et al., 2011, Hearnden et al., 2012). Furthermore, some of the commercially available topical formulations designed for oral mucosa such as topical anesthetics, does not guarantee effectiveness, especially considering the palatal mucosa (Franz-Montan et al., 2012b, Meechan, 2002, Meechan et al., 2005).

Besides local or systemic effect, a great challenge in topical drug delivery is to achieve deep tissues of oral cavity, such as the dental pulp.

Several efforts enrolling different drug delivery technologies, such as tablets, wafer/film, spray, mouthwash, gel, pastes, and different mucoadhesive dosage forms were developed in order to achieve success in transbuccal delivery, especially to treat oral diseases (Hearnden et al., 2012, Paderni et al., 2012). More recently, the microneedles, known as a minimal invasive successful transdermal drug delivery device, provided an efficient tool to achieve oral mucosa vaccination (Wang and Wang, 2015, Wang et al., 2015, Ma et al., 2014).

One of the most stablished *in vitro* method to evaluate the feasibility and to determine the best formulation composition during pre-clinical stage of novel designed topical formulations or devices is known as buccal absorption or permeation studies performed mainly in vertical diffusion cells (Chinna Reddy et al., 2011, Nair et al., 2013, Squier, 1991). Concerning the mucosal barrier, isolated pig buccal or esophageal epithelia are the most widely used as non-keratinized models (Diaz Del Consuelo et al., 2005, Diaz-Del Consuelo et al., 2005, Kulkarni et al., 2009, Kulkarni et al., 2010, Kulkarni et al., 2011). One of the best advantages of those tissues is the similarity to human mucosa in terms of histological organization, permeability, and lipid composition (Lesch et al., 1989, Wertz and Squier, 1991,

de Vries et al., 1991a). Nevertheless, when the target site is located under the bone, i.e. the dental pulp, these barriers does not represent the real condition. Therefore, it could not be a reliable method to predict the effectiveness of topical formulations designed for deep tissues.

In this context, the objective of the present study was to propose a new barrier model using porcine palatal mucosa with or without bone. In addition, it aimed to adapt the Franz-type vertical diffusion cells and observed the experimental conditions necessary for the new barriers during drug permeation studies. Additionally, the proposed method was used to test the *in vitro* efficiency and feasibility of microneedles as a physical permeation enhancer at oral mucosa.

2. MATERIALS AND METHODS

2.1. Materials

Lidocaine and prilocaine hydrochloride, calcein, ammonium hydroxide, and phosphoric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Methylene blue was obtained from Labsynth (Diadema, SP - Brazil), and acetonitrile and ethanol from J.T. Baker (Center Valley, PA, U.S.A). Conventional silicone glue was used (Cascola®, Henkel Ltda., Itapevi, SP, Brazil) was used. The commercial topical formulation used was a eutectic mixture of 2.5% lidocaine and 2.5% prilocaine (EMLA®, Astra-Zeneca, Cotia, Brazil). Aqueous solutions were prepared using ultrapure water obtained from a Direct-Q® Water Purification System (Millipore Corporation, Billerica, MA, USA).

2.2. Adapted Franz Type Vertical Diffusion Cells, Clamps and Experimental Procedure

The complete apparatus of the adapted Franz-type vertical diffusion cell is represented in Fig. 1. It was based on the model first described by Franz (Franz, 1975). The vertical diffusion cells were composed of a donor and a receptor chamber maintained together by a specially developed metallic joint metal clamps (3 mm-thick). The receiving compartment contained one sampling port opened to allow manual sample collection from the receptor solution. The diffusion permeation area was around 0.78 cm^2 with an acceptor compartment volume of 4 mL.

To ensure a complete sealing of the system, conventional silicone glue was applied on the entire contact surface of the receptor compartment 24-h before experiment starts, in order to achieve its complete polymerization. Following that, the silicone surface was cut and adjusted, to allow a better contact with mucosa and bone tissues. In addition, the region was involved with a plastic paraffin film (Parafilm®, Laboratory Film. Bemis®. Neenah, WI, USA) before positioning the clamps.

Following the sealing procedure, the acceptor chambers were filled with degassed phosphate buffer saline solution (PBS containing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, pH 7.4.) maintained under constant magnetic stirring (1080 rpm) (IKA® do Brasil. Model RO 10PS32, Campinas, SP, Brazil). As the cells were out of the temperature control water jacket, they were placed into an outer bath receipt at 37 °C.

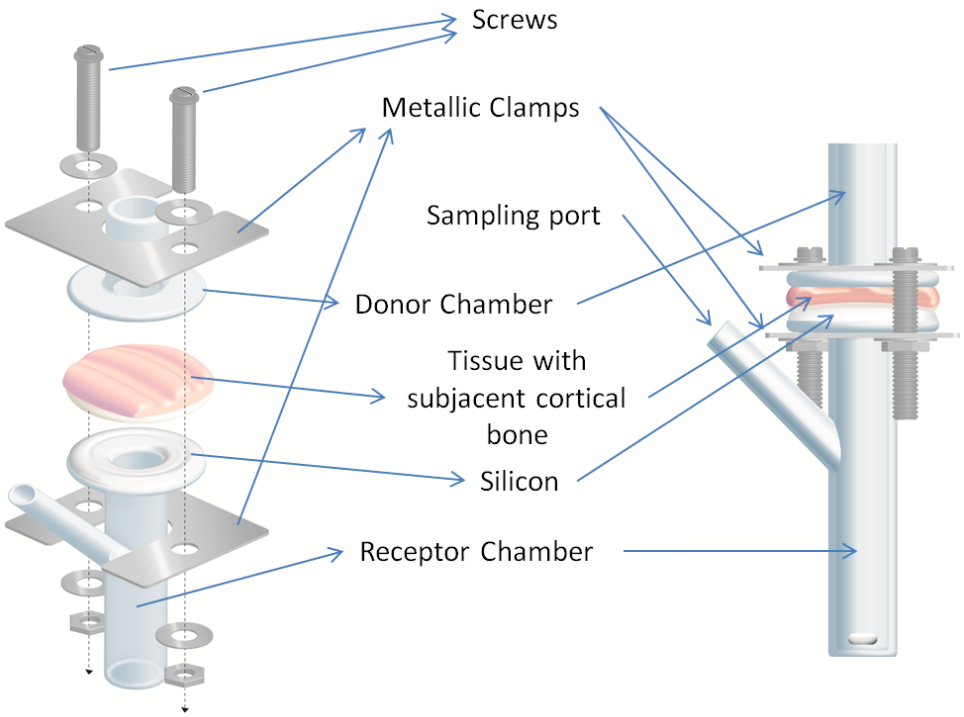


Fig. 1. Diagrammatic representation of the complete apparatus of the adapted Franz-type vertical diffusion cell used in this study.

2.3. Preparation of Porcine Oral Mucosa

Palate tissues from pigs were selected due to the easy removal of samples containing soft tissues and a thin layer of cortical bone, and the reproducibility of the model. Fresh porcine maxillae (from 5 months-old pig, weighing around 75 – 80 kg) were obtained immediately after the animal slaughter in a local slaughterhouse (Frigorífico Angelelli Ltda, Piracicaba, SP, Brazil). The maxillae were transported in ice-cold PBS buffer within 30 min.

The palatal mucosa site used in the present study is schematized in Fig. 2. The posterior region was used due to reduced palatine roughness. Mucosa samples with and without the subjacent bone were collected.

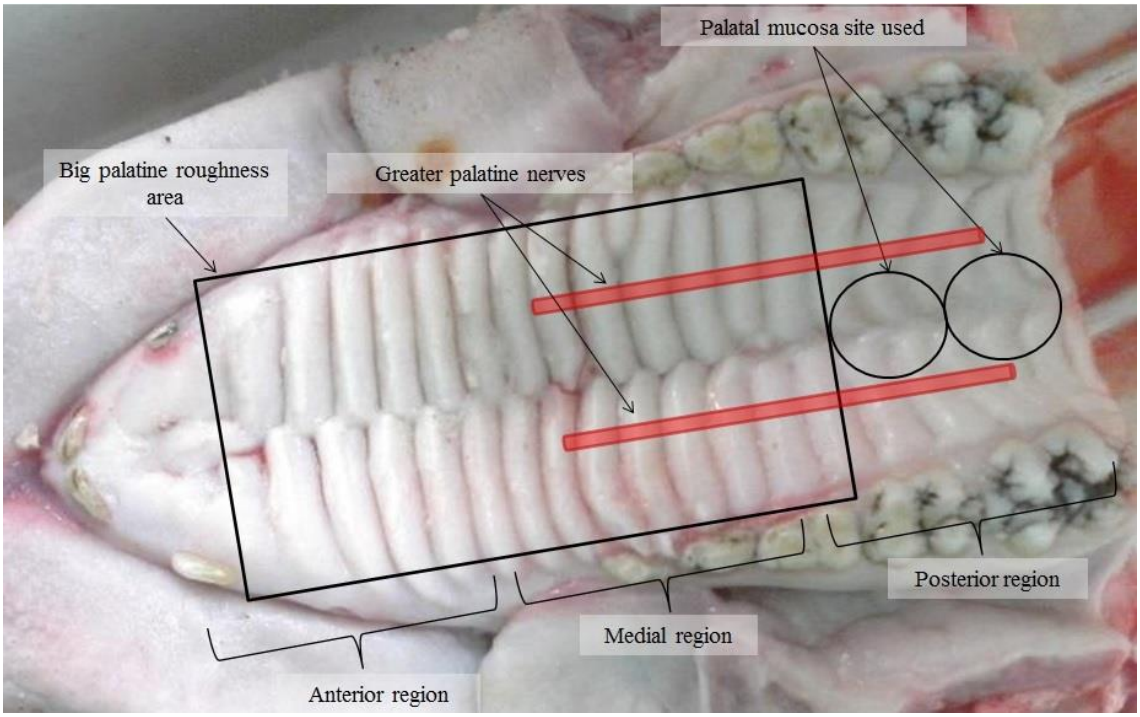


Fig. 2. Porcine maxilla and illustrative schematization of the conditions adopted to select the palatal mucosa site used in the study.

The tissues with or without bone were cut in circular shape by using a scalpel (Fig. 3A). Palatal mucosa without bone was separated from the underlining tissue and rinsed with saline. For the barrier with bone, a high-speed air turbine drill (Fig. 3B) with abundant water flow was used to remove the barrier from the surrounding bone. This separation was carefully performed in order to avoid the separation of the mucosa from the underlining bone. Following its separation, the excess of superficial bone was removed (Fig. 3C) to allow a homogenous surface and rinsed with saline. For both tissues, the epithelium was preserved and gently handled. Mucosa with any visual surface damage was discarded.

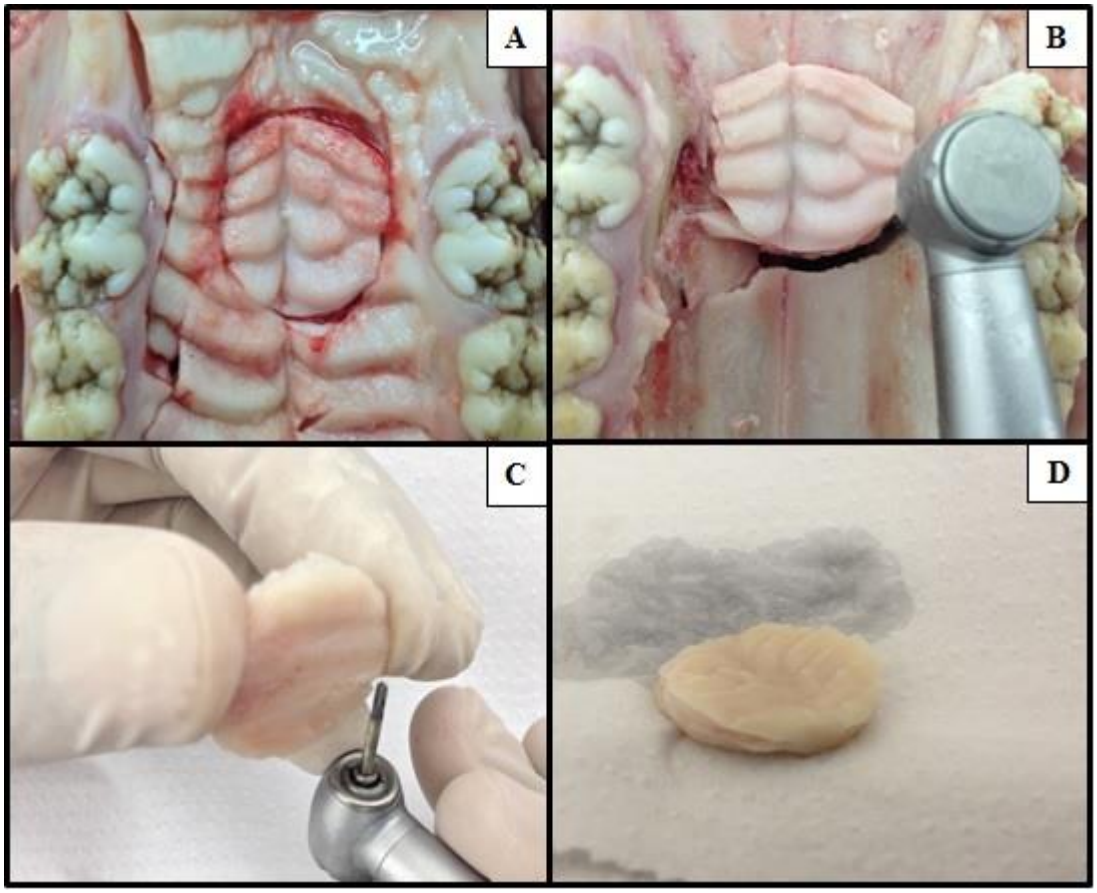


Fig. 3. Sequence of procedures performed to separate tissues of porcine palatal mucosa with or without bone. (A) The mucosa was cut in circular shape using scapel. (B) Removal of tissue from surrounding bone by high-speed air turbine drill. (C) Homogenization of bone surface. (D) Final view of palatal mucosa with bone.

2.4. Validation of the Mechanical Elements of the Diffusion Cell

Dimension of Permeation Area and Volume of the Receptor Compartment

The dimension of the permeation area of all cells was evaluated by a digital caliper (King Tools, São Paulo, SP, Brazil). The total volume of the receptor compartment of each cell was evaluated by mass weight. All cells were weighted empty and when full of distilled and deionized water. Assuming that the density of water is 1 g/mL, the total volume of each cell was the difference between empty and full cells (Gratieri et al., 2010) (n=11).

Stirring Efficiency

The receptor chamber was filled with buffer solution (phosphate buffer saline – PBS - diluted 1 time) following to the addition of a magnetic stir bar (0.5 cm) set at 1080 rpm. 50 µL of 1% methylene blue solution (w/v) was dropped into the receptor compartment and the time necessary to completely dissolve the dye was assessed visually (Gratieri et al., 2010) (n=10).

Temperature Control in the Different Chambers

In order to verify the ability of the water bath to maintain a controlled temperature throughout the experiment, the cells were mounted with a plastic paraffin film (Parafilm®) between the compartments, which were filled with PBS buffer. The temperature was measured every 5 minutes in both chambers, during 50 minutes, by using a digital thermometer (Einstich Thermometer®, Testo GmbH & Co Lenzkirchen/Schwarzwald, Germany) (Gratieri et al., 2010) (n=10).

Confirmation of Uniaxional Flux

To ensure that the permeation was occurring only in the vertical direction (donor to receiver chamber), but not horizontally through the tissue, 1 mL of 1% (w/v) methylene blue solution was used in the donor compartment of the mounted cells containing the mucosa ("with" and "without" bone) samples, prepared according to the procedure described below. The cells were kept under magnetic stirring (1080 rpm) and after 24h, the cells were disassembled and the mucosa surface areas were examined for staining with methylene blue (n=10/group).

2.5. Confirmation of Tissue Integrity and Demonstration of Microneedles Perforations

The samples with and without bone were analyzed histologically to confirm tissue integrity after preparation process. In addition, the barriers were submitted to topical application of a commercially available handheld device containing microneedles

(Dermaroller®, Deutschland, S.A.R.L., Germany) for 4 times, according to the scheme observed in Fig 4A. Histological images were obtained in order to confirm micro perforations created. Figures 4B, C, D and E show details of microneedles with 0.2, 0.5 and 1 mm in length (Dermaroller®) used in the present study.

Following preparation process and microneedles application, pieces of mucosa were fixed with 10% buffer formaldehyde solution. The samples with bone were decalcified in a solution of trichloroacetic acid (10%). The samples were dehydrated in successive ethanol series (50%, 70%, and 100%), diaphanized, and embedded in paraffin. Histological sections (5 μm) were obtained with a microtome Lupetec MRPO3 (Lupetec Ltd, São Carlos, SP, Brazil), and stained with hematoxylin and eosin. Histological sections were analyzed in an optical microscope (Model DMLP, Leica Microsystems GmbH, Wetzlar, Germany) coupled to a digital camera (Leica MPS 60) connected to an image processing software (Optika View, Optika®, Ponteranica, BG, Italy).

It was obtained two tissue samples of each palatal mucosa from at least three different animals for either “with” and “without” bone barriers (n=6/group). At least five slides were prepared and examined for each tissue sample.

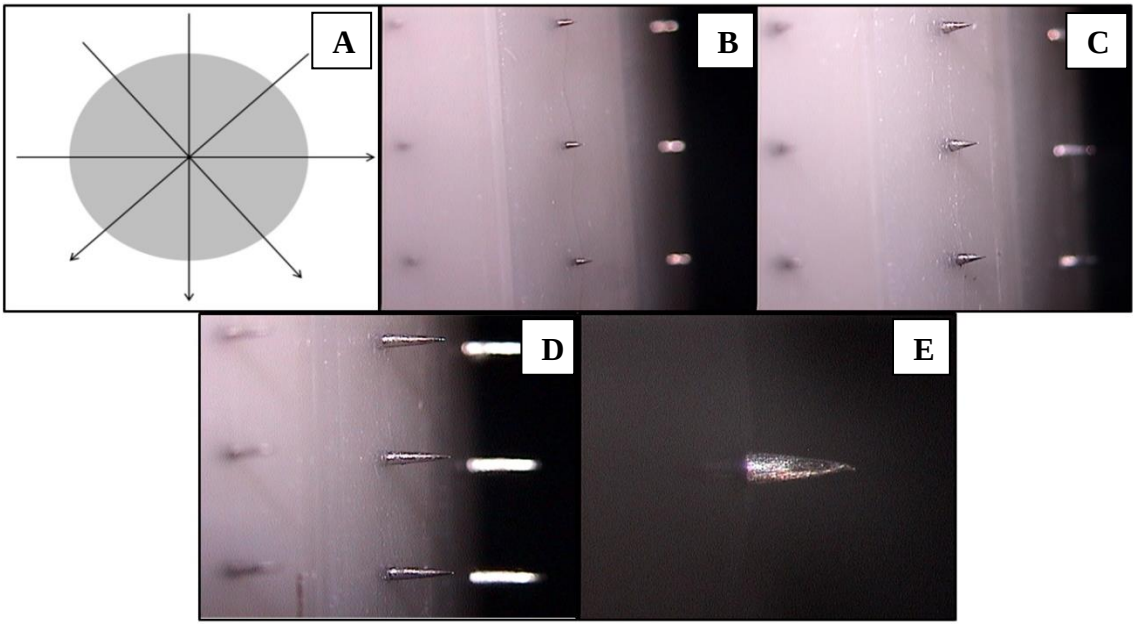


Fig. 4. (A) Application method of the commercially available handheld devices containing microneedles used in the present study (Dermaroller®) (adapted from (Badran et al., 2009). Details of microneedles with 0.2 mm (B), 0.5 mm (C) and 1 mm (D) length – mag. 0.8x. Single microneedle measuring 0.2 mm (E) – mag. 5x.

2.6. Analysis of Microchannels Created by Microneedles

Two procedures were performed to confirm the creation of microchannels by the microneedles. In the first test, methylene blue staining was used to check for creation of microchannels (Kalluri et al., 2011). The number of perforations created by the microneedles in three different lengths (0.2, 0.5 and 1 mm) was quantified in 1 cm^2 after the application of the device for 1, 3 and 5 times, in triplicates. Following microneedle application, the pieces of palatal mucosa were immersed into a 1% (w/v) methylene blue solution for 1 min, removed and cleaned with swabs moistened in alcohol. The samples were analyzed in a stereo microscope (Optech, Thame, Oxfordshire OX9, UK) coupled to a digital camera (Leica MPS 60), connected to an image processing software (Optika View). One sample from palatal

mucosa from at least three different animals for each microneedle length (n=5/microneedle length) was evaluated.

In a second assay, we analyzed how the permeation procedures could affect the perforation area made by microneedles. In addition, we evaluated if the presence of bone attached to the mucosa could influence the microchannel area. The adapted Franz cells were mounted with palatal mucosa barriers ("with" and "without" bone) between donor and receiver compartment right after microneedles application (0.2, 0.5 and 1 mm), as described in Fig 4A. PBS buffer was used as receptor medium under magnetic stirring (1080 rpm) and 37 ºC. After 12 h, the cells were dismounted, and mucosa samples were immersed in 0.35% calcein solution (w/v) during 1 min, and gently cleaned with swabs embedded with alcohol (Kalluri et al., 2011).

Fresh palatal mucosa barriers were submitted to microneedle application and were immediately analyzed (0 h) to compare perforations before (0 h) and after (12 h) permeation experiment. Samples were analyzed in a fluorescence microscope (Zeiss Axiovert 40 CFL coupled to a camera AXIO CAM MRC and a fluorescent lamp HBO 50 connected to a software Zen Pro 2011, Zeiss, Oberkochen, Germany). The perforation area was quantified in μm^2 at the magnification of 20×. Three samples (from three different animals) were tested per group and a minimum of 9 perforations were analyzed per sample (n=27/group).

2.7. Permeation experiments

One first set of permeation assays was performed with 5%lidocaine hydrochloride solution (w/v - prepared in distilled and deionized water) as a model hydrophilic drug (Franz-Montan et al., 2016), in order to test the efficiency of those barriers and permeation conditions described. For this set, the commercially available handheld device containing microneedles

with 1.0 mm in length (Dermaroller®) was applied on the palatal mucosa ("with" and "without" bone) as shown in Fig. 4A prior to the permeation assays.

Permeation of lidocaine hydrochloride across the two different palatal barriers was carried out for 12 hours in the adapted Franz-type diffusion cells described above. The acceptor chambers were filled with degassed PBS solution (pH = 7.4) maintained under constant magnetic stirring (1080 rpm) at 37 °C. Before the experiment was started, degassed buffer was placed in the donor compartments and the assembled cells were allowed to equilibrate for 60 minutes in a water bath.

Following the equilibration period, the buffer in the donor compartment was substituted by 1 mL of the lidocaine hydrochloride solution occluded with Parafilm® (83.3 mg/cm^2 of lidocaine hydrochloride). Sink conditions were maintained during the permeation assays, as described elsewhere by our research group (Franz-Montan et al., 2016). Samples of 300 µL were periodically collected from the acceptor compartment and analyzed by HPLC. The volume was replaced with the same amount of fresh buffer, taking account of dilution effects.

In a second set of permeation assays, the *in vitro* ability of microneedles to act as a permeation enhancer at the palatal mucosa was evaluated with the barriers and conditions described. A commercial topical formulation composed of lidocaine and prilocaine base (EMLA®) was used. The choice of this formulation was based on its increased *in vivo* efficacy as a topical anesthetic at the palatal mucosa (Svensson and Petersen, 1992, Al-Melh and Andersson, 2007, Primosch and Rolland-Asensi, 2001, Franz-Montan et al., 2012a, Franz-Montan et al., 2015). The association of EMLA cream and microneedles at the oral cavity could increase anesthetic penetration, improving the anesthesia effectiveness.

The *in vitro* permeation experiments with EMLA were performed across the two different palatal mucosa barriers and conditions already described. For this set of experiments,

the commercially available handheld device containing microneedles with 0.2 and 0.5 mm in length (Dermaroller®) were used. The choice of microneedles size was based in their lower probability to promote pain in *in vivo* condition. Due to their smaller length, it is more unlikely to reach the free nerve endings located at the *lamina propria*, right below the epithelium, which presents thickness between 250-600 µm depending on the oral cavity site (Squier and Brogden, 2011). In addition, we observed no difference in the number of perforations with longer microneedles, as discussed latter.

The experiment was conducted at the same way, with minor modifications. Following the equilibration period, the buffer solution in the donor compartment was substituted by 300 mg of EMLA cream occlusively (12.5 mg/cm^2 of lidocaine and 12.5 mg/cm^2 of prilocaine). The acceptor chamber was filled with degassed PBS buffer + 30% ethanol to ensure sink conditions. The solubility of lidocaine and prilocaine (18.8 ± 0.11 and 21.89 ± 0.03 mg/mL, respectively) was calculated by saturation of the local anesthetics in this medium, prior to undertaking the permeation assays.

In both set of experiments, a graphic was obtained with the cumulative amount of lidocaine hydrochloride or lidocaine and prilocaine across the two different palatal barriers plotted as a function of permeation time. The slope of the linear portion of the curve provided the steady-state flux (J_{ss}) of across the barrier (in µg.cm^{-2}.h^{-1}).

2.8. Quantitative analysis

Lidocaine and prilocaine analysis was performed by high-performance liquid chromatography (HPLC) (Thermo Electron Surveyor HPLC with a LC Pumps Plus, a UV/VIS detector and automatic injector, San Jose, CA, USA).

Lidocaine and prilocaine were quantified simultaneously in the following chromatographic conditions: a mobile phase composed of a 60:40 (v:v) mixture of

acetonitrile and buffer (25 mM NH_4OH, adjusted to pH 7.0 with H_3PO_4) at a flow rate of 1 mL/min, an injection volume of 20 µL and detection wavelength of 254 nm. For the separation of both local anesthetics, a C18 reverse phase column (Phenomenex, Gemini, 5µ, 150 X 4.60mm) was used. Data collection was performed using Thermo Scientific ChromQuest Software Platform (Thermo Scientific Inc., Pittsburgh, PA).

The specificity of the analytical method was checked to confirm that no component of the pig mucosa and bone would interfere in the local anesthetics quantification. A calibration curve (n=3, analyzed in triplicate, on three consecutive days) was constructed using six different concentrations (between 5 and 200 µg/mL) prepared from a stock solution of lidocaine and prilocaine in mobile phase. Linearity was evaluated by linear regression of the peak area against the concentration of the drug (r^2=0.99998 for lidocaine and prilocaine).

The intraday and between-day precision and accuracy were evaluated by quantification of low (5 µg/mL), medium (50 µg/mL), and high (200 µg/mL) lidocaine and prilocaine concentrations in triplicate on three consecutive days. For lidocaine, the method showed precision (RSD) <4% and accuracy between 97.83% and 102.87% for the intra- and inter-day evaluations, and a limit of detection and quantification of 0.52 and 1.74 µg/mL, respectively. For prilocaine, the precision (RSD) was <3% and accuracy between 95.56% and 102.32% for the intra- and inter-day evaluations, and the limit of detection and quantification were 0.19 and 0.62 µg/mL, respectively.

2.9. Statistical analysis

The number of perforations was compared by one-way analysis of variance (ANOVA) and Tukey- Kramer's (*post-hoc*) tests. The area of perforations and *in vitro* flux of local anesthetics were compared by Kruskall Wallis and Dunn's (*post-hoc*) tests or unpaired

Student's t test. The statistical analysis was performed by using the GraphPad Prism® package (GraphPad Software, Inc. La Jolla, CA, USA), being the significance level set at 5%.

3. RESULTS

3.1. Validation of the Mechanical Elements of the Diffusion Cell

The receptor compartment presented volume (mean±SD) of 4.55 ± 0.28 mL and a permeation area of 0.78 ± 0.10 cm^2, which were uniform among the 11 cells analyzed. The time necessary to achieve complete dissolution of the stain methylene blue in the receptor compartment was 36.6 ± 13.1 s.

In the receptor chamber, the time necessary to reach 37 ºC was less than 5 minutes. However, the temperature at the donor chamber stabilized at 32 ºC after 10 minutes, but it never reached 37 ºC.

Fig. 5 illustrates pieces of palatal mucosa with and without bone after a 24-h period of permeation assay with methylene blue stain solution at the donor compartment. Only the permeation area is stained in blue and the surrounding area was not dyed. The flux was uniaxional in the vertical way in both palatal mucosa (with or without bone), which demonstrates the efficacy of the adapted cells in promoting adequate sealing, with no sign of leakage.

Fig. 5. Images of porcine palatal mucosa after a 24-h permeation experiment obtained with 1% methylene blue solution applied at the donor compartment to illustrate uniaxional flux. Left image palatal mucosa without bone and right, palatal mucosa with bone (n=10/group).

3.2. Confirmation of Tissue Integrity and Demonstration of Microneedles Perforations

Histological analyses was performed for the preparation method for the porcine palatal mucosa with or without bone used in the *in vitro* permeation studies. Fig. 6 shows histological sections of porcine palatal mucosa with (Fig. 6A and 6B) and without bone (Fig. 6C and 6D).

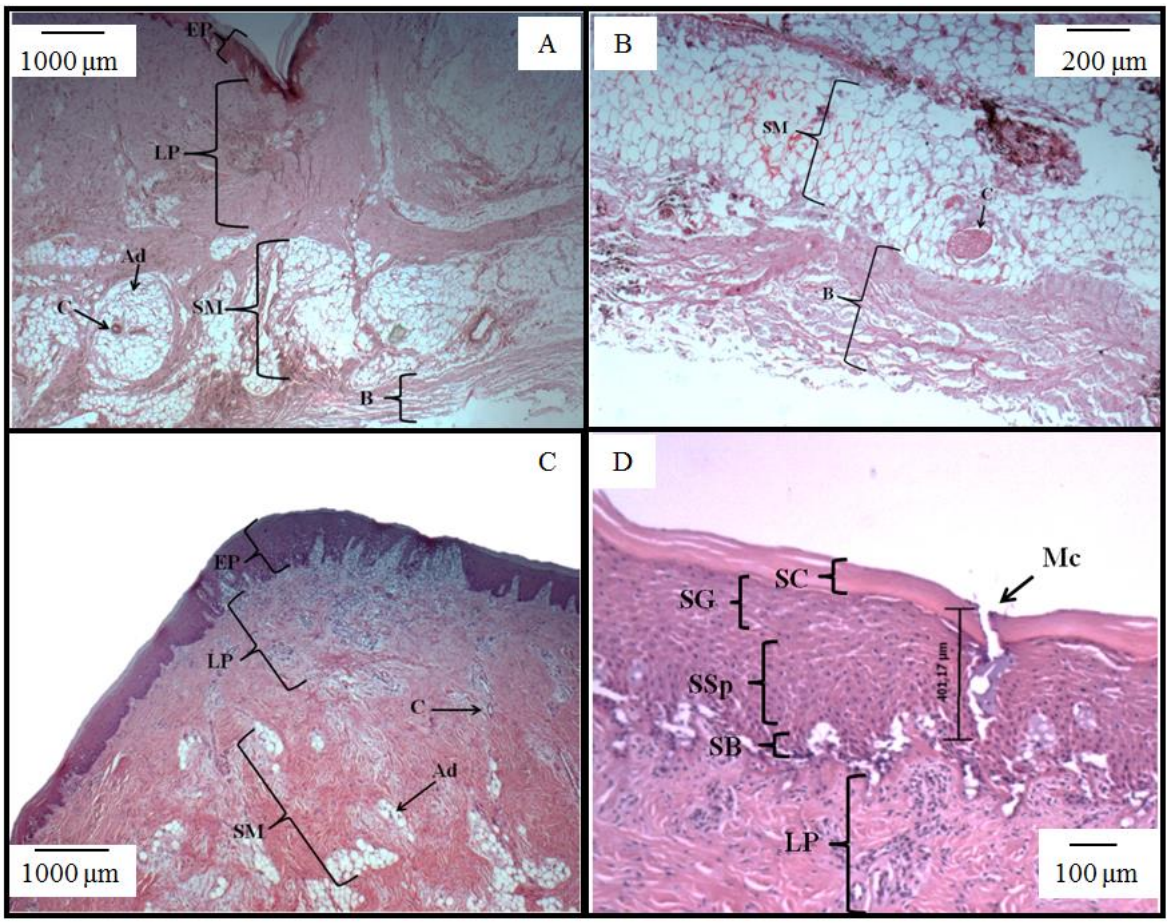


Fig. 6. Histological sections of fresh porcine palatal mucosa with or without bone. (A) palatal mucosa with bone (magnification of 2.5x); (B) close view on the cortical layer (mag. 5x); (C) palatal mucosa without bone (mag. 2.5x); (D) details of a microchannel created by microneedles (0.5 mm length) at the surface of the palatal mucosa (mag. 5x). EP – epithelium; LP – lamina propria; SM – submucosa; B – Bone; Ad – adipocytes; C – capillary; Mc –

microchannel; SC – *stratum corneum;* SG – *stratum granulosum*; SSp – *stratum spinhosum*; SB – *stratum basale.*

The histological images revealed that the palatal mucosa with or without bone were successfully removed with no histological damage. Both palatal mucosa tissues presented intact stratified squamous epithelium with all the expected layers (*stratum basale, spinhosum,* and *granulosum*) with a homogeneous keratinized layer (*stratum corneum*), typical from the masticatory mucosa. The *lamina propria* and submucosa layers are also observed in both tissues, being the first consisting of a dense collagenous tissue, and the second rich in fat. Worthy of note, bone was present, as shown in Fig. 6A and 6B.

Fig. 6D shows details of microchannel creation around 0.4 mm, which corresponds to the length of the microneedle used (0.5 mm). Disruption of *stratum corneum* layer and epithelium by microneedles is clearly demonstrated.

3.3. Analysis of Microchannels Created by Microneedles

Fig. 7 illustrates the microchannels created by the application of microneedles with 0.2 mm (Fig. 7A), 0.5 mm (Fig. 7B), and 1.0 mm (Fig. 7C) rolled 5 times and stained by 1% methylene blue solution. The creation of microchannels by different microneedles length applied on mucosa surface is confirmed, corroborating with the details observed in Fig. 6D.

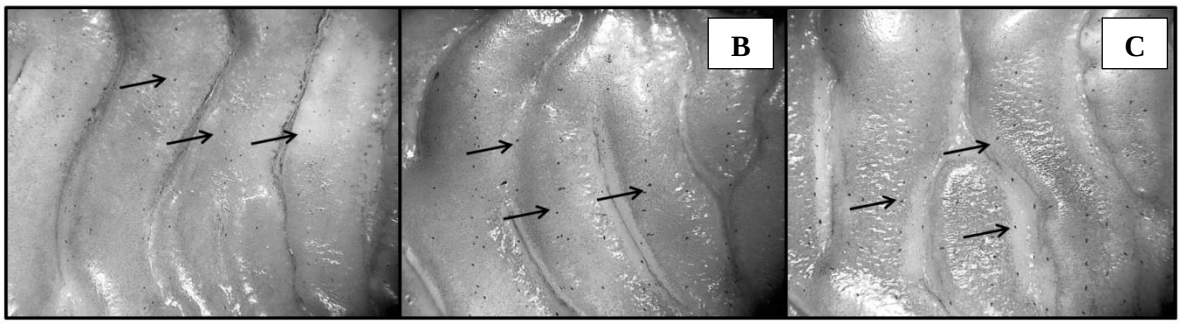


Fig. 7. Top view images of porcine palatal mucosa (no bone) after application of microneedles for 5 times over mucosa surface to illustrate the creation of microchannels. Microneedles lengths of 0.2 mm (A), 0.5 mm (B) and 1 mm (C).

The number of perforations on palatal mucosa surface made by the application of microneedles varying in length is shown in Fig. 8. In general, as expected the number of microchannels per square centimeter increased proportionally to the number of application times. There were no statistically differences among the different microneedles lengths with the same times of application regarding the number of perforations (1 time, p = 0.0723; 3 times, p = 0.6297; and 5 times, p = 0.0916). Therefore, mucosa perforations occur independently of the microneedle length.

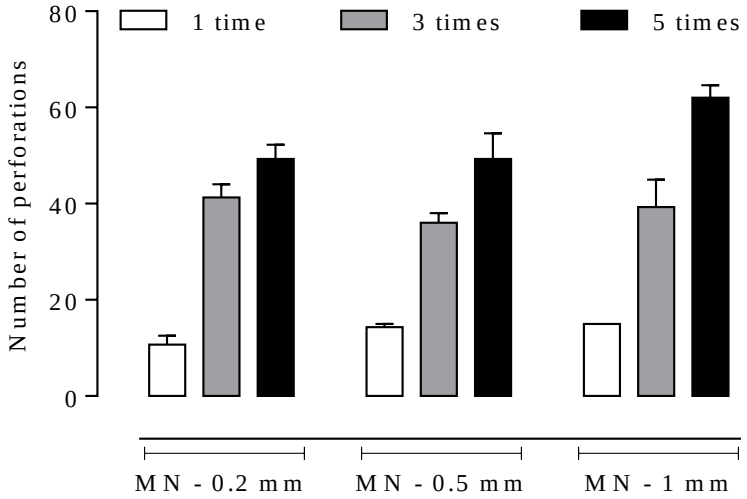


Fig. 8. Mean (±SD) number of perforations made on palatal mucosa without bone by different microneedles lengths as a function of number of passes.

Fig. 9 shows the pattern of perforation created by the microneedles with different length on the surface of porcine palatal mucosa stained by calcein.

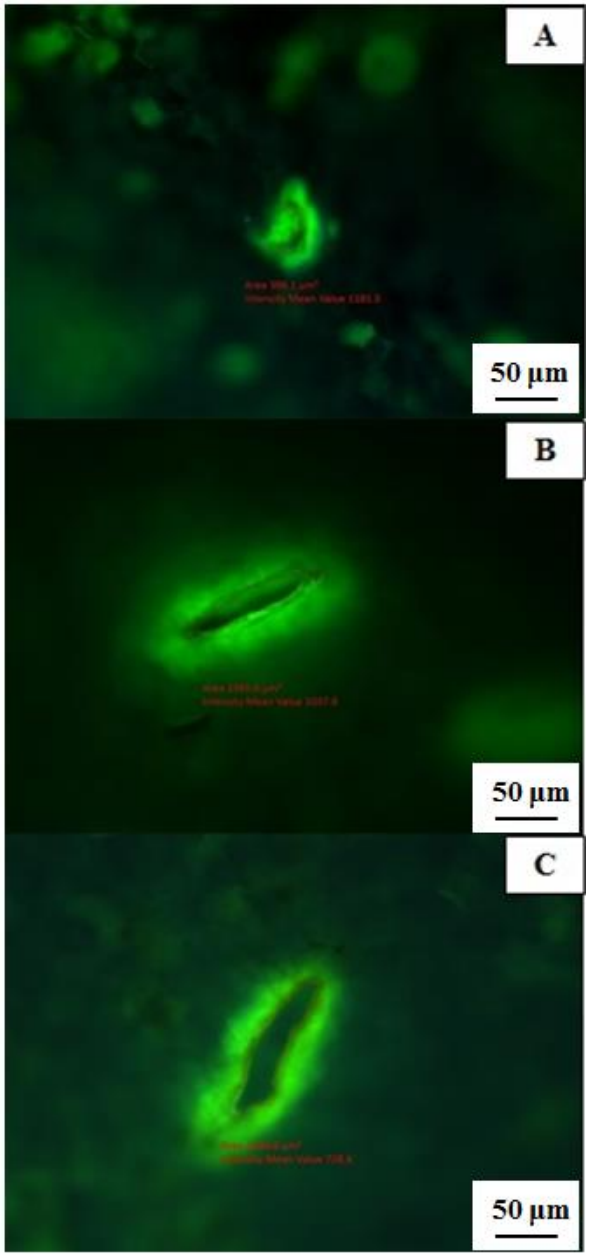


Fig. 9. Top view fluorescent images to illustrate the pattern of perforation created by the microneedles with different length on the surface of porcine palatal mucosa stained by calcein. (A) microneedles with 0.2 mm length (area 386.1 μm^2), (B) 0.5 mm (area 1593.6 μm^2), and (C) 1 mm (area 2609.0 μm^2). Mag. 20x for all figures.

Figure 10 shows the area of perforations created by microneedles treatment by the three lentghs (0.2 mm, 0.5 mm, and 1 mm) on the surface of the palatal mucosa with and without bone were assessed before (0 h) and after (12 h) the permeation.

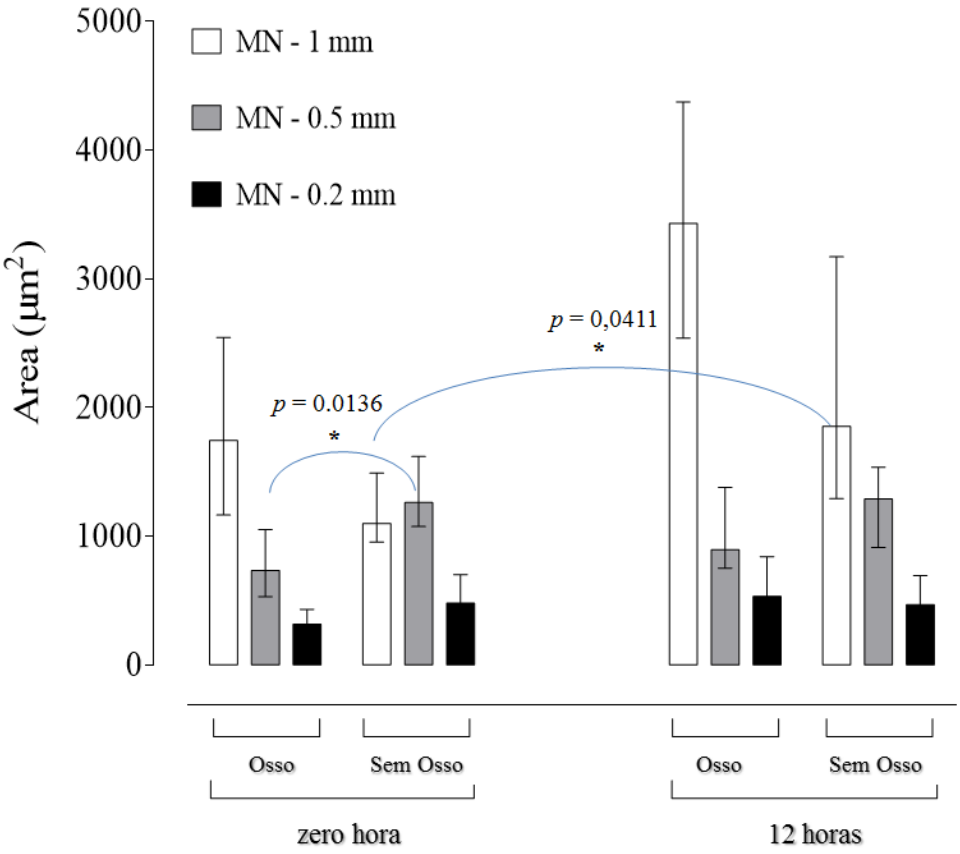


Fig. 10. Mean (±SD) area of perforations created by microneedles treatment in 3 different lentghs (0.2, 0.5, and 1.0 mm) at the surface of the palatal mucosa with and without bone assessed before (0 h) and after (12 h) the permeation experiment.

The presence of bone attached to the mucosa barrier did not affect the microchannel area created by microneedles of 0.2 mm (p = 0.2982) and 1.0 mm (p = 0.0771) on surface of porcine mucosa when comparing the areas prior (0h) to permeation studies. Except for microneedles of 0.5 mm which showed significantly larger areas in the presence of bone (p = 0.0136).

In general the permeation conditions evaluated during 12h did not promote significant alterations at microchannels area ($p < 0.05$), except when microneedle of 1 mm was used on palatal mucosa without bone, which presented a significant larger microchannels area after 12h of permeation assay ($p = 0{,}0411$).

3.4. Permeation studies

The efficacy of the standardized barriers and permeation conditions presented were confirmed, as observed in Fig. 11, which shows the permeation profile of lidocaine hydrochloride across porcine palatal mucosa without bone. The microneedle treated mucosa, slight increased the drug permeation. The steady-state flux of lidocaine hydrochloride was significantly higher in the microneedle treated mucosa, showing its efficacy as a permeation enhancer in the *in vitro* model ($p = 0.0137$) (Fig. 12).

However it was not possible to quantify lidocaine hydrochloride permeated across porcine palatal mucosa with bone as its amount was under limit of detection. The presence of bone increased the efficacy of the barrier, since no drug was permeated during 12 h. Considering this, permeation parameters (*Jss* and Q_{12}) was not obtained, and the linear portion of the permeation profile was not recognized.

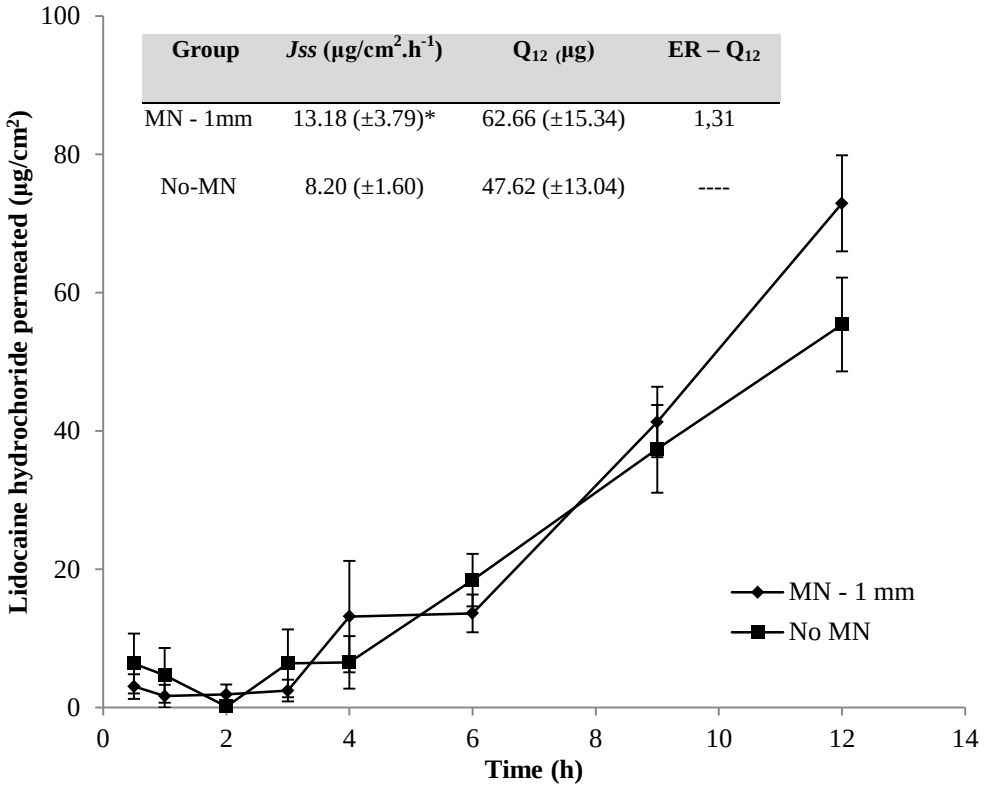
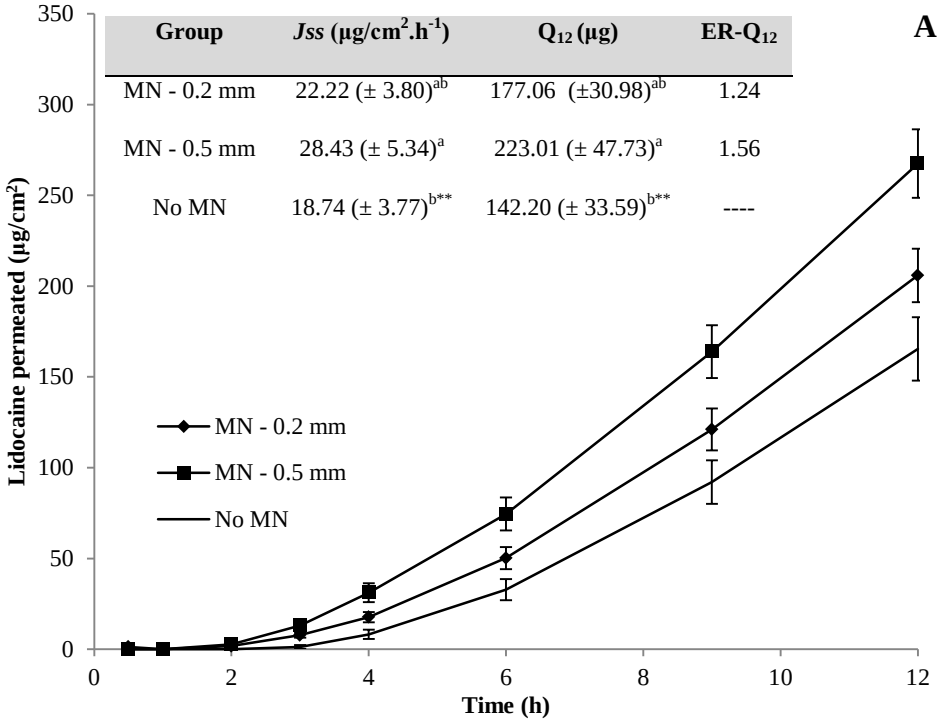
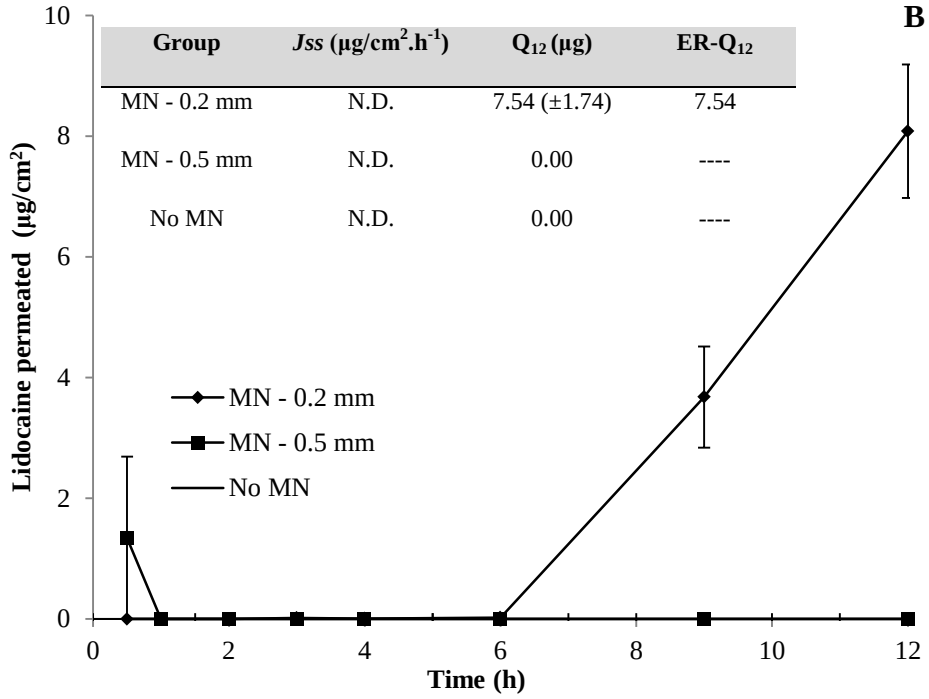


Fig. 11. Permeation profile (mean ±SEM, n = 10-12) and steady state flux (*Jss*) of lidocaine hydrochloride across intact (No MN) or microneedle pre-treated (MN – 1 mm) porcine palatal mucosa without bone, obtained with 5% lidocaine hydrochloride solution under infinite dose condition. Individual table's presents mean (±SD) of the steady state flux (*Jss*). Q_{12} is the total amount of drug permeated in 12 h of experiment. Enhancement ratio (ER) was obtained between the Q_{12} of lidocaine hydrochloride using microneedle treated mucosa in comparison to passive permeation. Unpaired t-test, * $p < 0.05$).

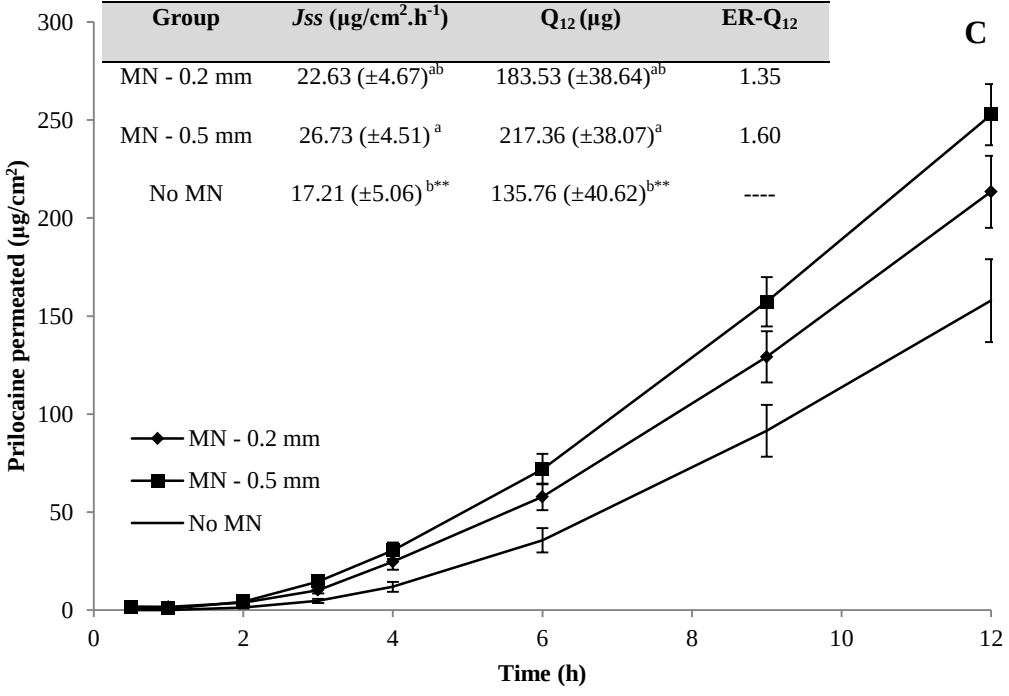
Fig. 12 shows the permeation profile of lidocaine (Fig. 12A, 12B) and prilocaine (Fig. 12C, 12D) from EMLA across palatal mucosa with (Fig. 12B, 12D) or without (Fig. 12A, 12C) bone. The *in vitro* ability of microneedles to enhance the permeation was confirmed only when the palatal mucosa without bone was used. It was observed a slight increasing in the permeation profile and a significant higher steady-flux of both lidocaine (p =

0.0047) and prilocaine (p = 0.0095) across this barrier when 0.5 mm microneedle treated mucosa was compared with the untreated group. However, this difference was not observed when 0.2 mm microneedle was used (p > 0.05). Nevertheless, in accordance to the previous permeation study performed with lidocaine hydrochloride (Fig. 11B), the presence of bone reduced permeation of both drugs. It was not possible to calculate the steady-state flux, since a typical linear interval was not observed (Fig. 12B, 12D).





Group	*Jss* (µg/cm².h⁻¹)	Q_{12} (µg)	ER-Q_{12}
MN - 0.2 mm	N.D.	7.54 (±1.74)	7.54
MN - 0.5 mm	N.D.	0.00	----
No MN	N.D.	0.00	----



Group	*Jss* (µg/cm².h⁻¹)	Q_{12} (µg)	ER-Q_{12}
MN - 0.2 mm	22.63 (±4.67)^{ab}	183.53 (±38.64)^{ab}	1.35
MN - 0.5 mm	26.73 (±4.51)^{a}	217.36 (±38.07)^{a}	1.60
No MN	17.21 (±5.06)^{b**}	135.76 (±40.62)^{b**}	----

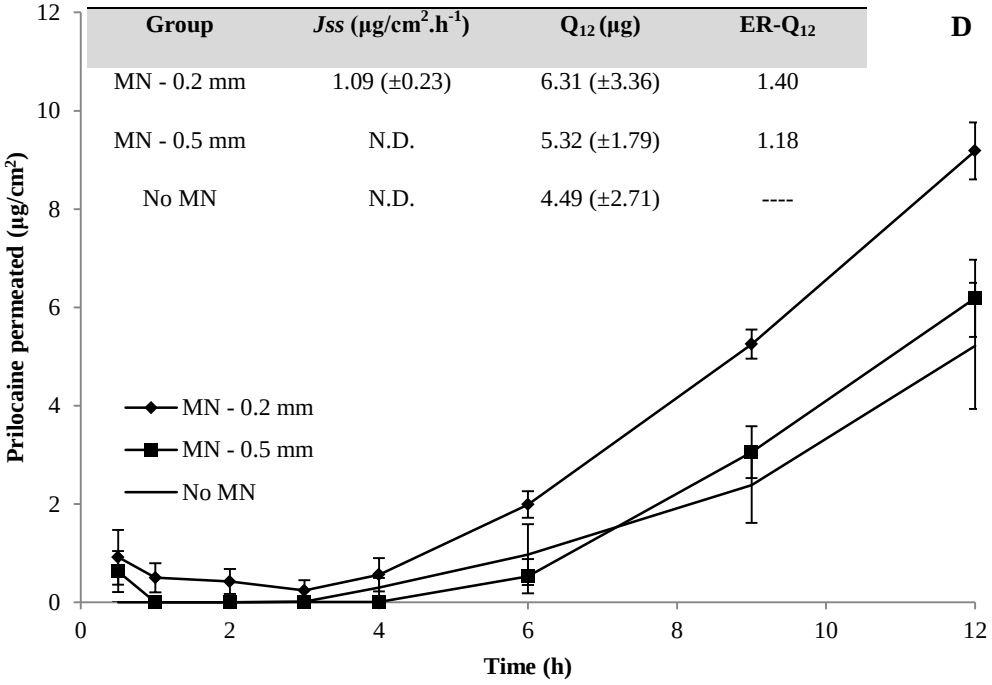


Fig. 12. Permeation profile (mean ± SEM, n = 10-12) and steady state flux (*Jss*) of lidocaine (A, B) and prilocaine (C, D) across intact (No MN) or microneedle pre-treated (MN – 0.2 mm; MN – 0.5 mm) porcine palatal mucosa without (A, C) and with (B, D) bone obtained with EMLA® cream under infinite dose condition. Individual tables' presents mean (±SD) of the steady state flux (*Jss*). Q_{12} is the total amount of drug permeated in 12 h of experiment. Enhancement ratio (ER) was obtained between the Q_{12} of lidocaine or prilocaine using microneedle treated mucosa in comparison to passive permeation. N.D. – not defined, permeated amount under limit of detection. ANOVA/Tukey–Kramer, $p < 0.01$.

4. DISCUSSION

Some adapted models using diffusion cells were previously described in the literature, such as the continuous-flow chambers for the measurement of permeability of small tissue samples with reduced amount of formulation (Squier et al., 1997), and a modified Franz-cell especially designed to evaluate ocular delivery and iontophoresis technique through porcine cornea (Gratieri et al., 2010). In the present study, we described an adapted Franz type vertical diffusion cell to accomplish permeation studies with thick porcine oral mucosa barriers, since the commercial available cells usually limits the thickness of tissues by their clamps.

The most traditional model to evaluate *in vitro* transbuccal permeability of topical drugs is the Franz-type vertical diffusion cells (Chinna Reddy et al., 2011, Nair et al., 2013, Squier, 1991). This cell design was first reported by Franz in the 70's for transdermal studies (Franz, 1975). Even though the diffusion cell model evaluated in the present study is very similar to the Franz's described cell in terms of design, volume and permeation area, we proposed some changes in methodology, which required validation. The diffusion cell evaluated here does not present a water circulation jacket, thus the ability to control the temperature throughout the experiment should be confirmed in both compartments. Our system reached a stable temperature in 5 min, and it was able to maintain it, despite the difference of 5 °C between donor and receiver chamber. Meanwhile, a delay in 15 min to achieve the experimental temperature and only about 1 °C of difference between the chambers was observed (Gratieri et al., 2010). These variations could be attributed to the differences in design and volume of cells, and to the thicker thickness associated to the samples used in this study, that could interfere with the heat exchanges between the chambers. Although in most of the transbuccal permeation studies the temperature is set at 37 °C to resemble the *in vivo* condition, it is worth noticing that the temperature at the surface of

palatal mucosa (~28 °C) is usually lower than the body temperature (~37 °C) (Pallagatti et al., 2012). Thus the reduced temperature found at the donor chamber could better mimetic the *in vivo* condition.

An additional concern was if the metal clamps were able to promote an adequate sealing of the system, due to the irregularity and thickness of the tissue, especially in the presence of bone. The use of especial designed metal clamps and silicon glue were able to prevent leakage between the chambers, as confirmed by the uniaxional flux. Moreover, it was demonstrated that the modified cells proposed here presented uniformity of its components (permeation area and volume) and they were useful, robust and easy to perform permeations assays across the mucosal barriers.

Even though the permeability across the oral mucosa is higher than across skin, there is a misconception that this surface is highly permeable. The high impermeability of oral mucosa is usually attributed to its epithelium. The masticatory mucosa regions (such as palate and gingiva), which are covered by a keratinized and stratified squamous epithelium, are considered to be the least permeable regions (Harris and Robinson, 1992, Lesch et al., 1989). The lower permeability is related to the lipid composition (sphingomyelin, glucosylceramides, ceramides, and other nonpolar lipids organized in a lamellar phase) of its intercellular material derived from the membrane-coating granules (Squier et al., 1991, Squier, 1984, Squier and Hopps, 1976, Squier and Hall, 1984) and also attributed to the basal layer (Alfano et al., 1977, de Vries et al., 1991a). In the present study, microcopy images confirmed the presence of an intact epithelium from all the oral mucosa palatal samples prepared, demonstrating the presence of the main permeation barrier. Moreover, an undamaged connective tissue and bone were also observed (Fig. 7).

Despite most of transbuccal studies are performed only with the porcine esophageal or buccal epithelium barrier, there was a lack of a stricter barrier model to test the

in vitro permeation of topical formulations designed to act across bone structures. The present study was the first attempt to demonstrate preparation methods of porcine palatal mucosa with a consider thickness (~2.5-3.5 mm) and high barrier efficacy to be used during pre-clinical studies with such formulations. A similar study was conducted by Kulkarni and colleagues (Kulkarni et al., 2009), who demonstrated the importance of the connective tissue to the buccal epithelium barrier, as a non-keratinized model.

In the present study, a masticatory mucosa with the presence of connective tissue and bone was presented as a keratinized and relatively impermeable model. The presence of a thick connective tissue with or without bone conferred a more efficient barrier to permeation. This was confirmed by a steady state flux of lidocaine hydrochloride almost 5 times lower across pig palatal mucosa without bone (~8 $\mu g/cm^2.h^{-1}$) than pig palatal epithelium (~37 $\mu g/cm^2.h^{-1}$) previously obtained in similar experimental conditions (Franz-Montan et al., 2016). Moreover, it was not possible to calculate lidocaine flux across the palatal mucosa with bone as permeated amount of lidocaine was under the limit of detection. As expected, the presence of bone conferred more resistance to the drug permeation.

Microneedles have been extensively used to disrupt the epithelium barrier and increase drug absorption across skin. Different types of microneedles systems have been described, and we decided to test a metal commercially available handheld device (Dermaroller®), which is extensively used for esthetical treatment in Dermatology (Doddaballapur, 2009), and in transdermal drug delivery (Kalluri et al., 2011, Badran et al., 2009).

Although the literature have demonstrated the *in vivo* effectiveness of metal microneedles as drug delivery device in oral mucosa (Wang and Wang, 2015, Wang et al., 2015, Ma et al., 2014, Ma et al., 2015), to our knowledge *in vitro* studies involving microneedles and oral mucosa was not performed so far. In the present study, histological,

fluorescent and dye evaluation with methylene blue confirmed that microneedles were able to perforate the stratum corneum barrier and create microchannels at oral mucosa as observed in Figs 7D, 8, 10. Similar results were obtained when the same commercially available handheld device was applied *in vitro* at the skin (Kalluri et al., 2011).

The number of perforations promoted by microneedle application at the mucosa surface was proportional to the number of passes of the microneedle device. Similar results were reported at the skin surface (Kalluri et al., 2011). In addition, we observed that the number of perforations was not dependent of microneedles length. Based on these results, a 0.2 mm microneedle device could be considered an appropriate system for intra oral use, since it is more unlikely to reach the free nerve endings at the *lamina propria* considering the mean thickness of palatal epithelium (~250 µm) (Squier and Brogden, 2011).

Moreover we decided to verify if the *in vitro* permeation conditions could affect the microchannels throughout the 12-h of experiment, since skin usually recovers its barrier function around 4 to 5 h after microneedles application in the *in vivo* assay (Kalluri et al., 2011). In general, the microchannels remained constant during the *in vitro* conditions evaluated here, as observed by fluorescent images and evaluation of the pore areas. However, it is possible that the microchannels closure are more likely to occur in the *in vivo* conditions as a result of healing process (Kalluri et al., 2011).

As expected, the permeation enhancement due to the use of microneedles in the *in vitro* assay was confirmed in the adapted diffusion cell. As observed in Figs. 12 and 13, the mucosa pre-treated by microneedles had increased permeation profile, independently of the microneedles length (0.2, 0.5 and 1.0 mm). Similar results were obtained when skin pre-treated with microneedles showed increased permeation of lidocaine (Nayak and Sudha, 2006), calcein (Oh et al., 2008) and docetaxel (Qiu et al., 2008) .

The increased permeation profile and steady-state flux was more evident for the local anesthetics in its base form (EMLA) (Fig. 13) than for lidocaine hydrochloride (Fig. 12). This was probably associated to their lipophilic nature, which can help to permeate across a fat-rich tissue, evidenced in Fig. 6B, mostly through the paracellular route (Senel and Hincal, 2001), rich of non-polar lipids.

A statistically difference between the steady state flux of mucosa pre-treated with 0.2 mm or 0.5 mm microneedles was not observed. Besides, no difference regarding the number of perforations was observed when comparing different microneedle lengths (Fig. 9). It seems that the permeation enhancement ability is more likely to be associated with microchannels presence and not with their depth.

5. CONCLUSIONS

Histological and permeability evaluation suggest that porcine palatal mucosa with or without bone are reliable and adequate model barriers in order to perform *in vitro* permeation studies when targeting deep tissues at oral cavity. The adapted Franz diffusion cell is a valid model when thick barriers are used.

Microneedles were efficient and feasible to physically enhance the drug permeation through oral mucosa, independently of the microneedle length.

The present study represents a step forward in methods to perform *in vitro* permeation studies to evaluate new designed topical formulations and permeation enhancers focusing on deep tissues of oral cavity.

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Conflicts of interest: There are no conflicts of interest associated with this study.

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2.2 Artigo 2: Influence of salivary washout on drug delivery to the oral cavity using coated microneedles: an in vitro evaluation

Running head: Use of coated microneedles in the oral cavity

Artigo será submetido ao periódico *Pharmaceutical Research*

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Abstract

Purpose: To determine whether in buccal tissues, after insertion and removal of coated microneedles, the presence of saliva over the insertion site can lead to loss of the deposited drug, and saliva can influence its *in vitro* permeation across the tissue.

Methods: Microneedles were coated with sulforhodamine (SRD), which was used as a model drug, and inserted in to porcine buccal mucosa *in vitro*. Fluorescence microscopy was used to study microneedle coating quality and the diffusion of SRD through the mucosa. Permeation experiments were conducted for simulated dynamic or static salivary flow by adding 100 µL/h or 100, 200 or 300 µL of phosphate buffered saline (PBS) in the donor compartment of the Franz diffusion cells, into which buccal tissue after insertion of SRD-coated microneedles was placed.

Results: Microscopy showed that microneedles were uniformly coated with SRD and that SRD was successfully delivered in to the mucosa. Some SRD remained in the tissue even after 24 h, despite presence of PBS on top of the coated microneedle insertion site. Either of the simulated salivary flow conditions (dynamic and static) affected the permeation parameters by means of increasing drug permeation, increasing lag time, and increasing drug loss to the donor chamber, as compared to when the mucosal surface was just kept moist.

Conclusion: Salivary washout can result in loss of drug that has been deposited in oral cavity mucosal tissues using coated microneedles, and presence of fluid over the coated microneedle insertion site can increase flux across the tissue. Thus, it is advisable to include salivary flow during *in vitro* studies related to the use of coated microneedles for drug delivery to the oral cavity in order to not obtain misleading results.

ABBREVIATIONS

Dyn-Flow - dynamic-simulated salivary flow

ER - enhancement ratio between steady-state flux of St-100, St-200, St-300, and Dyn-Flow in comparison to *Moist condition*

$\boldsymbol{J_{ss}}$ – flux of SRD across buccal mucosa

$\mathbf{Q_{24}}$ - total amount of SRD permeated after 24 h of experiment

SRD – sulforhodamine

St-100 - static-simulated salivary flow with 100 µL

St-200 - static-simulated salivary flow with 200 µL

St-300 - static-simulated salivary flow with 300 µL

$\mathbf{T_L}$ – Lag time

INTRODUCTION

The oral cavity mucosa has been noted as an interesting site for drug delivery of topically applied formulations. The transmucosal route offers several advantages such as fast onset of action (increased blood supply); absence of drug degradation as seen in the gastrointestinal tract; absence of hepatic first pass metabolism; reduced dose and toxicity; and potential to achieve local or systemic therapeutic effects (Hassan et al., 2010, Patel et al., 2011).

Despite the higher permeability of the oral mucosa in comparison to that of the skin (Squier et al., 1991, Lesch et al., 1989), it's outermost layer, the stratified squamous epithelium, represents a significant challenge in drug delivery because it acts as an important barrier to drug penetration. Microneedles represent a new approach for topical drug delivery for either local or systemic effects. This system consists of micron-scaled needles, designed to penetrate the barrier and enhance drug delivery in a minimally invasive and painless manner (Gill et al., 2008). Coated microneedles have typically been reported in the literature for drug delivery through the skin (Gill and Prausnitz, 2007a, Gill and Prausnitz, 2007b, Ma and Gill, 2014). However, recently, coated microneedles have also been used to successfully deliver drug across the oral cavity mucosal barrier for different purposes such as immunization (Ma et al., 2014, Zhen et al., 2015, Wang et al., 2015) and oral cancer treatment (Ma et al., 2015).

Besides the presence of an effective barrier to penetration, the oral cavity is a moist environment with a salivary flux, which constantly washes the oral mucosa, dilutes the drug, and can reduce the contact of a topically applied formulation and its bioavailability, a phenomenon known as "saliva wash out" (Patel et al., 2011, Paderni et al., 2012, Chinna Reddy et al., 2011). Thus, keeping the formulation on its application site for longer duration, and minimizing its loss due to salivary flow, is a great challenge.

In spite of the presence of saliva in the oral cavity, the efficiency of coated microneedles for drug delivery in to oral cavity tissues has been reported to be comparable to that in the skin, which is a dry surface. Ma et al. have reported delivery efficiencies of $63.9\% \pm 6.9\%$ and $91.2\% \pm 1.6\%$ into the lip and tongue of a rabbit, respectively (Ma et al., 2014). However, McNeilly et al. have reported a much lower delivery efficiency of 31.7 ± 3.7% into the mouse buccal tissue (McNeilly et al., 2014). While these studies have quantified the amount of drug delivered into the mucosa using coated microneedles, it remains unclear whether the drug that is deposited into the tissues can be backwashed due to saliva that bathes the insertion site, and whether presence of saliva can affect the diffusion of the deposited drug deeper into the tissue.

Thus, we were motivated to determine the effect of saliva on the drug that is deposited into the oral cavity tissues via coated microneedles. *In vivo* studies to determine this effect can be complicated and tough to interpret. This is because drug lost, if any, from salivary flow will be ingested by the animal, and thus cannot be quantified directly. Thus, we simulated the salivary flux condition *in vitro* using a Franz diffusion setup. The Franz diffusion is a classical experiment that is widely used to evaluate drug release and permeation across different barriers such as the skin or other different mucosal tissues including the oral buccal tissues. Usually, both pig buccal mucosa and skin are used to simulate the respective human tissues due to their high similarities in terms of permeability, structure and composition (Lesch et al., 1989). Typically, *in vitro* permeability studies using a Franz diffusion setup involve addition of a buffer or a formulation containing the drug over the tissue, in conjunction with either a permeability enhancing agent added to the formulation or after pretreatment of the tissue to increase its permeability. However, coated microneedles are unique because they directly deposit the drug into the tissues. Thus, further assessment of diffusion of this deposited drug across the tissue should not involve addition of fluid in the donor chamber in the case of skin.

On the contrary, for oral cavity tissues, saliva should be simulated in the donor chamber. Nonetheless, to our knowledge, there are no studies that have examined *in vitro* permeability across either the oral cavity mucosa or the skin for drug that has been deposited in the tissues using coated microneedles.

Therefore, the objective of the present study was to test the hypothesis that it is possible to perform an *in vitro* permeation study in a Franz-type vertical diffusion cell with porcine buccal mucosa into which drug has been delivered using coated microneedles, and, to simulate salivary flow *in vitro* to evaluate the influence that saliva has on drug loss and drug permeation across the buccal tissue.

MATERIALS AND METHODS

Microneedles

According to a previously described method (Ma and Gill, 2014, Ma et al., 2014), a wet etch process was used to fabricate 2D microneedle patches comprising of 57 microneedles (700-µm long and 200-µm wide) from a 50 µm-thick stainless sheet (SS304). As described previously (Gill and Prausnitz, 2007a), each microneedle of the 2D patch was bent “out of plane” manually under a microscope.

Microneedles were coated using a micro-precision dip coating process (Gill and Prausnitz, 2007a, Ma et al., 2015, Ma et al., 2014). Briefly, an automated x-y linear computer-controlled device on which microneedle arrays were positioned, was used to dip microneedles into the coating solution. The coating solution was composed of 1% (w/v) of carboxymethylcellulose sodium salt (low viscosity, USP grade, CarboMer, San Diego, CA, USA), 0.5% (w/v) Lutrol F-68 NF (BASF, Mt. Olive, NJ, USA) and 0.25% (w/v) sulforhodamine (SRD) (Molecular Probes, Eugene, OR, USA) (Ma et al., 2014).

Preparation of porcine buccal mucosa

Porcine buccal mucosa was obtained from Innovative Research (Novi, MI, USA). The excess of underlying tissue was manually removed with scalpels and scissors, until the samples had about ~ 1.5 mm thickness, which was measured with a caliper. After preparation, the samples were kept frozen (- 80 °C) for no longer than 3 weeks.

Before all experiments, to ensure tissue integrity, electrical impedance across mucosa was measured using a LCR Meter (LCR200, EXTECH Instruments, Nashua, NH, USA). First the mucosal tissue was cut to size and mounted on the Franz diffusion cell with phosphate buffered saline (PBS) in the donor and in the acceptor chambers. Next the two electrodes were placed in the donor and acceptor chambers, respectively. Mucosa was considered reliable with resistivity higher than 2 kohm.cm^2. This resistivity value was obtained based on a previous study by de Vries et. al (de Vries et al., 1991b), which we verified through pilot studies. In our pilot studies, porcine buccal mucosa were prepared and punctured with hypodermic needles. Impedance values of these tissues before and after puncture were measured. Resistivity values of non-punctured tissues were greater than 2 kohm.cm^2, while the punctured tissues had lower values.

Characterization of coated microneedles and delivery into porcine buccal mucosa *in vitro*

Fluorescence stereomicroscope (Olympus SZX16 fitted with DP73 CCD camera, Olympus America Inc fitted) was used to visually inspect uniformity of coatings on the microneedle surface and to inspect microneedles before and after insertion into the porcine buccal mucosa. For insertion, microneedles coated with SRD were manually pressed into the porcine buccal mucosa and held in place for 5 min. After a 5-min period, microneedles were removed and inspected under a fluorescent stereomicroscope. The surface of the porcine buccal mucosa after insertion was also visualized under the microscope. The porcine buccal

mucosa was next placed in OCT compound (Tissue-Tech, 4583, Sakura Finetek, Torrance, CA, USA), and frozen (-80°C). The samples were sliced into 10-µm thick sections using a cryostat (CM 1950, Lec, Buffalo grove, IL, USA). Fluorescence microscopy images were obtained for these sections using an inverted fluorescent microscope (Nikon Ti eclipse fluorescent microscope) fitted with a CCD camera (Andor DR-328G-c10-SIL, Andor Technology, South Windsor, CT, USA).

Determination of delivery efficiency of coated microneedles

Transmucosal delivery efficiency (DE) of coated microneedles was determined according to a previously described methodology (Gill and Prausnitz, 2007a, Ma et al., 2014, Ma et al., 2015). The amount of drug delivered into the mucosa was calculated by subtracting SRD that remained on microneedles after mucosal insertion (C_2 – in µg/mL) and SRD that remained on top of the mucosal surface (C_3 – in µg/mL) from the total amount of SRD that was coated on microneedles (C_1– in µg/mL), and DE was found according to the equation:

$$DE = \frac{C_1 - (C_2 + C_3)}{C_1} \, x \, 100$$

For all these measurements freshly prepared SRD-coated microneedles were used. Briefly, an unused patch of SRD-coated microneedles was inserted in 1 mL of deionized water to quantify the amount of SRD on the coating (C_1). Next, another set of coated microneedles were applied on top of the mucosal surface for 5 min, and SRD that remained on microneedles was obtained by placing the used patch in 1 mL of deionized water to determine C_2. The amount of drug left on the tissue surface was gently removed with a moistened swab followed by its immersion in 500 µL of deionized water to quantify C_3. Samples were analyzed using a fluorescence spectrophotometer (Cary Eclipse, Agilent

Technologies, Santa Clara, CA, USA) at the excitation and emission wavelengths of 565 and 586 nm, respectively, together with a standard curve of SRD.

Permeation set up

Permeation experiments were performed in jacketed Franz-type vertical diffusion cells (PermeGear, Inc., Hellertown, PA, USA) with a permeation area of 1.77 cm^2 and receptor chambers with a volume of 7 ml. The jacket was coupled to a water bath (Fisher Scientific®) at 37 °C. Buccal mucosa was submitted to the application of coated microneedles for 5 min and mounted in the diffusion cells. PBS was used both as a receptor medium and as simulated-saliva in the donor chamber. The donor chamber was covered with parafilm (Parafilm "M". Laboratory Film. Bemis®. Neenah, WI, USA) to minimize water loss.

Histological verification of SRD-presence in the buccal mucosa

After inserting SRD-coated microneedle arrays into a porcine buccal mucosa for 5 min, it was mounted in the Franz diffusion cells as described above, and 100 µL PBS was added in the donor compartment and 7 mL in the receptor compartment. Mucosa was removed after 0.5, 1, 3, 6, 9, or 24 h, gently rinsed in PBS, padded dry with paper towels, embedded in OCT, sectioned into 10 µm-thick slices using a cryostat, and imaged using an inverted fluorescent microscope as described above.

SRD permeation study using simulated saliva conditions

For flux measurement studies, SRD-coated microneedles were inserted into the mucosa for 5 min. Following removal of the microneedle patch, the tissues were mounted in the diffusion cells between the donor and the receptor chambers. The water jackets of the diffusion cells were warmed to 37 °C at least 30 min before mounting the mucosa. Dynamic

and static conditions of salivary flow as described below were implemented on the donor side. To measure permeation of SRD across the buccal mucosa into the receptor chamber, a volume of 300 µL was collected from the receptor chamber at 0.5, 1, 2, 3, 4, 6, 9, 12 and 24 h, and replaced with the same volume of fresh buffer solution.

Dynamic condition

The dynamic-simulated salivary flow (Dyn-Flow) used was 100 µL/h, which was created by replacing the total volume (100 µL) of PBS buffer from the donor chamber every hour, during a 24 h experiment.

Static conditions

For comparison, we also used the static conditions of simulated saliva. These conditions were: 100 µL (St-100), 200 µL (St-200) and 300 µL (St-300), wherein the stated volume of PBS was added into the donor compartment and allowed to remain there for the entire duration of the study time point.

Moist condition

To study the influence of saliva, a 'Moist' condition was created on top of the buccal mucosa in the donor chamber. To create this moist and high humidity environment, a moistened gauze was placed in close proximity of the mucosal surface (without physical contact) in the donor chamber, and the free space inside the donor chamber was simultaneously reduced. This was done by cutting the barrel of a syringe to the required length so that when hung inside the donor chamber using the flange of the barrel as a stopper, it would hang about 3 mm above the buccal surface (**Fig. 1**). A 10 mL syringe-barrel (BD®, Inc. Franklin Lakes, NJ, USA) was found to closely fit the inside of the donor chamber. The

hollow lumen of the syringe barrel was stuffed with gauze, which was then moistened and saturated with water (no dripping). This assembly was hung inside the donor chamber, and the top was sealed with parafilm in order to prevent water evaporation. Altogether, this system allowed us to keep the top of the buccal mucosa mounted in the Franz cell in a moist state.

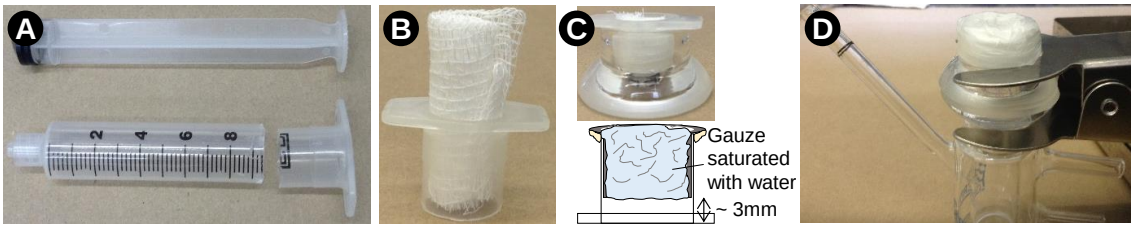

Fig 1. 'Moist case': Modification of a vertical Franz-diffusion cell to minimize free space in the donor chamber and to increase humidity above the buccal tissue. **(A)** A syringe barrel was cut to size so that the barrel hangs leaving about 3 mm from the bottom of the donor chamber. **(B)** A gauze was rolled and stuffed into the barrel. **(C)** The extra gauze on the top was cut and the barrel was hung in the donor chamber using the flanges on the barrel. **(D)** Final assembly of the Franz diffusion cells. Water was added to saturate the gauze and the top of the donor chamber was wrapped with parafilm to seal it.

Data analysis

SRD was quantified in the sample collected from the receptor chambers by fluorescence spectrophotometry as described above in the delivery efficiency measurement section. The dilution effect caused by addition of fresh buffer during sample collection was accounted for in the calculations. The cumulative amount of SRD that permeated across the buccal mucosa was expressed on a per unit area basis of the buccal surface as ng/cm^2, and was plotted as a function of time. The linear portion of this curve was fitted with a linear regression. The steady-state flux (J_{ss}) was obtained from the slope of this line (ng/cm^2/h), while the lag time (T_L) was determined as the x-intercept of this regression fit.

The buffer from the donor chamber (simulating saliva) was also analyzed in order to verify bidirectional flux of the model drug and to calculate drug loss into the simulated saliva. In the static conditions, the amount of SRD was quantified at the end of the experiment, and in the dynamic flow, the total amount of SRD in the donor was the sum of SRD detected at all collection times.

Statistical Analysis

Statistical analysis of data were performed using the GraphPad Prism® package (GraphPad Software, Inc. La Jolla, CA, USA.), using parametric tests (ANOVA – Tukey) and non-parametric tests (Kruskall Wallis – Dunn) with significance level set to less than 0.05.

RESULTS

Microneedle coatings and delivery efficiency into porcine buccal mucosa

The images obtained of coated microneedles (**Figs. 2A and 2B**) show that all the microneedles of the patch were coated with SRD, and that the coating on each microneedle was uniform. After microneedle insertion into buccal mucosa, it was observed that almost no coating was left on the microneedle surface (**Fig. 2C**). Observation of the microneedle insertion site showed that all microneedles penetrated and delivered their coatings in to the tissue (**Fig. 2D**). This is evident from the 57 fluorescent dots that can be seen on the surface of the buccal mucosa, and their arrangement, which recreates the pattern of the 57 microneedles on the patch. Slicing up the buccal mucosa into thin sections, and examining them under a fluorescent microscope showed that SRD was deposited into the tissue, and it was not merely superficially located (**Fig. 2E**).

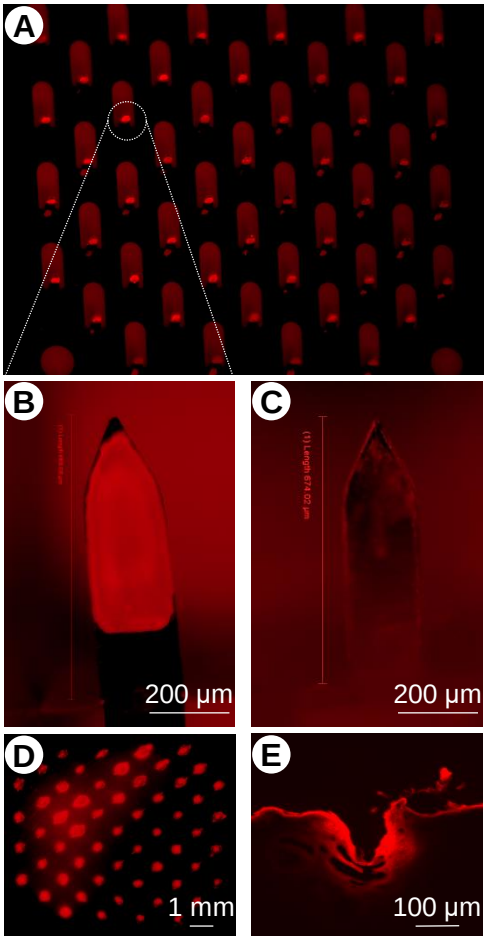

Fig 2. Fluorescence micrographs of microneedles and tissue sections. (**A)** A microneedle patch uniformly coated with SRD. **(B)** An individual microneedle of the patch demonstrating coating uniformity. (**C)** An individual microneedle of the patch after insertion into porcine buccal tissue. (**D)** Tissue surface after microneedle insertion. **(E)** A histological cross-section of buccal mucosa after application of the microneedle patch.

After visual confirmation of SRD delivery, we next quantified the amount of SRD that was delivered into the buccal mucosa. Quantification of the amount of SRD coated on microneedle patches showed that each microneedle patch was coated with 15.0 ± 2.2 µg of SRD. The transmucosal delivery efficiency of SRD into buccal mucosa was almost 75%, while 14% and 11% remained on the mucosal surface and microneedles, respectively (**Fig. 3**).

This result corroborates with the image of microneedle obtained immediately after its insertion (**Fig. 2C**) where it is seen that almost no SRD can be observed on its surface.

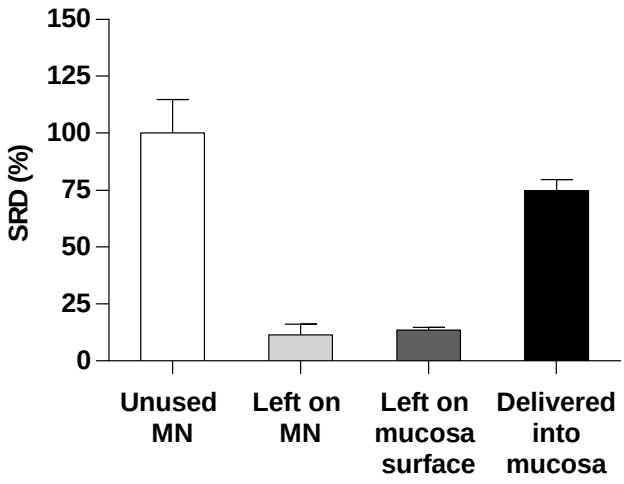

Fig. 3. Delivery efficiency of SRD-coated microneedles into porcine buccal mucosa. Mean ± SD; n = 6 for Unused MN; n = 8 for delivery efficiency insertions.

Detection of SRD in buccal mucosa 24 h post insertion

Prior to initiating flux measurement studies, we wanted to first establish that SRD that is deposited into buccal mucosa does not get completely backwashed into PBS placed in the donor chamber. Therefore, we histologically confirmed presence of SRD in buccal mucosa after allowing it to remain in contact with PBS for different periods of time, up to 24 h. SRD fluorescence could be detected at microneedle insertion sites up to 24 h (**Fig. 4**), suggesting that despite presence of fluid on top of the insertion site, all of the deposited SRD is not lost into the donor chamber.

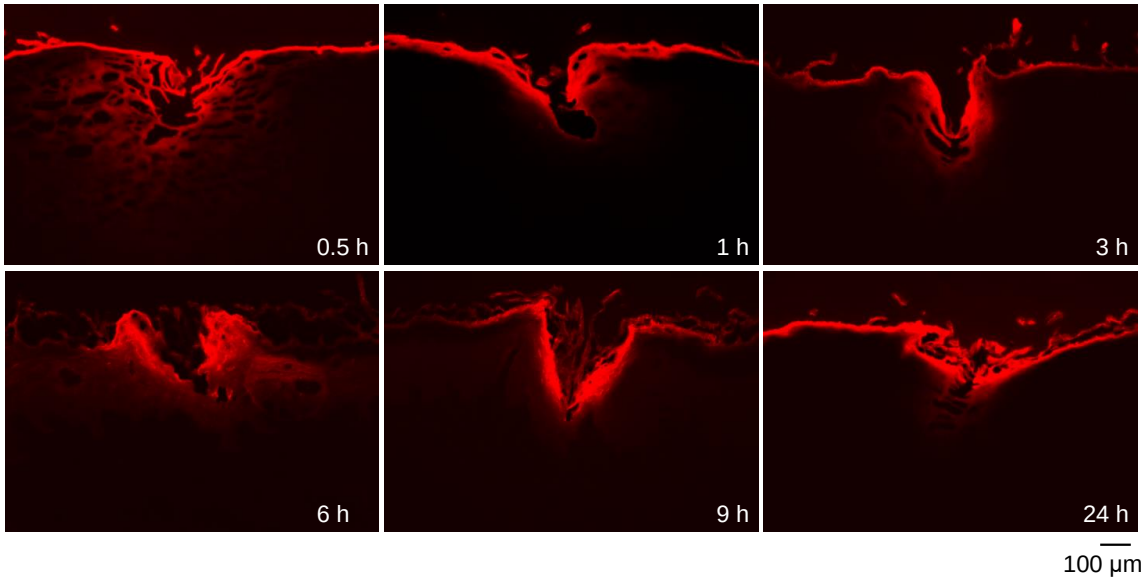

Fig. 4. Fluorescence micrographs of histological sections of porcine buccal mucosa after insertion of SRD coated microneedles, and allowing the treated site to remain in contact with 100 µL PBS for 0.5, 1, 3, 6, 9 or 24 h. Images represent studies done in triplicate.

Permeation Studies

In vitro permeation of drug deposited into porcine buccal mucosa using SRD-coated microneedles was successfully performed in the Franz-type vertical diffusion cells. Moreover, permeability of the model drug, SRD, was compared under different saliva flow conditions in order to evaluate whether or not the presence of saliva could alter its permeation. Permeation profiles of SRD under static (100, 200 and 300 µL of saliva) and dynamic flow (100 $\mu L.h^{-1}$) can be seen in **Figs. 5A** and **5B**, respectively. The transport of SRD across buccal mucosa was clearly increased when either of the saliva flow – static or dynamic was simulated, as compared to the 'moist' condition.

Table 1 shows permeation parameters: steady-state flux, lag time, and the cumulative amount of SRD permeated per cm^2 of buccal mucosa after 24 h for the different saliva flow

conditions. The steady state permeation fluxes and lag times were calculated using the linear portion of the graphs of the cumulative amount of SRD permeated through buccal mucosa (ng/cm^2) plotted against time (h). The linear intervals were between 6 and 24 h for St-100 and Dyn-Flow, and between 4 and 24 for Moist, St-200 and St-300. The values of the regression coefficients for all the individual curves exceeded 0.96.

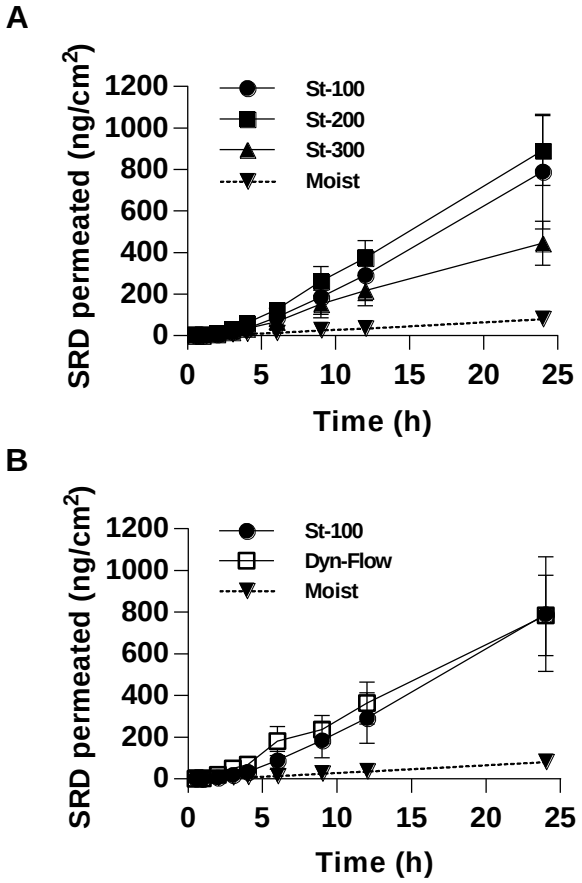

Fig. 5. Permeation profiles of SRD across porcine buccal mucosa obtained after application of SRD-coated microneedles: **(A)** under different static simulated salivary flow groups (mean±SEM, n=6-9); **(B)** under dynamic simulated salivary flow group (mean ± SEM, n = 6-9).

Table 1. Calculated lag time (T_L), steady-state flux (J_{ss}), enhancement ratios (ER) and cumulative amount of SRD permeated per cm^2 of buccal mucosa after 24 h (Q_{24}) for permeation of SRD across porcine buccal mucosa under static and dynamic saliva flow conditions (n=6-9).

	T_L (h)	J_{ss} (ng/cm^2/h)	J_{ss} (IQR)	ER*	Q_{24} (ng)	Q_{24} (IQR)
St-100	5.02 ± 1.92[a]	43.47 (16.08 – 68.13)[a]	52.06	14.63	1367.60 (491.95 – 2456.08)[a]	1964.13
St-200	3.36 ± 1.00[ab]	46.18 (30.50 – 52.83)[a]	22.33	15.54	1752.56 (1063.97 – 1992.74)[a]	928.77
St-300	3.32 ± 1.24[ab]	19.55 (10.45 – 26.77)[ab]	16.32	6.58	891.03 (350.97 – 1041.74)[ab]	690.77
Moist	1.74 ± 1.20[b]	2.97 (2.19 – 4.74)[b]	2.55	-------	115.82 (90.83 – 180.13)[b]	89.30
Dyn-Flow	3.40 ± 1.93[ab]	33.68 (18.14 – 45.36)[a]	27.22	11.34	1293.60 (636.70 – 1881.69)[a]	1224.99

*ANOVA/ Tukey-Kramer for T_L – data presented in mean ± SD. Kruskall-Wallis/Dunn for J_{ss} and Q_{24} - data presented in median (minimum – maximum), IQR – interquartile range. Alphabets 'a' and 'b' signify statistical differences among the groups in the column only if the alphabets differ ($p < 0.05$). For example, for T_L, St-100 and St-200 each differ from the Moist case. Each permeation parameter was analyzed separately. *Enhancement ratio between steady-state flux of St-100, St-200, St-300, and Dyn-Flow in comparison to Moist.*

In general, the presence of PBS under static or dynamic conditions increased the lag time, however only the static condition with 100 µL PBS as simulated saliva (St-100) presented a statistically longer lag time when compared with the 'Moist' group (ANOVA/Tukey, $p = 0.0321$).

A higher steady-state flux for all the saliva-simulated groups was observed in comparison to the negative control group (Moist) (Kruskal Wallis/Dunn, $p = 0.0003$), except for the static condition with 300 µL PBS, which although had a higher steady state flux than the 'Moist' control, but the two were not statistically different (Kruskal Wallis/Dunn, $p >$

0.05). The flux in the presence of PBS as simulated saliva was at least 6.58 fold higher than the ‘Moist’ group for St-300, and this increase was almost 15 fold for St-100 and St-200 simulated saliva cases.

Similar results were observed for cumulative amount of SRD permeated per cm^2 of buccal mucosa after 24 h, where all the saliva simulated groups presented a higher amount of SRD permeation as compared to the negative group (Moist) (Kruskal Wallis/Dunn, $p = 0.0008$), except for the static condition with 300 µL PBS, which although higher, was not statistically different from the ‘Moist’ group (Kruskal Wallis/Dunn, $p > 0.05$).

To assess the loss of SRD out of the mucosa due to simulated salivary conditions, the amount of SRD in the donor chamber was quantified. **Fig. 6A** shows the amount of SRD collected from the donor chamber at the end of 24 h of the experiments for St-100, St-200 and St-300. For the dynamic group the amount of SRD at the end of 24 h was the sum of SRD amount from every hourly collection.

The static-simulated saliva flow with 100 µL PBS (St-100) presented the smallest loss of SRD to the donor chamber in comparison to all groups (Kruskall-Wallis/Dunn, $p < 0.05$), except when compared to the static condition with 200 µL PBS (St-200), which although was higher than St-100, but it was not statistically different (Kruskall-Wallis/Dunn, $p > 0.05$). The dynamic condition (Dyn-Flow) presented the highest loss of SRD to the donor when compared to all the other groups (Kruskall-Wallis/Dunn, $p < 0.001$), except when compared to the static condition with 300 µL PBS (St-300), which although was lower than Dyn-Flow, but it was not statistically different (Kruskall-Wallis/Dunn, $p > 0.05$).

Fig. 6B summarizes the relationship between the total amount of SRD that got permeated into the receptor chamber, was lost to the donor chamber, and was retained in the mucosa at the end of 24 h of the experiment. Increasing the volume of saliva in the static

condition led to an increase in the amount of SRD lost to the donor chamber (St-100 ~14%; St-200 ~36%; St-300 ~37%), reduced the amount of SRD retained in the mucosa (St-100 ~75%; St-200 ~53%; St-300 ~57%), and reduced the amount that permeated into the receptor chamber (St-100 ~12%; St-200 ~11%; St-300 ~6). The simulation of salivary flow (Dyn-Flow) demonstrated that almost 90% of SRD was lost to the donor chamber, about 9% permeated across, and less than 1% was retained in the mucosa.

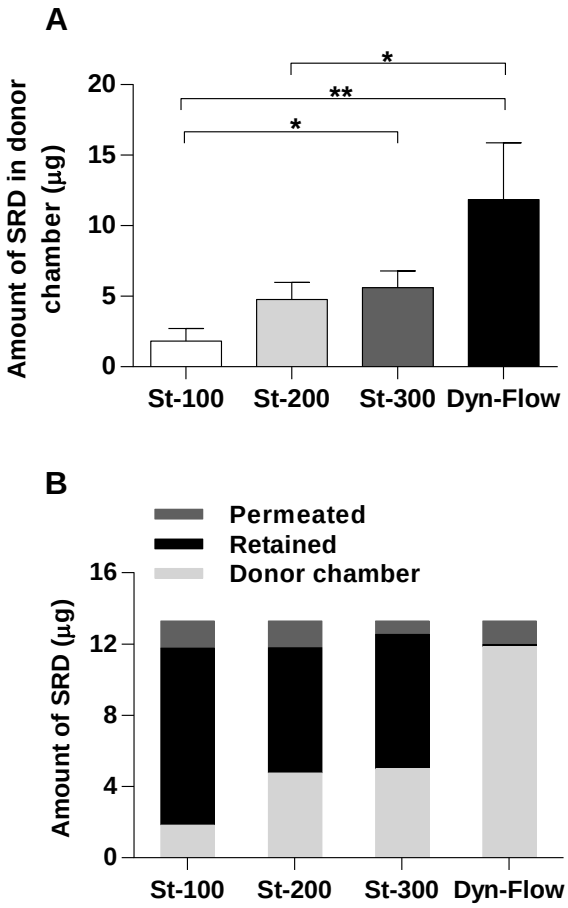

Fig. 6. Salivary washout effect as determined by measuring loss of SRD into donor chamber. **(A)** Amount of SRD collected from donor chamber after 24 h of permeation experiment under different static or dynamic simulated salivary flow groups. (ANOVA/Tukey, *$p < 0.05$, **$p < 0.001$). Mean ± SD (n = 8-9). **(B)** Total amount of SRD that permeated across the buccal mucosa in 24 h (Permeated), retained in the mucosa (Retained), or lost to the donor chamber

(Donor) under different simulated saliva conditions (St-100, St-200, St-300 and Dyn-Flow). Mean ± SD (n = 8-9).

DISCUSSION

Previously, in an *in vivo* experiment in a rabbit, we have shown that despite the moist environment in the oral cavity, coated microneedles can deliver the coated drug with high efficiency into the lip and tongue of rabbits (Ma et al., 2014). This delivery efficiency was comparable to the delivery efficiency seen in the skin, which is a dry surface. However, unlike the skin, in the oral cavity tissues, after the drug has been deposited using coated microneedles, it is possible that due to salivary flow, the drug deposited in the tissues may diffuse out into the saliva resulting in drug-loss. There is a lack of studies evaluating the influence of saliva on permeation using coated microneedles. Thus, in this experiment, we were motivated to establish how presence of saliva affects diffusion of the deposited drug into deeper parts of the mucosal tissue, and whether deposited drug is susceptible to salivary washout. To our knowledge this is the first study examining this effect for coated microneedles.

The choice of SRD as a model drug was based on its good water solubility (0.1 g/mL). This is because, drugs that have good water solubility can be more easily removed from their deposition site into the saliva, while poorly water soluble drugs would have low solubility in saliva, and would thus not be readily washed away in the saliva. Furthermore, SRD has previously been evaluated in the context of coated microneedles for *in vitro* transdermal delivery (Gill and Prausnitz, 2007a, Gill and Prausnitz, 2007b) and *in vivo* oral transmucosal delivery in rabbits (Ma et al., 2014).

For transbuccal permeation studies that involve evaluation of novel formulations, porcine buccal mucosa in the thickness range of 200 to 500 μm is often used (Kulkarni et al., 2009). However, because our goal was to use microneedles measuring 700 μm long to directly deposit drug in to the buccal tissue, we used thicker tissues measuring about 1.5 mm in thickness for our studies. Use of tissues measuring 500 μm or lower in thickness, would have resulted in microneedle perforations spanning their entire thickness, and would have caused abnormally high flux rates.

As expected, the quality of the microneedle coatings obtained here were similar to those reported previously (Ma et al., 2015, Gill and Prausnitz, 2007b, Ma et al., 2014), which also used stainless steel microneedle arrays, and the same coating method and model drug. Overall, the microneedle coatings prepared in this study were without gaps or structural damage.

The *in vitro* delivery efficiency of SRD into porcine buccal mucosa after a 5-min application was 75%. This result corroborates with our previous *in vivo* delivery test in rabbits, which demonstrated a delivery efficiency of 63.9% for the inner lip and 91.2% for the dorsum of the tongue after a 2-min application (Ma et al., 2014). These results were expected because even though we used a porcine buccal tissue *in vitro* and an application time of 5 min, in both studies the type of microneedle, coating method, solution and drug (SRD) were the same. A similar deliver efficiency (85.6%) was demonstrated for doxorubicin coated-microneedles after a 5 min application time in porcine buccal mucosa *in vitro* , using the same microneedle device and coating method (Ma et al., 2015).

The permeability of oral mucosa has been studied in humans (Lesch et al., 1989), pigs (Squier et al., 1991, Vries et al., 1991) and other species (Squier and Hopps, 1976). The similarities in permeability between pig and human oral tissues (Lesch et al., 1989) make the

porcine model acceptable for evaluating *in vitro* permeation of newly-designed drug delivery systems. The main permeability barrier of the oral cavity mucosa is the epithelium due its lipid composition, i.e. ceramides for keratinized tissues and glycosylceramides for non-keratinized tissues (Squier et al., 1991). The ability of coated microneedles to physically penetrate this barrier and deposit the drug into the mucosal tissue to improve the drug effectives has already been demonstrated *in vivo* (Ma et al., 2014).

The salivary flow rate can be divided in to two types, stimulated and unstimulated. The stimuli can be physical or sensorial and the maximum stimulated flow rate is up to 7 mL/min (Humphrey and Williamson, 2001). The average unstimulated salivary flow is about 0.3 mL/min during awake periods, but near to zero during sleeping time (Humphrey and Williamson, 2001). The presence of a constant salivary flow in the oral cavity, in conjunction with a largely water-like property of saliva (> 99% water with dissolved electrolytes and proteins) (Humphrey and Williamson, 2001), creates a favorable environment in the oral cavity to wash hydrophilic drugs, such as SRD, away from its application site. In the present study, the simulated dynamic salivary flow was 100 μL/h, i.e. 1.66 μL/min. Collins and Dawes in 1987 reported a similar salivary flow rate. They demonstrated that the total area of the human oral mucosa was about 220 cm^2, and considering an unstimulated salivary flow of 0.3 mL/min, the salivary flow would then be 2.4 μL/min for an area measuring 1.77 cm^2 (area of oral mucosa used in the present study) (Collins and Dawes, 1987). In this study we also compared how static salivary volume maintained over the buccal tissue compares to the dynamic flow. We observed that the dynamic flow of saliva causes significant backwash of drug (**Fig. 6**), with about 90% of SRD being lost into the PBS of the donor chamber. In contrast, although increasing the static volume of PBS in the donor chamber from 100 μL to 300 μL led to an increase in loss from 14% to 37%, it was significantly lower than the dynamic flow case. This data thus suggests that even with the use of coated microneedles,

which can deposit drug with high efficiency into the oral cavity mucosal tissues, it may be important to still cover the insertion site with a protective mucoadhesive covering or patch to reduce drug loss due to salivary washout. The molecular weight of the drug may also affect back-diffusion from the tissue into the saliva, thus larger hydrophilic drugs molecules such as proteins also need to be investigated.

With respect to diffusion of SRD across the buccal tissue, we observed that presence of fluid on top of the buccal tissue actually increased the flux as compared to the Moist case when the buccal surface was just kept moist. Addition of 100 μL PBS into the donor chamber led to about 15 fold higher flux as compared to the 'Moist' case. This effect could be explained by considering that coated microneedles deposit the drug into the tissue when the coating is delaminated from the microneedle surface. Presence of a small amount of moisture can help achieve this delamination, however, the material left behind in the tissue may only be partially solubilized and could be in a highly concentrated state. Addition of liquid on top of the insertion site could help provide fluid to solubilize the drug and enhance its diffusion, as was seen in St-100, St-200, St-300, and Dyn-Flow cases.

The lag time increased in the presence of saliva. As observed in **Table 1**, there was an overall tendency for the lag time to increase when simulated salivary conditions were used as compared to the Moist condition. In the Moist case, a high solute (SRD) concentration in the tissue at initial stages could have caused the diffusion to be faster resulting in a lower lag time, but then due to limited availability of moisture in the tissue the solute mobility could have reduced resulting in a low cumulative flux. In contrast, for simulated saliva cases, the presence of liquid on top of the mucosa could have partially removed the drug and reduced the solute concentration in the tissue causing lower diffusion rate (higher lag times) than the Moist case, but by providing a continuous solvent phase for solute diffusion, the presence of simulated-saliva could have led to a greater cumulative diffusion of the solute in 24 h.

In an *in vivo* situation, the effect of saliva washout could be even more severe due to the daily activities such as swallowing, chewing, and speaking (Paderni et al., 2012). However, *in vivo*, micropores created by the microneedles could close after microneedle removal due to tissue elasticity and healing process, which might help to protect the inserted drug from the washing effect. It is well established in the literature that *in vivo* pore-lifetime is short for the skin, and the pores reseal in about 2 h (Brogden et al., 2012, Milewski et al., 2010, Gupta et al., 2011a) after removal of microneedles from the skin. However, the closure time of micropores in the oral cavity mucosa after microneedle insertion and removal has not yet been evaluated.

The literature already demonstrates a strong correlation between *in vitro* permeation parameters of semi-solid formulations across pig epithelium mucosa and *in vivo* topical anesthetic efficacy in human volunteers (Franz-Montan et al., 2013, Franz-Montan et al., 2015), suggesting that *in vitro* permeation studies can help to predict *in vivo* efficacy. Likewise, studies with coated microneedles involving *in vitro* permeation in the presence of salivary flow could provide insight into *in vivo* pharmacokinetics from delivery to the oral cavity, and could provide guidelines for better design of the delivery system. However, additional studies are necessary to select the best *in vitro* condition of salivary flow that can more reliably predict permeation parameters to simulate *in vivo* results. For example, a continuously flowing fluid stream atop the buccal mucosa could be used to better simulate the salivary flow. However, care would have to be taken to not only simulate the salivary flow rate, but also the thickness of the saliva film over mucosal tissues.

CONCLUSION

This is the first study to demonstrate *in vitro* the effect of salivary washout on drug delivered to the oral cavity using coated microneedles. Using Franz-diffusion cells we investigated the effect of saliva on drug washout into the donor chamber and drug permeation across porcine buccal tissue into the receptor chamber. It was found that salivary flow (dynamic and static cases) affects permeation dynamics by means of increasing drug permeation, and increasing lag time as compared to the control group ("Moist"). Salivary flow also resulted in an increase in drug loss to the donor chamber, i.e. it promoted a bidirectional flux. Future studies are necessary to choose a better optimized *in vitro* salivary flow simulation so that a more suitable *in vitro* permeation model can be developed to better reflect *in vivo* pharmacokinetics of drugs delivered to the oral cavity mucosa using coated microneedles.

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CONFLICTS OF INTEREST: HSG is a co-inventor on a patent related to microneedle coating technology.

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3 DISCUSSÃO

Estudos de permeação representam uma importante ferramenta para o desenvolvimento de novas formulações tópicas durante a fase pré-clínica. No entanto, as barreiras comumente utilizadas (epitélio de mucosa bucal e esôfago) não representam modelos aplicáveis aos fármacos de interesse odontológico, especialmente em decorrência da grande variabilidade estrutural da mucosa oral. Esses modelos são bastante utilizados quando novas formulações tópicas em desenvolvimento objetivam efeito sistêmico.

Recentemente, nosso grupo de pesquisa validou o preparo e armazenamento do epitélio dos diferentes tipos de mucosa oral: mucosa mastigatória (gengiva e palato), de revestimento (bucal) e especializada (língua) visando o auxílio no desenvolvimento de novas formulações tópicas de interesse em efeito local (Franz-Montan et al., 2016). No entanto, este modelo é de pouca aplicabilidade quando a formulação deve penetrar profundamente em tecidos como a polpa dental.

O presente estudo padronizou o método de preparo de dois tipos de barreira de mucosa palatina de porcos, com ou sem osso subjacente. Este modelo de barreira terá enorme aplicabilidade quando formulações em desenvolvimento visando efeito na polpa dental como antibióticos, anti-inflamatórios, analgésicos e anestésicos locais forem testadas *in vitro.* Além disso, foi padronizado as condições experimentais para realização de permeação *in vitro* com essas barreiras uma vez que a espessura dos tecidos poderia por exemplo, promover vazamentos e impedir o controle adequado de temperatura ao longo tempo de experimentação.

A fim de se testar a eficiência desta barreira, decidimos avaliar a aplicabilidade de microagulhas como promotores de absorção, uma vez que seu uso em Dermatologia já está bastante avançado (Doddaballapur, 2009), e sua aplicabilidade em mucosa oral é bastante restrita (Ma and Gill, 2014, Ma et al., 2014, Ma et al., 2015, Wang et al., 2015). Além disso, não há, do nosso conhecimento, nenhum relato de avaliação *in vitro* com microagulhas em mucosa oral. Conforme já esperado, o pré-tratamento da mucosa com esse sistema foi eficiente em aumentar a permeação *in vitro* de anestésicos locais contidos em uma formulação comercial (EMLA®), utilizando o modelo de barreira e célula adaptada descrita no presente trabalho.

Nesse contexto, decidimos também avaliar a possibilidade de realização de estudos de permeação *in vitro* com microagulhas revestidas, uma vez que os relatos de uso

desse sistema em mucosa oral, foram avaliados diretamente em estudos *in vivo* (Ma and Gill, 2014, Ma et al., 2014, Ma et al., 2015, Wang et al., 2015). Para isso, foi necessário padronizar as condições experimentais uma vez que a reduzida quantidade de fármaco representa um fator limitante desse sistema.

Poucos estudos utilizam saliva (artificial ou natural) em estudos de permeação através da mucosa oral (Giannola et al., 2007b, Giannola et al., 2007a) apesar de ser conhecida a sua influência em *drug delivery* utilizando microagulhas (Ma et al., 2014). No entanto, não existem relatos que avaliaram a influência da saliva em ensaios de permeação *in vitro* com microagulhas revestidas. De uma maneira geral, o presente estudo demonstrou um aumento do fluxo e do *time lag* da droga modelo na presença de fluxo salivar, demonstrando sua importância em simular as condições reais da cavidade oral em permeação *in vitro*. Desta forma, o presente estudo sugeriu pela primeira vez condições experimentais para testes de permeação com microagulhas revestidas, bem como sua eficiência em aumentar a difusão através de mucosa oral *in vitro*.

A presente tese propõe metodologias com grande aplicabilidade para realização de estudos *in vitro* visando o melhoramento de formulações tópicas para mucosa oral, bem como a redução do uso de animais em pesquisa.

4 CONCLUSÃO

O preparo de barreiras de mucosa palatina de porco com ou sem osso, bem como a célula adaptada foram padronizados e permitiram a realização de experimento de permeação *in vitro*, demonstrando a viabilidade do método. O uso de microagulhas nessas barreiras foi eficaz em aumentar a permeação de drogas modelo nas condições avaliadas, demonstrando que o pré- tratamento da mucosa oral com microagulhas pode ser eficiente em melhorar a ação de fármacos aplicados topicamente na cavidade oral e pode representar um avanço em *drug delivery*. A presença de fluxo salivar demonstrou ser importante para simular as condições reais da cavidade oral na avaliação da permeação *in vitro* de microagulhas revestidas, pois pode ter um papel importante na retirada do fármaco de seu sítio de aplicação.

Nesse contexto o presente estudo representa um aprimoramento na realização de experimentos de permeação *in vitro* visando a melhora de formulações tópicas e promotores de absorção para uso em Odontologia.

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