Department of Pharmaceutics[1], School of Pharmacy, Potchefstroom University for Christian Higher Education, Potchefstroom, and Pulmonary Unit[2], Pretoria Academic Hospital, Pretoria, Republic of South Africa

Intranasal toxicity of selected absorption enhancers

N. Haffejee[1], J. Du Plessis[1], D. G. Müller[1], C. Schultz[2], A. F. Kotzé[1] and C. Goosen[1]

The intranasal toxicity of selected absorption enhancers (LPC, DMβCD, *N*-trimethyl chitosan chloride (TMC) and chitosan hydrochloride) were determined *in vivo* by investigating the acute microscopic toxic potential on the morphology of rat nasal epithelium with transmission electron microscopy (TEM) and *in vitro* by measurement of the ciliary beat frequency (CBF), of human ciliated nasal epithelium. TEM evaluations showed that LPC (1% w/v) caused severe epithelial damage and pyknosis. No damage to the rat nasal epithelium was caused by the other absorption enhancers. CBF measurements showed that LPC resulted in total loss of ciliated cells while DMβCD, TMC and chitosan hydrochloride did not cause any major changes in CBF.

1. Introduction

The nasal route of administration is one of several transmucosal routes under investigation for the systemic delivery of drugs. The pharmacokinetic rationale for employing the nasal route, is to accommodate drugs that are extensively metabolized in the gut wall or that are subject to extensive first pass elimination in the liver when administered orally [1]. Nasal drug delivery often requires the coadministration of absorption enhancers to achieve therapeutic blood levels, particularly for large, hydrophilic drugs such as peptides and proteins [2–4]. Efficacy and safety of nasal absorption enhancers depend on many different parameters, such as their influence on nasal epithelial membrane barriers, the enzymatic activities in the nasal cavity and the mucociliary clearance. The influence of the enhancers on the absorption of the drug across the nasal membrane is related to a direct effect on the drug and/or influence on the mucus layer and/or the nasal mucosal epithelium. With respect to the mucus and mucous membranes, enhancers may act by alteration of the properties of the mucus layer, by opening the tight junctions between epithelial cells or by increasing membrane fluidity, either by creating disorders in the phospholipid domain in the membrane or by facilitating the leaching of proteins and lipids from the membrane. They improve peptide and protein absorption probably by one or a combination of mechanisms [5].

Ciliary beat frequency (CBF) is under cellular control, and is on average 10–15 Hz in the respiratory tract of mammals [6]. The average beat frequency of human nasal cilia is 13 Hz *in vitro* at 37 °C [7]. Cilia are motile fingerlike appendages extending from the surface of the nasal epithelial cells [8] and move in a well-organized and coordinated way to propel the overlying mucus layer toward the throat. The inspired dust, allergens and bacteria are entrapped in the mucus and are then removed. The combined action of the mucus layer and cilia is called mucociliary clearance. The function of the nasal mucociliary clearance is to remove foreign substances and particles from the nasal cavity, thus preventing them to reach the lower airways as well as to protect the respiratory system from damage by inhaled substances [9]. Because the self-cleaning capacity of the nose by the ciliary epithelium is necessary to remove dust, allergens and bacteria, it should not be influenced by nasal medication. Ciliary movement is a major factor for mucociliary clearance in the upper airways [10]. The occurrence of respiratory diseases is often related to impairment of mucociliary clearance. It is therefore of great importance to determine the effects of nasal drug delivery systems and their individual components on nasal mucociliary clearance. Impairment of the mucociliary clearance by these systems can be prohibitive of their therapeutical use. Merkus et al. have studied the *in vitro* effects of a variety of well-known and effective absorption enhancers on ciliary beat frequency using chicken embryo tracheal tissue and human adenoid tissue [5]. These results suggest that a good correlation exist between the effects of the enhancers on nasal morphology and CBF. The measurement of CBF *in vitro* is a very accurate and reproducible technique to determine potential toxic effects on ciliated epithelium [11–14] and is a good *in vitro* screening method to establish the potential toxicity of drugs and excipients.

The following enhancers were chosen to determine and compare their acute nasal toxicity effects: L-α-lysophosphatidylcholine (LPC) (phospholipid), dimethyl-β-cyclodextrin (DMβCD) (cyclodextrin), *N*-trimethyl chitosan chloride (TMC) and chitosan hydrochloride. These absorption enhancers are frequently used in drug delivery studies. TMC, a partially quaternized derivative of chitosan, has intensely been studied and described for its absorption enhancing effects by Kotzé et al. [15]. They also studied the absorption enhancing properties of chitosan hydrochloride and determined that this polymer is a highly effective absorption enhancer in acidic environments [16]. LPC has been shown in a study done by Fisher et al. to be an effective nasal enhancer of human growth hormone (hCG) in rats, rabbits and sheep [17]. Previous studies have shown that nasal formulations of estradiol and progesterone containing dimethyl-β-cyclodextrin (DMβCD) as solubilizer and enhancer appeared to increase substantially the bioavailability of these steroid hormones in rabbits, rats and men [3, 18–20]. These DMβCD-hormone formulations exerted only minor effects on the ciliary beat frequency of human adenoid tissue *in vitro*. Although there is extensive proof of the absorption enhancing properties of TMC and chitosan hydrochloride, toxicity data on these polymers is limited. Therefore, in this study, the acute toxicity of the above selected nasal absorption enhancers was investigated on a morphological (histopathology) and physiological (CBF) level after intranasal administration.

2. Investigations, results and discussion

2.1. Transmission electron microscopy (TEM)

The acute local effects of nasal absorption enhancers on the morphology of the rat nasal epithelium were studied with transmission electron microscopy (TEM). The nasal cavity was not affected by administering *physiological sal-*

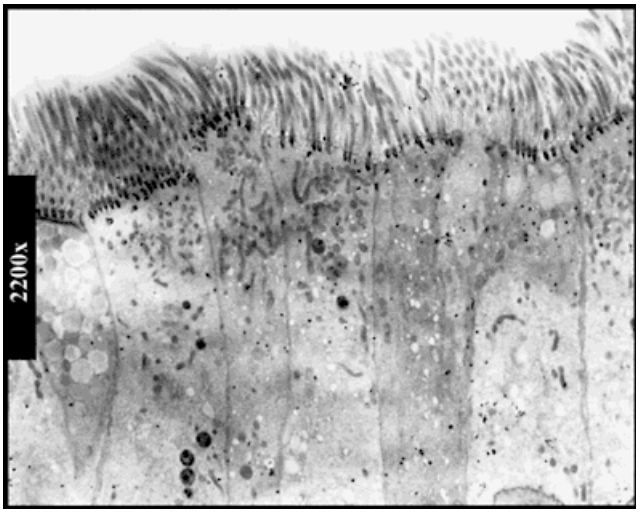

Fig. 1: TEM micrograph of the rat nasal epithelium treated with physiological saline (control, 2200x)

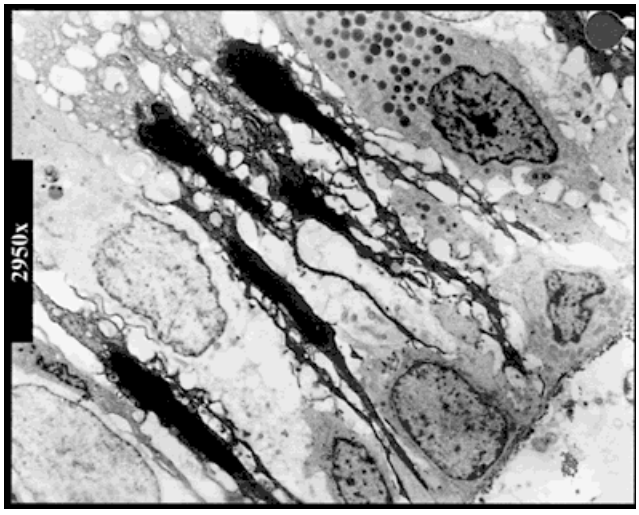

Fig. 2c: TEM micrograph of the rat nasal epithelium treated with LPC 1% w/v with visible epithelial disruption and pyknosis (2950x)

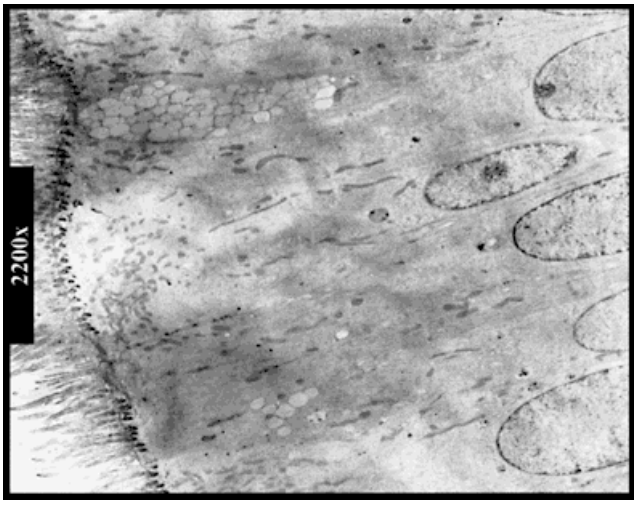

Fig. 2a: TEM micrograph of the rat nasal epithelium treated with LPC 0.0625% w/v (2200x)

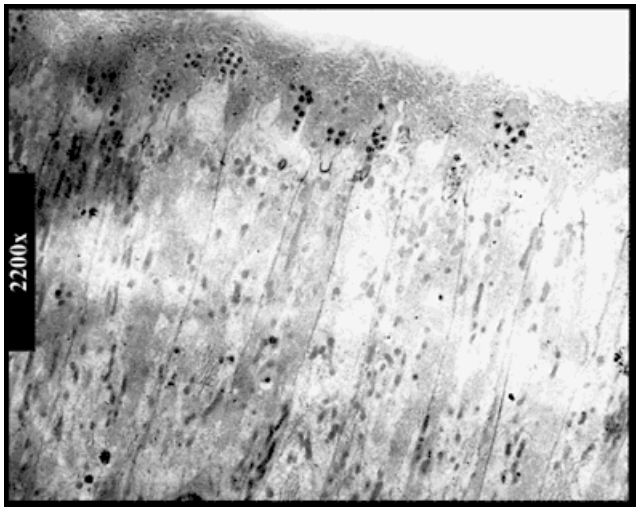

Fig. 2d: TEM micrograph of the rat nasal epithelium treated with LPC 1% w/v with loss of cilia (2200x)

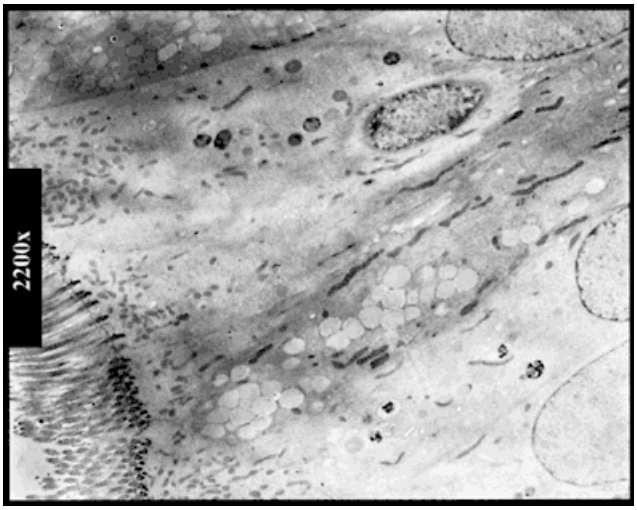

Fig. 2b: TEM micrograph of the rat nasal epithelium treated with LPC 0.125% w/v (2200x)

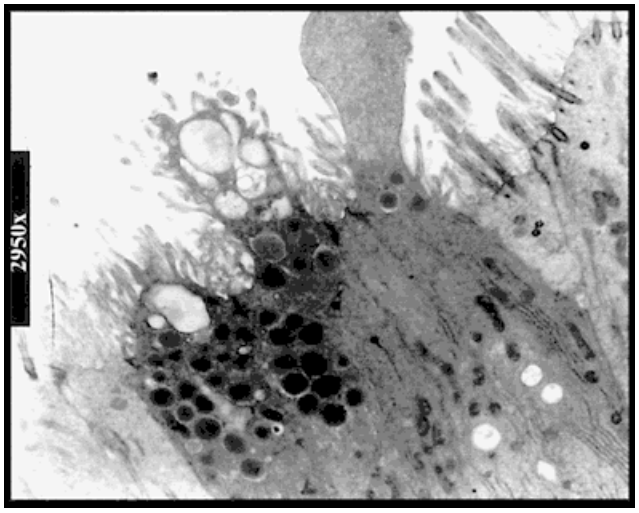

Fig. 2e: TEM micrograph of the rat nasal epithelium treated with LPC 1% w/v with mucus discharge from a goblet cell (2950x)

ine (Fig. 1) and therefore the untreated side of the nasal cavity was consistently used as the control side. LPC 0.0625% w/v (Fig. 2a) and 0.125% w/v (Fig. 2b) caused no change in the respiratory epithelia or the ciliary integrity. After intranasal administration of LPC (1% w/v) severe damage occurred. Pyknosis (recognised by darker colouring of the nucleus and shrinkage) and epithelial disruption were seen (Fig. 2c). Cilia were irregular or absent (Fig. 2d) and abundant mucus extrusion was observed (Fig. 2e). The histological effects of LPC on animal tissue have also been investigated by several research groups, using different contact times and was also reported to cause severe epithelial disruption [21, 22]. *DMβCD* 1% w/v (Fig. 3a), 3% w/v (Fig. 3b) and 5% w/v (Fig. 3c) resulted in no change in the epithelia and the effects observed were comparable to that obtained with physiological saline. The shape of the cells and integrity of the cilia were maintained. *TMC* 0.125% w/v (Fig. 4a) caused no change in the respiratory epithelia and the shape of the cells were normal. The effects caused by TMC 0.25% w/v (Fig. 4b) were comparable to that obtained with physiological saline. TMC 1% w/v caused no change in the arrangement of the cilia (Fig. 4c) or the shape of the cells and nuclei (Fig. 4d). TMC 3% w/v (Fig. 4e) and 5% w/v (Fig. 4f) caused no change in the respiratory epithelia and the effects were graded similar to that obtained with physiological saline. After intranasal administration of *chitosan hydrochloride* 0.125% w/v (Fig. 5a) and 0.25% w/v (Fig. 5b) no changes were recorded in the respiratory nasal epithelia and the effects were comparable to that obtained with physiological saline. Chitosan hydrochloride 1% w/v resulted in slight disarrangement in the shape of the nuclei (Fig. 5c) but no effect on the arrangement of the cilia (Fig. 5d).

LPC 1% w/v showed severe epithelial damage at higher concentrations. TEM evaluation showed a slight disarrangement in the shape of the nuclei obtained after administration of chitosan hydrochloride 1% w/v. It is possible that this due to a beginning reaction, which caused morphological changes in the shape of the nuclei and required further evaluation. All the other absorption enhancers studied resulted in no damage to the nasal epithelium of the rat.

Fig. 3a: TEM micrograph showing no change of the rat nasal epithelium exposed to DMβCD 1% w/v (3200x)

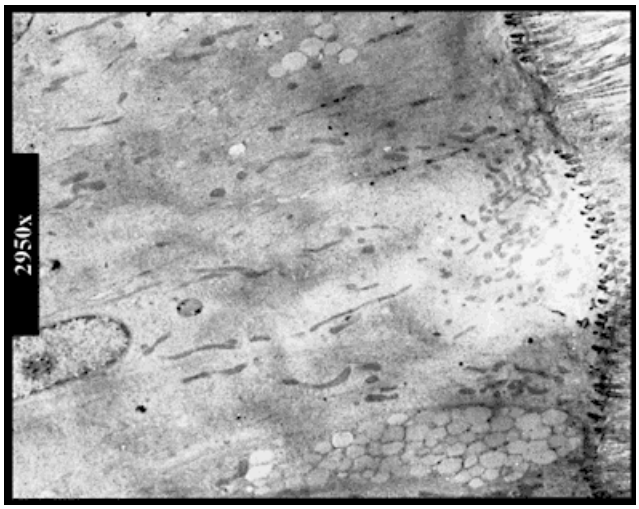

Fig. 3b: TEM micrograph showing no change of the rat nasal epithelium exposed to DMβCD 3% w/v (2950x)

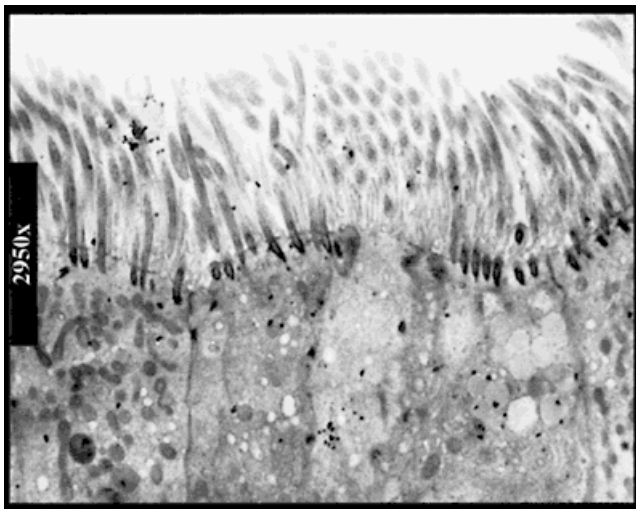

Fig. 3c: TEM micrograph showing no change of the rat nasal epithelium exposed to DMβCD 5% w/v (2950x)

2.2. Ciliary beat frequency (CBF)

The results of the *in vitro* nasal CBF measurements on human ciliated nasal epithelium are presented in the Table. The mean CBF for the various absorption enhancers ranged from 10.03 Hz (DMβCD) to 12.74 Hz (control). These values are within the CBF range (10–15 Hz) for humans measured *in vitro* [6]. However, no ciliated epithelia could be found after treatment with LPC 1% w/v due to disruption and loss of ciliated cells and it was therefore impossible to do CBF measurements. The percentage reduction in the CBF after administration of DMβCD, TMC and chitosan hydrochloride is also listed in the Table. It is apparent that DMβCD 5% w/v affected CBF, causing a decrease of 21.3% w/v in CBF, but was still within the control CBF range. TMC and chitosan hydrochloride affected CBF to a similar degree but was also within the control CBF range.

From the CBF results obtained in this study, it is apparent that DMβCD 5% w/v, TMC 5% w/v and chitosan hydrochloride 1% w/v have no ciliostatic activity, however LPC 1% w/v was found not to be suitable for intranasal use. Ciliary beat frequency measurements have been shown to be a good indicator for the effects of substances on nasal tissue morphology and are useful to screen compounds.

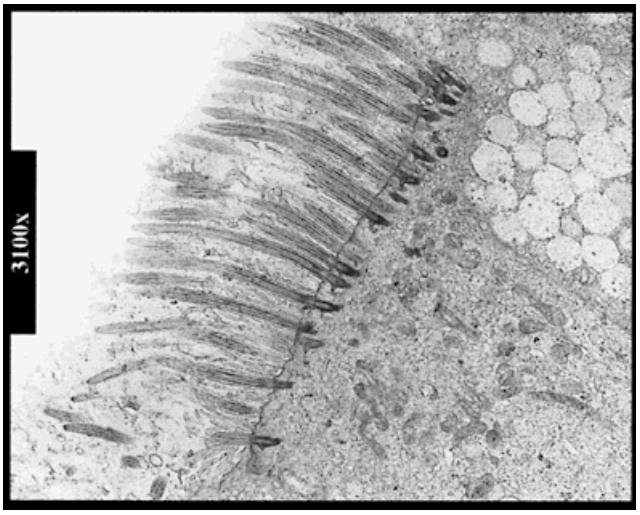

Fig. 4a: TEM micrograph of the nasal epithelium after intranasal administration of TMC 0.125% w/v (3100x)

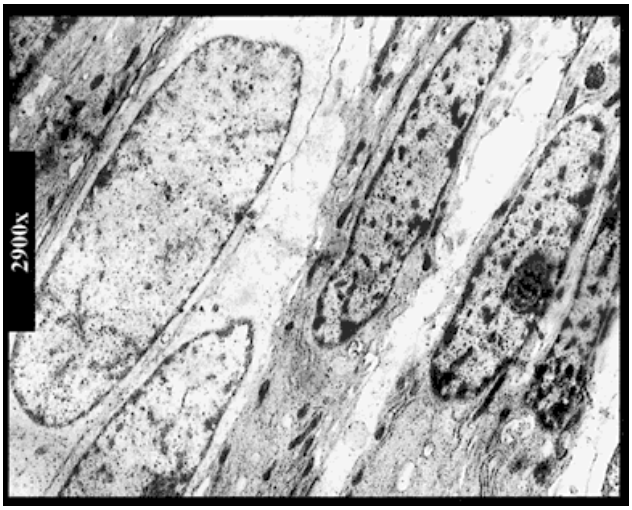

Fig. 4b: TEM micrograph of the nasal epithelium after intranasal administration of TMC 0.25% w/v (2900x)

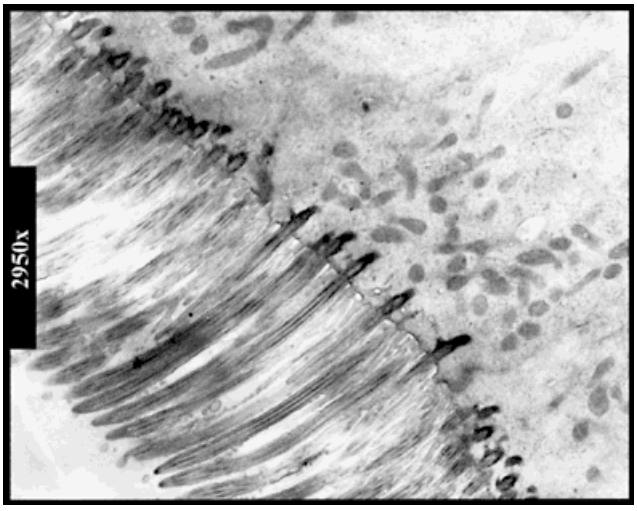

Fig. 4c: TEM micrograph of the rat nasal epithelium treated with TMC 1% w/v, showing no change in the arrangement of the cilia (2950x)

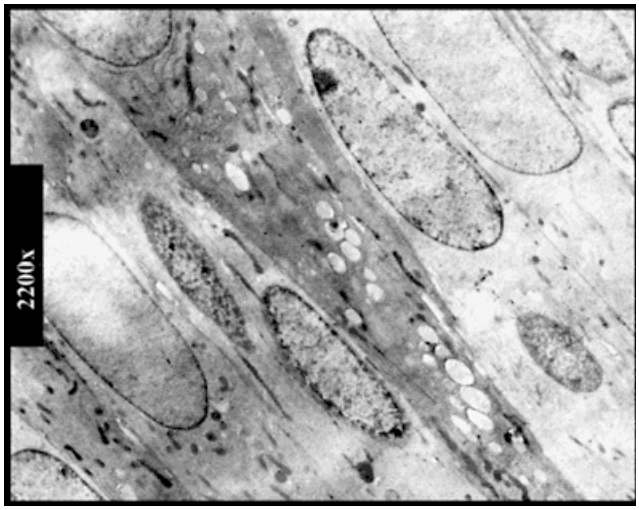

Fig. 4d: TEM micrograph of the rat nasal epithelium treated with TMC 1% w/v, showing no change in the shape of the cells (2200x)

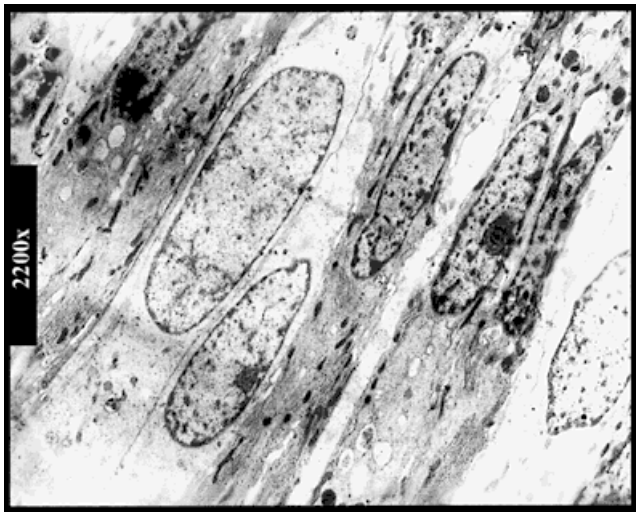

Fig. 4e: TEM micrograph of the rat nasal epithelium after 15 min exposure to TMC 3% w/v (2200x)

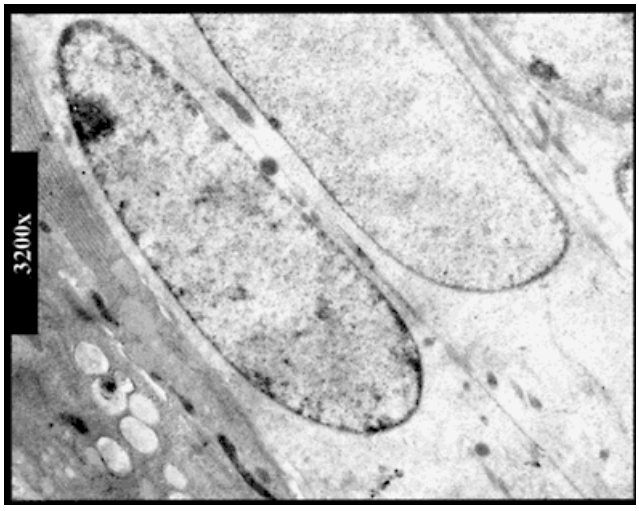

Fig. 4f: TEM micrograph of the rat nasal epithelium after 15 min exposure to TMC 5% w/v (3200x)

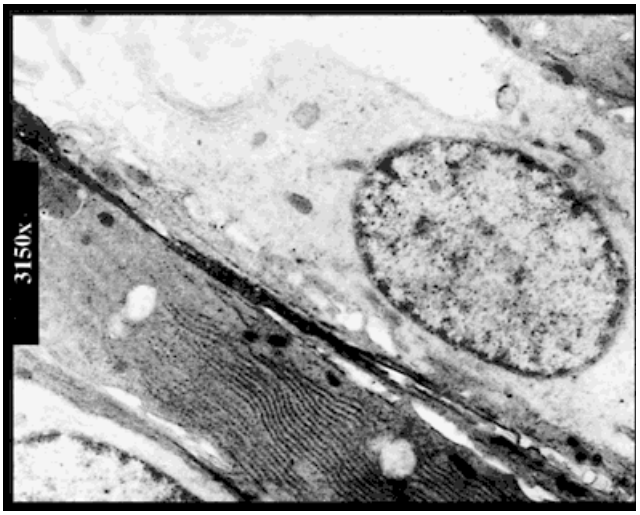

Fig. 5a: TEM micrograph of the rat nasal epithelium exposed to chitosan hydrochloride 0.125% w/v (3150x)

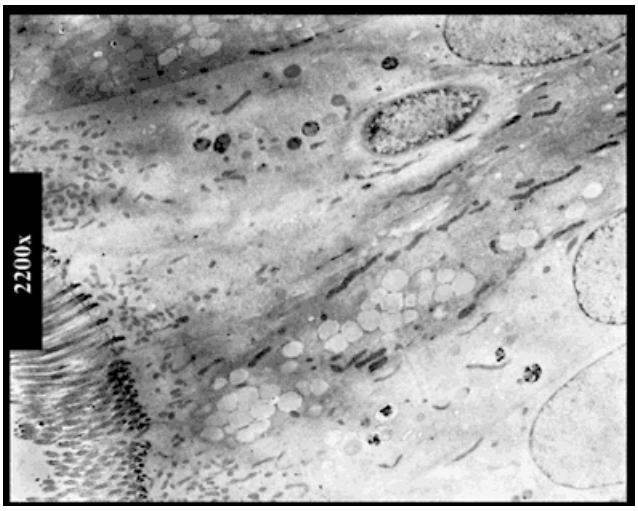

Fig. 5b: TEM micrograph of the rat nasal epithelium exposed to chitosan hydrochloride 0.25% w/v (2200x)

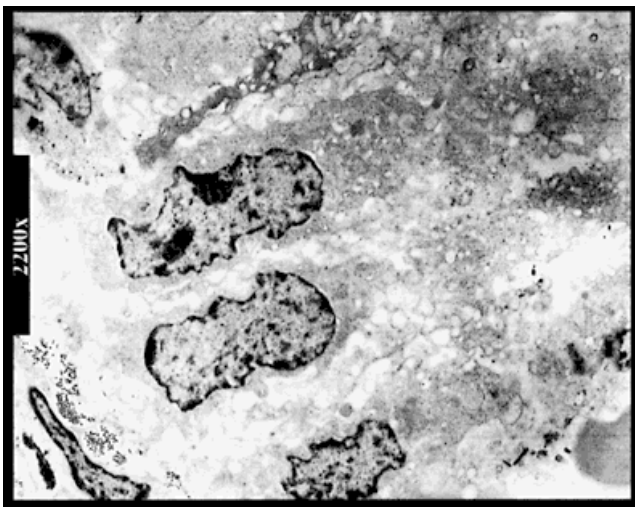

Fig. 5c: TEM micrograph of the rat nasal epithelium treated with chitosan hydrochloride 1% w/v, with slight disarrangement in the shape of the nuclei. (2200x)

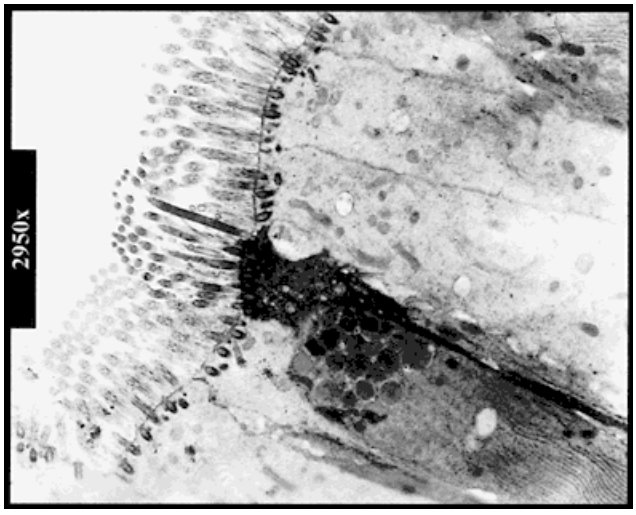

Fig. 5d: TEM micrograph of the rat nasal epithelium treated with chitosan hydrochloride 1% w/v, with no disarrangement in the shape of the nuclei. (2950x)

Table: CBF measurements after exposure of human nasal cilia to DMβCD, TMC, and chitosan hydrochloride

	Minimum Hz	Maximum Hz	Mean Hz ± S.D.	Reduction in CBF (%)
Control	8.3	16.8	12.74 ± 3.07	–
DMβCD (5% w/v)	6	13.6	10.03 ± 2.95	21.3
TMC (5% w/v)	6.1	13.6	11.13 ± 2.35	12.6
Chitosan HCl (1% w/v)	7.5	12.9	11.12 ± 1.50	12.7

No ciliated epithelia could be found in the solution after treatment with LPC 1%, due to the disruption and loss of ciliated cells and it was therefore impossible to do CBF measurements

Before a drug or a pharmaceutical excipient can be included in a nasal formulation, the effect on CBF has to be tested at an early stage.

3. Experimental

3.1. Reagents

Dimethyl-β-cyclodextrin (DMβCD) and L-α-lysophosphatidylcholine (LPC) were purchased from Sigma (St. Louis, MO, USA). Chitosan hydrochloride was acquired from Pronova Biopolymer (Drammen, Norway, degree of deacetylation, 83%). *N*-trimethyl chitosan chloride (TMC) was supplied by the Pharmaceutics Department (PU for CHE, Potchefstroom, RSA, degree of deacetylation 80%, degree of quaternization 12.28%) [23]. Physiological saline (0.9% NaCl) was obtained from Adcock Ingram Critical Care Ltd. (Aeroton, Johannesburg, RSA). Todd's fixative (containing 25% glutaraldehyde, 1.25% paraformaldehyde, 0.03% $CaCl_2$ and 0.03% picric acid) was supplied by Merck (Darmstadt, Germany). Fluothane® (containing halothane and thymol 0.01%) was supplied by Zeneca SA Pty. Ltd. (Woodmead, RSA). Spurr's resin was purchased from Ladd Research Industries (Burlington, USA). Cacodilate buffer, uranyl acetate and osmium tetroxide were purchased from Sigma (St. Louis, MO, USA). Hanks' balanced salt solution was obtained from Bio-Whittaker (Walkersville, Maryland). The nasal cytology brush was purchased from Hobbs Medical, Inc. (Traunstein, Germany). All other agents used were of analytical grade.

3.2. Preparation of absorption enhancer solutions

3.2.1. Transmission electron microscopy

All solutions were prepared in physiological saline (0.9% NaCl) in Millipore water. Solutions were adjusted to a pH of 6.8 (within the physiological pH range of the nose) with 0.1 M solutions of either NaOH or HCl. The following absorption enhancers and concentrations (w/v) were studied: LPC 0.0625% w/v, 0.125% w/v, 1% w/v; DMβCD 1% w/v, 3% w/v, 5% w/v; TMC 0.125% w/v, 0.25% w/v, 1% w/v, 3% w/v, 5% w/v; chitosan hydrochloride 0.125% w/v; 0.25% w/v, 1% w/v. These concentrations were based on those frequently used in nasal drug delivery studies in ani-

mals and humans. Both higher and lower concentrations from the concentrations reported in the literature for each respective absorption enhancer, were chosen to study if the effects were concentration dependent.

3.2.2. Ciliary beat frequency

All solutions were prepared in physiological saline (0.9% NaCl). However, for CBF measurements the pH of the solutions was not adjusted to pH 6.8 to prevent potential adverse reactions caused by HCl and NaOH. The absorption enhancers subjected to CBF measurements were LPC (1% w/v), DMβCD (5% w/v), TMC (5% w/v) and chitosan hydrochloride (1% w/v).

3.3. Animal studies

Male Sprague-Dawley rats, weighing between 200–250 g were used. All animal procedures were approved by the Ethical Committee of the Potchefstroom University (Potchefstroom, RSA). Each formulation was administered to 6 rats. Six rats also received an intranasal dose of physiological saline to measure the effects due to administration of the formulation vehicle without the absorption enhancer. Rats were kept in a controlled temperature environment of 21 ± 2 °C.

Each rat was separately sedated by placing it in a closed glass container filled with a 0.02% v/v halothane-medical oxygen mixture by spraying 0.6 ml Fluothane (halothane) on paper towel below a metal grid. Each rat was removed at loss of consciousness. The anesthesia apparatus consisted of two 5 l plastic bags connected to two ends of a three-way valve. Rubber latex was connected to the middle opening of the three-way valve and was fitted securely over the head of the rat to maintain anesthesia. The plastic bags were filled with 4% halothane (0.24 ml) for induction and 2% (0.12 ml) for maintenance in medical oxygen respectively. Sodium lime (10 mg) was placed in both bags to absorb the exhaled carbon dioxide. The intranasal dose (20 µl) was applied unilaterally into the left nostril using a calibrated Eppendorf micropipette. This volume was chosen to avoid leaking into the right side of the nasal cavity and to ensure a constant absorption enhancer dose. Rats were kept in the supine position during dosing and for a 15 min exposure time to the absorption enhancer. Previous histological studies with nasal absorption enhancers have shown that the acute effects of the enhancer are manifested after an exposure time of 15 min [24, 21]. Moreover, under physiological circumstances the mucociliary clearance removes substances from the nasal cavity to the oesophagus in about 10–15 min. Before the end of the allowed exposure time, rats were deeply anesthetized with Fluothane® (halothane) in the closed glass container. After the respiration and heartbeat of the rat seased, the nasal cavity of the rat was dissected with surgical scissors to remove the left nostril epithelium for transmission electron microscopic evaluation. The epithelium of the right nostril was used as a control side. The epithelium was immediately placed in Todd's fixative [25].

3.4. Transmission electron microscopy (TEM)

Specimens were fixed in Todd's fixative for 2–6 h. It was then rinsed three times for 10 min with 0.05 M cacodilate buffer and post fixed with 1% osmium tetroxide for 1 h. The epithelia were three times rinsed with distilled water for 10 min and stained with 2% uranyl acetate for 30 min. Epithelia were again rinsed three times with distilled water for 10 min and dehydrated in a graded acetone series (50%, 70%, 90%, 100%, 100%) for 15 min each. The solution was replaced with a 1 : 1 mixture of acetone and Spurr's resin [26] for 3 h and then replaced with 100% Spurr's resin for 5 h. The solution was subsequently replaced with 100% Spurr's resin for 2 h and polymerized at 70 °C for 8 h. Cross sections (approximately 150 nm) were cut with a Reichert-Jung ultramicrotome. The sections were collected on 200 mesh copper (Cu/Pd) grids and stained for 3 min with 2% uranyl acetate. Each grid was rinsed with distilled water and dried on filter paper. The sections were then stained with lead citrate [27], rinsed with distilled water and dried on filter paper. Cross sections of the epithelia were investigated with a Philips CM 10 transmission electron microscope (PW 6020/10, Eindhoven, The Netherlands) at a tension of 100 Kv.

3.5. Ciliary beat frequency (CBF)

3.5.1. Nasal brushing technique

Nasal brushing is a non-invasive technique, which is relatively simple to perform. The nostril of a healthy human volunteer was inspected via a Welch Allyn Diagnostic set and a nylon nasal cytology brush of 2 mm in diameter was inserted via the Welch Allyn Diagnostic set (window removed). The nasal mucosa was harvested from the inferior nasal turbinate. With this sampling technique it is possible to obtain single ciliated cells and strips of ciliated epithelium and brushing can be repeated several times [7]. After nasal brushing the ciliated epithelia was immediately immersed in approximately 2 ml Hanks' balanced salt solution (37 °C) in a glass test tube.

3.5.2. Ciliary beat frequency measurements

Ciliary beat frequency (CBF) measurements were performed on human ciliated nasal epithelium at the Pulmonary Unit of the Pretoria Academic Hospital (Pretoria, RSA) using the analogue contrast enhancement technique, as described previously [14]. This technique involves transfer of the microscope image of the specimen onto a television monitor via a video camera, followed by electronic 'stretching' of the video signal such that the contrast is dramatically increased to allow visualisation of the specimen far beneath the resolution limits of the optical microscope. Television signals relating to differences in light intensity resulting from ciliary motion at any specific point on the monitor screen as pre-determined by positioning an electronic mouse-operated probe over ciliated cells are analysed by an on-line microcomputer. The differences in light intensities are computed directly into units of Hertz. Ciliated human epithelial tissue was obtained by nasal brushing of healthy volunteers. The epithelium was placed in 2 ml of Hanks' balanced salt solution (37 °C) in a glass test tube. The strips of human nasal epithelium were then removed from the brush by brisk agitation. One ml of the latter solution was withdrawn with a micropipette and placed in a glass test tube and 1 ml of the specific absorption enhancer (dubble dilution technique) was added. The solution was kept at 37 °C for 15 min. Ciliated cells were aspirated with a pasteur pipette and placed on a microscope slide containing 800 µl of Hanks' balanced salt solution. A border was made with high vacuum grease on the microscope slide to prevent spillage of the specimen. A coverslip (22 × 22 mm) was then placed over the strips of human nasal epithelium and the coverslip was gently pressed over the high vacuum grease to seal the microscope preparation. This ensured that the coverslip did not press directly onto the sample and interfere with the ciliary beat activity. The sealed slide preparation was then placed under an Olympus BH-2 light microscope (Japan) of which the microscope table was connected to a thermostat to maintain a temperature of 37 °C. The CBF was monitored and displayed as a frequency count. For a control CBF value, seventy five CBF readings (during a period of 40 min) were recorded at 5 different sites on an untreated strip of epithelium. A mean and a standard deviation were obtained for each specimen and the percentage change in CBF was calculated.

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Prof. Dr. Jeanetta du Plessis
Department of Pharmaceutics
School of Pharmacy
Potchefstroom University for CHE
Private Bag X6001
Potchefstroom 2520
South Africa
fmsjdp@puknet.puk.ac.za