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Pre-clinical evaluation of novel mucoadhesive bilayer patches for local delivery of clobetasol-17-propionate to the oral mucosa

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Abstract

Oral lichen planus (OLP) and recurrent aphthous stomatitis (RAS) are chronic inflammatory conditions often characterised by erosive and/or painful oral lesions that have a considerable impact on quality of life. Current treatment often necessitates the use of steroids in the form of mouthwashes, creams or ointments, but these are often ineffective due to inadequate drug contact times with the lesion. Here we evaluate the performance of novel mucoadhesive patches for targeted drug delivery. Electrospun polymeric mucoadhesive patches were produced and characterised for their physical properties and cytotoxicity before evaluation of residence time and acceptability in a human feasibility study. Clobetasol-17-propionate incorporated into the patches was released in a sustained manner in both tissue-engineered oral mucosa and *ex vivo* porcine mucosa. Clobetasol-17 propionate-loaded patches were further evaluated for residence time and drug release in an *in vivo* animal model and demonstrated prolonged adhesion and drug release at therapeutic-relevant doses and time points. These data show that electrospun patches are adherent to mucosal tissue without causing tissue damage, and can be successfully loaded with and release clinically active drugs. These patches hold great promise for the treatment of oral conditions such as OLP and RAS, and potentially many other oral lesions.

Introduction

Oral lichen planus (OLP) and recurrent aphthous stomatitis (RAS, also termed aphthous ulcers) are common debilitating lesions that affect the mucosal lining of the oral cavity. OLP, a chronic inflammatory disease, affects 1-3% of the world's population causing bilateral, white striations, papules or plaques, whereas RAS presents as painful, round, shallow ulcerations of the mucous membrane, causing substantial morbidity in a reported 25% of the world's population at some point in their lifetime [1, 2]. The pathogenesis of both conditions is not entirely understood and consequently they lack effective clinical management. Current treatment is dependent on immune-modulating steroids to reduce inflammation and pain that are delivered either systemically, which although effective, rapidly induces unacceptable side effects leading to cessation of treatment or alternatively delivered topically by mouthwashes or gels. These topical dosage forms are generally considered suboptimal due to the continuous flow of saliva and mechanical stresses within the oral cavity that result in the active substance being washed away, leading to shorter exposure times and unpredictable drug distribution [3]. For localised controlled delivery, it is necessary to prolong and improve the contact time between the drug and the mucosal lesion, and this has driven the development of a number of mucoadhesive delivery systems including particulates [4, 5], tablets [6, 7], films [8-10] and patches [11]. Oral patches are usually laminates consisting of an impermeable backing layer and a drug-containing bioadhesive layer for mucosal attachment, and have typically been prepared using solvent casting [12] or hot melt extrusion techniques [13]. Recently, investigations by others and us have focussed on electrospinning as an innovative method to produce mucoadhesive patches [14-17]. Electrospinning is a highly versatile fibre and membrane manufacturing method that enables the unique combination of polymers, solvents and other molecules in ways that offer the ability to tune the physical structure and biological functionality of the resulting structures, which cannot easily be achieved with other conventional manufacturing techniques [18]. Furthermore, electrospinning produces patches that structurally can be composed of both nano- and microscale fibres, creating a high porosity and surface area for drug bioavailability and enabling a high level of interaction with the epithelium of the oral mucosa.

We recently reported the successful fabrication of a novel electrospun dual-layer mucoadhesive system comprising of an outer hydrophobic polycaprolactone (PLC) backing layer and an inner, mucoadhesive component formed by electrospinning polyvinylpyrrolidone (PVP) and Eudragit® RS100, as fibre-forming polymers. Particles of polyethylene oxide (PEO), were also added to the inner layer, to enhance the mucoadhesive properties of the structure [14]. Combining Eudragit® RS100, a copolymer of ethyl acrylate, methyl methacrylae and trimethylammonioethyl methacrylate

chloride with the PVP was shown to reduce membrane solubility and allowed control over the structural integrity of the patches upon hydration. This combination of materials produced a highly flexible, nano-fibre-forming matrix with a large surface area that showed strong mucoadhesive properties in an *ex vivo* model [14]. The system, once loaded with drugs, has the potential to provide greater therapeutic efficacy via highly localised and controlled drug delivery to the mucosal surface

Several recent reviews on OLP and RAS management suggest that the best treatment remains high-potency topical corticosteroids, acting to modulate the dysregulated immune response [19, 20]. Among those studied, clobetasol-17-propionate has been shown to be a highly effective topical steroid, with 95% improvement in patients with OLP after 2 months of therapy [21] and complete remission with no major side effects in patients with persistent RAS [22]. Clobetasol-17-propionate is currently only available formulated as topical preparations (mouthwash, mousse, ointment or emollient cream) that have low aqueous solubility and minimal oral bioavailability [23].

To summarise, oral lichenoid reactions and recurrent aphthous stomatitis together represent unmet clinical needs in oral medicine. While steroids are generally the drugs of choice, site-specific targeted delivery is a major challenge in the wet environment of the human mouth. The aim of this study was to examine the physico-chemical and mucoadhesive properties of our recently developed, electrospun patch [14] designed to address this problem, and to evaluate the clinical acceptability of the system at three intraoral locations (buccal, gingivae and tongue) in a human healthy volunteer study. Drug release from the patches was determined for clobetasol-17-propionate by measuring dissolution rates in an *in vitro* tissue-engineered oral mucosa system and an *ex vivo* porcine mucosa model. Finally, the clobetasol-17-propionate loaded patches were evaluated for residence time and drug release in an *in vivo* animal model.

2. Materials and Methods

2.1 Manufacture of mucoadhesive patches

2.1 Materials

Polyvinylpyrrolidone (MW 2,000 kDa; PVP) was a gift from BASF (Cheadle Hulme, UK). Eudragit RS100® was a gift from Evonik Industries AG (Essen, Germany). Poly(ethylene oxide) (MW 2,000 kDa; PEO), poly(caprolactone) (MW 80 kDa; PCL), and clobetasol-17-propionate (analytical standard, CP) were purchased from Sigma Aldrich (Gillingham, UK). Ethanol (EtOH), dichloromethane (DCM) and dimethylformamide (DMF) were purchased from Fisher Scientific (Loughborough, UK).

2.2.1 Fabrication of mucoadhesive patches

Electrospun materials were fabricated commercially (Bioinicia, Spain) or in-house using electrospinning equipment as previously described [14]. Briefly, a KDS200 syringe pump (KdScientific, USA) with an Alpha IV Brandenburg power source (Brandenburg, UK) was used. Plastic syringes (1 ml; Becton Dickinson, UK) were used to contain and drive the solutions into 15-gauge blunt metallic needles (Intertronics, UK). The applied voltage was 17 kV, the flow rate was 1 - 5 ml/h, and the distance from the tip to the collector was set at 19 cm. Polymeric solutions were prepared by dissolving PVP (10 wt%) and Eudragit RS100 (12.5 wt%) in 97 vol% EtOH (prepared in dH_2O) and the solutions kept under continuous stirring at room temperature until the polymers were completely dissolved. PEO (20 wt%) was then added to the polymeric solutions and stirred for a minimum of 30 minutes. Clobetasol-17-propionate was incorporated into the solutions by dissolving the required amount of the drug into EtOH prior to the addition of the polymers. Typically, electrospun membranes containing 1, 5 and 20 µg were produced and stored in a desiccator after manufacture. Before use, each batch of membranes was tested for total clobetasol-17-propionate content following total dissolution using HPLC and in all instances drug content was within ± 5% of the loaded dose.

2.2.2 Preparation of backing layer

A hydrophobic backing layer was prepared by electrospinning a 10 wt% solution of PCL on top of the drug delivery layer. The solution was prepared by adding PCL to a blend of DCM and DMF (90:10 vol% DCM:DMF), keeping the solutions under continuous stirring at room temperature until the

polymer had completely dissolved. A thermal treatment (70°C for 10 minutes) was applied to the samples in order to enhance the attachment between both layers by gently clamping the two layers together and heating in a dry oven.

2.3 Mucoadhesive patch characterisation

2.3.1 Determination of film thickness, mass uniformity and pH

The assessment of weight and patch thickness was completed on randomly selected patches from three independent batches. For determination of mass, patches were weighed on an electronic digital balance. Patch thickness was measured at 3 different randomly selected points using Vernier callipers and the pH determined by dissolving the patches in dH_2O for 5 minutes and measurements recorded using a pH meter (Hanna Instruments, Rhode Island, US).

2.3.2 Swelling index

Patches were cut from the electrospun membranes (1.5×1.5 cm), weighed, and submerged into 5 ml of dH_2O. After definite time intervals (30 seconds – 60 minutes) the patches were removed, excess moisture absorbed using tissue paper and reweighed. Increase in patch weight was determined at each time interval until a constant weight was observed. The degree of swelling was calculated using the formula:

$$\frac{(W_t - W_0)}{W_0} \times 100$$

where, W_t is the weight of the patch at time t and W_0 is the weight of the patch at time zero.

2.3.3 Scanning electron microscopy

Materials were imaged using a Philips XL20 scanning electron microscope (SEM). Samples were sputter coated with gold and imaged using an emission current of 15 kV. All images were processed using GNU Image Manipulation Program (GIMP, http://www.gimp.org) and Fiji20 software tools.

2.3.4 Differential thermal analysis

Differential thermal analyses (DTA) of the bioadhesive patches and of clobetasol-17-propionate analytical standard (Sigma Aldrich, UK) were performed in a Perkin-Elmer Diamond DTA/TG system. Samples (10-15 mg) were loaded into platinum crucibles and heated from 50°C to 325°C at a rate of 10°C/minute in a nitrogen atmosphere. The DTA patterns were processed using Perkin Elmer Pyris software and Microsoft Excel software.

2.3.5 X-ray diffraction analysis

X-ray diffraction (XRD) analyses of the electrospun membranes and of clobetasol-17-propionate analytical standard (Sigma Aldrich, UK) were performed in a PANalytical X'Pert³ powder spectrometer. Samples of the electrospun membranes (1 x 1 cm) were loaded on sample holders using Apiezon putty so that the surface of the specimen was level with the top of the specimen holder. Clobetasol-17-propionate was loaded on sample holders designed to hold powder samples. All samples were analysed on reflection mode using Cu radiation, scanning angles ranging from 5° 2θ to 70° 2θ, and step sizes of 0.013° 2θ. The XRD spectra were processed using PANalytical Data Collector software and Microsoft Excel software.

2.4.1 Cell culture

Cell culture of immortalized oral keratinocytes FNB6-TERT immortalized oral keratinocytes (Beatson Institute for Cancer Research, Glasgow, United Kingdom; commercially available at Ximbio, London, United Kingdom) originally isolated from the buccal mucosa [24] were cultured in Green's Medium consisting of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium in a 3:1 (v/v) ratio supplemented with 10% (v/v) fetal calf serum (FCS), 0.1 mM cholera toxin, 10 ng/ml epidermal growth factor, 0.18 mM adenine, 5 mg/mL insulin, 5 mg/ml transferrin, 2 mM glutamine, 0.2 nM triiodothyronine, 0.625 mg/mL amphotericin B, 100 IU/ml penicillin, and 100 mg/mL streptomycin. Normal oral fibroblasts (NOF) were isolated from the connective tissue of biopsies obtained from the buccal oral mucosa from patients during routine dental procedures with written, informed consent (ethical approval number 09/H1308/66) as previously described [25] and cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin.

2.4.2 Tissue-engineered oral mucosal equivalents

Oral mucosal models were constructed as previously described [26]. NOF were added to rat tail collagen at a concentration of 2.5 x 10^5 cells/ml before adding 1 ml to 12 mm cell culture transwell inserts (0.4 mm pore; Merck Millipore, Darmstadt, Germany) and allowed to set in a humidified atmosphere at 37°C for 2 h. Inserts were submerged in growth media and incubated for 2 days, after which 2.5 x 10^5 FNB6 cells per model were seeded onto the surface. After a further 5 days, the models were raised to an air-to-liquid interface and cultured for 10 days to allow a fully stratified epithelium to form before use.

2.4.3 Cytotoxicity and permeation studies using tissue-engineered oral mucosal

To assess cytotoxicity a standard *in vitro* skin irritation test was performed according to OECD standards (OECD 439)[27]. Briefly, placebo or clobetasol-17-propionate loaded patches (1, 5 and 20 µg) were applied, with gentle pressure, to the models and incubated for 1 h before removing, washing in PBS and the models cultured for a further 42 h in fresh medium. At this point, the models were washed in PBS and incubated in 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Poole Dorset, UK) in PBS (0.5 mg/ml) for 3 hours. The solution was removed and 0.1 M HCl in 2-propanol added (2 ml) to each model with gentle agitation to dissolve the formazan crystals. Absorbance at 570 nm was measured using a spectrophotometer (Tecan, Männedorf, Switzerland). Data was processed using Microsoft Excel and expressed as viability relative to the negative control. For *in vitro* drug permeating studies, cell culture media was refreshed and placebo or clobetasol-17-propionate loaded patches (1, 5 and 20 µg) applied to tissue-engineered models. After 1 h incubation, the patches were removed, washed in PBS and weighed. The models were bisected and dissolved in collagenase IV (2 mg/ml) for 1 h. Both the dissolved model and receptive medium were analysed by high performance liquid chromatography (HPLC) to determine clobetasol-17-propionate content. HPLC analysis was performed using a Waters 2690 HPLC with a Zorbax RX-C18 250 mm x 4.6 mm column and a mobile phase composed of acetonitrile (ACN)/water: CP (45% of ACN in water for 15 minutes, ramping to 100% ACN after 16 minutes) at 1 ml/min. UV was measured using Waters 486 UV/dis detector at 240 nm. For each concentration, single injections were made to obtain the peak area for constructing the calibration curve.

2.4.4 Histological analysis

For histological processing, the insert containing the tissue-engineered models were removed from the culture medium, washed with PBS and fixed in 10% buffered formalin overnight. The entire model (connective tissue and epithelium) was removed from the transwell insert along with the polycarbonate filter, subjected to routine histological processing, and paraffin-wax embedded. Five-micrometre sections were cut by using a Leica RM2235 microtome (Leica microsystems) and stained with haematoxylin and eosin.

2.5 In vivo residence time and patch acceptability

Twenty-six volunteers who satisfied the inclusion and exclusion criteria (Supplementary Figure 1) were recruited with written, informed consent after approval from the University of Sheffield Ethical Committee. Following the international standard of Good Clinical Practice, placebo patches (25.4 x 12.7 mm) were applied to the lateral tongue, buccal and gingival mucosa for 5 seconds with applied pressure. Patch adhesion was monitored every 10 minutes for 2 hours and residence time recorded for each location. The residence time was taken as the time for the patch to completely dislodge from the site where the patch had been placed. At the end of the study, an acceptability questionnaire was completed by all volunteers to collect information regarding parameters of the patch such as irritancy, comfort, taste, dry mouth and salivation. Food and drink intake was not allowed for 1 hour prior to beginning the study and until the study was complete.

2.6 In vitro drug dissolution

The release of clobetasol-17-propionate from the mucoadhesive patches manufactured with a range of concentrations (1, 5 and 20 µg) was determined using Erweka DT80 dissolution apparatus in conjugation with paddle stirrers, according to Ph. Eur. method 2.9.3. In brief, the patches were attached to supports and lowered into the dissolution vessels containing dissolution medium (0.5 M phosphate buffer saline and 0.5% sodium dodecyl sulphate, pH 6.8 at 37°C). The medium was stirred at a constant rate of 100 ± 2 rpm and at pre-determined intervals (15-360 minutes) samples of dissolution fluid (2 ml) were removed and replaced with an equal volume of fresh, pre-warmed dissolution fluid. The concentration of clobetasol-17-propionate in the samples of dissolution fluid were analysed by reverse phase HPLC with reference to a previously constructed calibration curve (r^2>0.99).

2.7 Ex vivo drug permeation through the oral mucosa

Mucosa (2.5 x 2.5 cm), freshly prepared from whole porcine cheeks (Citoxlab Scantox A/S, Lille Skensved, Denmark) were mounted in a Franz cell (7 ml receiver volume of PBS, exposure area of 2.3 cm^2, 37°C), wetted with PBS (50 µl) and patches (1.2 x 1.2 cm) applied with gentle pressure to the mucosal surface. After three hours, the patches were removed, a 1 ml sample of the acceptor buffer collected and the mucosa rinsed with PBS to remove residual clobetasol-17-propionate present on the surface. To calculate the amount of drug within the mucosa, the mucosa pieces were first heated to 65 °C for three minutes to enable removal of the epithelial layer of the mucosa, which was subsequently cut into smaller pieces and placed in acetonitrile (1 ml) and treated with ultrasound for 10 minutes before filtering (0.22 µm cellulose acetate filter) for analysis. Both the collected receiver buffer and acetonitrile were analysed for the concentration of clobetasol-17-propionate by HPLC using a Kinetex C18-XB 100x4.6, 5µ column at 40 °C, in MilliQ water: acetonitrile, Isocratic elution; (ratio 30:70) with an injection volume of 5 µl and flow rate of 0.5 ml/min, coupled to a UV-detector (237 nm) and a MS-detector (Electrospray: Negative, SIM Ions: 501.2, 503.0. Fragmentor: 70 drying gas flow: 12 L/min, drying gas temperature: 250 °C, nebulizer pressure 35 psig, vaporizer temperature 200 °C, capillary voltage 4000 V).

2.8 In vivo residence time and local tolerance of clobetasol-17-propionate loaded patches in minipigs.

All animal studies were conducted at CiToxLAB Scantox A/S (Lille Skensved, Denmark) in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use and European Medicine Agency guidelines; (EMA/CPMP/ICH286/1995, December 2009; CPMP/ICH/384/95, June 1995 and CPMP/SWP2145/00, March 2001). Six female Göttingen SPF minipigs (Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark) weighing 12-18 kg were used. A phase 0 study was conducted to determine experimental residence time. Patches were applied to the cheek of three anaesthetised (1 ml/10 kg body weight of Zoletil 50®Vet; Virbac, France) minipigs and the patches visually examined for patch detachment for up to 240 minutes. Residence time was recorded as the time when the patch had completely detached from the mucosa. In phase 1 and 2, patches were applied to each cheek of six anaesthetised minipigs randomised to one of two study groups for treatment with either 5 or 20 µg clobetasol-17-propionate-loaded mucoadhesive patches. To determine local systemic tissue pharmokinetics (phase 1), 3 ml blood samples were collected prior to patch application and also at 30, 60, 120 and 240 minutes (the time of patch removal) and additionally at 360 minutes post patch application (2 hours after patch removal). Samples were

centrifuged (10 minutes at 1600 g, 4°C) and plasma removed and stored at -70°C prior to analysis. To determine local systemic tissue pharmokinetics (phase 2), after an eight-day washout period, patches were applied and two tissue biopsies (8 mm biopsy punch) taken from each patch application site at 30, 60, 120 and 240 hours post application. Biopsies were weighed, snap frozen and stored at -70°C prior to analysis. Clobetasol-17-propionate concentrations in plasma and biopsy samples were determined using protein precipitation followed by solid phase extraction, evaporation and reconstitution with analysis of the supernatant by LCMS/MS using multiple reaction monitoring; data are expressed as µg/biopsy.

2.9 Data analysis

Results are presented as mean ± standard deviation unless otherwise stated. ANOVA with the Tukey multiple comparisons post-hoc test was used to compare differences between groups. Statistical analysis of all data was carried out using Graphpad prism version 7.0 (Graphpad software Inc., San Diego, California, USA) and results were considered statistically significant if $p<0.05$. All experiments were conducted at least in triplicate.

3. Results

3.1 Mucoadhesive characteristics and evaluation of mucosal toxicity of the placebo patch

Mucoadhesive patches were manufactured by electrospinning PVP (10 wt%), RS100 (12.5 wt%) and PEO (Mw 2,000 kD; 20 wt%) to yield a patch with final dry mass ratio of 1:1.25:2 for PVP:RS100:PEO with a PCL backing layer to create a dual-layer system. Patches assessed from 3 different batches were observed to have uniformity of mass with an average weight of 55.3 ± 5.18 mg (Figure 1A) and an average thickness of 0.43 ± 0.028 mm (Figure 1B). The values for surface pH were consistently in the range of 8.2 ± 0.38, close to that of saliva, indicating that the patches are suitable for application to the oral mucosa (Figure 1C). The degree of swelling was rapid with the patches taking on 50% of their weight within 3 minutes followed by a steady swelling rate up to one hour, when the patch had increased in weight by 65%. (Figure 1D). SEM images revealed a smooth PLC backing layer that was tightly adherent to the mucoadhesive layer, which displayed electrospun fibres homogeneous in number, diameter and alignment (Figure 1E).

Before testing in a volunteer human study, cytotoxicity of the placebo patch was evaluated in tissue-engineered models of the oral mucosa following OECD guidelines. MTT analysis revealed that the placebo patches did not reduce viability compared to the media only control after a 1 hour incubation period and can therefore be classified as non-irritant according to OECD guidelines (Figure 1G). This data was supported further by histological examination of the tissue-engineered mucosal models that revealed no damage or loss of integrity of the epithelium after incubation with the patches (Figure 1H).

3.2 In vivo mucoadhesive performance and acceptability of the placebo patch

In vivo residence time and patch acceptability was assessed in 26 healthy adult volunteers (15 male 11 female) aged between 21 and 64 years (mean 34 ± 3.8); all volunteers were non-smokers. Residence time was recorded for three locations within the oral cavity; upper labial gingiva, lateral border of tongue and buccal mucosa (Figure 2A-C) to a maximum of 120 minutes. Residence times were highest for the gingival applied patches followed by those on the buccal mucosa with 96% and 46% of patches remaining adherent for the full 120 minutes, respectively. No patches remained attached to the tongue for the full 120 minutes. Average residence times were 118 ± 5, 43 ± 26 and 96 ± 26 minutes for gingiva, tongue and buccal mucosa, respectively (Figure 2D). In terms of participants' perception of the patch, 96% of volunteers responding positively with good, very good or excellent when asked to rate the overall adherence of the patches and over 88% of volunteers felt little or no irritation whilst wearing the patches (Table 1).

With regards to patch specifics, 88% of volunteers thought the size of the patches were appropriate with over 65% stating that they thought the patch appearance was good/very good or excellent. All volunteers agreed that the patches had none or a weak taste that was neither pleasant nor unpleasant. Over 85% of volunteers thought that the method of application was acceptable and that removal was easy. The majority of volunteers (>70%) stated that overall the patches were not bothersome to wear on the gingiva and buccal mucosa but the tongue was more bothersome with 53% finding it moderately so. Some participants (23%) reported moderate or somewhat interference with speech, although over 60% stated only minor effects on saliva production and swallowing. 84% of volunteers responded positively stating that they would be willing to wear the patch twice-a-day to treat an oral lesion if required (Table 1).

3.3 Physiochemical characterisation of clobetasol-17-propionate loaded mucoadhesive patches

Clobetasol-17-propionate-loaded patches, assessed from 3 different batches, were observed to have an average weight of 67.4 ± 5.1, 59.0 ± 3.7 and 53.0 ± 3.2 mg for the 1, 5 and 20 μg clobetasol loaded patches, respectively; weight differences were not significant (Figure 3A). Average thickness of the patches was 0.51 ± 0.05, 0.36 ± 0.02 and 0.45 ± 0.032 for the 1, 5 and 20 μg clobetasol loaded patches, respectively (Figure 3B). The values for surface pH were consistently between 8.0 and 8.1 for the different clobetasol-17-propionate concentrations (Figure 3C).

The degree of swelling for the clobetasol17-propionate loaded patches was slightly slower, although not-significantly, than for the placebo patches with the patches taking on 50% of its weight within 24 minutes for the 1 μg patch and 14 minutes for both the 5 and 20 μg patches. All patches increased in weight to approximately 70% of their own weight within 60 minutes (Figure 3D). SEM images revealed no change in ultrastructure with the addition of clobetasol-17-propionate with the electrospun fibres remaining homogeneous in alignment, diameter and number (data not shown).

The DTA curve for clobetasol-17-propionate shows a clear peak at 226°C, which corresponds to the melting point for clobetasol-17-propionate (www.drugbank.ca/drugs/DB01013). The curves of the electrospun membranes did not present a peak at this location but both materials presented a peak at 73°C that is not present in clobetasol-17-propionate alone (Figure 3E). Both electrospun materials produced very similar XRD patterns. The pattern produced by clobetasol-17-propionate alone showed several peaks, evidence of a significantly more crystalline structure, peaks that were absent in the pattern of the electrospun material containing 2.31 wt% of drug (Figure 3F), suggesting that the clobetasol-17-propionate is in an amorphous form within the electrospun fibres.

3.4 In vitro drug dissolution

No difference was observed in the clobetasol-17-propionate release profile from patches loaded with 1, 5 or 20 µg of the drug. All the drug loaded patches slowly released the clobetasol-17-propionate in a sustained manner over a 6 hour period with approximately 20%, 50% and 80% released after 30, 180 and 360 minutes, respectively (Figure 4A). Reproducibility between batches was high with no difference in the percentage of clobetasol-17-propionate propionate released observed between two independently manufactured 5 µg patches (Figure 4B).

3.5 In vitro drug loaded patch cytotoxicity and in vitro and ex vivo drug permeation analysis

Clobetasol-loaded mucoadhesive patches were applied to the epithelial surface of a tissue-engineered oral mucosa for one hour (Figure 5A) and then mucosal equivalents tested for cytotoxicity using the OECD irritancy assay. There was a small but non-significant reduction in tissue engineered mucosal viability to 76.8 ± 10.3, 71.2 ± 18.4 and 74.6 ± 24.4 for the 1, 5 and 20 µg patches, respectively, which is above the 50% threshold and therefore considered to be a non-irritant in accordance with the OECD guidelines (Figure 5B). In addition, histological analysis revealed no epithelial damage after application and removal of the clobetasol-17-propionate-loaded patches compared to placebo controls (Figure 5C).

To ascertain drug release and permeation in physiologically relevant tissues, tissue-engineered oral mucosal equivalents and *ex vivo* porcine mucosa were employed. Drug permeation in to the tissue-engineered oral mucosal equivalents was assessed after a one hour incubation period by tissue homogenization followed by HPLC analysis. The amount of clobetasol-17-propionate found in the epithelium increased as the initial loading concentration increased with 66, 121 and 312 nM/mg detected in the epithelium after 1 hour (Figure 5D). Interestingly, clobetasol was only detected in the receptor medium when a 20 µg patch was applied to the epithelium, the amount detected was low at 16 nM (data not shown).

Clobetasol-17-propionate permeation into *ex vivo* porcine mucosa was also investigated for three different doses (1.25, 5 and 25 µg) but for a longer time period of three hours. HPLC analysis revealed that the drug was able to permeate into porcine buccal mucosa in a dose-dependent manner with significantly ($p<0.01$) more clobetasol-17-propionate delivered into the mucosa for the 25 µg patch (1484 ± 690.8 µg/g of patch) than for the 1.25 and 5 µg patches (124 ± 63 and 237 ± 68 and µg/g of patch respectively) (Figure 5E).

3.7 *In vivo residence time and local physiochemical permeation of clobetasol-17-propionate in mini-pig mucosa*

In vivo adhesion to the buccal mucosa for the clobetasol-17-propionate patch (5 µg) showed an average residence time of 184 ± 45 minutes in mini-pigs (Figure 6A). Local tissue physiochemical analysis revealed that clobetasol-17-propionate permeation into mini-pig buccal mucosa for the 5 µg patch was low (~10 ng/biopsy) after 30 minutes that was sustained for up to 240 minutes. In contrast, release from a 20 µg patch was significantly greater ($p<0.01$) after 30 minutes. However, levels of clobetasol-17-propionate released into the oral mucosa then declined and were not significantly different to the 5 µg patch at later time points (Figure 6B). Plasma analysis revealed that systemic exposure was below the level of detection (20 pg/ml) at the time points investigated (up to six hours).

Discussion

Oral lesions, including those such as OLP and RAS, are prevalent in society and can impart a significant burden on quality of life. These lesions are usually treated using topically applied corticosteroids but current drug delivery systems are inadequate and new ways of delivering these therapeutic agents directly to lesions are required. Controlled delivery of drugs to the oral mucosa is challenging because of moist mucosal surfaces, salivary flow and abrasive forces within the oral cavity. To overcome these obstacles we recently developed an innovative dual-layered electrospun mucoadhesive patch [14]. Here, we expand this work and report the first use and acceptability of our optimised, drug-free electrospun mucoadhesive patch in humans. We also show drug loading and both *in vitro* and *in vivo* drug release profiles of these dual-layer patches.

The use of electrospun nanofibers manufactured from a variety of polymers is becoming increasingly popular as a way of improving adhesion of patches to biological surfaces and to control drug release. This is because electrospun nanofibers have increased surface area, high porosity and are amenable to incorporation of bespoke polymer characteristics compared to current film formulations [28]. We recently developed a complex mucoadhesive electrospun dual-layer system comprised of FDA approved polymers that consists of a bioadhesive layer containing hybrid PVP, Eudragit®RS100, PEO nanofibers and a hydrophobic protective backing layer made from thermally-treated PCL nanofibers [14]. These patches show a high level of consistency for weight, thickness and nano-fibre structure. In addition, the pH of the patches was ~ 8.2, slightly more alkali than that of saliva (pH 5.6–7.9) but deviation not significant enough for these patches to cause irritation or cytotoxicity.

Nano-fibre swelling is a crucial property for bioadhesion. Successful mucoadhesion of electrospun patches critically relies upon the rapid hydration and subsequently gelation of the nano-fibres at the moist mucosal surface [29]. Our electrospun patch displayed extremely quick and sustained swelling over 60 minutes, a profile suitable for rapid and prolonged mucoadhesion. Indeed, when applied with gentle finger pressure, our malleable electrospun patches adhered rapidly to human gingival and buccal mucosa, and tongue epithelium, common sites for OLP and RAS lesions. *In vivo* residence time, recorded for up to 120 minutes, in human volunteers with healthy mucosa was longest for gingivae (118 minutes) then buccal mucosa (93 minutes) and then tongue (43 minutes); data that suggest adhesion strength is linked to the tissue-specific mechanical stresses or degree of epithelial keratinisation. Very few studies have examined the adhesion of electrospun patches to human oral mucosa *in vivo*. Although, Samprasit *et al* showed rapid swelling properties of their thiolated-chitosan sulphate (CS) and polyvinyl alcohol (PVA) blended electrospun patches and adhesion to *ex vivo* porcine mucosa; these patches only achieved a residence time of 5 minutes when applied to

human buccal mucosa [11] and suggest that the polymer blend as well as increased surface area provided by electrospinning is critically important for adhesion to human mucosa. Several similar human *in vivo* adhesion studies have been performed using adhesive films comprised of various polymer formulations and blends where different degrees of *in vivo* residence times have been observed, with times being either comparable to or below those presented in this study [30-32]. The adhesion studies described herein were performed in the absence of food or water intake. Although we have no empirical evidence, it is possible that the consumption of food or water whilst wearing the oral patch may reduce its adhesiveness and therefore impact on drug release. Therefore, we envisage that individuals using these patches will be asked to refrain from food and liquid intake for the duration of treatment.

Overall perception of the adhesiveness of our electrospun patches from healthy volunteers was rated as good, very good or excellent with the majority stating that the patches were appropriately sized, had an acceptable appearance and displayed either no taste at all or a weak neutral taste. Moreover, the majority of volunteers did not feel that the patches interfered with their speech, saliva production or swallowing, indicating that our patches are highly acceptable for human use.

The best treatment for many oral lesions remains use of topical corticosteroids, with clobetasol-17-propionate arguably showing greatest efficacy [19-22]. Clobetasol-17-propionate has been successfully incorporated into other polymer nanosystems including lecithin/chitosan nanoparticles [33] polymer-coated nanocapsules [34] lipid nanoparticles [35] but these systems are all aimed at drug delivery to skin. Therefore, we chose to incorporate clobetasol-17-propionate within the electrospun adhesive layer of our patches as the pharmacologically active agent for oral delivery.

Addition of clobetasol-17-propionate to the patches had no effect on any of the physiochemical properties investigated including weight, thickness, pH and swelling index. Both XRD and DTA analysis show that within electrospun patches the clobetasol-17-propionate is in an amorphous rather than crystalline state. Similar observations have been reported for a number of electrospun polymer combinations containing a myriad of agents such as the anti-microbials clotimazole [15] and α-mangostin [11], non-steroidal anti-inflammatory drugs, ibuprofen [36] and aceclofenac [37] and the corticosteroid budesonide [38]. In contrast, Vacanti *et al* and Hsu *et al* both observed that the corticosteroid dexamethasone remained in the crystalline state in their electrospun polymer systems [39, 40], suggesting that either not all corticosteroids will convert to the amorphous state or, more likely, that the polymer blend and manufacturing conditions are crucial for this process to occur. It is well appreciated that the amorphous state of a compound possesses several advantages including enhanced solubility and increased dissolution rate to its crystalline counterpart, therefore the

presence of the amorphous form of clobetasol-17-propionate in our electrospun system offers a distinct advantage for increased drug delivery.

The selected doses of clobetasol-17-propionate used in this study were intended to replicate the current dosing regimens of gels and creams used in the topical delivery for treatment of dermal inflammatory disease. Dermal dosing typically is imprecise, based on the fingertip-unit that is equivalent to 0.4-0.5 g covering 100-150 cm^2. Current formulations for dermal use contain 0.05% clobetasol-17-propionate, which once applied as a fingertip unit, results in approximately 1.33-2.5 $\mu g/cm^2$. To replicate this dosage, 3.1 cm^2 patches were fabricated with 0.0004%, 0.002% or 0.008% clobetasol-17-propionate to create patches that contained a total drug content of 1, 5 and 20 μg/patch, respectively.

In vitro release profiles of patch-loaded clobetasol-17-propionate demonstrated fast but sustained release with approximately 80% of the drug liberated within 360 minutes. The polymer composition of electrospun mats or patches is crucial in determining drug release kinetics. Dott *et al*, showed that *in vitro* release of the antihistamine diphenhydramine by PVA electrospun patches was rapid with 86% released after 3 minutes [17]. Similarly, Vacanti *et al* showed that 50% of dexamethasone was release from PCL electrospun fibers *in vitro* after 20 minutes and 100% after 90 minutes, whereas release of this steroid was much slower with poly(L -lactic) acid fibers with 100% being released after 1 month [39]. Rapid, *in vitro* burst release drug profiles have also been observed for CS/PVA single or blended electrospun fibres [11, 15, 41]. The initial burst release is not only related to the physicochemical properties and concentration of the drug but also polymer formulation of the electrospun fibres [28]. Indeed, Kathikeyan *et al* showed that addition of Eudragit RS100 to zein electrospun nanofibres significantly prolonged release of aceclofenac by several hours compared to zein alone nanofibres [37], implying that inclusion of Eudragit RS100 in our electrospun fibre polymer blend allows for improved sustained *in vitro* drug release compared to previous drug-loaded electrospun systems.

Tissue engineered models of the oral mucosa are increasingly being used as surrogate models to assess tissue irritancy, toxicity and transepithelial drug delivery [42]. Application of clobetasol-17-propionate-loaded electrospun patches containing up to 20 μg/ml did not show any toxic or irritant effects on tissue engineered oral mucosal models as assessed using the OECD irritancy test and by histological examination of tissue, suggesting that even relatively concentrated forms of clobetasol-17-propionate do not cause tissue damage on contact with the epithelium. Moreover, tissue

profiling for clobetasol-17-propionate content by HPLC in both *in vitro* tissue engineered and *ex vivo* porcine mucosa show a dose-dependent release of steroid into the tissue, with the 20 μg/ml clobetasol-containing patch showing the greatest release into these tissue. Quicker drug release was obtained using PVA electrospun patches containing diphenhydramine on *ex vivo* porcine mucosa where 78% of drug permeated the mucosal tissue within 3 minutes [17]. In contrast, sumatriptan (a drug used in the treatment of migraine)-loaded PVA electrospun porcine sublingual drug delivery was just 1%, whereas PCL or CS electrospun patches loaded with the non-steroidal anti-inflammatory drug Naproxen were able to release up to 50% of their cargo to the sublingual mucosa within 5 hours [41]. Once again these data show that both the polymer nanofibre blend as well as physiochemical properties of the drug are essential for efficient mucosal drug delivery.

Finally, we applied clobetasol-17-propionate-loaded electrospun patches to mini-pig buccal mucosa as an *in vivo* model of drug delivery. Interestingly, *in vivo* buccal residence time in minipigs was similar to that observed in humans. Here, marked levels of clobetasol-17-propionate were detected in the mucosal epithelium after just 30 minutes application using the 20 μg/ml loaded patch where upon levels declined by 60 minutes but remained constant for up to 240 minutes. Although these data may not be directly related to the human setting since porcine mucosa epithelium is 3 times thicker than in humans [43], they clearly show release of steroid from the electrospun patch into the epithelium *in vivo*.

Previous studies examining the delivery of clobetasol-17-propionate to the dermis using a tape-strip pig ear model showed that the steroid was retained in the stratum cornea with little present in the rest of the epithelium [44, 45]. Since the buccal oral mucosa does not possess a stratum corneum, it is likely that the clobetasol will pass without hindrance into the entire oral epithelium. In support of this we did not observe substantial retention of clobetasol-17-propionate in the mucosa over time in our mini-pig *in vivo* studies. A further reason for the disappearance of clobetasol17-propionate from the mucosa may be due to its metabolism into undetectable metabolite forms by xenobiotic cytochrome p450 enzymes that are likely to be expressed in the epithelium [46, 47].

One of the main risks with using long-term, highly potent corticosteroid therapy is the potential for these compounds to induce suppression of the hypothalamic-pituitary-adrenal (HPA) axis if high plasma levels are maintained. It is difficult to determine maximal dose ranges due to person-to-person variability, and although there is currently no cut-off concentration for clobetasol-17-propionate, data suggest that dosages as low as 25 g of 0.05% cream applied to the skin per week may affect the HPA axis [48]. The serum absorption of 0.05% clobetasol-17-propionate-containing emulsion on normal skin was previously found to be between 1 to 6 ng/ml [49], suggesting that

topical delivery of clobetasol-17-propionate may reach serum levels that could potentially cause off-target effects. The oral mucosa is more permeable than skin and so up-take is likely to be greater for oral delivery. Indeed, Varoni *et al* observed that patients with oral lesions taking long term 0.05% clobetasol-17-propionate treatment (ointment or within hydroxyethylcellulose gel) had serum levels of around 1.5 ng/ml potentially placing them at high risk [23]. However, in a volunteer study, these authors showed that although clobetasol-17-propionate was able to pass more quickly through damaged than healthy oral mucosa when applied topically (0.05% in 4% hydroxyethylcellulose gel), the serum levels of the drug were just 0.2 ng/ml. We could not detect clobetasol-17-propionate in serum samples taken from mini-pigs wearing clobetasol-loaded patches (20 μg) applied to the buccal mucosa over 4 hours, and although this needs to be confirmed in humans, these data suggest that electrospun patch-delivered clobetasol-17-propionate will not affect the HPA axis.

While the high surface area: volume ratio of electrospun fibres is a potentially attractive feature for site specific drug delivery, this approach is not possible without adhesion to the mucosal surface. Indeed, the moist environment in the human mouth presents a major challenge that, until now, has prevented the successful direct delivery of drugs to oral lesions via adhesive devices. The data presented here demonstrates that the combination of drug loaded electrospun fibres with a hygroscopic polymer facilitates long term adhesion that leads to successful local delivery of a potent steroid. This work therefore demonstrates the utility of a new class of adhesive devices to address the challenge of local drug delivery to mucosal surfaces including within the oral cavity. It is predicted that these devices have the potential to introduce a step change in improved healthcare in oral medicine, and clinical evaluation is strongly recommended.

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Author Contributions

Helen E. Colley, Martin E. Santocildes-Romero, Jens Hansen, Paul V. Hatton, Lars Siim Madsen, Sarah Baker and Craig Murdoch conceived and designed the research. Martin E. Santocildes-Romero, Helen E Colley, Zulfahmi Said and Katy D'Apice performed the experiments, analysed the data, conducted statistical analysis and interpreted the results. The manuscript was written and figures prepared by Helen E. Colley and further edited by Craig Murdoch, Martin E. Santocildes-Romero, Martin H. Thornhill, Paul V. Hatton, Lars Siim Madsen, Sarah Baker and Jens Hansen. Jens Hansen and Lars Siim Madsen contributed essential reagents. Jens Hansen, Martin H. Thornhill, Paul V. Hatton, Craig Murdoch and Helen E. Colley contributed essential expert knowledge. All authors are aware of the content and have read and edited the manuscript.

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Figure Legends

Figure 1: Mucoadhesive placebo patch characteristics and evaluation of mucosal toxicity. Electrospun mucoadhesive placebo patches were characterised from three different batches for (A) weight, (B) thickness, (C) pH and (D) swelling (n=8). Scanning electron microscope micrographs of the (Ei) PCL backing layer, (Eii) a cross section of the patch showing adherence of the impermeable PLC (lower most layer) backing layer to the underlying mucoadhesive layer (upper most layer; PVP 10 wt%, RS100 12.5 wt% and PEO 20 wt%) (Eiii) with mucoadhesive layer fibres homogeneous in diameter and alignment. (F) Cytotoxicity testing of the placebo patch using tissue-engineered oral mucosa equivalents revealed that they do not cause cytotoxicity compared to media only controls (SDS treatment used as positive control). Histological examination also confirmed that there was no evidence of damage or loss of integrity to the epithelium after (Gi) a 1 hour incubation period compared to (Gii) media only control. The swelling data is presented as mean ± SEM. n=6 Scale bars= 20 and 100 μm.

Figure 2: *In vivo* mucoadhesive performance of placebo patch. Mucoadhesive patches were placed on the (A) gingiva, (B) lateral tongue or (C) buccal mucosa of healthy human volunteers for 5 seconds with applied pressure and (D) residence time measured every 10 minutes for up to 2 hours. Volunteers' responses when asked to rate the perception of overall (E) patch adherence and (F) irritation to the mouth (n=26).

Figure 3: Clobetasol-17-propionate loaded patch characterisation. Electrospun mucoadhesive patches loaded with clobetasol-17-propionate (1, 5 and 20 μg) were characterised from three different batches for (A) weight, (B) thickness, (C) pH and (D) swelling (E) Differential thermal analysis and (F) X-ray diffraction patterns of soluble clobetasol-17-propionate, a placebo patch and a clobetasol loaded patch. The weight, thickness and pH data is presented as mean ± SEM (n=8) and the swelling data is presented as mean ± SEM (n=5).

Figure 4: Dissolution of clobetasol-17-propionate from the mucoadhesive patches. (A) Clobetasol-17-propionate dissolution from patches loaded with differing concentrations of the drug (1, 5 and 20 μg) revealed a sustained release profile over a six-hour period. (B) Reproducibility between

manufacturing batches was high with no difference in the percentage of clobetasol-17-propionate released observed between the patches.

Figure 5: Cytotoxicity and *in vitro/ex vivo* clobetasol-17-propionate permeation into oral mucosa. (A) Cytotoxicty testing of the patches using tissue-engineered oral mucosa equivalents using a MTT assay (B) revealed that the although the drug loaded patches reduced viability by approximately 25% they were not considered cytotoxic and histological examination confirmed that there was no evident damage or loss of integrity to the epithelium after an one hour incubation period from either the (Cii) 1 µg, (Ciii) 5 µg or (Civ) 20 µg when compared to (Ci) placebo patch. Clobetasol-17-propionate levels extracted from (D) tissue-engineered oral mucosal equivalents or (E) *ex vivo* porcine oral mucosa determined using HPLC after a one or three hour adhesion period of the drug loaded patches (1, 5 and 20 µg or 1.25, 5 and 25 µg), respectively (n=4) ** $p<0.01$. Scale bar = 100 µm.

Figure 6: *In vivo* residence time and local tissue release of clobetasol-17-propionate patches. (A) Average residence time of clobetasol-17-propionate loaded patches to the buccal mucosa in minipigs over a 4 hour time period (n=3). (B) Clobetasol extracted from the buccal mucosa (ng/biopsy) of minipigs after 30, 60, 120 and 240 minutes from 5 µg (B) and 20 µg (C) loaded patches with a surface area of 3.12 cm^2 (n=6).

Table 1: Response of healthy human volunteers to various parameters of the patches.

Supplementary figure 1: Inclusion and exclusion criteria for the human volunteer study.

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