

Title: The effect of ingredients commonly used in nasal and inhaled solutions on the secretion of mucus in vitro.

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Abstract:

Hypersecretion of mucus is associated with impaired mucociliary clearance that can influence the retention of active pharmaceutical ingredients in the airway but is also linked with recurrent airway disease. Therefore, the effect on mucin secretion of a range of ingredients used in solutions delivered to the nose and lung was studied. Mucin secretion from explants of ovine epithelium was quantified using an enzyme-linked lectin assay (ELLA) or sandwich ELLA depending on the compatibility of the ingredients with the assay. Benzalkonium chloride (0.015% w/w), Methocel™ E50 premium LV (1.0% w/w), propylene glycol (1.5% w/w), potassium sorbate + propylene glycol (0.3% w/w + 1.5% w/w) and polysorbate 80 (0.025% w/w), used at common working concentrations, all increased the secretion of mucin from the explants ( $P < 0.05$ ). Ethylenediamine tetraacetic acid-disodium salt (EDTA) (0.015% w/w), Avicel® RC591 (1.5% w/w), fluticasone furoate (0.0004% w/w, concentration in

solution) and dimethyl sulfoxide (DMSO) (0.2% w/w) did not affect mucin secretion. Compounds increasing mucin secretion could alter the rate of mucociliary clearance and the mucus could provide a barrier to drug absorption. This could predispose patients to disease and affect the activity of delivered drugs, decreasing or increasing their clinical efficacy.

Key words:

Mucus

Avicel® RC591

Benzalkonium chloride

Dimethyl sulfoxide (DMSO)

Ethylenediamine tetraacetic acid-disodium salt (EDTA)

Fluticasone furoate

Methocel™ E50 premium LV

Polysorbate 80 (Tween 80)

Potassium sorbate

Propylene glycol

Nasal drug delivery

Enzyme-linked lectin assay

Abbreviations:

API – active pharmaceutical ingredient

ASL – airway surface liquid

ATP – adenosine triphosphate

BKC – benzalkonium chloride

CBF – ciliary beat frequency

DMEM – Dulbecco's Modified Eagle medium

ELLA – enzyme-linked lectin assay

EDTA - ethylenediamine tetraacetic acid-disodium salt

FF – fluticasone furoate

HRP-HPA – horseradish peroxidase-labelled *Helix pomatia* agglutinin

IN – intranasal

LLOQ – lower limit of quantification

MC – mucociliary clearance

MEM – Minimum Essential medium

PBS – phosphate buffered saline

PG – propylene glycol

KS – potassium sorbate

PS 80 – polysorbate 80

S-ELLA – sandwich enzyme-linked lectin assay

## 1. Introduction

The epithelium of the nose and upper airway is directly exposed to the external environment and is therefore constantly at risk of exposure to infectious and noxious agents. The primary innate defence mechanism that protects the airways against inhaled environmental stimuli is mucociliary clearance (MC). Airway surface liquid (ASL) and cilia, the functional elements of the mucociliary system, integrate efficiently to eliminate debris laden mucus to the gastrointestinal tract by swallowing or to the external environment by expectoration. The efficiency of this defence mechanism depends on the ciliary input and the amount, depth, composition and viscoelastic properties of the ASL (composed of the mucous gel and the underlying periciliary fluid) (Chilvers and O'Callaghan, 2000; Houtmeyers et al., 1999b; Lansley, 1993; Merkus et al., 1998).

Intranasal (IN) administration provides a non-invasive route of drug delivery. Therapeutic agents delivered to the nasal cavity act locally, in the case of local conditions such as allergic rhinitis. They can also act systemically or provide a direct route to the brain. MC clears the drug formulations and undesirably decreases their residence time. This can limit the duration of action of drugs intended for a local effect and the time available for absorption for those drugs intended to act systemically or in the brain, reducing efficacy. Any adverse effect of the formulation on MC could compromise its ability to clear inhaled substances from the airways and leave the individual susceptible to disease e.g. respiratory tract infections, inflammation and damage. For example, mucociliary transport is compromised in allergic rhinitis (AR), and this predisposes patients to other respiratory diseases (Guan et al., 2018; Kirtsreesakul et al., 2009; Schuhl, 1995; Vlastos et al., 2009). It is

therefore important to understand the effect of IN and pulmonary formulations on MC.

IN formulations can be screened for their effect on MC by measuring their effects on individual components of the mucociliary apparatus such as ciliary beat frequency (CBF), mucus viscoelasticity, mucus secretion or by observing their effects on mucociliary transport rate as a whole, either in-vivo or ex-vivo. The effect of a number of drugs and excipients administered IN on MC and CBF has been studied widely and is the subject of a recent review (Jiao and Zhang, 2019). However, there is a lack of data on the effect of IN formulation variables on the secretion of mucus. The aim of this study was to gain an understanding of how the ingredients of nasal sprays could affect the secretion of mucus within the nasal cavity.

Mucin secretion studies have traditionally relied on in-vitro, in-situ or ex-vivo experimental models. Intact parts of a respiratory organ (e.g. ferret trachea (Abanses et al., 2009; Liu et al., 1999) and human bronchi (Roger et al., 2000a)) or explants of the surface epithelium of a respiratory organ (e.g. human turbinates (Lethem et al., 1993), canine tracheae (Davis et al., 1992) and ovine tracheae (Clancy et al., 2004) are typical models. The former are used to study mucins emanating from surface epithelial secretory cells and submucosal glands. While explants are used to study mucins released solely from the surface epithelial secretory cells (goblet cells) (Davis, 2002). The use of mucin-secreting cell culture models is an area of research that bears much potential (Lechanteur et al., 2018). Both human bronchial epithelial primary cells (Abdullah et al., 2012) (Hill and Button, 2012; Kemp et al., 2004) and cell lines e.g., UNCN3T, Calu-3 and SPOC1 (rat) have successfully demonstrated mucin secretion (Abdullah et al., 2003; Abdullah and Davis, 2007; Abdullah et al., 1996; Fulcher et al., 2005; Lee et al., 2021; Randell et al., 1996). However the

culture and differentiation procedures for primary cultures are labour-intensive and cell lines rarely express motile cilia.

Owing to the scarcity of *in-vitro* cell culture models with a well-characterised mucociliary system (BéruBé et al., 2009), this investigation was conducted in sheep tracheal epithelial explants cultured on collagen-coated nitrocellulose permeable supports at an air liquid interface, which maintained mucociliary function. This model permits the measurement of surface epithelial mucin secretion (predominantly from goblet cells) without any interference from submucosal gland secretion. Further, as an accepted model of human mucociliary clearance, sheep are used to conduct *in-vivo* mucociliary measurements to study its response to therapeutic agents (Nickolaus et al., 2020; Sabater et al., 2005; Sabater et al., 1999).

A prerequisite for this investigation was a valid assay that could quantify the amount of mucins secreted from ovine tracheal explants in the presence of the pharmaceutical ingredients of interest.

An enzyme-linked lectin assay (ELLA) was chosen to quantify mucin secretion (Argüeso and Gipson, 2006; Harrop et al., 2012; McGuckin and Thornton, 2000). ELLAs rely on the binding of mucins, which act as antigens, to lectins, which are established probes in the analysis of glycoconjugates (Thompson et al., 2011). In direct ELLAs, detection and quantification is achieved by labelling the lectins with fluorophores or enzymes that act on substrates to produce a measurable colour. In indirect ELLAs, labelled secondary conjugates (e.g. streptavidin) are employed to bind lectin-ligand conjugates (e.g. lectin-biotin). ELLAs are generally highly sensitive and selective quantitative methods of mucin analysis that can handle

large numbers of samples, although they often require optimisation of conditions (Harrop et al., 2012).

Initially, it was intended to use the ELLA employed by Clancy et al. (Clancy et al., 2004). However, some of the studied IN excipients were found to interfere with the assay rendering it invalid for measuring mucins in samples containing these excipients. Thus, a sandwich ELLA was developed and validated to achieve the quantitative determination of airway mucins in matrices containing these excipients.

Benzalkonium chloride (BKC) (0.015% w/w), Methocel™ E50 premium LV (1.0% w/w), propylene glycol (PG) (1.5% w/w), potassium sorbate (KS) + PG (0.3% w/w + 1.5% w/w) and polysorbate 80 (PS 80) (0.025% w/w), used at common working concentrations, all increased the secretion of mucin from the explants ( $P < 0.05$ ).

EDTA (0.015% w/w), Avicel® RC591 (1.5% w/w), fluticasone furoate (FF) (applied in solution rather than in suspension and, therefore, at a lower than expected concentration of 0.0004% w/w) and DMSO (0.2% w/w) did not affect mucin secretion.

## 2. Materials and Methods

### 2.1 Preparation of epithelial explants

Airway epithelium was isolated from sheep trachea and explanted onto permeable nitrocellulose supports as described previously (Davis et al., 1992). Briefly, sheep tracheae were collected from a local abattoir and transported to the laboratory in Dulbecco's Modified Eagle medium (DMEM) (or Minimum Essential medium (MEM)) containing 1 U/mL penicillin and 1 µg/mL streptomycin (all PAA Laboratories, GE Healthcare, Buckinghamshire, UK). The posterior membrane was removed and each trachea was cut between and across the cartilage rings to give 3 cm<sup>2</sup> pieces of tissue. These were pinned onto a Sylgard® elastomer solid base and a sterile solution of collagenase (0.1 U/mL)/dispase (0.8 U/mL) (Roche Diagnostics Ltd., Burgess Hill, UK) in phosphate buffered saline (PBS) (Sigma Aldrich, Gillingham, UK) was then injected under the epithelium (into the lamina propria of the mucosa). The tissue preparation was then covered in a freshly prepared sterile MEM (or DMEM) containing 0.1 mg/mL DNase and 0.15 mg/mL dl-dithiothreitol (MEM+) (both Sigma Aldrich) and incubated at 37 °C in a 5 % CO<sub>2</sub> : 95 % air atmosphere for 45 minute. A coverslip was used to scrape the epithelial sheet from the submucosal layer. The sheet was suspended in fresh MEM+, cut into ~ 5-10 mm diameter pieces with fine scissors and floated (cilia uppermost) onto collagen-coated (PureCol Bovine Collagen, CellSystems GmbH, Troisdorf, Germany) nitrocellulose membranes (produced in-house). The explants were allowed to attach to the membrane at the air-liquid interface by overnight incubation at 37 °C in 5 % CO<sub>2</sub>:95 % air atmosphere. The culture medium beneath the explants was a 1:1 mixture of 3T3-conditioned medium and Ham's F12 medium buffered with 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (15 mM) (PAA Laboratories) and containing penicillin



(1 U/mL), streptomycin (1 µg/mL), insulin (10 µg/mL), endothelial cell growth supplement (7.5 µg/mL), apo-transferrin (5 µg/mL), hydrocortisone (36 ng/mL) and triiodothyronine (20 ng/mL) (all Sigma Aldrich). The explants were used experimentally after 18 hours.

## **2.2 Mucin secretion studies**

Discs (5-7 mm) of ovine tracheal epithelial explants were prepared, placed in warmed Ham's F12 medium and allowed to equilibrate at 37 °C in 5 % CO<sub>2</sub> : 95 % air for two hours. During this time, the medium was discarded and replaced with fresh warm (37 °C) medium every 20 minutes for the first hour and every 10 minutes during the second hour. Following equilibration, the medium was collected and replaced every 10 minutes to provide four samples collected under baseline conditions. The drug or excipient (Table 1) (kind gifts from GSK, R&D, Ware, UK) was applied to the explant in pre-warmed Ham's F12 medium (37 °C) and samples were collected and replaced at the end of each 10 minute period to provide four samples containing mucins collected under treatment conditions for each explant disc. Finally, each explant disc was exposed to warm (37 °C) Ham's F12 medium containing 100 µM adenosine triphosphate (ATP) (mucin secretagogue) (Roche Diagnostics Ltd.) for 3 x 10 minute periods, in which the medium was collected and replaced at the end of each 10 minute period to provide three positive-control samples. Baseline, treatment and positive-control samples were assayed for their mucin content using an enzyme-linked lectin assay (ELLA) or sandwich ELLA (S-ELLA)

## **2.3 Quantification of mucin secretion using an enzyme-linked lectin assay (ELLA) or sandwich ELLA (S-ELLA)**

Mucin secretion was either quantified using an ELLA or a S-ELLA. The S-ELLA was used when the substances under test interfered with the ELLA. During the course of the study, the availability of the lectin from the suppliers was not consistent necessitating further adaptation of the ELLA from a direct ELLA to an indirect ELLA. Each adaptation was optimised, and the mucin concentration of all samples was compared with a calibration of mucin standards run alongside each assay undertaken.

### **2.3.1 ELLA**

The ELLA was adapted from the procedure developed by Clancy et al. (Clancy et al., 2004). Briefly, 96-well high binding plates were loaded with 100 µl/well of sample containing mucin (the blank wells received Ham's F12 medium) in triplicate. The plates were then incubated either at 37 °C for 90 minutes or overnight at 4 °C then at 37 °C for 45 minutes. Each well was washed three times with PBS containing 0.05% w/v gelatin and 0.5% v/v Tween 20 (both from Sigma Aldrich) at 37 °C and then blocked by incubating with PBS containing either 0.1 % w/v gelatin (for assays subsequently using Horseradish-Peroxidase-labelled *Helix Pomatia* agglutinin (HRP-HPA) (Sigma Aldrich)) or 0.5% w/v gelatin (for assays subsequently using HRP-HPA (EY Laboratories, San Mateo CA) or HPA-biotin (Sigma Aldrich)) at 37 °C for 1 hour.

Following further washing, wells were loaded with 100 µl/well of HRP-HPA lectin (1.25 µg/mL in the appropriate block buffer) (direct ELLA). When the HRP-HPA lectins became no longer available, they were replaced with HPA-biotin lectin (100 µl/well of a 0.625 µg/mL solution) changing the ELLA from a direct to an indirect

ligand binding assay. The lectin-loaded plates were incubated at 37 °C for one hour. Following incubation with HPA-biotin (indirect ELLA), plates were washed and then loaded with 1.25 µg/mL streptavidin-HRP (Sigma Aldrich) in PBS containing 0.5% w/v gelatin (100 µL/well) and incubated at 37 °C for 70 minutes.

Finally, plates were washed and developed by adding 150 µl/well of the substrate o-phenylenediamine dihydrochloride solution (made up as per manufacturer's instructions (Sigma Aldrich)) and allowing the plate to stand in the dark for 12 minutes (reaction-time if the Sigma Aldrich HRP-HPA lectin was used in the previous step) or 3-4 minutes (reaction-time if the EY Laboratories HRP-HPA lectin or the streptavidin-HRP was used in the previous step). The reaction was stopped by adding 50 µl of 20% v/v sulfuric acid to each well and absorbance read at 492 nm using a Labsystems Multiskan Ascent microplate reader (MTX Lab Systems Inc., Vienna, VA).

### **2.3.2 Sandwich ELLA (S-ELLA)**

The S-ELLA was adapted from a variety of lectin-based sandwich assays (Bamrungphon et al., 2007; Jackson et al., 2002; Kemp et al., 2004; Kishioka et al., 1997; Parker, 1998; Shinogi et al., 2001). High binding 96-well plates were coated with unlabelled HPA lectin (0.5 µg/mL in PBS) (Sigma Aldrich) by overnight incubation (100 µL/well) at 4 °C followed by 45 minutes incubation at 37°C. Each well was then washed with PBS containing 0.05% w/v gelatin and 0.5% v/v Tween 20, applied warm at 37 °C, loaded with 150 µl block buffer (0.1 % w/v gelatin in PBS; at 37 °C) and incubated at 37 °C for 1 hour. After being blocked, the HPA-coated plates were washed and loaded (100 µl/well) with the mucin samples in triplicate

(Ham's F12 medium in the blank wells). The plates were then incubated overnight at 4 °C followed by 45 minutes incubation at 37 °C.

Following washing, each well was loaded with 100 µL HPA-biotin lectin (0.625 µg/mL in block buffer) and incubated at 37 °C for 1 hour. Following further washing, each well was loaded with 100 µl streptavidin-HRP (1.25 µg/mL in block buffer) (Sigma Aldrich) before being incubated at 37 °C for 70 minutes

Finally, the wells were washed and developed by adding 150 µl/well of the substrate o-phenylenediamine dihydrochloride solution (made up as per manufacturer's instructions). The plate was allowed to stand in the dark for 3-4 minutes before the reaction was stopped by adding 50 µl/well 20% v/v sulfuric acid. The absorbance of each well was then read at 492 nm on a Labsystems Multiskan Ascent microplate reader.

For ELLA and S-ELLA, absorbance was compared with known mucin standards purified by two rounds of density gradient ultracentrifugation (Carlstedt et al., 1983) from the sputum of a volunteer with chronic obstructive pulmonary disease. Informed consent was obtained. Ethical approval was obtained from the University of Brighton, School of Pharmacy and Biomolecular Sciences Ethics Committee, under reference PABSREC 0509.

Samples, as well as calibration standards, were assayed in triplicate on the same plate and a calibration curve was generated for each plate. Calibration standards (0.39 – 50 ng/well) were constructed in Ham's F12, which was spiked with the test compound when assaying the treatment samples containing this compound (to achieve the same matrix between the samples and the standards). The calibration

curves were subsequently used for the conversion of the samples' absorbance values into mucin content values (ng/well).

All excipients were prepared at their working formulation concentration in Ham's F12. Table 1 lists all the compounds tested and explains any special procedure used during their preparation. It also indicates which ELLA procedure was used to quantify the mucin secretion response to each compound.

## **2.4 Selectivity of the direct ELLA in the presence of IN Pharmaceutical**

### **Excipients and fluticasone furoate**

To assess any interference with the assay, calibration curves constructed using dilutions of the mucin standard (0.39 – 50 ng/well) made up in Ham's F12 medium were compared to those constructed using standard dilutions made up in Ham's F12 medium that was spiked with the excipients/active pharmaceutical ingredient (API) (each independently). The excipients were assessed in solution at their working formulation concentrations. FF is practically insoluble in water and was therefore assessed at a solution concentration of 0.0004% w/w rather than at its suspension concentration (Table 1). The analysis was performed in triplicate and the two calibration curves to be compared were run simultaneously on the same 96-well microplate.

## **2.5 Data Analysis**

Owing to the difference in the number of goblet cells present in each explant, the baseline mucin secretion varied a great deal between explants. Thus, to compare different explants, the mucin content (ng/well) of samples from each explant was expressed as percentage of the mean baseline mucin secretion of that explant (% baseline). The mean baseline mucin secretion is the average mucin content (ng/well)

of the pooled four mucin secretion measurements sampled following the four successive 10-minute exposures to Ham's F12 containing no test compound, which preceded the exposure of any one explant to any particular treatment. Meanwhile, the mean response mucin secretion is the average mucin content (ng/well) of the pooled four mucin secretion measurements sampled following the four successive 10-minute exposures to a particular treatment. Statistical analysis was conducted using GraphPad Prism software. The mean baseline mucin secretion value (in units of ng/well) was paired to the mean response mucin secretion value (in units of ng/well) of the same explant, and for each set of explants exposed to the same treatment, the paired data was compared using Wilcoxon matched-pairs signed rank test. On occasions of data proving to be parametric, a paired t-test was employed for comparison.  $P \leq 0.05$  was considered statistically significant.

Only data from valid (physiologically functioning) explants was considered in this study. A valid explant was defined as any explant demonstrating the ability to secrete stimulated mucins via a clear increase in mucin secretion (at least a 1.5-fold increase above the mean secretion in the period prior to exposure) in response to ATP or the test preparation (positive control). In addition, valid explants were required to demonstrate baseline variability that did not mask the clarity of the ATP-stimulated mucin secretion response. 12% of explants did not meet these criteria and were excluded. All explants considered in this study demonstrated a % relative standard deviation (RSD) of  $\leq 145$  % among the four baseline measurements.

### 3. Results

#### 3.1 Selectivity of the ELLA and S-ELLA to Airway Mucins in the presence of Intranasal Excipients and fluticasone furoate

Despite the ELLA demonstrating excellent assay characteristics concerning its range, the linearity of its response to mucin concentration over the 7 – 8 standard concentrations of the calibration curve, its lower limit of quantification (LLOQ), accuracy and precision (Table 2), it was not capable of detecting mucins in matrices containing many of the IN pharmaceutical ingredients. Figures 1 and 2 demonstrate the compatibility of the excipients/API with the ELLA by comparing calibration curves of mucin standards prepared in Ham's F12 to those prepared in Ham's F12 containing these pharmaceutical ingredients. The ELLA was suitable for use with BKC, PG, FF and DMSO at the concentrations studied as recovery of the mucins in the calibration standards prepared in these matrices was comparable to recovery when prepared in Ham's F12 alone ( $P > 0.05$ ) (Figure 1). KS + PG and ethylenediamine tetraacetic acid-disodium salt (EDTA) partially blocked the ELLA ( $P < 0.05$ ) and Methocel™ E50 premium LV and PS 80 completely blocked the ELLA ( $P < 0.05$ ) (Figure 2).

It was therefore evident that airway mucins could not be assayed using the direct ELLA in the presence of those pharmaceutical ingredients that fully blocked the assay. This prompted the development of a sandwich ELLA with the prospect of airway mucin quantification in the presence of these compounds. The S-ELLA also demonstrated excellent assay characteristics concerning its range, the linearity of its response to mucin concentration over the 7 – 8 standard concentrations of the calibration curve, its LLOQ, accuracy and precision that were comparable to the

standard ELLA (Table 2). In addition, it was able to detect mucins in matrices containing PS 80 and Methocel™ E50 premium LV (Figure 3) that previously caused full blockade of the direct ELLA. This indicated the S-ELLA was able to quantitate airway mucins in the presence of these IN pharmaceutical ingredients.

The standard ELLA was used in the presence of compounds that only partially blocked the assay. This was achieved by spiking the calibration standards with the compound under test at a concentration identical to that in the sample.

### **3.2 Effect of excipients and fluticasone furoate on mucin secretion**

BKC (0.015% w/v) increased mucin secretion in all explants studied (nine explants from three animals). Figure 4 (a - c) shows the response of three representative individual explants to BKC and demonstrates that the magnitude of the maximal response to BKC varied between explants (a maximal response between 1.6-fold and 16-fold above the mean baseline mucin secretion was observed). In addition, the exposure time required to induce that response was also variable among explants. This resulted in the observed level of variability in figure 4(d), which is intrinsic to biological systems. However, it had no impact on the significance of the reported increase in mucin secretion in response to BKC ( $P < 0.05$ ) as demonstrated by comparing the mean baseline mucin secretion of the individual explant to the mean response mucin secretion of the same explant in the nine tested explants (Figure 5). The variability of the response magnitude among explants can also be seen in this figure.

Other excipients causing a significant increase in mucin secretion were Methocel™ E50 premium LV (1.0% w/w), PG (1.5% w/w), KS+PG (0.3% w/w / 1.5% w/w) (Figure



5) and PS 80 (0.025% w/w) (Figure 6) ( $P < 0.05$ ). Similar to BKC, variation in the nature of the response was observed.

Methocel™ E50 premium LV (1% w/w) caused an increase in mucin secretion in all explants studied, with a maximal response ranging between a 1.6-fold and a 13-fold increase above the mean baseline secretion (Figure 5). This response was significantly different to baseline mucin secretion ( $P < 0.05$ ).

PG, (1.5% w/w) caused an increase in mucin secretion, with a maximal response between 2-fold and 20-fold above the mean baseline secretion, in six (out of seven) explants after 20 to 30 minutes exposure (i.e. at the 60 and the 70 minute time-points) to PG. This increase appeared to be transient as it declined at the 80 minute time-point in all six explants despite the continued exposure to PG. A paired analysis demonstrated that mucin secretion in response to PG was significantly different to baseline mucin secretion ( $P < 0.05$ ).

Due to solubility issues, KS (0.3% w/w) was tested in the presence of PG (1.5% w/w), which was used as a cosolvent. The combined effect of the two compounds on mucin secretion was studied in twelve explants from three animals (Figure 5). An increase in mucin secretion, with a maximal response between a 1.8-fold and a 24-fold increase above the mean baseline secretion, was observed in ten explants, which varied in the exposure time required before observing this maximal response. This response was significantly different to baseline mucin secretion ( $P < 0.05$ ). There was no significant difference between the effect of PG alone and in combination with KS ( $P > 0.05$ ).

The effect of PS 80 (0.025% w/w) on mucin secretion was studied in seven explants from three animals (Figure 6). An increase in mucin secretion, with a maximal

response between a 1.3-fold and a 3.2-fold increase above the mean baseline secretion, was elicited in all explants. This response, although lower than those described previously, was significantly different to baseline mucin secretion ( $P < 0.05$ ).

The remaining compounds studied, (EDTA (0.015% w/w), Avicel® RC591 (1.5% w/w), FF (0.0004% w/w), DMSO (0.2% w/w)) had no significant effect on mucin secretion ( $P > 0.05$ ). Certain trends were observed but the variability of the responses meant that no firm conclusions could be drawn.

EDTA (0.015% w/w) caused an increase in mucin secretion, with a maximal response between a 1.6-fold and a 9-fold increase above the mean baseline secretion, in six (out of eight) explants (Figure 6); this occurred at different time points following exposure to EDTA. While these data suggest that EDTA increases mucin secretion, the response was not significantly different to baseline mucin secretion ( $P > 0.05$ ).

The effect of Avicel® RC591 (1.5% w/w) on mucin secretion was variable. An increase in mucin secretion was observed in four explants (from two animals), with a maximal response ranging between 1.5-fold and 1.8-fold above the mean baseline secretion. However, no change or a decrease in mucin secretion was observed in the remaining explants (Figure 6). In comparing the mean response of each explant with itself, the response was not significantly different to baseline mucin secretion ( $P > 0.05$ ).

Similar variability was observed when FF (0.0004% w/w, concentration in solution) was applied to the explants (Figure 7). An increase in mucin secretion was observed in seven explants from three animals, with a maximal response ranging between 1.9-fold and 3.9-fold above the mean baseline secretion. While a decrease was

observed in three explants from a fourth animal. The response was not significantly different to baseline mucin secretion ( $P>0.05$ ).

FF was solubilised in DMSO to give a final DMSO concentration of 0.2% w/w.

Therefore, the effect of DMSO (0.2% w/w) on mucin secretion was studied in five explants from two animals (Figure 7). The response was variable and not significantly different to baseline mucin secretion ( $P>0.05$ ). Therefore, neither FF nor FF in combination with DMSO significantly altered baseline mucin secretion.

#### **4. Discussion**

The initial objective of the study was to establish an ELLA that was fit for purpose. A human mucin standard was used for the quantitative determination of ovine mucins as a marker of ovine airway mucous secretion. Due to species differences between mucins (Royle et al., 2008), this determination was a relative rather than a definitive quantitation, in which human mucins acted as a scalar for the relative measurement of ovine mucins. This is a common approach in the quantitation of physiological macromolecules when there is difficulty in obtaining the endogenous matrix (Lee and Hall, 2009).

Despite the abundance of ELLAs in the public domain (Bamrungphon et al., 2007; Jackson et al., 2002; Kemp et al., 2004; Kishioka et al., 1997; Piqué and De Servi, 2018; Shinogi et al., 2001), to date, no ELLA has been used in the presence of pharmaceutical excipients. As excipients can have a wide range of physicochemical properties, it is important to validate any assay with each ingredient. Major interference with the direct ELLA (using HRP-HPA lectin) occurred when matrices containing some of the IN excipients were analysed. It was not clear whether the interfering excipients were preferentially binding to the high-binding plates or whether they were capable of altering the mucin properties (e.g. solubilising it) and hence interfering with its ability to bind to the plates. Initially, the latter possibility appeared more likely, owing to the negatively charged nature of mucins (sulphated and sialylated O-glycan structure) with an affinity for positively charged molecules (electrostatic interactions), in addition to the hydrophobic and H-bonding interactions that could attract mucins to other molecules (Bansil and Turner, 2006; Harrop et al., 2012). However, as the issue of the interfering excipients was ultimately resolved using the sandwich ELLA, in which the mucins were captured by the unlabelled-

HPA-lectin for subsequent detection with the labelled-lectin, it seemed more likely that the excipients were preferentially binding to the plate in the direct ELLA precluding the retention of the mucins on the plate. Regardless of the mechanism, the sandwich ELLA was able to determine quantitatively airway mucins in the presence of the interfering IN pharmaceutical excipients, a prerequisite for studying the effect of these ingredients on airway mucin secretion.

All the ELLAs validated here were highly sensitive with an LLOQ of as little as 0.2 ng/well and a useful range of up to 50 ng/well in all the ELLAs (the average amount of mucin released was ~ 10 ng/well). The biotin-streptavidin detection system clearly enhanced the assay sensitivity owing to the high affinity between streptavidin and biotin (Holmberg et al., 2005), which is associated with signal amplification. It is worth noting that the concentration of the capture lectin (unlabelled HPA) in the sandwich ELLA should not be too high as this compromised the assay sensitivity. This was presumably because fewer binding sites on the mucins remained available to bind the labelled HPA lectin. The assays here also demonstrated adequate accuracy over the assay range using a linear function based on a log-log data transformation.

The sandwich ELLA procedure presented here is the first report, to our knowledge, of a sensitive and selective quantitative method used to analyse airway mucins in the presence of certain pharmaceutical excipients.

Mucins are secreted constitutively at a low basal rate in healthy airways, which increases in the presence of secretagogues. The secretagogue ATP was used as a positive control (Clancy et al., 2004; Roger et al., 2000b) in the current study and only explants responding to ATP were analysed.

BKC is a cationic surfactant, widely used as a preservative with proven efficacy in IN and ophthalmic formulations. It is also used in some inhalation solutions (e.g. Spiriva® Respimat®, Spiolto® Respimat®). BKC has been reported to affect the MC system. Its effect on ciliary activity has been studied in-vitro (Batts et al., 1990; Hofmann et al., 2004; Mallants et al., 2007; Merkus et al., 2001; Morimoto et al., 1998; Stanley et al., 1985; Stennert et al., 2008; van de Donk et al., 1980; Wang et al., 2012) and its effect on the rate of MC has been studied both in-vivo (McMahon et al., 1997; Morimoto et al., 1998; Naclerio et al., 2003; Riechelmann et al., 2004; Rizzo et al., 2006) and in-vitro (Batts et al., 1989; Inoue et al., 2012). The toxic effects of BKC were more pronounced in-vitro, particularly in the absence of a protective mucous layer. It was argued that in-vivo, the mucous layer, large surface area and MC itself, protect the respiratory mucosa against the noxious effects of BKC (Berg et al., 1995; Bernstein, 2000; Riechelmann et al., 2004; Verret and Marple, 2005). Despite a lack of consensus on its effect on the nasal mucosa (Berg et al., 1995; McMahon et al., 1997; Naclerio et al., 2003; Riechelmann et al., 2004; Rizzo et al., 2006), in 2017 the European Medicines Agency revised the label and package leaflet guidelines of BKC-containing pulmonary, ophthalmic and nasal formulations to indicate the concentration of BKC and the potential adverse effects. The current study is the first to report the effect of BKC (0.015% w/w), on mammalian mucin secretion. BKC elicited a significant increase in mucin secretion, which was probably to be expected from the many in-vitro studies reporting its effect on nasal cells, cilia and MC (Batts et al., 1989; Batts et al., 1990; Beasley et al., 1998; Berg et al., 1995; Bernstein, 2000; Cho et al., 2000; Graf, 2001; Ho et al., 2008; Lebe et al., 2004; Mallants et al., 2007; Rizzo et al., 2006) and its effect in the

slug mucosal irritation assay (Adriaens et al., 2001). The increase in secretion could serve to dilute the formulation and may help to explain its lower toxicity in-vivo.

The preservative, KS, can be used as a substitute for BKC in IN formulations. It has been reported to cause nasal irritation and lesions in the nasal mucosa of rats following long-term use (Cho et al., 2000; Lebe et al., 2004), but caused no significant changes in mammalian in-vitro CBF (Hofmann et al., 2004; Jiao et al., 2014; Wang et al., 2012). There are no previous studies of its effect on mucin secretion. In the current study, the effect of KS (0.3% w/w), was studied in the presence of the cosolvent, PG (1.5% w/w). PG helped solubilise sorbic acid (slightly soluble in water), the product of the conversion of KS in the test medium. This combination increased the secretion of mucin to a similar extent to PG alone. Therefore, it is possible that the effect is solely due to the cosolvent and not the preservative. How an increase in mucin secretion combined with a lack of effect on ciliary activity would affect mucociliary clearance should be explored.

Little is known about the effect of solubilizers e.g. PG and surfactants e.g. PS 80 in IN formulations and nebuliser suspensions (Pulmicort Respules and budesonide nebuliser suspension contain PS 80) on MC. PG is minimally ciliotoxic (Vetter et al., 2012) while in-vivo studies in mice indicated that it does not affect MC following acute exposure (20 minutes/day for one week). However, chronic exposure to PG (20 minutes/day for 3 weeks) stimulated MC (Laube et al., 2017). PS 80 induced a concentration-dependent ciliotoxicity (Dimova et al., 2003; van de Donk, H.J.M. et al., 1982). Despite this, PS 80 (1%) was able to restore mucociliary transport in a fluid depleted trachea (Ballard et al., 2006). PS 80 (0.025% w/w), was demonstrated here, for the first time, to induce a small, but significant, increase in mucin secretion.

Viscosity enhancers, such as Methocel™ E50 premium LV, a suspending agent comprising hydroxypropyl methyl cellulose (HPMC) polymer, and Avicel® RC591 (microcrystalline cellulose and sodium carboxymethylcellulose), are routinely included in IN formulations and, by reducing MTR (Lin et al., 1993; Pennington et al., 1988; van de Donk, H.J. et al., 1982; Yu et al., 1994) (Hu et al., 2009; Shah and Donovan, 2007; Ugwoke et al., 2000b; Zhou and Donovan, 1996), provide a conventional means of extending IN residence time and thus enhancing therapeutic efficacy. Carboxymethylcellulose was also reported to cause mild inhibition of rabbit CBF after short-term exposure (Ugwoke et al., 2000a). The effect of these compounds on mucin secretion is unknown. In the current study, Methocel™ E50 premium LV induced a significant increase in mucin secretion which might contribute to its effect on MTR (there are no studies of its effect on ciliary activity). Avicel® RC591 showed no effect on mucin secretion, therefore its effect on MTR would require a different explanation.

The chelator, EDTA, has routinely been included in IN formulations to aid stability and synergize preservation. It is also an excipient in certain nebuliser liquids e.g. (Bricanyl Respules, terbutaline nebuliser liquid, Pulmicort Respules, budesonide nebuliser suspension). The first report of EDTA-related respiratory diseases (asthma and/or rhinitis) (Laborde-Castérot et al., 2012) highlighted the limited state of knowledge on the respiratory effects of this excipient. Animal studies in-vitro have shown EDTA to impair mucus transport rate (MTR) (Batts et al., 1989) but not via any substantial effect on CBF (Batts et al., 1990; Merkus et al., 2001; van de Donk et al., 1980). However, in humans, no effect was observed on human nasal clearance (in-vivo) (Batts et al., 1991; Stanley et al., 1985; van de Donk, H.J. et al., 1982) or CBF (in-vitro) (Stanley et al., 1985). The current study provided evidence that EDTA



(0.015% w/w) does not affect respiratory mucin secretion, which was investigated here for the first time. This is consistent with the reported innocuous nature of EDTA to other components of the mucociliary system. Nevertheless, the association of EDTA with occupational rhinitis (Laborde-Castérot et al., 2012) suggests that further work is needed to assess the long-term effects of EDTA.

It is evident that the excipients with surface-active properties (BKC, PG, PS 80 and Methocel™ E50 premium LV) stimulated an increase in respiratory mucin secretion, which is consistent with the increase in mucin secretion induced by bile salts in different types of gastrointestinal epithelia (Klinkspoor et al., 1999). This effect is likely to be non-specific and, to date, the mechanism of detergent-induced mucin secretion has not been elucidated although Klinkspoor et al. proposed an effect at the apical membrane of the cells where mucin release is stimulated to protect the epithelium from the detergent effect of surfactants (Klinkspoor et al., 1999; Klinkspoor et al., 1996), which can cause cell lysis. Damage to the cell membranes would permit the release of cellular ATP into the extracellular space which would stimulate mucin release from airway goblet cells by activation of its specific nucleotide receptor, P2Y2 (Kim and Lee, 1991). Several other molecules are involved in the regulation of mucin exocytosis including soluble N-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE) complex and myristoylated alanine-rich C-kinase substrate (MARCKS) (Rogers, 2007), which may also be affected by a perturbation of the cell membrane.

Management of allergic rhinitis usually involves treatment with corticosteroids or antihistamines. This study examined the effect of the corticosteroid, FF on mucus

secretion. Despite agreement on the need to investigate the long-term effects of IN steroids on the nasal mucosa, research to date has reported significant improvement of compromised mucociliary function in patients with allergic rhinitis (Hofmann et al., 2004; Houtmeyers et al., 1999a; Klossek et al., 2001; Lee and Gendeh, 2003; Naclerio et al., 2003; Verret and Marple, 2005). However, little is known of the effect of corticosteroid IN formulations on airway mucin secretion. Topically applied prednisolone has been shown to reduce mucus secretion from a goblet cell line (Fergie et al., 2003) while other glucocorticoids have been shown to inhibit MUC5A production induced by TGF- $\alpha$  in another cell line (Takami et al., 2012).

FF is practically insoluble and is formulated as a suspension (0.05% w/w) for nasal use. As such, it was not possible to test FF at a solution concentration higher than 0.0004% w/w (~ 4  $\mu$ g/ml). It has been reported that the concentration of FF in artificial nasal fluid was  $0.196 \pm 0.006$   $\mu$ g/mL (Baumann et al., 2009), which is much lower than the concentration tested here. Therefore, studying the effects of FF at a concentration of 4  $\mu$ g/mL was deemed clinically relevant particularly in the absence of the in-vivo dilution effect of the airway secretions. No significant alteration in airway mucin secretion was detected following the exposure to FF, which supports the notion that glucocorticoids like FF do not have a direct effect on the process of mucin production and secretion (Evans and Koo, 2009). Glucocorticoids are recognised to be effective in the treatment of hypersecretory conditions, particularly asthma, by inhibiting the inflammatory process that releases mucin-stimulating mediators (Evans and Koo, 2009; Fahy and Dickey, 2010; Fokkens et al., 2012; Kanoh et al., 2011; Proud and Leigh, 2011; Riesenfeld et al., 2010; Takami et al., 2012). So it is of interest that mucin secretion was potentiated in nasal lavage fluid of healthy subjects (in-vivo) following three weeks treatment with IN glucocorticoids

(MacGregor et al., 1996). There is an ongoing debate regarding the long term effects of the IN delivery of these compounds (Verret and Marple, 2005), despite an absence of deleterious effects on the nasal mucosa following one-year exposure to FF (Fokkens et al., 2012).

DMSO can be used as a cosolvent/solubiliser/penetration enhancer in pharmaceuticals but is not an inactive ingredient present in approved drug products for the nasal and respiratory route. It was used at 0.2% w/w to solubilise FF in the current study. Therefore, it was necessary to assess its effect on mucin secretion in the absence of the drug. No significant effect was detected during this exposure. This concentration of DMSO also proved innocuous to ciliary activity (Pawsey et al., 2011).

In conclusion, the effect of a number of IN pharmaceutical ingredients on airway mucin secretion has been reported here for the first time. Some compounds significantly increased mucin secretion and might therefore be expected to alter mucociliary clearance. This could predispose patients to disease and affect the activity of delivered drugs, decreasing or increasing their activity.

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Test substance	Concentration in Ham's F12 % w/w	Type of ELLA used (d) direct; (i) indirect
Benzalkonium chloride (BKC)	0.015	ELLA (d)
Ethylenediamine tetraacetic acid (EDTA)	0.015	ELLA (d)
Propylene glycol (PG)	1.5	ELLA (i)
Potassium sorbate (KS)	0.3 (+ 1.5% w/w PG as a cosolvent)	ELLA (i)
Fluticasone furoate (FF)	0.0004 (~ 4 µg/ml) *	ELLA (i)
Dimethyl sulfoxide (DMSO)	0.2%	ELLA (i)
Polysorbate 80 (PS 80)	0.025	S-ELLA
Methocel™ E50 premium LV	1.0 **	S-ELLA
Avicel® RC591	1.5 ***	S-ELLA

**Table 1 Compounds studied for their effect on mucin secretion.**

\* FF is practically insoluble in water and was dissolved in DMSO (1 part in 500 parts; sparingly soluble) to yield a solution. This solution was then added to Ham's F12 to yield a final concentration of 0.0004% w/w FF and 0.2% w/w DMSO.

\*\*To achieve optimum dispersion, the polymer was initially wetted with water; 10% of the total diluent volume, and agitated at 60-70°C for ~ 1 hour using a magnetic stirrer before making up to the final volume in Ham's F12

\*\*\* To achieve optimum dispersion, the polymer was homogenised in water; 20% of the total diluent volume, before making up to the final volume in Ham's F12

Parameter	ELLA	S-ELLA
Linearity	0.99 for all lectins (P≤0.0001)	0.99
log Absorbance vs. log mucin concentration (R <sup>2</sup> )		(P<0.0001)
Lower limit of quantification (LLOQ)	0.6 ng/well (Sigma Aldrich HPA) 0.4 ng/well (EY HPA)	0.28 ng/well
10x SD of the blank samples	0.2 ng/well (biotin-streptavidin lectin)	
Accuracy* % relative error (RE)	< 15% (20-25 ng/well and 50 ng/well) < 21.5% (near LLOQ)	< 11% (20-25 ng/well and 50 ng/well) 22% (near LLOQ)
Precision* % relative standard deviation (RSD)	< 3% (50 ng/well) < 5% (20-25 ng/well) ≤ 9.5% (near LLOQ)	< 2% (50 ng/well) < 4% (25 ng/well) ≤ 5% (0.78 ng/well)
Assay range	0.2/0.4/0.6** – 50 ng/well	0.28 – 50 ng/well

**Table 2 Validation parameters of the ELLA and S-ELLA**

\* The recommended-acceptance-range of accuracy and precision of immunoassays is ± 25% at the LLOQ, and ± 20% elsewhere

\*\* LLOQ is dependent on source of lectin

## Figures

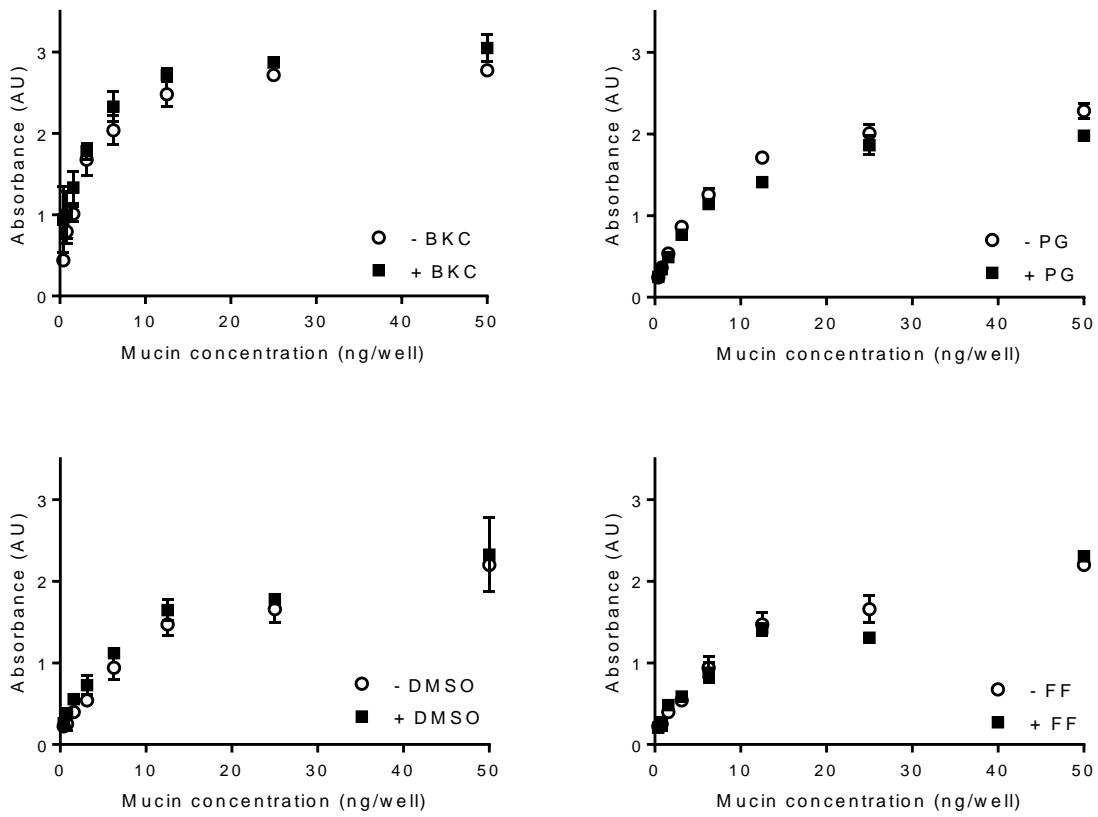


Figure 1: Selectivity of the ELLA for airway mucins in the presence of BKC (0.015% w/w), PG (1.5% w/w), DMSO (0.2% w/w) and FF (0.0004% w/w). Mean  $\pm$  SD; n = 3. None of the substances interfered with the assay.

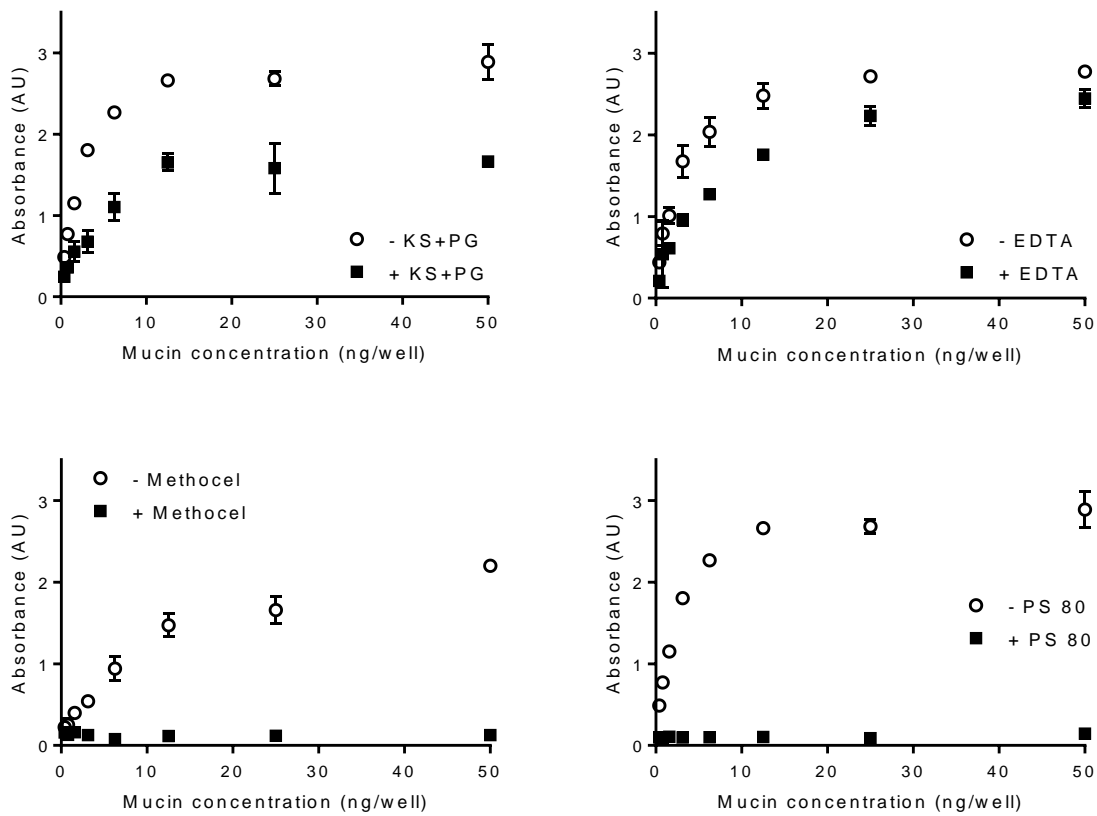


Figure 2: Selectivity of the ELLA for airway mucins in the presence of KS-PG (0.3% w/w / 1.5% w/w), EDTA (0.015% w/w), Methocel™ E50 premium LV (1.0% w/w) and PS 80 (0.025% w/w). Mean  $\pm$  SD; n = 3. Partial interference was observed with KS-PG and EDTA, and complete interference with Methocel™ E50 premium LV and PS 80.

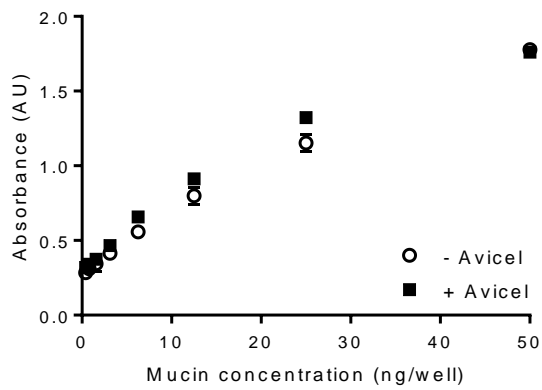
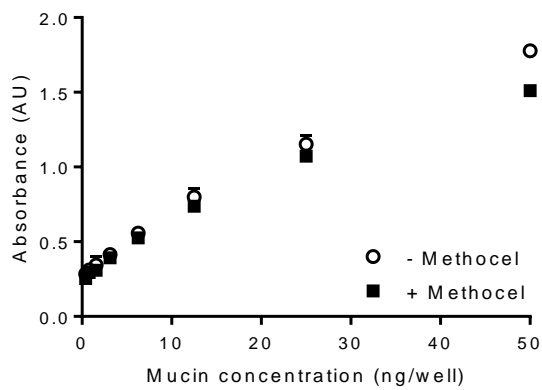
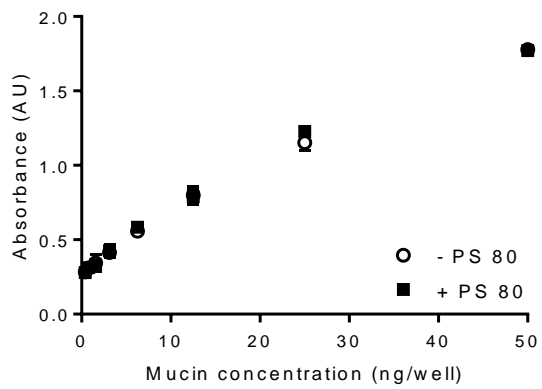


Figure 3: Selectivity of the Sandwich ELLA (S-ELLA) for airway mucins in the presence of PS 80 (0.025% w/w), Methocel™ E50 premium LV (1.0% w/w) and Avicel®RC591 (1.5% w/w). Mean ± SD; n=3. None of the substances interfered with the assay.



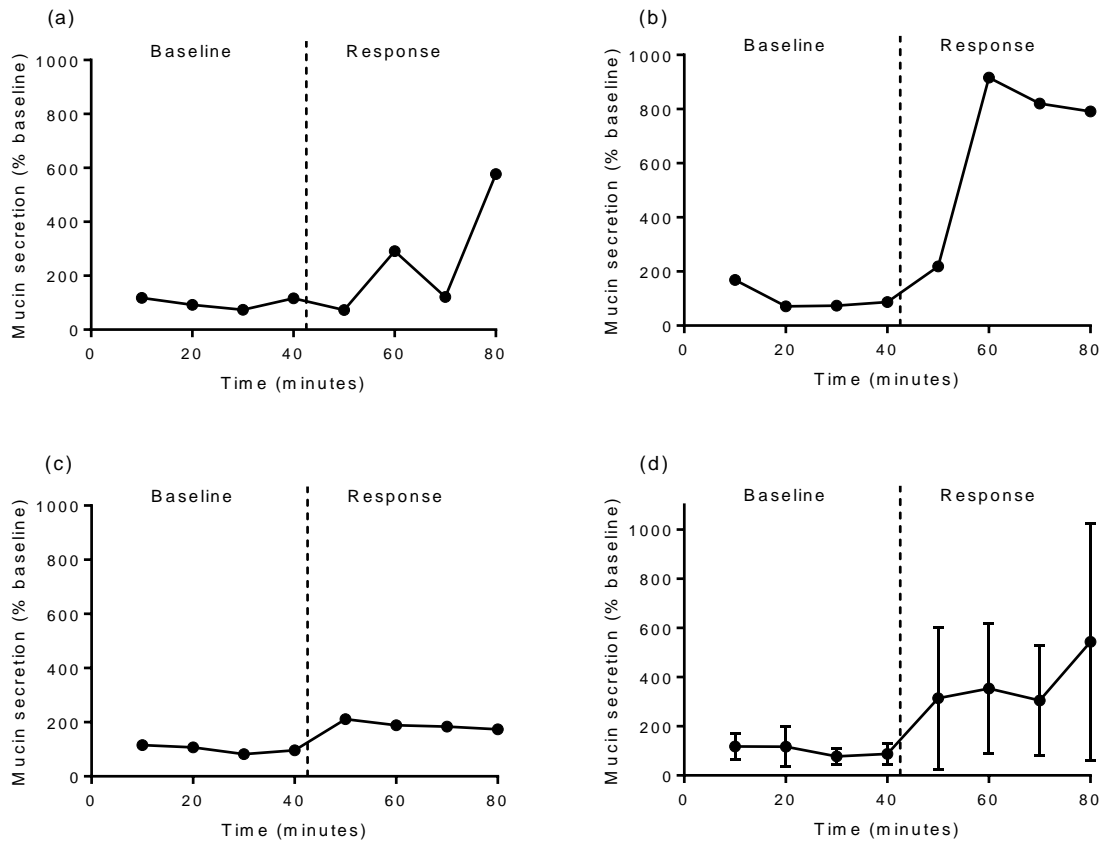


Figure 4: The effect of BKC (0.015% w/w) on mucin secretion. (a) - (c) Examples of mucin secretion from individual explants in response to BKC exposure. (d) The average mucin secretion response upon exposure to BKC Mean  $\pm$  SD; n=9 explants from 3 animals.

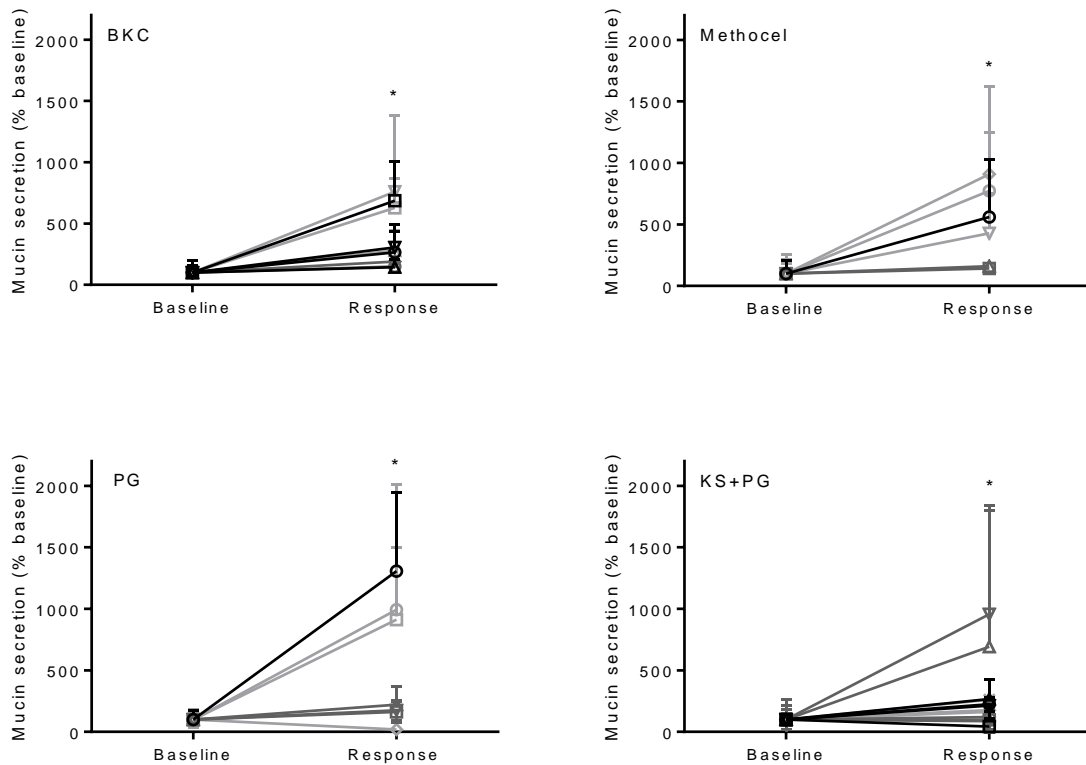


Figure 5: The effect of BKC (0.015% w/w), Methocel™ E50 premium LV (1.0% w/w), PG (1.5% w/w) and KS+PG (0.3/1.5% w/w) on mucin secretion. The four baseline mucin secretion measurements of each explant were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant. Mean  $\pm$  SD; n=9 explants from 3 animals (BKC), n=6 explants from 3 animals (Methocel™ E50 premium LV), n=7 explants from 3 animals (PG), n=12 explants from 3 animals (KS+PG). For each substance, the different animals are indicated in a different shade of grey. \* P<0.05.

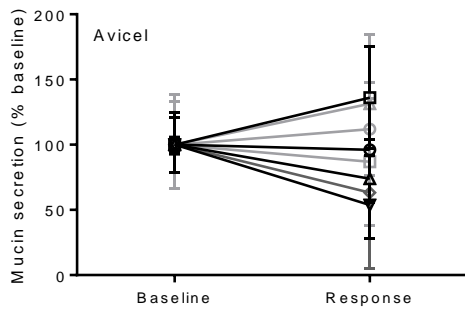
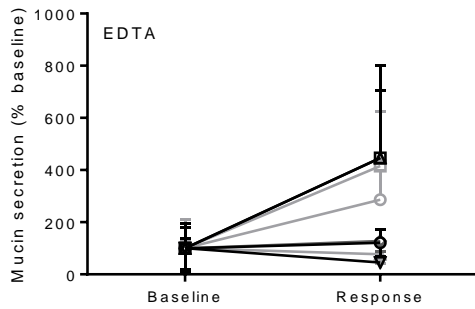
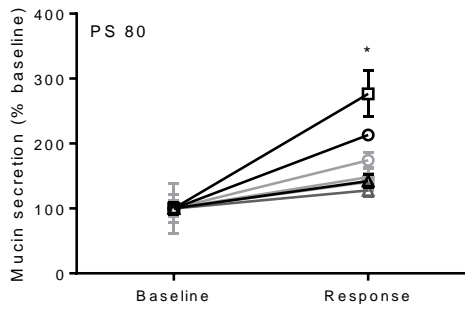


Figure 6: The effect of PS 80 (0.025% w/w), EDTA (0.015% w/w) and Avicel® RC591 (1.5% w/w) on mucin secretion. The four baseline mucin secretion measurements of each explant were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant. Mean  $\pm$  SD; n=7 explants from 3 animals (PS 80), n=8 explants from 3 animals (EDTA), n=8 explants from 3 animals (Avicel® RC591). For each substance, the different animals are indicated in a different shade of grey. \* P<0.05.

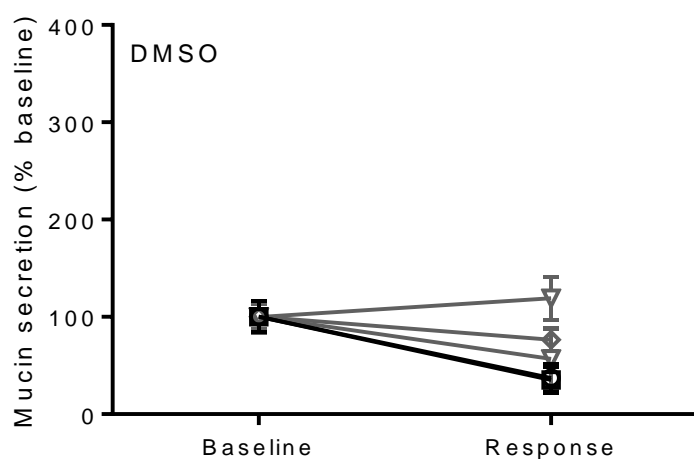
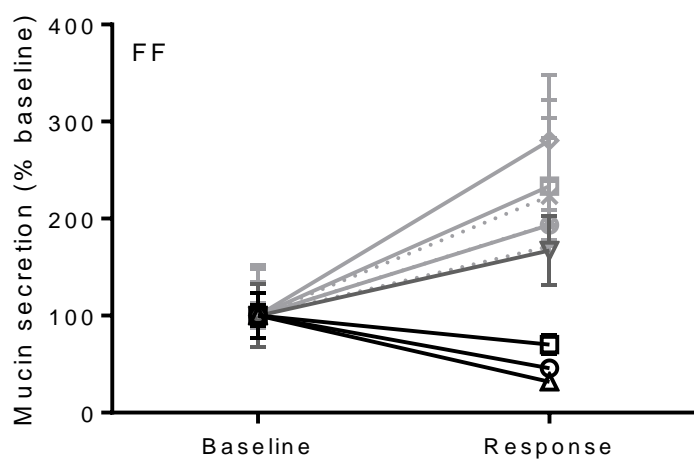


Figure 7: The effect of FF (0.0004% w/w) and DMSO (0.2% w/w) on mucin secretion. The four baseline mucin secretion measurements of each explant were pooled and the mean (equivalent to 100 % of the baseline) was compared to that of the pooled four response measurements of the same explant. Mean  $\pm$  SD; n=10 explants from 4 animals (FF), n=5 explants from 2 animals (DMSO). For each substance, the different animals are indicated in a different shade of grey or type of line.

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