

Effects of anaesthetic and sedative agents
on human respiratory cilia *in vitro*

A Thesis presented for the degree of Doctor of Medicine
at the University of Leicester

J.H.Raphael
Department of Anaesthesia
1996

UMI Number: U085610

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI U085610

Published by ProQuest LLC 2015. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346



X75 3580259

Contents

Synopsis.....	6
Presentation & Publications.....	7
Declaration.....	9
Acknowledgements.....	10
List of Experiments.....	11
List of Figures.....	13
List of Tables.....	16
List of Abbreviations.....	19
<u>Chapter 1. Pulmonary infections in anaesthesia and intensive care.....</u>	20
Incidence of postoperative and intensive care pulmonary infections.....	20
Risk factors for postoperative and intensive care pulmonary infections.....	21
Association of general anaesthesia and sedation with pulmonary infections.....	23
Local anaesthesia and postoperative pulmonary infections.....	32
Reducing the risk factors for postoperative pulmonary infections.....	33
<u>Chapter 2. The mucociliary system and anaesthesia.....</u>	39
The mucociliary system: an important defence against respiratory infections.....	39
Respiratory mucus.....	41
Effects of anaesthetic agents on respiratory mucus.....	43
Cilia.....	44
Interactions of mucus and cilia: importance of ciliary beat frequency.....	53
Effects of anaesthetic agents on ciliary beat frequency.....	57
<u>Chapter 3. Materials & Methods 1:</u>	
<u>Measurement of ciliary beat frequency.....</u>	58
Introduction.....	58
Methods of measurement of ciliary beat frequency previously described.....	59

Perfusion system for the <i>in vitro</i> measurement of ciliary beat frequency during exposure to volatile liquids: an overview.....	61
Perfusion chamber.....	62
Expt 3.1. Calibration of chamber temperature controller.....	65
Expt 3.2. Equilibration time and flow characteristics of the perfusion chamber.....	67
Perfusion system.....	74
Expt 3.3. Measurements of osmolality and pH.....	76
Expt 3.4. Calibration of perfusion flow controller.....	77
Expt 3.5. Delivery of halothane to the perfusion chamber.....	78
Microscopy.....	80
Photoelectric transduction.....	83
Ciliary beat frequency analysis.....	84
Expt 3.6. Ciliary beat frequency of nasal brushings from awake volunteers..	85
Discussion.....	88

Chapter 4. Materials & Methods 2:

<u>Human ciliated respiratory tissue samples</u>	94
Introduction.....	94
Cytological specimens of human respiratory cilia.....	95
Expt 4.1. Nasal ciliary beat frequency measured <i>in vitro</i> after an <i>in vivo</i> bolus of propofol.....	97
Nasal turbinate explants.....	101
Preparation of turbinate explants.....	103
Expt 4.2. Ciliary beat frequency of turbinate explants over 10 days in a controlled environment.....	105
Expt 4.3. Ciliary beat frequency of turbinate explants during six hours in a perfusion chamber.....	108
Response of turbinate explants to known cilioactive agents.....	111

Expt 4.4. Effects of forskolin on turbinate explant ciliary beat frequency.....	111
Expt 4.5. Effects of phenol on turbinate explant ciliary beat frequency.....	114
Discussion.....	117

Chapter 5. Inhalation anaesthetic agents and ciliary beat frequency

<u>1: Effects of high concentrations</u>	120
Introduction.....	120
Expt 5.1: Effects of 3 MAC of halothane, enflurane and isoflurane on human respiratory ciliary beat frequency.....	122
Expt 5.2: Duration of effect of halothane, enflurane and isoflurane on human respiratory ciliary beat frequency.....	131
Discussion.....	139

Chapter 6. Inhalation anaesthetic agents and ciliary beat frequency

<u>2: Effects of low concentrations</u>	143
Introduction.....	143
Expt 6.1. Effects of 0.5 - 3 MAC of halothane, enflurane and isoflurane on <i>in vitro</i> ciliary beat frequency.....	144
Ciliary effects of prolonged use of isoflurane.....	149
Expt 6.2. Effects of 48-72 hours of 0.2-0.4 % isoflurane on ciliary beat frequency <i>in vitro</i>	151
Discussion.....	154

Chapter 7. Effects of morphine on human respiratory cilia.....

Introduction.....	157
Expt 7.1. Effects of morphine on human respiratory ciliary beat frequency <i>in vitro</i>	159
Opioid receptors on nasal cilia.....	163
Expt 7.2. Binding of tritiated diprenorphine, [³ H]DPN, to human nasal ciliated epithelial cell membranes.....	165
Discussion.....	167

<u>Chapter 8. Effects of propofol on human respiratory cilia</u>	170
Introduction.....	170
Expt 8.1. Short-term effects of propofol on human respiratory ciliary beat frequency <i>in vitro</i>	171
Expt 8.2. Effects of incubation of cilia with a sedative concentration of propofol for three days.....	175
Expt 8.3. Dose- and time-dependent effects of incubation of cilia with propofol.....	180
Expt 8.4. Neurotoxicity of propofol in SH-SY5Y cells measured by inhibition of thiazoyl blue reduction.....	184
Discussion.....	188
<u>Chapter 9. Effects of midazolam on human respiratory cilia</u>	192
Introduction.....	192
Expt 9.1. Short-term effects of midazolam on human respiratory ciliary beat frequency <i>in vitro</i>	193
Expt 9.2. Long-term effects of midazolam on human respiratory cilia <i>in vitro</i>	197
Expt 9.3. Neurotoxicity of midazolam in SH-SY5Y cells measured by inhibition of thiazoyl blue reduction.....	201
Discussion.....	207
<u>Chapter 10. Conclusions & clinical recommendations</u>	210
<u>Chapter 11. References</u>	219
Appendix: List of Reagents	233

Synopsis

Chest infections developing after surgery and in patients in the intensive care unit occur commonly, and the incidence has been largely unchanged over the years. This has occurred despite the widespread use of antibiotics and of methods to improve mucus clearance through more effective coughing. One of the most important defences against respiratory infections is mucociliary clearance. A variety of anaesthetic and sedative drugs impair this and therefore may be a factor in the development of these respiratory infections. The mechanisms by which these agents impair mucociliary clearance have not been elucidated. The presence of cilia and their beating frequency is one of the most important determinants and the investigation of anaesthetic and sedative agents upon these factors forms the subject of this thesis.

We have developed an *in vitro* method for investigating the effects of anaesthetic and sedative agents on ciliary beat frequency. This comprises a perfusion system together with new techniques of ciliated tissue sample preparation.

The inhalation anaesthetic agents, halothane, enflurane and isoflurane caused a reversible dose-dependent reduction in ciliary beat frequency that was significant at clinical concentrations. In contrast, short term exposure to intravenously administered anaesthetics and sedatives, propofol, midazolam and morphine, did not cause any significant change in ciliary beat frequency.

The long term effects of intensive care sedatives gave differing results. 48-72 hours exposure to a sedative concentration of isoflurane did not affect ciliary beat frequency or cilia survival. In contrast 72 hours incubation with either propofol or midazolam impaired cilia survival. In the case of propofol the effect was only apparent with tissue that had been stored for several days and with fresh tissue there was no effect on cilia survival. In the case of midazolam there was a dose-dependent reduction in the number of functioning cilia following incubation of fresh tissue although this effect was significant only above clinical concentrations.

Presentation & Publications

Presentation

Effects of morphine on human nasal cilia beat frequency *in vitro*.
Proceedings of the Anaesthetic Research Society, London meeting,
November 11th, 1994.

Abstracts

Raphael JH, Selwyn DA, Key A, Mottram SD, Langton JA. Effects of morphine on human nasal cilia beat frequency *in vitro*. British Journal of Anaesthesia 1995;74:484P-485P.

Hann HCL, Raphael JH. An investigation into the effects of midazolam and propofol on human respiratory cilia beat frequency *in vitro*. British Journal of Anaesthesia 1996;76:591P.

Full Publications

Selwyn DA, Gyi A, Raphael JH, Key A, Langton JA. A perfusion system for the *in vitro* measurement of human cilia beat frequency. British Journal of Anaesthesia 1996;76:111-115.

Raphael JH, Selwyn DA, Mottram SD, Langton JA, O'Callaghan C. The effects of 3 MAC of halothane, enflurane and isoflurane on the cilia beat frequency of human nasal epithelium *in vitro*. British Journal of Anaesthesia 1996;76:116-121.

Selwyn DA, Raphael JH, Lambert DG, Langton JA. Effects of morphine on human nasal cilia beat frequency *in vitro*. British Journal of Anaesthesia 1996;76:274-277.

Raphael JH, Strupish J, Selwyn DA, Hann HCL, Langton JA. Recovery of respiratory ciliary function following depression by inhalational anaesthetic agents: an *in vitro* study using nasal turbinate explants. British Journal of Anaesthesia 1996;76:854-859.

Declaration

This research was conducted while I was a lecturer (honorary registrar and senior registrar) in the University Department of Anaesthesia at the Leicester Royal Infirmary between 1994 and 1996.

The human tissue samples used in the experiments have been obtained with the ethical approval of the Leicestershire Ethics Committee.

The work undertaken in this thesis is original and has either been undertaken by myself or by others working closely under my supervision, whose contributions are recognised in the publications listed. Data from three of the 23 experiments (expts 4.2, 8.1 and 9.1) have been previously submitted by H.Hann for a Batchelor of Science degree at the University of Leicester.

This thesis was written and composed by myself and all books and papers quoted in this thesis were consulted by me personally.

Acknowledgements

I should like to express my gratitude to the following for their assistance with this work:

Dr C. O'Callaghan, Senior Lecturer in Child Health at the University of Leicester for introducing me to the investigation of cilia.

Dr J. Langton, Consultant Anaesthetist, Derriford Hospital, Plymouth and formerly Senior Lecturer in Anaesthesia at the University of Leicester for his constant advice and encouragement during this project.

Mr J. Strupish, Chief Technician to the department for his enthusiastic support and for many stimulating discussions.

Mr A. Key and Dr E. Pallett for their technical support with hardware.

Mr S. Mottram, for his assistance with software and statistical advice.

Mr R. Hirst for performing the gas chromatographic analysis.

Dr J. Willets for assistance with the toxicological studies.

Dr D. Lambert, Lecturer in Anaesthetic Pharmacology at the University of Leicester for assistance with the opioid receptor binding investigation.

Professor G. Smith for providing the facilities to undertake this work and for welcoming me into his department.

The financial support of Zeneca which enabled the purchase of the video camera required for this investigation is gratefully appreciated.

Finally, I should like to thank Helen, Alice and Eleanor for allowing me the time, which should have been theirs, to pursue this work.

List of Experiments

Expt 3.1 Calibration of chamber temperature controller.....	65
Expt 3.2 Equilibration time and flow characteristics of the perfusion chamber.....	67
Expt 3.3 Measurements of pH and osmolality.....	76
Expt 3.4. Calibration of perfusate flow controller.....	77
Expt 3.5 Delivery of halothane to the perfusion chamber.....	78
Expt 3.6 Ciliary beat frequency of nasal brushings from awake volunteers.....	85
Expt 4.1 Nasal ciliary beat frequency measured <i>in vitro</i> after an <i>in vivo</i> bolus of propofol.....	97
Expt 4.2 Ciliary beat frequency of turbinate explants over 10 days in a controlled environment.....	105
Expt 4.3 Ciliary beat frequency of turbinate explants during six hours in a perfusion chamber.....	108
Expt 4.4 Effects of forskolin on turbinate explant ciliary beat frequency.....	111
Expt 4.5 Effects of phenol on turbinate explant ciliary beat frequency.....	114
Expt 5.1 Effects of 3 MAC of halothane, enflurane and isoflurane on human respiratory ciliary beat frequency.....	122
Expt 5.2 Duration of effect of halothane, enflurane and isoflurane on <i>in vitro</i> human turbinate explant ciliary beat frequency.....	131

Expt 6.1 Effects of 0.5 - 3 MAC of halothane, enflurane and isoflurane on ciliary beat frequency <i>in vitro</i>	144
Expt 6.2 Effects of 0.2-0.4 % isoflurane for 48-72 hours upon <i>in vitro</i> ciliary beat frequency.....	151
Expt 7.1 Effects of morphine on human respiratory ciliary beat frequency <i>in vitro</i>	159
Expt 7.2 Binding of tritiated diprenorphine, [3H]DPN, to human nasal ciliated epithelial cell membranes.....	165
Expt 8.1 Short-term effects of propofol on human respiratory ciliary beat frequency <i>in vitro</i>	171
Expt 8.2 Effects of incubation of cilia with a sedative concentration of propofol for three days.....	175
Expt 8.3 Dose- and time-dependent effects of incubation of cilia with propofol.....	180
Expt 8.4 Neurotoxicity of propofol in SH-SY5Y cells measured by inhibition of thiazoyl blue reduction.....	184
Expt 9.1 Short-term effects of midazolam on human respiratory ciliary beat frequency <i>in vitro</i>	193
Expt 9.2 Long-term effects of midazolam on human respiratory cilia <i>in vitro</i>	197
Expt 9.3 Neurotoxicity of midazolam in SH-SY5Y cells measured by inhibition of thiazoyl blue reduction.....	201

List of Figures

Fig 2.1 The two layers of respiratory mucus and the relation of the cilia.....	41
Fig 2.2 Electron micrograph of respiratory ciliated epithelium.....	44
Fig 2.3 Mucociliary interaction showing how the claws projecting from the cilia interact with the mucus.....	45
Fig 2.4 A cross section of a cilium showing microtubular arrangement of the axoneme.....	46
Fig 2.5 The bending of the ciliary axoneme.....	47
Fig 2.6 Ciliary motion: effective, rest and recovery strokes.....	48
Fig 2.7. Schematic representation of control of ciliary beating.....	52
Fig 2.8 Interactions of factors affecting mucociliary transport.....	53
Fig 2.9. Relationship of ciliary beat frequency to mucus transport.....	56
Fig 3.1 Photograph of perfusion chamber.....	63
Fig 3.2 Section through perfusion chamber.....	64
Fig 3.3 Photographs of the perfusion chamber as dye is washed in.....	69
Fig 3.4 Median density for inlet, middle and outlet cuts across the perfusion chamber, no ciliated sample present.....	71
Fig 3.5 Median density for inlet, middle and outlet cuts across the perfusion chamber, ciliated sample present.....	73
Fig 3.6 Schematic diagram of perfusion system.....	75
Fig 3.7 Differential interference contrast microscopy.....	81
Fig 3.8 Nomarski prisms.....	82

Fig 3.9 Ciliary beat frequency from nasal brushings of volunteers measured with the perfusion system.....	87
Fig 4.1 Ciliary beat frequency measured before and after propofol anaesthetic induction.....	100
Fig 4.2. Photograph of ciliated edge of nasal turbinate explant.....	104
Fig 4.3 Ciliary beat frequency of nasal turbinate explants over ten days.....	107
Fig 4.4 Ciliary beat frequency of turbinate explants over six hours in the perfusion chamber.....	110
Fig.4.5 Effects of 100 μ M forskolin on ciliary beat frequency of turbinate explants.....	113
Fig 4.6 Effects of 40 ppm phenol on ciliary beat frequency of nasal turbinate explants.....	116
Fig 5.1 Effects of 3 MAC halothane on ciliary beat frequency.....	126
Fig 5.2 Effects of 3 MAC enflurane on ciliary beat frequency.....	128
Fig 5.3 Effects of 3 MAC isoflurane on ciliary beat frequency.....	130
Fig 5.4 Recovery of ciliary beat frequency after 3 MAC halothane.....	134
Fig 5.5 Recovery of ciliary beat frequency after 3 MAC enflurane.....	136
Fig 5.6 Recovery of ciliary beat frequency after 3 MAC isoflurane.....	138
Fig 6.1 Effects of inhalational agent concentration on ciliary beat frequency.....	148
Fig 6.2. Gas chamber for prolonged exposure of ciliated tissue to isoflurane	150
Fig. 6.3 Effects of prolonged 0.2-0.4% isoflurane on ciliary beat frequency.	153
Fig. 7.1 Effects of morphine on ciliary beat frequency.....	162
Fig. 7.2 Specific and non-specific opioid binding.....	164

Fig 8.1 Effects of short-term propofol on ciliary beat frequency.....	174
Fig 8.2 Effects of propofol incubation for 72 hours on ciliary beat frequency <i>in vitro</i>	178
Fig 8.3 Survival of cilia incubated with 20 μ M propofol.....	179
Fig 8.4 Effect of propofol concentration on survival of human respiratory cilia incubated for up to 48 hours.....	182
Fig 8.5 Effect of propofol concentration on survival of human respiratory cilia incubated for up to 72 hours.....	183
Fig 8.6 Effects of propofol on SH-SY5Y cell viability after 72 hours exposure.....	187
Fig 9.1 Effects of short-term midazolam on ciliary beat frequency.....	196
Fig 9.2 Effects of midazolam incubation for 48 hours on cilia survival <i>in vitro</i>	199
Fig 9.3 Effects of midazolam incubation for 72 hours on cilia survival <i>in vitro</i>	200
Fig 9.4 Effects of midazolam on SH-SY5Y cell viability after 48 hours exposure.....	205
Fig 9.5 Effects of midazolam on SH-SY5Y cell viability after 72 hours exposure.....	206

List of Tables

Table 2.1 Regulation of ciliary beat frequency and effects on second messengers.....	51
Table 3.1 Calibration of chamber heating element.....	66
Table 3.2 Median density for inlet, middle and outlet cuts across the perfusion chamber , no ciliated sample.....	70
Table 3.3 Median density for inlet, middle and outlet cuts across the perfusion chamber, ciliated sample present.....	72
Table 3.4 pH and osmolality of the medium during perfusion with 3 MAC halothane.....	76
Table 3.5 Relation of flow rate to drip count through perfusion system.....	77
Table 3.6 Concentration of halothane , upstream and downstream of perfusion chamber with vaporiser set to 4 %.....	79
Table 3.7 Ciliary beat frequency of nasal epithelial brushings from unanaesthetised volunteers.....	86
Table 4.1. Comparison of ciliary beat frequency measured before and after propofol anaesthetic induction.....	99
Table 4.2. Ciliary beat frequency of turbinate explants over 10 days.....	106
Table 4.3 Ciliary beat frequency of turbinate explants over six hours in the perfusion chamber.....	109
Table 4.4. Effects of 100 μ M forskolin on ciliary beat frequency of turbinate explants.....	113

Table 4.5 Effects of 40 ppm phenol on ciliary beat frequency of nasal turbinate explants.....	116
Table 5.1. Effects of 3 MAC halothane on ciliary beat frequency.....	125
Table 5.2 Effects of 3 MAC enflurane on ciliary beat frequency.....	127
Table 5.3. Effects of 3 MAC isoflurane on ciliary beat frequency.....	129
Table 5.4. Recovery of ciliary beat frequency after 3 MAC halothane.....	133
Table 5.5. Recovery of ciliary beat frequency after 3 MAC enflurane.....	135
Table 5.6. Recovery of ciliary beat frequency after 3 MAC isoflurane.....	137
Table 6.1 Effects of halothane concentration on ciliary beat frequency.....	147
Table 6.2 Effects of enflurane concentration on ciliary beat frequency.....	147
Table 6.3 Effects of isoflurane concentration on ciliary beat frequency.....	147
Table 6.4 Effects of prolonged 0.2-0.4% isoflurane on ciliary beat frequency.....	153
Table 7.1 Effects of morphine on ciliary beat frequency.....	161
Table 7.2. Experimental protocol for binding of [³ H]DPN to cilia.....	166
Table 7.3 Binding of [³ H]DPN to human nasal ciliated cell membranes.....	166
Table 8.1 Effects of short-term propofol on ciliary beat frequency.....	173
Table 8.2 Fluorimetric assay of propofol in dishes incubating cilia.....	177
Table 8.3 Ciliary beat frequency of cilia incubated with 20 μ M propofol.....	177
Table 8.4 Effects of propofol on cilia survival.....	181
Table 8.5 Effects of 72 hours incubation with propofol on MTT reduction in SH-SY5Y cells.....	186
Table 9.1 Effects of short-term midazolam on ciliary beat frequency.....	195

Table 9.2 Effects of midazolam on cilia survival.....	198
Table 9.3 Effects of 48 hours incubation with midazolam on MTT reduction in SH-SY5Y cells.....	203
Table 9.4 Effects of 48 hours incubation with midazolam on MTT reduction in SH-SY5Y cells.....	204

List of Abbreviations

ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
Ca ²⁺	ionised calcium
CI	confidence interval
dpm	decays per minute
DPN	diprenorphine
FEV ₁	forced expiratory volume in the first second
FRC	functional residual capacity
FVC	forced vital capacity
GC	gas chromatography
HBSS	Hanks balanced salts solution
HPLC	high pressure liquid chromatography
IC ₅₀	50% inhibitory concentration
ITU	intensive therapy unit
MAC	minimum alveolar concentration
MANOVA	multiple analysis of variance
M199	medium 199
NO	nitric oxide
NSB	non-specific binding
PEFR	peak expiratory flow rate
rpm	revolutions per minute
SD	standard deviation
SEM	standard error of the mean
TrisHCl	tris[hydroxymethyl] aminomethane hydrochloride
VC	vital capacity

Chapter 1

Pulmonary infections in anaesthesia and intensive care

Incidence of postoperative and intensive care pulmonary infections

Advances in anaesthesia over the past few decades have produced a very low level of mortality associated with anaesthesia, with a recent report finding only three deaths out of nearly half a million procedures resulted from anaesthesia alone (Buck, 1987). Consequently, greater attention focuses upon the morbidity associated with anaesthesia and especially upon serious forms of morbidity. An important and common type of morbidity is that due to pulmonary infections. In a recent prospective study of 127 patients having upper abdominal surgery there was an incidence of postoperative pulmonary infections of 21% (Dilworth, 1992). Furthermore, Dilworth found that the mean length of postoperative hospital stay was significantly extended by three days in those developing a pulmonary infection.

The high incidence of postoperative pulmonary infections has remained largely unchanged for over 60 years despite changes in anaesthetic techniques and the widespread introduction of the use of antibiotics for wound prophylaxis during this time. A prospective study of over 7000 patients from Massachusetts in 1930 and 1931 revealed an overall incidence of pulmonary infections of 6% and 14% respectively in patients undergoing laparotomies (King, 1933). Nearly 40 years later, a study was undertaken in which 785 postoperative patients were prospectively followed (Wightman, 1968). In this investigation the overall incidence of pulmonary infections was 6%, but in the sub-group of 118 patients having upper abdominal procedures the incidence was 19%. Ten years later, in a

prospective study of 520 patients undergoing thoracic and abdominal surgery an 18% incidence of postoperative pneumonia overall was found (Garibaldi, 1981).

The development of pulmonary infections in patients in the intensive therapy unit (ITU) is also a common occurrence. In a European multi-centre study, 38% of patients admitted to the ITU with no evidence of pulmonary infection developed pneumonia subsequently (Ruiz-Santana, 1987). Furthermore, the mortality from hospital-acquired (nosocomial) pneumonia in the ITU ranges from 20-50 % (Stevens, 1974; Stamm, 1977; Riuz-Santana, 1987). The importance of pneumonias acquired in the ITU was demonstrated in Riuz-Santana's study which found a mortality from nosocomial pneumonia in ITU of 47%, but found that only 17% of the patients with pneumonia acquired before admission to the ITU died. In a separate study, nosocomial pneumonia was shown to prolong ITU stay, with survivors staying 12 days on average compared with 4.3 for a matched group who did not develop this complication (Craig, 1984).

Risk factors for postoperative and intensive care pulmonary infections

The causes of postoperative pulmonary infections are multifactorial and the selectivity of the data collected in prospective studies limits the known associations to thoracic and upper abdominal surgery, cigarette smoking, chronic bronchitis, airflow obstruction, low serum albumin, prolonged pre-operative hospital stay and the duration of anaesthesia and surgery. These associations may be explained by the provision of an environment in the lungs after surgery that is suitable for bacterial invasion as factors that promote the retention of bronchial secretions will predispose to the development of pulmonary infections.

Thoracic and upper abdominal surgery result in an inability to cough effectively and predispose to the retention of secretions. Indeed an association between postoperative pneumonia and vertical abdominal incision was found (Dilworth, 1992) which presumably relates to the greater impairment to coughing in these circumstances. The anterolateral abdominal wall muscles contract during the forced expiration of coughing and the adequacy of coughing is reduced considerably by these incisions that cut across multiple muscle fibres.

In patients with underlying respiratory disease involving excessive secretions, such as smokers and patients with chronic bronchitis, the postoperative retention of secretions is likely to be worse. There is an association with postoperative pneumonias and a history of smoking, chronic bronchitis and airflow obstruction (Dilworth, 1992).

If there has been an infective process in the respiratory tract before operation then the development of pneumonia in this stagnant mucus environment will be more likely. An association was found with low serum albumin (Garibaldi, 1981) which perhaps reflects the malnourished state with depressed immunity and an increased risk for developing infection. This study also demonstrated an association with prolonged pre-operative hospital stay, despite controlling for underlying disease, which may be due to the development of nosocomial infections.

In the ITU, effective coughing is impaired by the presence of an endotracheal tube that prevents glottic closure. Prolonged intubation is associated with lower respiratory tract infections. Riuz-Santana found that intubation for longer than 72 hours was associated with a 9% incidence of pulmonary infection compared with 46% in those intubated for more than 14 days (Riuz-Santana, 1987).

Another risk factor for the development of pulmonary infections in the ITU relates to the widespread use of broad spectrum antibiotics. These may selectively eradicate the normal bacterial flora and lead to colonisation of the oropharynx and to the subsequent development of a pulmonary infection.

Association of general anaesthesia and sedation with pulmonary infections

The techniques and agents used to produce anaesthesia for surgical operations and sedation in the ITU have a number of effects upon the respiratory system that predispose to the development of pulmonary infections through the retention of respiratory secretions. Effects upon both the mechanics of the lung and upon the movement of mucus may contribute to the retention of secretions and to the development of pneumonia and these will be considered in turn.

Effects of anaesthesia on lung mechanics

The resting lung volume or functional residual capacity (FRC) of the lungs is reduced during and for some time after general anaesthesia (Bergman, 1963). The reduction in FRC is mainly due to decreased outward chest wall recoil from the loss in normal inspiratory muscle tone that occurs in the anaesthetic state (Wahba, 1991). The reduction in FRC results in effects on both the small airways and on the coughing mechanism which predispose to the development of pulmonary infections. The reduction in FRC results in reduced small airways calibre and reduced alveolar size and stability. The changes in FRC are regionally distributed being more pronounced in basal lung units and are associated with atelectasis in these regions (Strandberg, 1986).

In many patients the reduction in FRC falls below the closing capacity of the lungs, the lung volume at which the terminal airways that are devoid of cartilaginous support close. Since this airways closure occurs in those regions of the lungs with the least negative intrapleural pressure, it also occurs in the dependent regions of the lungs. This basal airways closure results in gas trapping and as this gas becomes absorbed into the blood perfusing these dependent areas of the lung, collapse of lung tissue may occur in these regions. The collapsed areas of the lung are associated with retention of bronchial secretions (Gamsu, 1976) and this predisposes to the development of pulmonary infections.

The clearance of respiratory secretions depends in part on the act of coughing. Coughing involves an inspiratory phase, a compressive phase with a closed glottis that builds up a large positive pressure in the lungs and an expiratory phase when the glottis opens. It thus depends upon lung volume, respiratory muscle power and co-ordination, and upon laryngeal competence. Therefore, the reduced lung volumes consequent upon a reduction in the FRC will affect the ability to cough effectively.

Agents used in general anaesthesia may reduce muscle power and thus reduce all the phases of coughing; however, the short-acting anaesthetic agents and muscle relaxants in modern anaesthetic practice would not be expected to reduce this power for any prolonged length of time. The analgesia utilised peri-operatively may affect the ability to cough as opioids have an anti-tussive effect. However, anaesthetic techniques that avoid opioids for major abdominal surgery and rely upon epidural and spinal local analgesia may have effects that also impair coughing. These local anaesthetic techniques are associated with a reduction in power of the upper abdominal muscles which will manifest as a reduction in the intrapulmonary pressures generated during coughing (Egbert, 1961).

There may be also be important effects upon coughing due to the impairment of laryngeal muscular function that is associated with the administration of anaesthesia and ITU sedation. In a study of volunteers with normal laryngeal function and in patients with vocal cord palsies, it was demonstrated that the time to reach the cough peak flow rate correlated with laryngeal function (Murty, 1991). In a subsequent study undertaken in the period during recovery after a short anaesthetic with minimal invasion of the airway, the time to reach the cough peak flow rate was increased; however, this was only significant for the first twenty minutes after awakening from anaesthesia, implying that the effects of anaesthetics on this parameter in the recovery period are probably short-lived (Lamb, 1993).

Other anaesthetic factors associated with anaesthesia and ITU sedation may affect laryngeal function, in particular, the effects of tracheal intubation. Siedlecki and workers investigated laryngeal function in 30 patients after anaesthesia that included endotracheal intubation (Siedlecki, 1974). They assessed laryngeal competence by giving the patients contrast medium to swallow after regaining full consciousness and found that eight of these aspirated the medium. In a separate study, Tomlin challenged patients two or more hours after anaesthesia and found aspiration in nine of 41 whose tracheas had been intubated (Tomlin, 1968). In a more recent investigation, Stanley and colleagues found only one out of 20 patients aspirated dye immediately after regaining consciousness (Stanley, 1995) and they hypothesised that this lower incidence may be due to the shorter acting muscle relaxants in modern anaesthetic practice.

The effects of prolonged intubation upon laryngeal function relevant to the situation in the ITU was assessed by Burgess and workers in an investigation of patients undergoing cardiac surgery and postoperative ventilation (Burgess, 1979). They studied 64 patients divided into three groups and challenged them

with radio-opaque dye immediately following extubation, at four hours or at eight hours after extubation. The mean duration of intubation was 18 hours and they found a 33% incidence of aspiration immediately after extubation, 20% in the four hour group and 5% in the eight hour group.

Thus a variety of factors in the peri-operative and intensive care periods related to the administration of anaesthesia and sedation affect lung mechanics which predispose to the retention of secretions and affect the ability to cough and clear these secretions.

Effects of anaesthesia on respiratory mucus transport

In the period after surgery and anaesthesia there is a reduction in the rate of transport of mucus; however, the role of anaesthesia in this is not clear.

Gamsu and workers investigated mucus clearance after general anaesthesia in a study using tantalum, a radio-opaque marker that adheres to mucus (Gamsu, 1976). They compared the clearance rate of tantalum from the lungs of patients receiving general anaesthesia with an awake control group undergoing tracheography. In one group having intra-abdominal surgery the tantalum was retained for up to six days and the average retention was three times longer than in the control group; however, in another group undergoing peripheral orthopaedic surgery there was no significant difference in tantalum clearance compared with the controls. This supports the important role that surgical incision has upon respiratory mucus clearance and pulmonary defence, but does not provide evidence for any significant effects of the anaesthetic agents themselves.

Supportive evidence for a role of the anaesthetic agents in impeding mucus clearance is provided from studies of women undergoing gynaecological surgery. Lichtiger and workers measured mucus clearance by tracking Teflon discs placed on the tracheal mucosa with a fibroscope. The tracheal mucus velocity in the anaesthetised women was 7.7 mm min^{-1} compared with 20 mm min^{-1} in awake volunteers (Lichtiger, 1975). In a separate study of gynaecological patients using a similar technique to measure mucus transport rate, Annis and workers also found a significant reduction in mucus transport during anaesthesia compared with the awake values (Annis, 1976).

In these human studies a variety of factors were operating that diminish mucus transport. Both the technique of anaesthetic administration as well as the drugs administered may be implicated. These two factors are considered separately.

1. Effects of anaesthetic techniques on mucus transport

A variety of factors associated with the administration of anaesthesia for surgery and ITU sedation impair respiratory mucus transport.

Wolfe and workers investigated the effects of positive pressure ventilation in a study on dogs using powdered tantalum and serial chest radiography. They found a threefold decrease in clearance in those animals ventilated with air for 24 to 36 hours compared with a group breathing spontaneously (Wolfe, 1972). In contrast, Konrad and colleagues measured bronchial transport of radioactive-labelled albumin microspheres with a scintillation counter in fourteen patients undergoing mechanical ventilation for an average duration of five and a half hours and found no effects on mucus transport rates compared with measurements made in nine of them at the commencement of mechanical ventilation (Konrad, 1992). The

explanation for this difference may be that *short-term* ventilation does not affect mucus transport rate.

It is common practice to administer concentrations of oxygen greater than that in room air to both anaesthetised patients and patients in the ITU. The effects of increased oxygen concentrations on mucus transport appear to be deleterious.

Using differential lung ventilation in dogs, Wolfe compared the effects of ventilating one lung with 100% oxygen and the other with air and found that clearance rates were one and a half times longer in the group receiving 100% oxygen (Wolfe, 1972).

Sackner and colleagues investigated the effects of breathing high concentrations of oxygen in a study on human volunteers in which mucus transport was measured by the movement of Teflon discs placed on the trachea and followed with a fibroscope (Sackner, 1975a). Compared with a control value of 22.9 mm min⁻¹, the tracheal mucus velocity fell to 17.8 mm min⁻¹ after breathing 90-95% oxygen for three hours. These workers went on to investigate the effects of graded concentrations of oxygen on tracheal mucus velocity in dogs by similar techniques. They found that the tracheal mucus velocity fell by 42% after the administration of 75% oxygen for nine hours, by 20% after administration of 50% oxygen for 24 hours and by 51% after 30 hours (Sackner, 1976).

In spontaneously breathing anaesthetised patients, the partial pressure of carbon dioxide rises above normal. The effect of elevated carbon dioxide levels upon mucus transport was investigated in a study with anaesthetised dogs breathing normoxic gas mixtures containing 7.5% carbon dioxide and the tracheal mucus transport rate measured by the movement of radiopertechnate droplets was found to have decreased by 31% of the control values (Marin, 1969).

Endotracheal intubation bypasses the humidifying tissues of the nose and consequently inadequately humidified gases are often delivered to the lungs. Burton investigated the effects of dry gases upon mucus clearance in anaesthetised dogs. India ink was placed at the carina and its movement followed by repeat bronchoscopy. The marker reached the vocal cords within 30 minutes in those animals receiving completely humidified gases via an endotracheal tube compared with a maximal movement of only 2.5 cm in 30 minutes in those receiving inadequately humidified gases (Burton, 1962).

The endotracheal tube itself may affect mucus transport. Sackner investigated the effects of endotracheal tubes on mucus clearance in dogs anaesthetised with warm humidified air (Sackner, 1975b). Teflon discs were placed on the tracheal mucosa with a fiberoptic bronchoscope and their movement filmed through the fibrescope. With a low compliance cuffed endotracheal tube there was a 26% reduction in mucus velocity after one hour which fell to 74% by four hours. This compared with a group using an uncuffed endotracheal tube in which there was no significant difference in mucus velocity over a four hour period.

The effect of endotracheal tubes on mucus transport is of even greater concern in the ITU where patients are intubated for longer periods. In this situation it is common to advocate the use of highly compliant cuffs on the endotracheal tubes to minimise tissue trauma. Interestingly, high compliance cuffed tubes lowered tracheal mucus velocity by 37% after one hour and by 52% after four hours (Sackner, 1975b).

2. Effects of anaesthetic and sedative agents on mucus transport

In animal studies in which temperature and humidity of inspired gases and endotracheal tube cuff pressure were controlled a variety of the agents used in anaesthesia and sedation have been shown to reduce mucus transport rates *in vivo*.

Forbes and Horrigan used a radioactive droplet method and external scintillation counters in dogs and found a depression in tracheal mucociliary flow of 59-77% of control values during exposure to 1.2 MAC of the inhalation anaesthetic agents, halothane and enflurane (Forbes, 1977).

The intravenous barbiturate anaesthetics also appear to reduce mucus transport. Landa and workers administered 30 mg kg^{-1} of the intravenous barbiturates, pentobarbitone or thioamylal to sheep. Using a cine-broncho-fibrescopic technique they followed Teflon discs placed on the tracheal mucosa and found a reduction in disc movement from average control values of 17.3 mm min^{-1} to 11.1 mm min^{-1} (Landa, 1975). Forbes and Gamsu using tantalum bronchography in dogs showed a similar reduction in mucociliary clearance maintaining anaesthesia with thiopentone compared with 1.2 MAC of halothane (Forbes, 1979a).

The effects of the opioids on mucociliary clearance were investigated in cats using barium sulphate insufflated into the lungs and serial chest radiography. The clearance was delayed threefold after the administration of 0.5 mg kg^{-1} of morphine (Van Dongen, 1953). Forbes and Horrigan also studied the effects of morphine on mucus transport rate in dogs and found that 6 mg kg^{-1} of morphine produced a similar reduction to 1.2 MAC of the inhalation anaesthetics (Forbes, 1977).

The removal of bronchial secretions which is necessary to the prevention of pulmonary infections depends upon coughing and the transport of mucus, both of which may be impaired by the effects of anaesthesia. Under normal conditions mucus clearance is achieved in the absence of coughing by the functions of the cilia interacting with the overlying mucus; however, if this is impaired then mucus clearance comes to depend upon coughing. It is therefore with the combination of both impaired mucociliary clearance and impaired coughing that retention of secretions is most likely to occur.

The high incidence of chest infections associated with upper abdominal surgery, may be related to an impairment in coughing due to the muscular dysfunction and pain of surgical incision upon a background of impaired mucociliary clearance due to general anaesthesia. In smokers and those with chronic bronchitis, the increased mucus load already requires the act of coughing to clear secretions and it would be anticipated that any further decompensation to mucociliary clearance would lead to significant mucus retention in these patients.

The effects of prolonged intubation in the ITU compromises effective coughing and may compound any depression in mucociliary clearance due to the techniques of ventilation or the sedatives used. Thus impairment to coughing both in the ITU and after abdominal surgery renders patients more dependent on mucociliary clearance to maintain bronchopulmonary toilet.

The role of reduced mucociliary clearance upon the subsequent development of postoperative and ITU pulmonary infections is unknown; however, mucociliary clearance is known to be impaired by some anaesthetic agents and it is one of the most important defences against respiratory tract infections.

Local anaesthesia and postoperative pulmonary infections

One might anticipate that if general anaesthesia is associated with the retention of pulmonary secretions and the development of chest infections then regional anaesthetic techniques might be associated with a lower incidence. There have been no data collected prospectively on the use of different anaesthetic agents or techniques and the subsequent development of postoperative pulmonary infections so that any associations remain speculative.

Retrospective analyses are unsatisfactory due to sample bias as it would be anticipated that those patients who have had regional anaesthetic techniques may have had pre-operative respiratory disease. Furthermore, most regional techniques for abdominal procedures are largely confined to lower abdominal operations, where the incidence of pulmonary infection is lower compared with upper abdominal operations, so that a large study would be required to be sufficiently powerful to detect a real difference between the groups.

In hypothesising that general anaesthesia impairs respiratory defences, consideration must be given to the physiological effects of spinal or epidural analgesia using local anaesthetics which may do likewise. High spinal anaesthesia has been shown to reduce the ability to cough due to weakness of the abdominal muscles that are required for the forced expiratory phase of coughing (Egbert, 1961). Since epidural analgesia for lower abdominal procedures may reduce the power of some of the abdominal muscles one might expect an impairment in coughing ability too.

Those at greatest risk from the effects of depressed mucociliary clearance are patients with excessive mucus production. In a retrospective study restricted to patients with chronic obstructive pulmonary disease (Tarhan, 1973), the authors

found that for operations in which general anaesthesia (119 cases) and spinal or epidural analgesia (121 cases) were used there were six deaths following general anaesthesia and none following epidural or spinal analgesia. The causes of death in this subgroup were not defined; however, this was a statistically significant difference, and would suggest that at least for patients with chronic pulmonary disease, the impairment of respiratory function may be less after regional techniques than after general anaesthesia.

Reducing the risk factors for postoperative pulmonary infections

Attempts to reduce postoperative pulmonary complications have involved efforts to improve the clearance of secretions by promoting more effective coughing. This has involved the prophylactic use of chest physiotherapy before and after surgery; however, there is no satisfactory data to support the use of widespread prophylactic chest physiotherapy. Dilworth compared a group of patients undergoing upper abdominal surgery treated conventionally with a group given regular chest physiotherapy together with prophylactic antibiotics and found a similar incidence of postoperative respiratory infections in both (Dilworth, 1992).

More recently techniques to optimise patient analgesia in the postoperative period and the introduction of laparoscopic surgical techniques that minimise the extent of abdominal surgical incision have been introduced and their impact on promoting more effective coughing and the clearance of secretions is discussed.

Effects of postoperative analgesia

It is generally believed that good postoperative analgesia improves respiratory function and the effectiveness of coughing after abdominal surgery. This would be expected to reduce the incidence of postoperative respiratory infections; however, studies investigating the effects of analgesia upon the various respiratory parameters that are associated with the adequacy of coughing appear to show only a limited benefit.

Pflug compared the effects of intramuscular morphine administered as requested by the patient with epidural bupivacaine infusions in 40 patients having upper abdominal or hip fracture surgery (Pflug, 1974). Although pain scores were not recorded it is anticipated that pain relief would have been better in the epidural group. They found reductions in the vital capacity (VC) and peak expiratory flow rate (PEFR) of the lungs in both groups and although this was less pronounced in the epidural group the difference between the two treatment groups was not statistically significant.

Schulze studied 24 patients undergoing open cholecystectomy half of whom received conventional postoperative analgesia with nicomorphine and paracetamol as requested and half received optimal analgesia with epidural bupivacaine and morphine as well as systemic indomethacin (Schulze, 1988). Although there was a significant difference in the pain scores of the two groups with the epidural group being virtually pain free, there was a large decrease in peak flow rates in both groups with minimal difference between the two groups.

Brismar and workers found that the administration of intrapleural local analgesia gave excellent pain relief after open cholecystectomy, with complete analgesia in 143 of 159 administrations; however, the forced vital capacity (FVC) and forced

expiratory volume in the first second (FEV_1) measured on the day after surgery had still declined to 60% of preoperative values (Brismar, 1987).

There is only limited data on the effects of analgesia upon the development of postoperative chest infections. Spence and Smith studied 21 patients having upper abdominal surgery who were given either on-demand intramuscular morphine or continuous local anaesthetic epidural infusion for postoperative analgesia (Spence, 1971). They found that 7 out of 10 patients given morphine developed pneumonia at 48 hours compared with 2 out of 11 in the epidural group.

In a study of patients undergoing cholecystectomy, Cuschieri compared the effects of systemic intramuscular morphine given to 25 patients with epidural bupivacaine given to a further 25. He found that analgesia was better in the epidural group and that only one patient in this group developed a chest infection compared with five in the morphine group (Cuschieri, 1985).

A recent report recognised the inadequacy of postoperative analgesia in the United Kingdom (Royal College of Surgeons of England and College of Anaesthetists, 1990) and has led to the development of acute postoperative pain services. Wheatley and workers described their experience with such a service providing optimal analgesia after surgery by the use of patient-controlled analgesic devices delivering opioids intravenously and epidural analgesic infusions of local anaesthetics and opioids (Wheatley, 1991). Although not a primary outcome measure, they retrospectively analysed all patient records in their hospital to determine the incidence of postoperative lower respiratory tract infections before and after the introduction of this pain service. They found that the incidence fell from 1.3 % of 4290 patients to 0.4 % of 4667 having general surgical operations.

It would appear that optimal analgesia reduces the incidence of postoperative chest infections; however, respiratory indices related to mucus clearance by coughing, the FVC, VC and PEFR, are not returned to normal. This is probably because factors other than incisional pain affect these parameters such as pneumoperitoneum and bowel distension.

Effects of laparoscopic surgery

Laparoscopic techniques of surgery have become more popular in recent times due to their aesthetic appeal, shorter hospital stay and reduced postoperative pain. A major advantage of laparoscopic surgery is believed to be the avoidance of the pulmonary consequences of an abdominal incision; however, the incidence of postoperative chest infections after these operations compared with open abdominal procedures is still awaited. Currently, the most frequently performed laparoscopic upper abdominal operation is laparoscopic cholecystectomy and comparative studies with open abdominal surgery have largely been restricted to this procedure.

Lung function following open upper abdominal surgery is typically restrictive, with more rapid and shallower breathing, reduced VC and FRC. There is a shift from abdominal to rib cage breathing due to loss of the diaphragmatic contribution to tidal volume. Two major factors determine the magnitude of restrictive lung function seen after upper abdominal surgery: incisional pain and reflex inhibition of diaphragmatic function (Wahba, 1991).

Although the pain of abdominal incision is less with laparoscopic techniques, there is still a reduction in FRC and VC in the days following operation, presumably due mainly to the impairment in diaphragmatic function. The peritoneal insufflation that necessarily accompanies laparoscopic procedures

affects respiratory function by causing cephalad displacement of the diaphragm which reduces the FRC. Although FRC has not been directly measured in this situation, total lung compliance has been shown to fall significantly after laparoscopic surgery (Feinstein, 1993).

In a study measuring abdominal and thoracic volume changes and transdiaphragmatic pressures during respiratory manoeuvres, Couture and workers showed that the breathing pattern after laparoscopic cholecystectomy is similar to the open operation measured 18 hours after surgery (Couture, 1994). In the published studies the impairment in lung function was less and the return to normal function was faster after laparoscopic cholecystectomy compared with open operation. In a study using forced expiratory flow during the 25-75% of the VC breath, an effort-independent measurement, the reduction following open cholecystectomy was 50% which compared with 25% after laparoscopic cholecystectomy on day two. Although the rate of recovery was more rapid in the case of the laparoscopic procedure, the values were only 80% of control on day three, suggesting that there is some residual pulmonary dysfunction 72 hours after surgery (Wahba, 1995).

Notably, these studies have been restricted to relatively healthy patients. There is a wide scatter in the reported reductions in FRC after laparoscopic cholecystectomy and more marked reductions occurred in older patients, the obese and in smokers. Thus the beneficial effects of laparoscopic surgery upon those most at risk from pulmonary complications may prove to be limited and although laparoscopic techniques of abdominal and thoracic surgery are becoming more popular, there will always be a need for open abdominal operations.

In view of the limited benefits that are likely to accrue from attempts to optimise gross mucus clearance by improvement in the ability to cough, alternative strategies may be worth investigating. It is notable that anaesthetic and sedative agents impair mucociliary clearance, which is one of the most important defences against respiratory tract infection. This has received little attention in the past and merits investigation to elucidate the mechanisms involved which may ultimately provide therapeutic options.

Chapter 2

The mucociliary system and anaesthesia

The mucociliary system: an important defence against respiratory infection

The mucociliary system is composed of a fluid, mucus, secreted by cells lining the airways which is propelled cephalad by ciliary beating. This system is an important defence against inhaled insoluble particulate matter and its importance in defence against respiratory infections is demonstrated by pathological conditions in which it is defective.

Primary hereditary abnormalities of the cilia have been described in association with susceptibility to chronic respiratory infections. In 1933 Kartegener described a syndrome of bronchiectasis, sinusitis and situs inversus, and patients with this condition were found to have cilia with ultrastructural abnormalities of the dynein arms and reduced or absent motility (Afzelius, 1976). In another inherited abnormality, Sturgess described two siblings with chronic sinobronchial infections with a different structural abnormality of the cilia involving one microtubular doublet (Sturgess, 1980).

A variety of secondary causes of ciliary damage have been described which are associated with susceptibility to respiratory infections. Viral upper respiratory tract infections render patients susceptible to subsequent bacterial infection. This is related in part to loss of ciliated cells as demonstrated in nasal epithelial biopsies taken during upper respiratory tract infections with rhinoviruses (Wilson, 1985); however, acquired ciliary microtubular defects were observed in nasal epithelial biopsies of children with viral infections (Carson, 1985). In an

animal study using chick nasal mucosa, Bang and workers investigated the effects of a laryngotracheitis virus on the nasal mucosa. They showed that the mucosa was destroyed and that the normal linear pattern of mucus movement by the cilia was disorganised after infection (Bang, 1966). As a result the functional clearance of mucus was abolished and the mucus accumulated in the nasal fossa.

Many patients with chronic bronchitis are susceptible to acute respiratory infections. Measurements of mucociliary clearance in these patients are complicated by the associated airways obstruction and increased incidence of coughing; however, these patients demonstrate alterations in both the mucus and the cilia that would be expected to impair mucociliary clearance.

In a study on sputum from patients with bronchitis, Dulfano and Adler showed that this had a higher viscosity than frog mucus controls and had lower transport rates over the frog palate (Dulfano, 1975). In another study of the cilia from patients with chronic bronchitis, an increased proportion of the cilia was found to have an abnormal ultrastructure (Lungarella, 1983).

The effects of chronic bronchitis upon respiratory ciliary beat frequency is still disputed. In two studies of patients with chronic bronchitis, ciliary beat frequency *in vitro* did not differ from normal (Yager, 1978; Konietzko, 1981); however, in a rat model of bronchitis, there were small decreases in overall ciliary beat frequency compared with normal rats (Iravani, 1976). In this latter study, areas of static and reversed ciliary beating were found and this work suggests that effects on cilia could be missed if biopsy samples alone are examined. The mucociliary system can usefully be considered to comprise two components, a transport element, the mucus and a motor element, the cilia. These will now be considered separately.

Respiratory mucus

Respiratory mucus consists of a variety of glycoproteins suspended in an aqueous solution. It is produced in the lungs by submucosal glands innervated by efferent postganglionic parasympathetic fibres and by Goblet, Clara and serous cells.

A normal healthy adult expels 10 ml of mucus each day from the trachea representing a layer 10 μm deep propelled continuously at 0.2 mm sec^{-1} . The mucus layer overlying the cilia consists of two parts: a watery sol layer in which the cilia beat and an overlying viscid gel layer which is propelled by mechanical coupling to the ciliary beating (Fig. 2.1). The mucus overlying the cilia traps particulate matter which is carried by mucociliary transport to the pharynx to be expectorated or swallowed.

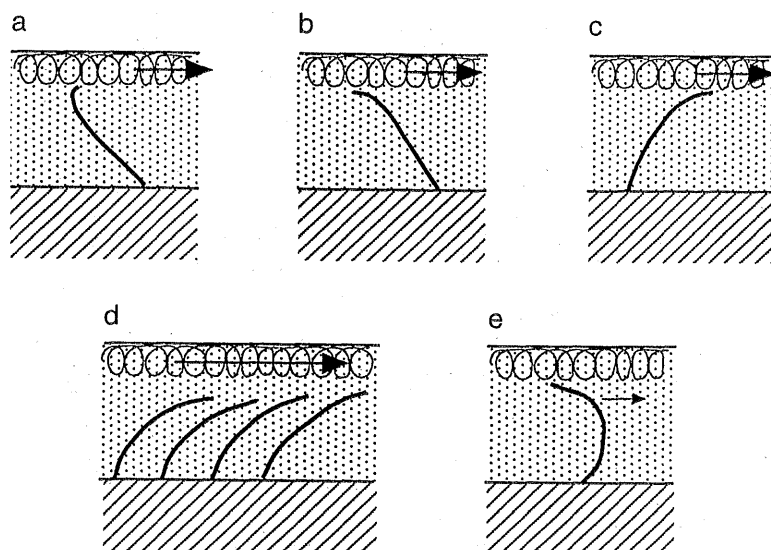


Fig. 2.1 The two layers of respiratory mucus and the relation of the cilia

The physical properties of the mucus determine the efficiency of mucus clearance. Mucus exhibits both viscous and elastic properties. Mucus is not a Newtonian fluid and its viscosity decreases as the applied force increases (Sleigh, 1988); thus, the more forceful the cilia beat the more easily the mucus moves. This may explain the importance of the ciliary beat frequency in mucus transport, because disproportionate decreases in mucus transport rates are found with modest decreases in ciliary beat frequency (Hee, 1985). Mucus is also elastic and its modulus of elasticity increases as the rate of shear increases (Sleigh, 1988). Thus at low frequencies viscosity dominates and at high frequencies elasticity dominates. The loose and random coiling of the glycoprotein molecules produces viscoelasticity with sufficient relaxation times to prevent recoil from negating the forward impulse of each effective stroke. The viscosity of mucus provides the long relaxation time and also enhances the ability of mucus to trap particles, but if it is too high then the cilia cannot penetrate the mucus. The elasticity is important in the efficiency of transport through energy transfer.

Although the viscoelastic properties of the glycoprotein molecules are important, viscosity and elasticity are taken from linear theories and may not apply for large deformations of non-linear materials. After secretion the mucus hydrates and the cohesion of these masses allows the molecules to be drawn out as threads. The thread-like property of the mucus, termed spinnability, is an appropriate property of mucus at the macroscopic level and high spinnability (the ability to be drawn out into long threads) correlates with high transport rates (Sleigh, 1988).

Submucosal gland secretion is increased by cholinergic drive; however, water flow across the respiratory epithelium is important too in lubricating the airways. This water flow is driven by chloride flux into the airway lumen and it may also alter the physical properties of the mucus and affect its rate of transport.

Effects of anaesthetic agents on mucus

Observations that mucus transport rates are depressed in anaesthetised animals and humans (Forbes, 1977; Lichtiger, 1975) has led to speculation that general anaesthesia may alter the volume or physical properties of respiratory mucus.

In a study using tracheostomised dogs it was found that pentobarbitone caused an increase in mucus elasticity and viscosity and reduced volume of mucus secretion (King, 1979). These workers went on to demonstrate that mucus from the anaesthetised dogs was transported across the frog palate at 20% below the control values in awake animals. Using a fiberoptic bronchoscopic technique in sheep, Landa found a 35% reduction in tracheal mucus velocity with barbiturate anaesthesia (Landa, 1975). The greater reduction in mucus transport rates in the latter study suggests that the effects of anaesthetics on mucus transport may not be confined to the mucus and that effects on the cilia are possible too.

Using canine tracheal epithelia, Pizov investigated the effects of halothane on ion transport, an important determinant of water movement across this tissue (Pizov, 1992). The current required to reset the electrical potential difference across the epithelia to zero, an index of ion transport, was reduced by halothane. Such an inhibitory effect on fluid transport across the epithelium could affect mucociliary coupling and reduce mucus transport.

In a direct investigation of human mucus rheology studying respiratory mucus from awake volunteers and anaesthetised patients, no significant differences in the physical properties of the mucus or its transport rate across mucus-depleted frog palate were found (Rubin, 1990). There is therefore only limited and indirect evidence to support an important effect of anaesthetic agents on human respiratory mucus and attention is now drawn to the cilia.

Cilia

Cilia are rhythmically-beating hair-like projections from the surface of the cell found throughout zoological species from unicellular protozoa to highly evolved metazoa. They may propel the organism itself as in the case of ciliated protozoa or move the adjacent fluids past the ciliated cells as in the ciliated gills of molluscs and in the movement of overlying mucus by respiratory tract cilia of mammals.

In the human, cilia are found in the upper and lower respiratory tract, male and female genital tracts, ependymal lining cells, olfactory cells and vestibular-cochlear apparatus of the central nervous system. Most of the airways as far as the respiratory bronchioles are covered by ciliated epithelium. Each ciliated cell has around two hundred cilia which are 6 μm in length and 0.2 μm in width (Fig.2.2).

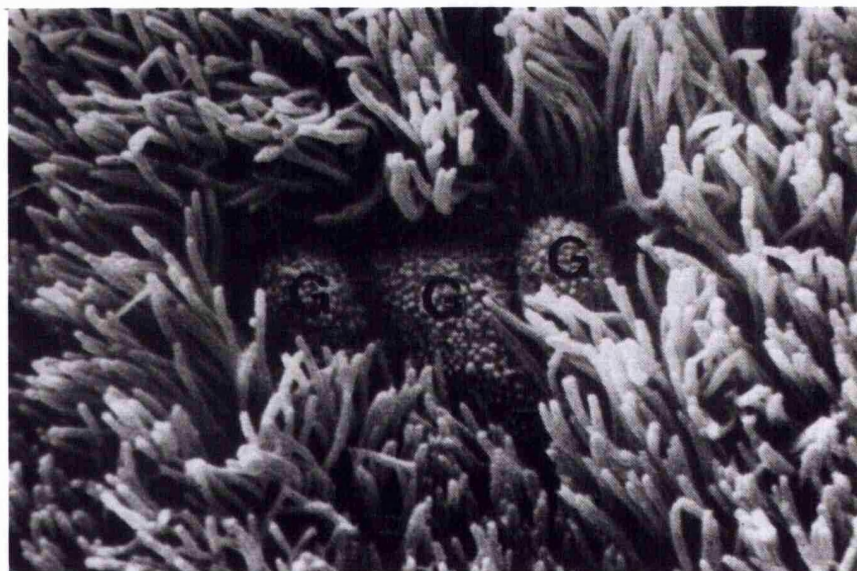


Fig 2.2 Electron micrograph of respiratory ciliated epithelium

(G = Goblet cell)

Cilia have a common structure of longitudinally arranged microtubules enclosed by a projection of the plasma membrane. Respiratory cilia differ from others in possessing a crown of 3-7 short claws 25-35 nm long projecting from a dense cap at their tips which presumably anchors the overlying mucus (Fig. 2.3).

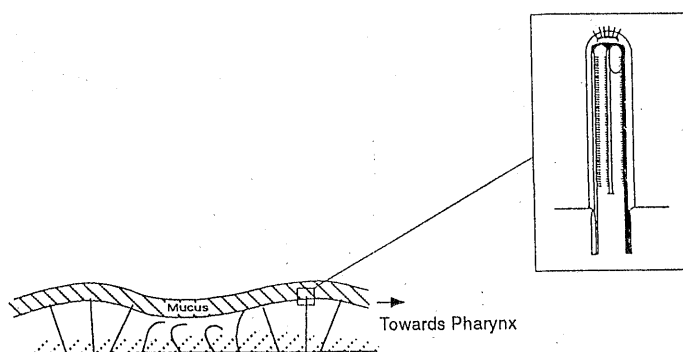


Fig. 2.3 Mucociliary interaction showing how the claws projecting from the cilia interact with the mucus

The cilium consists of an array of microtubules called the axoneme (Fig. 2.4). This is composed of nine pairs of filaments ('A' and 'B' microtubules) arranged in a ring around a central pair of filaments. Adjacent doublets are connected by nexin links and the peripheral microtubules are linked to the central pair by radial spokes. The outer A microtubules possess projections called dynein arms where an adenosine triphosphate (ATP) hydrolysis system is located.

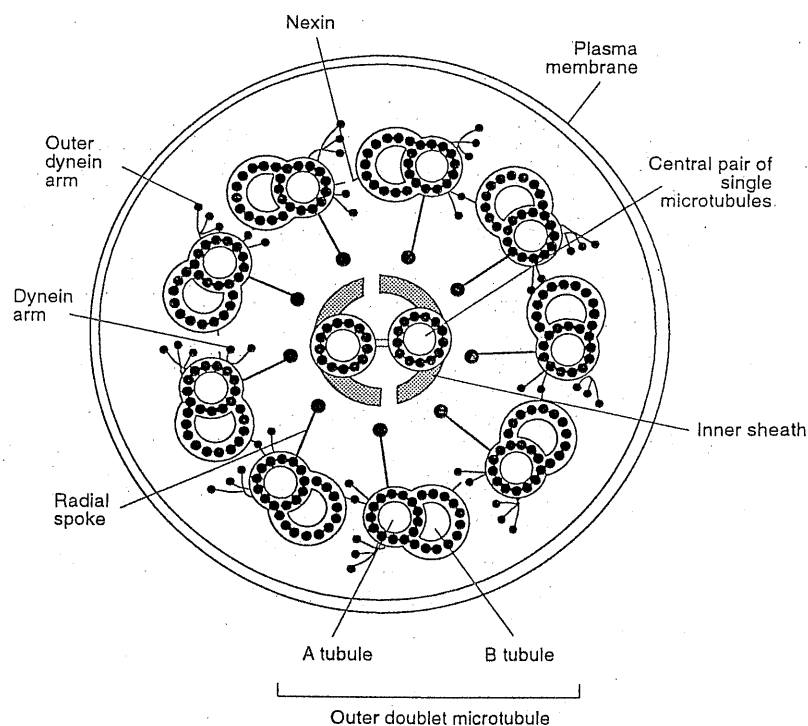


Fig. 2.4 A cross section of a cilium showing microtubular arrangement of the axoneme

Movement of the cilium occurs with the hydrolysis of ATP causing repetitive molecular bridging between an outer 'A' microtubule and an adjacent 'B' microtubule belonging to another pair (Fig. 2.5). As a result the tubules slide upon one another. Because one of the microtubules of the peripheral doublets is shorter than the other, the anchorage provided by the radial spokes converts the axonemal sliding into ciliary bending.

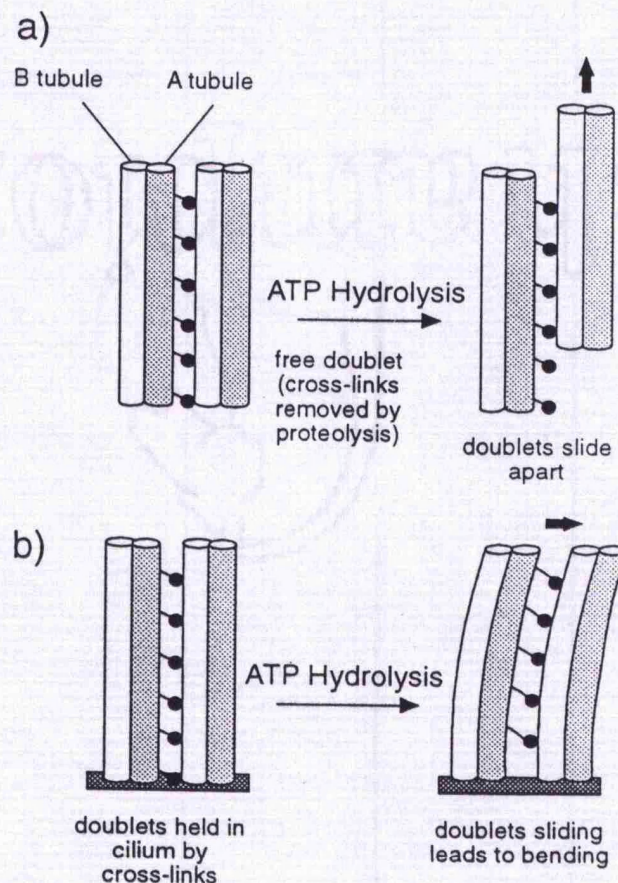


Fig. 2.5 The bending of the ciliary axoneme

a) Microtubule doublets slide against each other

b) Anchoring of both doublets converts this sliding into bending

Cilia propel fluids because the cyclical movements they perform are asymmetric (Fig. 2.6). In the effective stroke the cilia remain extended moving in an arc perpendicular to the cell surface, whereas in the recovery stroke the cilium bends along its length and swings around near the cell surface to reach the starting position for the next effective stroke.

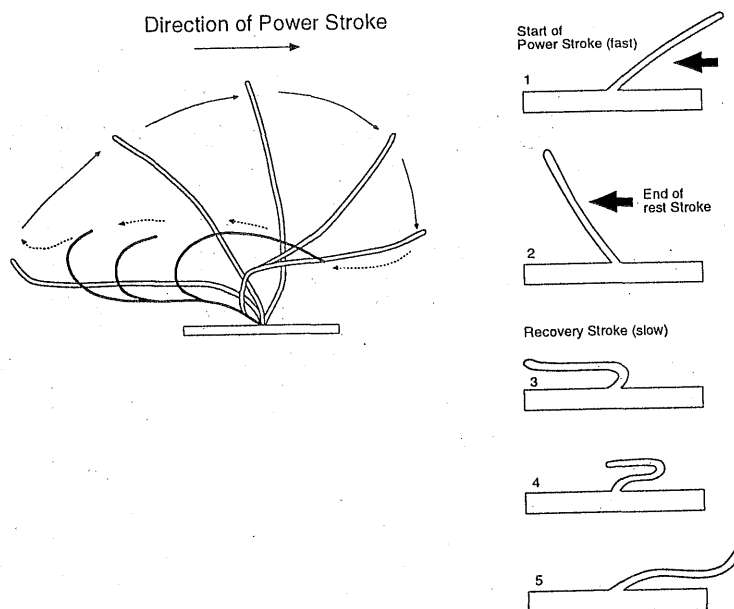


Fig. 2.6 Ciliary motion: effective, rest and recovery strokes

In comparing the effective with the recovery strokes of ciliary motion, the former acts perpendicular rather than tangential to the cell surface, faster than the recovery stroke and is in contact with and therefore influences a larger volume of fluid, producing a net transport of fluid by the ciliary beat cycle.

Adjacent cilia will share influence over an area of fluid and the cilia adjust their frequency and phase to minimise this interference producing limited areas of co-ordinated motion termed metachronal waves. Although such metachronal waves are limited in area by irregularities of the epithelial surface and the presence of non-ciliated cells, they are important in increasing the efficiency of mucus transport by the beating cilia. This may partly explain the reduced mucus transport rates in conditions where regenerated ciliated epithelial cells beat in different directions that is seen in smokers (Iravani, 1976), or where there are larger gaps between ciliated cells due to cell shedding in respiratory infections.

Control of ciliary function

There are limited examples of nervous control of ciliary activity. Transection of branchial nerve of the lamelli-branch mollusc, *mytilis*, reduces ciliary activity of the gill filaments; however, except for the frog palate, vertebrate cilia are not known to be innervated. The control of human respiratory tract ciliary function is currently unknown. Nerves in the tracheal mucosa do not penetrate the basement membrane and nerves found around the basal end of ciliated cells do not make synaptic contact with the cells and appear similar to the free sensory nerve endings common to other epithelia (Rhodin, 1966). Thus there is no morphological evidence of neural control of human cilia directly. Furthermore, lung clearance in five patients with autonomic failure, a model of a denervated mucociliary escalator, failed to show any slowing (Jenkins, 1980).

Extracellular ions affect the cilia but there are again notable species differences. In studies on the ciliated protozoan, *Opalina*, alterations in the ionic composition reversed the direction of ciliary beating by altering the direction of membrane currents, an effect not seen with respiratory tract cilia (Sleigh, 1966). This may represent evolutionary change as ciliated protozoa require to change speed and direction in response to environmental changes in ionic composition and have more sophisticated requirements than respiratory tract cilia which are only required to beat incessantly to move mucus out of the lungs.

There are species differences in the susceptibility to changes in osmotic pressure and mechanical stimulation. In a study using rat tracheal cilia, modest osmotic changes incurred by replacing Ringer's lactate with 0.9% sodium chloride were associated with a 10% difference in ciliary beat frequency (van de Donk, 1980); whereas a study with human bronchial cilia found the ciliary beat frequency to be constant between 160-400 mOsm l^{-1} (Luk, 1983). Mechanical stimulation has been shown to affect the cilia of the frog palate and pharynx. The cilia are quiescent when unstimulated but become active in the presence of mucus or particulate matter. This is not found with human respiratory tract cilia.

Regulation of ciliary activity is dependent on the intracellular concentration of the second messengers, cyclic adenosine monophosphate (cAMP) and ionised calcium (Ca^{2+}) which affect axonemal dynein (Tash, 1983). In respiratory tract cilia, increases in both cAMP and Ca^{2+} increase ciliary beat frequency (Lansley, 1992). A variety of neurotransmitters and hormones have been shown to affect respiratory tract cilia by affecting these messengers (table 2.1 and fig. 2.7). The best documented evidence suggests that ciliary beat frequency is increased when cAMP, inositol triphosphate (IP_3) and intracellular Ca^{2+} are increased; and ciliary beat frequency is decreased when cAMP is reduced or cGMP is increased. Sodium channel blockade may also reduce ciliary beat frequency.

Table 2.1 Regulation of ciliary beat frequency (CBF) and effects on second messengers

Receptor/Agent	2nd Messenger	Effect on CBF
Beta -Adrenoreceptor ¹	+cAMP	+
Alpha-Adrenoreceptor ¹	+IP ₃ , -cAMP?	?-/+
Muscarinic ²	+IP ₃	+
Substance P ²	+IP ₃	+
Prostaglandin E ₁ , E ₂ ^{3,4}	+IP ₃ , +/-cAMP	+
Nicotinic ⁵	Na/K/Ca channel	+
Ionomycin ⁶	+Ca ²⁺	+
ATP ⁷	?+Ca ²⁺	+
Atrial Natriuretic Factor ⁸	+cGMP	-
Endothelin ⁹	+IP ₃	+
Local anaesthetics ¹⁰	block Na channel	-
Angiotensin ¹¹	+IP ₃	+
Pyocyanin ¹²	-cAMP	-
NO synthetase inhibitors ¹³	?	+

References

1 Ingels, 1992; 2 Aiello, 1991; 3 Khan, 1995; 4 Bonin, 1992; 5 Hahn, 1992;
6 Lansley, 1992; 7 Korngreen, 1994; 8 Tamaoki, 1991a; 9 Tamaoki, 1991b;
10 Ingels, 1994; 11 Kobayashi, 1990; 12 Kanthakumar, 1994; 13 Jain, 1993.

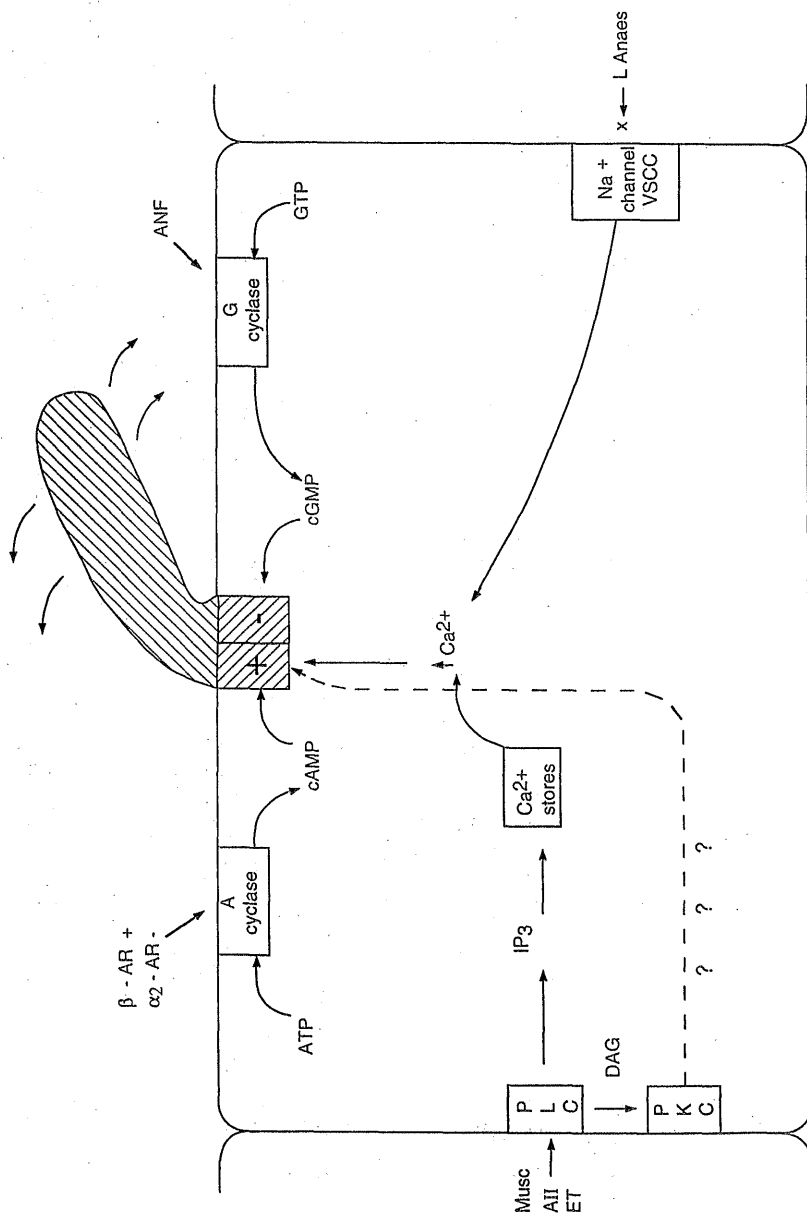


Fig. 2.7 Schematic representation of control of ciliary beating

Interactions of mucus and cilia: the importance of ciliary beat frequency

Mechanical clearance of the airways by the mucociliary system depends on the interaction of the cilia and the mucus (Fig. 2.8).

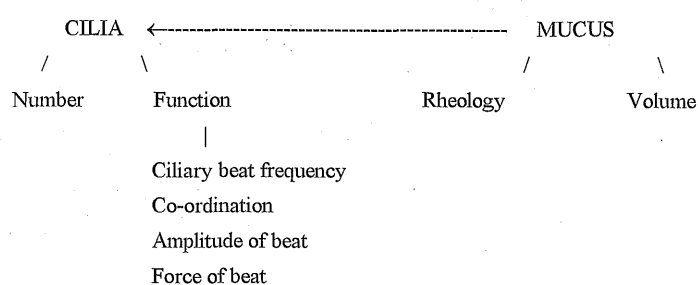


Fig. 2.8 Interactions of factors affecting mucociliary transport

The depth and physical properties of the mucus determine its contribution to mucociliary transport. The variables of ciliary function that determine the efficiency of mucociliary transport are the number of the cilia, their beating frequency, amplitude, force of beat and their co-ordination.

These variables are not independent and may interact, for example, some ions can stimulate or depress ciliary activity and may also affect water flux across the epithelium and control the depth of the sol layer. Furthermore, the system itself may interact with the variables. For example, if mucociliary transport decreases, bronchial secretions stagnate, their physical properties change and this affects the efficiency of the ciliary movement of the mucus.

Both the amplitude of ciliary beating and ciliary co-ordination can only be expressed qualitatively and the ciliary beat frequency is the only parameter of ciliary function that has been reliably quantified and this must serve as an index of ciliary function.

The ciliary beat frequency correlates with ciliary beat amplitude. In a study using ciliated cells from human maxillary sinuses, investigators qualitatively graded ciliary beat amplitude from video tape recordings and found a strong positive correlation between ciliary beat frequency and ciliary beat amplitude (Rautiainen, 1992).

The ciliary beat frequency may also be related to ciliary co-ordination. Ciliary co-ordination is a function of the forces in the fluid between the cilia. Adjacent cilia may adjust their frequency to coincide with one another to minimise any interference in the entrained fluid around them. The extent of ciliary coordination is however limited due to the areas of epithelial irregularity that result in greater than average distances between the cilia. As a consequence, groups of adjacent cilia are co-ordinated, but the adjacent groups are not, resulting in metachronal waves. The volume of influence of a cilium determines the degree to which it will affect adjacent cilia and the extent of the metachronal waves. Clearly, the spacing of the cilia is a determinant of fluid influence on adjacent cilia; however, Sleight, Blake and Liron argue that the sphere of influence may also depend upon the rate of ciliary beating. When the cilia beat more quickly, the proportion of time that the cilia spend in the resting phase is reduced and greater forces are generated around the cilia. As a result the fluid forces may propagate across some smaller discontinuities of the ciliated surface and the metachronal fields may increase in extent (Sleight, 1988).

Ciliary beat frequency is an important determinant of mucus transport rate. Forty years ago, Hill investigated the movement of carborundum particles across frog oesophagus and rat trachea (Hill, 1957). She described a hyperbolic relationship between mucus transport rates and particle velocity such that there were disproportionately larger decreases in mucus transport rates at lower particle velocities. It was hypothesised that this relationship arose because the transfer of power from the beating cilia to the mucus was most effective within a narrow range of ciliary beat frequencies.

Puchelle and Zahm studied the relationship between the transport rate of sputum from bronchitic patients across mucus-depleted frog palate and the rheological properties of the sputum (Puchelle, 1984). Not surprisingly, the rheological properties of increased viscosity, increased elasticity and reduced spinnability were associated with reduced transport rates; however the most important determinant of mucus transport rate found by step-by-step multiple regression was ciliary beat frequency.

The exact relationship between ciliary beat frequency and the rate of mucus transport is complicated due to the interplay of multiple factors. In a study of 20 patients, Katz could not find a direct relationship between the ciliary beat frequency of human nasal mucosa and tracheal clearance rates using a radioisotope technique. However, in this study the range of mucus transport rates and ciliary beat frequencies in the patients studied was small, limiting the chances of finding any relationship if it should exist (Katz, 1987).

Duchateau investigated the relationship between nasal ciliary beat frequency measured photometrically from biopsies *in vitro* with nasal mucociliary clearance of dye and saccharine *in vivo* in 31 healthy volunteers (Duchateau, 1985). The range of mucus transport rates was twice that of Katz's study and a good

good correlation was found between the logarithm of the mucus clearance rate and the ciliary beat frequency. Hee and Guillerm similarly described a non-linear relationship between ciliary beat frequency and mucus transport rates in sheep (Hee, 1985) such that modest reductions in ciliary beat frequency were associated with large reductions in mucus transport rates (Fig. 2.9). These studies suggest that ciliary beat frequency has an important role in determining the transport rate of mucus.

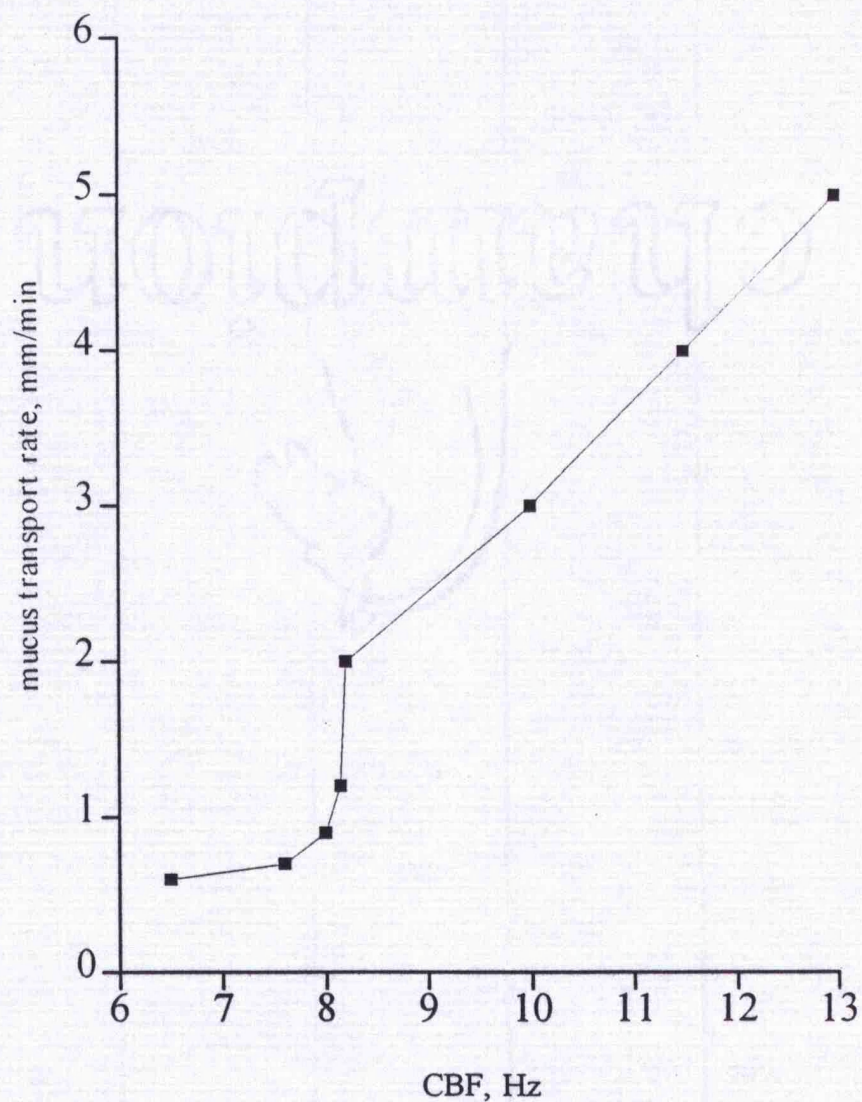


Fig. 2.9 Relationship of ciliary beat frequency to mucus transport
(from Hee, 1985)

Effects of anaesthetic agents on ciliary beat frequency

The depressant effects of anaesthetic agents on cilia were recognised twenty years ago in a study of the changes in the swimming velocity of the ciliated protozoan, *Tetrahymena pyriformis*, exposed to a variety of these drugs (Nunn, 1974).

The first studies of these agents on respiratory cilia was undertaken by Manawadu and colleagues who investigated the effects of halothane on ferret tracheal cilia in a semiquantitative way by noting the presence or absence of ciliary activity at different sites (Manawadu, 1979). Lee and Park investigating respiratory cilia, demonstrated suppression of ciliary beat frequency of rabbit tracheal specimens with halothane and enflurane (Lee, 1980).

Although ciliary structure is similar in different species, ciliary function and in particular, mechanisms of control are not uniform (Lansley, 1992). The use of human, rather than non-human, tissue for studying the effects of pharmacological agents in clinical use is therefore preferable. The only previous work on the effects of anaesthetic agents on human respiratory cilia is a pilot study from our department showing a depression of ciliary beat frequency with halothane *in vitro* (Gyi, 1994).

There is at present a lack of information on the effects of anaesthetic and sedative agents on human respiratory ciliary beat frequency, which is an important determinant of mucociliary clearance, and this forms the subject of this thesis.

Chapter 3

Materials and Methods

1: Measurement of ciliary beat frequency

Introduction

Ciliary movement of mucus in the respiratory tract depends upon the number of ciliated cells, the ciliary frequency of beating, the degree to which the cilia are co-ordinated and the amplitude of the ciliary beat. There are a variety of methods described for the measurement of respiratory ciliary motion, but quantitative methods are restricted to the measurement of only one parameter, the ciliary beat frequency.

In the last chapter evidence was presented that the ciliary beat frequency may be related to other parameters of ciliary function, the amplitude of the ciliary beat and the degree of ciliary co-ordination. Furthermore, the ciliary beat frequency may be an important determinant of mucus transport in view of the logarithmic relationship between the ciliary beat frequency and mucus transport rate. As a result modest changes in the ciliary beat frequency may be associated with much larger changes in the mucus transport rate.

The measurement of human respiratory ciliary beat frequency is restricted to *in vitro* methods at present. The techniques that have previously been described are inappropriate for the investigation of anaesthetic agents which include highly volatile liquids.

We have therefore developed *in vitro* apparatus that has involved modifications of previously described systems to measure ciliary beat frequency of tissue samples exposed to the volatile inhalation anaesthetics and to other drugs. In this chapter the measurement techniques for ciliary beat frequency that have previously been reported are described; and the system we have developed and the experiments undertaken to validate it are presented.

Methods of measurement of ciliary beat frequency previously described

The movement of the mammalian respiratory cilia is too fast for the unaided human eye to accurately observe and quantify. The ciliary beat frequency of this tissue has been measured in a variety of ways which are largely restricted to *in vitro* techniques.

The recording of ciliary motion on film and its replaying at a slower speed has been described for the measurement of ciliary beat frequency (Dalhamn, 1954). Although this photographic method is accurate, it is expensive, laborious and fails to measure the ciliary beat frequency directly as there is no real time analysis.

Other early methods have involved direct observation of the cilia aided by technical devices such as the stroboscope (Gray, 1930) and an auditory clicking device synchronised with visually perceived motion (Bleeker, 1971). Although these are both simple methods they have disadvantages in being prone to human error as they depend upon the perceptual abilities of the observer. It is also difficult to obtain an exact "freeze" of movements due to the metachronicity and phase difference between the different groups of cilia. Furthermore, the movements of the individual cilia themselves are not exactly periodic.

Indirect methods for the measurement of ciliary activity have therefore been developed. Early methods were limited in not being restricted to ciliary beat frequency. The speed of rotation of clusters of ciliated cells (Corssen, 1959) depended not only on the ciliary beat frequency but upon the weight of the cell mass and the presence of mucus and damaged cells in the cell cluster. Dalhamn and Rylander recorded the rhythmic changes of the intensity of the light reflected off the ciliated epithelial surface due to the moving cilia with the use of a photosensitive cell and they found that this corresponded with the ciliary beat frequency (Dalhamn, 1962).

Modifications to this principle of photoelectric conversion with the detection of transmitted rather than reflected light due to the moving cilia (Yager, 1978) have resulted in the transmitted light technique. This has become the most widely used technique for the measurement of ciliary beat frequency.

The advantages of the transmitted light technique are that it is reproducible, convenient and requires minimal subjective assessment; however, it is subject to the effects of extraneous vibrations, cannot detect individual cilia and is limited to an *in vitro* technique.

Laser light scattering spectroscopy is a more recent and sophisticated light technique that is not restricted to *in vitro* use (Wong, 1988). This involves the analysis of fluctuations in the intensity of laser light scattered by the moving cilia. The constructive and destructive interference between the Doppler-shifted back scattered photons from the moving cilia with the non-Doppler-shifted background photons is processed to compute ciliary beat frequency. The laser technique is accurate and suitable for *in vivo* work in animals; however, because of its invasive nature it is unsuitable in non-anaesthetised humans and is also restricted in use by its expense.

The measurement of *human* respiratory ciliary beat frequency is restricted to *in vitro* methods. The transmitted light technique is the most widely used technique for the measurement of ciliary beat frequency and the most reliable method for *in vitro* studies. We have modified this technique to enable us to investigate the effects of the volatile inhalation anaesthetic agents.

Perfusion system for the *in vitro* measurement of ciliary beat frequency during exposure to volatile liquids : an overview

To investigate the effects of drugs on ciliary beat frequency requires a chamber to house the tissue and a perfusion system to deliver the drugs. A variety of chambers and perfusion systems have been described but none is suitable for the investigation of highly volatile anaesthetic liquids and we have therefore developed our own system.

The perfusion system and chambers are designed to deliver the drugs in a controlled and blinded manner. The perfusion system has a controlled flow rate and incorporates a preheating facility and the use of metallic tubing to minimise losses of the volatile liquid anaesthetics. The perfusion chambers are designed to allow a wide plane of movement for the microscope objective to optimise viewing of the cilia but retain a small volume to minimise drug equilibration times. A thermostatically-controlled heating element is attached to the chamber to maintain a constant temperature.

For the measurement of ciliary beat frequency we have utilised the transmitted light technique with some modifications to the optics, light transmission and analysis.

The cilia are imaged by differential interference contrast microscopy mounted on a concrete block to reduce extraneous vibrations that interfere with photometric techniques of measurement. An extra-long working objective is used to provide for optimal detection of ciliary movement throughout the depth and field of view. Using a video camera, the image is transmitted to a high resolution video monitor. A pinhole photodiode is fixed to the monitor screen to detect the interference in light due to the moving cilia. The changing voltage signals from the photodiode are sampled by an analogue-to-digital converter and the data analysed by software to provide a power spectrum using fast Fourier transforms. The peak of the power spectrum is taken to represent the ciliary beat frequency.

Perfusion Chamber

Many chambers have been described in the literature for different microscopic investigations (Braga, 1989); however, none is suitable for the delivery of the highly volatile inhalation anaesthetic agents and few for the optical requirements appropriate to the study of ciliated cells and explants. We have developed a chamber adapted to these requirements (Figs 3.1 and 3.2).

The perfusion chamber is made from aluminium and is constructed in two halves which each accommodate a cover slip. The chamber contains an integral perfusion channel to allow for fluid exchange. The sample of ciliated tissue is placed between a lower fixed coverslip and an upper removable coverslip.

The lower coverslip is irreversibly sealed with acrylic glue. The microscope objective views through the upper coverslip. This upper coverslip has a large diameter of 45 mm providing a wide plane of movement for the microscope objective to optimise viewing of the cilia.

The chamber depth is restricted to 0.85 mm, which is large enough to accommodate the tissue samples but small enough to limit the internal volume of the chamber to 1.1 ml. This minimises the time taken for changes in drug concentration of the delivery bottles to equilibrate with that in the chamber .

A silicone impregnated rubber washer is placed above the upper coverslip and the whole chamber is closed and sealed with an annular threaded screw and a composite locking device. A thermostatically-controlled heating element is attached to the base of the chamber to maintain the temperature of the chamber contents.

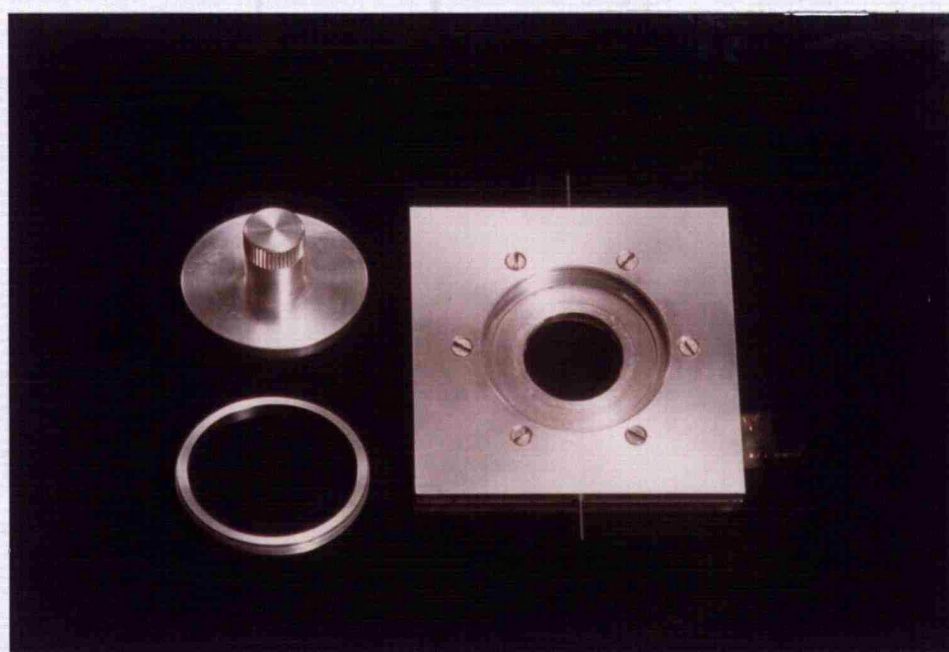


Fig. 3.1 Photograph of perfusion chamber

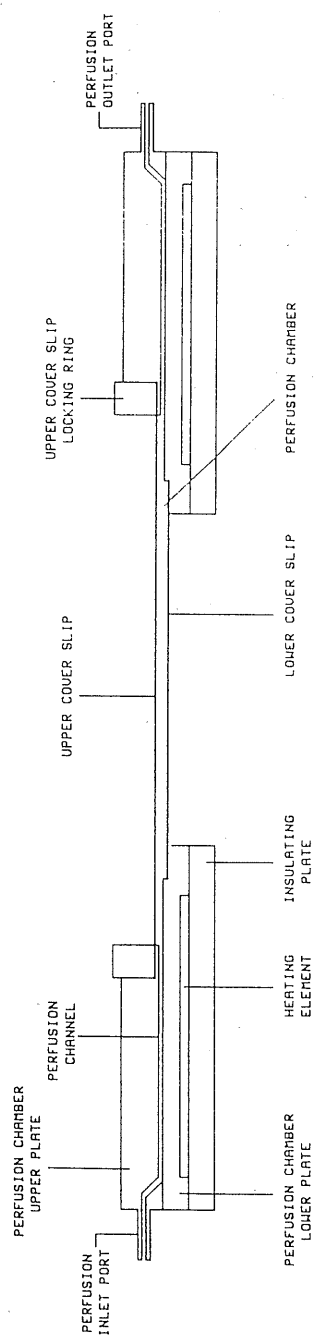


Fig. 3.2 Section through perfusion chamber

Expt 3.1 Calibration of chamber temperature controller

Introduction

Ciliary beat frequency is dependent upon temperature, being faster at higher temperatures (Mercke, 1974; Konietzko, 1981), making this an important parameter to control for in pharmacological experiments.

The small chamber dimensions in our system do not allow for the siting of a temperature sensing device within it. We have incorporated temperature control into our experimental design by the attachment of a thermostatically-controlled heating element to the underside of the perfusion chamber. These incorporate an integrated circuit temperature sensor (LM35CZ, RS components, UK) with a linear voltage output and accuracy of ± 0.1 °C .

The aim of this experiment was to calibrate the temperature controller for estimates of the temperature within the chamber.

Methods

To estimate the temperature within the perfusion chamber at different settings of the heating element, a type K thermocouple (No. 158-626, RS components, UK) with a temperature range 0-200 °C and accuracy of ± 0.1 °C, was glued to the middle of the lower coverslip of a chamber. The modified chamber was perfused at 0.5 ml min^{-1} with Hanks Balanced Salts Solution (HBSS) preheated to 40 °C.

The heating element was set at a particular temperature and the temperature measured by the thermocouple was recorded. After the temperature sensors had stabilised the heating element was reset and the process repeated with the heating

element temperature ranging from 35 to 39 °C. The temperature was measured on three days with different ambient temperatures simulating the range of room temperatures common in the laboratory.

Results

The results are shown in table 3.1. There was mean(SD) difference in temperature between the heating element and temperature of the glass coverslip of 0.3(0.1) °C. The 95 % confidence intervals for the difference in temperature measured by the two devices were 0.3 - 0.4 °C.

Table 3.1 Calibration of chamber heating element, figures in °C

heating element	thermocouple temperature		
	ambient temp 21.2	ambient temp 24.3	ambient temp 29.7
35.0	34.6	34.7	34.8
35.5	35.0	35.1	35.3
36.0	35.7	35.7	35.8
36.5	36.1	36.2	36.2
37.0	36.6	36.6	36.4
37.5	37.2	37.3	37.2
38.0	37.5	37.7	37.7
38.5	37.9	38.1	38.1
39.0	38.6	38.8	38.8

Expt 3.2 Equilibration time and flow characteristics of the perfusion chamber

Introduction

The primary aim of this experiment was to investigate the estimated time it would take for changes in delivered drug concentration to equilibrate within the perfusion chamber.

We were also interested to determine if flow streaming occurred in the chamber and produced regions of differing drug concentration with a ciliated tissue sample in the chamber. A dye and densitometer technique was used to represent a change in the delivered drug concentration.

Methods

The perfusion chamber was mounted on a light box (RS 556-272) and a camera (Pentax, Japan) with a Vivitar 28-135 mm macrozoom lens was mounted at a fixed height of 41.5 mm above the light box.

Two concentrations of Dylon ivory black dye of 0.625 g L^{-1} and 5 g L^{-1} were prepared. The chamber was perfused with the two concentrations of dye in turn flowing at a rate of 0.5 ml min^{-1} .

In order to investigate the wash-in and wash-out characteristics of the chamber, the lower dye concentration initially perfused the chamber and was then switched to the higher dye concentration for five minutes after which perfusion was switched back to the lower concentration for a further five minutes.

Photographs were taken throughout the experiment at 20 second intervals using a shutter speed of 1/15 second with an aperture of f11 onto ISO100 film (Kodak, USA).

The experimental protocol was repeated with a sample of ciliated tissue placed in the chamber.

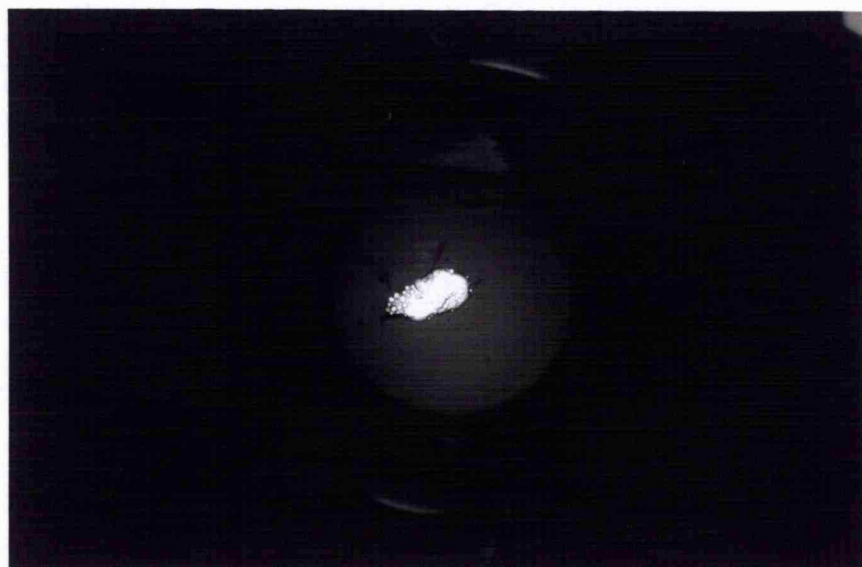
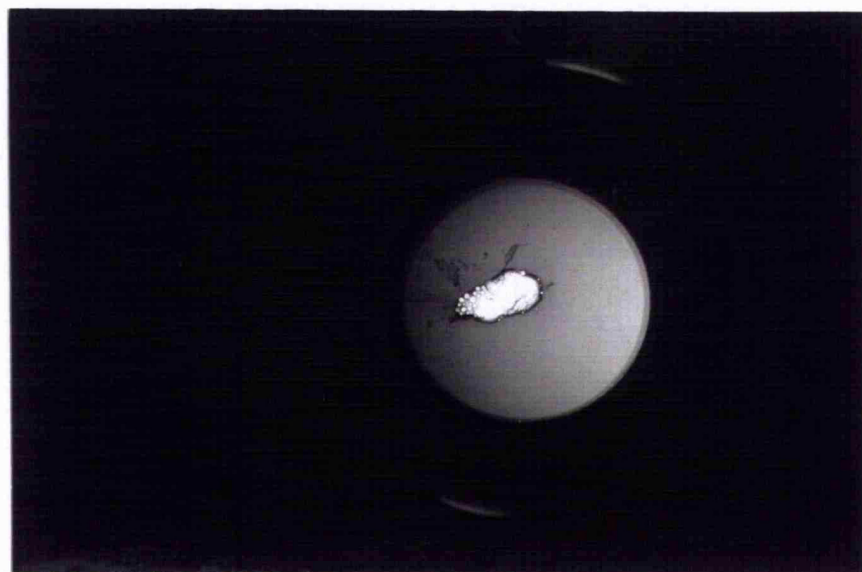
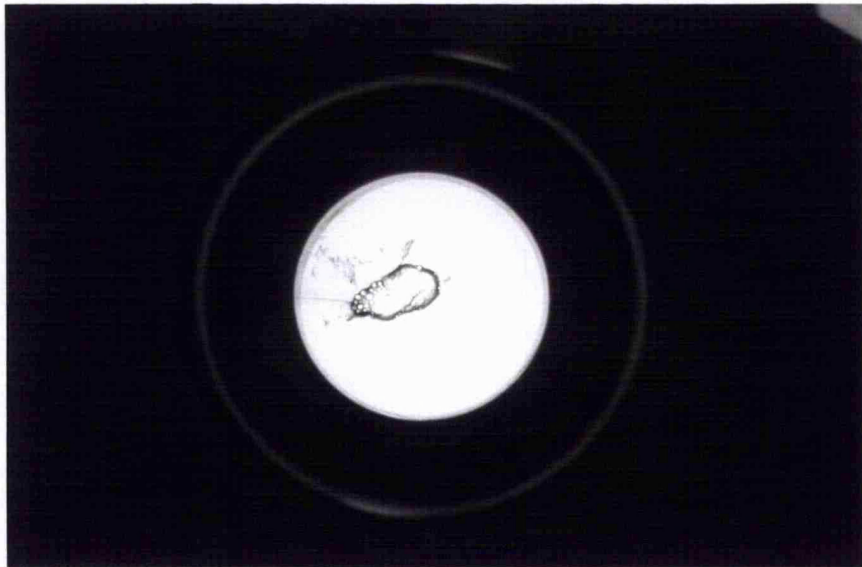
The change in colour as the second dye concentration washed into and out of the chamber was analysed across the chamber photographs. The photographs of the chamber were divided into three parts representing that third nearest the inlet to the chamber, that in the middle and that nearest the outlet.

A densitometer (LKB, Bromma) was used to measure the absorbance of light in each section. This measured thousands of points in each section and computer software provided a median value for the absorbance of light in each of these sections at each time point.

Results

Examples of the photographs are shown in fig. 3.3 With reference to tables 3.2 and 3.3 and figs. 3.4 and 3.5, the change in density of the perfusion dye (median density, arbitrary units) is plotted against time in seconds. The time taken for the change in dye concentration to equilibrate within the chamber was between 180 and 220 seconds with the dye flowing at 0.5 ml min^{-1} .

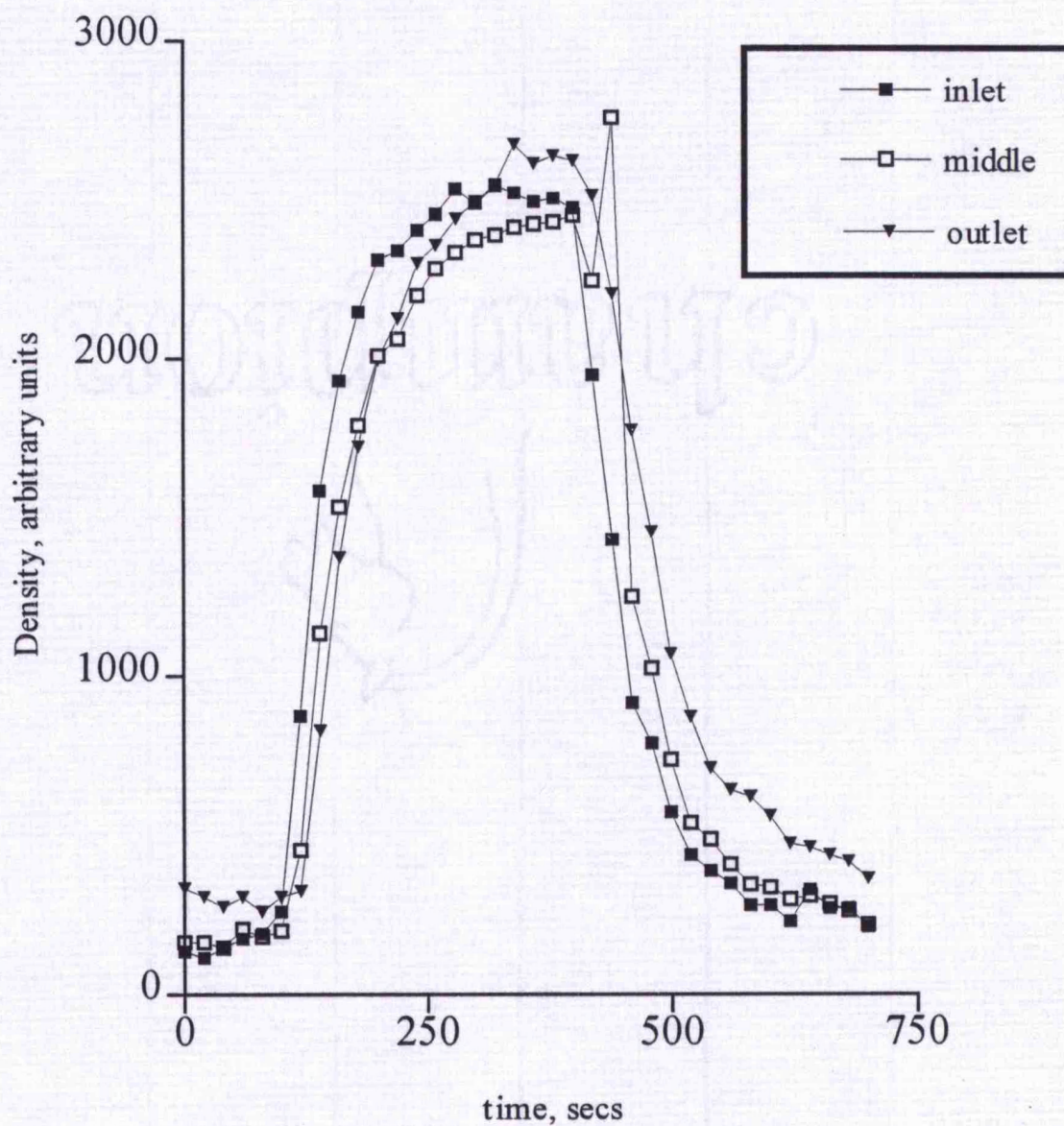
Fig. 3.3 Photographs of the perfusion chamber as dye is washed in



**Table 3.2 Median density(arbitrary units) for inlet, middle and outlet cuts
across the perfusion chamber , no ciliated sample**

<u>time, secs</u>	<u>inlet</u>	<u>middle</u>	<u>outlet</u>
0	135	165	335
20	115	165	307
40	145	147	275
60	175	205	305
80	190	180	260
100	260	200	307
120	875	455	325
140	1585	1135	830
160	1930	1535	1375
180	2147	1790	1722
200	2312	2010	1997
220	2340	2062	2125
240	2405	2200	2300
260	2455	2285	2357
280	2535	2335	2440
300	2490	2375	2495
320	2545	2390	2550
340	2522	2415	2675
360	2495	2425	2615
380	2505	2430	2640
400	2475	2452	2625
420	1950	2247	2515
440	1430	2760	2205
460	915	1250	1775
480	790	1025	1455
500	575	740	1070
520	440	542	872
540	390	490	712
560	350	410	645
580	280	345	625
600	280	337	565
620	230	300	475
640	330	310	465
660	270	285	440
680	270	265	420
700	212	220	365

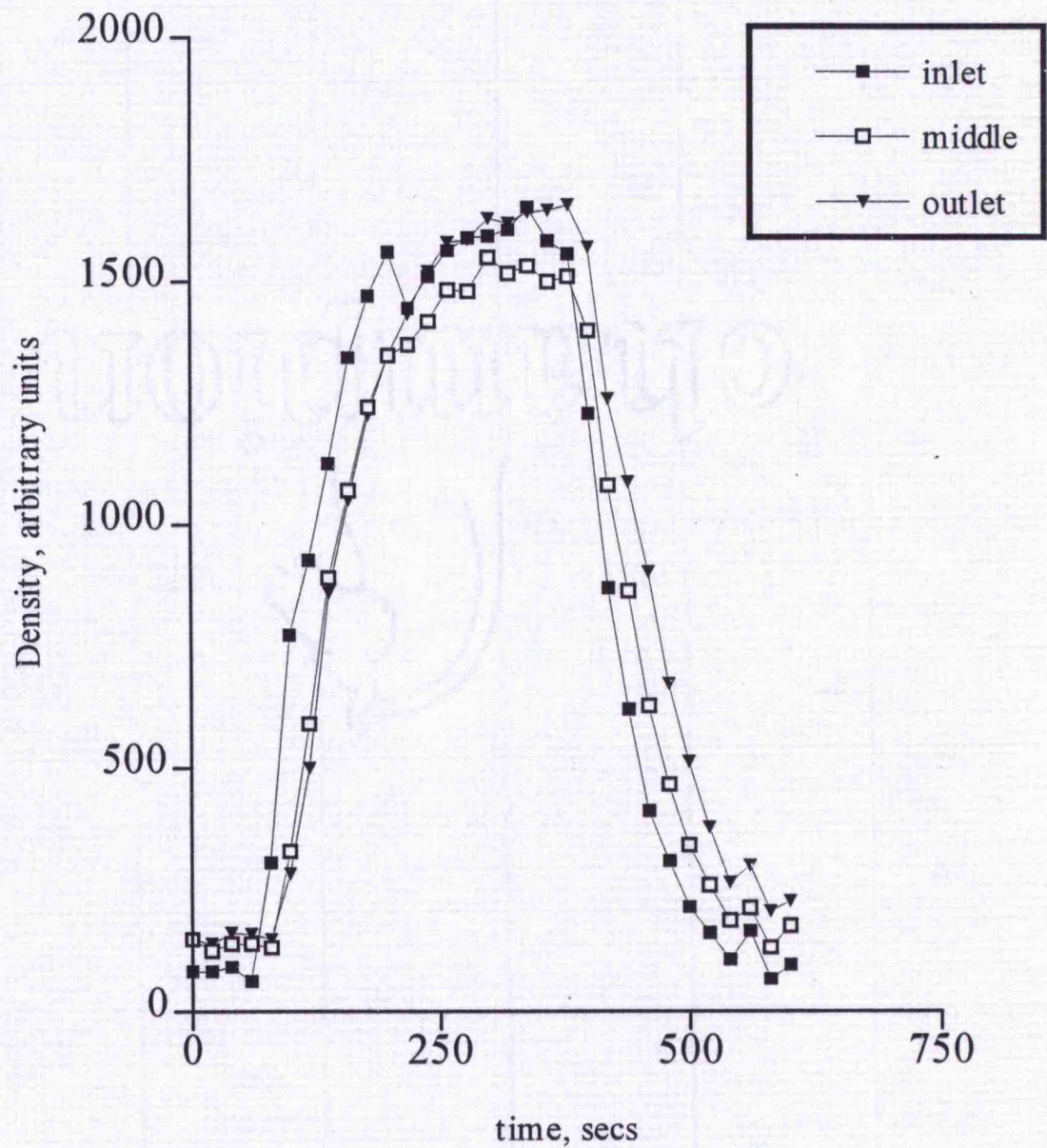
Fig 3.4 Median density (arbitrary units) for inlet, middle and outlet cuts across the perfusion chamber, no ciliated sample present



**Table 3.3 Median density(arbitrary units) for inlet, middle and outlet cuts
across the perfusion chamber , ciliated sample present**

<u>time, secs</u>	<u>inlet</u>	<u>middle</u>	<u>outlet</u>
0	83	150	150
20	83	127	140
40	93	140	163
60	63	140	160
80	307	133	150
100	773	330	283
120	927	590	500
140	1125	890	860
160	1343	1070	1051
180	1470	1240	1223
200	1560	1347	1348
220	1445	1370	1433
240	1510	1417	1520
260	1563	1483	1580
280	1590	1480	1585
300	1593	1550	1630
320	1607	1517	1620
340	1653	1531	1643
360	1583	1500	1647
380	1557	1510	1657
400	1227	1398	1570
420	870	1080	1257
440	620	863	1087
460	413	627	903
480	310	467	673
500	215	343	513
520	163	260	380
540	107	188	265
560	167	213	300
580	67	133	207
600	97	177	227

Fig 3.5 Median density (arbitrary units) for inlet, middle and outlet cuts across the perfusion chamber, ciliated sample present



Perfusion system

The chamber is perfused with a buffered medium because ciliary beat frequency is affected by changes in hydrogen ion concentration (Van de Donk, 1980). The medium used is Hanks Balanced Salts Solution (HBSS), a solution that is free of amino acids. We elected to use an amino acid free solution because of the theoretical possibility of interactions with perfused drugs.

Medical air flows from a gas cylinder via a flowmeter at 1000 ml min^{-1} and passes into the delivery bottles of HBSS via sintered diffusers. A plenum anaesthetic vaporiser can be placed between the flowmeter and the delivery bottle.

To minimise loss of the volatile agents and maintain temperature within the perfusion chamber, the delivery bottles of HBSS are preheated by being immersed in a water bath maintained at 40°C by a thermostat. The heated medium then flows through a countercurrent heat exchanger surrounding the delivery tubing to minimise heat losses.

Metal tubes are used to connect the delivery bottles to the chamber to prevent loss of the inhalation agents by diffusion through plastic tubing. The outlet from the chamber passes to a drainage bottle, the perfusate flowing under the effect of gravity. The flow rate of the perfusate is measured by a photoelectric drop counter incorporated in the entrance to the drainage bottle.

The apparatus is shown schematically in Fig 3.6.

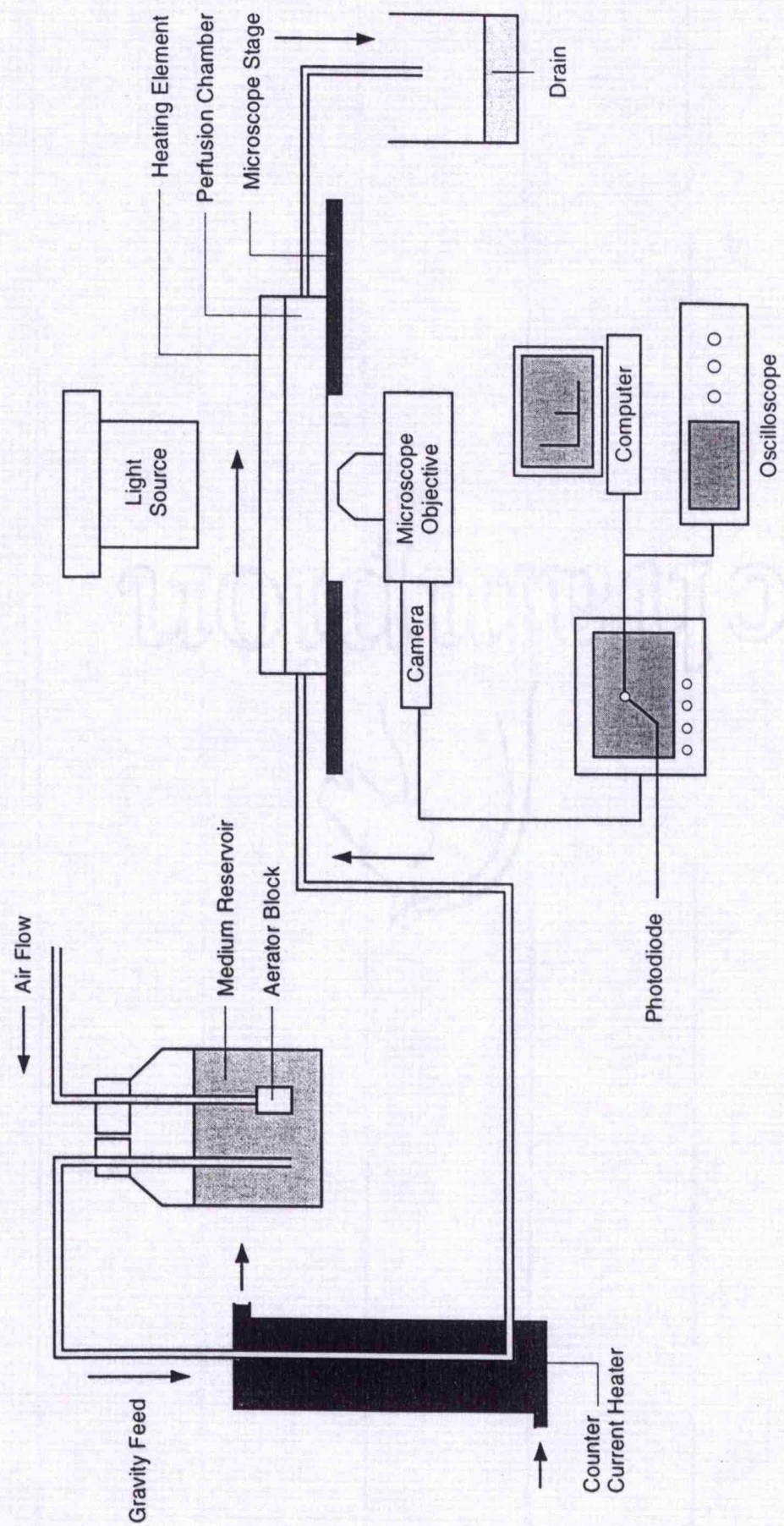


Fig. 3.6 Schematic diagram of perfusion system

Expt 3.3 Control of medium pH and osmolality

Ciliary beat frequency is known to be affected by changes in osmolality and pH (Van de Donk, 1980; Luk, 1983). In this experiment we investigated the range in pH and osmolality that would be expected with the perfusion system.

Methods

The perfusion system was set up with HBSS medium heated to 40 °C flowing at 0.5 ml min⁻¹. Halothane was used as a test inhalation agent and was passed into the delivery bottles at a concentration of 3 MAC, carried by air flowing at 1000 ml min⁻¹. The system was used for five hours without changing the delivery bottle to represent the longest duration of any subsequent experiment. Medium from the delivery bottle was sampled before and after the experiment. The experiment was repeated on five occasions. pH was measured with a pH meter and osmolality was measured by freezing point depression.

Results

The buffering of the medium was sufficient to prevent any change in pH. There was no significant difference in osmolality during the course of the experiment

Table 3.4 pH and osmolality of the medium during perfusion with 3 MAC halothane for five hours, mean(range). (n=5)

	Before perfusion	After perfusion
pH	7.20 (7.18-7.21)	7.20 (7.19-7.21)
osmolality, (mOsm/kg)	280 (270-288)	288 (276-292)

Expt 3.4 Calibration of Perfusate Flow Controller

There is evidence from animal species that the load applied to cilia influences their activity (Sleigh, 1988) and therefore in using perfusion systems it is possible that the rate at which fluid passes the cilia may affect their beat frequency. We have incorporated a photoelectric drip counter to regulate flow through the chambers under the effect of gravity and in this experiment we aimed to calibrate the counter.

Methods

We varied the height of the delivery bottle and recorded the drip counter rate and the weight of the perfusate in the collecting beaker measured with a calibrated electronic balance (Ohaus, CT 200S, NJ, USA) over a given time.

Results

There was a linear relationship between the flow rate and drip count as shown in table 3.5.

Table 3.5 Relation of flow rate to drip count through perfusion system

Drip count, drops/minute	Flow rate, ml/min
16	0.4
19	0.5
22	0.6
25	0.7

Expt 3.5 Delivery of halothane to the perfusion chamber

Introduction

Two physical properties of the inhalation anaesthetic agents, volatility and adhesion to plastics, were considered when designing the system for their delivery to the perfusion chamber. In the early stages in the development of this system, we had found that the use of plastic connecting tubing between the delivery bottles and the chamber resulted in a significant loss of the agent before reaching the chamber. We therefore substituted this for metallic connecting pipes. The aim of this investigation was to determine if we could successfully deliver volatile inhalation agents to the chamber.

Methods

The outputs from three Tec 3 vaporisers for halothane, enflurane and isoflurane with an air flow of 1 L min^{-1} were calibrated using a vapour analyser (Capnomac, Datex).

To confirm the ability of the system to successfully deliver volatile inhalation agents to the chamber, we used halothane as a test volatile agent. We set a calibrated Fluotec 3 vaporiser to 4 % and allowed 15 minutes for equilibration. We sampled the perfusate from the reservoir bottle by aspirating with air-tight syringes and obtained samples downstream of the perfusion chamber by attaching the syringes to a T-piece. The samples were extracted into known quantities of *n*-heptane. These samples were then analysed by gas chromatography (Perkins Elmer 8410) with a DB-17 column, using helium as carrier gas and detection by flame ionisation that had been standardised for halothane (Routledge, 1962).

Results

Measurements from the gas chromatograph indicate that there was no significant loss of the test volatile agent during perfusion through the system (Table 3.6). From the measured aqueous concentrations of halothane in the delivery bottle and downstream of the perfusion chamber, the equivalent concentrations in % atm. were calculated and the estimated delivered concentrations were derived from a standard curve and were equivalent to 3.62 % (95 % C.I. 3.02 to 4.22) for the delivery bottle samples and 4.3 % (95 % C.I. 3.2 to 5.4) for the samples downstream of the perfusion chamber.

Table 3.6 Concentration of halothane, mean(SEM), upstream and downstream of perfusion chamber with vaporiser set to 4 %

	Delivery Bottle (n=4)	Downstream of chamber (n=7)
Aqueous conc (μ M)	785 (56.2)	933.4(120.5)
Equivalent conc (% atm)	2.79 (0.2)	3.32 (0.43)
Estimated delivered conc (%atm)	3.62 (0.3)	4.3 (0.55)

Microscopy

The transmitted light technique for the measurement of ciliary movement depends upon the photoelectric detection of movements of the cilia due to their interference with a light beam. One of the limiting factors in photoelectric detection is the quality of the microscopy image produced. With reflected light or brightfield optics, the amplitude of the photoelectric signal is determined by the optical capacity of the cilia to reflect or absorb the incident light.

An improvement on this, with the signal amplitude greatly enhanced, involves the use of phase contrast microscopy (Teichtahl, 1986). This depends on phase shift of light due to the refractive index or depth of the object and detects transparent objects by the recombination of the phase-shifted light due to the object with the background illumination to produce a high contrast image. The image brightness is also related to the plane of focus and brightness inverts as objects pass through the focal plane. As a result the complicated movements of the cilia during their beating cycle result in variations in phase contrast throughout the cycle. The cilia lie almost parallel to the cell surface in the rest phase and present a minimal optical path, whereas in the active and recovery phases the path difference is increased as the cilia becomes more nearly upright and also move out of the plane of focus. Thus the use of phase contrast optics is beneficial in detecting the movements of the cilia.

Braga used a variant of phase contrast microscopy called differential interference microscopy to good effect (Braga, 1986). We have used this optical method together with the aid of Nomarski prisms and long range objectives using the Diaphot 200 (Nikon, UK). This system produces high quality images and allows the chamber contents to be viewed throughout its depth. The principles of differential interference microscopy as used in this system are described overleaf.

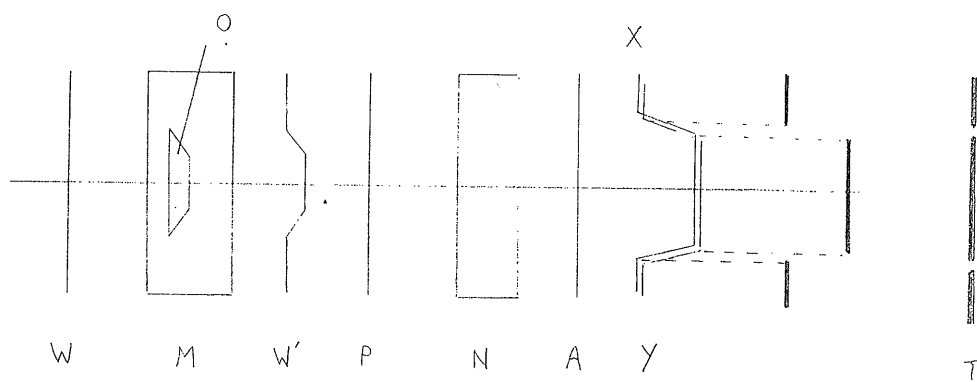


Fig. 3.7 Differential interference contrast microscopy

If we consider a transparent object, O , contained in a transparent medium, M , with a greater refractive index than O , then a flat incident wavefront, W , undergoes a change due to object O to produce wavefront, W' . After passing through a polariser, P , the coherent waves are then split by a birefringent prism, N , into two wavefronts, X and Y , whose shift determines the distance between the flat and angled wavefronts. An analyser produces the visualised image, I , with differing contrast between the flat wavefront and the region of the angled wavefront. In the case of ciliated tissue the differences in refractive index between the cilia and the background produce a change in form of the incident light waveform and the light from the angled wavefront produced by the edges of the cilia is of a different light intensity to the flat wavefront.

The Nikon Diaphot uses Nomarski prisms which are modified birefringent prisms with the optical axis of the crystal tilted to bring the interference fringes to the outside of the prism. As a result, the localised surface of interference fringes is further away from the specimen permitting the use of a longer range objective (Fig. 3.8).

In addition the light passes through a second prism with opposite optical path difference so as to maintain a constant shift for every wavelength and incident angle of the incident light and therefore offers a brighter image increasing the resolving power.

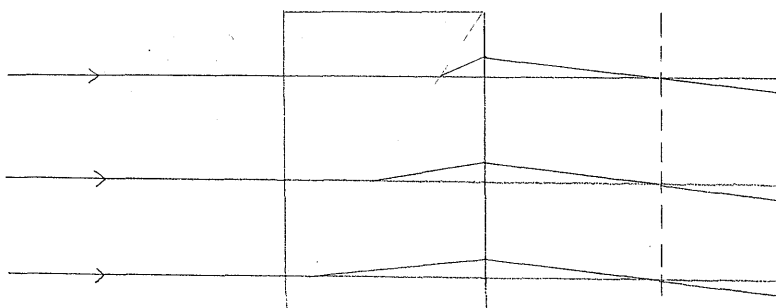


Fig. 3.8 Nomarski prisms.

Photoelectric transduction

Using a 60x objective with a 10x ocular, the image obtained from the microscope is transmitted via a video camera (Panasonic vw-CL 110-AE) to a high resolution video monitor (Sony KX-14CPI). The final magnification of the image obtained on the monitor was calibrated using a human hair. This has a diameter of 0.06mm and measured 18cm on the screen, giving a magnification of 3000x.

A pinhead photodiode with diameter 1 mm^2 is used to detect the changing intensity of the transmitted light. The photodiode was calibrated by driving a sinusoidal wave of frequency 1-100 Hz through a light emitting diode and looking at the output signal. The voltage across the diode is amplified to provide a signal on an oscilloscope greater than 0.2 V and low pass filtered with a cut off above 25 Hz. This is used to reduce interference from artifactual noise due to vibration since human respiratory ciliary beat frequency has always been reported below this frequency. The frequency signal is synthesised from a large number of discrete samples and the signal can be reconstructed from samples as long as the signal is no more than half the sampling rate. This is the Nyquist limit, otherwise there is an ambiguous shift frequency called aliasing. Since the monitoring screen flickers at 50 Hz this filtering prevents the effects of aliasing from signals at or above half the mains frequency.

The pinhead photodiode is fixed to the monitoring screen to eliminate artefacts due to observer movement found with hand held probes. By moving the microscope stage, the ciliated edge is made to appear immediately beneath the photodiode on the screen. The fluctuations in light intensity due to the moving cilia produce changing voltage signals from the photodiode that are displayed on a Gould 20 MHz cathode ray oscilloscope to confirm the detection of ciliary movement.

Ciliary Beat Frequency Analysis

The voltage changes across the photodiode are simultaneously passed via an analogue-to-digital converter to a computer (Dell 386sx) that samples the voltages at a frequency of 200 Hz. The voltage signals are collected over a period of 15 seconds and divided into three sequential five second intervals for analysis. This reduces the resolution to 0.2 Hz but provides three measurements to be averaged. The reduced resolution is still well below the accuracy of the measurement technique and the collection and averaging of three measurements increases the confidence of the calculated value for the ciliary beat frequency.

The data are transferred to an RM Centra V466 computer and analysed by software in Mathematica 2.2 to provide a power spectrum using fast Fourier transforms. The peak of the power spectrum is taken to represent the ciliary beat frequency. Mean frequencies below 2 Hz are filtered out to eliminate artefacts due to sudden movements of the light pen or microscope stage. Spectra with an interquartile range of 10 Hz or more are rejected as the peaks of these are considered to be unrepresentative of ciliary beat frequency.

The measurement of ciliary beat frequency by the transmitted light technique theoretically applies to a single cilium; however, in studying ciliated epithelial cells, multiple cilia interfere with the light path to the photodiode. The magnification and size of the photodiode results in three to five cilia in a given plane affecting the photodiode; however, the ciliated edges are three dimensional and cilia throughout the depth of the field interfere with the light path. To provide an accurate measure of the ciliary beat frequency from multi-ciliated cells it is necessary for the cilia that influence the light path to be co-ordinated. In our experiments we have selected continuous ciliated edges and not single cells or small clumps of cilia from which to measure the ciliary beat frequency.

Expt 3.6 Ciliary beat frequency of nasal brushings measured with the perfusion system

Introduction

The aim of this experiment was to assess the ability to measure ciliary beat frequency using the chamber and perfusion system described above. We used a recognised ciliated tissue sample, obtained from nasal brushings from awake volunteers, and compared the measurements of ciliary beat frequency with that found by other workers. We also assessed the effects of perfusion through the chamber upon the cilia over a period of time by measuring the ciliary beat frequency of the samples over a period of four hours.

Methods

We recruited ten volunteers, (7 male, 3 female, aged 22 - 35 years). All were non-smokers, in good health, on no medications and with no history of an upper respiratory tract infection in the previous four weeks. We obtained samples of human respiratory ciliated cells by the methods first described by Rutland and Cole (Rutland, 1980). This involved passing a bronchoscopy brush over the nasal turbinates of unanaesthetised volunteers to remove ciliated epithelial cells. The brush was then agitated in HBSS to remove the tissue from the brush. The ciliated cells were then pipetted onto the lower coverslip of the perfusion chamber within one hour of removal from the subject. The chamber was sealed, heated to 37 °C and perfused with HBSS flowing at 0.5 ml min⁻¹.

Measurements of ciliary beat frequency were made from acceptable ciliated edges which were deemed to be those that were free of mucus, not part of single cells and at least 60 µm long. The ciliary beat frequency was measured hourly

over a four hour observation period by recording the ciliary beat frequency from a minimum of seven to a maximum of 13 edges within the defined time bands.

The ciliary beat frequency was measured by the modification of the transmitted light technique as described previously. Data were analysed by repeated measures analysis of variance with significance taken at $p < 0.05$.

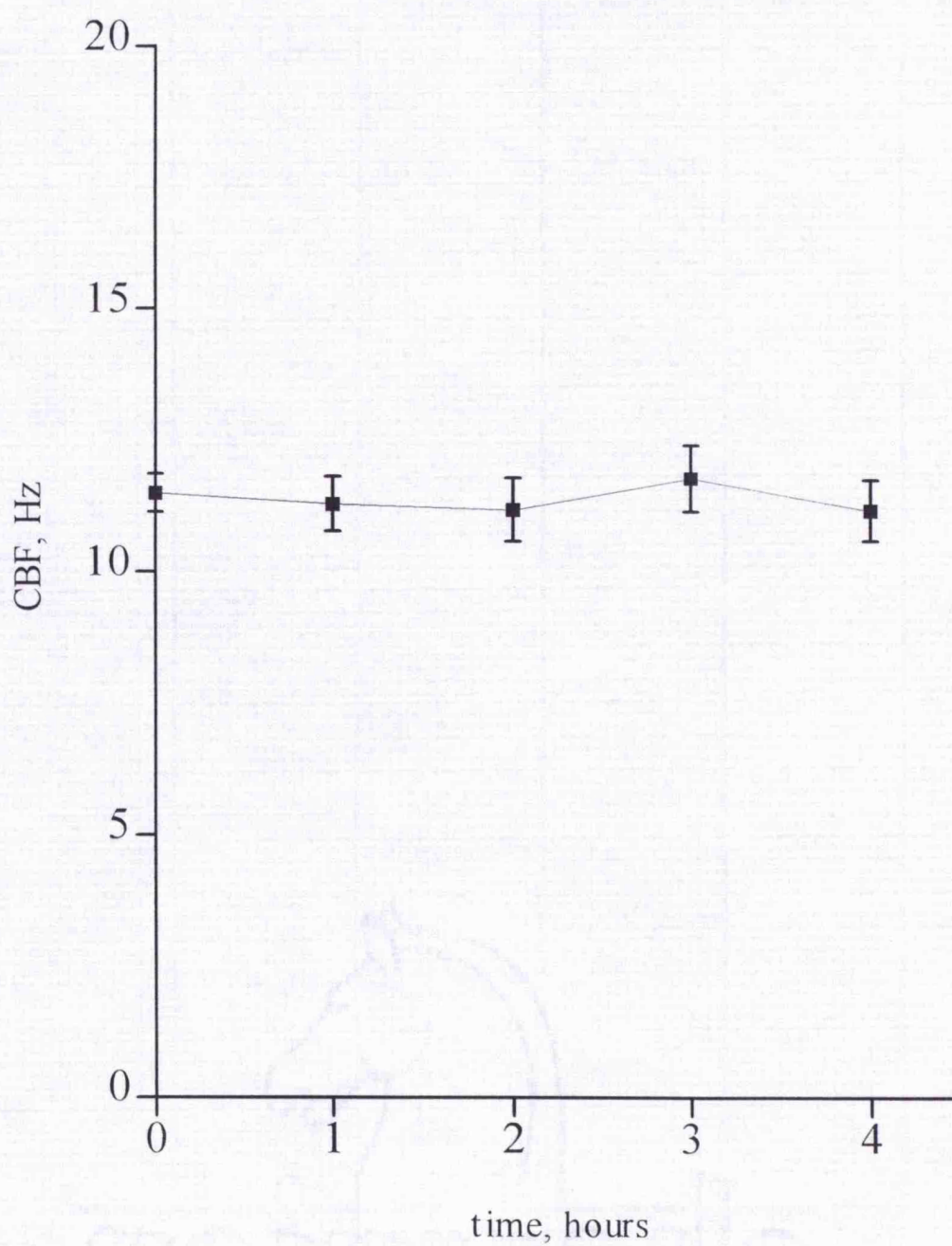
Results

The mean (SEM) ciliary beat frequency of the nasal brushings from ten healthy subjects as measured by this system was 11.4 (1.2) Hz. There was no significant difference in the ciliary beat frequency measured in these ten subjects with this system over a four hour observation period (ANOVA for repeated measures, $p = 0.97$) (Table 3.7, Fig. 3.9).

Table 3.7 Ciliary beat frequency(CBF) of nasal epithelial brushings from unanaesthetised volunteers

time, hours	mean CBF, Hz	SEM CBF, Hz	Ciliated edges measured
0	11.5	0.36	47
1	11.3	0.52	48
2	11.2	0.60	47
3	11.8	0.62	48
4	11.2	0.58	48

Fig 3.9 Ciliary beat frequency (CBF), mean (SEM) from nasal brushings of volunteers measured with perfusion system



Discussion

We have developed a perfusion system for exposing ciliated tissue to drugs including the volatile inhalation anaesthetic agents under controlled conditions. In a series of experiments we have evaluated the control of temperature and the flow characteristics of the chamber, the control of pH and osmolality in the perfusion system and the control of flow rate. We have also investigated the delivery of volatile inhalation anaesthetic agents and finally assessed the ability of the system to measure ciliary beat frequency of nasal brushings.

Temperature control

In calibrating the chamber temperature controller (expt 3.1) we found only a small difference between the temperature of the heating element and the temperature recorded by the thermocouple attached to the glass coverslip of the chamber. Even on the days with lower ambient temperatures when heat loss from the chamber would be greater, the difference in temperature recorded with the heating element set to 37 °C measured only 0.4 °C.

The siting of the thermocouple adjacent to the glass coverslip would be expected to under-read the temperature of the fluid within the chamber whereas the temperature of the heating element would over-read the temperature within the chamber due to heat losses. Therefore the true temperature within the chamber is expected to lie between these two.

For subsequent experimental work we have set the heating element to 37.0 ± 0.1 °C. With the accuracy of the heating element sensor being ± 0.1 °C then the chamber contents would be expected to lie within the range 36.4 -37.2 °C.

Different relationships between ciliary beat frequency and temperature are described in the literature due to use of different tissue preparations. The first study on the effect of graded increases in temperature on ciliary function involved the study of mucociliary wave frequency by the measurement of reflected light on rabbit trachea *in vitro* (Mercke, 1974). A threefold increase in mucociliary wave frequency was found between 20 °C and 40 °C and the relationship between the two parameters was linear. Kennedy and Duckett studied the effects of temperature on rabbit trachea ciliary beat frequency using the transmitted light technique and found that ciliary beat frequency doubled between 28 °C and 37 °C. They found a sigmoid relationship between the two parameters with maximum change at around 32 °C and little change above 37 °C (Kennedy, 1981). In a study on human samples, Konietzko and colleagues found an exponential rise in ciliary beat frequency of tracheobronchial brushings with a threefold increase between 20 °C and 40 °C (Konietzko, 1981). More recently, the effect of temperature on nasal brushings was studied (Green, 1995) and although there was an increase with increasing temperature the change was minimal in the range 32 °C to 40 °C. The consensus from these studies is that within the range 36-37.5 °C there is a variation in ciliary beat frequency of less than 0.5 Hz.

Dye densitometry

Using a dye and densitometer technique and a flow rate of 0.5 ml min⁻¹ we have found that there is equilibration in the chamber with a change in dye concentration within four minutes. There was also no evidence of any flow streaming or areas of diminished perfusion in the chambers. The evaluation of the dye concentration with a ciliated tissue sample in the chamber also showed uniform perfusion. The design of the chamber is satisfactory for exposing the ciliated samples to different drug concentrations.

pH and osmolality

During perfusion with the test inhalation agent halothane at a concentration of 3 MAC for a period of five hours, there was no change in pH.

Other workers have shown that using rat trachea and human bronchial explants, the ciliary beat frequency changed significantly outside the range of pH 7-10, but was unchanged within this range (Van de Donk, 1980; Luk, 1983). The perfusion medium has sufficient buffering capacity for the delivery of the inhalation anaesthetic agents we have studied.

The mean change in osmolality during the course of these experiments was around 3 %.

In previous work, Luk and colleagues found the ciliary beat frequency to be constant between 80 and 200 mmol l⁻¹ of sodium chloride, whereas others have found effects with smaller changes in osmotic pressure. In a study with human nasal biopsies, ciliary beat frequency only remained unchanged between 315 and 335 mOsm l⁻¹ (Luk, 1983). Similarly, with rat trachea, changes of osmotic pressure of only 30 mOsm l⁻¹ were associated with a 10 % change in ciliary beat frequency. The modest changes in osmolality found in this experiment would not be expected to have a significant effect upon ciliary beat frequency.

Flow controller

Previous workers have used peristaltic pumps to control the flow rate perfusing the chamber (Braga, 1986); however, we found that this method led to periodic movements of the tissue samples in the chamber which interfered with measurement of ciliary beat frequency. We elected to perfuse the chambers by a

passive gravity feed system; however, we found that the flow rate at any given height difference between the delivery bottle and collecting beaker varied with different experiments. This was thought to be due to the differences in resistance to flow with the use of different connecting tubing and different chambers.

We therefore built a flow rate detector that measures the number of drops that pass a photodetector sited at the entrance to the collecting beaker, updating the count every minute. This allows the height of the delivery bottle to be varied so as to maintain a constant flow rate.

The determinants of drop size in this experiment are the viscosity of the medium and the flow rate. Since the experiment was performed at constant temperature with the same medium, the viscosity is held constant; and within the narrow range of flow rates measured, a significant difference in drop size would not be expected. Therefore there is a linear relationship between flow rate and drip count through the perfusion system. In subsequent experiments, the difference in height between the delivery and collecting bottles of the perfusion system was altered to maintain a drip count between 18 and 20 drops per minute. This corresponds to a flow rate of $0.5 \pm 0.03 \text{ ml min}^{-1}$.

Delivery of halothane

The calibration data for halothane gas chromatographic analysis indicate a reproducible but significant difference between the delivered and measured concentrations. This is caused by loss of some of the agent during sampling due to its volatility. The concentration of the volatile agent in the delivery bottle was less than downstream of the perfusion chamber and this is due to the greater difficulties achieving an airtight seal when sampling from the bottle. This problem could have been overcome by sampling from the delivery bottle with a

T-piece; however, the vaporiser had been calibrated, and our interest was in the drug concentration that reached the perfusion chamber as measured from the downstream samples.

The derived delivered concentration of halothane downstream of the perfusion chamber with the vaporiser set to 4 % was 4.3 % (95 % C.I. 3.2 to 5.4) and suggests that this system delivers volatile agents to the ciliated tissue in the sample chamber without significant loss.

Ciliary beat frequency of nasal brushings

In this experiment (expt 3.6) the ciliary beat frequency of human nasal ciliated epithelial cells measured *in vitro* with the perfusion system was found to be around 11 Hz. This is in close agreement with that found by previous workers using different apparatus ranging from 11 to 18 Hz (Braga, 1985; Rutland 1982; Teichtahl, 1986). The ciliary beat frequency remained stable in the perfusion chamber over a period of four hours.

Clinically significant differences in ciliary beat frequency are those changes that alter mucus transport rates by a significant amount. As discussed previously (chapter 2), there is a non-linear relationship between ciliary beat frequency and mucus transport rate and it appears that once the ciliary beat frequency is reduced by 20-25 % there is then a disproportionate relationship between these two variables such that modest changes in ciliary beat frequency are associated with larger reductions in mucus transport rate (Hee, 1985). In this thesis it is therefore assumed that reductions in ciliary beat frequency of less than 2 Hz below the baseline value are of limited clinical significance.

There is a variability of measurement with this system that has a standard error of 10 %. This experiment has a posthoc power of 80 % at the 5 % level of significance to detect a difference greater than 1.5 Hz . This system appears to provide a reliable and reproducible measure of ciliary beat frequency.

Chapter 4

Materials and Methods

2: Human ciliated respiratory tissue samples

Introduction

Although ciliary structure is similar in different species, ciliary function and in particular, mechanisms of control are not uniform (Lansley, 1992) and the use of human, rather than non-human, tissue for studying the effects of pharmacological agents in clinical use is therefore preferable.

Measurements of human ciliary beat frequency are restricted to *in vitro* experiments. Tissue samples are mainly obtained from cytological specimens obtained by brushing the respiratory tract and either bronchial or nasal brushings may be used (Rutland, 1980; Low, 1984).

Nasal brushings have the advantage of being minimally invasive and may be collected without local anaesthesia. We found that this method of sampling presented problems due to the discomfort associated with the procedure and even when local anaesthetic was used we had some difficulties recruiting volunteers. We have developed an alternative technique that overcomes this by sampling after the induction of anaesthesia with propofol.

Despite the advantages of this technique due to the improvements in compliance, it still presented limitations. In particular these cytological samples are only suitable for use on the day of sampling which prevents longer term *in vitro* investigations.

We have developed a second method of sample preparation involving the use of whole nasal turbinates that are excised during surgery. From these specimens we have been able to provide ciliated specimens that survive *in vitro* for a longer period. These methods of sample collection and preparation and the experiments undertaken to validate these tissues for measurements of ciliary beat frequency are described.

Cytological specimens of human respiratory cilia

The use of cytological specimens from tissue brushings of the respiratory tract has become the commonest method of providing human respiratory ciliated samples. A useful technical advance was made by Rutland and Cole (Rutland, 1980) who showed that samples taken from the ciliated epithelium overlying the inferior nasal turbinates provided useful specimens in a less invasive fashion than that required for those taken from further down the respiratory tract. Furthermore they were able to obtain these specimens without the need for local anaesthesia which itself may affect ciliary function (Ingels, 1994). In a later study, it was demonstrated that the ciliary beat frequency from these nasal brushings was found to be the same as that from samples further down the respiratory tract (Low, 1984) and therefore nasal samples were likely to be representative of cilia from more distal parts of the lungs.

Although we have used nasal brushings from awake volunteers as described by Rutland and Cole to validate our system (expt 3.6), it became apparent to us that this method of sampling had limitations. Although it is feasible to obtain these samples from volunteers, it is not without an unpleasant sensation and not surprisingly the recruitment of such volunteers began to fall off.

We evaluated nasal brushings removed from anaesthetised patients who had received a bolus dose of the intravenous anaesthetic, propofol, as a means of eliminating the unpleasantness of the procedure.

Expt 4.1 Nasal ciliary beat frequency measured *in vitro* after an *in vivo* bolus of propofol

Introduction

The aim of this study was to evaluate the use of nasal brushings for measurements of ciliary beat frequency obtained from anaesthetised patients. We investigated whether a bolus dose of propofol used to induce anaesthesia administered *in vivo* caused an alteration in ciliary beat frequency measured *in vitro* at one and two hours after its administration.

Methods

Six healthy non-smokers (three male, aged 22-40 years) scheduled for dental surgery under general anaesthesia were recruited into this study. A sample of ciliated nasal epithelial cells was obtained by passing a bronchoscopy brush over the inferior turbinates without any drugs being administered as described by Rutland and Cole (Rutland, 1980). The sample was then removed from the brush by agitating in HBSS. After the induction of anaesthesia with 2-3.3 mg kg⁻¹ of propofol, a second sample of ciliated epithelium was taken from the same patient in the same manner and placed in a separate bottle of HBSS.

The samples from any one patient were then loaded in paired perfusion chambers as described previously in chapter 3. Each chamber was perfused from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. One chamber contained the samples obtained in the awake state and the other containing the samples obtained after anaesthetic induction with propofol. The chambers were perfused with aerated HBSS flowing at 0.5 ml min⁻¹ and maintained at 36.8±0.4 °C (Fig. 3.6).

Ciliary beat frequency was measured using differential interference contrast microscopy and the transmitted light technique as described in detail in chapter 3.

Measurements commenced one hour after removal of the samples and were repeated a further one hour later. As many readings as possible were taken within the defined time bands. As a result between ten and 13 readings of ciliary beat frequency were taken from each chamber at each time point. Acceptable ciliated edges for measurement were defined as those free of mucus and at least 60 μm long. Measurements were not taken from individual cells or disrupted edges.

As detailed in chapter 3, the frequency of voltage changes elicited at the photodiode was converted to an analogue signal and transformed by fast Fourier analysis. The mean of the peak ciliary beat frequencies from the power spectra was computed for each sample at each time point. The data were analysed by multiple analysis of variance for repeated measures. Significance was taken at $p < 0.05$.

Results

The mean(SD) ciliary beat frequency before administration of propofol was 11.4 (2.3) Hz and between one and two hours after the administration of 2-3.3 mg kg^{-1} of propofol was 11.8 (2.0) Hz. This difference was not statistically significant (MANOVA for repeated measures, $p=0.70$).

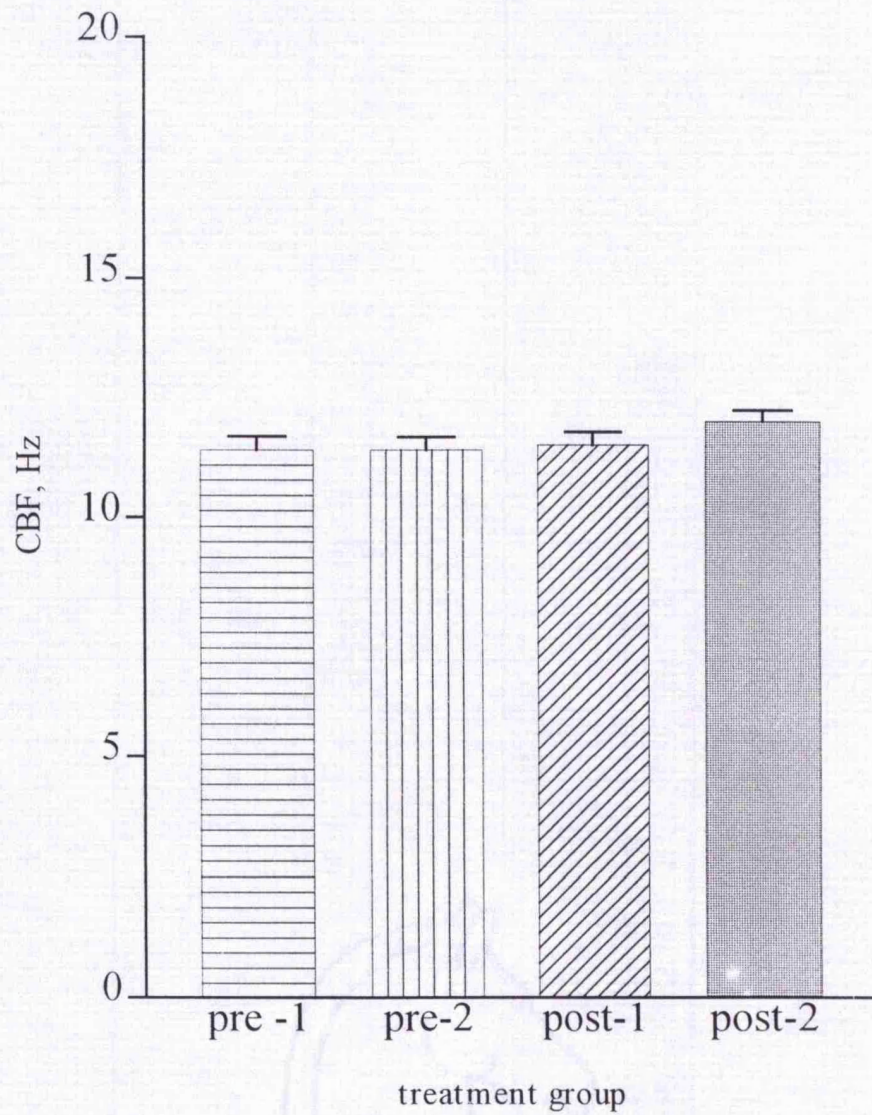
The mean(SD) ciliary beat frequencies at one and two hours after the administration of propofol were 11.5 (2.2) Hz and 12.0 (1.9) Hz respectively. This difference was not statistically significant (MANOVA for repeated measures, $p=0.47$). (Table 4.1; Fig 4.1).

Thus measurements of ciliary beat frequency taken at both one and two hours after the administration of 2-3.3 mg kg⁻¹ of propofol were not significantly different from those taken from the same subjects before administration of the drug. It appears that an induction dose of propofol has no effect on ciliary beat frequency measured one and two hours later *in vitro*.

Table 4.1. Comparison of ciliary beat frequency(CBF) measured before and after propofol anaesthetic induction

treatment	time point	CBF, Hz	SEM, Hz	ciliated edges measured
pre-propofol	1 hour	11.4	0.29	63
	2 hours	11.4	0.27	65
post-propofol	1 hour	11.5	0.28	61
	2 hours	12.0	0.23	66

Fig 4.1 Mean (SEM) ciliary beat frequency (CBF) measured pre- and post- propofol anaesthetic induction



1 and 2 refer to measurements made at one and two hours after removal of tissue from the patient

Nasal turbinate explants

Despite the improvements with the methods of sample collection following the administration of propofol a number of problems remained. The samples consist of exfoliated cells that are prone to sample movement which interferes with the measurement of ciliary beat frequency with the transmitted light technique (Yager, 1978); the tissue has to be fresh and sampled on the day of the experiment with obvious time constraints; and the quality of the tissue is variable and often in our experience does not provide satisfactory ciliated edges from which to measure ciliary beat frequency. We therefore developed a second method of ciliated sample preparation derived from nasal turbinates excised at surgical turbinectomy.

In view of the problems of requiring daily samples of ciliated cells from human subjects we began to investigate the possibility of culturing ciliated cells. We collected human nasal turbinates removed at surgical turbinectomy from which other workers have managed to produce a secondary culture and which provides a supply of such cells for about three weeks (Devalia, 1990). However, we noted that sections from the whole turbinates had long continuous edges of functioning cilia suitable for the measurement of ciliary beat frequency within our chambers and that these remained beating in culture medium for more than a week. We therefore abandoned attempts at tissue culture and explored the use of turbinate explants for ciliary beat frequency measurements.

Historically, samples of respiratory ciliated epithelium were provided by biopsy of the inferior nasal turbinates and used to diagnose such conditions as cystic fibrosis; however, such techniques fell into disfavour because they were performed under local anaesthesia with its attendant ciliotoxic effects and there

was a risk of haemorrhage with this procedure. Nevertheless we considered that biopsies from the inferior nasal turbinates had advantages over nasal brushings.

In addition to solving the need for a ready source of cilia in the laboratory and with a tissue less prone to sample movement in a perfusion system, this tissue provides a better model of *in vivo* ciliary behaviour as more of the adjacent tissue is retained. This source of tissue could also be maintained in the laboratory and therefore used to investigate the *in vitro* effects of drugs administered continuously over a period of days as is common with ITU sedatives.

The use of explants from excised turbinates overcomes the problems of haemorrhage from turbinate biopsies referred to earlier and the ability to store the tissue for use more than 24 hours after excision overcomes the short-lived effects of topical vasoconstrictors and local anaesthetics on ciliary function (Mason, 1995).

Clearly, turbinates that are surgically excised are abnormal and the possibility that their cilia may also be abnormal was considered. We therefore restricted ourselves to the use of tissue from turbinates removed from patients with a diagnosis of tissue hypertrophy causing nasal obstruction without any history of allergy. Previous workers have demonstrated that the mucus transport rate of such turbinates is normal implying normal mucociliary function (Mason, 1996).

Preparation of turbinate explants

The inferior nasal turbinates from patients with a primary diagnosis of turbinate hypertrophy scheduled for elective surgical turbinectomy were collected in medium 199 (M199). After thoroughly rinsing the turbinates in the medium to remove as much blood and mucus as possible, discs of the surface ciliated epithelium were removed using a 4 mm biopsy punch (Stiefel Laboratories, Wooburn Green, U.K.). The specimens were rinsed in a mixture made from 100 ml of M199 and 2 ml of antibiotic/antimycotic solution containing 10 000 U penicillin, 10 mg streptomycin and $23\mu\text{g ml}^{-1}$ of amphotericin B.

We placed the specimens in 10 ml of culture medium made from 100 ml of M199, 10 ml of fetal calf serum, 2 ml of the antibiotic/antimycotic solution and 1 ml of mixed additives containing insulin $250\mu\text{g ml}^{-1}$, transferrine $250\mu\text{g ml}^{-1}$ and hydrocortisone $36\mu\text{g ml}^{-1}$.

The specimens were then incubated at 37°C with occasional shaking for one hour to separate the mucus. The specimens were then transferred into multidishes with 3 ml of culture medium per well and incubated at 37°C in 5 % CO_2 in air. The medium was changed after 24 hours and every 3-4 days thereafter.

Fig 4.2 Photograph of ciliated edge of nasal turbinate explant



Expt 4.2 Ciliary beat frequency of turbinate explants over 10 days in a controlled environment

To investigate the survival characteristics of the cilia from the turbinate preparations over a period of time we initially measured the ciliary beat frequency from the samples over a period of ten days during which they were kept in a controlled environment in an incubator.

Methods

Nasal turbinate explants were obtained from 11 patients with a mean age of 28.3 years (range 21-56), all were non-smokers, not receiving regular medications, and were anaesthetised with propofol for induction, morphine or fentanyl for analgesia and isoflurane for anaesthetic maintenance; three were paralysed with suxamethonium or atracurium. All received 200 mg of cocaine and 1 mg of adrenaline applied topically to the nasal cavity before surgical excision.

We measured the cilia survival characteristics over a period of ten days from samples taken from the eleven patients by measuring the ciliary beat frequency one and two hours after removal from the incubator. Samples from any six of the patients were measured one, three, six, eight and ten days after surgical excision from the patient. We placed a sample in the perfusion chamber as described in chapter 3. The chamber was maintained at 36.8 ± 0.4 °C and perfused with aerated HBSS at 0.5 ml min^{-1} . Ciliary beat frequency was measured using differential interference contrast microscopy and the transmitted light technique as detailed in chapter 3. Ciliary beat frequency was measured from between ten and twenty edges from each turbinate on each of the days. Acceptable ciliated edges for measurement were defined as those free of mucus and at least 60 μm long.

Measurements were not taken from disrupted edges. As detailed in chapter 3, the frequency of voltage changes elicited at the photodiode was converted to an analogue signal and transformed by fast Fourier analysis. The mean peaks of the power spectra were obtained for each tissue sample on each day of measurement and the nested data were analysed by repeated measures analysis of variance. Significance was taken at $p < 0.05$.

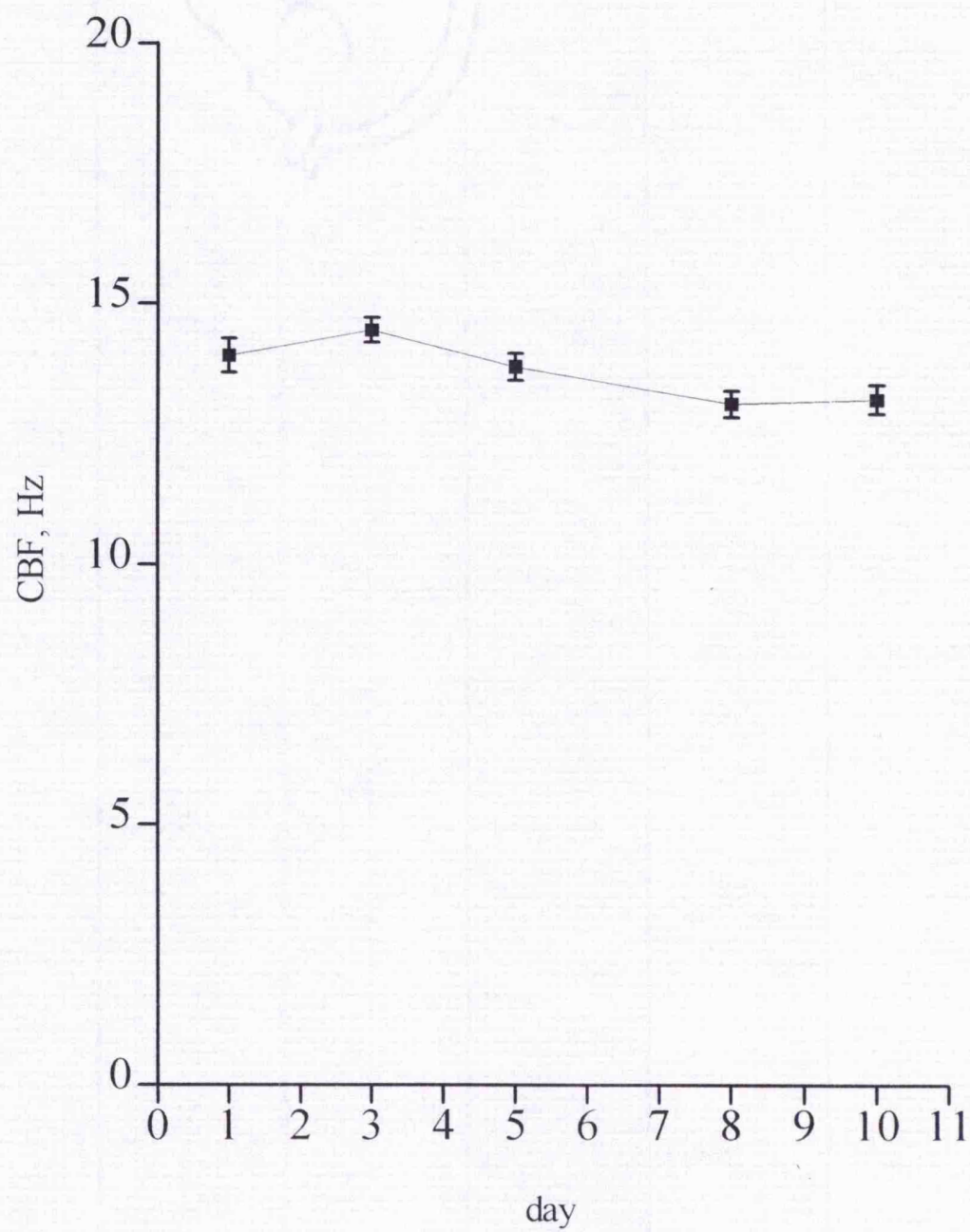
Results

The ciliary beat frequency of the turbinate samples did not change significantly over the ten day observation period whilst maintained in the incubator with a mean(SD) ciliary beat frequency ranging from a minimum of 13.1(2.8) Hz to a maximum of 14.4(2.4) Hz. (ANOVA for repeated measures, $p = 0.168$) (Table 4.2 and Fig. 4.3)

Table 4.2. Ciliary beat frequency(CBF) of turbinate explants over 10 days

day	mean CBF,Hz	SEM, Hz	Ciliated edges measured
1	14.0	0.33	109
3	14.5	0.24	99
5	13.8	0.26	123
8	13.1	0.26	112
10	13.2	0.28	97
all days	13.7	0.13	540

Fig 4.3 Ciliary beat frequency (CBF) of nasal turbinate explants over ten days, mean (SEM)



Expt 4.3 Ciliary beat frequency of turbinate explants during six hours in a perfusion chamber

To further evaluate the survival characteristics of the explants from the turbinates we measured the ciliary beat frequency of samples perfused in the chamber for a period of six hours to determine their behaviour under these conditions.

Methods

We measured the cilia survival characteristics of tissue samples from the same 11 patients as in the previous experiment whilst perfused in the controlled perfusion chamber. The chamber was perfused with aerated HBSS flowing at 0.5 ml min^{-1} and maintained at $36.8 \pm 0.4 \text{ }^{\circ}\text{C}$. The ciliary beat frequency was measured from the explants using differential interference contrast microscopy and the transmitted light technique as described in detail in chapter 3. Measurements were taken from between eight and ten ciliated edges hourly over a six hour observation period.

The analysis of the measurements of ciliary beat frequency is detailed in chapter 3. The frequency of voltage changes elicited at the photodiode was converted to an analogue signal and transformed by fast Fourier analysis. The peaks of the power spectra were meaned for each tissue sample at each time point of measurement and the nested data were analysed by repeated measures analysis of variance. Significance was taken at $p < 0.05$.

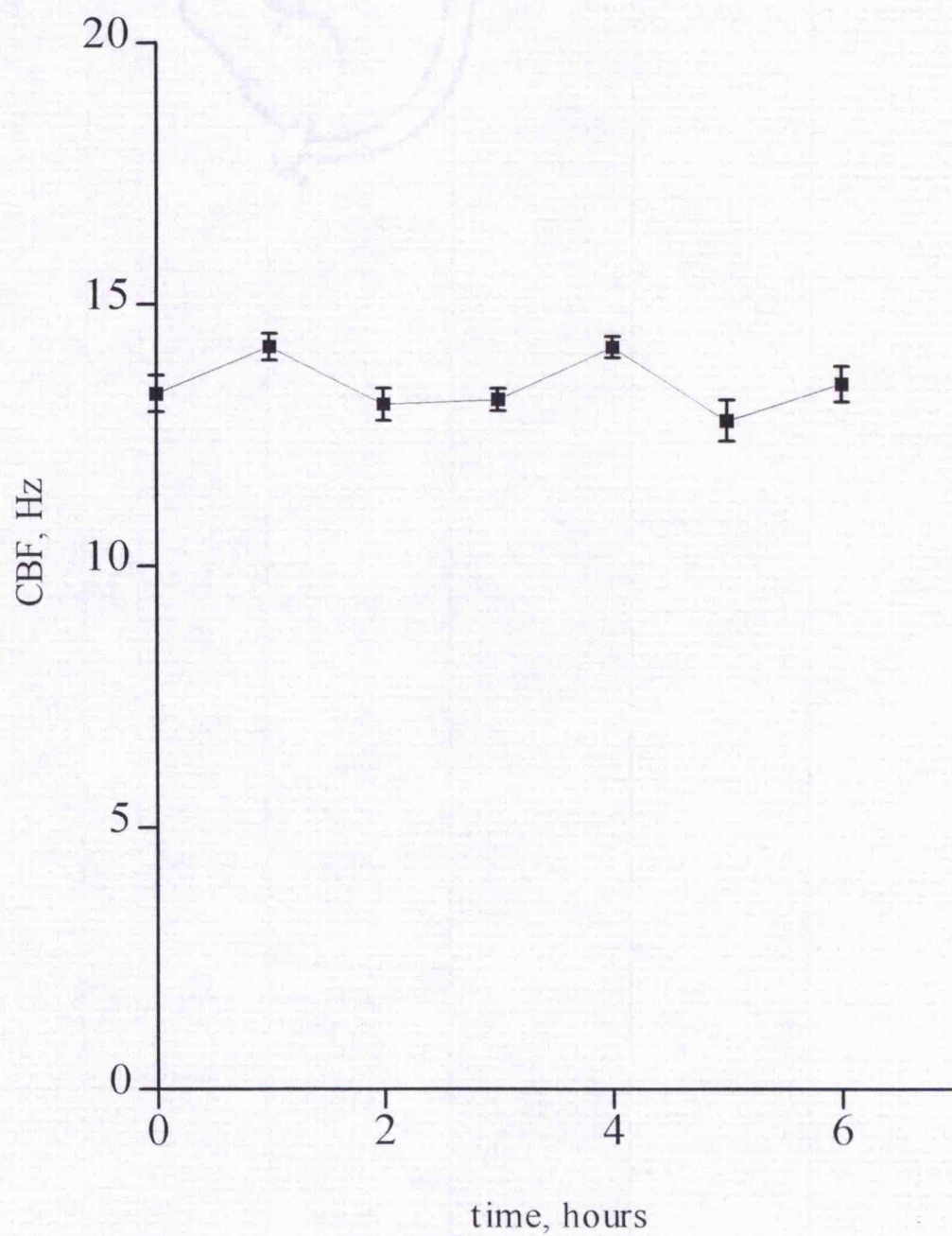
Results

The ciliary beat frequency of the turbinate samples did not change significantly over the six hour observation period in the perfusion chamber with a mean(SD) ciliary beat frequency ranging from a minimum of 13.1(2.6) Hz to a maximum of 14.2(2.1) Hz (ANOVA for repeated measures, $p=0.185$) (Table 4.3, Fig 4.4) .

Table 4.3 Ciliary beat frequency(CBF) of turbinate explants over six hours in the perfusion chamber

time, hrs	mean CBF,Hz	SEM, Hz	No. of readings
0	13.3	0.35	77
1	14.2	0.25	71
2	13.1	0.31	72
3	13.2	0.21	72
4	14.2	0.20	70
5	12.8	0.40	72
6	13.5	0.34	71

**Fig 4.4 Ciliary beat frequency (CBF) of
turbinate explants over six hours in the
perfusion chamber, mean (SEM)**



Response of turbinate explants to known cilioactive agents

The cilia of the nasal turbinate explants have a beat frequency within the normal range and tests of the mucus transport rate of hypertrophied nasal turbinates are also normal (Mason, 1995); however, this does not demonstrate that these cilia respond to pharmacological agents in the same way as the cilia from normal tissue in other parts of the respiratory tract. To determine further the suitability of this tissue preparation for studying pharmacological effects on ciliary beat frequency, we investigated whether known ciliodepressant and ciliostimulatory agents produced detectable changes in the ciliary beat frequency measured from the nasal turbinate explants.

Expt 4.4 Effects of forskolin on ciliary beat frequency of turbinate explants

The aim of this experiment was to determine if pharmacological effects known to increase ciliary beat frequency on cytological specimens increased the ciliary beat frequency measured from the turbinate explants. Ciliary beat frequency has been shown to be increased by a variety of agents that increase intracellular cAMP (Ingels, 1992; Lansley, 1992). We have therefore used the adenylyl cyclase activator, forskolin, as an example of a ciliostimulant.

Methods

Explants of ciliated epithelium from the human nasal turbinates of six healthy patients who had undergone nasal turbinectomy for a primary diagnosis of tissue hypertrophy were prepared as described previously. The explants were placed in paired perfusion chambers as described in chapter 3. Each chamber was perfused

from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. The chambers were perfused with aerated HBSS flowing at 0.5 ml min^{-1} and maintained at $36.8 \pm 0.4 \text{ }^{\circ}\text{C}$.

After 30 minutes the ciliary beat frequency was measured from between six and 10 ciliated edges by differential interference contrast microscopy and the transmitted light technique as described previously in chapter 3. Forskolin was added to one delivery bottle at a concentration of $100 \text{ }\mu\text{M}$, the observer being unaware as to which chamber received the drug and 30 minutes later the ciliary beat frequency was again measured from both chambers.

The analysis of the ciliary beat frequency is detailed in chapter 3. Briefly, the peaks of the power spectra obtained by fast Fourier transformation of the digitised voltages at the photodiode were meaned for each tissue sample at each time point and the nested data were analysed by repeated measures multiple analysis of variance with significance taken at $p < 0.05$.

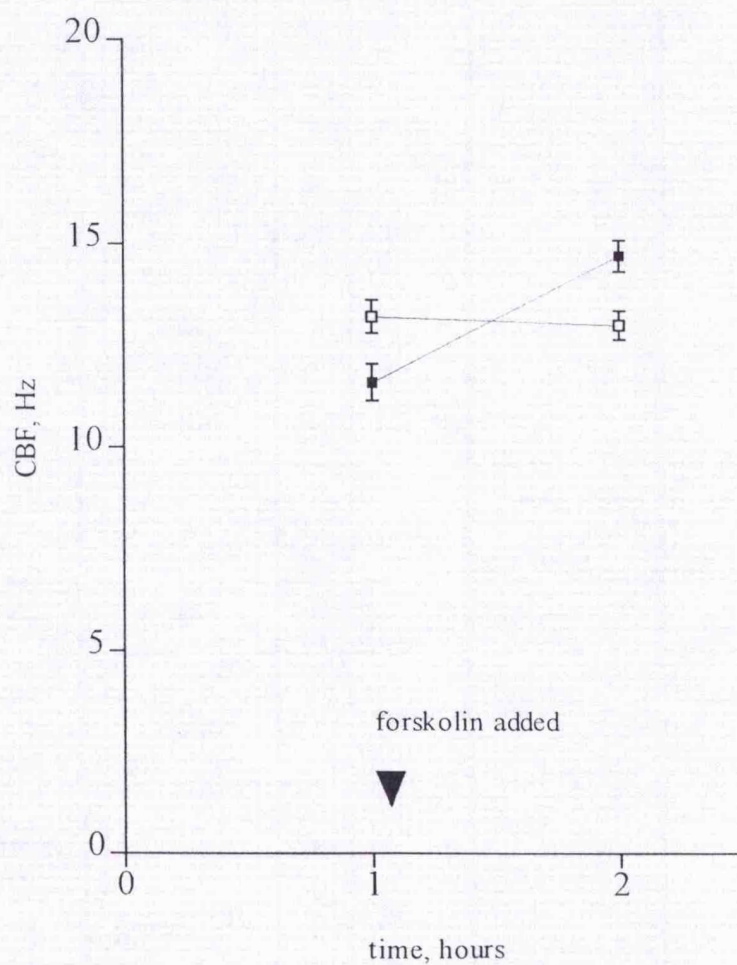
Results

The mean(SD) ciliary beat frequency of the samples exposed to forskolin was $14.7 (2.9) \text{ Hz}$ and that of the controls was $13.0 (2.7) \text{ Hz}$. This difference was statistically significant ($F=10.86$, $p=0.008$, MANOVA for repeated measures) (Table 4.4; Fig 4.5).

Table 4.4. Effects of 100 μ M forskolin on ciliary beat frequency of turbinate explants

Treatment	Time, hrs	CBF(SEM), Hz	No. of ciliated edges measured
control	1	13.2(0.41)	60
control	2	13.0(0.35)	59
pre-forskolin	1	11.6(0.45)	58
forskolin	2	14.7(0.39)	55

Fig. 4.5 Effects of 100 μ M forskolin on mean(SEM)ciliary beat frequency(CBF) of turbinate explants; forskolin ■, control □.



Expt 4.5 Effects of phenol on ciliary beat frequency of nasal turbinate explants

Background

Tobacco smoke contains a number of chemicals that have been shown to reduce ciliary activity *in vitro* using various ciliated specimens (Hee, 1985). Phenol is one of these cilio-toxins in cigarette smoke. It possesses a relatively low volatility which permits more reliable delivery of the agent to the tissue. We have investigated the effects of 40 ppm of phenol on the *in vitro* ciliary beat frequency of human nasal turbinate explants to determine the behaviour of this tissue preparation.

Methods

Turbinate explants were prepared as previously described from six male patients aged 18-47 years (mean 34.7) who had undergone elective surgical turbinectomy for a diagnosis of turbinate hypertrophy.

The samples were placed in paired perfusion chambers as described previously in chapter 3. Each chamber was perfused from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. The chambers were perfused with aerated HBSS flowing at 0.5 ml min^{-1} and maintained at $36.8 \pm 0.4^\circ \text{C}$ for a period of one hour to maintain stability. Then to one chamber was added phenol to provide a concentration of 40 ppm.

Ciliary beat frequency was measured by differential interference contrast microscopy and the transmitted light technique using the methods described in chapter 3.

The observer was blinded as to which chamber received the phenol. The ciliary beat frequency from between 10 and 12 ciliated edges was measured from each specimen.

The analysis of the ciliary beat frequency is detailed in chapter 3. The mean peaks of the power spectra were obtained for each tissue sample at each time point and the nested data were analysed by unpaired t-test with significance taken at $p < 0.05$.

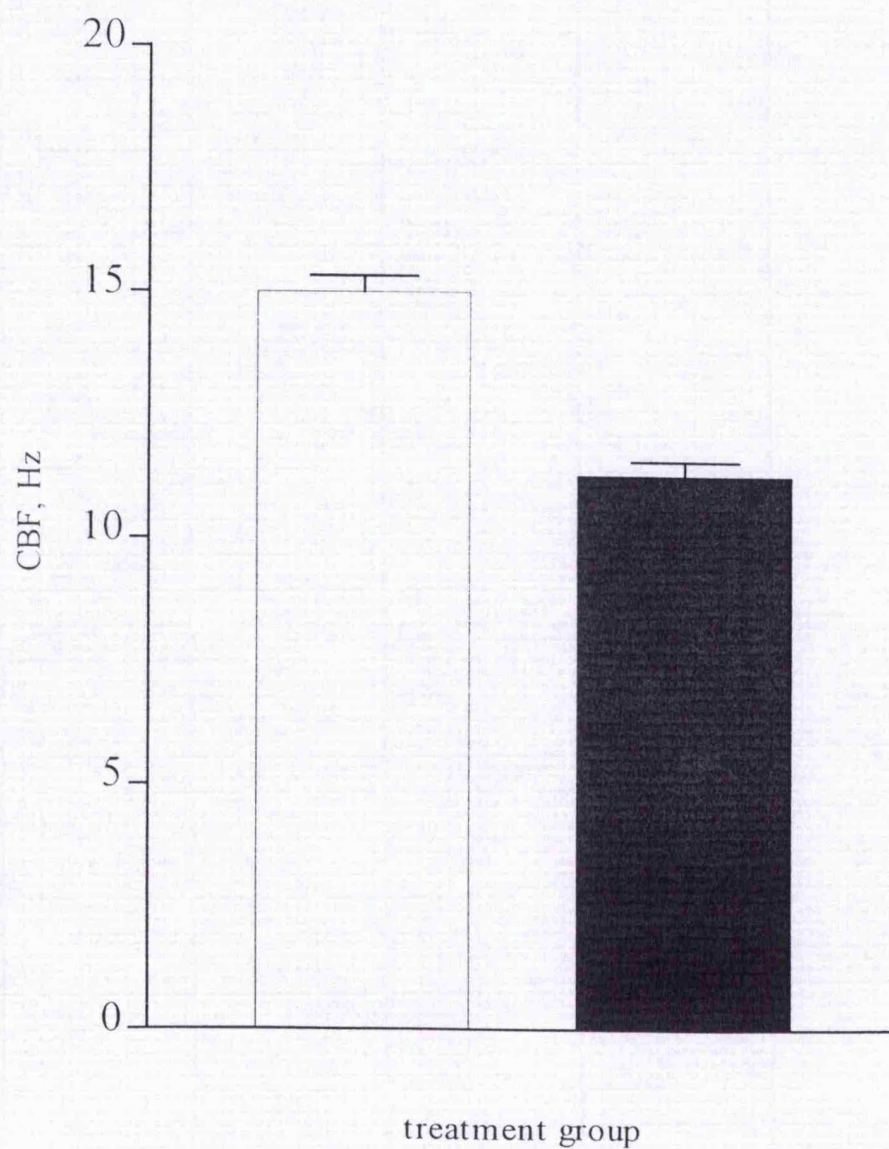
Results

The mean(SD) ciliary beat frequency in the specimens exposed to phenol was 11.3 (2.2) Hz compared with 15.0 (2.5) Hz in the controls. This difference was statistically significant (unpaired t-test, $p < 0.0001$) (Table 4.5; Fig. 4.6).

Table 4.5 Effects of 40 ppm phenol on ciliary beat frequency of nasal turbinate explants

	CBF, Hz	SEM, Hz	No. of ciliated edges measured
Air	15.0	0.33	57
Phenol	11.3	0.28	63

Fig. 4.6 Effects of 40 ppm phenol on ciliary beat frequency(CBF) of nasal turbinate explants; phenol ■, control □.



Discussion

We have evaluated two sources of human respiratory ciliated tissue for investigating the effects of drugs on ciliary beat frequency.

We first studied the effects of anaesthetic induction with propofol upon nasal cytological brushings and found that there was no change in the ciliary beat frequency compared with that obtained in the awake state. There is no previous work investigating the effects of propofol on ciliary beat frequency. Related work involving the effects of another intravenous anaesthetic, thiopentone, also found no effect on ciliary beat frequency compared with awake controls (Di Benedetto, 1991).

We also found that we were able to obtain better yields of ciliated epithelium from the patients under anaesthesia compared with those taken from them when awake. This is presumably because we were able to brush against the nasal turbinates more forcefully.

Thus cytological specimens obtained after the induction of anaesthesia with propofol provide a useful method of sampling for the measurement of ciliary beat frequency. It overcomes problems of recruiting volunteers for awake sampling which is restricted by the discomfort associated with the procedure and provides a better yield of tissue.

We also prepared samples of ciliated tissue from excised human nasal turbinates. The ciliary beat frequency of the samples maintained in a buffered incubator did not change significantly over a 10 day observation period and the samples also remained stable in the perfusion system for a period of up to six hours. The ciliary beat frequency of the samples was in close agreement with that found with

other human nasal and bronchial ciliated specimens (Yager, 1978). The studies on the assessment of human nasal turbinate explants have a posthoc power of 80 % at the 5 % level of significance to detect differences greater than 1.4 Hz. Alterations in ciliary beat frequency reported with ciliotoxic drugs are substantially greater than this (Hee, 1985) implying that this tissue preparation may be suitable to detect significant effects upon ciliary beat frequency.

To evaluate further the suitability of the samples from excised turbinates for studies of ciliary beat frequency we investigated the effects of known cilioactive agents. We found a significant increase in ciliary beat frequency with 100 μ M forskolin and a significant reduction in the ciliary beat frequency with 40 ppm phenol. There is no comparable work using forskolin on cilia; however other agents that elevate cAMP, such as salbutamol, have previously been shown to increase the ciliary beat frequency of other ciliated tissue preparations (Ingels, 1992). The reduction in ciliary beat frequency with 40 ppm phenol is comparable to that found by other workers using animal tissue. In a study using sheep tracheal cilia, Hee and Guillermin found a 50 % reduction in ciliary beat frequency with 50 ppm of phenol (Hee, 1985).

These experiments with forskolin and phenol provide further evidence that the tissue obtained from nasal turbinates is a reliable model for investigating pharmacological effects on ciliary beat frequency *in vitro*.

The two ciliated preparations we have developed are both useful to investigate ciliary function *in vitro* and therefore we have used both in our investigations. The nasal brushings obtained after the induction of anaesthesia with propofol provide a source of tissue suitable for receptor binding experiments and allow for the investigation of *in vivo* effects. The turbinate explants can be incubated in the laboratory to investigate long term effects of drugs.

The range of ciliary beat frequencies computed from the nasal brushings (11.2-12.0 Hz) is lower than that measured from the turbinate explants (11.6-15.0 Hz). This difference represents different patterns of interference with the light beam, the rate of which is deemed to represent the ciliary beat frequency. As discussed in chapter 3, the theory of measurement of ciliary beat frequency by the transmitted light technique is best applied to a single cilium; however, multiple respiratory cilia are imaged. The diameter of the light pen images between three and five cilia in any plane as well as the cilia throughout the depth of the field. Because adjacent cilia are co-ordinated and beat in synchrony the interference in the light path by the multiple cilia imaged with each position of the light pen provides a measure of the ciliary beat frequency. The depth and co-ordination of adjacent cilia may well be different in cytological specimens compared with explants. With turbinate explants there is a greater depth of ciliated tissue interfering with the light path but the greater ciliary continuity may provide better co-ordination. These factors may explain the different ciliary beat frequency that is computed. Nevertheless, for each specimen, whether nasal brushings or turbinate explants, the measure of ciliary beat frequency is within the range found by other workers (Rutland, 1982, Yager 1978, Teichtahl, 1986), is reproducible and predictably responsive to pharmacological agents.

Chapter 5

Inhalation anaesthetic agents and ciliary beat frequency

1: Effects of high concentrations

Introduction

The depressant effects of inhalation anaesthetic agents on mucus transport *in vivo* are well established in both animals and humans (Forbes, 1979a; Lichtiger, 1975); however, the mechanisms involved have not been elucidated. Mucus transport depends upon the volume and physical properties of the mucus and on the function of the beating cilia. There are few studies on the effects of anaesthetics on either of these parameters.

Due to technical limitations no direct measures of the effects of anaesthetics on mucus volume have been made. Using canine tracheal epithelia, Pizov and workers investigated the effects of halothane on ion transport, which is an important determinant of water flux across this tissue and an indirect measure of the mucus volume that is secreted (Pizov, 1992). They measured the current required to reset the electrical potential difference across the epithelia to zero as an index of ion transport and found that halothane reduced the required current and thus appeared to inhibit the ion transport. Such an effect could inhibit mucociliary coupling and reduce mucus transport.

Rubin and workers investigated the effects of anaesthesia on the rheological properties of mucus. They obtained mucus samples from awake subjects and from patients under general anaesthesia and did not find any significant differences in the viscosity, elasticity or spinability of the mucus. To determine

the functional characteristics of the mucus from these two groups they measured the rate of transport of the sampled mucus across mucus-depleted frog palate and could find no significant difference (Rubin, 1990).

There is more evidence to suggest an effect of anaesthetics on the cilia. The depressant effects of anaesthetic agents on cilia were recognised by Nunn 20 years ago investigating changes in the swimming velocity of the ciliated protozoan, *Tetrahymena pyriformis*, exposed to anaesthetic agents (Nunn, 1974). The first studies of these agents on respiratory cilia was undertaken by Manawadu and colleagues (Manawadu, 1979) who investigated the effects of halothane on ferret tracheal cilia in a semiquantitative way by noting the presence or absence of ciliary activity at different sites and demonstrated a reduction in the numbers of functioning cilia after prolonged exposure to halothane. Lee and Park investigating respiratory cilia, demonstrated suppression of cilia beat frequency of rabbit tracheal specimens with halothane and enflurane (Lee, 1980).

Although ciliary structure is similar in different species, ciliary function and in particular, mechanisms of control are not uniform (Sleigh, 1966; Wanner, 1977). The use of human, rather than non-human, tissue for studying the effects of pharmacological agents in clinical use is therefore preferable. The only published work to date on human respiratory cilia is a pilot study from our laboratory showing a depression of ciliary beat frequency with halothane on human ciliated tissue (Gyi, 1994).

We have investigated the *in vitro* effects of high concentration of the three commonly used inhalation anaesthetic agents, halothane, enflurane and isoflurane upon ciliary beat frequency using the perfusion system described in chapter 3.

Expt 5.1: The effects of 3 MAC of halothane, enflurane and isoflurane on human respiratory ciliary beat frequency

Introduction

In this first study of the inhalation anaesthetic agents, we have selected a concentration of the inhalation agents at the upper end of the clinical dose range so as to determine whether or not there was an effect of these agents on human ciliary beat frequency. The apparatus described in chapter 3 was employed. This is an improvement on previous techniques by allowing on line measurements of ciliary beat frequency during exposure to volatile agents in a controlled and blinded manner. We have developed alternative sources of cilia from the explants of inferior nasal turbinates as described in chapter 4; however, in this first experiment we have used nasal epithelial cell brushings because the brushings are a recognised ciliated tissue sample that allows a closer comparison with the previous work by Gyi in this field (Gyi, 1994).

In a separate study described in chapter 4 (expt 4.1), we have demonstrated that a single induction dose of propofol has no effect on the ciliary beat frequency of nasal brushings measured subsequently *in vitro* one hour later and have obtained our specimens in this manner.

Methods

Samples of ciliated epithelium were obtained from 18 non-smoking healthy patients, eight male, mean(range) age 34.3(22-78 years) who were not receiving any medications and had not suffered with an upper respiratory tract infection within the past four weeks. The patients received no preoperative medications.

Following an induction dose of 2-3.3 mg kg⁻¹ propofol, samples of ciliated respiratory epithelium were obtained by passing a bronchoscopy brush over the inferior nasal turbinates. The brush was agitated in HBSS to remove the tissue from the brush.

To investigate the effects of the volatile anaesthetic agents upon the cilia a perfusion system has been designed and built as described in chapter 3, comprising two perfusion chambers to house the ciliated tissue from one individual. Each chamber was perfused from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. The chambers were perfused with the HBSS under the effect of gravity at 0.5 ml min⁻¹. Air at 1000 ml min⁻¹ was either passed directly into one of the bottles of HBSS or passed through a Tec 3 vaporiser before passing into the other bottle. This divergence was achieved in a manner to which the observer was blinded as both delivery tubes were wound around one another within a countercurrent water jacket.

The bottles of HBSS were immersed in a water bath at 40 °C that also flowed through the countercurrent heat exchanger surrounding the delivery tubing. The perfusion chambers were heated to 36.8±0.4 °C by means of a thermostatically controlled heating element mounted on the underside of the chamber (Fig. 3.6).

Samples of ciliated epithelium from any one patient were mounted between the coverslips of two paired chambers and these were connected to the bottles containing the HBSS perfusate with one bottle receiving the volatile agent in air and the other air alone.

The analysis of ciliary beat frequency was performed using the transmitted light technique as detailed in chapter 3. This method detects ciliary movement by their interference with a light beam which is transduced from the voltage changes of a photodiode and mathematically processed to give a power spectrum of the frequencies due to ciliary beating.

Acceptable ciliated edges for measurement were defined as those free of mucus and at least 60 μm long. Measurements were not taken from individual cells or disrupted edges. Six patient samples were exposed to each of the volatile agents studied and readings were taken from both chambers before exposure to the volatile agent and one, two and three hours after one chamber was exposed to the volatile agent and the other continued to be exposed to air alone. The vaporiser was set on a concentration of 3 MAC for unpremedicated young adults at 37 °C . This represented 2.3 % for halothane, 5 % for enflurane and 3.6 % for isoflurane. We analysing as many acceptable ciliated edges as possible from a minimum of six to a maximum of ten from each chamber in each defined time band.

The mean of the peak ciliary beat frequencies from the power spectrum was computed for each sample at each time point. The data were assumed to represent a multivariate normal distribution and analysed by multiple analysis of variance for repeated measures. Significance was taken at $p < 0.05$.

Results

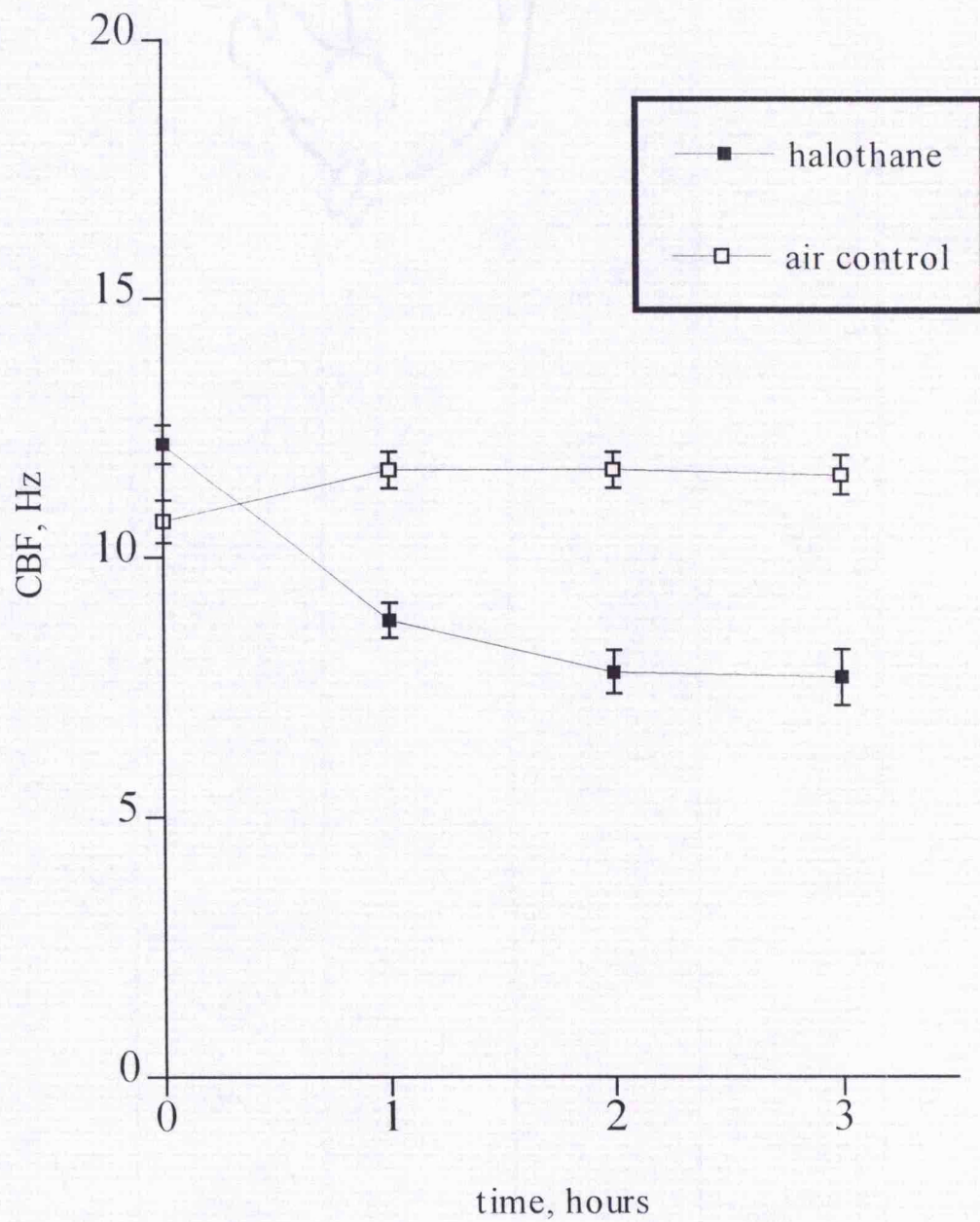
Halothane

Over the three hour observation period when the cilia were exposed to halothane the mean(SEM) cilia beat frequency in the group exposed to 3 MAC halothane in air was 9.3 (1.3) Hz compared with 11.4(1.0) Hz in the group exposed to air alone. There was a statistically significant difference between the groups exposed to halothane or air (MANOVA for repeated measures, $F = 7.8$, $p = 0.001$) (Table 5.1; Fig 5.1)

Table 5.1 Effects of 3 MAC halothane on ciliary beat frequency(CBF)

	Time, hours	Mean CBF, Hz	SEM, Hz	No. of readings
<hr/>				
<u>3 MAC halothane in air</u>				
Air	0	12.2	0.37	46
Halothane	1	8.8	0.34	47
Halothane	2	7.8	0.42	39
Halothane	3	7.7	0.54	37
 <u>Air control</u>				
Air	0	10.7	0.41	49
Air	1	11.7	0.35	49
Air	2	11.7	0.34	43
Air	3	11.6	0.39	39

Fig 5.1 Ciliary beat frequency (CBF), mean (SEM) during exposure to 3 MAC halothane



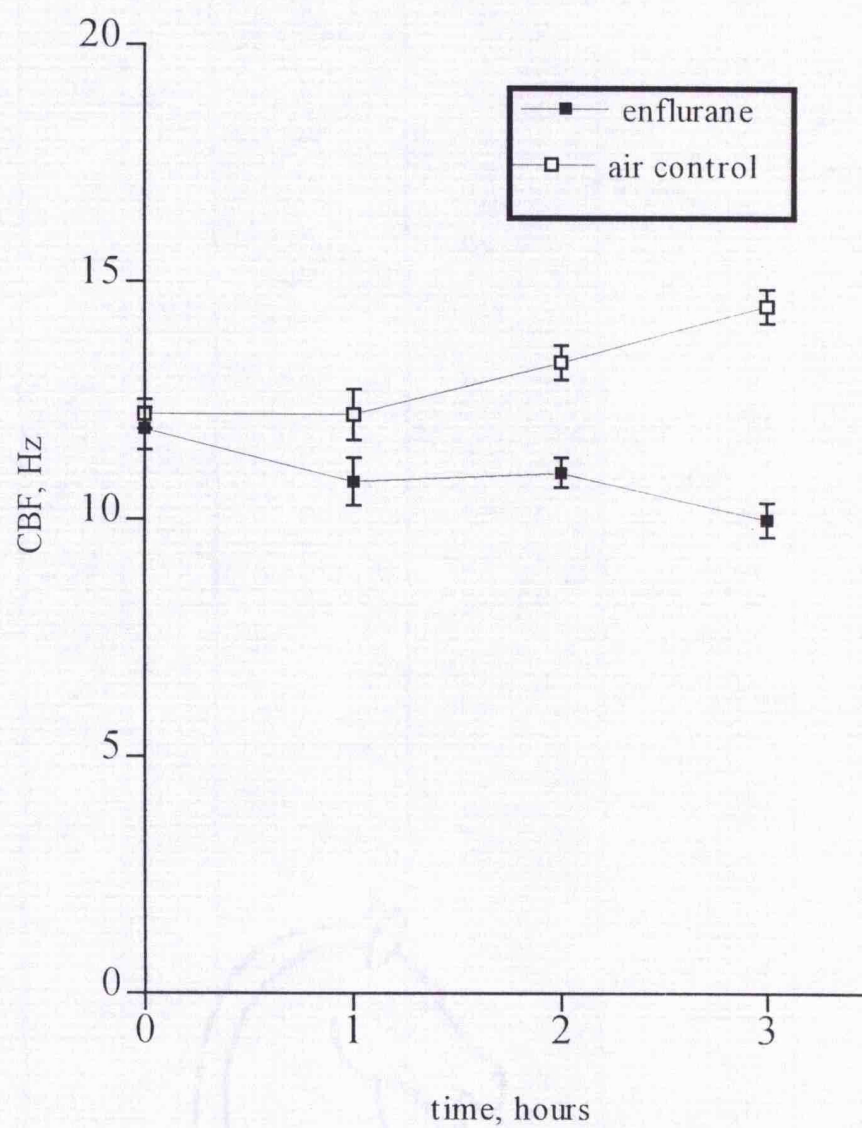
Enflurane

Over the three hour observation period when the cilia were exposed to the enflurane the mean(SEM) cilia beat frequency in the group exposed to 3 MAC enflurane in air was 10.9 (1.3) Hz compared with 13.1 (1.2) Hz in the group exposed to air alone. There was a statistically significant difference between the groups exposed to enflurane or air (Univariate F test , $F=7.4$, $p=0.02$) (Table 5.2; Fig. 5.2).

Table 5.2 Effects of 3 MAC enflurane on ciliary beat frequency(CBF)

	Time, hours	Mean CBF,Hz	SEM, Hz	No. of readings
<hr/>				
<u>3 MAC enflurane in air</u>				
Air	0	11.9	0.44	56
Enflurane	1	10.8	0.50	52
Enflurane	2	11.0	0.31	54
Enflurane	3	10.0	0.36	57
 <u>Air control</u>				
Air	0	12.2	0.31	58
Air	1	12.2	0.53	53
Air	2	13.3	0.37	53
Air	3	14.5	0.36	55
<hr/>				

Fig 5.2 Ciliary beat frequency (CBF), mean (SEM) during exposure to 3 MAC enflurane



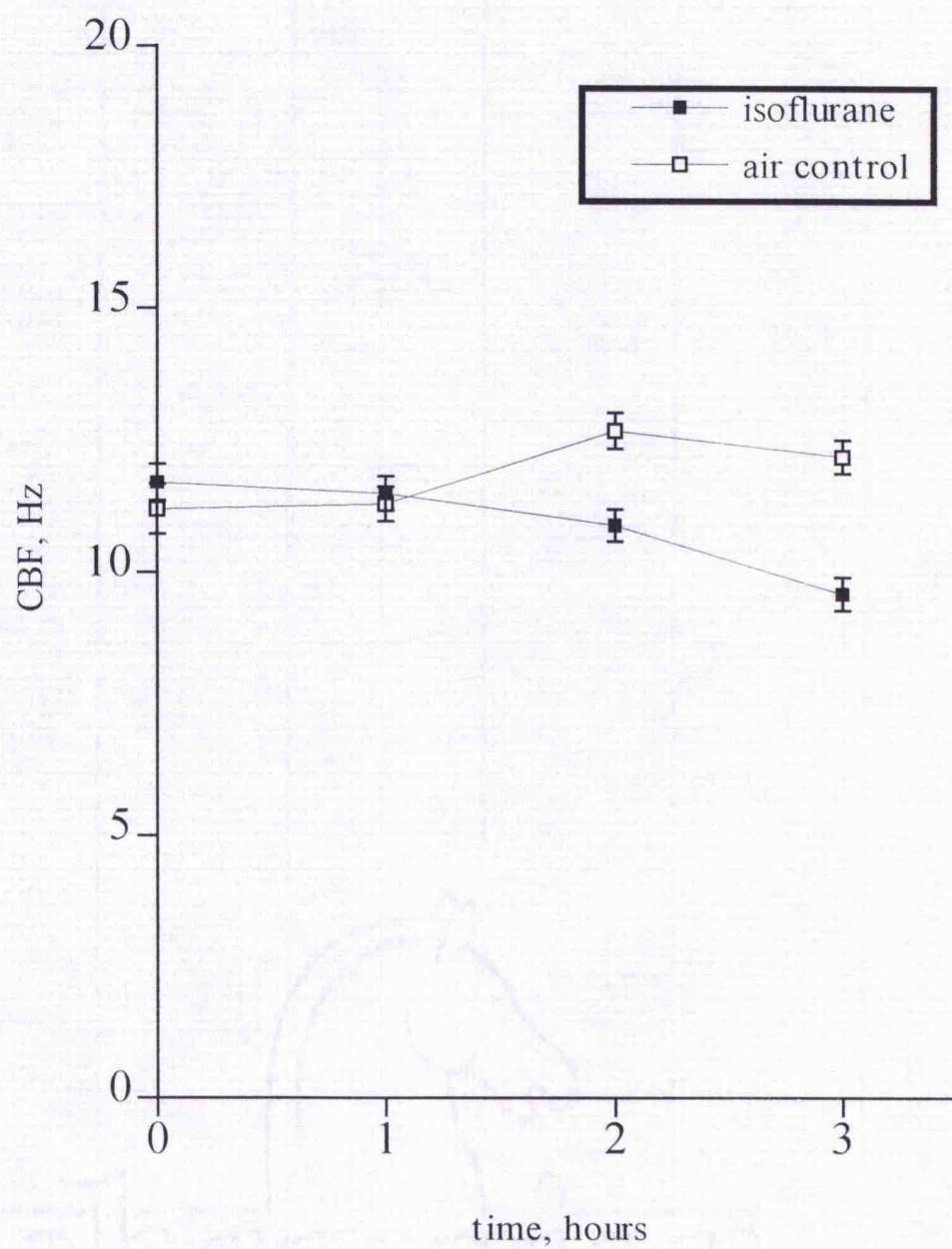
Isoflurane

Over the three hour observation period when the cilia were exposed to the isoflurane the mean(SEM) ciliary beat frequency in the group exposed to 3 MAC isoflurane in air was 10.8 (1.1) Hz compared with 11.6 (1.2) Hz in the group exposed to air alone. There was a statistically significant difference between the groups exposed to isoflurane or air (MANOVA for repeated measures , $F=4.6$, $p = 0.01$) (Table 5.3; Fig. 5.3).

Table 5.3 Effects of 3 MAC isoflurane on ciliary beat frequency(CBF)

	Time, hours	Mean CBF, Hz	SEM, Hz	No. of readings
<hr/>				
<u>3 MAC Isoflurane in air</u>				
Air	0	11.7	0.35	49
Isoflurane	1	11.5	0.33	51
Isoflurane	2	10.9	0.31	53
Isoflurane	3	9.6	0.32	48
 <u>Air control</u>				
Air	0	11.2	0.47	50
Air	1	11.3	0.33	50
Air	2	12.7	0.34	48
Air	3	12.2	0.32	46

Fig 5.3 Ciliary beat frequency (CBF), mean (SEM) during exposure to 3 MAC isoflurane



Expt 5.2 : The duration of effect of halothane, enflurane and isoflurane on *in vitro* human turbinate explant ciliary beat frequency

Introduction

In the previous study we have demonstrated depression of human nasal ciliary beat frequency with 3 MAC of three inhalation anaesthetic agents. In this study we aimed to investigate the duration of ciliary beat frequency depression at these concentrations by measuring the recovery characteristics of ciliary beat frequency after air washout. A different ciliated tissue preparation consisting of explants from human nasal turbinates was used. These preparations were developed in part to overcome the technical problems of sample movement in addition to providing a more accessible source of ciliated tissue as discussed in chapter 4.

Methods

Samples of human respiratory ciliated epithelium were obtained from the inferior nasal turbinates of nine patients undergoing turbinectomy surgery for a primary diagnosis of turbinate hypertrophy. The patients were mean(range) age 30.1(18-43) years, seven were male, and all were healthy non-smokers. The turbinates were removed under general anaesthesia and after topical preparation with 200 mg of cocaine and 1 mg of adrenaline.

The samples were prepared by the methods described in chapter 4, and the epithelial explants from the turbinates were incubated in buffered, temperature-controlled culture medium. The ciliary beat frequency of the tissue samples were measured after a variable storage period of between one and six days.

A sample from the patient's turbinates was mounted in a perfusion chamber as described previously in chapter 3. The chamber was perfused from a delivery bottle of HBSS under the effect of gravity at 0.5 ml min^{-1} . Air at 1000 ml min^{-1} was passed through a Tec 3 vaporiser before reaching to the delivery bottle.

The bottle of HBSS was immersed in a water bath at 40°C that also flowed through a countercurrent heat exchanger surrounding the delivery tubing. The perfusion chamber was heated to $36.8 \pm 0.4^\circ\text{C}$ by means of a thermostatically controlled heating element mounted on the underside of the chamber (Fig. 3.6). Ciliary beat frequency was measured with Nomarski Differential Interference microscopy and the transmitted light technique as described previously.

The vaporiser was set on a concentration of 3 MAC for unpremedicated young adults at 37°C . This represented 2.3% for halothane, 5% for enflurane and 3.6% for isoflurane. Samples from eight patients were exposed to halothane and isoflurane and samples from nine patients to enflurane. After exposure at 3 MAC to one of the three inhalation agents for a period of an hour, we recorded between six and 10 readings of the ciliary beat frequency. We then exposed the samples to air alone and measured the cilia beat frequency after exposure to air at half-hourly intervals for one and a half hours.

The mean of the peak cilia beat frequencies from the power spectrum was computed for each sample at each time point. The data were assumed to represent a sample from a multivariate normal distribution and analysed by analysis of variance for repeated measures. If this was significant then the data were analysed by unpaired t-tests with Bonferroni correction comparing the baseline ciliary beat frequency with that measured at subsequent time points. Significance was taken at $p < 0.05$.

Results

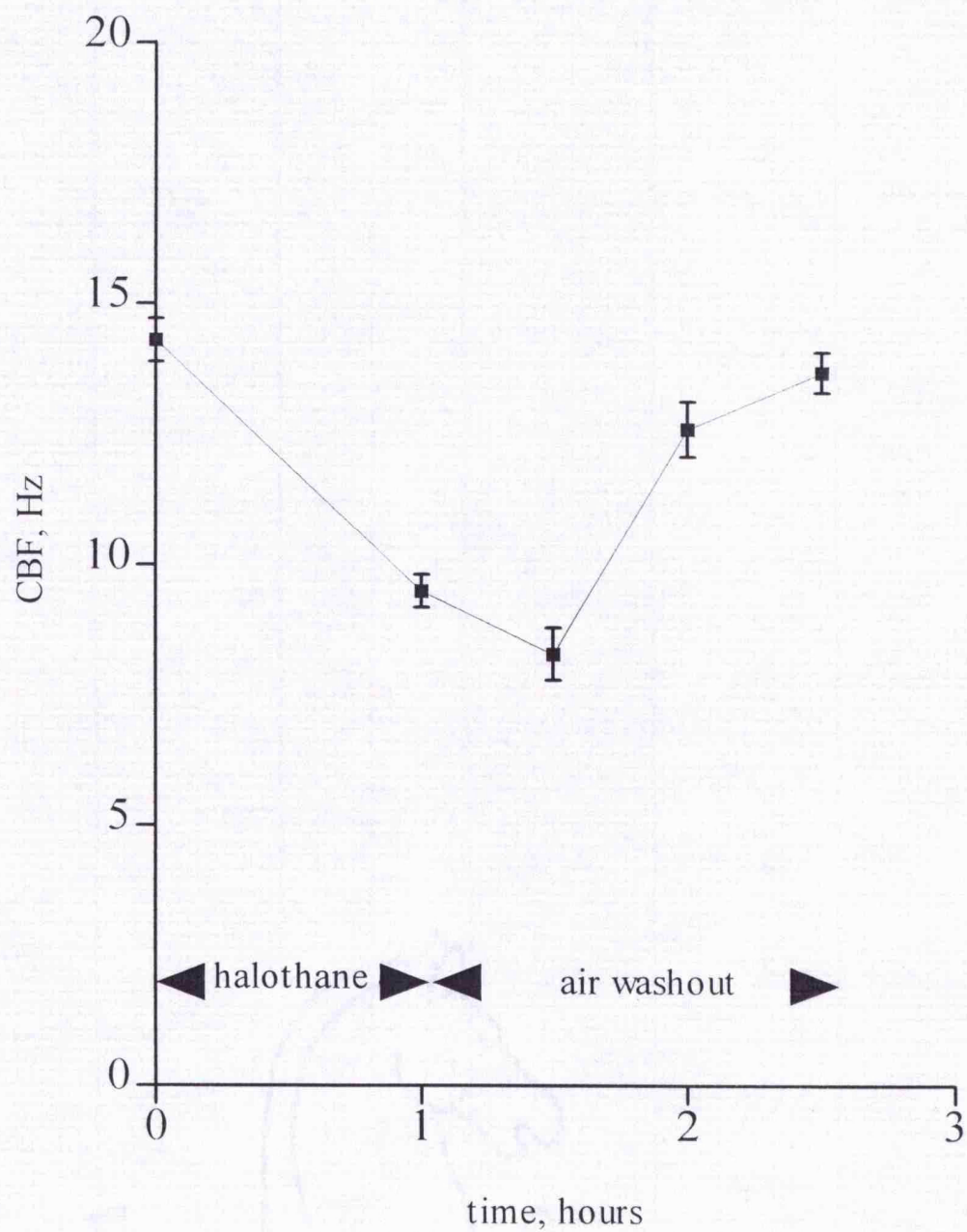
Halothane

Turbinate explants from eight patients were exposed to 3 MAC halothane and subsequently to air. The mean (SEM) ciliary beat frequency following exposure to halothane and a one hour period of airwashout was 12.6 (0.54) Hz and after 90 minutes of washout was 13.7 (0.38) Hz compared with a pretreatment value of 14.3 (0.42) Hz. After a one hour period of air washout the ciliary beat frequency remained significantly depressed (repeated measures ANOVA, $p < 0.001$, unpaired t-test with Bonferroni correction, $p = 0.01$ at one hour and $p = 0.31$ at one and a half hours of air washout compared with pretreatment control). (Table 5.4; Fig 5.4)

Table 5.4. Recovery of ciliary beat frequency (CBF) after 3 MAC halothane

	Time. hours	Mean CBF, Hz	SEM, Hz	No. of readings
Air	0	14.3	0.41	50
Halothane	1	9.5	0.32	42
Air	1.5	8.3	0.50	39
Air	2	12.6	0.53	43
Air	2.5	13.7	0.39	42

Fig 5.4 Ciliary beat frequency (CBF), mean (SEM) after 3 MAC halothane for one hour and airwashout for one and a half hours



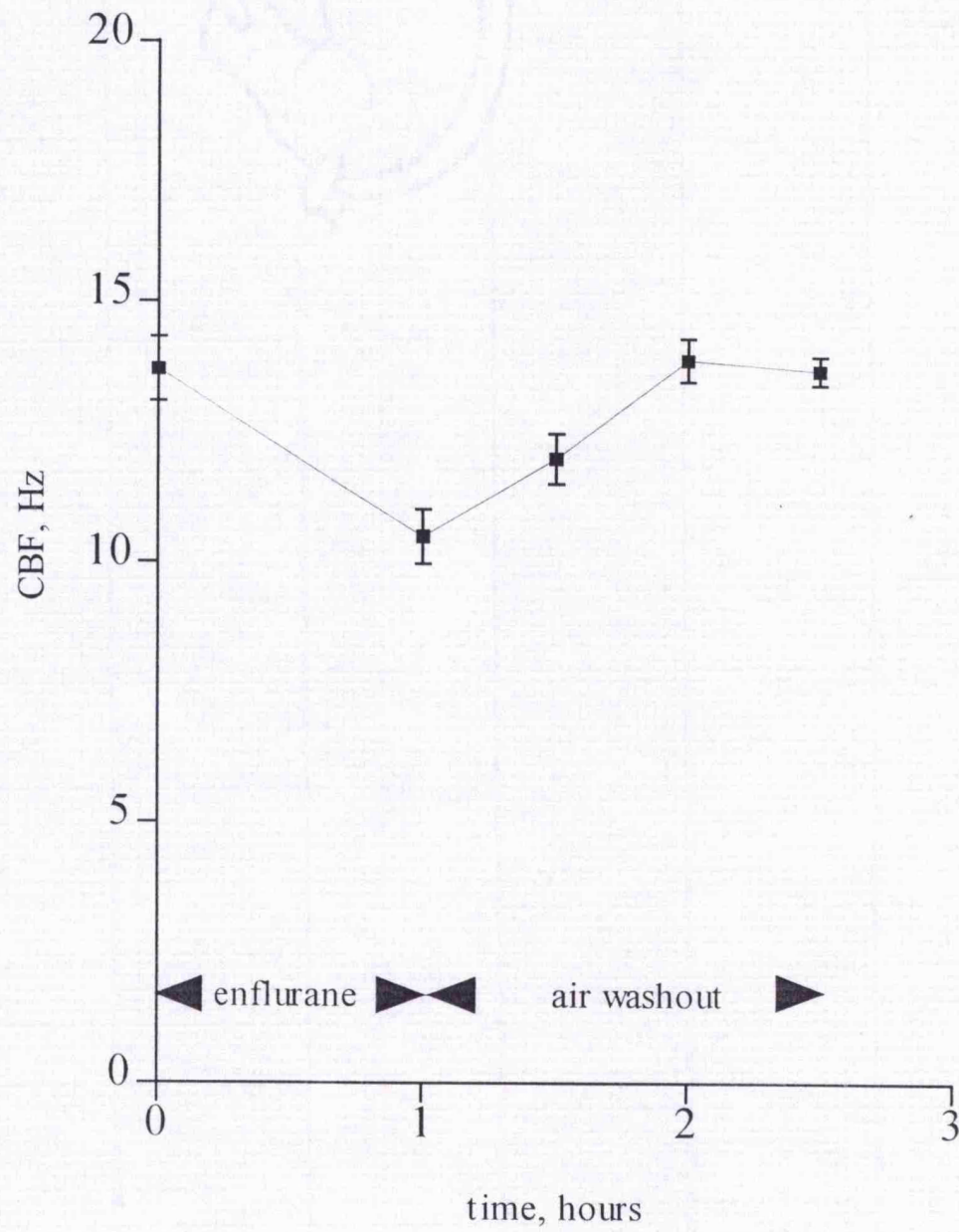
Enflurane

The mean (SEM) ciliary beat frequency following exposure to enflurane and a one hour period of air washout was 13.9 (0.42) Hz compared with a pretreatment value of 13.7 (0.62) Hz. The mean ciliary beat frequency had returned to values that were not significantly different from pretreatment values after an air washout period of one hour (repeated measures ANOVA, $p < 0.001$, unpaired t-test with Bonferroni correction, $p = 0.83$ at one hour of air washout compared with pretreatment controls.) (Table 5.5; Fig 5.5)

Table 5.5 Recovery of ciliary beat frequency (CBF) after 3 MAC enflurane

	Time. hours	Mean CBF, Hz	SEM, Hz	No. of readings
Air	0	13.7	0.62	44
Enflurane	1	10.5	0.52	42
Air	1.5	12.0	0.48	42
Air	2	13.9	0.42	45
Air	2.5	13.7	0.27	45

Fig 5.5 Ciliary beat frequency (CBF), mean (SEM) after 3 MAC enflurane for one hour and airwashout for one and a half hours



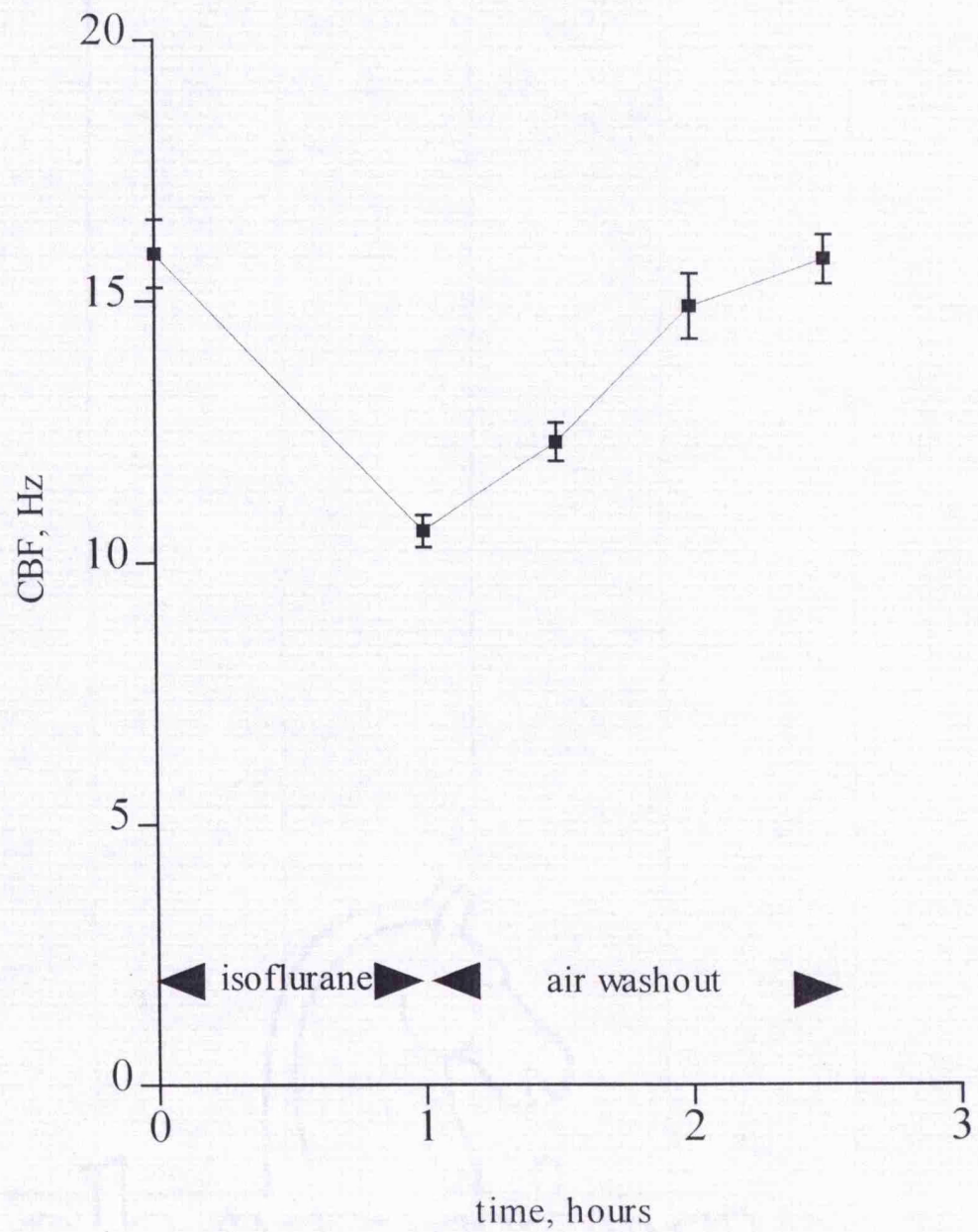
Isoflurane

The mean(SEM) ciliary beat frequency following exposure to isoflurane and a one hour period of air washout was 14.9 (0.62) Hz compared with a pretreatment value of 15.9 (0.63) Hz. The mean ciliary beat frequency had returned to values that were not significantly different from pretreatment values after an air washout period of one hour (repeated measures ANOVA, $p < 0.001$, unpaired t-test with Bonferroni correction, $p = 0.26$ at one hour of air washout compared with pretreatment controls.) (Table 5.6; Fig 5.6)

Table 5.6 Recovery of ciliary beat frequency (CBF) after 3 MAC isoflurane

	Time. hours	Mean CBF, Hz	SEM, Hz	No. of readings
Air	0	15.9	0.64	36
Isoflurane	1	10.6	0.30	40
Air	1.5	12.3	0.37	38
Air	2	14.9	0.62	37
Air	2.5	15.8	0.46	37

**Fig 5.6 Ciliary beat frequency (CBF) after 3
MAC isoflurane for one hour and
airwashout for one and a half hours**



Discussion

We have found a reversible depression of ciliary beat frequency *in vitro* after exposure to 3 MAC of the three inhalation agents halothane, enflurane and isoflurane. There was a progressive reduction in ciliary beat frequency during exposure to 3 MAC of these agents measured over a four hour observation period. The return to baseline values of ciliary beat frequency following exposure to 3 MAC of halothane, enflurane and isoflurane for one hour took one hour in the cases of enflurane and isoflurane and one and a half hours in the case of halothane.

There was an unexpected increase in ciliary beat frequency measured from nasal brushings of the control group paired with the samples exposed to enflurane. The increase in ciliary beat frequency in the control group may represent the movements of exfoliated collections of cells that becomes more apparent as the perfusate separates the tissue into smaller segments over time. In our experimental design we controlled for all the external factors known to affect ciliary beat frequency (temperature, flow rate, pH and osmolality) and when assessing the chamber we found ciliary beat frequency to remain stable for many hours (expt 3.6). By conducting controlled and blinded experiments we would anticipate that increase in ciliary beat frequency in the control group may have similarly occurred in the paired chamber but for the depressant effects on ciliary beat frequency of the volatile agent.

Previous work from our laboratory using a different method investigated the effects of halothane on nasal brushings (Gyi, 1994). In this study there was a depression of ciliary beat frequency with 1.8% halothane of 25% after one hour and 20% after 2 hours exposure, and with 5.7% halothane there were reductions of 20% and 40% after one and two hours exposure respectively. These are

comparable to our findings of a depression of 25% at one hour and 40% after two hours exposure to 2.3% halothane.

Using rabbit trachea, Lee found reductions in ciliary beat frequency using 3MAC of halothane of 22%. This compares with the reductions we have found at these concentrations of 25% with the nasal brushings and 33% with the turbinate explants. In the case of enflurane, these workers found reductions in ciliary beat frequency at 3 MAC of 26% which compares with our findings of 10% with the brushings and 26% with the turbinate explants (Lee, 1980).

The depression of ciliary beat frequency we have found with the turbinate preparations of 33, 25 and 33% with halothane, enflurane and isoflurane respectively differs from that found at one hour with nasal brushings, where we found reductions of 28, 10 and 2% with these three agents. This difference represents different patterns of interference with the light beam due to different tissue sources, the rate of which is deemed to represent the ciliary beat frequency. The light pen images between three and five cilia in any plane as well as the cilia throughout the depth of the field. Because adjacent cilia are coordinated and beat in synchrony the interference in the light path by the multiple cilia imaged with each position of the light pen provides a measure of the ciliary beat frequency. The depth and co-ordination of adjacent cilia may well be different in cytological specimens compared with explants. With turbinate explants there is a greater depth of ciliated tissue interfering with the light path but the greater ciliary continuity may provide better co-ordination. These factors may explain the different ciliary beat frequency that is computed. Interestingly the results we have found with the turbinate preparations are closer to those found by Lee using rabbit trachea (Lee, 1980) and might suggest that the turbinate samples may be a more physiological tissue to investigate ciliary function than exfoliated cells.

In this study, a supraclinical concentration of each volatile agent was used so as to determine the presence of any effects. This restriction to one concentration makes it impossible to quantitatively compare the agents. We have proceeded to evaluate the effects of a range of concentrations in chapter 6.

The modest reductions in ciliary beat frequency found with 3 MAC of the inhalation agents may appear to be of limited clinical significance; however, there is a disproportionate relationship between ciliary beat frequency and mucus transport rate as discussed in chapter 2 and reductions in mucus transport produced by similar concentrations of the inhalation agents is much greater. In a study of tracheal mucus transport in dogs using radioactive droplets and scintillation counters, Forbes found a mucus transport rate of 20% of controls at around 3 MAC of halothane and enflurane (Forbes, 1976 and 1977).

The progressive reduction in ciliary beat frequency that we found during inhalation agent exposure over a four hour measurement period merits further investigation and is addressed in chapter 6.

The return to baseline values of ciliary beat frequency following exposure to 3 MAC of halothane, enflurane and isoflurane for one hour took one hour in the cases of enflurane and isoflurane and one and a half hours in the case of halothane. Interestingly, ciliary beat frequency measured 30 minutes after ceasing inhalation agent exposure demonstrated some recovery in the cases of enflurane and isoflurane but a further reduction in the case of halothane.

The differences in recovery time and effects of early washout with the three agents may be related to their differing recovery characteristics from general anaesthesia which is dependent on their physical properties. Halothane has a greater lipid/water solubility coefficient than the other two and may take longer

to diffuse out from the fat soluble tissues of the preparation. The mechanism of continuing reduction in ciliary beat frequency after cessation of halothane is unknown but is an interesting finding.

The only comparable work is by Gyi and colleagues who investigated the effects of halothane alone on ciliary beat frequency. Their data failed to show a reversal of effect following air washout for 50 minutes (Gyi, 1994). This concurs with our findings at one hour.

There are no data available comparing the duration of reduction in mucus transport nor any clinical data comparing the incidence of infective respiratory problems with different inhalation agents.

The time for the reversal of depression in ciliary function that we have found may appear to be of little clinical significance especially in the light of the longer duration of depression of mucus transport rates demonstrated with the inhalation anaesthetics *in vivo*. Forbes measured tantalum bronchographic clearance in dogs anaesthetised with halothane and found that 1.2 MAC of halothane administered for two hours delayed the clearance of tantalum for more than four hours after the termination of anaesthesia (Forbes, 1979b). This may represent additional effects of the anaesthetics on the mucus itself. Alternatively, even relatively short periods of ciliary beat frequency depression which produce mucus stasis could alter the physical properties of the mucus and therefore impair mucus transport for a longer period.

Chapter 6

Inhalation anaesthetic agents and ciliary beat frequency

2: Effects of low concentrations

Introduction

We have previously shown a reversible depressant effect of 3 MAC of halothane, enflurane and isoflurane upon human respiratory ciliary beat frequency *in vitro* (expts 5.4-5.6). In this chapter we have investigated the effects of lower concentrations of these agents as used in clinical anaesthetic practice upon ciliary beat frequency. We have first investigated the effects upon ciliary beat frequency of short term exposure to the three inhalation anaesthetic agents *in vitro*.

In previous studies (expts 5.1-5.3) a time-dependent depression in ciliary beat frequency of tissue exposed to 3 MAC of the inhalation agents was found. This poses the question of whether prolonged use of clinical concentrations of these anaesthetic agents causes significant reductions in ciliary beat frequency.

Prolonged use of the inhalation agents occurs during occasional clinical anaesthetic practice and particularly during sedation in the ITU. The inhalation agent used in these circumstances is most commonly isoflurane (Spencer, 1992).

We have investigated the effects of upon ciliary beat frequency of low concentrations of isoflurane given over a few days in the laboratory to simulate the use of this agent for sedation in the ITU. This latter study has required the development of a gas chamber to expose the ciliated tissue to the inhalation agent in a controlled environment, the design of which is described.

Expt 6.1: The effects of 0.5 - 3 MAC of halothane, enflurane and isoflurane on ciliary beat frequency *in vitro*

Methods

Explants of human respiratory ciliated epithelium were obtained from the inferior nasal turbinates of 24 patients undergoing turbinectomy surgery for a primary diagnosis of nasal obstruction with no history of allergy. The explants were prepared and maintained as described previously in chapter 4. The patients had a mean (range) age of 35.5 (18-59) years, 6 were female, and none was a smoker. None was receiving any regular medications and all had the nose prepared topically with 1 mg of adrenaline and 200 mg of cocaine before surgical excision under general anaesthesia.

Exposure of the ciliated tissue to the inhalation agents was made using the perfusion system described in detail in chapter 3. This comprises two perfusion chambers to house the ciliated tissue from any one individual. Each chamber was perfused from a separate delivery bottle of HBSS flowing under the effect of gravity at 0.5 ml min⁻¹. Air at 1000 ml min⁻¹ was simultaneously passed to the two perfusion chambers via a Tec 3 vaporiser and then into one of the bottles of HBSS. This divergence was achieved in a manner to which the observer was blinded as both delivery tubes were wound around one another within a countercurrent water jacket. The perfusion chamber was thermostatically controlled at 36.8±0.4 °C.

The chambers were exposed to air alone and also to one of the three inhalation anaesthetic agents, halothane, enflurane and isoflurane at concentrations of 0.5, 1, 2 and 3 MAC. Each concentration of each inhalation agent was maintained for

one hour before measurements of ciliary beat frequency were made and a period of air washout of one and a half hours was allowed before re-introducing the inhalation agent again.

The two paired chambers received different concentrations of the same inhalation agent or a different inhalation agent. The observer was blinded as to which chamber received which agent or concentration of agent until the measurements of ciliary beat frequency had been performed.

After chamber exposure at a particular concentration of the inhalation agent or to air alone for a period of an hour between six and 10 measurements of the ciliary beat frequency were recorded from each specimen using the transmitted light technique as detailed in chapter 3.

This process was repeated for a maximum time of six hours and the ciliary beat frequency of turbinate samples from six patients were separately measured after exposure to the range of concentrations of each of the three inhalation anaesthetic agents under study.

The data were analysed by repeated measures multiple analysis of variance with significance taken at $p < 0.05$.

Results

The results are shown in tables 6.1-6.3 and displayed in figure 6.1.

There was a dose dependent depression of ciliary beat frequency for all three inhalation anaesthetic agents. The difference in ciliary beat frequency was significant at concentrations at and above 1 MAC for halothane and enflurane and at and above 0.5 MAC for isoflurane. (MANOVA for repeated measures for the effect of concentration $p < 0.0001$; unpaired t-tests with Bonferroni correction: halothane $p < 0.02$ at 1.0 MAC, enflurane $p < 0.0001$ at 1.0 MAC, isoflurane $p < 0.03$ at 0.5 MAC).

There was a statistically significant difference between the effects of the three agents on ciliary beat frequency (MANOVA for the effect of drug treatment, $p < 0.01$).

The difference between halothane and enflurane only reached statistical significance at a concentration of 1.0 MAC and was not significant at any of the other concentrations (t-test with Bonferroni correction, $p < 0.05$ at 1.0 MAC). The difference between enflurane and isoflurane reached statistical significance at 0.5 MAC and 3.0 MAC, but was not significant at the other concentrations (t-test with Bonferroni correction, $p < 0.005$ at 0.5 MAC and $p < 0.01$ at 3 MAC). The difference between halothane and isoflurane was statistically significant at all concentrations (t-test with Bonferroni correction $p < 0.05$ at 0.5 MAC, $p < 0.05$ at 1.0 MAC, $p < 0.05$ at 2 MAC, $p < 0.005$ at 3 MAC).

Table 6.1 Effect of halothane on ciliary beat frequency (CBF), Hz

MAC	number of measurements	CBF(mean)	CBF(SEM)
0	43	14.6	0.33
0.5	44	14.3	0.21
1	69	13.4	0.35
2	62	12.1	0.28
3	73	11.0	0.19

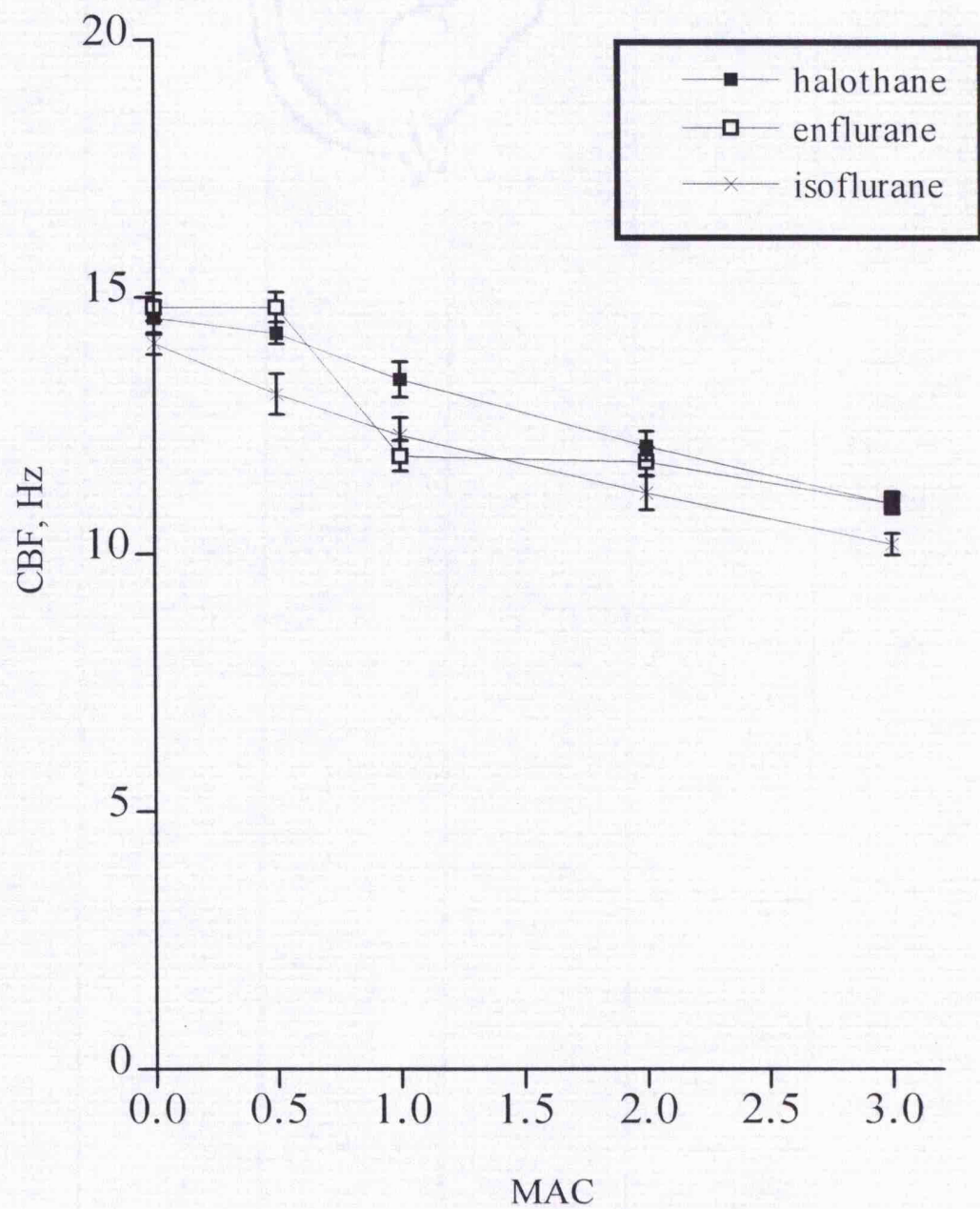
Table 6.2 Effect of enflurane on ciliary beat frequency (CBF), Hz

MAC	number of measurements	CBF(mean)	CBF(SEM)
0	49	14.8	0.27
0.5	61	14.8	0.29
1	62	11.9	0.29
2	64	11.8	0.28
3	73	11.0	0.22

Table 6.3 Effect of isoflurane on ciliary beat frequency (CBF), Hz

MAC	number of measurements	CBF(mean)	CBF(SEM)
0	31	14.1	0.21
0.5	47	13.1	0.40
1	61	12.3	0.35
2	56	11.2	0.33
3	71	10.2	0.21

Fig 6.1 Effects of MAC levels of inhalation agents on ciliary beat frequency (CBF) *in vitro*, mean (SEM)



Ciliary effects of prolonged use of isoflurane

The pharmacokinetics of the inhalation anaesthetic agent, isoflurane, make it suitable to be used for prolonged anaesthesia and for sedation in the ITU. It undergoes minimal metabolism and is therefore almost completely independent of renal or hepatic clearance. The effects of prolonged sedation with isoflurane for two to three days upon ciliary function is unknown. To investigate the effects of prolonged sedative concentrations of isoflurane on ciliary function we incubated respiratory cilia *in vitro* with isoflurane.

In order to expose the ciliated tissue to isoflurane for a period of 48-72 hours a specially-designed chamber was constructed (Fig 6.2). This consists of a metallic box with inlet and outlet ports. An electronically-timed solenoid valve controls the input to the chamber. This opens a valve for a period of 20 seconds every 30 minutes and the box is flushed during this period with a mixture of 5% CO₂ in air flowing from a size A cylinder at 5L min⁻¹ via a Tec 3 isoflurane vaporiser. The outlet from the chamber consists of a long and narrow tube to minimise diffusion of gas out of the chamber but prevent excessive increases in pressure within it. The outlet has an adapter for intermittent connection to a vapour analyser to check the concentration of isoflurane in the chamber. The samples of ciliated tissue are placed in the chamber in a multiwell flask that allows diffusion of the isoflurane, and a beaker of water is placed alongside to maintain humidity.

We tested the ability to maintain a continuous concentration of isoflurane in the chamber by intermittently connecting the chamber's outlet tube to a vapour analyser (Datex Capnomac). This sampled at 200 ml min⁻¹ and was connected for one minute at periodic intervals over a 72 hour period. The mean(range) concentration in the chamber was 0.3(0.2-0.4) %.

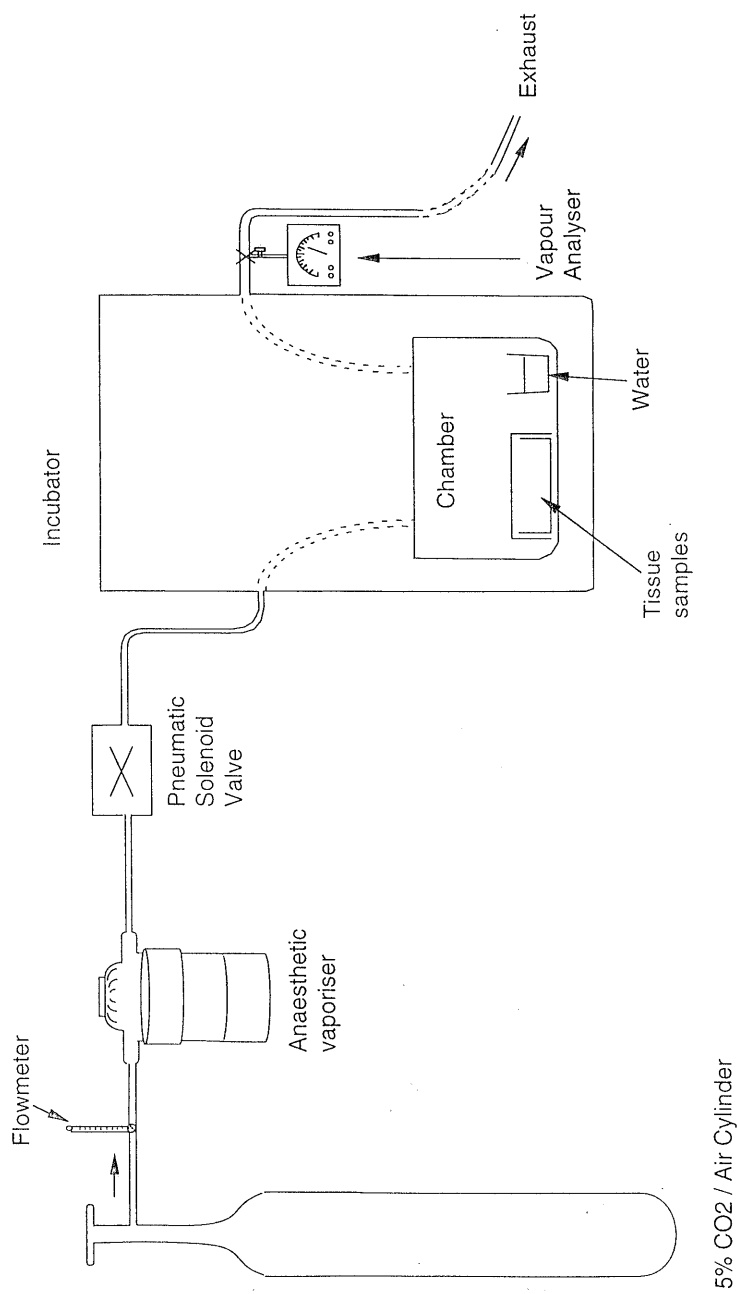


Fig 6.2. Gas chamber for prolonged exposure of ciliated tissue to isoflurane

Expt 6.2 Effects of 0.2-0.4% isoflurane for 48-72 hours upon *in vitro* ciliary beat frequency

Methods

Samples from the turbinates of six patients (five male) undergoing nasal turbinectomy for tissue hypertrophy were prepared as described previously (chapter 4). The patients had a mean(range) age of 37.8(26-56) years. None were smokers or had a history of allergy.

The following day the samples were checked for the presence of active ciliated edges under the microscope and then transferred to the multiwell flask in the gas chamber. The samples were then exposed to either 0.2-0.4 % isoflurane in CO₂/Air or to CO₂/Air only for 48-72 hours. The culture medium was changed after 24 hours.

After 48-72 hours the samples of ciliated tissue were transferred to paired perfusion chambers as described in chapter 3. Each chamber was perfused from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. The chambers were perfused with the HBSS under the effect of gravity at 0.5 ml min⁻¹.

To one chamber, containing the samples that had acted as controls in the incubator, air flowing at 1000 ml min⁻¹ was passed directly via a bottle of HBSS. To the other chamber, containing the samples that had been incubated with isoflurane, air flowing at 1000 ml min⁻¹ was passed via a Tec 3 isoflurane vaporiser and then into a separate delivery bottle of HBSS perfusing the

appropriate chamber. The observer was not blinded to the identity of the tissue in the perfusion chambers.

The vaporiser delivered a calibrated concentration of 0.2-0.4 %. The chambers were maintained at 36.8 ± 0.4 °C by a thermostatically-controlled heating element attached to the chamber.

Ciliary beat frequency was measured by the transmitted light technique using Nomarski Differential Interference microscopy with a Nikon Diaphot as previously described in chapter 3.

Between six and 10 measurements of ciliary beat frequency were taken from each explant. The peak of the power spectrum was taken to represent the commonest ciliary beat frequency for each recording. These data were analysed by unpaired t-test with significance taken at $p < 0.05$.

Results

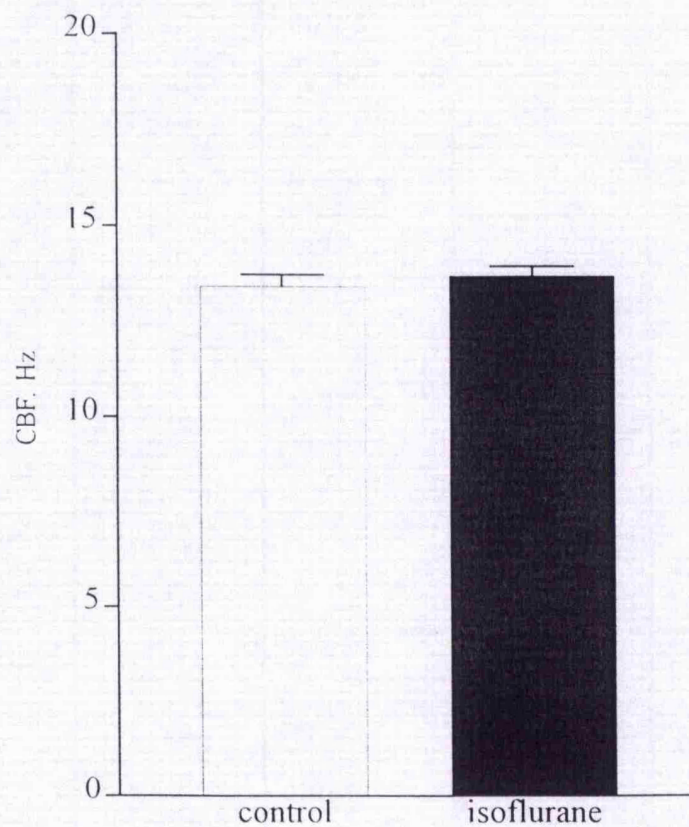
The results are shown in table 6.4 and fig 6.3.

The mean (SEM) ciliary beat frequency for the samples exposed to isoflurane was 13.7 (0.24) Hz and that of the controls was 13.4 (0.31) Hz. This difference was not statistically significant (unpaired t test $p = 0.26$).

Table 6.4 Effects of prolonged 0.2-0.4% isoflurane on ciliary beat frequency

Treatment	no. of measurements	mean CBF, Hz	SEM CBF, Hz
isoflurane	58	13.7	0.24
control	52	13.4	0.31

Fig. 6.3 Effects of prolonged 0.2-0.4% isoflurane on ciliary beat frequency



Discussion

We have demonstrated a dose dependent reduction in ciliary beat frequency measured *in vitro* during exposure to the three inhalation anaesthetic agents, halothane, enflurane and isoflurane.

The difference reached statistical significance at 0.5 MAC of isoflurane and at 1.0 MAC of halothane and enflurane. However, of more importance is a clinically significant reduction in ciliary beat frequency. It has been hypothesised earlier that a reduction in the ciliary beat frequency of around 2 Hz is likely to be of clinical importance in terms of significantly reducing mucus transport rate (chapter 2). On the basis of this, there is a clinically significant reduction in ciliary beat frequency with 1.0 MAC of enflurane and isoflurane and with 2.0 MAC of halothane. These represent concentrations of the anaesthetics used in clinical anaesthetic practice.

The differences between the effects of the three agents studied reached statistical significance at a number of the concentrations investigated; however, the differences in ciliary beat frequency during exposure to the three agents was modest and less than 2 Hz. On the basis of these results we have not found a clinically significant difference between the three agents on ciliary beat frequency.

The reduction of ciliary beat frequency we have found in this experiment using 3 MAC of the inhalation agents is around 25%. This is similar to the reductions of 23 - 33% found in the washout experiment with 3 MAC described in chapter 5 (expts 5.4-5.5).

Previous work from our laboratory using a different method investigated the effects of halothane on human nasal brushings (Gyi, 1994). In this study there was a depression of ciliary beat frequency with 1.8% halothane, equivalent to 2.5 MAC, of 25% after one hour.

There are few previous studies from other workers of the effects of inhalation anaesthetic agents upon ciliary beat frequency with which to compare this investigation; however, these other studies found similar reductions in ciliary beat frequency. Using rabbit trachea Lee found reductions in ciliary beat frequency with 1, 2, and 3 MAC of halothane of 7, 17 and 22% (Lee, 1980). This compares with the reductions we have found at these concentrations of 9, 17 and 25% respectively. In the case of enflurane, these workers found reductions in ciliary beat frequency at 1, 2 and 3 MAC of 9, 20 and 26% which compares with are findings of 20, 20 and 26 % reductions. There is no previous work investigating isoflurane.

We have not found any significant effects upon ciliary beat frequency *in vitro* of 0.2-0.4% isoflurane administered for a period of 48-72 hours. This experiment has a 80% power of detecting differences in ciliary beat frequency of more than 1.6 Hz at the 5 % level of significance. As discussed previously (chapter 2) it is unlikely that differences in ciliary beat frequency of less than 2 Hz are associated with clinically significant reductions in mucus transport rate.

We did not measure the isoflurane concentration of the medium bathing the turbinate explants in the incubator because of technical problems of obtaining air tight seals. We are confident that the CO₂ from the gas supply had readily diffused into the medium as shown by the colour indicator of the buffer in the medium. Therefore we have assumed that the isoflurane in the chamber had similarly equilibrated with the medium.

In summary we have found that concentrations of halothane, enflurane and isoflurane as used in clinical anaesthetic practice significantly reduce ciliary beat frequency *in vitro*, but sedative concentrations of isoflurane administered for two to three days *in vitro* do not affect the ciliary beat frequency.

Chapter 7

Effects of morphine on respiratory cilia

Introduction

Chest infections commonly develop following surgical operations and may complicate a patient's recovery whilst on the ITU. Opioids are potent analgesics that form the mainstay of perioperative pain relief and are widely used as sedatives in the ITU. One of the most important defences against respiratory tract infections is mucociliary clearance which has been shown to be reduced by opioids. A variety of such agents, both natural and synthetic, are available and one of the commonest used in these circumstances is morphine.

An ingenious study by Hibma and colleagues in 1942 demonstrated quite clearly that morphine reduces the rate of mucus transport in the lungs (Hibma, 1942). They studied a patient with a chronic bronchopleural fistula, instilling dye into the bronchus and timing its appearance in the sputum. Compared with control values morphine increased the time required for the dye to reach the sputum.

In a study of anaesthetised cats in which barium sulphate was insufflated into the lungs, Van Dongen found that the administration of 0.5 mg kg^{-1} of subcutaneous morphine delayed the clearance of the radiological marker by a factor of two to three times (Van Dongen, 1953).

Using a radioactive droplet technique, Forbes and Horrigan measured mucociliary flow in the trachea in dogs. Compared to those ventilated with 40 % nitrous oxide in air the addition of 6 mg kg⁻¹ of intravenous morphine reduced mucus transport rates to 70 % of controls. This reduction was similar to that found with 1.2 MAC halothane (Forbes, 1977).

Since mucociliary clearance is an important respiratory defence mechanism, its impairment by morphine may have relevance in the periods after surgery and in the ITU. The mechanisms by which morphine reduces mucus transport rates have not been elucidated. The rate of mucus transport depends upon the volume and physical properties of the mucus and upon the function of the cilia.

Using human nasal cytological brushings, we have investigated the effects of morphine upon respiratory ciliary function by the measurement of the ciliary beat frequency *in vitro*, and we have also determined opioid receptor binding in these specimens.

Experiment 7.1 Effects of morphine on human respiratory ciliary beat frequency *in vitro*

Methods

Samples of ciliated epithelium were obtained from seven non-smoking healthy patients, (six female), mean age (range) 42.4 (28-56) years. None of the patients was taking any medications, or had contracted an upper respiratory tract infection within the past four weeks. No premedication was given. The samples were acquired by passing a nylon bronchoscopic cytology brush over the inferior nasal turbinates after the patient had undergone anaesthetic induction with 2-3.3 mg kg⁻¹ of propofol. The tissue was removed from the brush by agitation in HBSS.

The ciliated samples from any one individual were placed between the coverslips of paired perfusion chambers as described in chapter 3. Each chamber was perfused from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. The HBSS was aerated at 1000 ml min⁻¹ and perfused the chambers under the effect of gravity at 0.5 ml min⁻¹.

To one delivery bottle was added morphine to provide a concentration of 10 µM. The connections from the delivery bottles to the perfusion chambers were achieved in a manner to blind the observer to which chamber was perfused with the morphine, as both delivery tubes were wound around one another within a countercurrent water jacket. The bottles of HBSS were immersed in a water bath at 40 °C that also flowed through the countercurrent heat exchanger surrounding

the delivery tubing. The perfusion chambers were maintained at 36.8 ± 0.4 °C by means of a thermostatically-controlled heating element on the underside of the chamber.

To confirm that morphine from the delivery bottle was reaching the chamber in a sufficient concentration we sampled the perfusate downstream of the perfusion chamber and measured the morphine concentration by high pressure liquid chromatography (HPLC). We used an 18C column, 25cm in length with phosphate buffer at pH 2 running at 1ml min^{-1} and electrochemical detection with a carbon electrode. The lower limit of this assay for morphine was of the order of 1 ng ml^{-1} (Venn, 1990).

For the measurement of ciliary beat frequency we used the photometric method as described previously in chapter 3, the voltage changes across the photodetector being digitised and mathematically processed to give a power spectrum. The peak of the power spectrum was taken to represent the ciliary beat frequency.

Acceptable ciliated edges for measurement were deemed to be those free of mucus and at least $60\text{ }\mu\text{m}$ long. Readings were taken from ciliated edges from both chambers before addition of the morphine and one, two, three and four hours after one chamber had morphine added by analysing as many acceptable ciliated edges as possible from a minimum of six to a maximum of twelve from each chamber in each defined time band.

At the end of each experiment it was determined which chamber had received the morphine and the samples were discarded. The data were analysed by two-way repeated measures analysis of variance with significance taken at $p < 0.05$.

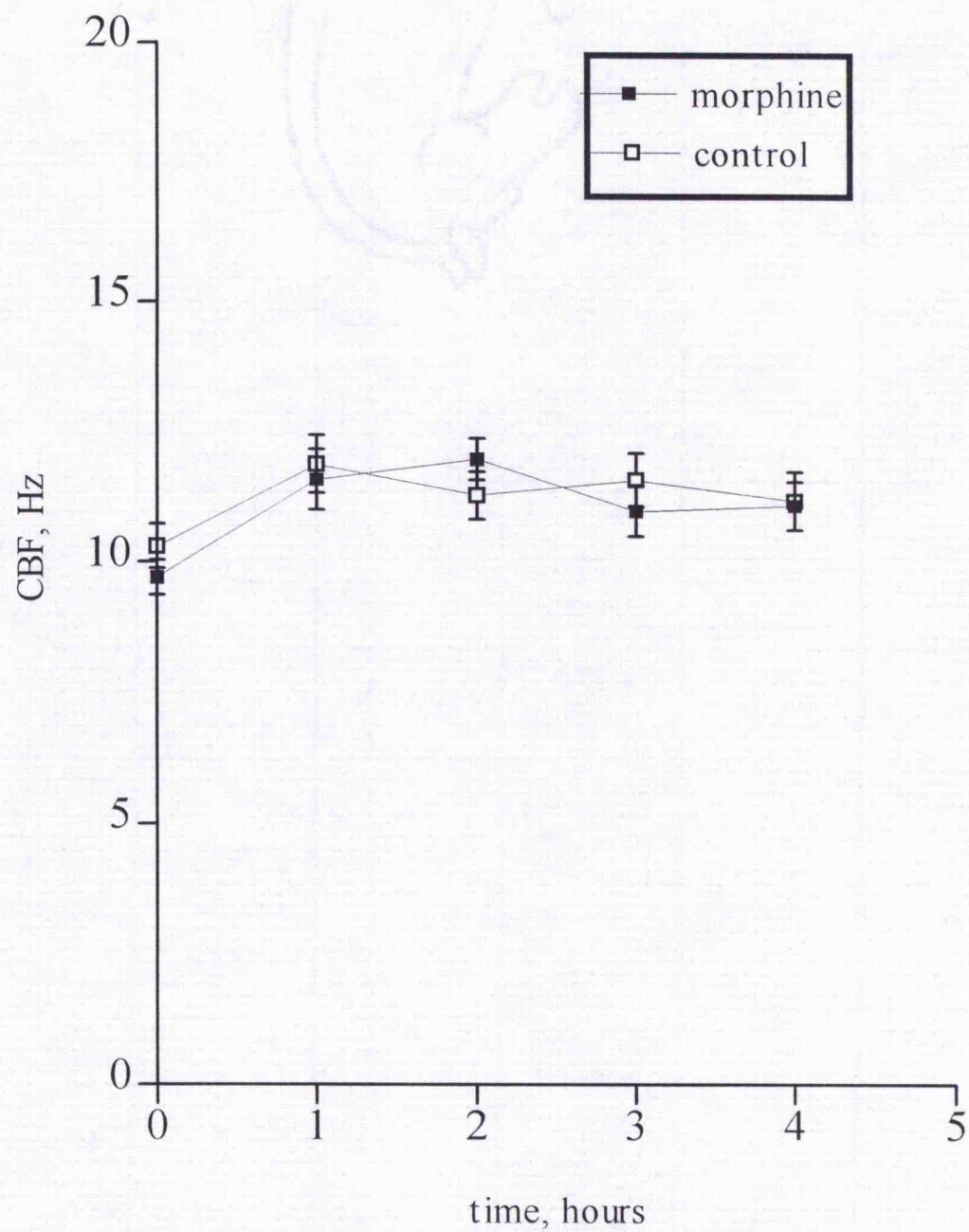
Results

These are summarised in Table 7.1 and displayed in Figure 7.1. There were a total of 739 measurements of ciliary beat frequency : 374 from the cilia exposed to morphine and 365 from the controls. The mean(SEM) ciliary beat frequency of the samples exposed to morphine was 11.1 (1.1) Hz and that of the controls was 11.3 (1.2) Hz (two-way repeated measures analysis of variance, $F=0.61$, $p=0.66$). There was no significant effect of morphine on ciliary beat frequency measured *in vitro* with human nasal cytological samples.

Table 7.1 Effects of morphine on ciliary beat frequency(CBF)

Time, hours	ciliated edges measured	CBF, mean(SEM), Hz
<u>Morphine</u>		
0	36	9.70(0.33)
1	37	11.6(0.58)
2	37	12.0(0.40)
3	38	11.0(0.49)
4	37	11.1(0.47)
<u>Controls</u>		
0	36	10.3(0.43)
1	36	11.9(0.56)
2	37	11.3(0.46)
3	36	11.6(0.52)
4	36	11.2(0.56)

Fig 7.1 Effects of morphine on mean (SEM) ciliary beat frequency (CBF)

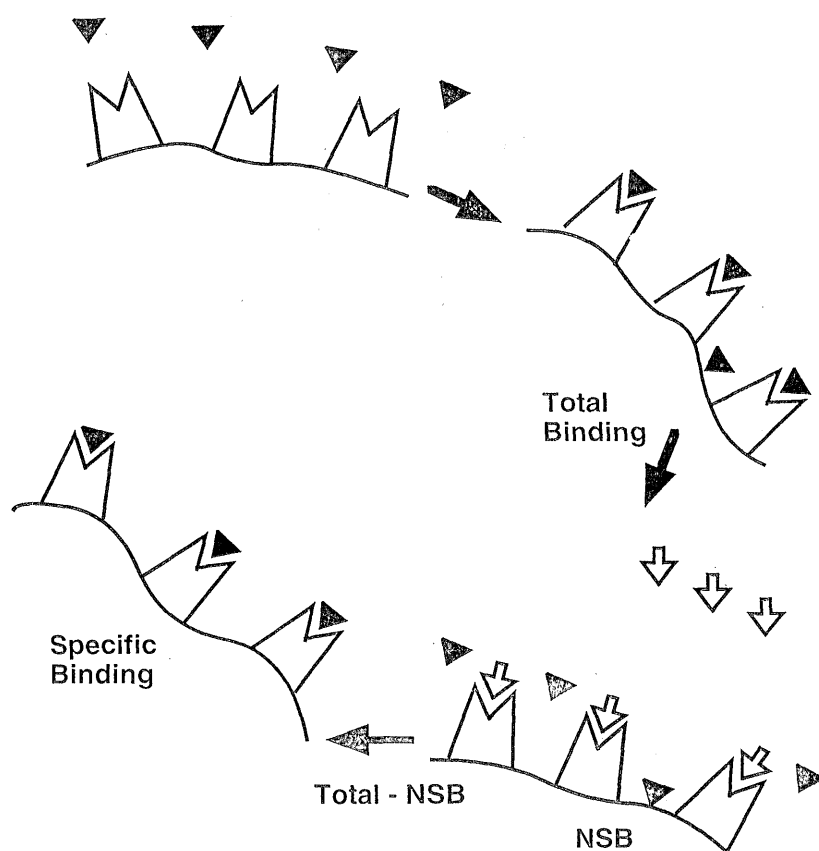





Opioid receptors on nasal cilia

To comprehensively investigate the effects of morphine upon respiratory cilia we determined whether opioid receptors were present on these cells using radioligand binding studies.

The non-specific opioid antagonist, tritiated diprenorphine ($[^3\text{H}]$ DPN) was used. This binds to all three types of opioid receptors in addition to non-opioid cellular proteins. To determine whether opioid receptors were present on cilia, the total binding of $[^3\text{H}]$ DPN to ciliated epithelial cell membranes and the non-specific binding to non-opioid cellular proteins were measured. The non-specific binding was measured by displacing the ligand from opioid receptors with excess of the opioid antagonist, naloxone. The presence of opioid receptors was determined by the difference between the total binding and non-specific binding representing specific opioid binding (Fig 7.2).

Fig 7.2 Specific and non-specific opioid binding



-  Opioid receptor
-  Radiolabelled ligand
-  Naloxone
- NSB** Non-specific binding

Expt 7.2 Binding of tritiated diprenorphine, [³H]DPN, to human nasal ciliated epithelial cell membranes

Methods

Nasal ciliated epithelial cells were collected on two separate occasions. This involved passing a bronchoscopy brush over the inferior nasal turbinates of 10 healthy patients on each occasion after they had received an anaesthetic induction with 2-3.3 mg kg⁻¹ of propofol. The samples were removed from the brushes by agitation in M199.

The tissue was washed in 50 mM Tris HCl/100mM NaCl buffered to pH 7.4. The tissue was homogenised using three 15 second bursts with a tissue tearor and then centrifuged at 13500 rpm for 10 minutes at 4 °C. This process of homogenisation and centrifugation was repeated three times and the cell membranes were then resuspended in 2 mls of the above buffer.

100 µL of the membranes were aliquoted into 12 test tubes. Binding studies were performed in 1 ml assay volumes of TrisHCl containing 200 µL of [³H]DPN. Non-specific binding (NSB) was defined in the presence of excess (10 µM) naloxone. The binding was equilibrated for 60 minutes at 37 °C (Table 7.2).

Bound and free radioactivity were separated by rapid vacuum filtration using a Brandel cell harvester onto Whatman GF/B filters and washed with three 4 ml aliquots of ice cold buffer. Radioactivity was extracted overnight. The 12 test tubes a control tube containing 200µL of the [³H]DPN were measured by liquid scintillation spectroscopy with Optiphase-X (LKB Wallac).

Table 7.2. Experimental protocol for binding of [³H]DPN to cilia, figures in μ L

Tube	Buffer	DPN	Naloxone	Membrane
Total binding	700	200	-	100
Non-specific binding	500	200	200	100

Results

The radioactivity of the controls containing 200 μ mol [³H]DPN was 321,900 and 370,796 dpm which corresponds to 3.7 - 4.3 nM of DPN. The results are presented in Table 7.3. The mean(SEM) dpm of the total and non-specific binding were 9036(328) and 9130(379) respectively. These values were not significantly different indicating no specific binding (paired t test, $t=0.22$, $p=0.83$).

Table 7.3 . Binding of [³H]DPN to human nasal ciliated cell membranes

No. of patients		DPN binding(dpm)	
		Total	NSB
Expt 1	10	8235(299)	8106(771)
Expt 2	10	9837(664)	10153(193)
Mean	20	9036(328)	9130(379)

Data are mean(SEM) for 6 determinations of total and NSB in each experiment. Mean values are for 12 determinations.

Discussion

There was no significant effect of morphine on ciliary beat frequency measured *in vitro* and there were no significant opioid receptor numbers on nasal ciliated epithelium.

The variability in our measurements of ciliary beat frequency gives a computed power of 80 % to detect differences greater than 2 Hz at the 5 % level of significance.

The concentration we investigated, 10 μ M represents a concentration at the upper limit of the range found in clinical practice, being the plasma concentration found in patients receiving up to 1500 mg of morphine per day for the treatment of cancer pain (Goucke, 1994).

We found a small initial rise in ciliary beat frequency in both the control and treatment groups. This may have been due to effects of the sampling of the tissue involved when placing it in the chamber or to a delay in the chamber medium equilibrating with temperature.

There are few comparable studies on the effects of morphine on ciliary beat frequency. Rutland and workers measured the ciliary beat frequency of human nasal brushings with a photometric technique after premedication with the opioid papaveretum and atropine (Rutland, 1982). They found the mean(SD) ciliary beat frequency in the premedicated patients was 11.4(1.4) Hz compared with that found in a group of awake and unpremedicated controls of 13.1(1.9) Hz and this difference was statistically significant ($p < 0.001$).

Roth and workers used a similar method to directly measured ciliary beat frequency *in vitro* from nasal brushings and compared the same patients before and after the administration of an opiate-containing premedication (Roth, 1991). The patients received a cocktail of 5-10 mg of intramuscular morphine, 1mg of intramuscular atropine and 2-3 ml of 2 % lignocaine topically applied to the nose. The authors found a small but significant reduction in ciliary beat frequency after the medication from a mean(SD) of 12. (1.6) Hz to 11.0(2.1) Hz.

In both these studies the addition of other drugs limits interpretation. The effects of lignocaine on ciliary beat frequency *in vivo* remain unclear. Although *in vitro* studies with lignocaine have demonstrated a depressant effect at concentrations as low as 0.25 % (Ingels, 1994); *in vivo* studies with concentrations as high as 4% have failed to show any effects (Rutland, 1982). Furthermore, in a study measuring mucus transport rates with Teflon discs in sheep, Landa and colleagues found no difference after the tracheal instillation of 10 mls of 2 % lignocaine (Landa, 1975).

Atropine is known to inhibit mucus transport rates in humans (Groth, 1991); however, this appears to be due to inhibition of mucus secretion and there is little direct evidence for a depressant effect on the cilia (Ingels, 1992).

The amount of [³H]DPN added to each aliquot of membrane corresponded to 3.7- 4.3 nM which is well above the binding affinity constant of 0.17 nM (Lambert, 1993) and thus the majority of the tissue receptors would be occupied. Using [³H]DPN, we have failed to demonstrate the presence of opioid receptors on nasal ciliated epithelial cell membranes. The sensitivity of this assay is dependent on the purity of the tissue source. We were careful to minimise contamination of the cytological brushings with blood since red blood cells possess opioid receptors.

The two studies on respiratory cilia , the measurement of ciliary beat frequency and opioid binding of nasal cilia do not support an effect of morphine on human respiratory ciliary function.

The mechanisms by which morphine reduces mucus transport are currently unknown. There may be effects upon the mucus or *in vivo* effects upon the cilia. Rubin and colleagues found that lower spinability was associated with narcotic induction in an investigation of the physical properties of mucus in anaesthetised volunteers and this would be associated with reduced transport rates (Rubin, 1990; Sleigh, 1988). In an *in vitro* study using human bronchi removed at lung resection morphine was shown to reduce mucus secretion stimulated by capsaicin (Rogers, 1989) and one could speculate that under anaesthesia, which involves procedures and agents that are irritant to the airways, morphine might similarly inhibit mucus secretion.

Although there is no evidence for the neural control of ciliary function in humans, there are free nerve endings adjacent to the basement membrane (Rhodin, 1966). Morphine may have an effect on cilia *in vivo* due to effects on neural connections which are absent from *in vitro* preparations.

Chapter 8

Effects of propofol on human respiratory cilia

Introduction

Propofol is an intravenous anaesthetic agent that was introduced into clinical practice in 1985. It is an alkyl phenol, 2,6-diisopropyl phenol, that exists as an oil at room temperature. It is formulated for clinical administration in an aqueous solution of soya bean oil, glycerol and egg phosphatide that is known as intralipid. It is a popular agent for inducing anaesthesia as it provides a smooth induction compared with other agents due to the depression of upper airway reflexes. It is metabolised to inactive glucuronide and sulphate conjugates and there are no significant alterations in pharmacokinetic parameters in patients with renal or hepatic disease. The drug has a large volume of distribution and clearance rate and as a result it is used as an anaesthetic induction agent especially for day case procedures in which rapid recovery is required, as an intravenous infusion for the maintenance of anaesthesia during surgery and as an intravenous infusion for sedation in the ITU because it is non-cumulative.

As discussed earlier (chapter 1), chest infections are common after surgery and in patients in the ITU. Although this is due to a number of factors, mucociliary clearance is an important respiratory defence that is known to be impaired by a variety of inhalation and intravenous anaesthetic agents. In previous chapters we have shown that the inhalation anaesthetic agents reduce ciliary beat frequency, an important determinant of respiratory mucus clearance. There are currently no data on the effects of propofol on mucociliary clearance and so we have investigated the effects of propofol on respiratory tract cilia.

Expt 8.1 Effects of short-term propofol on ciliary beat frequency *in vitro*

Methods

Ciliated explants from the nasal turbinates of 12 patients scheduled for nasal turbinectomy with a primary diagnosis of tissue hypertrophy were prepared as described previously (chapter 4). There was no history of allergy. The ciliated samples were placed between the coverslips of paired perfusion chambers as described in chapter 3. Each chamber was perfused from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. The HBSS was aerated at 1000 ml min^{-1} and perfused the chambers under the effect of gravity at 0.5 ml min^{-1} . The perfusion chambers were maintained at $36.8 \pm 0.4 \text{ }^{\circ}\text{C}$ by means of a thermostatically-controlled heating element on the underside of the chamber.

Measurements of ciliary beat frequency were obtained from the samples in each chamber using the transmitted light technique as detailed in chapter 3. The ciliary movements were recorded for a period of 15 seconds and the voltage changes across the photodetector were digitised and mathematically processed to give a power spectrum. The peak of the power spectrum was taken to represent the ciliary beat frequency.

Acceptable ciliated edges for measurement were deemed to be those free of mucus and at least $60 \text{ }\mu\text{m}$ long. Readings were taken from ciliated edges in both chambers to provide baseline readings. Propofol in 1% dimethyl sulphoxide (DMSO) was then added to one of the chambers to provide a concentration of $70 \text{ }\mu\text{M}$ and DMSO was added to the other chamber to provide a concentration of 1%. The connections from the delivery bottles to the perfusion chambers were

achieved in a manner to blind the observer to which chamber was perfused with the propofol, as both delivery tubes were wound around one another within a countercurrent water jacket.

The ciliary beat frequency was measured at 30 and 90 minutes during drug perfusion and then the delivery chambers were changed for those containing medium without propofol and after a half hour period of washout the ciliary beat frequency was again measured. We analysed as many acceptable ciliated edges as possible from a minimum of six to a maximum of twelve from each chamber in each defined time band.

At the end of each experiment it was determined which chamber had received the propofol and the samples were discarded. The data were analysed by MANOVA for repeated measures with significance taken at $p < 0.05$.

Results

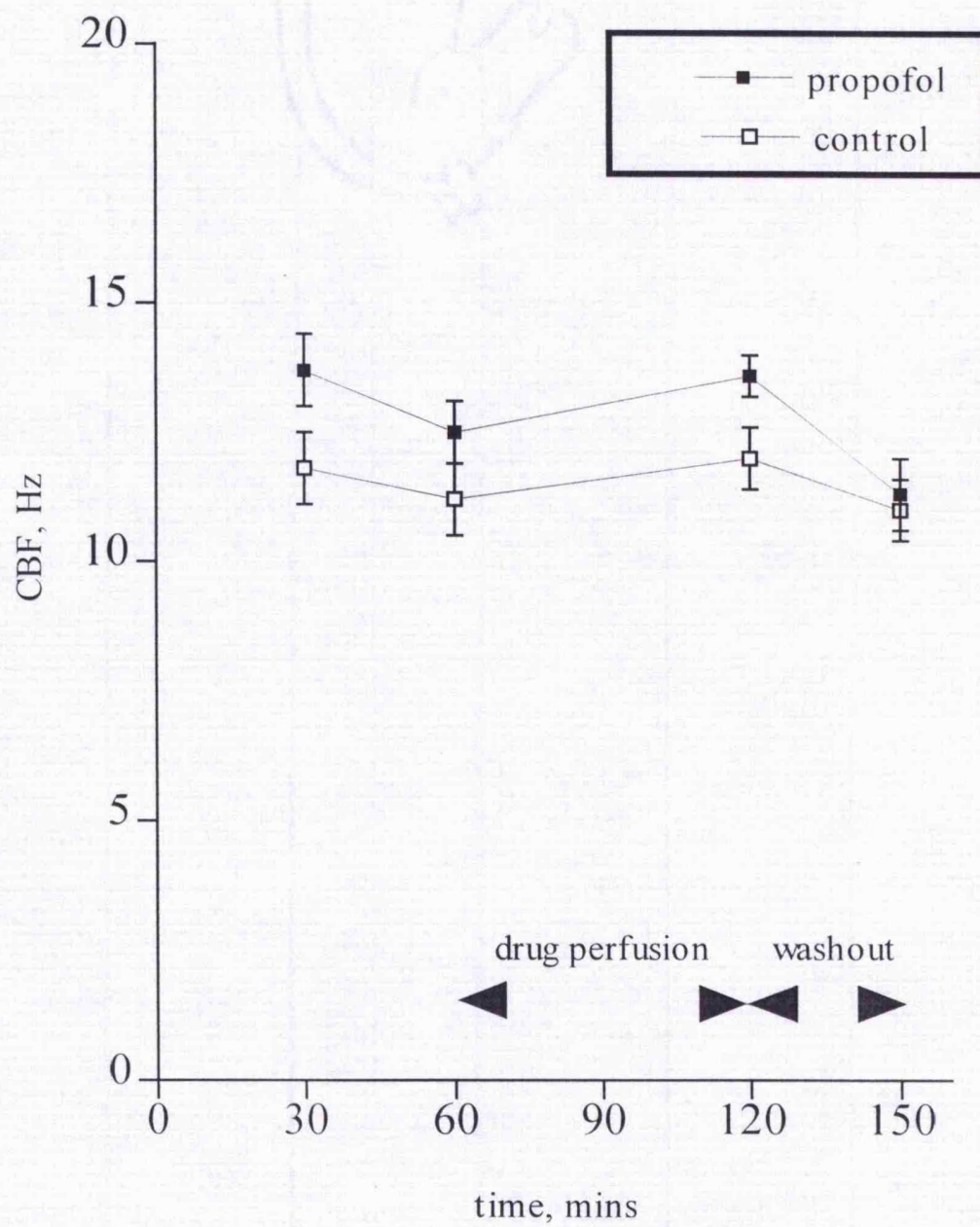
The results are summarised in table 8.1 and displayed in figure 8.1.

There was no significant effect of propofol on ciliary beat frequency compared to the controls (MANOVA for repeated measures, $p=0.310$)

Table 8.1 Effects of short-term propofol on ciliary beat frequency (CBF)

	time, mins	Mean CBF, Hz	SEM CBF, Hz	Ciliated edges measured
<hr/>				
<u>Control</u>				
	30	11.8	0.7	58
	60	11.2	0.7	60
	120	12.0	0.6	59
	150	11.0	0.6	59
<hr/>				
<u>Propofol</u>				
	30	13.7	0.7	59
	60	12.5	0.6	60
	120	13.6	0.4	55
	150	11.3	0.7	56
<hr/>				

Fig 8.1 Effects of propofol on mean (SEM) ciliary beat frequency (CBF) *in vitro*



Expt 8.2 Effects of incubation of cilia with a sedative concentration of propofol for three days

Introduction

Propofol is commonly used as a sedative over a number of days for patients who are ventilated on the ITU. To investigate the effects of prolonged exposure to propofol on cilia we incubated the ciliated turbinate explants with a sedative concentration of propofol *in vitro* for three days.

Methods

Human nasal turbinate explants were prepared and incubated in CO₂-buffered culture medium at 37 °C as described previously (chapter 4). Samples of variable storage period of up to six days were divided between two glass Petri dishes containing 30 ml of culture medium. To one dish was added 105 µL of propofol dissolved in 1 % DMSO to provide a concentration of 20 µM of propofol. To the other dish was added 105 µL of DMSO to act as a control. The dishes were incubated with CO₂- buffered medium at 37°C. At 24 hour intervals the medium was refreshed. We obtained four turbinate explants from each of ten patients. Two of the explants from any one patient were exposed to propofol and two to DMSO alone.

The propofol concentration after 24 hours of incubation was measured by fluorimetry (Plummer, 1987). Samples of the old medium and the fresh medium were assayed for propofol with a Perkins Elmer LS50B Fluorimeter using excitation and emission wavelengths of 276nm and 310 nm respectively.

72 hours after incubation with propofol or DMSO alone the ciliated tissue was transferred to paired perfusion chambers described previously for the measurement of ciliary beat frequency (chapter 3).

The chamber containing the tissue that had been incubated with propofol was perfused with HBSS to which was added propofol dissolved in DMSO to provide a concentration of 20 μ M propofol. The other chamber, containing the control tissue, was perfused with HBSS to which was added DMSO at 1/300 v/v %. The perfusate was flowing at 0.5 ml min⁻¹ and heated to 36.8 \pm 0.4 °C.

The ciliary beat frequency was measured as described previously by the transmitted light technique (chapter 3). Measurements were taken within 30 minutes after transfer to the perfusion chamber and up to ten readings of ciliary beat frequency were taken from each turbinate explant. Data were analysed by unpaired t-test with significance taken at $p < 0.05$

Results

The results of the fluorimetric analysis are shown in table 8.2. There was minimal loss of propofol from the containers.

There was no significant difference between the mean (SEM) ciliary beat frequency of the samples incubated with propofol 12.4(0.3) Hz compared with the controls 12.6(0.2) Hz (t-test, $z=1.07$, $p=0.28$) (Table 8.3)

A significant number of explants incubated with propofol were found to have no cilia present compared with the controls (Chi-squared test, $\chi^2=9.82$, $p < 0.01$) (Figure 8.2).

Table 8.2 Fluorimetric assay of propofol in dishes incubating cilia (n=3)

Standard	Sample	Control
264	234.2	-1.5
212	166	5.4
202	188.6	2.5

Table 8.3 Ciliary beat frequency of cilia incubated with 20 μ M propofol

	Number of edges measured	CBF, Hz	SEM, Hz
Propofol	105	12.4	0.3
Control	192	12.6	0.2

Fig 8.2 Ciliary beat frequency (CBF) of cilia incubated with 20 μ M propofol, mean (SEM)

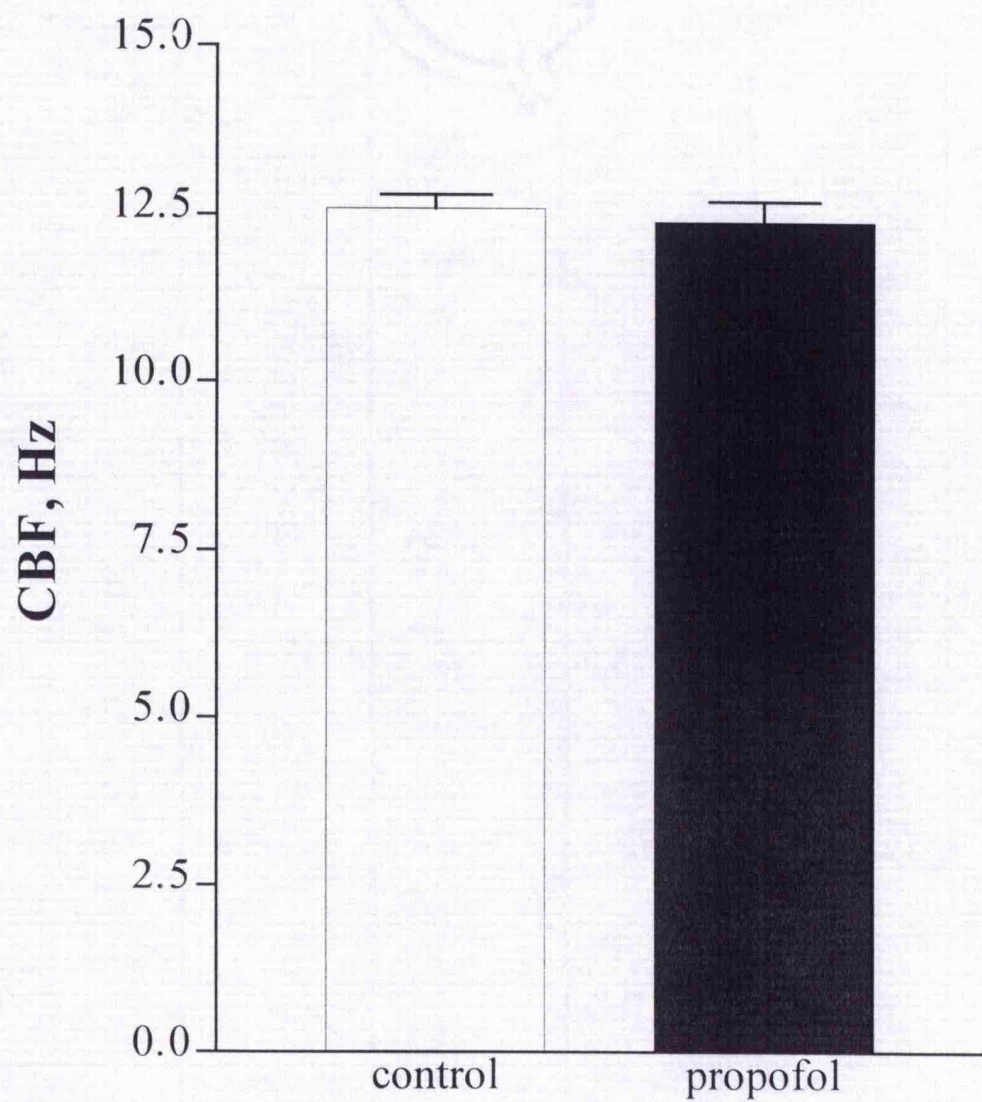
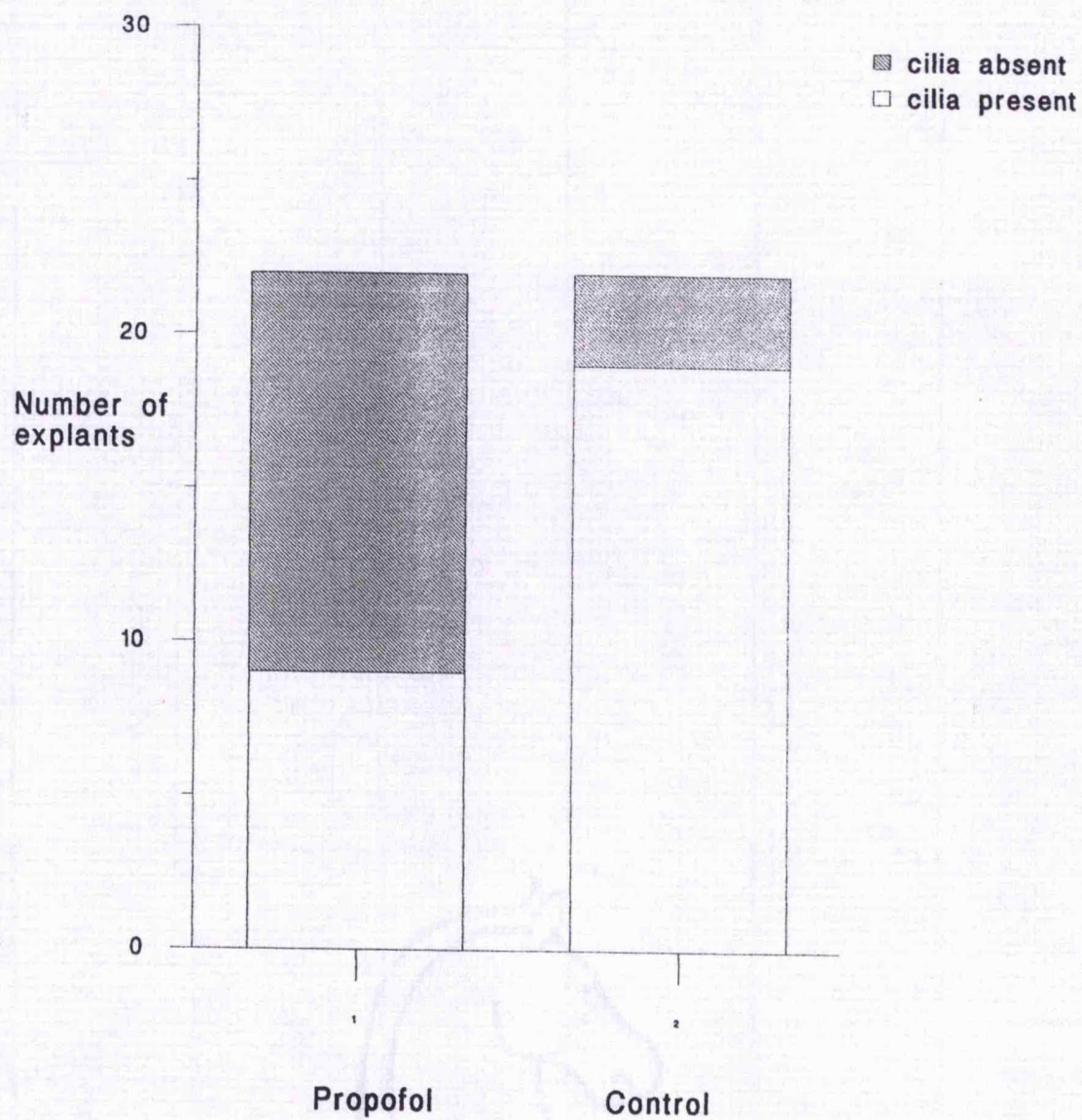


Figure 8.3 Survival of cilia incubated with 20 μ M propofol



Expt 8.3 Effect of propofol concentration on survival of human respiratory cilia incubated for up to 72 hours.

Introduction

In view of the unexpected effect of a sedative concentration of propofol that we found on the survival of the cilia, this was investigated further. We used a different method involving a dose-ranging study and used cilia survival as our primary endpoint.

Methods

Explants from human nasal turbinates were obtained from patients undergoing turbinectomy for a primary diagnosis of tissue hypertrophy and prepared as previously described in chapter 4. On the day after surgical removal the samples were checked for viable ciliated edges using the Nikon Diaphot microscope at 600x magnification and then incubated in glass test tubes with serial concentrations of propofol dissolved in less than 1% DMSO at 3, 10, 30 and 100 and 1000 μM together with culture medium as used for explant maintenance (chapter 4). A control group consisted of explants from the same patients incubated in 1% DMSO and culture medium. The explants were examined for the presence or absence of cilia at 48 and 72 hours after incubation and each explant was defined as either having or not having any visible cilia. Between 18 and 21 explants were exposed to each concentration.

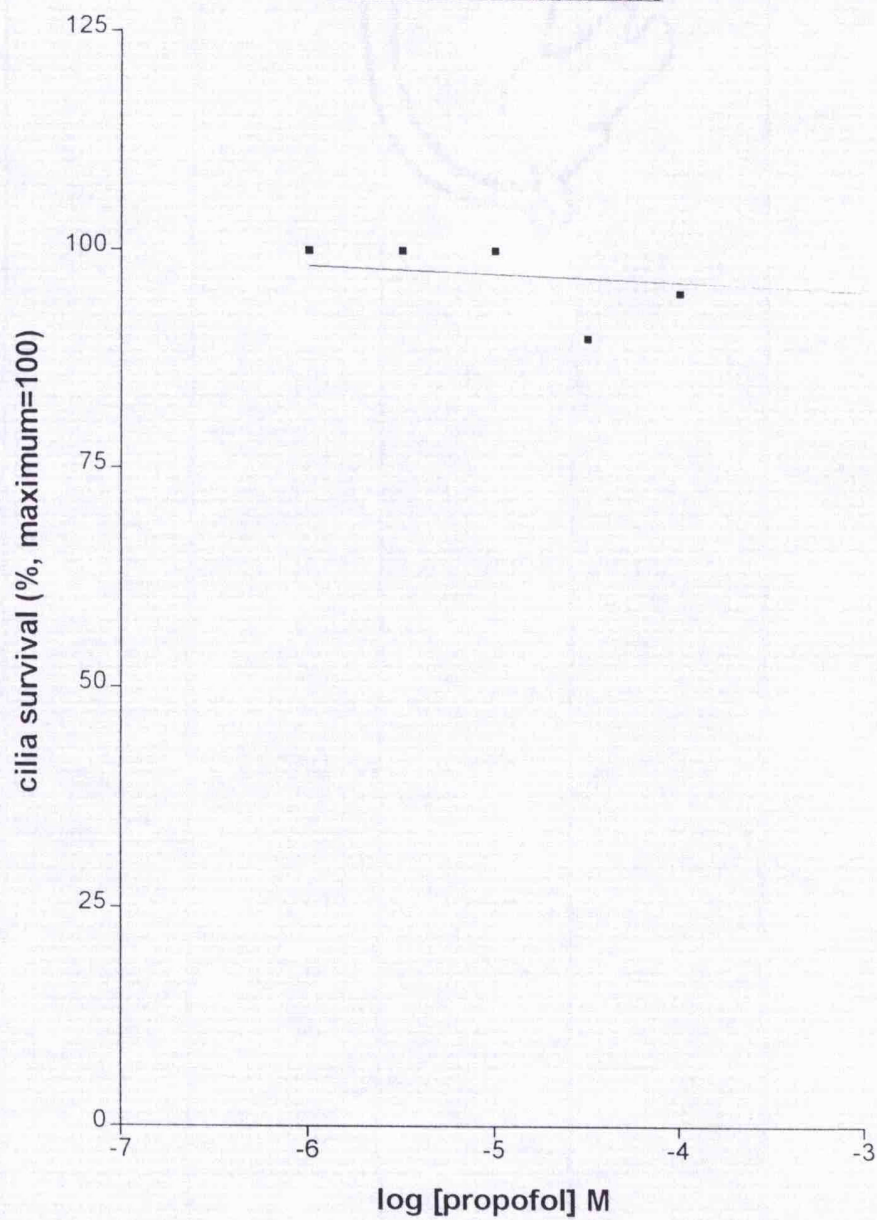
Results

There was no significant effect of propofol incubation on cilia survival (Table 8.4; Figs 8.4 and 8.5).

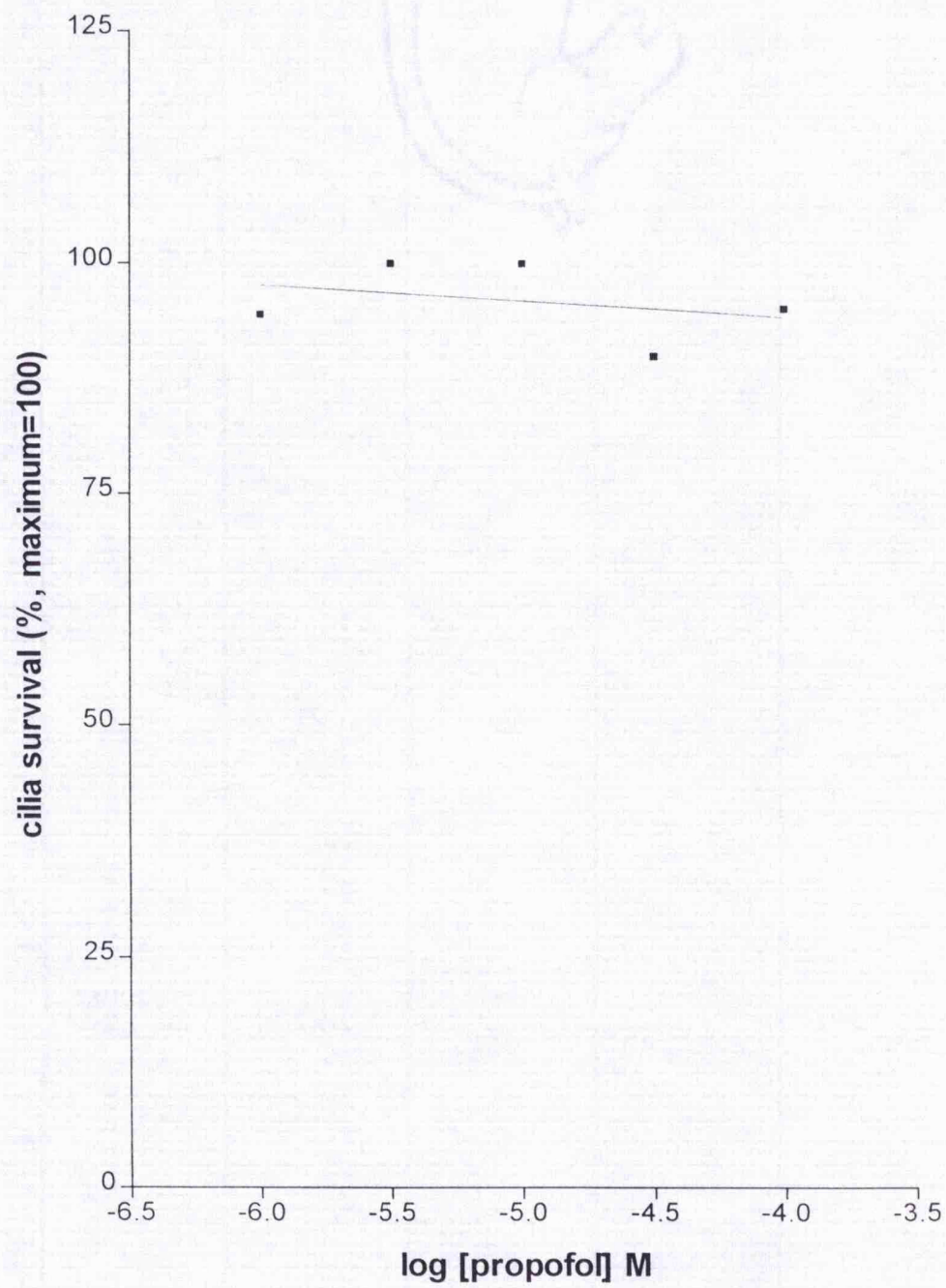
Table 8.4 Effects of propofol on cilia survival

Propofol, μ M	Surviving explants		Total number of explants
	48 hrs	72 hrs	
0	17	17	18
1	18	17	18
3	18	18	18
10	18	18	18
30	18	18	20
100	20	20	21

**Fig 8.4 Effects of propofol incubation for 48 hours
on cilia survival in vitro**



**Fig 8.5 Effects of propofol incubation for 72 hours
on cilia survival in vitro**



Expt 8.4 Neurotoxicity of propofol in SH-SY5Y cells measured by inhibition of thiazoyl blue reduction

Introduction

In view of the toxic effects we found upon the cilia in experiment 8.2, we have investigated the effects of long term incubation with a propofol upon another cell type, neuronal SH-SY5Y. This cell clone was available to us in the laboratory and afforded the opportunity to use a validated and sensitive method of cellular toxicity and mirror the *in vitro* ciliary system.

3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazoyl blue (MTT) is absorbed by cells and metabolised by the mitochondrial enzyme, succinate dehydrogenase. MTT is converted to an insoluble, coloured formazan dye and its reduction indicates mitochondrial integrity and activity which in turn reflects cell viability. The ability to reduce MTT compared to untreated controls enables the relative toxicity of compounds to be assessed. Mitochondrial activity (viable cell number) is proportional to the intensity of coloured dye measured spectrophotometrically (Willets, 1995).

Methods

Undifferentiated human SH-SY5Y neuroblastoma cells (a gift from J.L.Biedler) were maintained in 80 cm² culture flasks using minimum essential medium, supplemented with foetal calf serum (10 % v/v), L-glutamine 2 mM, penicillin(100 IU/ml), streptomycin (100µg/ml) and incubated at 37 °C, in 5% CO₂ / humidified air. Confluent cultures were routinely harvested by trypsinisation (0.05 %) and split at 1:10. Cells were harvested and seeded in 48 glass Petri dishes at a density of 1 x 10⁵ cells/well. Propofol at concentrations

ranging from 3 μ M to 1 mM was incubated with SH-SY5Y cells 3 days after seeding and incubated for up to 72 hours.

After 72 hours, MTT (0.12 mM, in HBSS) was added to all cells and left for one hour at 37 °C. The resulting dye was extracted with acidified isopropanol and the absorbance measured spectrophotometrically at 550 nm with reference at 620 nm on an Anthos 2001 plate reader.

Potential interaction between MTT and propofol was determined by the addition of the highest concentration of propofol alone, in media without cells. This represented background absorbance and was subtracted from all samples and MTT reduction was expressed as a percentage of the untreated cells (control minus propofol).

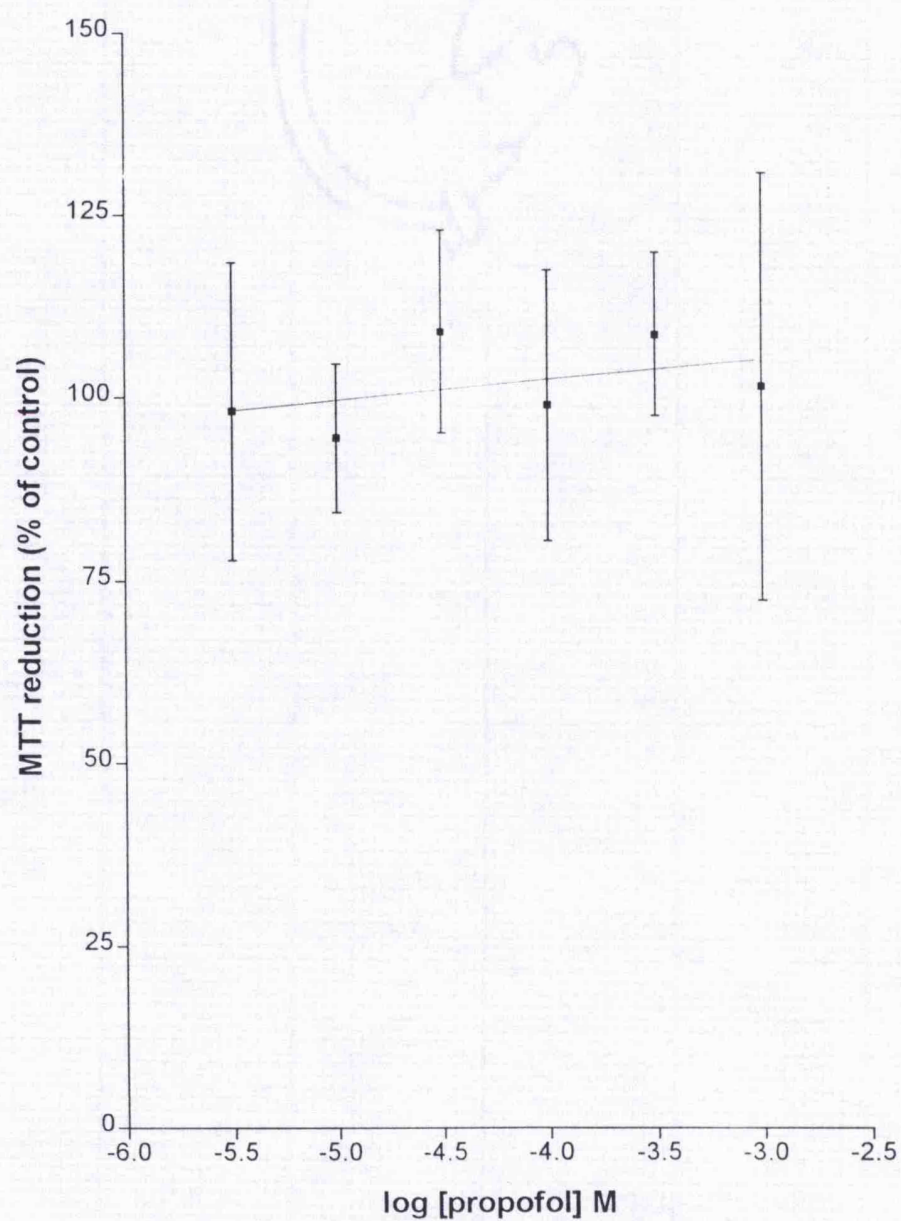
Results

The results are shown in table 8.5 and displayed in figure 8.6. Propofol did not cause a dose related inhibition of MTT reduction in SH-SY5Y cells after 72 hours incubation (ANOVA, $F=0.10$, $p=0.99$).

Table 8.5 Effects of 72 hours incubation with propofol on MTT reduction in SH-SY5Y cells. (C=control; results expressed as absolute values and as percentage of control).

Sample	1	2	3	4	5	6
C	0.763	0.820	0.680	0.336	0.611	0.500
%	100	100	100	100	100	100
3	0.307	0.363	0.924	0.56	0.565	0.547
%	40.2	44.3	135.9	166.7	92.5	109.4
10	0.786	0.384	0.663	0.401	0.572	0.533
%	103.0	46.8	97.5	119.3	93.6	106.6
30	0.434	0.823	0.841	0.540	0.613	0.563
%	56.9	100.4	123.7	160.7	100.3	112.6
100	0.316	0.727	0.740	0.601	0.479	0.489
%	41.4	88.7	108.8	178.9	78.4	97.8
300	0.668	0.687	0.732	0.519	0.564	0.636
%	87.5	83.8	107.6	154.5	92.3	127.2
1000	0.231	0.606	0.294	0.541	0.534	1.072
%	30.3	73.9	43.2	161.0	87.4	214.4

Fig.8.6 Effects of 72 hours incubation with propofol on MTT reduction in SH-SY5Y cells, % reduction(SEM) compared to controls



Discussion

We have investigated the effects of propofol on respiratory cilia *in vitro*, measuring the effects upon ciliary beat frequency and upon cilia survival. We have found that perfusion with 70 μM propofol for 90 minutes has no significant effects on respiratory ciliary beat frequency.

The average plasma concentration during total intravenous anaesthesia with propofol in which a bolus of 2 mg kg^{-1} was followed by a variable infusion of 0-10 $\text{mg kg}^{-1} \text{ hr}^{-1}$ was 35 μM (Frenkel 1993) and we have used a concentration of twice this. The variability found in this experiment results in a posthoc power of 80 % to detect differences in ciliary beat frequency of more than 2 Hz at the 5% level of significance. As discussed in chapter 2, reductions in ciliary beat frequency of less than 20 % or about 2 Hz are probably not associated with significant reductions in mucus transport rate.

Because of its hydrophobic properties the propofol was dissolved in DMSO. In clinical practice, propofol is solubilised in intralipid; however, it is not possible to deliver this mixture *in vitro*. It is possible that the intralipid may have additional effects upon the cilia that are not evident with the parent drug alone.

There is no previous work from others on the effects of propofol on ciliary beat frequency with which to compare our data. In a previous experiment (expt 4.1) we did not find any effect following a bolus dose of 2-3 mg kg^{-1} of propofol used to produce anaesthesia upon ciliary beat frequency measured one hour later. The plasma concentration after such a bolus is around 40 μM (Sebel, 1989)

Our results contrast with the effects we have found with other anaesthetics, the inhalation agents (chapters 5 and 6), where we found a depressant effect on the

ciliary beat frequency. Although the inhalational agents have been shown to reduce mucus transport rates there is no comparable work on the effects of propofol on this parameter. *In vivo* experiments with intravenous barbiturate anaesthesia have shown a reduction in mucus transport. In a study using a fiberoptic bronchoscopic technique in sheep anaesthetised with barbiturates there was a 35 % reduction in tracheal mucus transport (Landa, 1975). However, propofol is structurally unrelated to the barbiturates being a hindered phenol and is not possible to predict its effects upon mucus transport.

Since propofol is commonly administered for prolonged periods of time to provide sedation in the ITU we investigated this by incubating the cilia for up to 72 hours. We conducted two experiments on this that gave contrasting results. In the first experiment (expt 8.2) we found no difference in ciliary beat frequency but an unexpected absence of cilia in the specimens incubated with 20 μ M propofol compared with the controls.

We repeated the investigation into cilia loss using a different method investigating a range of concentrations of propofol (expt 8.3) but found no significant effects. The explanation for this may lie in the fact that the samples used in the first experiment were of a variable storage period of up to six days after surgical excision whereas those in the second experiment were used on the day after surgery. This difference arose because in the first experiment our primary end-point was the ciliary beat frequency, which does not alter during storage of up to ten days. It may therefore be that the cilia of fresh tissue is not susceptible to toxic effects of propofol whereas the cilia of tissue that has been stored for some time, and is presumably in less than optimal condition, is adversely affected by propofol.

To complement this unexpected finding of a toxic effect of propofol upon cilia , we investigated the cytotoxicity of the drug in an alternative tissue preparation that was available to us as a cell line.

There are variety of methods described for the measurement of cellular toxicity. Many detect the loss of membrane integrity by the leakage of proteins, or the exclusion of or take up of dyes; however, this requires a large amount of damage before it can be chemically detected. The most sensitive assess mitochondrial function as an index of cellular viability and such methods are suitable for replicating cell lines with significant levels of mitochondrial activity. In our laboratory we had access to human SH-SY5Y neuroblastoma cells and therefore used these for investigating the potential cellular toxicity of propofol.

The wavelength used to measure the formazan dye product of MTT was 550 nm as the highest absorbance for the dye occurs at this wavelength. The reference measurement was chosen where the formazan dye does not absorb to a high degree, to eliminate interference from the plate, cellular material or any compound that may overlap to increase the absorbance at 550 nm.

We did not find a toxic effect of propofol on these neuronal cells in concentrations up to 1 mM as measured by MTT reduction. This does not negate the findings on the cilia but the toxic effect we have found with propofol does not occur in this neuronal tissue.

The toxic effects of propofol that we found on stored cilia were conducted *in vitro* and one must be cautious as the situation *in vivo* is different. In particular, in life toxic agents are washed away by tissue perfusion whereas in our *in vitro*

experiment there was no such perfusion. Nevertheless this unexpected finding may have relevance in the ITU since many of these patients have critical illnesses with metabolic derangements that may make their tissues more susceptible to toxins. Furthermore, the tissue perfusion in such patients may be poor and the clearance of toxins limited.

Chapter 9

Effects of midazolam on human respiratory cilia

Introduction

Midazolam is a water soluble benzodiazepine with sedative properties. It is used as a sedative for minor surgical procedures and as a continuous intravenous infusion for longer term sedation in the ITU. Chest infections are common occurrences in both of these situations and the role of this sedative agent in such infections is unknown. Mucociliary clearance is one of the most important respiratory defences and a related benzodiazepine, temazepam, has been shown to reduce the respiratory mucus transport rate in healthy volunteers (Hasani, 1992).

We have conducted investigations into the effects of midazolam upon respiratory cilia. We have measured the ciliary beat frequency after 30 and 90 minutes exposure, simulating the use of the drug as a short-acting sedative for minor operative procedures. We have also evaluated the effects of exposure to midazolam for a few days upon the survival of the cilia, simulating its use as a long-term sedative in intensive care.

To complement this investigation on the long term incubation of cilia with midazolam we have also incubated a neuronal cell clone with the drug, allowing us to use a more sophisticated assessment of cellular toxicity by measuring mitochondrial function.

Expt 9.1 Short-term effects of midazolam on human respiratory ciliary beat frequency *in vitro*

Methods

Samples of ciliated epithelium were obtained from the explants of patients undergoing nasal turbinectomy for a primary diagnosis of tissue hypertrophy by the methods previously described in chapter 4.

The ciliated samples were placed between the coverslips of paired perfusion chambers as described previously (chapter 3). Each chamber was perfused at a constant rate of 0.5 ml min^{-1} from a separate delivery bottle of HBSS into the entry port of the chamber and out from its exit port into a collecting beaker. Air flowed through the HBSS at a rate of 1000 ml min^{-1} . The perfusion chambers were maintained at $36.8 \pm 0.4 \text{ }^{\circ}\text{C}$ by means of a thermostatically-controlled heating element on the underside of the chamber.

Measurements of ciliary beat frequency were obtained from the samples in each chamber using the transmitted light technique as detailed in chapter 3. The ciliary movements are recorded for a period of 15 seconds and the voltage changes across the photodetector are digitised and mathematically processed by fast Fourier transformation to give a power spectrum. The peak was taken to represent the ciliary beat frequency.

Acceptable ciliated edges for measurement were deemed to be those free of mucus and at least $60 \text{ }\mu\text{m}$ long. Readings were taken from ciliated edges from both chambers to provide baseline readings. Midazolam was then added to one of the chambers to provide a concentration of $20 \text{ }\mu\text{M}$.

The connections from the delivery bottles to the perfusion chambers were achieved in a manner to blind the observer to which chamber was perfused with the midazolam, as both delivery tubes were wound around one another within a countercurrent water jacket.

The ciliary beat frequency was measured at 30 and 90 minutes during drug perfusion. Then the delivery chambers were changed for those containing medium without midazolam and after a half hour period of washout the ciliary beat frequency was again measured. We analysed as many acceptable ciliated edges as possible from a minimum of six to a maximum of twelve from each chamber in each defined time band.

At the end of each experiment it was determined which chamber had received the midazolam and the samples were discarded. The data were analysed by MANOVA for repeated measures with significance taken at $p < 0.05$.

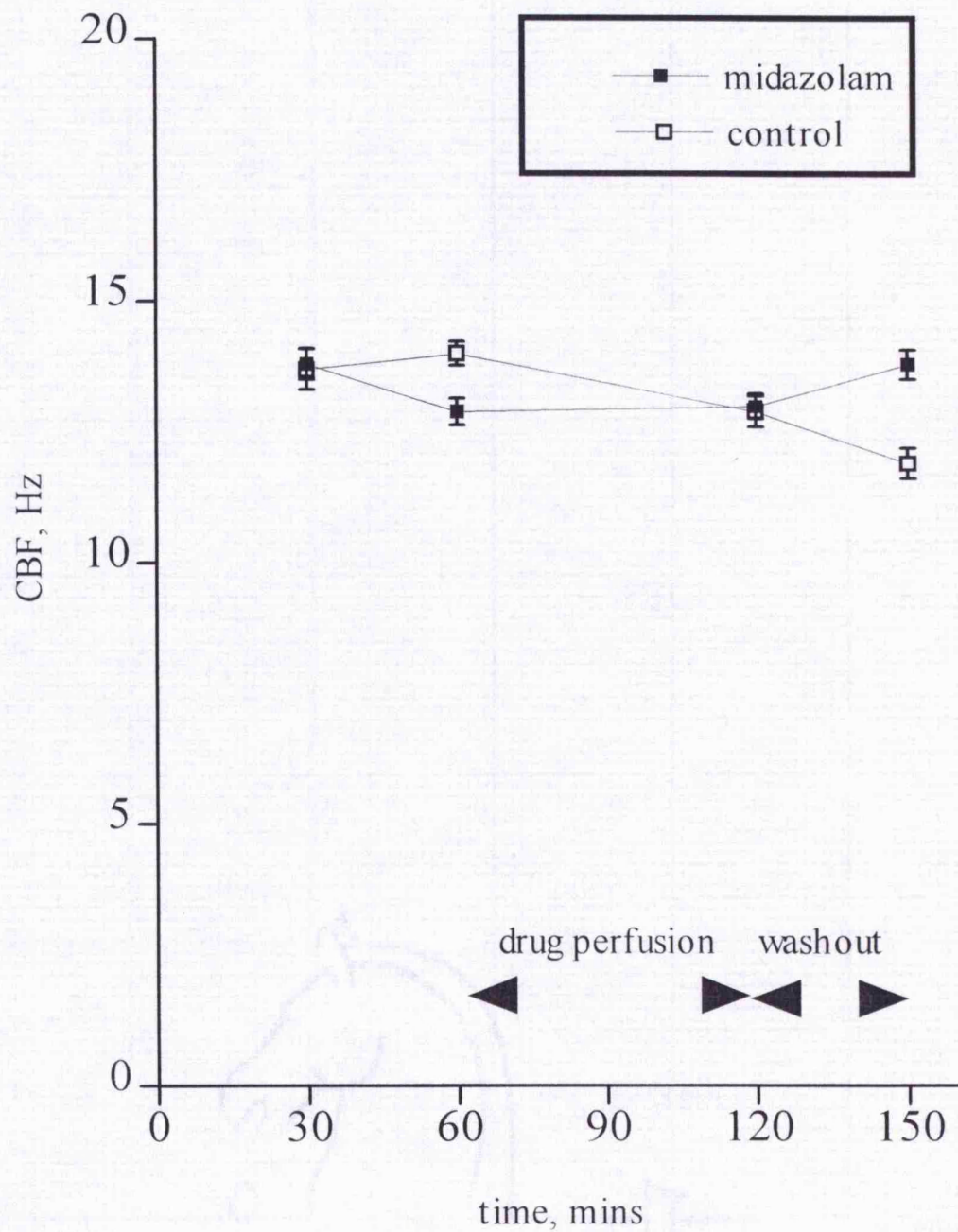
Results

The results are summarised in table 9.1 and displayed in figure 9.1. There was no significant effect of midazolam on ciliary beat frequency compared to the controls (MANOVA for repeated measures, $p=0.212$)

Table 9.1 Effects of short-term midazolam on ciliary beat frequency *in vitro*

time, mins	mean CBF, Hz	SEM CBF, Hz	Ciliated edges
<hr/>			
<u>Control</u>			
30	13.7	0.39	80
60	14.0	0.23	88
120	12.9	0.30	89
150	11.9	0.29	96
<hr/>			
<u>Midazolam</u>			
30	13.8	0.30	92
60	12.9	0.26	95
120	13.0	0.24	95
150	13.8	0.28	96
<hr/>			

Fig 9.1 Effects of midazolam on mean (SEM) ciliary beat frequency (CBF) *in vitro*



Expt 9.2 Long-term effects of midazolam on human respiratory cilia *in vitro*

Methods

Explants from human nasal turbinates were obtained from patients undergoing turbinectomy for a primary diagnosis of tissue hypertrophy and prepared as previously described in chapter 4. On the day after surgical excision the samples were checked for viable ciliated edges using the Nikon Diaphot microscope at 600x magnification.

The samples were incubated with serial concentrations of midazolam at 1, 3, 10, 30 and 100 μM together with culture medium as used for explant maintenance (chapter 4). A control group consisted of explants from the same patients was incubated with culture medium alone. The explants were examined for the presence or absence of cilia at 24, 48 and 72 hours after incubation and each explant was defined as either having or not having any visible cilia. 16 explants were exposed to each concentration.

Results

There was both a time-dependent and dose-dependent effect upon cilia survival as shown in table 9.2 and figures 9.2 and 9.3. The concentrations of midazolam for 50 % chance of cilia survival (IC_{50}) were 25 μM at 48 hours and 16 μM at 72 hours of incubation.

Table 9.2 Effects of midazolam on cilia survival

Midazolam, μ M	Surviving explants			Total number of explants
	24 hrs	48 hrs	72 hrs	
0	15	15	15	16
1	15	15	15	16
3	16	16	16	16
10	16	16	14	16
30	15	3	4	16
100	16	3	3	16

Fig 9.2 Effects of midazolam incubation for 48 hours on cilia survival

in vitro

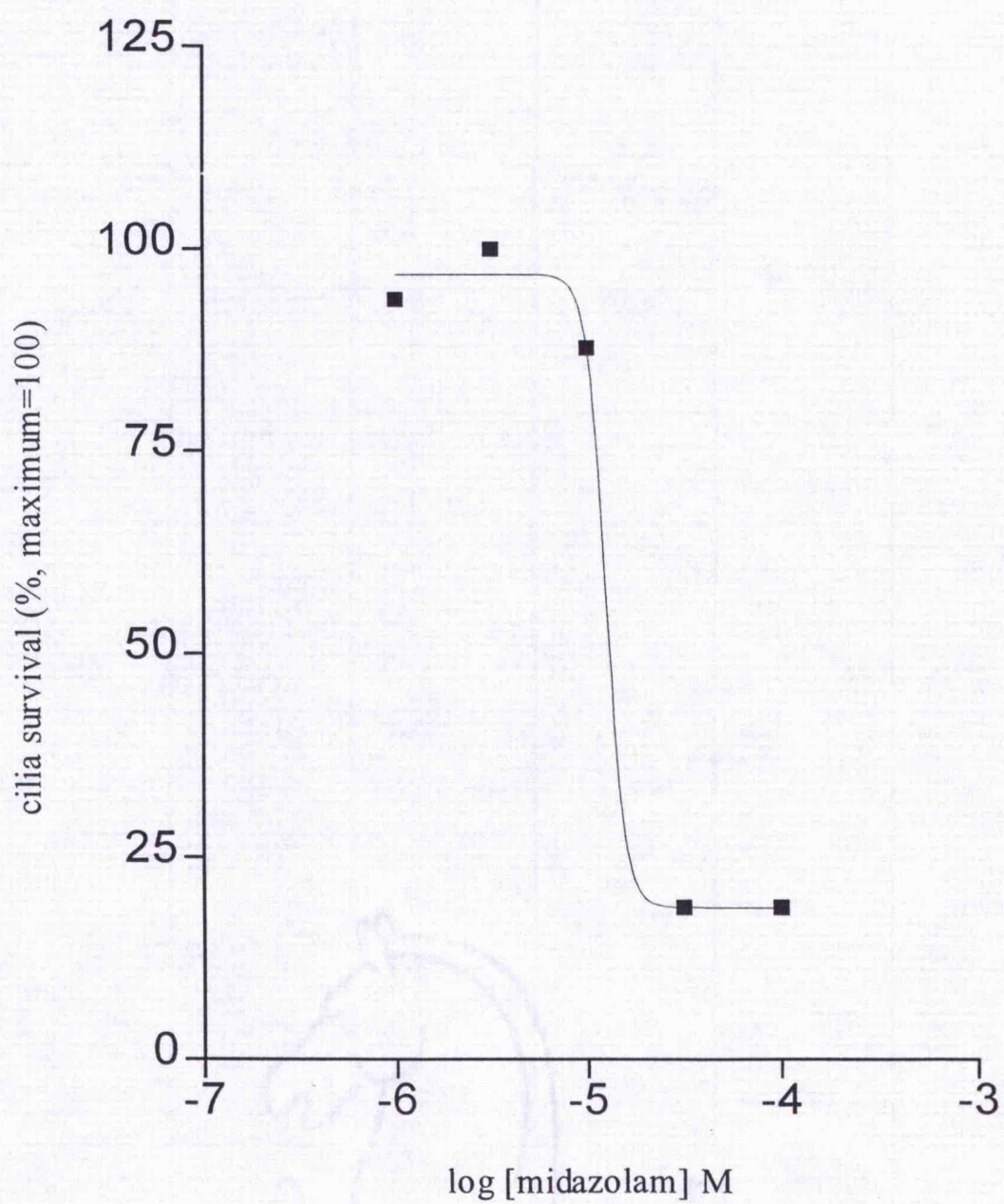
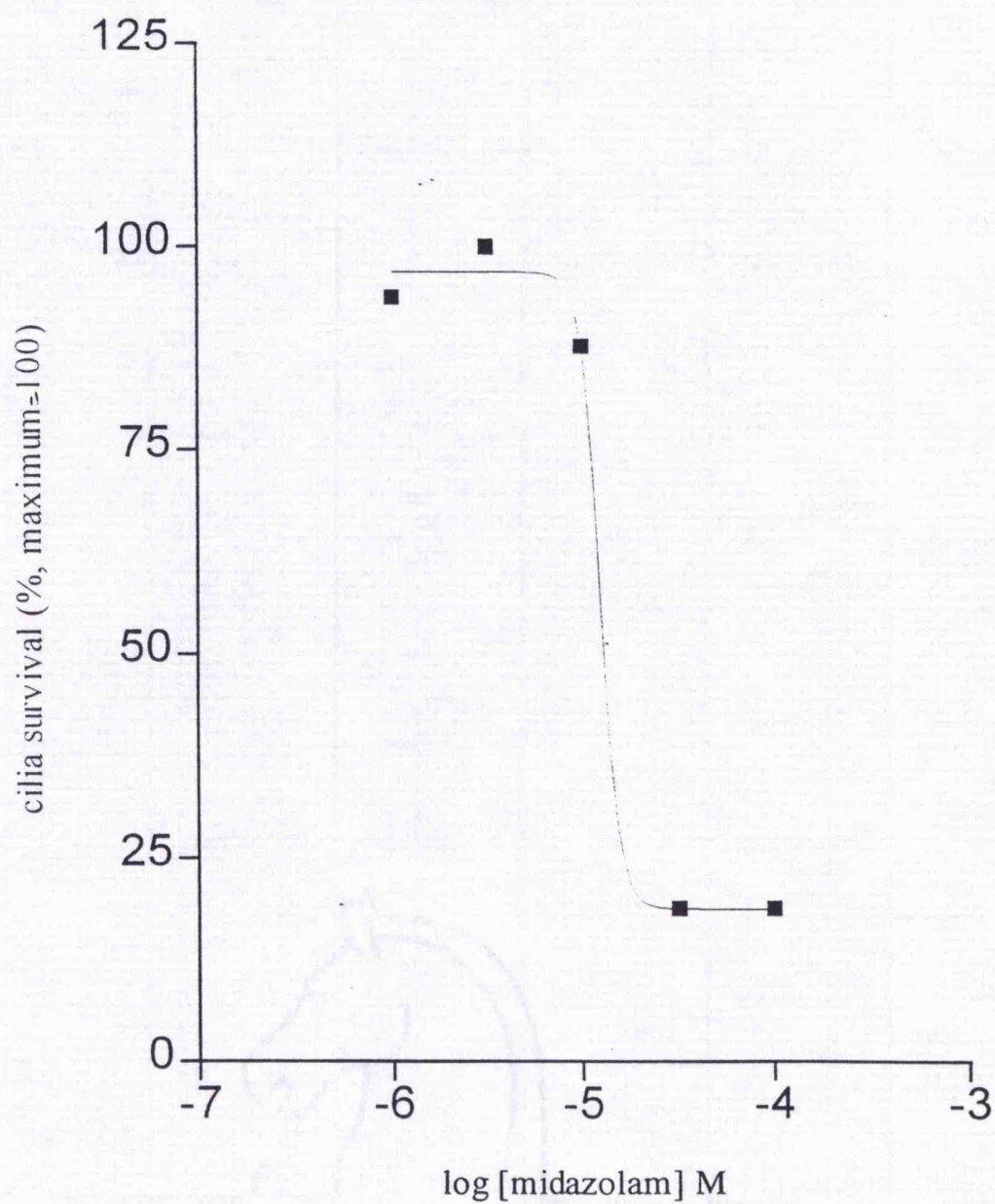


Fig 9.3 Effects of midazolam incubation for 72 hours on cilia survival
in vitro



Expt 9.3 Neurotoxicity of midazolam in SH-SY5Y cells measured by inhibition of thiazoyl blue reduction

Introduction

To investigate further the suggestion from the previous experiment of a toxic effect of midazolam on cilia we investigated its effects on another tissue available in our laboratory as a cell culture clone, neuronal SH-SY5Y cells. This afforded the opportunity to use a validated and sensitive method of cellular toxicity and mirror the *in vitro* ciliary system.

The background to the method is detailed in chapter 8, expt 8.4. Briefly the agent MTT is converted to a coloured dye by mitochondrial enzymes and its reduction indicates mitochondrial integrity and activity which in turn reflects cell viability. The ability to reduce MTT compared to untreated controls enables the relative toxicity of compounds to be assessed. Mitochondrial activity (viable cell number) is proportional to the intensity of coloured dye measured spectrophotometrically (Willets, 1995).

Methods

Undifferentiated human SH-SY5Y neuroblastoma cells (a gift from J.L.Biedler) were maintained in 80 cm² culture flasks using minimum essential medium, supplemented with foetal calf serum (10 % v/v), L-glutamine 2 mM, penicillin(100 IU/ml), streptomycin (100µg/ml) and incubated at 37 °C, in 5% CO₂ / humidified air. Confluent cultures were routinely harvested by trypsinisation (0.05 %) and split at 1:10. Cells were harvested and seeded in 24 well multitrays at a density of 1 x 10⁵ cells / well.

Midazolam at concentrations ranging from 3 μM to 1 mM was incubated with SH-SY5Y cells 3 days after seeding and incubated for up to 72 hours.

After 48 and 72 hours, MTT (0.12 mM, in HBSS) was added to all cells and left for one hour at 37 °C. The resulting dye was extracted with acidified isopropanol and the absorbance measured spectrophotometrically at 550 nm with reference at 620 nm on an Anthos 2001 plate reader.

Potential interaction between MTT and midazolam was determined by the addition of the highest concentration of midazolam alone, in media without cells. This represented background absorbance and was subtracted from all samples and MTT reduction was expressed as a percentage of the untreated cells (control minus midazolam).

Results

Midazolam caused a time and dose related inhibition of MTT reduction in SH-SY5Y cells. After 48 hours incubation there was a significant inhibition with 100 μM midazolam (ANOVA, $p < 0.001$; unpaired t-test with Bonferroni correction, $p < 0.01$). After 72 hours incubation there was a significant inhibition with 30 μM midazolam (ANOVA, $p < 0.001$; unpaired t-test with Bonferroni correction, $p < 0.005$). The mean(SEM) IC_{50} s were 53.3(4.65) μM at 48 hours and 27.9(4.29) μM at 72 hours respectively.

Table 9.3 Effects of 48 hours incubation with midazolam on MTT reduction in SH-SY5Y cells. (C=control; results expressed as absolute values and as percentage of control).

Sample	1	2	3	4	5	6
C	0.092	0.102	0.109	0.222	0.216	0.214
%	100	100	100	100	100	100
3	0.091	0.093	0.102	0.182	0.189	0.213
%	99.5	91.2	93.6	81.9	87.5	99.5
10	0.076	0.097	0.084	0.161	0.191	0.209
%	83.0	95.0	77.1	72.5	88.4	97.7
30	0.074	0.066	0.055	0.141	0.143	0.153
%	80.9	64.7	50.5	63.5	66.2	71.5
100	0.029	0.034	0.038	0.08	0.083	0.088
%	31.7	33.3	34.9	36	38.4	41.1
300	0.002	0.001	-	0.008	0.006	0.004
%	2.2	0.98	-	3.6	2.8	1.8
1000	0.013	0.008	0.006	0.002	0.003	0.004
%	14.2	7.8	5.5	0.9	1.4	1.8

Table 9.4 Effects of 72 hours incubation with midazolam on MTT reduction in SH-SY5Y cells. (C=control; results expressed as absolute values and as percentage of control).

Sample	1	2	3	4	5	6
C	0.187	0.169	0.171	0.273	0.270	0.256
%	100	100	100	100	100	100
3	0.124	0.127	0.137	0.218	0.223	0.235
%	66.3	75.1	80	79.9	82.6	91.8
10	0.126	0.128	0.127	0.227	0.228	0.239
%	67.4	75.7	74.3	83.2	84.4	93.4
30	0.064	0.075	0.070	0.162	0.132	0.152
%	34.2	44.4	40.9	59.3	48.9	59.4
100	0.023	0.025	0.028	0.050	0.050	0.058
%	12.3	14.8	16.4	18.3	18.5	22.7
300	0.002	0.001	0.003	-	-	-
%	1.0	0.59	1.8	-	-	-
1000	0.001	0.004	0.006	0.005	-	0.002
%	0.53	2.4	3.5	1.8	-	0.78

Fig 9.4 Effects of midazolam on SH-SY5Y cell viability after 48 hours exposure assessed by MTT reduction (% of control, SEM)

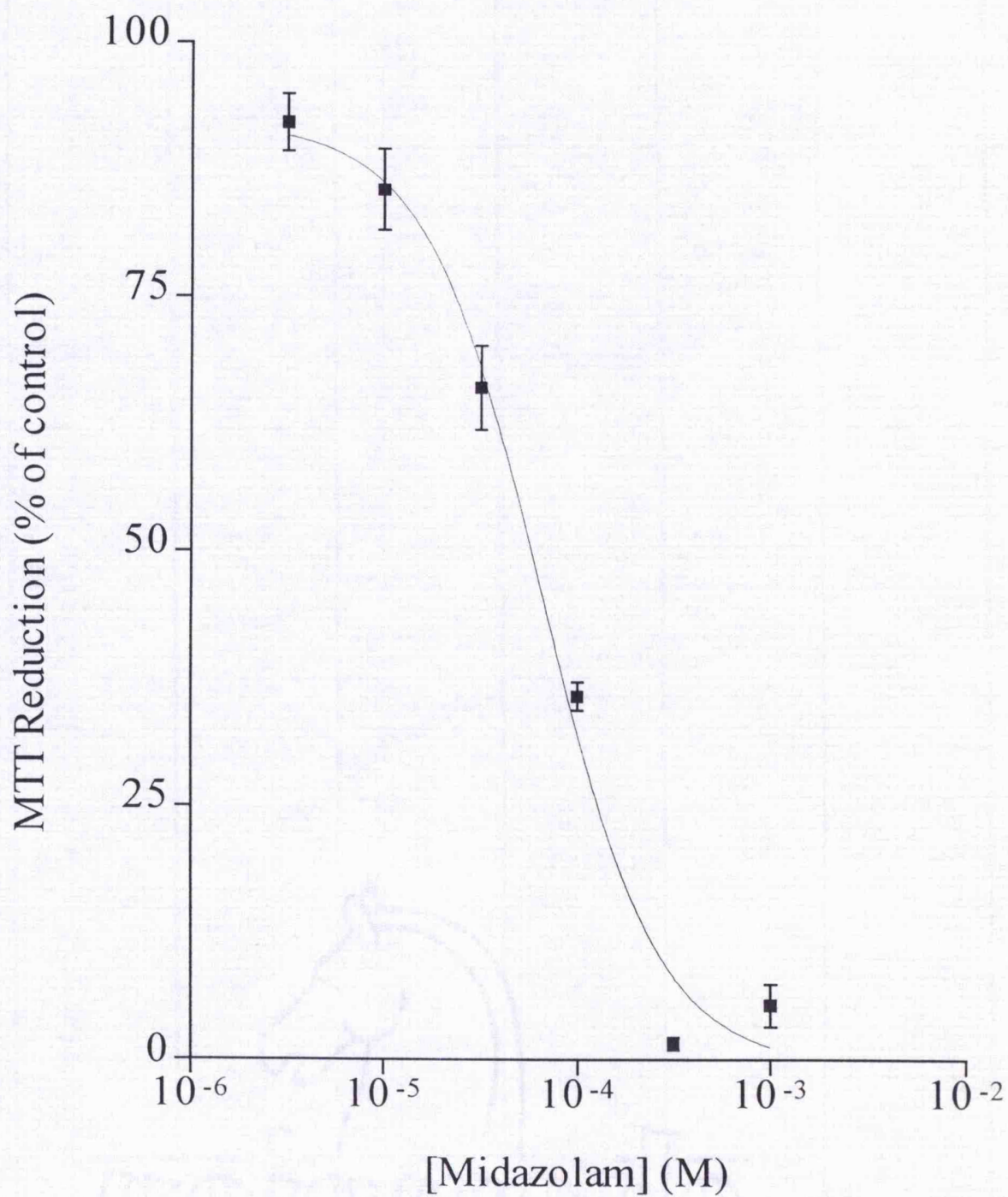
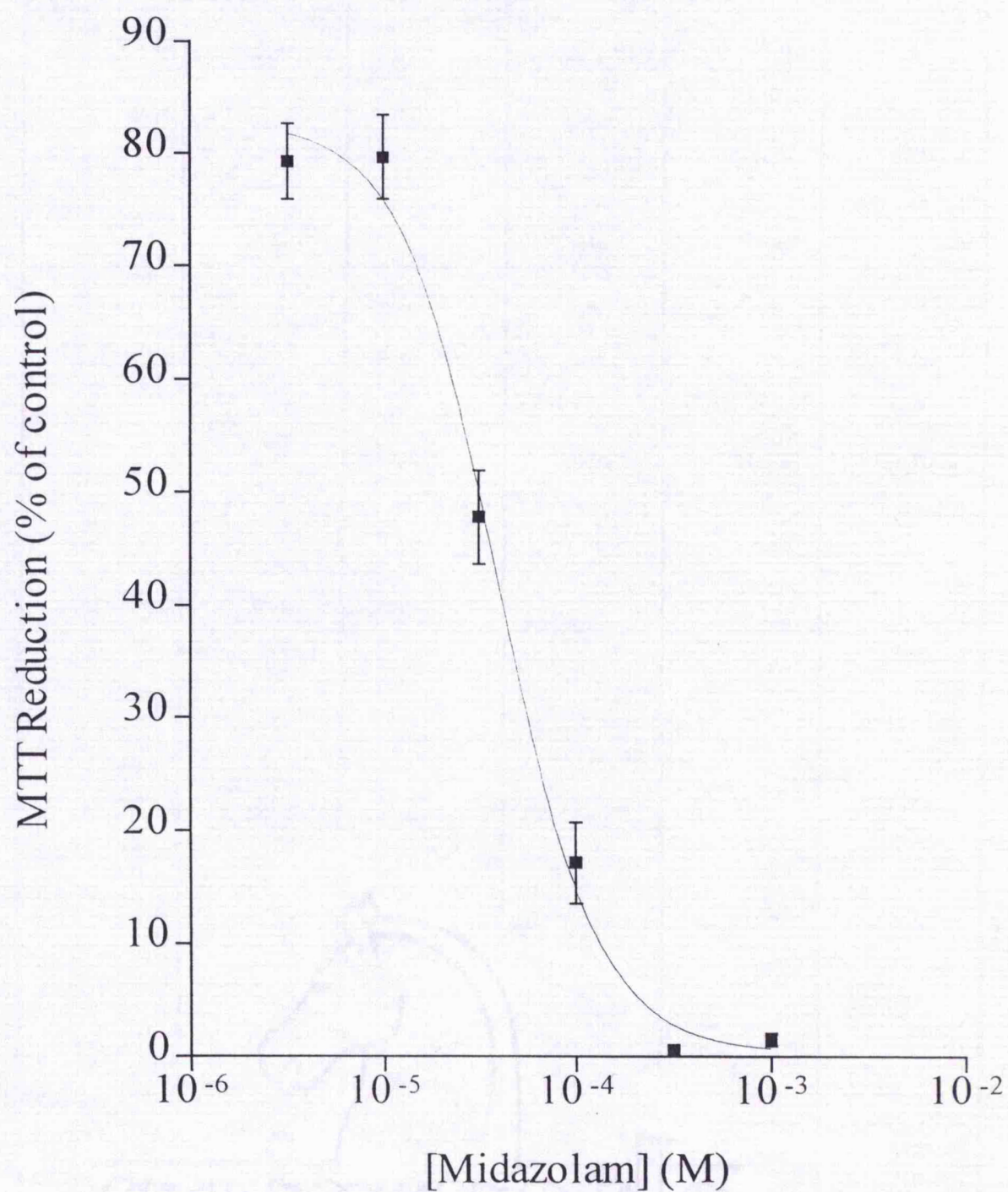


Fig 9.5 Effects of midazolam on SH-SY5Y cell viability after 72 hours exposure assessed by MTT reduction (% of control, SEM)



Discussion

We have found that perfusion with 20 μ M midazolam for 90 minutes has no significant effects on respiratory ciliary beat frequency *in vitro*. The plasma concentration during sedation is around 10 μ M (Frenkel, 1993) and we have used a concentration of twice this. This experiment has an 80 % power to detect differences in ciliary beat frequency of more than 1.5 Hz at the 5% level of significance. Reductions in ciliary beat frequency of less than 20 % or about 2 Hz are probably not associated with significant reductions in mucus transport rate as discussed in chapter 2.

There is no previous work on the effects of either this agent or related benzodiazepines on ciliary beat frequency with which to compare it. Our results contrast with the effects we have found with other anaesthetics, the inhalation agents, where we found a depressant effect on the ciliary beat frequency.

Investigations of the effects of benzodiazepines upon mucociliary clearance have been limited. Konrad and colleagues measured *in vivo* human bronchial mucus transport in patients anaesthetised and ventilated for five hours with midazolam, fentanyl, pancuronium and nitrous oxide (Konrad, 1992). Using radioactive albumin spheres and a scintillation camera they found no change in mucus transport rate. This contrasts with the earlier work by Forbes and Horrigan who found a reduction in mucus transport with nitrous oxide (Forbes, 1977) and others who found reductions in mucus transport with positive pressure ventilation (Wolfe, 1972; Forbes, 1979c).

The only work on the effects of a benzodiazepine alone on mucus transport is a volunteer study investigating temazepam using radioactive-labelled particles and

scintillation cameras. These workers found a 22 % reduction of *in vivo* mucus transport rate in eight healthy volunteers (Hasani, 1992).

We went on to investigate the effects of incubation with midazolam upon cilia survival simulating its use as a long term sedative in patients in the ITU. We have found a time-dependent and dose-dependent effect upon cilia survival *in vitro* after exposure to midazolam. The IC₅₀s were 25 μ M at 48 hours and 16 μ M at 72 hours of incubation; however, the dose-response curve is steep and at the concentration found in clinical practice, 10 μ M (Frenkel, 1993), only two of sixteen explants had no surviving cilia. This result contrasts with our findings with propofol in which there was no effect on the survival of cilia from fresh turbinate explants.

It is not possible to extrapolate these *in vitro* findings to that found in clinical practice. Indeed since the study has limitations in being conducted *in vitro* using a tissue removed from its normal environment with the inability to wash away any toxic metabolites, it may not be ciliotoxic *in vivo*; however, this surprising result may have importance in at least some intensive care patients with illnesses that also impair their perfusion. Furthermore, one could speculate that in ITU patients there are other factors that adversely affect the cilia, such as the use of high concentrations of oxygen and insufficient humidification. These factors may interact with any potential ciliotoxic effects of midazolam.

To complement this surprising effect of ciliotoxicity of midazolam, we investigated the cytotoxicity of the drug on SH-SY5Y cells using MTT as an index of cellular viability as discussed in chapter 8.

We have found a time and dose dependent inhibition of MTT reduction, an index of mitochondrial function, in human neuroblastoma cells measured *in vitro*

exposed to midazolam. However, the effect was significant after 72 hours incubation only with concentrations above 30 μ M midazolam, a concentration above that found in clinical practice. Neurotoxicity does not appear to be apparent at clinical concentrations.

Clearly this *in vitro* experiment does not reflect the situation *in vivo* in which potential toxins are removed from tissue by perfusion, metabolism and excretion; rather, it lends support to our *in vitro* findings with human ciliated tissue.

In summary we have found no effects upon ciliary beat frequency of short term exposure for 90 minutes to midazolam; however, incubation for 48 and 72 hours causes a significant loss of cilia although this is only apparent at concentrations above that found in clinical practice.

Chapter 10

Conclusions & Clinical recommendations

With the many advances in anaesthesia that have occurred over the past two decades, mortality due to anaesthesia is very low (Buck, 1987). Attention has therefore focused on anaesthetic-related morbidity. One of the most serious but common forms of morbidity associated with anaesthesia and surgery is postoperative respiratory infection. This problem has remained relatively frequent and is a particular problem for patients receiving intensive care. The cause of postoperative chest infection is multifactorial; however, attention to those factors that are considered most significant, pain relief, antibiotic prophylaxis and encouragement of coughing, have made little impact on the incidence of postoperative chest infection, which today remains as high as it was over 60 years ago (King, 1933).

One of the most important natural defences against the development of pulmonary infections is the mucociliary system which transports mucus and its adherent matter away from the lungs. It has been known for some time that anaesthetic and sedative agents impair the transport rate of mucus. Forbes and Horrigan conducted a study with dogs in which tracheal mucus transport rate was measured by a radioactive droplet technique and found reductions in mucus transport rate with halothane, enflurane and morphine (Forbes, 1977). Hasani and colleagues measured mucus transport rate in volunteers using radioactive albumin microspheres and found a significant reduction after the administration of the sedative, temazepam (Hasani, 1992). However, little attention has been paid to the mechanisms involved.

Mucus clearance depends upon the volume and physical properties of the mucus and upon the function of the cilia. Ciliary function depends upon the number of cilia and their movement. The only functional parameter of movement that is quantifiable is the ciliary beat frequency and this is an important determinant of ciliary function and a sensitive measure of mucus transport. The relationship between ciliary beat frequency and mucus transport rate appears to be non-linear such that modest reductions in ciliary beat frequency produce disproportionately large reductions in mucus transport rates (Hee, 1985; Duchateau, 1985). It would therefore appear that ciliary beat frequency is an important determinant of mucus transport rate.

Measurements of respiratory ciliary beat frequency are restricted to *in vitro* techniques and the transmitted light technique is the most widely used method (Teichtahl, 1976). Investigating the effects of anaesthetic agents upon the cilia produce specific problems due to the volatility of some of these agents. We have developed an *in vitro* method for the measurement of ciliary beat frequency which allows exposure to drugs including the volatile inhalation anaesthetic agents. Specially designed perfusion chambers and a delivery system have been built to allow for tissue exposure to drugs including the volatile inhalation anaesthetic agents. The perfusion chambers provide rapid equilibration with changes in delivered drug concentration. The use of metallic delivery tubing prevents significant volatile agent loss. The incorporation of temperature and flow controllers and the use of buffered medium provides for the delivery of drugs to ciliated tissue *in vitro* in a controlled environment. We have used the apparatus to measure the ciliary beat frequency of a recognised tissue source, human nasal cytological brushings obtained from awake volunteers, and it reliably measures the ciliary beat frequency during perfusion for up to 6 hours.

Although ciliary structure is similar in different species, ciliary function and in particular, mechanisms of control are not uniform and the use of human, rather than non-human, tissue for studying the effects of pharmacological agents in clinical use is therefore preferable.

Human ciliated tissue specimens have in the past been obtained by nasal brushings of awake volunteers; however, these present problems as the procedure is moderately uncomfortable. We have developed two alternative methods of sample collection. We have obtained samples after the induction of general anaesthesia with a bolus dose of propofol and found the ciliary beat frequency measured from one hour after sampling is similar to that measured from pre-anaesthetic samples from the same patients. Although these cytological samples are an improvement on awake sampling and have been used for a number of the investigations in this thesis they continue to have some limitations.

The exfoliated cells being traumatically obtained are of variable quality and often do not have continuous ciliated edges suitable for measurement; the cells are prone to sample movement which interferes with measurements using the transmitted light technique; and these preparations have to be used on the day they are obtained posing logistical restraints. We therefore developed a second tissue source. This is derived from the inferior nasal turbinates removed from patients at elective surgical turbinectomy with a primary diagnosis of tissue hypertrophy.

Historically *in vitro* samples of respiratory ciliated epithelium were provided by biopsy of the inferior nasal turbinates and used to diagnose such conditions as cystic fibrosis; however, such techniques fell into disfavour because they were performed under local anaesthesia with its attendant ciliotoxic effects and there was a risk of haemorrhage with this procedure. Nevertheless we considered that

biopsies from the inferior nasal turbinates had advantages over nasal brushings as they provide a better model of *in vivo* ciliary behaviour as more of the adjacent tissue is retained. We have developed a method of preparing explants from the turbinates and these provide samples with long continuous ciliated edges that survive in an incubator for up to 10 days.

The turbinate explants demonstrated beat frequencies within the normal range. The physiological responsiveness of the turbinate explants was examined by exposing them to agents which are known to either increase or decrease the ciliary beat frequency. Following exposure of the samples to forskolin (an adenylyl cyclase activator) there was an increase in the ciliary beat frequency, whereas following exposure to phenol, a known ciliotoxin, we were able to demonstrate a reduction in the ciliary beat frequency. The cilia of these turbinate explants appear to be representative of cilia from other parts of the respiratory tract.

We have used both these ciliated tissue preparations for investigations as the brushings allow for close comparison with previous work and are a suitable preparation for receptor binding studies and the turbinate explants can be used for *in vitro* studies of the long term effects of drugs.

We have found a dose-dependent reduction in ciliary beat frequency with the three inhalation anaesthetic agents, halothane, enflurane and isoflurane. The reduction in ciliary beat frequency of approximately 2 Hz after exposure to clinical concentrations of the three inhalation agents for a period of one hour may be clinically significant. There was no clinically significant difference in ciliary beat frequency when comparing the effects of the three agents studied.

The duration of these effects was investigated by exposing the tissue to the agent at a concentration of 3 MAC for one hour and then measuring the ciliary beat frequency during a 90 minute period of air washout. In these experiments we have found a reversible reduction in the ciliary beat frequency taking 90 minutes to recover in the case of halothane and 60 minutes in the cases of enflurane and isoflurane. This difference may be related to the physical properties of the agents, as halothane has a greater lipid/water solubility partition coefficient and may therefore take longer to diffuse out of the fat soluble tissue of the ciliated specimens.

In the first inhalation agent study we undertook, investigating the effects of 3 MAC of the agents on nasal brushings, we found a greater depression of ciliary beat frequency with increasing exposure time over a period of three hours. If a similar effect were to occur with time at clinical concentrations then the effects of prolonged use of inhalation agents either for operative procedures or for sedation in the intensive care unit may markedly reduce the ciliary beat frequency and associated mucus clearance rate. To investigate this, a specially-designed gas chamber was developed to accommodate prolonged delivery of the volatile agents and the effects of a low concentration of isoflurane of 0.2-0.4 %, as used for prolonged sedation in the ITU, was investigated *in vitro*. We did not find any significant effects of this upon ciliary beat frequency after 48-72 hours exposure.

Previous work has shown that morphine reduces mucociliary clearance, an important defence against respiratory tract infections (Hibma, 1942, Van Dongen, 1953) . To elucidate the mechanisms by which morphine impairs mucus transport we investigated the effects on the cilia and found no effects on the ciliary beat frequency and were able to support this with a study that

demonstrated no significant opioid receptor binding to the cilia. These *in vitro* studies do not support an effect of morphine on human respiratory ciliary function.

The mechanisms by which morphine reduces mucus transport rates may therefore be a function of effects upon the mucus itself or *in vivo* effects upon the cilia. Morphine has been shown to inhibit mucus secretion stimulated by the irritant capsaicin (Rogers, 1989) and one could speculate a similar effect in response to the irritancy of inhalation anaesthetic agents. Alternatively, morphine may have an effect on cilia *in vivo* due to effects upon neural connections which are absent from *in vitro* preparations.

Propofol is an intravenous anaesthetic agent used for the induction and maintenance of anaesthesia and for sedation in the ITU. We did not find any significant effects upon the ciliary beat frequency in the short term. Propofol can be used as an alternative to the inhalation agents for the maintenance of anaesthesia and therefore the difference we have found compared with the inhalation agents may have therapeutic benefits.

In studies of the long term effects of propofol upon the cilia *in vitro* we found adverse effects. Using turbinate explants that had been stored for a number of days we found that a sedative concentration of propofol caused significant loss of ciliated edges; however, with fresh tissue there were no such effects even with supraclinical concentrations. These *in vitro* findings must be viewed cautiously; however, it may be that propofol has a toxic effect on unhealthy cilia and may therefore be relevant to some critically ill ITU patients.

Midazolam is an intravenous agent used to provide anaesthesia as well as sedation in the ITU. A related benzodiazepine, temazepam, has been shown to

reduce mucus transport rates but the mechanisms are unknown (Hasani, 1992). We did not find any significant effects upon ciliary beat frequency during short term exposure to the drug.

Further experiments were designed to assess the *in vitro* effects upon the cilia of prolonged exposure to midazolam as used for ITU sedation. There was both a time-dependent and dose-dependent effect upon cilia survival with an IC_{50} of 25 μM at 48 hours and 16 μM at 72 hours of incubation. Although these values are close to the concentrations found in clinical practice, 10 μM (Frenkel, 1993), the dose-response curve is steep and only two of sixteen explants had no surviving cilia at the clinical concentration. One should be cautious in anticipating significant effects upon ciliary survival at clinical concentrations.

The clinical relevance of the reductions in ciliary beat frequency and in the loss of cilia that we have found with some of the anaesthetic agents is clearly limited by the *in vitro* nature of the investigations. We have evaluated cilia removed both from perfusion with blood in the body and from their coupling to mucus. The former may alter the toxic effects of the sedatives, that we found *in vitro*, since metabolites will be removed from the *in vivo* tissue. Since some of the patients in the ITU have impaired perfusion as part of their illness, these *in vitro* findings may be of clinical importance. Furthermore, one could speculate that in ITU patients there are other factors that adversely affect the cilia, such as the use of high concentrations of oxygen and insufficient humidification. These factors may interact with any potential ciliotoxic effects of the sedatives. The unexpected *in vitro* effects we have found merit further evaluation by *in vivo* study of mucus transport and ciliary presence during the course of prolonged sedation.

We have investigated cilia without adjacent mucus in order to eliminate any effects upon the mucus altering ciliary function. This experimental design allows us to delineate the effects of the drugs investigated on the cilia alone; however, the interaction between the two components of the mucociliary system *in vivo* may mean that we cannot extrapolate from ciliary effects to predict effects upon mucus transport. There is currently no information on the effects of propofol on mucus transport; however, in view of the differences between inhalation agents and propofol on ciliary beat frequency this would be worthy of investigation since these are alternative agents with which to maintain the anaesthetic state.

The remit of this thesis has been the development of methods for the investigation of anaesthetic agents upon ciliary beat frequency and the documentation of these effects. We have not investigated the mechanisms of these effects; however, a fruitful area for future work may be the investigation of the mechanisms by which anaesthetic drugs interfere with respiratory ciliary function.

The movement of the cilia depends upon the sliding of microtubules within the ciliary axoneme. Previous work supports effects of the inhalation anaesthetic agents on cellular microtubules (Allison, 1968). Alterations of intracellular calcium and cyclic nucleotides have been shown to alter beat frequency in respiratory tract cilia (Sanderson, 1992). A variety of receptor agonists and agents that act both on the cellular membrane and intracellularly have been shown to have effects upon ciliary beat frequency (Table 2.1). These agents mediate their effects upon ciliary beat frequency via alterations in intracellular 2nd messengers. It would appear that ciliary beat frequency is increased when cAMP, IP₃ and intracellular calcium are increased and that ciliary beat frequency is decreased when cAMP is reduced or cGMP is increased. In addition sodium channel blockade may reduce ciliary beat frequency.

Other workers using alternative tissue preparations have found that these intracellular mechanisms are affected by anaesthetic agents. Inhalational agents appear to have a variable effect on cAMP formation in different tissues which may reflect differences in the isoforms of adenylyl cyclase some of which are calcium sensitive, others being resistant (Lambert, 1993). There are no data on the effects of inhalation agents on respiratory tissue cAMP. Since the inhalation agents activate voltage-gated calcium channels, they may activate calcium-sensitive adenylyl cyclase through increasing intracellular calcium. The effects of anaesthetics upon IP_3 are not clear but indirect measures appear to favour a decrease. Reduced calcium release from IP_3 -sensitive stores with halothane suggests a reduction in IP_3 (Sill, 1991). Inhalation anaesthetic agents inhibit voltage-gated sodium channels (Urban, 1993).

In summary we have demonstrated differential effects of anaesthetic and sedative agents upon respiratory ciliary beat frequency and ciliary presence *in vitro*. The inhalation agents caused a dose-dependent and reversible decrease in ciliary beat frequency whereas the intravenous agents propofol, midazolam and morphine had no such effects. The long term use of these agents for sedation produced contrasting results. Incubation of ciliated tissue with midazolam caused loss of cilia; incubation with propofol only caused cilia loss with stored tissue and this effect was not found with fresh samples; and no such effects were seen with prolonged sedative concentrations of isoflurane.

Chapter 11

References

- Afzelius BA. A human syndrome caused by immotile cilia. *Science* 1976;193:317-9.
- Aiello E, Kennedy J, Hernandez C. Stimulation of frog ciliated cells in culture by acetylcholine and substance P. *Comparative Biochemical Physiology* 1991;99:497-506.
- Allison AC, Nunn JF. Effects of general anaesthetics on microtubules: a possible mechanism of anaesthesia. *Lancet* 1968;2:1326-1329.
- Annis P, Land J, Lichtiger M. Effects of atropine on velocity of tracheal mucus in anaesthetized patients. *Anesthesiology* 1976;44:74-77.
- Bang FB, Bang BG, Foard MA. Responses of upper respiratory mucosa to drugs and viral infections. *American Review of Respiratory Disease* 1966;93:142-149.
- Bergman NA. Distribution of inspired gas during anaesthesia and artificial ventilation. *Journal of Applied Physiology* 1963;18:1085-1089.
- Bleeker JDJW, Hoeksema PE. A simple method to measure the ciliary beat rate of respiratory epithelium. *Acta Otolaryngologica* 1971;71:426-429.
- Bonin SR, Phillips PP, McCaffrey TV. The effect of arachidonic acid metabolites on the ciliary beat frequency of human nasal mucosa in vitro. *Acta Otolaryngologica* 1992;112:697-702.
- Braga PC. Versatile perfusion chamber for high magnification microscopy. *Journal of Pharmacological Methods* 1989;22:1-6.
- Brismar B, Pettersson N, Tokics L, Strandberg A, Hedenstierna G. Postoperative analgesia with intrapleural administration of bupivacaine-adrenaline. *Acta Anaesthesiologica Scandinavica* 1987;31:515-520.

Burgess GE, Cooper JR, Marino RJ, Peuler MJ, Warriner RA.
Laryngeal competence after tracheal extubation. *Anesthesiology*
1979;51:73-77.

Buck, Devlin HB, Lunn JN. Report of the Confidential Enquiry into
Perioperative Deaths. Nuffield Provincial Hospital Trust and the King
Edward's Hospital Fund for London. London 1987.

Burton JDK. Effects of dry anaesthetic gases on the respiratory
mucous membrane. *Lancet* 1962;1:235-238.

Carson JL, Collier AM, Hu S-C S. Acquired ciliary defects in nasal
epithelium of children with acute viral upper respiratory infections.
New England Journal of Medicine 1985;312:463-468.

Corssen G, Allen CR. Acetylcholine: its significance in controlling
ciliary activity of human respiratory epithelium in vitro. *Journal of
Applied Physiology* 1959;14:901-904.

Couture JG, Chartrand D, Gagner M, Bellemare F. Diaphragmatic and
abdominal muscle activity after endoscopic cholecystectomy.
Anesthesia and Analgesia 1994;78:733-9.

Craig CP, Connelly S. Effect of intensive care unit nosocomial
pneumonia on duration of stay and mortality. *American Journal of
Infection Control* 1984;12:233-238.

Cuschieri RJ, Morran CG, Howie JC, McArdle CS. Postoperative pain
and pulmonary complications: comparison of three analgesic regimes.
British Journal of Surgery 1985;72:495-498.

Dalhamn T. A method for determination in vivo of the rate of ciliary
beat and mucous flow in the trachea. *Acta Physiologica Scandinavica*
1954;33:1-5.

Dalhamn T, Rylander R. Frequency of ciliary beat measured with a
photo-sensitive cell. *Nature* 1962;196:592-593.

Devalia JL, Sapsford RJ, Wells CW, Richman P, Davies RJ. Culture and comparison of human bronchial and nasal epithelial cells in vitro. *Respiratory Medicine* 1990;84:303-312.

Di Benedetto G, Manara-Shediac FS, Mehta A. Effect of cyclic AMP on ciliary activity of human respiratory epithelium. *European Respiratory Journal* 1991;4:789-795.

Dilworth JP, White RJ. Postoperative chest infection after upper abdominal surgery: an important problem for smokers. *Respiratory Medicine* 1992;86:205-210.

Duchateau GS, Graamans K, Zuidema J, Merkins FW. Correlation between nasal ciliary beat frequency and mucus transport rate in volunteers. *Laryngoscope* 1985;95:854-859.

Dulfano MJ, Adler KB. Physical properties of sputum: VII Rheologic properties and mucociliary transport. *American Review of Respiratory Disease* 1975;112:341-347.

Egbert LD, Tamersoy K, Deas TC. Pulmonary function during spinal anesthesia: the mechanism of cough depression. *Anesthesiology* 1961;22:882-885.

Feinstein R, Ghouri A. Change in pulmonary mechanics during laparoscopic cholecystectomy. *Anesthesia and Analgesia* 1993;76:S102.

Forbes AR. Halothane depresses mucociliary flow in the trachea. *Anesthesiology* 1976;45:59-63.

Forbes AR, Horrigan RW. Mucociliary flow in the trachea during anesthesia with enflurane, ether, nitrous oxide, and morphine. *Anesthesiology* 1977;46:319-321.

Forbes AR, Gamsu G. Depression of lung mucociliary clearance by thiopental and halothane. *Anesthesia and Analgesia* 1979a;58:387-389.

Forbes AR, Gamsu G. Mucociliary clearance in the canine lung during and after general anesthesia. *Anesthesiology* 1979b;50:26-29.

Forbes AR, Gamsu G. Lung mucociliary clearance after anesthesia with spontaneous and controlled ventilation. *American Review of Respiratory Disease* 1979c;120:857-861.

Frenkel C, Duch DS, Urban BW. Effects of IV anaesthetics on human brain sodium channels *British Journal of Anaesthesia* 1993;71:15-24

Gamsu G, Singer MM, Vincent HH, Berry S, Nadel JA. Postoperative impairment of mucous transport in the lung. *American Review of Respiratory Disease* 1976;114:673-679.

Garibaldi RA, Britt MR, Coleman ML, Reading JC, Pace NL. Risk factors for postoperative pneumonia. *American Journal of Medicine* 1981;70:677-680.

Goucke CR, Hackett LP, Ilett KF. Concentrations of morphine, morphine-6-glucuronide and morphine-3-glucuronide in serum and cerebrospinal fluid following morphine administration to patients with morphine-resistant pain. *Pain* 1994;56:145-149.

Gray, J. The mechanism of ciliary movement-VI : Photographic and stroboscopic analysis of ciliary movement. *Proceedings of the Royal Society* 1930;107:313-322.

Green A, Smallman LA, Logan ACM, Drake-Lee AB. The effect of temperature on nasal ciliary beat frequency. *Clinical Otolaryngology* 1995;20:178-180.

Groth ML, Langenback EG, Foster WM. Influence of inhaled atropine on lung mucociliary function in humans. *American Review of Respiratory Disease* 1991;144:1042-1077.

Gyi A, O'Callaghan C, Langton JA. Effect of halothane on cilia beat frequency of ciliated human respiratory epithelium in vitro. *British Journal of Anaesthesia* 1994;73:507-510.

Hahn HL, Kleinschrot D, Hansen D. Nicotine increases ciliary beat frequency by a direct effect on respiratory cilia. *Clinical Investigator* 1992; 70:244-251

Hann HCL. The use of human nasal turbinates for the in vitro measurement of cilia beat frequency and the effects of pethidine, midazolam and propofol. BSc Thesis, Leicester 1995

Hasani A, Spiteri MA, Lopez-Vidriero MT, Agnew JE, Clarke SW. Effect of temazepam on tracheobronchial mucus clearance. *Thorax* 1992;47:298-300

Hee J, Guillerm R. Discussion on smoke and mucociliary transport. *European Journal of Respiratory Diseases* 1985 ;66(Suppl 139):86-88.

Hibma OV, Curreri AR. A study of the effect of morphine, atropine and scopolamine on the bronchi. *Surgery, Gynecology and Obstetrics* 1942;74:851-854.

Hill JR. The influence of drugs on ciliary activity. *Journal of Physiology* 1957;139:157-166

Iravani J, Melville GN. Mucociliary function in the respiratory tract is influenced by physicochemical factors. *Pharmacology and Therapeutics, Part B: General and Systemic Pharmacology* 1976;2:471-492.

Ingels KJAO, Meeuwssen F, Graamans K, Huizing EH. Influence of sympathetic and parasympathetic substances in clinical concentrations on human nasal ciliary beat. *Rhinology* 1992;30:149-160.

Ingels KJAO, Nijziel MR, Graamans K, Huizing EH. Influence of cocaine and lidocaine on human nasal cilia. *Archives of Otolaryngology, Head and Neck Surgery* 1994;120:197-201.

Jain B, Rbenstein I, Robbins RA, Leise KL, Sisson JH. Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochemical and Physiological Research Communications* 1993;191:83-88.

Jenkins P, Pavia D, Bateman JRM, Clarke SW, Citron KM, Bannister R.
Mucociliary clearance in patients with chronic autonomic failure. *Thorax*
1980;35:690-693.

Kanthakumar K, Cundell DR, Johnson M, Wills PJ, Taylor GW, Cole PJ, Wilson
R. Effect of salmeterol on human nasal epithelial cell ciliary beating : inhibition
of the ciliotoxin, pyocyanin. *British Journal of Pharmacology* 1994;112:493-498.

Katz I, Zwass T, Baum GL, Aharonson E, Belfer B . Ciliary beat frequency and
mucociliary clearance: what is the relationship. *Chest* 1987;92(3):491-493

Kennedy JR, Duckett KE. The study of ciliary frequencies with an
optical spectrum analysis system. *Experimental Cell Research* 1981;
135:147-156

Khan R, Dolata J, Lindberg S. Effects of inflammatory mediators on
ciliary function in vitro. *Rhinology* 1995;33:22-25.

King DS. Postoperative pulmonary complications. *Surgery,*
Gynecology and Obstetrics 1933;56:43-50.

King M, Engel LA, Macklem PT. Effect of pentobarbital on rheology
and transport of canine tracheal mucus. *Journal of Applied Physiology*
1979;46:504-509.

Kobayashi K, Tamaoki J, Sakai N, Kanemura T, Horii S, Takizawa T.
Angiotensin II stimulates airway ciliary motility in rabbit cultured
tracheal epithelium. *Acta Physiologica Scandinavica* 1990;138:497-
502.

Konietzko N, Nakhosteen JA, Mizera W, Kasparek R, Hesse H.
Ciliary beat frequency of biopsy samples taken from normal persons
and patients with various lung diseases. *Chest* 1981;80:Suppl 855-857.

Konrad F, Schreiber T, Grunert A, Clausen M, Ahnefeld FW.
Measurement of mucociliary transport velocity in ventilated patients:
short-term effect of general anaesthesia on mucociliary transport.
Chest 1992;102:1377-1383.

Korngreen A, Priel Z. Simultaneous measurement of ciliary beating and intracellular calcium. *Biophysical Journal* 1994;67:377-380.

Lamb JM, Murty GE, Slater RM, Aitkenhead AR. Postoperative laryngeal function assessed by tussometry. *British Journal of Anaesthesia* 1993;70:478P-479P.

Lambert DG, Atcheson R, Hirst RA, Rowbotham DJ. Effects of morphine and its metabolites on opiate receptor binding, cAMP formation and [3H]noradrenaline release from SH-SY5Y cells. *Biochemical Pharmacology* 1993;46:1145-1150.

Landa JF, Hirsch JA, Lebeaux MI. Effects of topical and general anesthetic agents on tracheal mucous velocity of sheep. *Journal of Applied Physiology* 1975; 38:946-948.

Lansley AB, Sanderson MJ, Dirkse ER. Control of the beat cycle of respiratory tract cilia by Ca^{2+} and cAMP. *American Journal of Physiology* 1992;263:L232-L242.

Lee KS, Park SS. Effect of Halothane, Enflurane and nitrous oxide on tracheal ciliary activity in vitro. *Anesthesia and Analgesia* 1980;59:426-430.

Lichtiger M, Landa JF, Hirsch JA. Velocity of tracheal mucus in anesthetized women undergoing gynecologic surgery. *Anesthesiology* 1975;42(6):753-755.

Low PP, Luk CK, Dulfano MJ, Finch PJP. Ciliary beat frequency of human respiratory tract by different sampling techniques. *American Review of Respiratory Disease* 1984;130:497-498.

Lungarella G, Fonzi L, Ermini G. Abnormalities of bronchial cilia in patients with chronic bronchitis: an ultrastructural and quantitative analysis. *Lung* 1983;161:147-156.

Luk CK, Dulfano MJ. Effect of pH, viscosity and ionic strength changes on ciliary beating frequency of human bronchial explants. *Clinical Science* 1983;64:449-451.

Manawada BR, Mostow SR, LaForce FM. Impairment of tracheal ring ciliary activity by halothane. *Anesthesia and Analgesia* 1979;58:500-504.

Marin MG, Morrow PE. Effect of changing inspired O₂ and CO₂ levels on tracheal mucociliary transport rate. *Journal of Applied Physiology* 1969;27(3):385-388.

Mason JDT, Aspden TJ, Adler J, Jones NS, Illum L, Davis SS. Measurement of nasal mucociliary transport rates on the isolated human inferior turbinate. *Clinical Otolaryngology* 1995;20:530-535.

Merke U, Hakansson CH, Toremalm NG. The influence of temperature on mucociliary activity. *Acta Otolaryngologica* 1974;78:444-450.

Murty GE, Lancaster P, Kelly PJ. Cough intensity in patients with a vocal cord palsy. *Clinical Otolaryngology* 1991;16: 248-251.

Nunn JF, Sturrock JE, Wills EJ, Richmond JE, McPherson CK. The effect of inhalational anaesthetics on the swimming velocity of tetrahymena pyriformis. *Journal of Cell Science* 1974;15:537-554.

Pflug AE, Murphy TM, Butler SH, Tucker GT. The effects of postoperative peridural analgesia on pulmonary therapy and pulmonary complications. *Anesthesiology* 1974;41:8-17.

Pizov R, Takahashi M, Hirshman CA, Croxton T. Halothane inhibition of ion transport of the tracheal epithelium. *Anesthesiology* 1992;76: 985-989.

Plummer GF. Improved method for the determination of propofol in blood by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography* 1987;421:171-176

Puchelle E, Zahm JM. Influence of rheological properties of human bronchial secretions on the ciliary beat frequency. *Biorheology* 1984;21:265-272.

Rautiainen M, Matsune S, Shima S, Sakamoto K, Hanamure Y, Ohyama M. Ciliary beat of cultured human respiratory cells studied with differential interference microscope and high speed video system. *Acta Otolaryngologica* 1992;112:845-851.

Rhodin JAG. Ultrastructure and function of the human tracheal mucosa. *American Review of Respiratory Disease* 1966;93:1-15.

Rogers DF, Barnes PJ. Opioid inhibition of neurally mediated mucus secretion in human bronchi. *Lancet* 1989;1:930-931.

Royal College of Surgeons of England and College of Anaesthetists. Report of a working party of the commission on the provision of surgical services: Pain after surgery. 1990.

Roth Y, Aharonson EF, Teichtahl H, Baum GL, Piel Z, Modan M. Human in vitro nasal and tracheal ciliary beat frequencies: comparison of sampling sites, combined effect of medication, and demographic relationships. *Annals of Otology and Laryngology* 1991;100:378-384.

Rubin BK, Finegan B, Ramirez O, King M. General anaesthesia does not alter the viscoelastic or transport properties of human respiratory mucus. *Chest* 1990;98:101-104.

Ruiz-Santana S, Jimenez AG, Esteban A, Guerra L, Alvarez B, Corcia S, Gudin J, Martinez A, Quintana E, Armengol S, Gregori J, Arenzana A, Rosada L, Sanmartin A. ICU pneumonias: a multi-institutional study. *Critical Care Medicine* 1987;15:930-932.

Rutland J, Cole PJ. Non-invasive sampling of nasal cilia for measurement of beat frequency and study of ultrastructure. *Lancet* 1980;2:564-565.

Rutland J, Griffen W, Cole PJ. An in vitro model for studying the effects of pharmacological agents on human ciliary beat frequency: effects of lignocaine. *British Journal of Clinical Pharmacology* 1982;13:679-683.

Routledge CO, Siefen E, Alper MH, Flake W. Analysis of halothane in gas and blood by gas chromatography. *Anesthesiology* 1963;24:862-867.

Sackner MA, Landa J, Hirsch J, Zapata A. Pulmonary effects of oxygen breathing: a six hour study in normal men. *Annals of Internal Medicine* 1975a;82:40-43.

Sackner MA, Hirsch J, Epstein S. Effect of cuffed endotracheal tubes on tracheal mucus velocity. *Chest* 1975b;68:774-777.

Sackner MA. Effect of oxygen in graded concentrations upon tracheal mucus velocity: a study in anaesthetised dogs. *Chest* 1976;69:164-167.

Sanderson MJ, Dirksen ER. Mechanosensitive and beta-adrenergic control of the ciliary beat frequency of mammalian respiratory tract cells in culture. *American Review of Respiratory Disease* 1989;139:432-440.

Schulze S, Roikjaer O, Hasselstrom L, Jensen NH, Kehlet H. Epidural bupivacaine and morphine plus systemic indomethacin eliminates pain but not systemic response and convalescence after cholecystectomy. *Surgery* 1988;103:321-327.

Sebel PS, Lowden JD. Propofol: A new intravenous anaesthetic. *Anesthesiology* 1989;71:260-277.

Siedlecki J, Borowicz JA, Adamski M. Impairment of efficiency of laryngeal defensive reflexes after regaining full consciousness following endotracheal anaesthesia. *Anaesthesia Resuscitation and Intensive Therapy* 1974;2:247-252.

Sill JC, Uhl C, Eskuri S, VanDyke R, Tarara J. Halothane inhibits agonist-induced inositol phosphate signalling in A7r5 cultured vascular smooth muscle cells. *Molecular Pharmacology* 1991;40:1006-1013.

Sleigh MA. Some aspects of the comparative physiology of cilia. *American Review of Respiratory Disease* 1966;93:16-31.

Sleigh MA, Blake JR, Liron N. The propulsion of mucus by cilia. *American Review of Respiratory Disease* 1988;137:726-741.

Spence AA, Smith G. Postoperative analgesia and lung function: a comparison of morphine with extradural block. *British Journal of Anaesthesia* 1971;43:144-148.

Spencer EM, Willatts SM. Isoflurane for prolonged sedation in the intensive care unit: efficacy and safety. *Intensive Care Medicine* 1992;18:415-421.

Stamm WE, Martin SM, Bennett JV. Epidemiology of nosocomial infections due to gram-negative infections: aspects relevant to development and use of vaccines. *Journal of Infectious Diseases* 1977;136:S151-160.

Stanley GD, Bastianpillai BA, Mulcahy K, Langton JA. Postoperative laryngeal competence. The laryngeal mask airway and tracheal tube compared. *Anaesthesia* 1995;50:985-986.

Stevens RM, Teres D, Skillman JJ, Feingold DS. Pneumonia in an intensive care unit: a 30-month experience. *Archives of Internal Medicine* 1974;134:106-111.

Sturgess JM, Chao J, Turner JAP. Transposition of ciliary microtubules: another cause of impaired ciliary motility. *New England Journal of Medicine* 1980;303:318-322.

Strandberg A, Tokics L, Brismar B, Lundquist H, Hedenstierna G. Atelectasis during anaesthesia and in the postoperative period. *Acta Anaesthesiologica Scandinavica* 1986;30:154-158.

Tamaoki J, Kobayashi K, Sakai N, Kanemura T, Horii S, Isono K, Takeuchi S, Chiyotani A, Yamawaki I, Takizawa T. Atrial natriuretic factor inhibits ciliary motility in cultured rabbit tracheal epithelium. *American Journal of Physiology* 1991a;260:C201-205.

Tamaoki J, Kanemura T, Sakai N, Isono K, Kobayashi K, Takizawa T. Endothelin stimulates ciliary beat frequency and chloride secretion in

canine cultured tracheal epithelium. American Journal of Respiratory Cell and Molecular Biology 1991b;4:426-431.

Tarhan S, Moffitt EA, Sessler AD, Douglas WW, Taylor WF. Risk of anesthesia and surgery in patients with chronic bronchitis and chronic obstructive pulmonary disease. Surgery 1973;74:720-726.

Tash JS, Means AR. Cyclic adenosine-3',5' monophosphate, calcium and protein phosphorylation in flagellar motility. Biology of Reproduction 1983;28:75-104.

Teichtahl H, Wright PL, Kirsner RLG. Measurement of in vitro ciliary beat frequency: a television- video modification of the transmitted light technique. Medical and Biological Engineering and Computing. 1986;24:193-196.

Tomlin PJ, Howarth FH, Robinson JS. Postoperative atelectasis and laryngeal incompetence. The Lancet;1:1402-1405.

Urban BW. Differential effects of gaseous and volatile anaesthetics on sodium and potassium channels. British Journal of Anaesthesia 1993;71:25-38.

Van de Donk HJM, Zuidema J, Merkins FWHM. The influence of the pH and osmotic pressure upon tracheal ciliary beat frequency as determined with a new photo-electric registration device. Rhinology 1980;18:93-104.

Van Dongen K, Leusink H. The action of opium-alkaloids and expectorants on the ciliary movements in the air passages. Archives of International Pharmacodynamics 1953;93:261-276.

Venn RF, Michalkiewicz A. Fast reliable assay for morphine and its metabolites using high performance liquid chromatography and native fluorescence detection. Journal of Chromatography 1990;525:379-388.

Wahba RWM. Perioperative functional residual capacity. Canadian Journal of Anaesthesia 1991;38:384-400.

Wahba RWM, Beique F, Kleiman SJ. Cardiopulmonary function and laparoscopic cholecystectomy. *Canadian Journal of Anaesthesia* 1995;42:51-63 .

Wanner A. Clinical aspects of mucociliary transport. *American Review of Respiratory Disease* 1977;116:73-125.

Wheatley RG, Madej TH, Jackson IJB, Hunter D. The first year's experience of an acute pain service. *British Journal of Anaesthesia* 1991;67:353-359.

Wightman JAK. A prospective study of the incidence of postoperative pulmonary complications. *British Journal of Surgery* 1968;55:85-91.

Willets JM, Lambert DG, Lunec J, Griffiths HR. Studies on the neurotoxicity of 6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline (salsolinol) in SH-SY5Y cells. *European Journal of Pharmacology, Environmental Toxicology and Pharmacology Section* 1995;40228:1-8.

Wilson R, Roberts D, Cole P. Effects of bacterial products on human ciliary function in vitro. *Thorax* 1985;40:125-131.

Wolfe WG, Ebert PA, Sabiston DC. Effect of high oxygen tension on mucociliary function. *Surgery* 1972;72:246-252.

Wong LB, Miller IF, Yeates DB. Stimulation of ciliary beat frequency by autonomic agonists: in vivo. *Journal of Applied Physiology* 1988;65:971-981.

Yager J, Chen T-M, Dulfano MJ. Measurement of frequency of ciliary beats of human respiratory epithelium. *Chest* 1978;73(5):627-633.

Appendix: Sources of reagents

Antibiotic/antimycotic solution (10 000 units penicillin, 10 mg streptomycin, 25 µg amphotericin B), Sigma, UK

DMSO, Sigma, UK

DPN, Amersham, UK

Enflurane, Anaquest Ltd, Windlesham, Surrey, UK

Foetal calf serum, Life Technologies, Paisley, Scotland

Halothane, Zeneca Ltd, Macclesfield, Cheshire, UK

HBSS, Sigma, UK

HEPES, Sigma, UK

Isoflurane, Abbott Laboratories, Queensborough, Kent, UK

Isopropanol, Fisons Scientific Equipment, Loughborough, UK

L-glutamine, Life Technologies, Paisley, Scotland

MEM, Life Technologies, Paisley, Scotland

Midazolam, Roche, Hertfordshire, UK

Morphine, Sigma, UK

MTT, Sigma, UK

Naloxone, Sigma, UK

Optiphas X, EG&G, Crownhill, Milton Keynes

Propofol, Zeneca Ltd, Macclesfield, Cheshire, UK

Sodium chloride, Sigma, UK

Sodium Hydrogen Carbonate, Fisons Scientific Equipment, Loughborough, UK

TrisHCL, Sigma, UK

Trypsin, Life Technologies, Paisley, Scotland