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Validation of Animal Experiments on Ciliary Function *In Vitro*. I. The Influence of Substances Used Clinically

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In vitro studies of ciliary activity require specimens of healthy epithelium in relatively large quantities. Since human material is difficult to obtain, fresh chicken trachea samples have frequently been used in function experiments. The aim of the present study was to investigate whether several substances had comparable effects on the ciliary beat frequency (CBF) of chicken trachea and cryopreserved human respiratory epithelium obtained from the sphenoidal sinus. For this study, we used two topical anaesthetics: cocaine (3% and 7%) and lidocaine (2%). These anaesthetic substances were adjusted to pH 6 and pH 7. We also used two decongestants, namely xylometazoline 0.1% and oxymetazoline 0.1% and the β-blocking agent propranolol. Topical anaesthetics appeared to be more ciliostatic in solutions with pH 7 compared to pH 6. Complete ciliostatic effects were reversible, with the exception of the ciliostasis induced by propranolol. The effects of these substances on the CBF of fresh chicken trachea and cryopreserved human tissue did not differ significantly. These experiments show that chicken trachea constitutes a valid substitute for human material in studying ciliary activity *in vitro*. Moreover, the experiments provide evidence in support of the assumption that cryopreservation has no effect on ciliary reactivity as expressed by the CBF. *Key words: ciliary beat frequency, chicken, cryopreservation, human, interspecies differences.*

INTRODUCTION

Mucociliary transport is an important defence mechanism of the airways, and ciliary beat frequency (CBF) is a major parameter in mucociliary clearance (1). In studying CBF, human specimens are generally preferred. The reason is that interspecies differences may occur, and most formulations will eventually be administered in humans (2–4). However, chicken embryo trachea is widely used in investigations of the effects of substances on ciliary activity (5–8). The validity of using this mucosa as a substitute for human ciliated epithelium has not yet been extensively established (2). Therefore, the aim of this study was to test the validity of chicken embryo trachea as a substitute for human ciliated epithelium. For this purpose, we compared the effects of several substances on chicken embryo trachea with their effects on human ciliated epithelium. We selected substances that are frequently used in clinical settings and that have well-known effects on CBF, namely the topical anaesthetics lidocaine and cocaine, the decongestants xylometazoline and oxymetazoline, and the β-blocking agent propranolol (9–11).

MATERIALS AND METHODS

CBF measurements of chicken embryo trachea

CBF measurements were performed on ciliated epithelium of freshly harvested chicken embryo trachea, as described by Van de Donk et al. (12). Immediately after dissection, the chicken embryo trachea was sliced into small rings approximately 1 mm thick. The trachea slices were placed in stainless steel supporting rings and allowed to recover for 30 min in Locke-Ringer's solution (LR). LR is an isotonic solution of NaCl, 7.72 g (132 mmol); KCl, 0.42 g (5.63 mmol); $CaCl_2 \cdot 2H_2O$, 0.16 g (1.24 mmol); $NaHCO_3$, 0.15 g (1.79 mmol); glucose anhydrous, 1.00 g (5.55 mmol) in 1 litre of water. Then the tissue samples were put in a well containing 1.0 ml of LR and placed under an Olympus BH-2 light microscope. The microscope table was connected to a thermostat and the well was kept at a temperature of 33°C. A light beam passed through moving cilia, resulting in varying intensities of light. These variations were registered by a photocell after magnification by the microscope. This signal was analysed using a Fast Fourier Transform algorithm. After measuring the initial frequency, the tissue was placed in another well containing 1 ml of test solution. Then CBF was measured every 10 min during a period of 1 h. Data were calculated as relative frequency of the initial frequency measured in LR.

CBF measurements of cryopreserved human sphenoidal sinus mucosa

Mucosa of the sphenoidal sinus was used for these measurements. This material was obtained from patients who underwent transnasal surgery of the pituitary. Cryopreservation of the mucosa was done as described previously (13). Specimens were stored in a

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mixture of 69% Dulbecco's MEM (Gibco, Paisley, Scotland), 20% fetal calf serum, 10% dimethyl sulfoxide (Baker Chemicals, Deventer, The Netherlands) and 1% penicillin streptomycin. A slow-freezing method was used. Eventually ampoules with specimens were placed in liquid nitrogen. Prior to the experiments, the material was rapidly thawed by placing the ampoules in a 37°C water bath. The tissue was then cut into pieces of approximately 0.2 × 0.2 cm and placed in a solution of LR. After a few minutes the specimens were put in a well containing 1 ml LR and fixed by placing a small stainless steel ring on top of the well. Subsequently, CBF measurements were performed as described above.

Procedure

The influence of the selected substances on CBF was assessed. First, the effect of the topical anaesthetics cocaine HCl 3% (w/v) and 7% (w/v) adjusted to pH 6 and pH 7, and lidocaine HCl 2% (w/v) adjusted to pH 6 and pH 7 was recorded. Then the effects of the β-blocking agent propranolol HCl 1% (w/v) and the nasal decongestants xylometazoline HCl 0.1% (w/v) and oxymetazoline HCl 0.1% (w/v) were determined. Whenever a substance caused complete ciliostasis, the reversibility was examined after rinsing with LR and putting the sample back in a well with LR. For each substance a new sample of ciliated mucosa was used. All chemicals used in these experiments were of analytical quality.

Statistical analysis

The statistical analysis of the results comprised multivariate repeated measures of analysis of variance (MANOVA). A p value <0.05 was considered as significant.

RESULTS

The results of these experiments are shown in Figs. 1–8. Measurements in LR were used as a control (12). CBF remained around 100% of the initial frequencies in both human ($n = 7$) and chicken ($n = 8$) tissue. No significant difference in CBF could be demonstrated between human and chicken material ($p = 0.92$). The severe ciliostatic effect of the β-blocking agent propranolol HCl 1% was evident in this study, with no differences showing up between human ($n = 6$) and chicken ($n = 6$) ciliated mucosa (Fig. 1). This effect was not reversible.

Figs. 2 and 3 depict the effects on CBF of cocaine 3% in solutions of pH 6 ($n = 6$) and pH 7 ($n = 6$). The solution at pH 6 showed a statistical difference between the two tissues ($p = 0.02$), with a more severe inhibition of CBF in human tissue. The significant

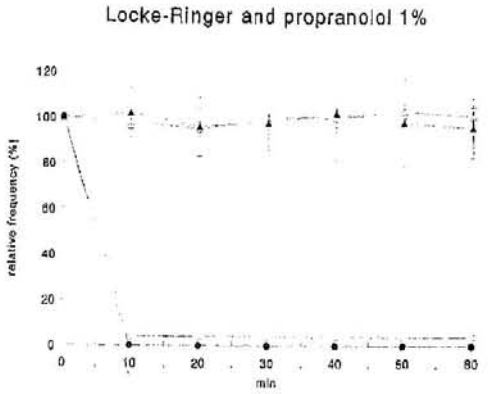

Fig. 1. The effect of LR on the CBF of human (▲) and chicken material (△), and the effect of propranolol 1% on the CBF of human (●) and chicken material (○). When CBF was 0 Hz, the tissue is rinsed and placed in LR (↑). Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies for LR were in human material 8.3 Hz (± 0.4 SEM) and in chicken material 17.1 Hz (± 1.0 SEM) and for propranolol 1% in human material 8.9 Hz (± 0.3 SEM) and in chicken material 18.3 Hz (± 0.6 SEM).

difference between groups and time existed solely at the measurement at 5 min ($p = 0.003$). After this moment there was no significant difference between groups and time. However, at pH 7 ciliostasis was observed between 5 and 20 min in both tissues. That effect turned out to be reversible when the samples were rinsed and put back in LR. No statistical differences were found between human and chicken material with pH 7 ($p = 0.09$).

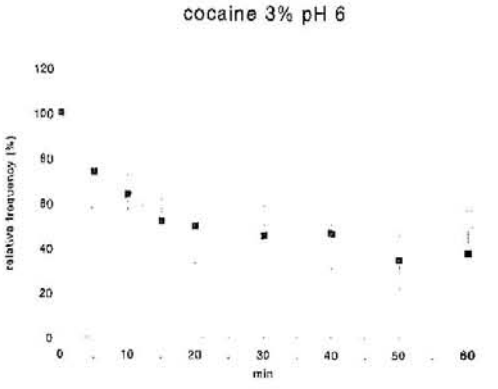

Fig. 2. The effect of cocaine HCl 3%, pH 6 on the CBF of human (■) and chicken (□) material. Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 10.0 Hz (± 0.8 SEM) and in chicken material 19.3 Hz (± 0.5 SEM).

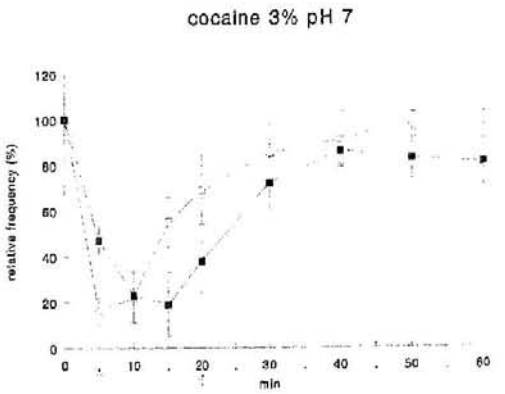

Fig. 3. The effect of cocaine HCl 3% pH 7 on the CBF of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue is rinsed and placed in LR (between ↑). Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 9.9 Hz (± 1.0 SEM) and in chicken material 20.7 Hz (± 0.8 SEM).

In cocaine 7% (pH 7), the chicken cilia stopped beating after 5 min, while human cilia stopped between 5 and 15 min (both $n = 6$). These effects were reversible as well. After the samples were rinsed and put back in LR the frequencies recovered up to 80% (chicken) and 95% (human material) of the initial frequencies. Cocaine 7% at pH 6 (both $n = 6$) had a moderate ciliostatic influence (Figs. 4 and 5). No significant differences were observed between the two tissues used in cocaine 7% at pH 6 ($p = 0.16$) and at pH7 ($p = 0.06$).

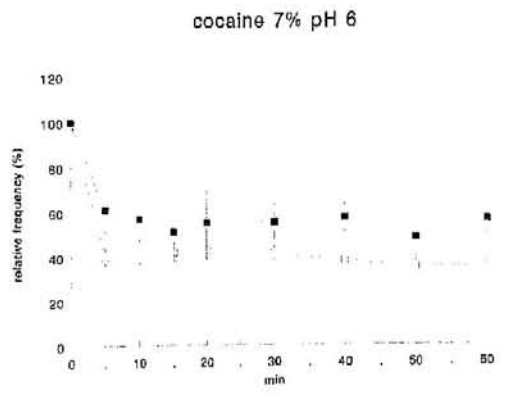

Fig. 4. The effect of cocaine HCl 7% pH 6 on the CBF of human (■) and chicken (□) material. Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 8.9 Hz (± 0.8 SEM) and in chicken material 19.4 Hz (± 1.0 SEM).

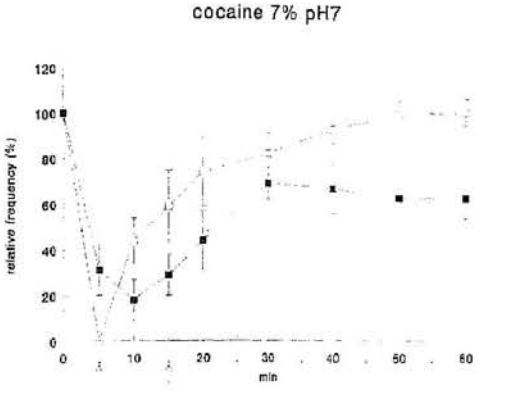

Fig. 5. The effect of cocaine HCl 7% pH 7 on the CBF of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue is rinsed and placed in LR (between ↑). Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 10.2 Hz (± 0.5 SEM) and in chicken material 19.2 Hz (± 0.5 SEM).

In Figs. 6 and 7, the results of lidocaine HCl 2% are presented at the same pH values as in the cocaine experiments. Again, at pH 7 ciliostasis was observed, in human tissue between 10 and 20 min, in chicken tissue between 5 and 15 min. Also here this effect appeared to be reversible, as in the experiments with cocaine. At pH 6 the inhibition of CBF resulting from lidocaine HCl 2% is as moderate as the one resulting from cocaine 3% and 7% at pH 6. No significant differences were found between animal and human ciliated mucosa (pH 6, $p = 0.27$; pH 7, $p = 0.31$, both $n = 6$). The results of xylometazoline

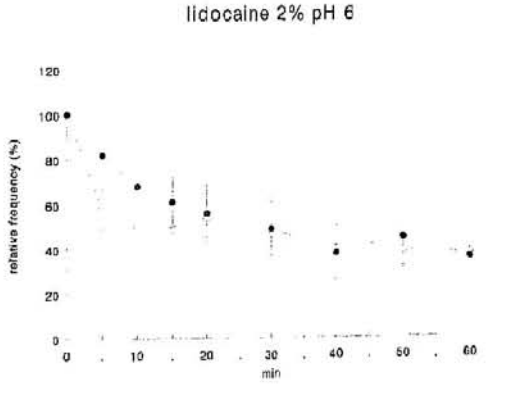

Fig. 6. The effect of lidocaine HCl 2% pH 6 on the CBF of human (■) and chicken (□) material. Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 9.6 Hz (± 0.6 SEM) and in chicken material 18.3 Hz (± 1.2 SEM).

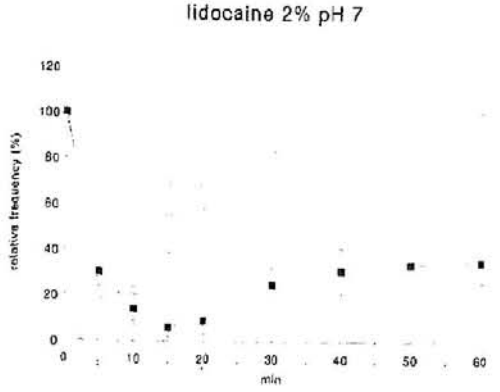

Fig. 7. The effect of lidocaine HCl 2% pH 7 on the CBF of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue is rinsed and placed in LR (between ↑). Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 11.4 Hz (± 0.2 SEM) and in chicken material 19.5 Hz (± 0.6 SEM).

0.1% and oxymetazoline 0.1% (human $n = 6$, chicken $n = 7$) are presented in Fig. 8. The effect of both decongestants was a moderate decrease in CBF, and no statistical differences were found between chicken and human tissue ($p = 0.29$ and $p = 0.15$, respectively).

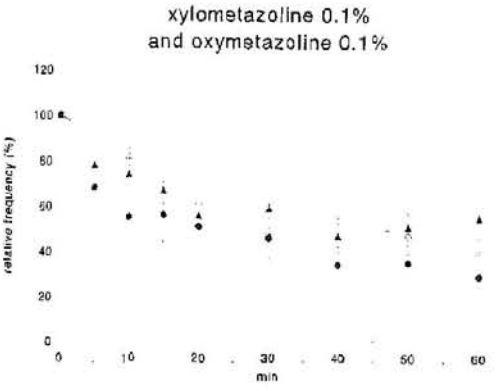

Fig. 8. The effect of xylometazoline 0.1% on the CBF of human (●) and chicken material (○), and oxymetazoline 0.1% on human (▲) and chicken material (△). Data are mean ± SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies for xylometazoline 0.1% were in human material 9.9 Hz (± 0.7 SEM) and in chicken material 18.0 Hz (± 0.9 SEM) and for oxymetazoline 0.1% in human material 9.0 Hz (± 1.0 SEM) and in chicken material 19.2 Hz (± 0.6 SEM).

DISCUSSION

Chicken embryo tracheas are widely used for determining the influence of substances on CBF (5–8). They are commonly used because this tissue is easy to obtain and easy to handle. Experiments have to be scheduled 3 weeks in advance because the eggs have to be hatched. Since the anatomy of cilia of humans and that of animals are quite similar, one would expect a comparable reactivity of human and animal ciliated epithelium (14). However, there are indications that CBF may differ between various species (4). To obtain human material, most biopsies used to be derived from nasal mucosa. A major problem is how to obtain sufficient quantities of healthy mucosa for experimental purposes (9, 15). It has been shown that mucosa of the sphenoidal sinus is an appropriate source of non-pathological ciliated epithelium (13). Previously, it had been demonstrated that cryopreservation has no effect on CBF of human ciliated epithelium (16–18). Moreover, the reactivity of CBF is apparently not influenced by cryopreservation. This has already been demonstrated by effects on β-adrenergic receptors (19). As a consequence, healthy human ciliated epithelium can be available for experimental purposes at any given time.

We determined whether chicken embryo tracheas are a valid substitute for human ciliated epithelium in function experiments. To that end we measured the influence of several substances on CBF of both species. Our experiments enabled us to assess more accurately the presence or absence of any influence of cryopreservation on the reactivity of human sphenoidal sinus mucosa. The substances used in these experiments are often applied in clinical settings. Lidocaine and cocaine are widely used topical anaesthetics. And xylometazoline and oxymetazoline are common nasal decongestants. Propranolol is applied in cardiac arrhythmia. All of these substances have well-known effects on CBF (9–11). The differences in the effect of the two pH solutions of lidocaine and cocaine are remarkable. But the same effects were observed on chicken embryo tracheas and on human ciliated epithelium.

The conclusion of this study is that none of these substances has a different influence on fresh chicken material than they have on cryopreserved human mucosa. Moreover, the reactivity of human ciliated sphenoidal sinus mucosa that has been stored in liquid nitrogen appeared to be comparable to that of fresh chicken trachea as far as CBF is concerned. Thus, there is evidence that chicken trachea is a valid substitute for human ciliated mucosa in conducting function experiments. Cryopreservation of human ciliated sphenoidal sinus mucosa does not influence its reactivity.

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