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# The effect of chronic nasal application of chitosan solutions on cilia beat frequency in guinea pigs

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**Abstract**

The cilia beat frequency (CBF) was evaluated in guinea pigs after daily nasal administration of various chitosan solutions for 28 days. The chitosans tested were of various molecular weights and degree of deacetylation. The effect of the chitosans on the CBF was measured on the excised nasal tissue and compared to the effect of sodium taurodihydrofusidate and cocaine by means of a microscope connected to a video camera mounted on the phototube and a data acquisition unit. The results showed that none of the chitosans tested has any effect on the CBF as compared to the controls. © 1997 Elsevier Science B.V.

*Keywords:* Cilia beat frequency; Guinea pigs; Chitosan; Nasal administration; Nasal toxicity

## 1. Introduction

For surfactant type absorption enhancers such as bile salts, fusidate derivatives and acylcarnitines, a strong correlation between the extent of morphological damage caused by their application to the mucosa and the degree of cilia toxicity observed has been reported on numerous occasions (Ennis et al., 1990; Hirai et al., 1981; Schipper et al., 1992). Therefore monitoring changes in the ciliary beat frequency (CBF) of respiratory tissue following the application of a test solution is a valuable tool for assessing the safety of potential nasal peptide absorption enhancers (Merkus et al., 1993).

Chitosan is a cationic polysaccharide derived from chitin by complete or partial deacetylation of the aminogroups in the glucosamine units.

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Table 1
Various characteristics of the five chitosans investigated in this toxicity study

| | Chitosan | | | | |
|---|---|---|---|---|---|
| | 113Cl | 211Cl | 210G | 313Cl | 411Cl |
| Molecular weight $\times 10^3$ (g/mol) | $<50$ | 130 | 205 | 340 | 350 |
| Deacetylation (%) | 85 | 50 | 83 | 84 | 50 |
| Intrinsic viscosity (ml/g, 25°C) | 153 | 621 | 976 | 1303 | 1086 |
| Salt type (% w/w) | Hydrochloride (16) | Hydrochloride (9) | Glutamate (42) | Hydrochloride (16) | Hydrochloride (9) |

Chitosan has been exploited for water purification due to its ability to bind some heavy metals. In the pharmaceutical industry chitosan has shown promise as a controlled release excipient for oral delivery systems. Recently, it was shown by Illum et al. (1994) that chitosan was able to enhance the transport of drugs such as peptides and proteins across mucosal membranes. It was later shown that the mechanism of transport enhancement was a combination of bioadhesion and a transient opening of the tight junctions in the membrane (Artursson et al., 1994). It was further shown that chitosan had no, or a transient, arresting effect on the mucociliary clearance rates as measured in vitro in the frog palate model and on human excised turbinates or in vivo in human volunteers using a saccharine clearance test (Aspden et al., 1997). Experiments in the perfused rat model showed negligible release of the cell markers LDH and 5-ND and of protein as compared to a positive control. Furthermore, histological sections of rat and human nasal tissue exposed to chitosan for extended time periods showed no difference to controls.

The aim of this study was to investigate changes in the nasal ciliary function of guinea pigs subjected to chronic dosing with a series of chitosans. The chitosans were applied once daily, over a 28 day period in an attempt to mimic the long-term effects of chitosan on the nasal mucosa. The method used was adapted from the ex vivo technique developed by Levrier et al. (1989). The main advantage of this method over the other animal models and CBF monitoring techniques is the close simulation of therapeutic use, where the normal physiological conditions of the mucosa are maintained throughout administration of the agent and the mucus layer is subsequently removed by the mucociliary clearance mechanism.

## 2. Materials

The chitosans in the study were of varying molecular weights and degree of deacetylation and donated by Pronova Biopolymer, Drammen, Norway (Table 1). Sodium chloride solution 0.9% w/v (Steriflex®, intravenous infusion, Fresenius, Hants, UK) was used throughout the study. Sodium taurodihydrofusidate (STDHF) was obtained from Leo Pharmaceuticals, Denmark. The 10% stock aqueous cocaine solution was supplied by the Pharmacy Dispensary, Queens Medical Centre, Nottingham. A concentrated solution of Patent Indigo dye ( $\approx 10\%$) was used as received. Ultrapure water (UHP, Elgastat, Bucks., UK) was used throughout the study and all other reagents were at least of analytical grade and were used as received.

## 3. Solution preparation

The chitosan salts (equivalent to 0.25% w/v chitosan base) were dissolved in 0.9% w/v saline overnight using a magnetic stirrer. Once fully dissolved the chitosan solutions were readjusted to pH 4 using 0.1 M HCl and made up to volume.

The cocaine solution was diluted with physiological saline to give solutions of 10, 5, 2.5 and 1% w/v.

The STDHF was dissolved in 0.9% w/v saline at a concentration of 1% w/v.

Saline (0.9% w.v) was adjusted to pH 4 using 0.1 M HCl.

## 4. Experimental methods

### 4.1. Nasal dosing

Male and female juvenile Dunkin–Heartley guinea pigs (JABU, Nottingham University with a starting weight of 300 g ( $\approx$ 500 g at the conclusion of the study) were used with five animals per group. Test solutions were applied in vivo to the nasal cavity of the guinea pigs using a 1 ml disposable insulin syringe (Becton Dickinson, UK) with a short length of polyethylene tubing (o.d. 0.8 mm, i.d. 0.4 mm) attached.

The guinea pigs were manually restrained in a supine position whilst an aliquot of study solution was instilled into each nostril. The animals were retained in this inverted position for 5 min to disperse the chitosan over the largest area of the nasal cavity possible. A separate group of animals dosed with 0.9% w/v saline pH 4 was used as a control.

The most suitable volume of test solution to instil into each nostril to ensure complete coverage of the respiratory epithelium was established by dosing unconscious guinea pigs with 30 and 50 $\mu$l aliquots of Patent Indigo dye. These were then immediately sacrificed with an intraperitoneal (i.p.) overdose of sodium pentobarbitone 200 mg/ml (Pentoject, Animalcare Ltd., York, UK), without disturbing the orientation of the animal's head. With minimal trauma, the nasal cavities were then immediately exposed and the distribution of the dye over the cavity noted.

The control guinea pigs were dosed with the cocaine solutions and the STDHF solution only once. The test solutions were left in contact with the surface of the nasal mucosa for 30 min before the animals were sacrificed with an overdose of anaesthetic and their average CBF determined.

The six groups of animals treated with the chitosans and the saline solution were dosed once daily in each nostril for 28 days. On the 29th day of the study the guinea pigs were sacrificed with anaesthesia and their CBF values determined.

### 4.2. Tissue removal

A midline incision through the skin covering the skull was made along the entire length of the bridge. The nasal cavity of the guinea pig was then exposed by carefully dismantling the skull to reveal the respiratory epithelium. Next, the tissue covering both sides of the nasal septum was removed. This was achieved by gently grasping the septum with forceps at the bridge of the nose and running a scalpel blade underneath it. Great care was taken not to damage the delicate epithelial surface. The extirpated tissue was then immediately immersed in pre-warmed Medium 199 enriched with Hanks salts (Gibco, UK) and incubated at 37°C until required (10–90 min).

Slides of these tissue samples were prepared by carefully sandwiching a piece of tissue between two round microscope cover slides separated by a rubber O-ring in a chamber and completely filling this with incubation medium.

The slides were left to equilibrate for 15 min at 37°C before ciliary movement was assessed. This prevented excessively high readings that could be attributed to the mechanical stimulation of the cilia that unavoidably occurs when manipulating the tissue. Ciliary activity was recorded ex vivo using the apparatus described below.

### 4.3. CBF detection apparatus

The beating cilia were visualised using an inverted binocular microscope (Olympus CK2-TRP) fitted with a thermostatted heating platform (Linkam Scientific Instruments) and a slide chamber. The magnifying power of the objective and ocular lenses was $\times 10$ and $\times 20$, respectively. Images of the beating cilia could either be analysed on line or recorded onto video tape for later examination.

The determination of CBF was achieved using a video camera (Hitachi KP-143) mounted on the phototube sited behind the eye pieces and connected to a video cassette recorder (Akai VS 425 EK). In practice, due to the small size of the tissue

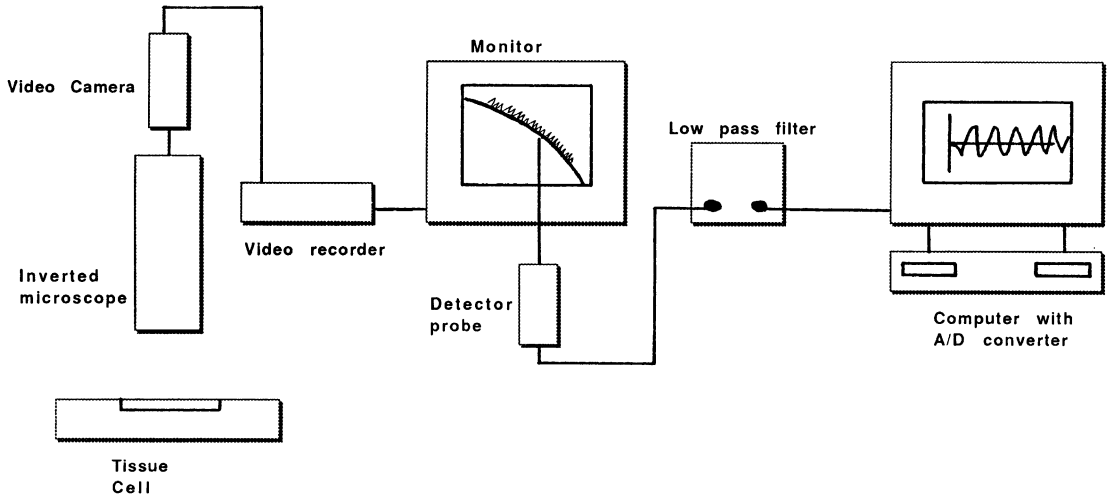


Fig. 1. Data acquisition and processing unit for measuring CBF.

samples under scrutiny and the vigorous beating of the cilia, the beat frequency of each area was determined directly on line as it was being monitored.

Recordings of ciliary movement were processed using a data acquisition unit which consisted of a photo-diode probe, a low pass filter and an Opus PC III computer with an onboard AC/DC converter. The photo cell converted the optical signal detected to an electrical signal which was then amplified to between 0 and 300 mV. The signal then passed through a filter which removed all frequencies above 30 Hz and adjusted the signal to between 0 and 5 mV. The electrical signal derived was then converted into a frequency spectrum using Fast Fourier Transform (FFT). The beating frequency of the cilia was assessed from the spectra displayed on the screen (Fig. 1).

### 4.4. CBF sampling

Initially the whole of the tissue area was scanned visually to provide a rough estimate of the proportion of actively beating cilia present over the whole sample. Once this subjective assessment of tissue quality had been made, six areas of the tissue sample, where the light contrast was sufficient to allow a reasonable signal to be detected, were randomly chosen from each piece of nasal tissue. These areas were used to determine the average ciliary beat frequency. The CBF of isolated cells has been reported to be lowered (Ingels et al., 1991), therefore ciliary activity was recorded only from groups of cells which exhibited a continuous layer of ciliated cells.

Five CBF values were determined from each site and the results were expressed as a mean of 30 values collected from tissues from each guinea pig. A sampling period of 30 s was used to obtain an average CBF value for each group of cilia under surveillance.

## 5. Results

### 5.1. Controls

The guinea pig that was dosed in one nostril with a 30 $\mu$l aliquot of Patent Indigo dye was found to be comprehensively stained in the anterior portion of the dosed side of the nasal cavity only. Dosing with 50 $\mu$l of dye to both nostrils of an unconscious guinea pig, revealed complete coverage of the epithelium of the nasal cavity. This verified that applying a 50 $\mu$l aliquot of test solution to each nostril ensures complete coverage of the nasal epithelium. The guinea pigs dosed with a 50 $\mu$l aliquot of the 1% w/v STDHF

Table 2
The effect on nasal CBF of dosing guinea pigs with various test solutions

| Test solution | Ciliary beat frequency (Hz ± S.D.) | | | | |
|---|---|---|---|---|---|
| | 1.0% Cocaine | 2.5% Cocaine | 5.0% Cocaine | 1.0% STDHF | Undosed[a] |
| Mean ($n = 3$) | 7.4 ± 1.9 | 2.3 ± 1.6 | 0 ± 0 | 0 ± 0 | 7.6 ± 0.8 |

[a] No anaesthetic was used to sacrifice.

solution exhibited no behavioural signs of distress after the nasal application was performed such as nose rubbing or eyes watering. However, a 30 min exposure resulted in the nasal septum becoming very fragile and difficult to excise. Examination of the few thin strips of tissue successfully extirpated, revealed an extremely abnormal mucosa with very few cilia, none of which were motile.

Problems encountered during nasally dosing of conscious guinea pigs with the STDHF and cocaine solutions included sneezing and vigorous struggling. Therefore it was decided to dose with 0.5 ml aliquots of the cocaine and chitosan test solutions. This is a volume grossly in excess of the 50 $\mu$l required, but ensures complete exposure of the nasal cavity to the test solution. Any excess will drain into the stomach and be harmlessly digested or excreted.

Due to the severe nasal epithelial damage caused by the STDHF solutions it was decided not to repeat this part of the study using the larger volume.

Application of a 5% w/v aqueous cocaine solution stopped the cilia beating throughout the 2 h observation period of the excised tissue. However, no obvious signs of epithelial degeneration, such as a colour change of the epithelium or fragmentation of the tissue, was seen. Applying a 2.5% cocaine solution reduced the CBF during this period with no apparent recovery, whilst solutions of 1% had no effect on the ciliary beat frequency (Table 2). These effects were expected, since the reversible CBF inhibitory effects of local anaesthetics is well documented (Van de Donk et al., 1982; Ingels et al., 1994).

### 5.2. Chitosans

Once administered to the nasal cavity, the guinea pigs showed no obvious signs of distress, such as runny noses or eyes, or rubbing of the nose immediately after dosing. The animals remained alert and healthy and exhibited behaviour typical of healthy animals throughout the 29 day study period. The tissue removed from the nasal cavities of the guinea pigs treated with the chitosan formulations, looked and showed characteristics similar to that removed from untreated animals.

Chronic application of all chitosans appeared to have little effect on nasal CBF when compared with the control groups of undosed animals sacrificed by severing the spinal cord and animals chronically dosed with saline only (Table 3).

The normality of the data set was assumed and the slight CBF differences found between the groups were analysed using both a one way analysis of variance (ANOVA) and an unpaired Student's $t$-test. It was found that none of the animals dosed with chitosan had ex vivo ciliary beat frequencies significantly different to those animals dosed with a solution of pH 4 saline ($P < 0.05$).

## 6. Discussion

There are several advantages of the ex vivo method described for assessing ciliotoxicity. These include the opportunity for simulating therapeutic dosage regimens under conditions where the animal's natural defence mechanisms, including mucus production and mucociliary clearance, remain uncompromised. The nasal cavity also experiences

Table 3
The effect on nasal CBF of chronically dosing guinea pigs with various grades of 0.25% chitosan solutions

| Animal | Ciliary beat frequency (Hz ± S.D.) | | | | | |
|---|---|---|---|---|---|---|
| | 113Cl | 211Cl | 210G | 313Cl | 411CI | Saline |
| 1 | 7.4 ± 0.7 | 7.6 ± 0.7 | 7.6 ± 0.6 | 7.7 ± 0.4 | 7.6 ± 0.4 | 7.8 ± 0.9 |
| 2 | 7.8 ± 0.4 | 7.7 ± 0.6 | 7.7 ± 0.4 | 7.4 ± 0.6 | 7.7 ± 0.5 | 7.7 ± 0.6 |
| 3 | 7.4 ± 0.7 | 7.5 ± 0.8 | 7.8 ± 0.5 | 7.6 ± 0.5 | 7.8 ± 0.4 | 7.5 ± 0.8 |
| 4 | 7.9 ± 0.8 | 7.3 ± 0.9 | 7.6 ± 0.5 | 7.6 ± 0.4 | 7.8 ± 0.5 | 7.8 ± 0.8 |
| 5 | 7.5 ± 0.4 | 7.8 ± 0.6 | 7.6 ± 0.8 | 7.5 ± 0.5 | 7.6 ± 0.6 | 7.6 ± 0.7 |
| Mean | 7.6 ± 0.6 | 7.6 ± 0.7 | 7.7 ± 0.6 | 7.6 ± 0.5 | 7.7 ± 0.5 | 7.7 ± 0.7 |

normal aeration and blood flow during dosing with this technique. The results obtained using this technique are also reproducible. This was demonstrated by Levrier et al. (1989) and confirmed by the results from this study.

A 28 day study was considered to be an adequate period of time to study the chronic effects of chitosan application on respiratory cilia, since under normal conditions, human ciliated cells have a life of 4–8 weeks (Herzon, 1983). After this time differentiation of the basal cells replaces any damaged and aged cells at the epithelial surface (Rautianen et al., 1994).

Guinea pigs with their docile nature were chosen for this chronic dosing study, since it was possible to subject these animals to daily nasal dosing, without resorting to repeated sedation. Daily sedation was not thought to be experimentally sound, as it has been shown that bronchial mucociliary clearance is depressed by inhaled anaesthetics such as halothane, nitrous oxide and enflurane (Forbes and Horrigan, 1977; Forbes and Gamsu, 1979). Phadhana-anek et al. (1989) also reported that inhaled anaesthetics reduced the number of ciliated trachael cells in humans but did not change the beat of the surviving cilia. Further, it was decided to avoid the use of daily injected barbiturates as the effect of long-term exposure on CBF is unknown.

In addition, a study by Joki and Saano (1994) concluded that out of six species studied, including rats and rabbits, the guinea pig proved the best model for studying ciliary activity due to the high signal quality produced and lack of variation in the CBF. In a more recent study by Joki et al. (1995) rat nasal tissue was compared with guinea pig nasal tissue and it was concluded that guinea pig cilia are longer (4.3 vs. 3.6 $\mu$m) and thicker (0.22 vs. 0.19 $\mu$m) than rat cilia. Guinea pig tissue was also reported to have a more dense and homogenous epithelial covering of ciliated cells. This could explain the superior CBF signal produced by the guinea pig preparations. However, the fragility of guinea pig tissue compared to rat tissue was also reported, 42% of the guinea pig samples examined showed signs of mechanical damage compared with only 14% of the rat samples. However, without sedation great difficulty was found in reliably administering the dose of test solution exclusively to one nostril. Although the guinea pigs remained non-aggressive when handling without sedation, they struggled vigorously when any of the solutions were administered. This resulted in part of the dose being lost due to sneezing and possibly swallowing. These problems became more frequent the longer the study progressed.

This method is a great improvement over techniques studying brushings or scrapings of small groups of cells where the samples are more likely to have been damaged by the collection process and therefore possess abnormally beating cilia, regardless of any effect caused by exposure to a test solution.

Ciliary abnormalities can also be analysed by ultrastructural observation using transmission electron microscopy (Braat et al., 1995; Jorissen and Bessems, 1995). However, this procedure is extremely time consuming due to the laborious process of serial sectioning involved. The results

are also difficult to obtain and interpret due to the low number of perfectly orientated sections that occur (Lamiot et al., 1990). The use of FFT to analyse the beating cilia, rapidly produces a reconstructed image containing less noise than the original image on the television screen. The largest peak from the power spectrum produced was taken to be the dominant CBF.

Studies by Eshel et al. (1985) and Kennedy and Duckett (1981) suggest that at room temperature the general characteristics of ciliary movement produce multipeak time dependent power spectra when 1 s samples are analysed using FFT. However, if the cilia are sampled for a longer period, then a CBF spectra with a dominant peak is produced. It is probable that the spectra recorded contain frequency components caused by the biphasic nature of ciliary beating and metachronal waves (Batts et al., 1990). However, this study was primarily concerned with differences in CBF found between each group of guinea pigs dosed with different test solutions; not true physiological CBF values. Therefore, it was decided to use the CBF from tissue monitored for 30 s to determine an average CBF. This makes comparisons between the groups less speculative and ambiguous and is considered an acceptable method of examining ciliotoxicity (Batts et al., 1990).

The main disadvantages of the method for assessing ciliotoxicity used in this study mainly concern the dosing procedure and the method of evaluating a representative ciliary beat frequency. Representative CBF determination is difficult using this technique since the excised tissue is very thick and vascular (0.5–1 mm width), particularly towards the centre, therefore light was not transmitted through all parts of the tissue. This meant that although vigorous movement in these areas could be seen on the video screen with the naked eye, it was almost impossible for the data acquisition unit to detect this beating, purely from the reflected light patterns. As a consequence, the majority of CBF values were determined from the translucent areas of the tissue, usually at the edges. An assumption was made that these frequencies were typical of ciliary action in the thicker areas of mucosa.

The chitosan solutions were prepared at pH 4 to mimic the characteristics of the formulations of chitosan used for nasal delivery. It has been confirmed by several groups that this pH does not adversely affect the nasal epithelium. Hirai et al. (1978) demonstrated that the permeability of dog nasal mucosa is not affected by pH unless it drops below three. Although using guinea pig tissue in vitro, Wharton (1931) reported a correlation between low pH and ciliotoxicity, in the pH range 5.8–8.3. In more detailed experiments, Van de Donk et al. (1980) and Luk and Dulfano (1983) reported that in vitro solutions between pH 7 and 9 are harmless to cilia but conditions outside this range result in a progressive deterioration in CBF. However all these studies used non-physiological, mucus free preparations. In addition, chitosan salts in solution have weak buffering properties and will quickly adjust to the pH of the mucus layer, which also possesses buffering properties (Fabriant, 1941).

To demonstrate that the very close CBF values determined were not an experimental artefact caused by the use of anaesthetic to sacrifice the animals, a control group of guinea pigs were humanely sacrificed without the use of anaesthetic, by severing the spinal cord. The CBF values determined from this group were not significantly different ($P < 0.05$) to those obtained from the groups sacrificed using anaesthetic.

This study shows that when chronically dosed in vivo none of the 0.25% w/v chitosan solutions affected the ability of nasal cilia to beat, when compared with the beating frequencies recorded for the nasal cilia of guinea pigs dosed with saline at pH 4 and also untreated guinea pigs nasal tissue.

It is difficult to compare the results from this study involving the chronic application of chitosan with other studies investigating ciliotoxicity. Most reported studies are concerned with the acute effects of various nasal absorption promoters and use mucus free, extirpated tissue samples wholly immersed in the test solution. In this environment the test solution can penetrate the nasal tissue from the normally inaccessible basolateral surface of the cells. In addition, the mucus blanket which physically protects the ciliated mu-

cosa and dilutes the test compound to, on average, a tenth of its original concentration, is absent (Streichenberger et al., 1992). Therefore, compounds are usually found to be much less toxic in in vivo studies compared to in vitro studies (Batts et al., 1990).

The ciliotoxicity caused by many nasal absorption promoters following acute exposure has been reported by numerous groups. A 1% w/v Laureth 9 solution dramatically decreased the CBF of human adenoidal tissue when applied in vitro (Hermens et al., 1990). This is not surprising considering the severe damage incurred by rat epithelial tissue exposed to this surfactant, previously reported by Daugherty et al. (1988) and Ennis et al. (1990). This latter group also reported a strong correlation between the effect of absorption promoters on nasal ciliary beating and their influence on nasal tissue integrity.

Different bile salts have been reported to have different effects on ciliary activity. 1% w/v solutions of the dihydroxy bile salts sodium deoxycholate and taurodeoxycholate applied to rat nasal mucosa produced gross morphological damage and hence totally disrupted ciliary movement and co-ordination (Hirai et al., 1981). By contrast, the more hydrophilic trihydroxy bile salts, glycocholate and taurocholate applied at a 1% w/v concentration to human adenoidal tissue and ciliated embryonal chicken trachael tissue only slightly reduced CBF values (Hermens et al., 1990; Duchateau et al., 1986). Jian and Li Wan Po (1993a,b) found that sodium cholate at 6 and 11 mM concentrations was ciliotoxic to rat tracheal rings. In the same paper, the ciliotoxicity of the peptidase inhibitor aprotinin, applied to rat tracheal cilia at concentrations of 1000 and 2000 KIU, was also reported.

Merkus et al. (1991) investigated the ciliotoxicity of cyclodextrins and reported that a 5% w/v solution of methyl-$\beta$-cyclodextrin induced ciliostasis 30–40 min after applying to ciliated chicken embryo trachea. This effect was reversible after rinsing with Locke ringer solution. However, it was later shown that the ciliostatic potential of 2% methylated-$\beta$-cyclodextrin was similar to that of 0.9% NaCl (Romeijn et al. (1996)). Schipper et al. (1992) further investigated this enhancer and found that for both chicken embryo trachea and human adenoidal tissue a 5% w/v solution gave the same results as Merkus et al. (1991). A 2% w/v solution which also produced an acceptable insulin bioavailability in rats when co-administered, reduced the original ciliary activity by 60% after 60 min exposure in both models. This group also showed that the fusidic acid derivative STDHF, at a 0.3% w/v concentration was less ciliotoxic than Laureth 9 and dihydroxy bile salts, but more ciliotoxic than trihydroxy bile salts at the same concentration. A 0.3% w/v solution of deoxycholate severely decreased human ciliary activity in vitro but taurocholate and glycocholate at the same concentration had a much less deleterious effect. At concentrations above 0.5% w/v, STDHF was found to induce irreversible ciliostasis. This correlates with the ciliotoxic effect found in vivo in this study when applied at 1% w/v.

The guinea pig study described in the present paper evaluates cilia beat frequencies in vitro free from the influence of mucus after removal of the nasal tissue from the guinea pigs. Therefore, it could be argued that the CBF values determined are not truly representative of guinea pig cilia beat frequencies in vivo. However, as already mentioned, this study was primarily concerned with the adverse effects on the capacity of the cilia to beat that could be attributed to the chronic application in vivo when the protective mucus layer was intact and not determination of the actual guinea pig CBF values in vivo.

Therefore, as a toxicity index, this study demonstrates the benign nature of all five chitosans investigated following chronic once daily application of a 0.25% w/v solution to guinea pig nasal mucosa.

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