INSIGHT INTO CILIARY ACTIVITY AND MUCOCILIARY TRANSPORT

WILBERT M. BOEK

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Printing of this thesis was financially supported by:

van Gansewinkel BV (integrale afvalbehandeling), GlaxoWellcome BV, Astra Pharmaceutica BV, Hoechst Marion Roussel BV, Mediprof BV, Schering Plough BV, Moduvice Medical Products, Janssen-Cilag BV, Horen Nederland, Phonak BV, Entermed BV, VVAA, Smith Kline Beecham Farma BV, Beter Horen, Pfizer BV, Abbott BV, Smith & Nephew BV, GN ReSound bv, Philips Hearing Instruments, Garant Optiek (hoortoestellen), Veenhuis Medical Audio BV, Sanofi~Synthelabo BV, ACM Ooms Allergie BV, HCI Cryogenics BV

ISBN-Nummer: 90-393-2397-6

Drukwerk: Drukkerij Elinkwijk B.V., Utrecht

INSIGHT INTO CILIARY ACTIVITY AND MUCOCILIARY TRANSPORT

Inzicht in trilhaaractiviteit en mucociliair transport

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. H.O. Voorma, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op dinsdag 2 mei 2000 des namiddags te 4.15 uur

door

Willibrordus Maria Boek

geboren op 13 mei 1964 te 's-Hertogenbosch

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Co-promotor: Dr. K. Graamans

The research presented in this thesis was performed at the Mucociliary Transport Laboratory, Department of Otorhinolaryngology, University Hospital Utrecht, the Netherlands.

Part of the research was financially supported by Glaxo Wellcome BV, Zeist, the Netherlands.

CONTENTS

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

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At the end of the seventeenth century, two Dutch scientists, Antoni van Leeuwenhoek and Antonius de Heide, independently discovered cilia and ciliary movements (1). Cilia are the motors of a transport system, which is an important defense mechanism of the upper and lower respiratory tract. Thus, for obvious reasons, ciliary activity and mucus transport have become a subject of interest in scientific research. Primary ciliary dyskinesia, an inherited disorder, is one of the most convincing illustrations of the importance of this system. Chronic sinusitis and bronchiectasis are the most important symptoms of this condition. Kartagener (1933) described this syndrome in combination with situs inversus (2). Later, it was demonstrated that this syndrome may include rhinitis, nasal polyposis, chronic and recurrent otitis media, male infertility and absence or hypoplasia of the frontal sinuses (3).

Two pathways can be taken to assess mucociliary function: investigation of mucociliary transport (MCT) as a whole; and study of ciliary activity by measuring ciliary beat frequency (CBF). Various methods are used to study mucociliary transport. Saccharine, a sweetener in the form of tablets or liquid, is placed on the mucosa of the head of the inferior turbinate or on the anterior nasal septum. The saccharine clearance time is set at the first perception of a sweet taste (4). One variation on this method involves using a dye such as methyleen blue or charcoal particles. In this variant, the investigator records how long it takes for the dye to become visible in the pharynx (5). Another way to measure MCT is to follow radiopaque particles along their course through the nasal cavity by means of roentgenographic registration (6). Gamma scintigraphy, using technetium-99m or iodine-111, provides good information about the deposition, dispersion and clearance of the radiolabelled particles detected by a gamma camera. In investigations on mucociliary clearance, spraying of the radioisotope is better than local deposition somewhere in the upper airways (7). CBF can be measured in various ways. As Lucas (1933) observed, microscopic light is reflected by ciliary activity (8). Nowadays, according to this principle, CBF can be measured with video images. Another widely applied method to determine CBF is the use of a photosensitive cell that converts light fluctuations from beating cilia into an electric signal (9). The light can be reflected from a surface with beating cilia. A more widespread method is transillumination, whereby the light source is placed below a specimen of ciliated epithelium. The light fluctuations of about 200 cilia are converted into an electric signal

with a highly sensitive photodetector. Then a fast Fourier transformation is used to calculate the frequency from the electric signal. One major disadvantage of these methods is that they are applied in an *in vitro* setting.

In vitro studies of ciliary activity require a large quantity of ciliated epithelium. Many studies use animal material to determine ciliary activity and to assess the effects of various formulations. Tracheas of chicken embryos are widely used in function experiments on ciliary activity (10-13). Since interspecies variations have been reported, human specimens are preferred in experiments, as most formulations eventually will be used in humans (14). However, a validation of this ciliated tissue as a substitute for human material is still lacking.

Since the upper respiratory tract in humans is easily accessible, most biopsies are taken there, especially from the nasal cavity. Nasal bleeding and discomfort are associated with obtaining biopsy material, particularly since local anesthetics cannot be used because of their ciliostatic effect. Forceps biopsies are preferred to brushing or curette techniques, even though they cause more discomfort and may lead to nasal bleeding (15). Some studies have used ciliated epithelium of the adenoid, obtained from patients who have undergone an adenoidectomy. The fact that these patients were eligible for this surgery means that their ciliated tissue was probably pathological. This highlights an important drawback of that easily obtainable tissue. Cell suspension cultures of human upper airway epithelium are also used for research purposes. However, this material can only be obtained by a complicated and time-consuming procedure. Moreover, the usefulness of this kind of ciliated cells is variable (16,17). For *in vitro* studies on ciliary activity, the ideal situation would be a constant availability of non-pathological, human ciliated mucosa.

The exact correlation between CBF and MCT is not known. It seems logical to assume that ciliary activity and mucociliary transport are positively correlated. Convincing evidence is still lacking, however. Since *in vivo* evaluation of MCT is more difficult than measuring CBF *in vitro*, most experiments have been performed *in vitro*. It is questionable whether the results of *in vitro* experiments can be transposed to *in vivo* situations. The ciliary activity is not the only determinant of MCT. Rheological properties of mucus as well as nervous and humoral influences on the mucosa are also important factors. Only when MCT and CBF are affected similarly would there be a strong indication that CBF is a determinant of MCT.

In the research on ciliary activity, physiologic saline (NaCl 0.9%) is frequently used as a control medium or as a solvent. Moreover, it is recommended as a lavage for the treatment of rhinitis and sinusitis, and after nasal and sinus surgery (18,19). However, its influence on MCT has not yet been clearly revealed. Hypertonic saline solutions are used in patients with impaired MCT, as they have been shown to improve transport in these patients (20,21). The impact of hypertonic solutions on the ciliated mucosa and on CBF has not been investigated yet. To our knowledge, this interesting field of the influence of saline solutions on ciliary activity still lies fallow.

OUTLINE OF THIS THESIS

The primary objective of this study is to address the problem of acquiring sufficient amounts of valuable ciliated epithelium for research on *in vitro* ciliary activity. Secondly, this study seeks to determine whether conclusions on ciliary reactivity *in vitro* may be transposed to reactions of MCT *in vivo*.

Chapter 2 describes a method of preserving non-pathological human ciliated epithelium that had been harvested from the sphenoid sinus during pituitary surgery. We investigated whether cryopreservation of this healthy mucosa would influence CBF.

Chapter 3 and 4 deal with the validation of chicken embryo tracheas as a substitute for human ciliated mucosa in experiments on ciliary activity. To this end we assess the influence of a variety of substances on CBF using human and chicken material, and then compare the results.

In *Chapter 5*, we question whether the results of experiments on *in vitro* ciliary activity apply to *in vivo* mucociliary transport. Formulations with well-known *in vitro* effects are used for experiments in young healthy subjects. The influence of these formulations on MCT is documented.

Chapter 6 concerns the influence of "physiologic" and hypertonic saline solutions on CBF.

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CHAPTER 2

CILIARY BEAT FREQUENCY OF HUMAN SPHENOID SINUS MUCOSA AFTER CRYOPRESERVATION

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Eur Arch Otolaryngol (1998) 255: 135-137

ABSTRACT

In vitro studies of human ciliary activity require relatively large quantities of specimens of healthy ciliated epithelium. For this reason we have investigated whether cryopreserved healthy mucosa taken from the sphenoid sinus during pituitary surgery would meet the demands of this type of study. The sinus mucosa from ten patients was immersed in two different cryopreservatives. One solution contained 10% dimethylsulfoxide (DMSO) as cryoprotector. The other contained glycerol as a part of human sperm preservation medium (HSPM). The ciliary beat frequency (CBF) was measured sequentially by a photoelectric method: when specimens were fresh and then at intervals of 1 week, 1 month and 3 months after cryopreservation in liquid nitrogen and thawing. Mean CBF values recorded after thawing did not differ significantly from the values measured before cryopreservation. Prior to cryopreservation and after thawing, CBF did not change during a period of 4 h. Epithelia preserved in DMSO demonstrated that the low mean CBF (5.4 Hz) found was caused by a reversible ciliostatic effect of the medium. After thawing and rinsing with a neutral medium, CBF showed normal values. We conclude that sphenoid sinus mucosa is an appropriate source of ciliated mucosa for *in vitro* experiments. Since non-pathological ciliated epithelium can be maintained in a "mucosa bank," our finding make further studies of CBF of normal human respiratory epithelium *in vitro* a realistic goal.

INTRODUCTION

The ciliary beat is an important defense mechanism in the respiratory tract. As found clinically, an impairment in ciliary movement is frequently associated with such respiratory diseases as sinusitis and bronchitis and even otitis media (7,9). For this reason ciliary (patho)physiology has been the subject of a variety of research programs in which samples of viable human ciliated epithelium are used. The aim of this study was to find new ways to easily obtain healthy human ciliated epithelium and to determine the effect of cryopreservation on ciliary beat frequency (CBF) of this tissue.

MATERIALS AND METHODS

Mucosal specimens

Mucosa was removed from the sphenoid sinuses of ten patients during transnasal surgery of the pituitary. A major advantage of this mucosa was its availability in rather large quantities, since during pituitary surgery all of the mucosa was removed from the sinus. None of the patients had concurrent pathology of the nose or the paranasal sinuses (which in general would be a contraindication for the operation). Asymptomatic septal deviations were considered to be non-pathological. Patients were operated upon under general anesthesia. In addition, 3% cocaine with 1:20.000 Adrenaline was administered topically to the turbinates and meatal mucosa by means of gauze placed in the nasal cavity.

Freezing technique

Mucosal specimens were washed in a 0.9% saline solution and stored in two different freezing media, each containing a different cryoprotector (dimethylsulfoxide or glycerol). The first half of the tissue obtained was stored in dimethylsulfoxide (DMSO) medium containing 69% Dulbecco's MEM (Gibco, Paisley, UK), 20% foetal calf serum (FCS), 10% DMSO and 1% penicillin/streptomycin. The other half was stored in human sperm preservation medium (HSPM), containing 15% glycerol (5). The samples were placed in six screw-capped 2-ml ampoules containing 1.8 ml freezing medium and then cooled to 0 °C. Ampoules were subsequently placed in an isolating polystyrene box, leaving the screw caps uncovered with the polystyrene material. Immediately afterwards, the box was placed in a

freezer and cooled to -80 °C. After 24 h, ampoules were transferred to liquid nitrogen (at -196 °C). Storage times were 1 week, 1 month and 3 months, at which times ampoules were thawed rapidly in a 33 °C water bath. By using this procedure, CBF was measured after each freezing period. A new sample was used for each measurement.

CBF measurements

CBF was measured by the photoelectric method described previously by Ingels et al. (3). Samples were examined in a perfusion chamber mounted in an aluminium frame. The chamber was kept at 33 °C by means of an electronic heating device in order to approximate the physiological temperature of the nasal mucosa (2). A Leitz phase-contrast microscope (Wetzlar, Germany) was adapted with disc interference contrast optics using a 100× oil immersion objective and a 10× ocular in the measuring tube. A variable circular diaphragm was mounted in the same tube along with a beam splitter and a visaflex house enabling a control view of the measured area. Light was provided by a 12 V/250 W halogen lamp.

A highly sensitive photodetector converted the light fluctuations caused by the beating action of the cilia into an electrical signal. This signal was digitalized in an IBM-XT personal computer using a 12-bit A/D converter with a sample frequency of 200 Hz. A fast Fourier transform analysis (FFT) of the signal recorded was performed over a period of 20 s, starting arbitrarily at the fifth second. CBF was determined from the first harmony.

CBF measurements were performed before the samples were frozen and again after 1 week, 1 month and 3 months of cryopreservation in both freezing media. Measurements were carried out once every hour during a period of 4 h.

Influence of DMSO

In order to determine the influence of DMSO as cryopreservative on CBF, measurements on specimens maintained in DMSO were carried out every 10 min for 1 h. During CBF measurements, specimens were mounted in Dulbecco's MEM with 10% foetal calf serum. CBF was thus determined on fresh material as controls and after 1 month of cryopreservation. Thawed specimens after cryopreservation were thoroughly rinsed twice in Dulbecco's MEM with 10% foetal calf serum in order to wash out the cryopreservative.

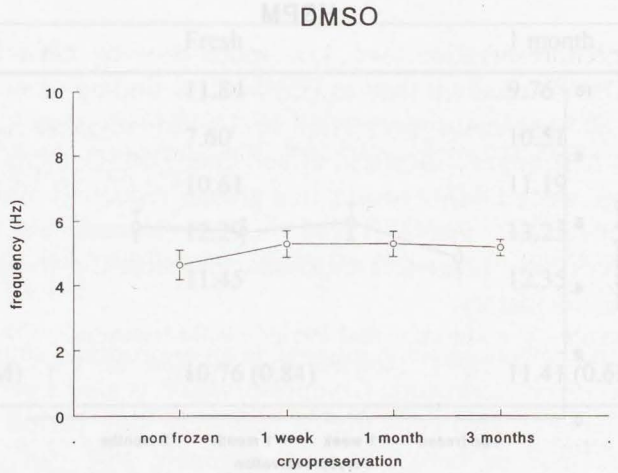

Fig. 1 Effect of cryopreservation on samples mounted in DMSO medium. No significant effect occurred on mean CBF (± SEM), $P = 0.67$

Statistical analysis

Statistical analysis of the results was carried out by repeated measures of analysis of variance (ANOVA). A P value < 0.05 was considered to be significant.

RESULTS

Mean CBF in DMSO medium before freezing was 4.6 Hz and was 5.3 Hz, after 1 week of cryopreservation (Fig 1). This count was stable after 1 and 3 months. These frequencies did not differ significantly from the frequencies of the fresh samples (ANOVA, P=0.67). The mean CBF in specimens placed in HSPM was 4.9 Hz prior to freezing. After 1 week of cryopreservation, this was 6.0 Hz and was 5.8 Hz after 3 months (Fig 2). These frequencies also did not differ significantly from those observed in fresh samples (ANOVA, P=0.18). Rinsing the samples, after cryopreservation in DMSO medium, resulted in mean values of 10.8 Hz and 11.4 Hz after freezing (Table 1). These values were not significantly different (ANOVA, P=0.78). No moving

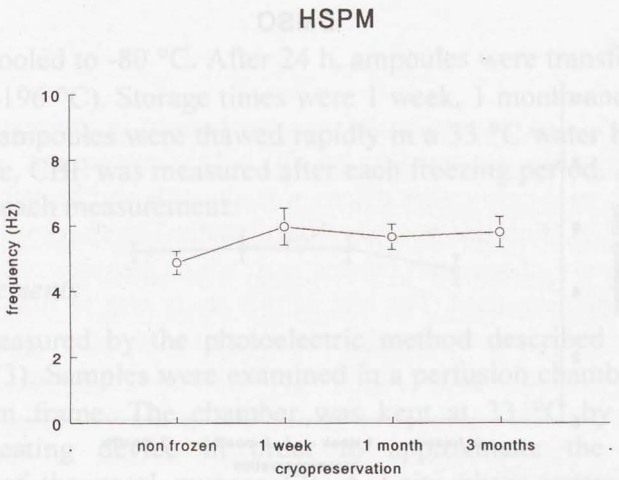

Fig. 2 Samples mounted in HSPM showing that cryopreservation resulted in no significant effect on mean CBF (± SEM), $P = 0.18$

cilia were found in only 6 of the 60 cryopreserved specimens after 1 and 3 months of cryopreservation. These six specimens were all preserved in HSPM. Compared to HSPM, cryopreservation in DMSO medium appeared to result in cells with more moving cilia. However, it seemed that a greater number of solitary ciliated cells and small clumps of ciliated cells were visible in both cryopreservation media after thawing than in samples that had not been frozen at all.

DISCUSSION

To allow *in vitro* studies of ciliary activity, a large number of mucosal biopsies are required. When studying the ciliary beat of human tissues, human specimens are preferred, since interspecies variations exist (8). Human material can be obtained from both the upper and lower airways. Because of the ready accessibility of the upper respiratory tract, most investigators use biopsies from the nasal mucosa. It is, however, difficult to obtain sufficient quantities of healthy mucosa for experimental purposes (3,4). Cryopreservation of ciliated mucosa has been used only occasionally, but observations on ciliary motility after cryopreservation seem to be encouraging (11). However, in the experiments described by Di Benedetto et

Sample	Fresh	1 month
1	11.84	9.76
2	7.60	10.51
3	10.61	11.19
4	12.29	13.25
5	11.45	12.35
Mean (± SEM)	10.76 (0.84)	11.41 (0.63)

Tabel 1 CBF of samples preserved in DMSO prior to freezing and 1 month after cryopreservation. CBF measurements were carried out after rinsing in Dulbecco's MEM with 10% FCS. Samples were mounted in this medium during measurements. CBF values are the means of seven measurements in 1 h. Mean CBF is expressed as the mean ± in Hz

al. (1) and Wulffraat et al. (10), storage times were relatively short and the amount of material available had been obtained from volunteers but was rather small. We prolonged storage times in order to investigate the viability of ciliated cells over a longer period. In addition, we used a new source of ciliated human epithelium, the sphenoid sinuses, which provided us with a relatively large amount of material.

Our study revealed that the CBF of ciliated epithelium does not change significantly after storage in liquid nitrogen. There was no difference in CBF after tissues had been stored for periods varying from 1 week to 3 months. It has been demonstrated that biological time stops when living tissues are stored below -103 °C (6). Therefore, we believe that samples can be kept even longer than 3 months without affecting the frequency of the ciliary beat. Additionally, measurements were also carried out in Dulbecco's MEM with 10% FCS, a medium without a cryopreservative. The resulting frequencies were found to occur in a more physiological range when compared to frequencies measured in cryopreservatives containing either DMSO or HSPM.

After thawing, more solitary ciliated cells and clumps of ciliated cells were observed compared to mucosa that was not cryopreserved. However, proper rows of beating cilia were also found in sufficient quantities for measuring

CBF. After cryopreservation, we saw more moving cilia in specimens preserved in DMSO medium than in HSPM. Our finding of no moving cilia in 6 out of 60 specimens preserved in HSPM suggests that the latter substance has a stronger ciliostatic effect than DMSO medium. This was surprising to us, since DMSO results in a greater reduction in the motility of human spermatozoa than HSPM (12). Nonetheless, since the cryopreservatives have reversible ciliostatic properties, a thorough washout after thawing is mandatory.

We conclude that sphenoid sinus mucosa is an appropriate source of ciliated mucosa for *in vitro* experiments. Cryopreservation is also a good method for maintaining viable ciliated cells over a relatively long period of time. This means that a "mucosa bank" is now available that can provide large quantities of healthy human ciliated epithelium for *in vitro* experiments.

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CHAPTER 3

VALIDATION OF ANIMAL EXPERIMENTS ON CILIARY FUNCTION IN VITRO - I - THE INFLUENCE OF SUBSTANCES USED CLINICALLY

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Acta Otolaryngol (Stockh) (1999) 119: 93-97

ABSTRACT

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 been used frequently 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 anesthetics: cocaine (3% and 7%) and lidocaine (2%). These anesthetic substances were adjusted to pH 6 and pH 7. We also used two decongestants, namely xylometazoline 0.1% and oxymetazoline 0.1%, and the ß-blocking agent propranolol. Topical anesthetics 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.

INTRODUCTION

Mucociliary transport is an important defense 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 anesthetics lidocaine and cocaine, the decongestants xylometazoline and oxymetazoline, and the ß-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 of 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); NaHCO3, 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 analyzed by a Fast Fourier Transform algorithm. After measuring the initial frequency, the tissue was placed into 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 mixture of 69% Dulbecco's MEM (Gibco, Paisley, United Kingdom), 20% foetal 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 sample. 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 anesthetics 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 ß-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 Figures 1-8. Measurements in LR were used as a control (14). 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 ß-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.

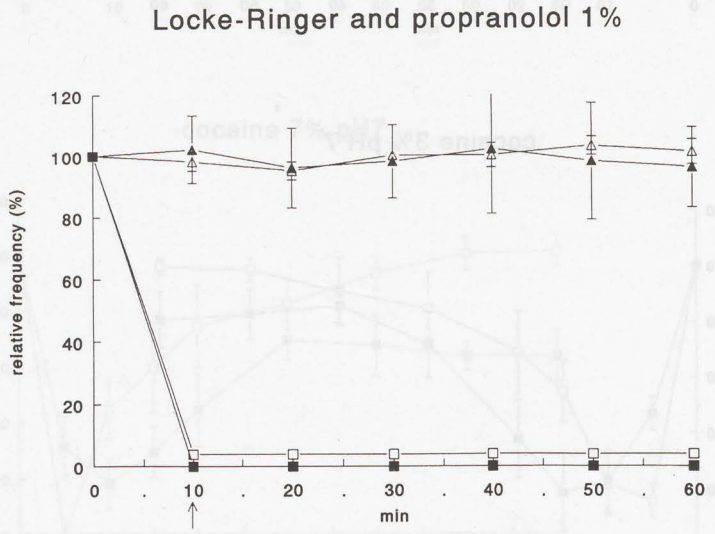


Fig. 1 The effect of LR on the CBF of human (▲) and chicken material (△), and the effect of propranolol 1% on the CBF of human (■) and chicken material (□). When CBF was 0 Hz, the tissue is rinsed and placed in LR (↑). Data are mean ±SEM (standard error of the mean). Values are percentages of the mean 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).

Figures 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 difference between groups and time existed

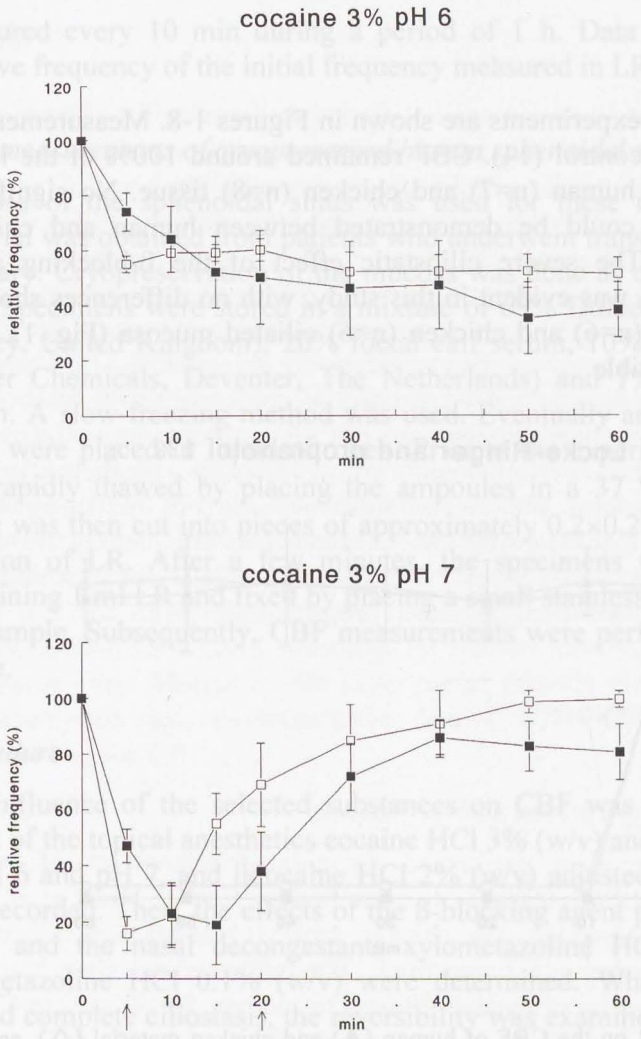


Fig. 2 and 3 The effect of cocaine HCl 3% pH 6 and pH 7 on the CBF of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue is rinsed and placed in LR (between ↑). Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 10.0 Hz (±0.8 SEM) pH 6 and 9.9 Hz (±1.0 SEM) pH 7 and in chicken material 19.3 Hz (±0.5 SEM) pH 6 and 20.7 Hz (±0.8 SEM) pH 7.

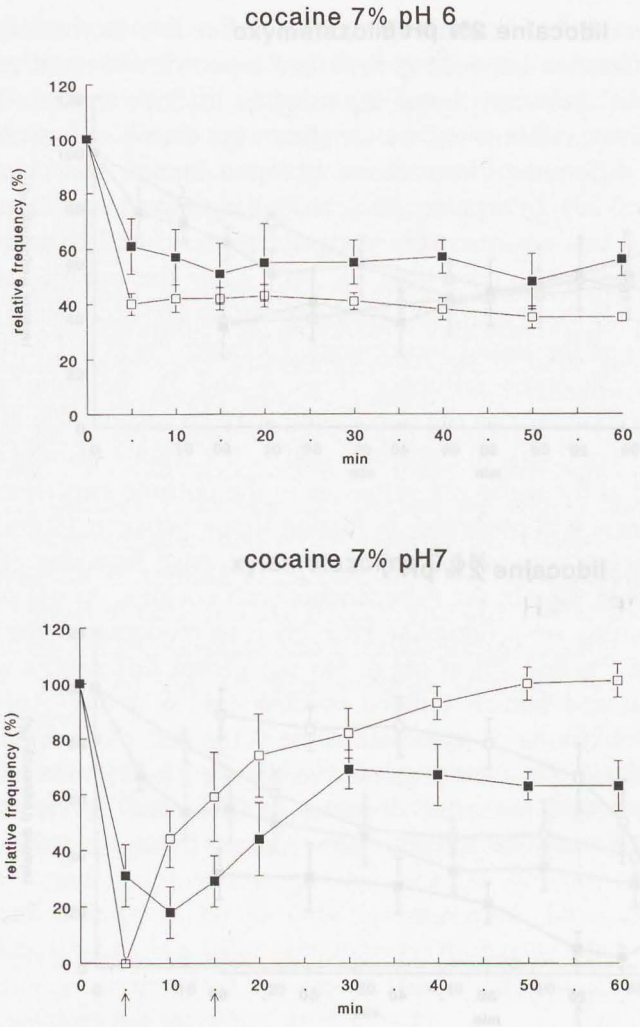
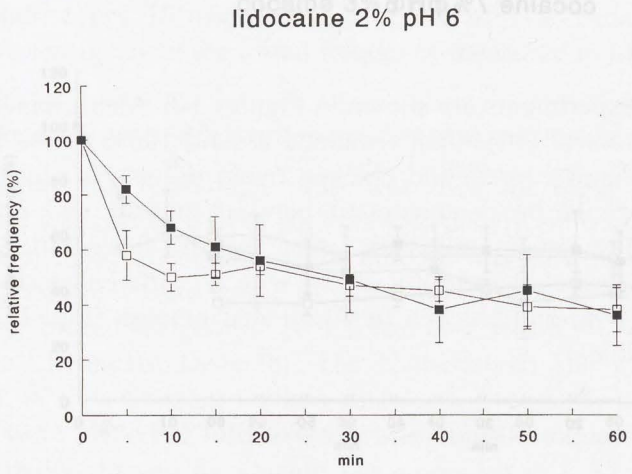


Fig. 4 and 5 The effect of cocaine HCl 7% pH 6 and pH 7on the CBF of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue is rinsed and placed in LR (between ↑). Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 8.9 Hz (±0.8 SEM) pH 6 and 10.2 Hz (±0.5 SEM) pH 7 and in chicken material 19.4 Hz (±1.0 SEM) pH 6 and 19.2 Hz (±0.5 SEM) pH 7.



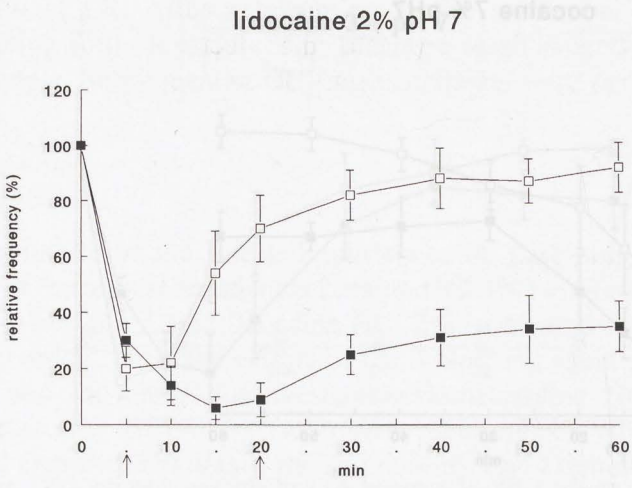
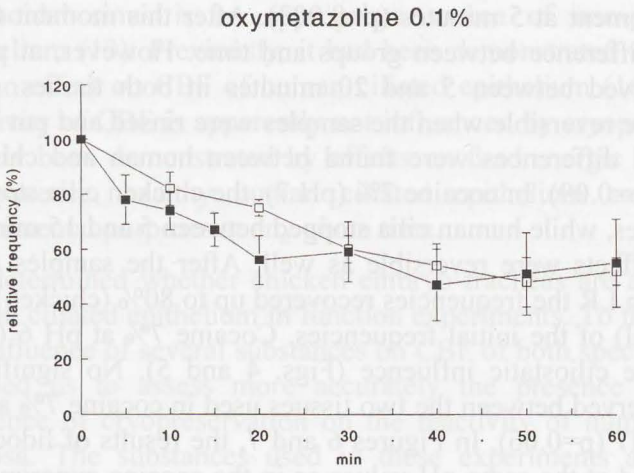


Fig. 6 and 7 The effect of lidocaine HCl 2% pH 6 and pH 7 on the CBF of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue is rinsed and placed in LR (between ↑). Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 9.6 Hz (±0.6 SEM) pH 6 and 11.4 Hz (±0.2 SEM) pH 7 and in chicken material 18.3 Hz (±1.2 SEM) and 19.5 Hz (±0.6 SEM) pH 7.



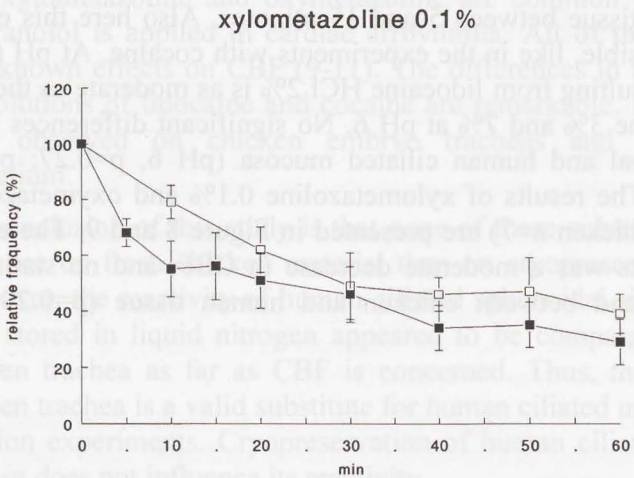


Fig. 8 and 9 The effect of xylometazoline 0.1% and oxymetazoline 0.1% on the CBF of human (■) and chicken (□). Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies for xylometazoline 0.1% were in human material 9.9 Hz (±0.7 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).

solely at the measurement at 5 minutes (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 minutes 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). In cocaine 7% (pH 7), the chicken cilia stopped beating after 5 minutes, while human cilia stopped between 5 and 15 minutes (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). In Figures 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 minutes, in chicken tissue between 5 and 15 minutes. Also here this effect appeared to be reversible, like 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 0.1% and oxymetazoline 0.1% (human n=6, chicken n=7) are presented in Figure 8 and 9. 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).

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 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 ß-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 anesthetics. 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 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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CHAPTER 4

VALIDATION OF ANIMAL EXPERIMENTS ON CILIARY FUNCTION IN VITRO - II - THE INFLUENCE OF ABSORPTION ENHANCERS, PRESERVATIVES AND PHYSIOLOGIC SALINE

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Acta Otolaryngol (Stockh) (1999) 119: 98-101

ABSTRACT

Ciliary beat frequency (CBF) is one of the most important parameters of mucociliary clearance. Previously, we demonstrated that mucosa from chicken embryo trachea is a good substitute for human ciliated epithelium to study the effects on CBF of substances that are used clinically. In this study, we examined the effect on CBF of four excipients for nasal drug formulations: the absorption enhancers methylated ß-cyclodextrin 2% and sodium taurodihydrofusidate 1%, the preservative benzalkonium chloride 0.01%, and physiologic saline. We also examined the effect on CBF of the cryopreservative dimethylsulfoxide, which is used to protect ciliated epithelium prior to storage in liquid nitrogen. Results obtained with chicken embryo trachea were compared with those of cryopreserved human mucosa taken from the sphenoidal sinus. For all of the substances tested, the effects on CBF of chicken material were comparable to those measured on human material. Benzalkoniumchloride had a stronger ciliostatic effect on human tissue. After 60 min, however, the effect of that substance on CBF was similar in both tissues. We conclude that chicken embryo trachea can be used as a substitute for human ciliated mucosa when studying ciliary activity in vitro.

INTRODUCTION

It is uncertain whether or not the ciliary reactivity of mucosa from chicken embryo tracheas is similar to that of mucosa from the human upper respiratory tract. This issue is relevant since specimens of the tracheas of chicken embryos are frequently used as a substitute for human material in a variety of experiments (1-4). We therefore compared the reactivity of ciliary beat frequency (CBF) in chicken embryo trachea with that observed in human material. The latter was examined in the form of cryopreserved mucosa from the sphenoidal sinus. The first part of this study deals with the validity of using the epithelium of chicken embryo trachea as a substitute for human ciliated epithelium. The validity was tested by using local anesthetics, decongestants, and propanolol (5). The aim of the present study is to determine how two nasal absorption enhancers, namely methylated ß-cyclodextrin (MßCD) and sodium taurodihydrofusidate (STDHF), and the preservative benzalkoniumchloride, and physiologic saline affect CBF. These substances have been frequently used in experiments with ciliated mucosa. In addition the study is intended to show the effect of dimethylsulfoxide (DMSO) on CBF. DMSO is used in a cryopreservation medium to protect human sinusoidal mucosa, before the mucosa is stored in liquid nitrogen (6).

MATERIALS AND METHODS

CBF measurements of chicken embryo trachea and cryopreserved human sphenoidal sinus mucosa

The methods used here are identical to the ones we described previously in the report on the first part of this validation study (5).

Procedure

The influence on CBF of two nasal absorption enhancers was measured. One is methylated ß-cyclodextrin (MßCD), in a concentration of 20 mg/ml (2% w/v) in Locke-Ringer's solution (LR). The other is sodium taurodihydrofusidate (STDHF) in a concentration of 10 mg/ml (1% w/v) in LR. In addition we assessed the effect on CBF of physiologic saline, 0.9% NaCl in Millipore water, and the benzalkoniumchloride, at a concentration of 0.01% w/v in LR. The chosen concentrations of these excipients are

commonly used in nasal drug formulations. Moreover, the effect on CBF of DMSO 10% was investigated. Whenever a substance caused complete ciliostasis, the reversibility of that effect was examined after rinsing with LR and putting the sample back in a well with LR. For each other substance a new sample of ciliated mucosa was used. All chemicals used in the experiments were of analytical quality.

Statistical analysis

The results were evaluated by multivariate repeated measures of analysis of variance (MANOVA). A P value < 0.05 was considered statistically significant.

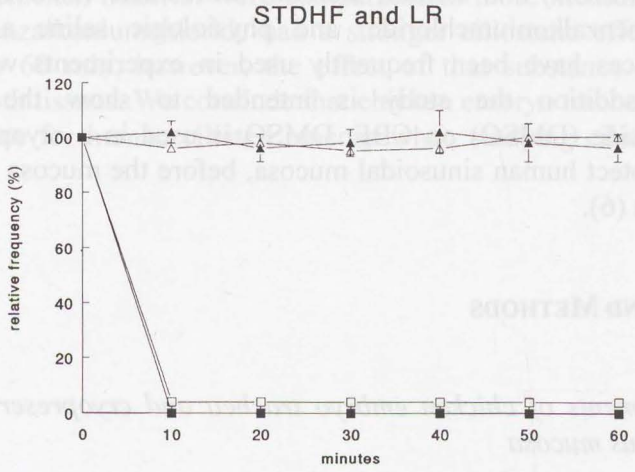

Fig. 1 The effect of Locke-Ringer on CBF of human (▲) and chicken material (△) material, and STDHF 1% of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue was rinsed and placed in LR (□). Data are mean ±SEM (standard error of the mean.). Values are percentages of the mean initial frequencies. 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 STDHF in human material 8.6 Hz (±0.4 SEM) and in chicken material 19.1 Hz (±0.5 SEM).

RESULTS

The results of these experiments are shown in Figures 1-5. Measurements in pure LR were chosen as a control, since this solution is used to maintain the viability of the ciliated tissue (7). CBF remained around 100% of the initial frequencies in both human (n=7) and chicken (n=8) tissue. No significant difference could be demonstrated between the frequencies measured in human and chicken material (P=0.92). The severe ciliostatic effect of the absorption enhancer STDHF 1% was manifest in this study, with no differences between human (n=7) and chicken (n=4) ciliated mucosa (Fig. 1). This effect was not reversible. Figure 2 shows the moderate ciliostatic effect of the enhancer MßCD 2%. No significant difference was observed between chicken (n=6) and human (n=7) material (P=0.07) for this substance either.

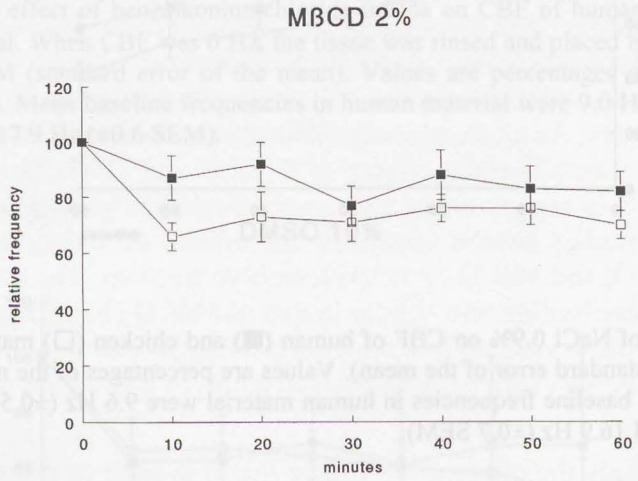

Fig. 2 The effect of MßCD 2% on CBF of human (■) and chicken (□) material. Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 8.6 Hz (±0.6 SEM) and in chicken material 18.4 Hz (±0.6 SEM).

Physiologic saline was mildly ciliostatic as well (Fig. 3). Again, no significant difference was found between animal (n=10) and human (n=8) ciliated mucosa (P=0.23). The preservative benzalkoniumchloride 0.01% was more ciliostatic, as demonstrated in Figure 4. Its effect was statistically

significant greater (P=0.02) in human (n=8) than in chicken (n=8) trachea. The effect of DMSO 10% on chicken tissue (n=7) was compared to its effect on cryopreserved human ciliated mucosa (n=5) (Fig. 5). This substance produced also a mild inhibition of CBF. No significant difference was found (P=0.13).

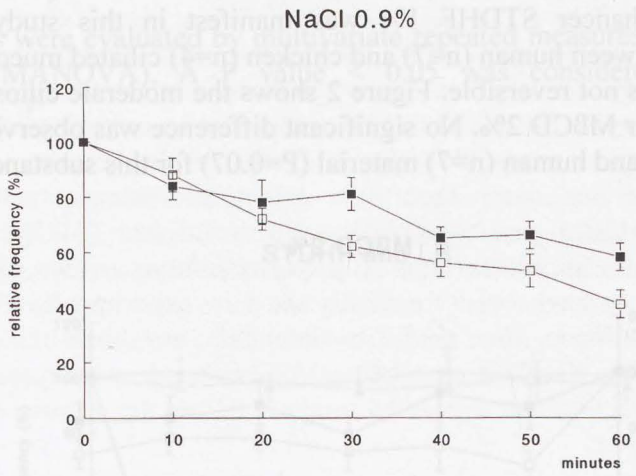

Fig. 3 The effect of NaCl 0.9% on CBF of human (■) and chicken (□) material. Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 9.6 Hz (±0.5 SEM) and in chicken material 16.9 Hz (±0.7 SEM).

DISCUSSION

CBF is a valid indicator of mucociliary transport (8). Animal experiments are frequently used to measure the effects on CBF because it is difficult to obtain sufficient quantities of healthy human ciliated mucosa (9,10). This study design allows us to assess the in vitro influence of these substances on normal non-pathological human mucosa and to compare the results with data from chicken experiments. Experiments with human material are usually performed on adenoids (10,11), but it is a disadvantage that this is pathological tissue. The need to perform an adenoidectomy nearly always

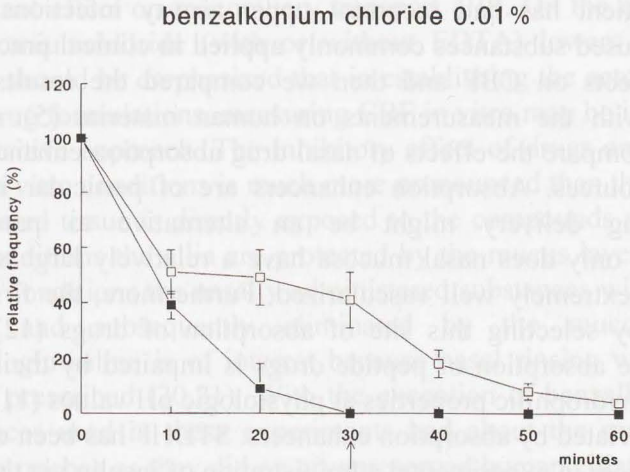

Fig. 4 The effect of benzalkoniumchloride 0.01% on CBF of human (■) and chicken (□) material. When CBF was 0 Hz, the tissue was rinsed and placed in LR (↑). Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 9.0 Hz (±0.5 SEM) and in chicken 17.9 Hz (±0.6 SEM).

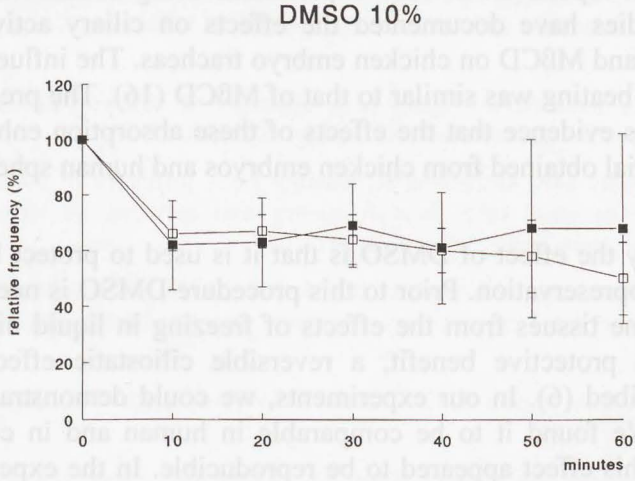

Fig. 5 The effect of DMSO 10% on CBF of human (■) and chicken (□) material. Data are mean ±SEM (standard error of the mean). Values are percentages of the mean initial frequencies. Mean baseline frequencies in human material were 9.4 Hz (±1.2 SEM) and in chicken material 19.9 Hz (±0.9 SEM).

means that the patient has had recurrent upper airway infections. In a previous study, we used substances commonly applied in clinical practice to determine their effects on CBF and then we compared the results from chicken material with the measurements on human material (5). In the present study, we compare the effects of nasal drug absorption enhancers on tissue from both sources. Absorption enhancers are of particular interest because nasal drug delivery might be an alternative to parenteral administration. Not only does nasal mucosa have a relatively large surface area, but also it is extremely well vascularized. Furthermore, the first-pass effect is avoided by selecting this site of absorption of drugs (12). One drawback is that the absorption of peptide drugs is impaired by their large molecular size and hydrophilic properties at physiologic pH values (11). This problem can be alleviated by absorption enhancers. STDHF has been used as an absorption enhancer in experimental administration of insulin via the nose (13). Dimethyl-ß-cyclodextrin (DMßCD) has served as an absorption enhancer in experiments on the nasal administration of steroid hormones and peptide drugs such as insulin (14,15). For obvious reasons, absorption enhancers are supposed to have some influence on the properties of the nasal mucous membranes. As a consequence, some effect on the ciliary activity is to be expected. The ciliostatic effects of STDHF and DMßCD have been well documented in reports on in vitro experiments using human adenoids (11,15). Other studies have documented the effects on ciliary activity of STDHF, DMßCD, and MßCD on chicken embryo tracheas. The influence of DMßCD on ciliary beating was similar to that of MßCD (16). The present in vitro study provides evidence that the effects of these absorption enhancers are similar in material obtained from chicken embryos and human sphenoidal sinuses.

The reason to study the effect of DMSO is that it is used to protect human mucosa during cryopreservation. Prior to this procedure DMSO is needed to protect cells in some tissues from the effects of freezing in liquid nitrogen (17,18). One such protective benefit, a reversible ciliostatic effect, has already been described (6). In our experiments, we could demonstrate that effect on CBF. We found it to be comparable in human and in chicken tissue. Moreover, this effect appeared to be reproducible. In the experiment reported here, we administered DMSO for the second time to human ciliated mucosa that had been thawed and washed out after having been stored in a cryopreservation medium containing DMSO.

The effect of benzalkoniumchloride was studied because this preservative is frequently used in nasal preparations. Some authors deny that it has a

significant effect on mucociliary transport (19). On the other hand, in vitro benzalkoniumchloride (with or without EDTA) lowers CBF considerably (12). It should be emphasized that in establishing the actual local toxicity of nasal drug formulations, measuring CBF in vitro may be in some situations a too sensitive approach. The inhibitory effect of drugs and excipients under these in vitro conditions is much more pronounced than that in vivo. In vitro, the ciliated tissue is directly exposed to the compounds studied, whereas in vivo conditions the cilia are protected by the mucus layer. Moreover, under in vivo conditions the nasally administered substances will be diluted by the mucus and subsequently eliminated by the mucociliary clearance. Physiologic saline is of interest because nasal rinsing with this solution is widely prescribed (20,21). With the exception of benzalkoniumchloride the substances used in these experiments had about the same effect on fresh chicken trachea as they did on cryopreserved human ciliated epithelium. The ciliostatic effect of benzalkoniumchloride was stronger on human tissue. After 60 min, however, the effect of this substance on CBF was comparable in tissue from both sources. We conclude that the effects in vitro on CBF of chicken trachea will be similar to the effects measured in experiments on cryopreserved human ciliated epithelium.

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CHAPTER 5

NASAL MUCOCILIARY TRANSPORT: NEW EVIDENCE FOR A KEY ROLE OF CILIARY BEAT FREQUENCY

W.M. Boek, K. Graamans, J.R. Natzijl, P.P. van Rijk, E.H. Huizing

Submitted

ABSTRACT

Mucociliary transport (MCT) is an important defense mechanism of the respiratory tract. Nonetheless the factors determining MCT are only partially understood. Ciliary beat frequency (CBF) is assumed to be one of the main parameters, although the experimental evidence remains inconclusive. This study measures the effects on MCT of two cilio-inhibiting compounds (xylometazoline 0.1% and NaCl 0.9%) and a cilio-enhancer (salbutamol 0.1%). The measurements were performed by a technetium-99m nebulizing scintigraphic method. The experiments were carried out in 15 healthy young volunteers. Xylometazoline 0.1% appeared to slow down ciliary transport, though the decrease was not significant ($P = 0.44$). NaCl 0.9% did reduce MCT significantly ($P = 0.033$). Salbutamol 0.1% resulted in a highly significant increase of MCT ($P = 0.009$). Xylometazoline brings about drastic changes in the nasal cavity, both anatomically and physiologically. Any comparison of MCT before and after using this vasoconstrictive agent has to take this effect into account. This study demonstrates a significant similarity in the effects of NaCl and salbutamol on CBF in vitro and on MCT in vivo. The evidence from our experiments suggests that CBF is a determining factor in the MCT rate in the nose.

INTRODUCTION

Mucociliary transport (MCT) plays a vital role in the defense of the upper as well as the lower respiratory tract. MCT is influenced by ciliary activity and the properties of the mucus layer. Measuring ciliary beat frequency (CBF) with a photoelectric device is a direct method to assess ciliary activity (1,2). MCT can be measured in various ways. The most accurate method of establishing the MCT rate is to register radioactivity in the nasal cavity after nebulizing with a solution containing technetium-99m (^{99m}Tc) (3).

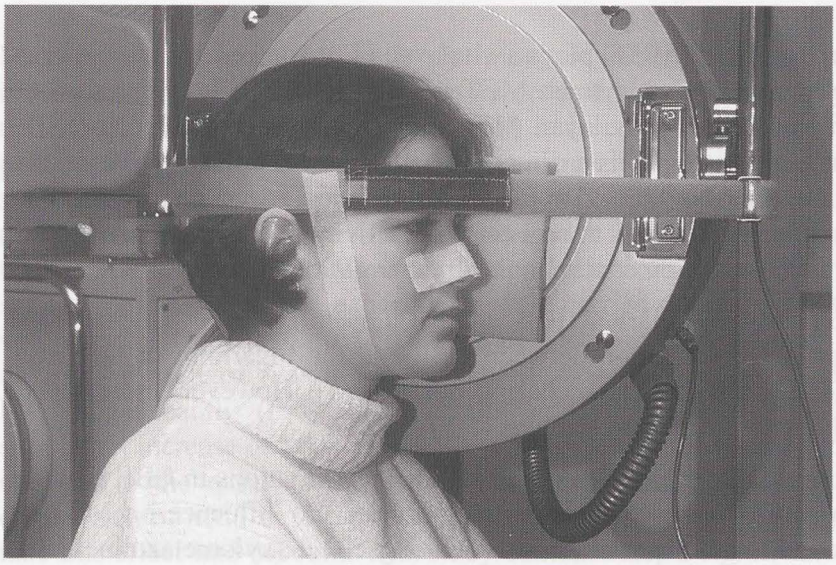
The relation between ciliary activity and MCT is still unclear. Duchateau et al. found a linear correlation between CBF and the logarithm of the MCT time as measured with the saccharine dye test (4). However, more recent studies did not confirm those findings (5-7).

The objective of this study is to establish whether variations in CBF produce changes in MCT. Our experiments demonstrated the influence on MCT of two compounds with a proven cilio-inhibiting effect (xylometazoline 0.1% and NaCl 0.9%) and one with a proven cilio-enhancing effect (salbutamol 0.1%). These experiments were conducted in vivo, using a ^{99m}Tc nebulizing method (1, 8-14). The hypothesis states that these substances are expected to impair or stimulate MCT according to their known effects.

MATERIALS AND METHODS

Test subjects

Fifteen healthy volunteers (12 women and 3 men, mean age 21.8 years, range 18 - 25) were included in this study. The criteria for exclusion were acute rhinitis 4 weeks prior to the experiments, a history of vasomotor rhinitis, chronic purulent rhinitis, allergic rhinitis, nasal surgery, major anatomic abnormalities of the nose, smoking, and any medication except contraceptives. The study was approved by an Independent Ethical Committee and performed according to the standards of Good Clinical Practice. Each subject gave their informed written consent according to the "Declaration of Helsinki" of the World Medical Association.



Test substances

The effect on MCT of three substances with well-documented influence on CBF in vitro was investigated: two cilio-inhibitors, xylometazoline 0.1% (w/v) and NaCl 0.9% (w/v) and one cilio-enhancer, salbutamol 0.1% (w/v).

Measurement of mucociliary transport time

Each subject took part in three experiments. At every session, a baseline MCT was established. Then one of the test compounds was applied, followed by a second measurement. The experiments were performed in an air-conditioned room where temperature and humidity were constant. The subjects were seated in this room 30 min prior to the experiment. Measurements were carried out between 9:00 and 12:00 a.m. in order to eliminate the influence of circadian and nasal rhythms. During the experiments, the subjects were allowed to drink only water. Each person had an interval of at least one week between each of the three experiments.

The most patent nasal cavity was first sprayed with 0.2 ml (2.2 MBq) ^{99m}Tc Albures labelled albumin solution (Amersham Cygne, Solco(r) Albures, Eindhoven, the Netherlands) using a pump spray (Glaxo Wellcome, Zeist, the Netherlands). A gamma camera ADAC Transcam (ADAC, Milpitas, CA, USA) with a low-energy, high-resolution (LEHR) collimator was positioned lateral to the subject's head. ^{99m}Tc markers had been placed on top of the nose and on the mastoid tip. The markers served as points of reference for subject positioning. Immediately after spraying of the radiopharmaceutical, dynamic imaging was started. The imaging went on for 20 min. The subjects were then allowed to blow their nose. Thirty minutes later, a new image was made in order to detect any residue of the radiopharmaceutical in the nasal cavity. When no residue was found, 0.2 ml of one of the test solutions was sprayed into the same nasal cavity. The subjects then waited 15 min before further measurement in order to allow the substance to interact with the ciliated epithelium. Subsequently a second measurement with the ^{99m}Tc Albures was carried out. Dynamic imaging with 30 sec frames was performed over a period of 20 min. The distance between the accumulation of radioactivity in the vestibule and the area where the front of the transported radiopharmaceutical reached the posterior margin of the soft palate was measured on a composite image. A region of interest was drawn at the posterior margin of the soft palate. Histograms were generated to determine the time of arrival of the first front of the radiopharmaceutical. Subsequently two persons independently of each other could calculate the rate in mm/min. The mean of their results was taken as basis for further analysis.

Statistical analysis

A logarithmic transformation was used to approximate the data to normal distribution because of the non-parametric distribution of transport time. A paired Student's t-test was used to compare the log-transport times before and after administering a test substance. A P value < 0.05 was considered to be statistically significant.

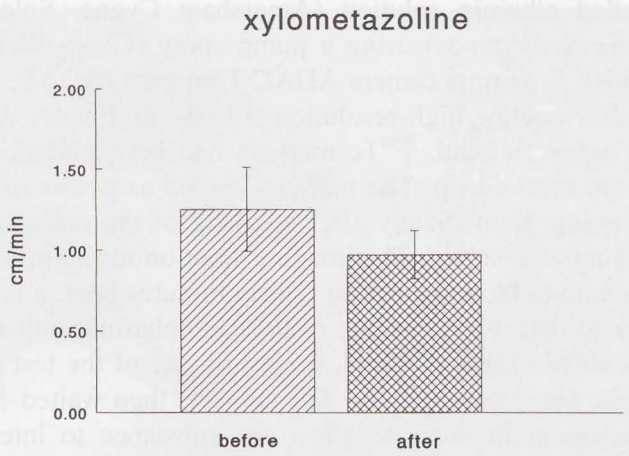


Fig 1 Mucociliary transport rates before and after nebulizing xylometazoline 0.1%. Data are mean ± SEM, n = 13. The difference is not significant (P = 0.44).

RESULTS

One subject had to be excluded because no MCT was found in any of his tests even though he met the criteria for enrolment in this study. In another subject, only two of the tests could be used because no MCT was visible during one experiment, probably due to rhinitis.

The results of the experiments are shown in Figs. 1 - 3. Xylometazoline 0.1% (n =13) reduced mean MCT from 12.5 mm/min (± 2.6 SEM) to 9.7 mm/min (± 1.5 SEM). This reduction was not significant (P = 0.44). In the NaCl 0.9% experiment (n =14), a significant decrease from 7.9 mm/min (± 1.5 SEM) to 4.5 mm/min (± 1.6 SEM,) was measured (P = 0.033). Salbutamol 0.1% (n = 14) enhanced MCT from 8.0 mm/min (± 1.4 SEM) to 12.5 mm/min (± 1.1 SEM). This increase is highly significant (P = 0.009).

The correlation coefficients of the interobserver differences in the three experiments were 0.88 (xylometazoline), 0.89 (NaCl), and 0.68 (salbutamol).

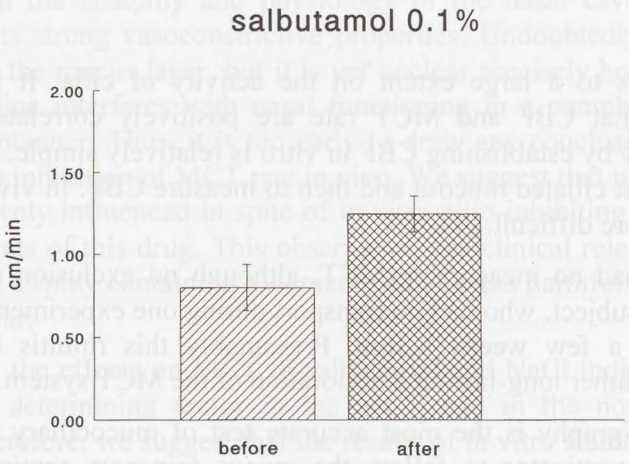


Fig. 2 Mucociliary transport rate before and after nebulizing salbutamol 0.1%. Data are mean ± SEM, n = 14. The difference is highly significant (P = 0.009).

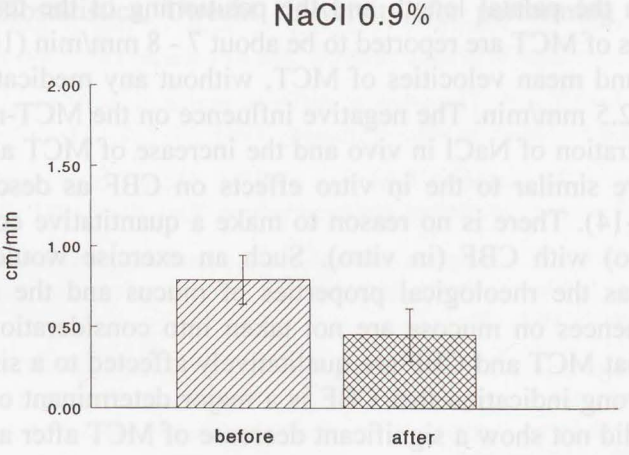


Fig 3 Mucociliary transport rate before and after nebulizing NaCl 0.9%. Data are mean ± SEM, n = 14. The difference is significant (P = 0.033).

DISCUSSION

MCT depends to a large extent on the activity of cilia. It is logical to hypothesise that CBF and MCT rate are positively correlated. Studying ciliary activity by establishing CBF in vitro is relatively simple, as it is fairly easy to harvest ciliated mucosa and then to measure CBF. In vivo evaluation of MCT is more difficult.

One person had no measurable MCT, although no exclusion criteria were present. The subject, who had no transport during one experiment, did have a mild rhinitis a few weeks before. Presumably this rhinitis had actually resulted in a rather long-lasting deterioration of the MCT system.

Gamma scintigraphy is the most accurate test of mucociliary clearance. It allows the investigator to follow the mucus transport continuously. The saccharine-dye test is inferior because it only provides a random indication of MCT (15). In most studies, a droplet of dye, saccharine, or ^{99m}Tc is placed on the head of the inferior turbinate. By nebulizing ^{99m}Tc, it is possible to observe not only the transport rate but also the behaviour of the nasal mucosa as a whole. Moreover, this method eliminates the influence of individual differences in the palatal length and the positioning of the test substance. Normal values of MCT are reported to be about 7 - 8 mm/min (16,17). In this study, we found mean velocities of MCT, without any medication, ranging from 7.9 to 12.5 mm/min. The negative influence on the MCT-rate recorded after administration of NaCl in vivo and the increase of MCT after spraying salbutamol are similar to the in vitro effects on CBF as described in the literature (11-14). There is no reason to make a quantitative comparison of MCT (in vivo) with CBF (in vitro). Such an exercise would imply that factors such as the rheological properties of mucus and the nervous and humoral influences on mucosa are not taken into consideration. However, our finding that MCT and CBF are qualitatively affected to a similar extent, provides a strong indication that CBF is a major determinant of MCT. Our experiments did not show a significant decrease of MCT after administering xylometazoline. Some reduction of the mean MCT rate was found. However, this outcome was caused by the measurements in one subject who had a very fast MCT rate during that experiment. As a consequence the mean value was drastically influenced. Xylometazoline 0.1% contains benzalkoniumchloride as a preservative. Both compounds have a well-known cilio-inhibiting effect in vitro (9). In view of that property, our finding is the opposite of what was to be expected. Furthermore, the outcomes of the experiments with NaCl and salbutamol would lead us to expect an inhibitory effect. Xylometazoline

changes both the anatomy and physiology of the nasal cavity drastically because of its strong vasoconstrictive properties. Undoubtedly it has some influence on the mucus layer, but it is yet unclear precisely how that works. Xylometazoline interferes with nasal functioning in a complex and as yet unspecified manner. Thus, it is too early to draw any conclusions from this finding of no inhibition of MCT rate in vivo. We suggest that nasal clearance is not negatively influenced in spite of in vitro cilio-inhibiting effects of the two ingredients of this drug. This observation has clinical relevance since it suggests that a spray containing xylometazoline is less harmful than has been assumed so far.

In summary, the effects on MCT of salbutamol and NaCl indicate that CBF is indeed a determining factor in the MCT rate in the nose of healthy subjects. Therefore, we suggest that the results of in vitro studies of CBF can be applied to MCT in vivo.

Acknowledgement

We are grateful for the financial support provided by Glaxo Wellcome, Zeist, the Netherlands. We would like to thank C.L.J.J. Kruitwagen, MSc, at the Center for Biostatistics, Utrecht University, for performing the statistical analysis.

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CHAPTER 6

PHYSIOLOGIC AND HYPERTONIC SALINE SOLUTIONS IMPAIR CILIARY ACTIVITY IN VITRO

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Laryngoscope (1999) 109: 396-399

ABSTRACT

Physiologic saline (NaCl 0.9%) is commonly used in treating acute and chronic rhinosinusitis. Moreover, physiologic saline is used as a control medium, vehicle, or solvent in studies on ciliary beat frequency (CBF). Hypertonic saline (NaCl 7% and 14.4%) has been applied in attempts to enhance mucociliary transport in patients with cystic fibrosis or asthma and in healthy subjects. Therefore the objective of this study is to document in vitro effects of saline solutions in different concentrations on CBF. The effects on CBF of cryopreserved mucosa of the sphenoidal sinus were measured by a photoelectric method. Initial frequencies, measured in Locke-Ringer's solution (LR), were compared to CBF after exposure to NaCl in concentrations of 0.9, 7.0 and 14.4% (w/v). NaCl 0.9% has a moderately negative effect on CBF. The 7% solution leads to a complete ciliostasis within 5 min, though this effect turns out to be reversible after rinsing with LR. A hypertonic solution of 14.4% has an irreversible ciliostatic effect. LR is an isotonic solution that has no effect on CBF. Therefore it is probable that this solution is more appropriate than saline for nasal irrigation and nebulization or antral lavage. Moreover, the results of this study suggest that mucolytic effects induced by hyperosmolarity should preferably be attained with hypertonic saline 7% in patients with cystic fibrosis or asthma. At this concentration, the ciliostatic effect is reversible, whereas irreversible changes are to be expected at higher concentrations.

INTRODUCTION

Saline solutions are a mainstay of the therapeutic armamentarium for patients with upper airway infections. Nasal irrigation with physiologic saline (NaCl 0.9%) is recommended in acute and chronic rhinosinusitis (1-3). The international consensus report on the diagnosis and management of rhinitis recommends saline lavage for the treatment of rhinitis (4). Antral lavage with NaCl 0.9% is a traditional treatment of chronic sinusitis (5). Moreover, physiologic saline is used as a control medium and a solvent in studies on ciliary beat frequency (CBF) (6-8). Hypertonic saline (NaCl 7%, 14.4%) has been applied in attempts to enhance mucociliary transport in patients with cystic fibrosis, asthma, and in healthy subjects (9-11). However, the effect of saline solutions on CBF is not well known. CBF is one of the most important parameters of mucociliary clearance (12). Therefore, the aim of this study was to investigate the effects of 0.9%, 7%, and 14.4% of saline solution on CBF.

MATERIALS AND METHODS

Tissue preparation

Cryopreserved mucosa of the sphenoidal sinus was used as a source of ciliated epithelium of the human airways. This material was obtained from patients who underwent transnasal surgery of the pituitary. The mucosa is non-pathological since concomitant pathology of the nose or the paranasal sinuses was a contraindication for the operation. Moreover, cryopreservation has no deleterious effect on CBF (13). The freezing of the mucosa took place as described previously (13). A slow freezing method was used. Eventually ampoules with specimens were placed in liquid nitrogen. Prior to the experiments, these specimens were quickly thawed by placing the ampoules in a 37 °C water bath. The tissue was thoroughly rinsed twice in Locke-Ringer's solution (LR). It was then cut into pieces of approximately 0.2×0.2 cm and placed in a perfusion chamber filled with LR. This is an isotonic solution of NaCl, 7.72 g (132 mmol); KCl, 0.42 g (5.63 mmol); CaCl2.2H2O, 0.16 g (1.24 mmol); NaHCO3, 0.15 g (1.79 mmol); glucose anhydrous, 1.00 g (5.55 mmol) in 1 litre of water. Previously, it had been shown to have no effect on CBF (13).

CBF measurements

CBF was measured by a photoelectric method described previously by Ingels et al. (14). The samples were examined in a perfusion chamber mounted in an aluminium frame. In order to approximate the physiologic temperature of nasal mucosa, the chamber was kept at 33 °C by means of an electronic heating device (12). A phase-contrast microscope (Leitz, Wetzlar, Germany) was adapted with disc interference contrast optics using a 25× objective and a 10×ocular in the measuring tube. A variable circular diaphragm was mounted in the same tube along with a beam splitter and a visaflex housing to allow a control view of the measured area. Light was provided by a 12V/250 W halogen lamp. A highly sensitive photodetector converted the light fluctuations, which were caused by the beating action of cilia, into an electrical signal. This signal was digitised in an IBM-XT personal computer using a 12-bit A/D converter with a sample frequency of 200 Hz. A fast Fourier transform analysis of the recorded signal was performed over a period of 20 sec, starting arbitrarily at the 5th sec. CBF was determined from the first harmonic.

Experimental design

The initial frequency was measured 15 min after placing the sample in the perfusion chamber containing LR. Then 2 ml of one of the saline solutions, at a temperature of 33 °C, was gently poured into the perfusion chamber. Afterwards, CBF was measured every 10 min during the course of 1 h. In this way, the possibility of mechanical influences was excluded (14). Whenever a solution caused complete ciliostasis, the possibility that this effect was reversible was tested by replacing the contents of the perfusion chamber with LR. In the event that ciliostasis was observed within the first 5 min, the saline solution was replaced by LR and measurements were performed every 10 min after the perfusion with LR. The effects of NaCl concentrations of 0.9% (w/v), 7.0% (w/v), and 14.4% (w/v) in Millipore water were examined. Specimens of the mucosa of 8 patients were used.

Statistical analysis

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

RESULTS

The initial frequencies were measured in LR, since this medium has proven to have no influence on CBF (15). Mean initial frequencies were 11.4 Hz (8.3-13.9), 11.0 Hz (6.5-15.6), and 10.4 Hz (6.6-13.2) in the 3 experiments with NaCl at concentrations of 0.9%, 7%, and 14.4% respectively. Fig. 1 depicts the effect of NaCl 0.9%. Mean frequency was reduced to 46% (42 - 52) within 10 min. This reduction is significant compared to the initial frequency ($P<0.001$). The effect of NaCl 7% is demonstrated in Fig. 2. After 5 min, no moving cilia were observed. After perfusion with LR, this effect turned out to be reversible. CBF returned to 70% (65 - 74) of its initial frequency, and rows of cells covered with moving cilia could be distinguished. This indicates that most of the ciliated cells had recovered. However, no recovery was observed when NaCl 14.4% was used (Fig. 3). At that concentration no moving cilia could be detected, even after 5 min. In the experiment using the higher concentration, beating cilia were observed after rinsing the perfusion chamber with LR though just in 4 out of 8 samples. Moreover, only some solitary cells in those four samples had beating cilia and they were beating slowly.

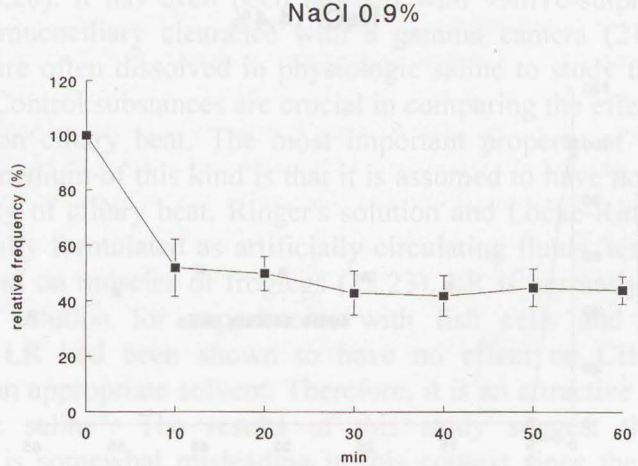

Fig. 1 Effect of NaCl 0.9% on the CBF of human sphenoidal mucosa. Data are mean ± SEM.

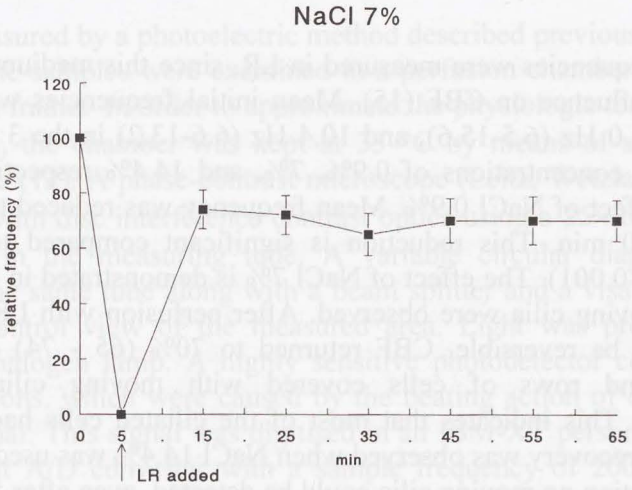

Fig. 2 Effect of NaCl 7% on the CBF of human sphenoidal mucosa. As soon as CBF measured 0 Hz, the saline solution was replaced by LR (↑). Data are mean ± SEM.

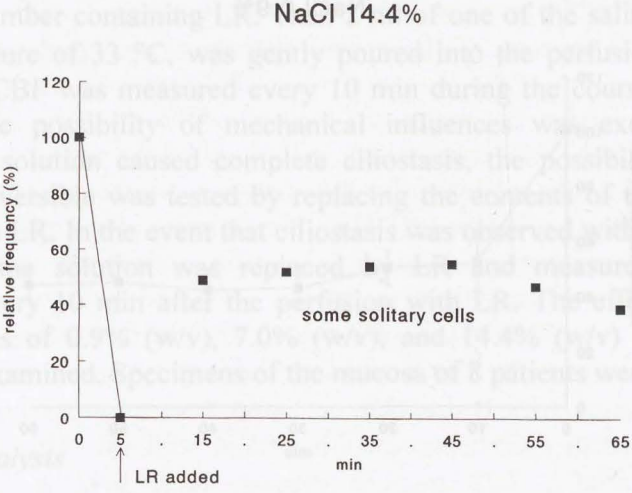

Fig. 3 The effect of NaCl 14.4% on the CBF of human sphenoidal mucosa. As soon as CBF measured 0 Hz, the saline solution was replaced by LR (↑). Data are mean ± SEM.

DISCUSSION

Ciliary beat is an important defense mechanism of the respiratory tract. Impairment of ciliary movement is frequently associated with respiratory diseases such as rhinitis, sinusitis, bronchitis, and otitis media (16,17). Nasal irrigation and nebulization as well as antral lavage with physiologic saline are recommended in rhinitis and sinusitis (1-4,18). Moreover, a combination of treatment is suggested, using antibiotics and sinus irrigation with saline, prior to functional endoscopic sinus surgery (19). The use of physiologic saline is invariably part of a variety of therapeutic strategies in patients with rhinosinusitis. However this study clearly demonstrates that physiologic saline decreases CBF in vitro. Therefore, physiologic saline might impair mucociliary clearance in vivo as well (12). This effect would be highly undesirable in therapeutic situations. Notwithstanding this possibility, positive results of irrigation with physiologic saline have been documented (1-4,18). We assume that the benefits come from evacuating debris and pus rather than from enhancing or protecting mucociliary transport. These irrigations might have a more beneficial result when a solution without ciliostatic effects is used.

Physiologic saline has been used as a control substance in studies on ciliary activity (6,8,20). It has even been labelled with 99mTc-sulphur colloid to investigate mucociliary clearance with a gamma camera (21). Moreover, substances are often dissolved in physiologic saline to study their effect on CBF (7,8). Control substances are crucial in comparing the effects of various substances on ciliary beat. The most important property of any solution, vehicle, or medium of this kind is that it is assumed to have no influence on the frequency of ciliary beat. Ringer's solution and Locke-Ringer's solution were originally formulated as artificially circulating fluids, tested on hearts of rabbits and on muscles of froglegs (22,23). LR is currently applied as a physiologic solution for experiments with fish cells and tissues (24). Previously, LR had been shown to have no effect on CBF (13). It is considered an appropriate solvent. Therefore, it is an attractive alternative to "physiologic saline". The results of this study suggest that the term physiologic is somewhat misleading in this context since the solution has unwanted effects on CBF.

Hypertonic saline solutions are used to enhance mucociliary clearance in patients with cystic fibrosis, asthmatics and healthy subjects. These solutions are used because they may have mucolytic properties (9-11). For such purposes, a concentration as high as 14.4% has been applied. The main

reason is that a large amount of nebulized fluid may trigger an asthma attack, whereas a small amount of fluid for nebulization is sufficient when using a substance with a high osmolarity. NaCl 7% has been applied on patients with cystic fibrosis. The results show a significant positive effect on mucociliary clearance (10). Patients with asthma or cystic fibrosis have an impaired mucociliary function. In this in vitro study a saline solution of 7% appeared to have a reversible ciliostatic effect, whereas the 14.4% solution had an irreversible negative effect on the ciliary activity. Moreover, cytotoxic effects occurred as a result of this high concentration. Granted, these patients did benefit from a short-term therapeutic effect. However, a high osmolarity may eventually be detrimental to their mucociliary clearance because of its adverse effects on ciliary activity as well as on the viability of the respiratory epithelium.

CONCLUSION

NaCl 0.9% appeared to have a negative effect on CBF in an in vitro situation. As a consequence, its role in the therapy of rhinosinusitis should be reconsidered. A solution with a composition that more closely approximates the extracellular fluids would be more deserving of the adjective "physiologic". LR is a good example. It does not affect CBF. Nor is there any reason to assume that LR would have adverse local or systemic side effects. Moreover, it is inexpensive and easy to produce. Therefore, tests using LR instead of NaCl 0.9% for nasal irrigation and nebulization as well as antral lavage should be started. We recommend that NaCl 0.9% should not be used in experiments on mucociliary activity as a solvent, vehicle, or control medium. Moreover, we conclude that a concentration of 7% is preferable if the application of saline solution is being considered in asthmatics and patients with cystic fibrosis. That preference is based on the evidence that, at least under in vitro conditions, the negative effects on CBF are reversible at this concentration.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Studying ciliary activity and mucociliary transport

Ciliary activity causes the transport of mucus in the airways, which is an essential defense mechanism of the respiratory tract. Mucociliary transport (MCT) can be studied by measuring ciliary activity - *in vitro* - as well as by recording MCT - *in vivo*. Ciliary activity in the airways has been a subject of interest for several decades. The movement of cilia is usually studied *in vitro* by measuring ciliary beat frequency (CBF) with a photoelectric method. Many biopsies are taken and many animals are sacrificed for *in vitro* experiments that are performed to measure CBF. Yet the findings of these studies are debatable, since interspecies differences have been reported. Biopsies of human resources have several drawbacks. Tissue harvested during surgery is often pathological. Moreover, biopsies from volunteers have to be taken without local anesthesia since anesthetics affect ciliary activity. So, there is still a need for non-pathological ciliated tissue, preferably of human origin. There are various ways to measure MCT. By placing a dye, saccharine or solutions that is radiopaque or radioactive in the nose the investigator can follow the transport of these vehicles, usually in an *in vivo* setting. The methods and resources for this kind of research are discussed in *Chapter 1*.

Development of a human "mucosa bank"

Relatively large quantities of healthy ciliated epithelium are required for *in vitro* experiments. Moreover, these specimens should be available at any time. Therefore, we looked for a way to preserve ciliated mucosa (*Chapter 2*). First, we investigated whether cryopreservation of healthy mucosa would influence CBF. This mucosa was taken from the sphenoidal sinus during surgery on pituitary tumours. It was immersed in two different cryopreservatives containing dimethylsulfoxide and glycerol. These substances are components of a human sperm preservation medium that serves as a cryoprotector. CBF was measured sequentially by a photoelectric method when the specimens were fresh and then at intervals of 1 week, 1 month, and 3 months after cryopreservation in liquid nitrogen and thawing. The mean CBF values recorded after thawing did not differ significantly from the values measured before cryopreservation. We conclude that sphenoidal sinus mucosa is an appropriate source of ciliated epithelium for *in vitro* experiments. With this source and this preservation method we have

relatively large quantities of non-pathological ciliated human mucosa at our disposal at any moment.

Chicken trachea can be used as a substitute for human ciliated mucosa

Chapter 3 considers whether *in vitro* experiments on animal material are comparable to experiments on human tissue. Since healthy human ciliated epithelium is difficult to obtain, fresh chicken trachea samples have often been used in function experiments. We investigated whether several substances had comparable effects on CBF in the mucosa of chicken trachea and of human material obtained and preserved as described in *Chapter 2*. The study examined influence of substances that are used clinically: two topical anesthetics, namely cocaine (3% and 7%) and lidocaine (2%), adjusted to pH 6 and pH 7; two decongestants, xylometazoline 0.1% and oxymetazoline 0.1%; and the ß-blocking agent propranolol. The topical anesthetics 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 CBF in fresh chicken trachea and cryopreserved human tissue did not differ significantly. These experiments show that chicken trachea samples are a valid substitute for human material used to study ciliary activity *in vitro*. Moreover, the experiments provide evidence that cryopreservation does not have a significant effect on ciliary activity, as expressed by CBF.

In continuation of *Chapter 3*, experiments with four excipients for nasal drug formulations are described in *Chapter 4*. In this study, we examined the effect on CBF of several substances: the absorption enhancers methylated ß-cyclodextrin 2% and sodium taurodihydrofusidate 1%; the preservative benzalkoniumchloride 0.01%; and physiologic saline (NaCl 0.9%). We also examined the effect on CBF of the cryopreservative dimethylsulfoxide (DMSO). The latter substance is used to protect ciliated epithelium prior to storage in liquid nitrogen. Results obtained with chicken embryo trachea were compared with those of cryopreserved human mucosa from the sphenoidal sinus. For all of the substances tested, the effects on CBF of chicken material were comparable to those measured on human material. Moreover, DMSO appeared to have a reversible negative effect on CBF. We conclude that chicken embryo trachea can be used as a substitute for human ciliated mucosa in studies on ciliary activity *in vitro*.

CBF plays a key role in nasal mucociliary clearance

Chapter 5 embraces the idea that CBF is a determining factor in mucociliary transport. Convincing experimental evidence is still lacking, however. The effects of two ciliostatic compounds (xylometazoline 0.1% and NaCl 0.9%) and of a cilio-enhancer (salbutamol 0.1%) were measured by a technetium-99m nebulizing scintigraphic method. The experiments were carried out in 15 young healthy volunteers. Xylometazoline appeared to slow down MCT, though the decrease was not significant. NaCl reduced MCT significantly. Salbutamol resulted in a highly significant increase of MCT. This shows that there is a significant similarity in the effects of NaCl and salbutamol *in vitro* on CBF and *in vivo* on MCT. Xylometazoline brings about drastic changes in the nasal cavity, both anatomically and physiologically. Any comparison of MCT before and after using this vasoconstrictive agent has to take this effect into account. This study demonstrates a significant similarity in the effects of NaCl and salbutamol on CBF in vitro and on MCT in vivo. The evidence from our experiments suggests that CBF is a determining factor in the MCT rate in the nose.

Physiologic saline inhibits CBF

During our previous studies, it had appeared that NaCl 0.9% affected CBF. *Chapter 6* deals with this clinically relevant issue. NaCl 0.9% is commonly used in the treatment of acute and chronic rhinosinusitis. Moreover, NaCl 0.9% is used as a control medium, vehicle, or solvent in studies on CBF. Hypertonic saline (NaCl 7% and 14.4%) has been used to enhance mucociliary transport in patients with cystic fibrosis or asthma and in experiments on healthy subjects. Therefore, our study focused on the *in vitro* effects on CBF of saline solutions in concentrations of 0.9, 7.0, and 14.4%. Changes in the CBF of cryopreserved mucosa from the sphenoidal sinus were measured by means of a photoelectric method. Initial frequencies, measured in Locke-Ringer's solution (LR), were compared to CBF after exposure to NaCl in concentrations of 0.9, 7.0, and 14.4%. LR is an isotonic solution that has no effect on CBF. NaCl 0.9% had a moderately negative effect on CBF. The 7% solution led to a complete ciliostasis within 5 min; this effect appeared to be reversible after rinsing with LR. A hypertonic solution of 14.4% had an irreversible ciliostatic effect. LR is probably more appropriate than saline for nasal irrigation and nebulization or antral lavage. Moreover, the results suggest that the mucolytic effects induced by hyperosmolarity should be attained with hypertonic saline 7% in patients with cystic fibrosis or asthma. At this concentration, the ciliostatic effect is

reversible, whereas irreversible changes may be expected at higher concentrations.

PRACTICAL AND CLINICAL IMPLICATIONS OF THIS STUDY

Access to sufficient amounts of ciliated mucosa is a well-known problem in ciliary research. During pituitary surgery, mucosa of the sphenoidal sinus is usually thrown away. Using this ciliated epithelium for research has two advantages: a relatively large amount of tissue can be harvested; and this material is not pathological. The CBF of this mucosa is not affected by cryopreservation and can therefore be stored for later use. Now, research laboratories have sufficient amounts of non-pathological human ciliated mucosa at their disposal, and this material is available at any time.

Over the past few decades, chicken ciliated tissue has frequently been used in studies of ciliary activity. We have demonstrated a similarity in the reactivity of human and chicken material, as expressed by *in vitro* measurements of CBF. This means that the results of experiments with chicken ciliated tissue can be transposed to human reactions *in vitro*.

A positive relationship between reactivity in the *in vitro* situation (CBF) and under *in vivo* conditions (MCT) was demonstrated. This parallel is important because it means that many more solutions can be tested *in vitro*. Moreover, the number of measurements can easily be multiplied *in vitro*, which may make the conclusions more reliable. A cilio-inhibiting and a cilio-enhancing solution had parallel effects on MCT (*in vivo*) and CBF (*in vitro*). Therefore, we concluded that the results of *in vitro* experiments on CBF may be transposed to human situations *in vivo*.

Physiologic saline is apparently an innocuous solution. It is widely used in both clinical and experimental settings. However, we demonstrated that physiologic saline has a negative effect on CBF. Therefore, we suggest using Locke-Ringer's solution in nasal and antral lavages and in ciliary function experiments. The latter formulation has no effect on CBF *in vitro*. Hypertonic saline of 7% is preferred to a solution of 14.4% for the purpose of enhancing mucociliary transport in patients with cystic fibrosis or asthma. In our experiments, the 14.4% solution caused an irreversible damage to the ciliated mucosa, whereas 7% saline had a negative but reversible effect.

CONCLUSIONS

1. Ciliary beat frequency is a determining factor in mucociliary transport.
2. Results from experiments *in vitro* concerning ciliary beat frequency may be transposed to mucociliary transport *in vivo*.
3. Sphenoidal sinus mucosa is an appropriate source of non-pathological ciliated tissue for *in vitro* experiments.
4. Cryopreservation has no effect on ciliary activity as expressed by CBF.
5. Since non-pathological ciliated mucosa and a cryopreservation method have become available, it is now possible to establish a "mucosa bank."
6. The cryopreservative dimethylsulfoxide has a reversible ciliostatic effect.
7. Chicken embryo trachea can be used as a substitute for human ciliated mucosa for *in vitro* studies of ciliary activity.
8. "Physiologic" saline has a negative influence on ciliary activity.
9. In attempts to enhance mucociliary transport by means of hypertonic saline solutions in severe pathological situations, a saline concentration of 7% is preferred to 14.4% because a 7% solution has a reversible ciliostatic effect.

HOOFDSTUK 8

SAMENVATTING EN CONCLUSIES

SAMENVATTING

Onderzoek naar mucociliair transport en trilhaaractiviteit

Transport van mucus in de luchtwegen wordt veroorzaakt door beweging van de trilharen, hetgeen een belangrijk afweermechanisme vormt. Dit mucociliair transport (MCT) kan worden onderzocht zowel door metingen van de activiteit van de trilharen zelf - *in vitro* - als door metingen van het mucustransport - *in vivo*. Deze trilhaaractiviteit wordt meestal vastgesteld aan de hand van metingen van de trilhaarslagfrequentie *in vitro*, een en ander met behulp van een foto-elektrische registratietechniek. Voor het verrichten van een voldoende aantal metingen is vaak een groot aantal biopten nodig, bij voorkeur van niet-pathologisch trilhaardragend epitheel. Dierlijk weefsel wordt nogal eens gebruikt omdat humaan trilhaardragend epitheel, hetgeen principieel de voorkeur verdient, beperkt beschikbaar is. Voor het *in vivo* meten van het MCT zijn verschillende technieken bekend. Door het aanbrengen in de neus van radiopake of radioactieve oplossingen, kleurstof of zoetstof is het transport te volgen. Methodologische aspecten die betrekking hebben op de hier genoemde onderzoeken worden vermeld in *hoofdstuk 1*. Bovendien wordt hier de mogelijke correlatie tussen trilhaarslagfrequentie *in vitro* en MCT *in vivo* besproken. In dit hoofdstuk komt tot slot de vraag aan de orde of diverse zoutoplossingen wellicht invloed hebben op de activiteit van trilharen.

Invriezen van trilhaardragend weefsel; mogelijkheid tot humane "mucosa bank"

Voor *in vitro* onderzoek naar invloeden op de trilhaarslagfrequentie is relatief veel trilhaar-dragend weefsel nodig, dat idealiter beschikbaar moet zijn op elk gewenst moment. Om deze reden hebben wij onderzocht of het mogelijk is ingevroren trilhaardragend epitheel te gebruiken. De vraagstelling hierbij was of invriezen de trilhaarslagfrequentie zou beïnvloeden (*hoofdstuk 2*). Trilhaardragend weefsel werd verkregen uit de sinus sfenoidalis tijdens transsfenoidale hypofyse chirurgie. Het weefsel werd geplaatst in twee verschillende invriesmedia, namelijk dimethylsulfoxide en glycerol. De laatstgenoemde stof wordt ook gebruikt bij het invriezen van sperma en is één van de ingrediënten van het "human sperm preservation medium". Invriezen geschiedde in vloeibaar stikstof. De trilhaarslagfrequentie werd vers, d.w.z. na afname, gemeten en vervolgens na 1 week, 1 maand en 3

maanden invriestijd. De gemiddelde trilhaarslagfrequentie bleek niet te veranderen na invriezen. Ingevroren trilhaardragend epitheel uit de sinus sfenoidalis blijkt derhalve geschikt voor *in vitro* experimenten. Met het op zetten van een "*mucosa bank*" voor trilhaaronderzoek kan op elk gewenst moment relatief veel, niet-pathologisch trilhaardragend epitheel beschikbaar zijn.

Kuikentrachea als vervanging voor humaan materiaal

Vers trilhaardragend weefsel van kuikentrachea's is veelvuldig gebruikt in functionele experimenten, vooral omdat voldoende humaan weefsel moeilijk te verkrijgen is. In *hoofdstuk 3* wordt beschreven of de resultaten van *in vitro* metingen van de trilhaarslagfrequentie van dit dierlijk weefsel te vergelijken zijn met die van menselijk weefsel. De invloed van verscheidene stoffen op de trilhaarslagfrequentie van weefsel afkomstig van kuikens en dat van mensen werd vergeleken. De invloed van de locaal anesthetica cocaïne (3% en 7%) en lidocaine (2%) in pH 6 en pH 7, twee neussprays (xylometazoline 0,1% en oxymetazoline 0,1%) en de ß-blokker propanolol werden onderzocht. De anesthetica bleken de trilhaarslagfrequentie te remmen. Dit effect was meer uitgesproken bij pH 7. Een complete stilstand van de trilhaarslag bleek reversibel na spoelen met Locke Ringer's oplossing (LR), behalve bij propanolol. Er werd geen significant verschil gezien tussen de reacties van ingevroren humane mucosa en die van vers trilhaardragend kuikenweefsel. Deze experimenten maken aannemelijk dat vers trilhaardragend weefsel van kuikentrachea's een goed substituut is voor humaan materiaal in het *in vitro* onderzoek van de trilhaarslag-frequentie. Bovendien wordt in dit onderzoek nogmaals bevestigd dat invriezen van humaan weefsel geen effect heeft op de reactiviteit van de trilhaarslagfrequentie. In *hoofdstuk 4* wordt onderzoek naar de invloed van vier stoffen op de trilhaarslag van zowel kuiken- als humaan epitheel beschreven. De effecten werden onderzocht van drie toevoegmiddelen van neussprays (methyl ß-cyclodextrine 2%, sodiumtaurodihydrofusidate 1% en benzalkonium-chloride 0,01%) en van fysiologisch zout (NaCl 0,9%). Bovendien werd de invloed van het invriesmedium dimethylsulfoxide (DMSO) op vers kuikenweefsel onderzocht. DMSO wordt gebruikt bij het invriezen van trilhaardragend slijmvlies. Resultaten verkregen met slijmvlies van kuikentrachea's werden vergeleken met die van humaan trilhaarepitheel. Bij al deze stoffen werd een vergelijkbare reactie van de trilhaarslag-frequentie gezien. Benzalkonium-chloride had aanvankelijk een sterker effect op humaan weefsel: echter na 60 minuten was het effect op de

trilhaarslagfrequentie gelijk. Bovendien bleek dat het negatief effect op de frequentie van de trilhaarslag van DMSO reversibel was. Geconcludeerd werd dat kuiken-trachea's vooralsnog bruikbaar zijn als vervanging voor humaan trilhaarepitheel bij het bestuderen van de trilhaaractiviteit *in vitro*.

De sleutelrol van de trilhaarslagfrequentie bij het mucociliair transport in de neus

In *hoofdstuk 5* wordt de relatie tussen de frequentie van de trilhaarslag en het MCT behandeld. Algemeen wordt verondersteld dat de trilhaarslagfrequentie bepalend is voor de snelheid van het transport van mucus in de neus. Overtuigend bewijs hiervoor ontbreekt nog. Het effect van twee trilhaarslag-vertragende middelen (xylometazoline 0,1% en NaCl 0,9%) en van één trilhaarslag-stimulerend middel (salbutamol 0,1%) werd gemeten door middel van scintigrafie na sprayen van technetium-99m in de neus. De experimenten werden verricht bij 15 gezonde proefpersonen. Xylometazoline vertraagde het MCT, zij het statistisch niet significant. NaCl vertraagde het MCT wel significant en salbutamol versnelde het MCT hoog significant. Deze resultaten laten zien dat er een parallel bestaat tussen de effecten van NaCl en salbutamol op de *in vitro* trilhaarslagfrequentie en op het *in vivo* MCT. Deze parallel kon niet worden aangetoond bij xylometazoline. Waarschijnlijk is dit het gevolg van de anatomische en fysiologische veranderingen die in de neus door xylometazoline ontstaan. Het onderzoek maakt aannemelijk dat de resultaten van *in vitro* studies naar de trilhaarslag-frequentie vertaald kunnen worden naar het *in vivo* MCT.

Het remmend effect van een fysiologische zoutoplossing op de trilhaaractiviteit

Hoofdstuk 6: fysiologische zoutoplossing (NaCl 0,9%) wordt algemeen gebruikt bij de behandeling van acute en chronische rhinosinusitis en bij onderzoek naar de trilhaarslag-frequentie als (controle-) medium of oplosmiddel. Hypertone zoutoplossingen (NaCl 7% en 14,4%) worden toegepast ter bevordering van het MCT bij patiënten met mucoviscidosis of astma. Het effect van deze verschillende concentraties zoutoplossingen op de *in vitro* trilhaarslagfrequentie werd onderzocht. Hierbij werd de trilhaarslagfrequentie van ingevroren slijmvlies afkomstig uit de sinus sfenoidalis gemeten met behulp van een foto-elektrische techniek. Uitgangswaarden, verkregen in LR, werden vergeleken met de trilhaarslagfrequentie na blootstelling aan NaCl concentraties van

respectievelijk 0,9%, 7% en 14,4%. NaCl 0,9% bleek een matig negatief effect te hebben op de trilhaarslagfrequentie. De oplossing van 7% leidde tot stilstand van de trilhaarslag. Dit effect was evenwel reversibel na spoeling met LR. Een irreversibele stilstand werd veroorzaakt door de oplossing van 14,4%.

LR is een isotone oplossing die geen effect heeft op de trilhaarslagfrequentie. Vergeleken met fysiologische zoutoplossing lijkt LR daardoor beter geschikt voor neus- en kaakspoelingen en vernevelingen. Bovendien wijzen de resultaten van dit onderzoek erop dat beter NaCl 7% kan worden aangewend in plaats van de oplossing van 14,4% bij patiënten met mucoviscidosis of astma.

PRAKTISCHE EN KLINISCHE IMPLICATIES VAN DEZE STUDIE

Het verkrijgen van voldoende bruikbaar trilhaardragend materiaal is een bekend en universeel probleem bij trilhaaronderzoek. Tijdens transsfenoidale hypofyseoperaties wordt gewoonlijk de mucosa van de sinus sfenoidalis verwijderd en niet meer gebruikt. Het gebruik van dit aldus verkregen epitheel heeft belangrijke voordelen: er komt een verhoudingsgewijs grote hoeveelheid niet-pathologisch trilhaardragend weefsel beschikbaar. Invriezen blijkt geen significant effect te hebben op de trilhaarslagfrequentie. Laboratoria kunnen aldus een *"mucosa bank"* opzetten en beschikken over voldoende hoeveelheden humaan trilhaar-epitheel op elk gewenst moment.

Sinds enkele decennia worden kuikentrachea's gebruikt bij trilhaaronderzoek. Het in dit proefschrift beschreven onderzoek toonde aan dat er *in vitro* geen verschil bestaat tussen de reacties van humaan en kuiken weefsel.

Een positieve relatie tussen reactiviteit in de *in vitro* situatie (de trilhaarslagfrequentie) en die in de *in vivo* situatie (het MCT) werd aangetoond. Door deze waarneming wordt het belang van *in vitro* onderzoek onderstreept. Dit onderzoek maakt aannemelijk dat resultaten van *in vitro* experimenten kunnen worden getransponeerd naar *in vivo* situaties.

Fysiologisch zout is een ogenschijnlijk onschuldige oplossing die veelvuldig wordt toegepast, zowel klinisch als experimenteel. Fysiologisch zout heeft echter een significant remmend effect op de trilhaarslagfrequentie. Omdat Locke-Ringer's oplossing geen effect heeft op deze trilhaarslagfrequentie geniet deze of een soortgelijke oplossing de voorkeur bij neus- en kaak-spoelingen. Ter stimulering van het MCT bij patiënten met mucoviscidosis

of astma wordt een hypertone zoutoplossing van 7% aanbevolen. In onze experimenten bracht een oplossing van 14,4% irreversibele schade toe aan het epitheel. Dit in tegenstelling tot een oplossing van 7%: het effect hiervan was weliswaar ook ciliostatisch, maar dit bleek reversibel.

CONCLUSIES

1. De trilhaarslagfrequentie is een belangrijke determinant van het mucociliair transport.
2. Resultaten van *in vitro* onderzoek naar de trilhaarslagfrequentie mogen worden vertaald naar de *in vivo* situatie van het mucociliair transport.
3. Mucosa van de sinus sfenoidalis is een geschikte bron van niet-pathologisch trilhaardragend epitheel voor *in vitro* experimenten.
4. Invriezen heeft geen effect op de trilhaarslagfrequentie.
5. Doordat ingevroren niet-pathologisch humaan trilhaardragend epitheel beschikbaar kan zijn bestaat de mogelijkheid tot het creëren van een "*mucosa bank*".
6. Het invriesmedium dimethylsulfoxide heeft een reversibel trilhaarslag-vertragend effect.
7. Kuikentrachea's kunnen worden gebruikt als vervanging voor humaan trilhaardragend epitheel bij *in vitro* onderzoek naar de trilhaaractiviteit.
8. "Fysiologisch" zout heeft een remmende invloed op de trilhaarslagfrequentie.
9. Wanneer men het mucociliair transport tracht te stimuleren door toediening van hypertone zoutoplossingen, heeft een concentratie van 7% de voorkeur boven een van 14,4%.

DANKWOORD

DANKWOORD

Het schrijven van een proefschrift is niet mogelijk zonder de hulp van anderen. Graag wil ik mijn dank uitspreken aan eenieder, die op enige wijze heeft bijgedragen aan mijn onderzoek en de totstandkoming van dit proefschrift.

Prof. Dr. E.H. Huizing, uw kritisch en niet aflatend wetenschappelijk inzicht heeft me scherp gehouden. Ik zie het als een voorrecht u als opleider te hebben meegemaakt.

Dr. K. Graamans, beste Kees, jij bent de begeleider die elke promovendus zich zou wensen. Je hebt steeds weer inspirerende ideeën en weet de gang in het onderzoek te houden, zelfs tijdens een voltijds klinische opleidingsperiode. Daarnaast was je ook nog bereid om onze jongste de fles te geven.

Frits Meeuwsen en Ferry Hendriksen, jullie waren een onmisbare steun, met onvoorwaardelijke hulp en adviezen. De verstrooiende momenten hebben een dierbare herinnering achtergelaten.

Prof Dr. G.J. Hordijk, aan u dank ik de mogelijkheid tot het doen van research, in een continu veranderende opleidingsstructuur, waarin het een opleider niet makkelijk wordt gemaakt.

Stefan Romeijn, Coos Verhoef en prof. Dr. F.W.H.M. Merkus, jullie hebben me op weg geholpen in het ciliair onderzoek. De samenwerking verliep bijzonder prettig. Dank voor al het werk.

Associate prof. Nesil Keles, dear Nesil, thank you very much for all your work and participation in our Nobel-winning research project. You did a great job.

Hanny Natzijl, bedankt voor het uitstekend uitvoeren van in vivo proeven en de bijbehorende berekeningen.

Alice van Dongen, Dr. P.P. van Rijk en Maarten Gerrits, dank ik voor de mogelijkheid tot onderzoek bij nucleaire geneeskunde en de vruchtbare discussies.

De (oud)arts-assistenten KNO waren een steun tijdens het onderzoek, maar bovenal wil ik hen bedanken voor het onvergetelijk maken van de opleidingstijd.

De stafleden KNO van het AZU dank ik voor een prima Utrechtse opleiding.

De KNO-maatschap Apeldoorn, dank voor de beschikbaar gestelde tijd. Eigenlijk horen jullie hiermee thuis in de lijst van sponsors!

Medewerkers van de KNO research, Joh(a)n de Groot, Sjaak Klis, Maarten, Nathalie, Patricia en anderen dank ik voor de raad en daad en de immer bestaande bereidheid om hulp te bieden.

Kas Kruitwagen, bedankt voor de statistische berekeningen én het geduld om deze ook nog uit te leggen.

Hubert van Strien en Leonard van Schelven, helaas is onze "in vivo scoop" geen werkelijkheid geworden.

De paranymfen Haiko Bloemendal en Raphael Hemler: nu weten we allemaal precies wat het inhoudt. Bedankt voor jullie hulp.

Hanneke en Ad, jullie gaven mij de kans om te komen waar ik nu ben.

Lieve Maarten, Anne en Pieter, dank jullie wel voor de broodnodige afleiding, de hulp bij het typen van de letters en het brengen van koffie.

Lieve An, hier is het dan. Dankzij jouw aanhoudende steun is de tijd van het onderzoek en de promotie vrijwel zonder stress verlopen. Ik vind het leuk dat je ook een essentiële bijdrage leverde bij de tekst en lay-out. We leveren mooie boekjes af!

CURRICULUM VITAE

Wilbert Boek werd op 13 mei 1964 geboren te 's-Hertogenbosch. In 1982 behaalde hij het eindexamen Gymnasium-ß aan het Sint Jans lyceum in dezelfde stad. In 1983 begon hij met de studie geneeskunde.

In 1991 deed hij zijn co-assistentschap interne geneeskunde in het Thomas Jefferson University Hospital te Philadelphia, USA. Vervolgens verrichtte hij onderzoek naar de "nerve growth factor", op de afdeling neurochirurgie (Prof. Peter Black) in het Brigham and Woman's Hospital, Harvard Medical School, Boston, USA.

In 1992 verrichtte hij zijn dienstplicht grotendeels in het Centraal Militair Hospitaal op de afdeling Keel-, Neus- en Oorheelkunde. Tot oktober 1994 bleef hij, als beroepsmilitair, hier werkzaam. Na 15 maanden als AGNIO op de afdeling Keel-, Neus- en Oorheelkunde in het Academisch Ziekenhuis Utrecht werkzaam te zijn geweest, startte in januari 1995 de opleiding tot KNO-arts, onder leiding van Prof. Dr. E.H. Huizing en Prof. Dr. G.J. Hordijk. De B-stage werd in het Ziekenhuiscentrum Apeldoorn doorgebracht (J.B. Antvelink). Tijdens de opleiding was het mogelijk om aan het onderzoek te werken dat heeft geleid tot dit proefschrift. In december 2000 zal de auteur zijn opleiding tot KNO-arts voltooien.

De auteur is getrouwd met Anneke Lips. Zij hebben drie kinderen, Maarten, Anne en Pieter.

STELLINGEN

behorend bij het proefschrift

INSIGHT INTO CILIARY ACTIVITY AND MUCOCILIARY TRANSPORT

Universiteit Utrecht

2 mei 2000, 16.15 uur

WILBERT M. BOEK

1. De snelheid van het mucociliair transport in de neus wordt in belangrijke mate bepaald door de trilhaarslagfrequentie.

2. Mucosa van de sinus sfenoidalis is een geschikte bron voor *in vitro* onderzoek van trilhaaractiviteit.

3. Het diepgevroren bewaren van trilhaardragend epitheel heeft uiteindelijk geen effect op de trilhaarslagfrequentie.

4. Doordat nu ingevroren niet-pathologisch humaan trilhaardragend epitheel ter beschikking staat, kan een "*mucosa bank*" worden opgericht voor experimenteel onderzoek.

5. Bij *in vitro* onderzoek naar de trilhaaractiviteit is epitheel van kuikentrachea's een goed alternatief voor humaan materiaal.

6. Bij neus- en kaakspoelingen wordt gewoonlijk fysiologisch zout (NaCl 0,9%) gebruikt. De term "fysiologisch" is in dit verband niet correct.

7. Bij onderzoek van een foetaal groeivertraagd kind moet, na perinatale asfyxie, niet alleen gedacht worden aan encephalopathie en convulsies maar ook aan mogelijke schade aan het gehoor (Audiology 1999;38:291-5).

8. Schizofrene patiënten zijn te herkennen aan de specifieke geur van hun zweet (Science, 1969;166:398-9).

9. Het is verbazingwekkend dat in de gezondheidszorg bezuinigingen vooral gericht zijn op zorg en zorgverleners, terwijl in de laatste jaren juist de kosten van het management fors zijn gestegen.

10. Organisatie van geneeskundige zorg *per uur* komt de patiënt niet ten goede aangezien het beloop per ziektegeval sterk varieert.

11. Het toenemend aantal motoren op de weg in Nederland zal slechts in de zomermaanden iets bijdragen aan het fileprobleem.

12. Ieder Boekje betekent een hoogtepunt.