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Toxicological investigations of the effects carboxymethylcellulose on ciliary beat frequency of human nasal epithelial cells in primary suspension culture and in vivo on rabbit nasal mucosa

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Abstract

The objective of this study was to investigate the safety of a mucoadhesive carboxymethylcellulose (CMC) formulation for intranasal administration of apomorphine. The effect of different concentrations of CMC on ciliary beat frequency (CBF) was studied using a human nasal epithelial suspension cell culture system. The CBF was determined by computerized microscope photometry. The in vivo rabbit nasal mucosal tolerance of the mucoadhesive polymer was investigated using light microscopy. Twice daily, six rabbits received CMC powder in one nostril and CMC/apomorphine powder in the alternate nostril for 4 weeks. Two control rabbits received air puffs in one nostril and nothing in the alternate nostril. The rabbits were subsequently sacrificed and the stained nasal sections examined microscopically. CMC showed both concentration- and time-dependent inhibitory effects on the CBF. Only mild-to-moderate cilio-inhibition was recorded with the different concentrations of the polymer. CMC (both with and without apomorphine) caused mild-to-moderate inflammation after 4 weeks. Necrosis, squamous metaplasia or ciliary degeneration was not observed. Based on: (1) the mild-to-moderate cilio-inhibition induced by different concentrations of CMC; and (2) the mild-to-moderate nasal mucosal inflammation caused by CMC with and without apomorphine, we conclude that this polymer can be considered as a safe carrier for short-term intranasal administration. However, further investigations are required for its use in the treatment of chronic diseases such as with apomorphine in Parkinson's disease. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nasal drug delivery; Ciliary beat frequency; Mucosal inflammation; Mucoadhesion; Carboxymethylcellulose; Apomorphine

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1. Introduction

The nasal route of drug administration has frequently been described as a promising alternative to parenteral administration of therapeutic peptides and proteins, as well as drugs used in emergency clinical situations. In this respect, quite a lot of research efforts have been directed to nasal drug delivery of a variety of drugs (Behl et al., 1998). However, the nasal drug administration has some limitations such as low permeability to large molecular mass and hydrophilic compounds, local enzymatic activity, and rapid clearance by the actively beating cilia (Cornaz and Buri, 1994). A number of approaches are being used to counter the various limitations of nasal drug administration. The three major approaches that have been attempted are: (1) the use of chemical enhancers to improve absorption; (2) incorporation of enzyme inhibitors; and (3) increasing drug local residence time using mucoadhesive polymers.

The use of mucoadhesive polymers can solve a number of problems encountered in controlled drug delivery. It localizes the formulation at a particular region of the body thereby improving bioavailability of drugs with low bioavailability. The increased contact time and localization of the drug due to the strong interaction between the polymer and mucus is essential for the modification of tissue permeability. Furthermore, enzymatic activity can be locally inhibited to improve the bioavailability of drugs that are subject to enzymatic degradation. This has been demonstrated for some mucoadhesive polymers such as Carbopol 934P® and polycarbophil that inhibit the proteolytic enzyme trypsin, thus increasing the stability of co-administered peptides (Leußen et al., 1994). Agents can be delivered locally to modulate antigenicity (Jiménez-Castellanos et al., 1993). The mucus coat covering a number of anatomical regions in the body provides the opportunity for the mucoadhesion approach to drug delivery via the buccal, sublingual, vaginal, rectal, ocular, gastrointestinal and nasal routes.

Nasal absorption enhancement has frequently been associated with tissue incompatibility with the nasal mucosa. These include: (1) epithelial necrosis (Chandler et al., 1991; De Fraissinette et al., 1995; Zhou and Donovan, 1996); (2) non-necrotic mucosal inflammations (Björk et al., 1991; Pereswetoff-Morath et al., 1996; Ugwoke et al., 1999a); and (3) cilio-inhibition (Romeijn et al., 1996; Aspden et al., 1997; Agu et al., 1999). The successful use of absorption enhancers depends not only on their absorption enhancement efficacy, but also, especially in the treatment of chronic diseases, on the safety of the absorption enhancer. The major areas of concern are: (1) local irritation of the mucosa; (2) effect on mucociliary clearance; (3) epithelial damage; and (4) rate of recovery of the damaged mucosa.

The objective of this study was to perform toxicity tests of carboxymethylcellulose (CMC) powder delivery systems of apomorphine. In the first part of this study, human nasal epithelial cells in suspension culture were used to investigate the ciliotoxicity of CMC alone. In the second part of this study, the in vivo rabbit nasal mucosal tolerance of CMC and CMC loaded with apomorphine was assessed using light microscopy. Both toxicity models we used for this toxicity investigation assess different indicators of nasal toxicity. We chose the cell culture model for the study of cilio-toxicity due to the importance of maintaining optimal ciliary beating in protecting the lower respiratory tract from infections. This model has been previously validated for cilio-toxicity investigations (Agu et al., 1999). On the other hand, the in vivo model would allow us to assess the degree of tissue inflammation based on neutrophil infiltration of the mucosal tissue. The use of both models would therefore be complementary to each other.

2. Materials and methods

2.1. Preparation of formulations

Apomorphine.HCl (Alpha Pharma, Zwevegem, Belgium) was dissolved in purified water (Elgastat Maxima SC, ELGA, High Wycombe, UK) in lyophilization vials by sonication (Branson 2200, Branson Ultrasonics Co., Danbury, CT, USA). Carboxymethyl cellulose (Blanose, type 7H3SXF, Aqualon, Surrey, UK) was dispersed in the solu-

tion, frozen overnight (−35°C) and lyophilized (Alpha, Christ, Osterode, Germany) at −10°C for 24 h followed by, 0°C for 24 h. The powder was sieved (180 μm mesh) and stored at room temperature until used. CMC without apomorphine was administered without freeze-drying. CMC contained about 15% (w/w) of apomorphine·HCl.

2.2. Sequential monolayer-suspension cell culture procedure

Human nasal epithelial tissues (without secondary ultrastructural abnormalities) obtained from patients undergoing elective surgery were cultured by following the sequential monolayer-suspension culture protocol as described previously (Jorissen et al., 1989). The tissues were washed three times with physiological saline supplemented with streptomycin (100 μg/ml) and penicillin (100 IU/ml; both purchased from Boehringer Mannheim, Germany). Subsequently, the cells were dissociated enzymatically during a period of 16–24 h using 0.1% Pronase (Sigma, St. Louis, MO, USA) in DME F12 1/1 (Life Laboratories, Paisley, UK) under continuous rotation at 4°C. The Pronase was deactivated with NU-serum prior to washing the cells with the monolayer culture medium {DME F12 1/1 supplemented with 2.0% Ultroser G (Life Laboratories, Paisley, UK), 10 ng/ml cholera toxin, (Sigma, MO, USA), streptomycin (50 μg/ml), and penicillin (50 IU/ml)}. The cells were washed three times with this monolayer medium and centrifuged ($70 \times g$ for 5 min) and preplated on plastic for 1 h at 37°C and 5% CO_2 to reduce fibroblast contamination.

To obtain the sequential monolayer-suspension culture, the cells were plated at a density of 5.0×10^5 cells/cm^2 in T75 tissue culture flasks (Falcon, Oxnard, CA, USA) coated with 0.2% collagen gel (extracted from rat tails) and containing 15 ml monolayer culture medium. The medium was changed the following day and subsequently three times a week. After 3 weeks of cell growth and loss of cilia in monolayer culture, the cells were dissociated from the collagen gel with 200 IU/ml-collagenase type IV (Worthington Biochemical Corporation, Freehold, NJ, USA). The resulting cell clusters were washed three times in the monolayer medium, centrifuged (70 g for 5 min), and transferred into several T25 tissue culture flasks. The cells were kept on a gyrotory shaker at 80 rpm for 1 week to avoid cell adhesion to the plastic surface. The following weeks, the medium consisted of 10% NU-serum in DME F12 1/1 (Life Laboratories, Paisley, UK). This medium was changed daily during the first week in suspension culture and three times per week thereafter. Ciliogenesis occurred during this period. The cells were incubated at 37°C in a 5% CO_2 atmosphere.

2.3. Ciliary beat frequency measurements

The ciliary beat frequency was determined by computerized microscope photometry (Jorissen et al., 1992).

The ciliary beat frequency (CBF) of cells in suspension culture was measured in T25 tissue culture flasks. The same group of cell aggregates prior to a particular treatment served as the control following that treatment. The CBF of different cells was measured before and after exposure to the test solution. To perform the measurements, the control and test solutions were removed prior to measurements leaving the cell aggregates adhered to the tissue flask except for 1% (w/v) dispersion of CMC where the high dispersion viscosity prevented movement of the cell aggregates. The reversibility of cilio-inhibition was not performed when using 1% (w/v) dispersion of CMC due to the inability to separate the cell aggregates from these viscous dispersions. Reversibility of toxicity due to compounds was determined by washing the cells with DME F12 1/1 of pH 5.0 after 90 min exposure to the test substance. CBF measurements were started 15 min after the addition of the wash medium. The effect of CMC was studied at the concentration: 1.0, 0.25 and 0.1% (w/v).

All measurements were conducted at room temperature. A sampling frequency of 500 Hz was used to record a signal for a period of 1 min. The recorded signal was analyzed by performing time spectral analysis using Fast Fourier Transform on the waveform obtained. The signal recorded

within 60 s was divided into periods of 5.12 s and analyzed in ten consecutive periods. The highest peak of the first harmonic within these time segments was taken to represent the CBF within that time slot. The degree of CBF change caused by the different concentrations of the tested substances/conditions was classified as follows (Ugwoke et al., 1999a):

- no effect: less than 10% or statistically insignificant;
- mild: 10–20% cilio-stimulation/inhibition and statistically significant;
- moderate: 20–50% cilio-stimulation/inhibition and statistically significant; and
- severe: greater than 50% and statistically significant.

Reversibility of effect after washing out the test substance was determined with equation below:

$$\% \text{ Reversibility} = \frac{\text{CBF after washing} - \text{CBF following treatment with test substance}}{\text{Control CBF} - \text{CBF following treatment with test substance}} \times 100$$

Reversibility was classified as follows:
% Reversibility greater than 75: reversible
% Reversibility 25–75: partially reversible
% Reversibility less than 25: irreversible
Negative values indicate cilio-inhibition or cilio-stimulation both before and after washing.

2.4. Data treatment and statistics for the CBF studies

In each of the conditions investigated, the CBF of 30 individual cells was measured (in three batches of ten cells per batch) for both the control and treated groups. A total of ten CBF data points were obtained per cell for each concentration investigated in all the groups. The mean CBF of the treated groups was expressed as a percentage of the control group $\pm$ S.E.M. Statistical tests for significance between control and treated populations were determined by Mann–Whitney (non-parametric) test for unpaired data for results obtained with the suspension culture at 95% confidence interval.

2.5. Drug administration

Male New Zealand white rabbits were used. The animals were procured from and cared for throughout the period of the experiment by the Animalium Department of the University of Leuven. They were housed individually in stainless steel cages, fed a commercial laboratory rabbit diet and had free access to water. The rabbits weighed (mean $\pm$ S.D.) 2.8 ± 0.23 kg at the beginning and 2.9 ± 0.22 kg at the end of the study period. Prior to drug administration each rabbit was anaesthetized with Penthotal® (Abbott, Ottignies, Belgium; 20 mg/kg) and Rompun® (Bayer, Leverkussen, Germany; 6–9 mg/kg) for easy drug administration and to minimize physical injuries to the nasal mucosa. The formulations were insufflated intranasally with a home made device consisting of an air filled 10-ml syringe compressed to 2 ml and an electrically actuated valve expulsing the drug through a plastic tip (internal diameter 2.3 mm, external diameter 3.0 mm) inserted about 1 cm into the nostril. Six rabbits received, twice daily for 4 weeks, CMC (8.5 mg) in one nostril and CMC/apomorphine (10 mg) in the other. These were compared to two control rabbits that received air in one nostril and nothing in the other.

2.6. Tissue collection and fixation for light microscopy

The rabbits were euthanized with a lethal dose (40 mg/kg, i.v. injection) of Nembutal® (Sanofi, Brussels, Belgium). With a scalpel the skin of the head was parted from the tip of the nose to the neck to reveal the skull. A pair of scissors was inserted into one nostril (about 1.5 cm), cut open and then inserted within the sutura internasalis and a gentle twist applied parting the sutura internasalis up to the forehead (os frontale). A pair of forceps was inserted into the os frontale and pulled apart opening the nasal cavity. Both the ventral and medial nasal conchae were then removed and fixed immediately in 6% formaldehyde solution. The specimens were decalcified in 10% (v/v) formic acid overnight, cut into two to three pieces and embedded in paraffin wax. Sec-

tions, 4–5-μm thick, were cut and stained with hematoxylin and eosin (H&E).

3. Results and discussion

3.1. Ciliary beat frequency

The effect of CMC on CBF was both concentration- and time-dependent as shown in Fig. 1. The effect on CBF obtained with 1.0% (w/v) dispersion was moderate both after 15 and 60 min, while 0.25% (w/v) dispersion showed no inhibition after 15 min and mild inhibition after 60 min. With the 0.1% (w/v) concentration, mild inhibitory effect was observed both after 15 and 60 min. The cilio-inhibition that was observed with the 0.1% (w/v) dispersion, was partially reversible, while washing had no effect on the cilio-inhibition caused by the 0.25% (w/v) dispersion. The reversibility of the 1.0% (w/v) dispersion was not ascertained because of the gel viscosity.

The knowledge of effects of drugs and excipients on ciliary beating is very important in the development of intranasal drug delivery systems, especially for drugs to be used in chronic illnesses. The importance of this type of study lies on the fact that interference with the mucociliary clearance can lead to lower airway infections. If the drug or formulation components inhibit the ciliary beating, this effect must be completely reversible upon removal of the noxious substances. It is expected that complete reversibility of CBF will occur in vivo as a result of mucus turnover, which will reduce the mucoadhesive strength of the polymer. The use of models in CBF toxicity investigations could in the future be performed not only for immediate effects but also for chronic effects on CBF given that models such as used in this study can maintain ciliary beating for upwards of 6 months (Jorissen et al., 1989).

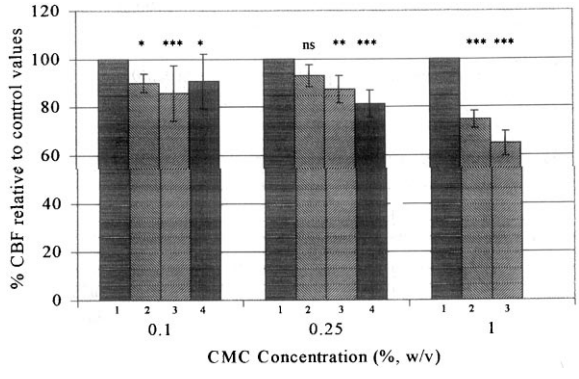

Fig. 1. Effects of different concentrations of CMC on CBF, showing time- and concentration-dependent inhibition of CBF after 15 min (2), and 60 min (3), compared to the control (1) and following washing (4). The levels of statistical significance were indicated as ns: $P > 0.05$, not significant; $*P < 0.05$, significant; $**P < 0.01$, very significant; and $***P < 0.001$, extremely significant.

CMC caused decreased CBF probably by mechanical interference with the cilia. Upon washing out the polymer, the CBF recovered partially with the 0.1% (w/v) dispersion. It was not possible to study the combined effects of both CMC and apomorphine due to insolubility of the formulation in the medium, DME F12 1/1 or in normal saline. In comparison to Carbopol 971P, another mucoadhesive polymer, the effect of CMC on CBF was similar, both in concentration- and time-dependence (Ugwoke et al., 1999a). However, CMC induced a slightly higher degree of cilio-inhibition at 1.0% (w/v) and 0.1% (w/v) concentrations. Unlike this previous report, the cilio-inhibition caused by 0.25% CMC was not affected by washing, while that of Carbopol of equal concentration was partially reversible. A slow onset of adhesion may have been responsible for the observation of no cilio-inhibition by the 0.25% (w/v) dispersion after 15 min, unlike the 0.1% (w/v) dispersion that has more free chains available for adhesion. The high viscosity of the 1.0% (w/v) dispersion may have physically prevented ciliary beating, even with polymer chains not fully extended.

3.2. Rabbit nasal mucosal tolerance

The animals remained healthy throughout the duration of the experiments with no general weight-loss. The nostrils treated with CMC, and CMC/apomorphine still contained the swollen gel at the anterior part of the nasal cavity more than at the posterior part by the time of sacrifice, 24 h after the last treatment. No gross necrosis or

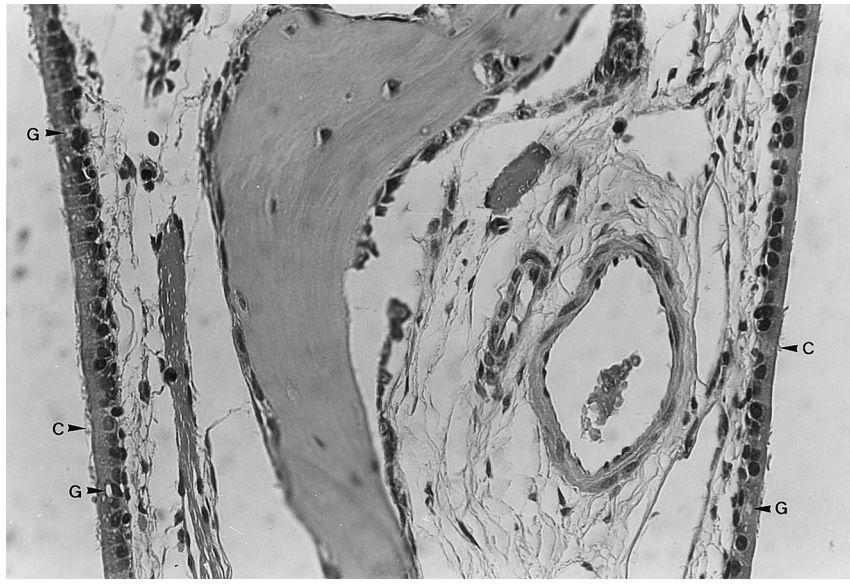

Fig. 2. Light micrograph of anterior conchae cross-section from a control rabbit that received no treatment showing the epithelial layer bearing cilia (C) and goblet cells (G) (original magnification 500 ×).

change in the architecture of the respiratory mucosa was observed. During the in vivo assessment of inflammation, both ventral nasal conchae and medial nasal conchae were collected as specimens. This enabled the determination of the contribution of initial dehydration to mucosal irritation (ventral nasal conchae), and also if the irritation was wide spread throughout the path where the formulation came in contact with during its clearance towards the nasopharynx. Cutting the conchae into two to three pieces before embedding them in paraffin enabled inflammation over a wider region to be assessed.

Epithelium from both ventral nasal conchae and medial nasal conchae of control rabbit was characterized by ciliated pseudostratified columna epithelium with goblet cells (Fig. 2). The subepithelial structures contained some seromucous glands and nerves. In a few cases some neutrophil infiltration of the epithelium and stroma was observed but this was not large in number and was judged as normal. The light microscopic study showed normal (control) respiratory epithelium with no observable differences between specimens from the nostrils that received puffs of air and those that received nothing. Cases with minor mucosal inflammation could be attributed to mucosal reaction to noxious agents that are ever present within our surroundings.

Specimens from nostrils treated for 4 weeks with CMC (Fig. 3) and CMC/apomorphine (Fig. 4) showed signs of mild-to-moderate inflammation characterized by epithelial and goblet cell hyperplasia, and neutrophil infiltration of the stroma, epithelium, and in a few cases the lumen. These inflammatory reactions were generally very focal in nature, the same inflammatory reaction (both in type and severity) not being seen in all parts of a sliced section. The varying degrees of focal inflammation were all applied to epithelial hyperplasia, goblet cell hyperplasia and neutrophil infiltration of the epithelium, stroma, and lumen. Also the ventral nasal conchae were affected more than the medial nasal conchae. The degree of inflammation after 1 week of treatment with Carbopol 971P® (Ugwoke et al., 1999a) was more than that following 4 weeks of treatment with CMC. It was not possible to ascertain from the microscopic examinations if apomorphine increases the severity of inflammation induced by CMC.

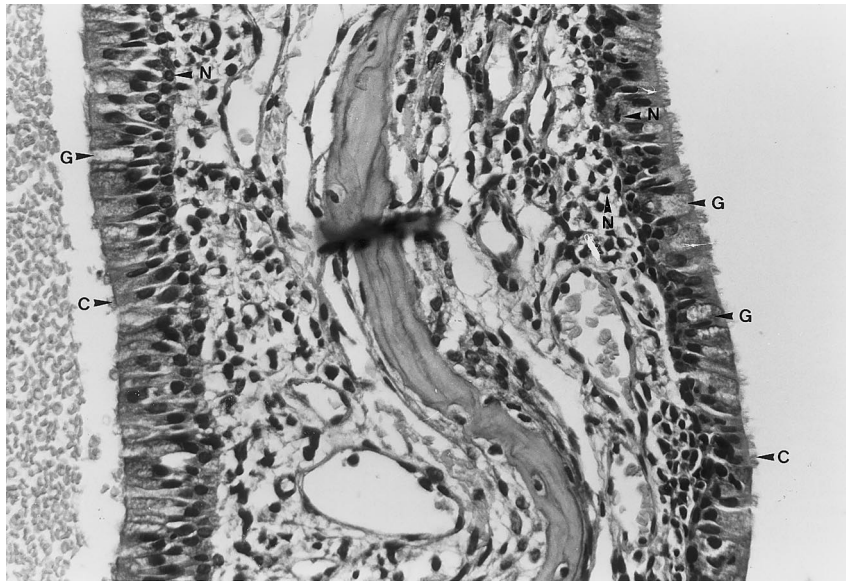

Fig. 3. Light micrograph of ventral nasal conchae cross-section, following administration of CMC (8.5 mg) for 4 weeks showing mild inflammation. They show mild inflammation of the epithelial layer bearing cilia (C) and goblet cells (G), and few neutrophils (N) infiltrating the epithelium (original magnification 500 ×).

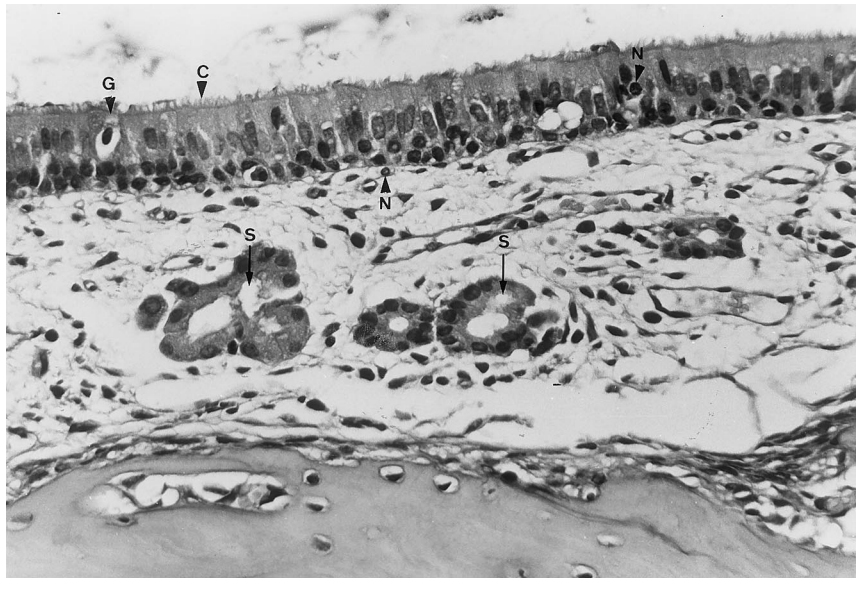

Fig. 4. Light micrograph of ventral nasal conchae cross-section, following administration of CMC/apomorphine (10 mg) for 4 weeks showing mild inflammation. They show mild inflammation of the epithelial layer bearing cilia (C) and goblet cells (G), seromucus glands (S), and neutrophils (N) infiltrating the epithelium and stroma (original magnification 500 ×).

It was an interesting observation that CMC caused no ciliary erosion in spite of the long period the gels remained in the nasal cavity. The ventral nasal conchae were more severely affected than the medial nasal conchae both with CMC- and CMC/apomorphine-treated specimens. This may be attributed to local dehydration caused by the polymer during its swelling and adhesion as suggested by Björk et al. (1991). Furthermore, slowing down of the mucociliary clearance led to more prolonged contact time between the epithelium of these regions with the polymer and as such the observed increased level of inflammation.

No case of epithelial necrosis with loss of both ciliated and non-ciliated columnar epithelial cells, goblet cells and basal cells observed with some absorption enhancers as chenodeoxycholate, laureth-9 and lysophosphatidylglycerol (Richardson et al., 1991; De Fraissinette et al., 1995; Zhou and Donovan, 1996) was observed with CMC. The inflammation caused by CMC may be related to the quantity and frequency of administration and/or the very prolonged residence time of the formulation within the nose. The polymer was still in the nasal cavity long after drug release was complete. As mucoadhesive drug delivery systems, it performed somewhat too well since drug release was not prolonged as was their residence times in the nose (Ugwoke et al., 1999b). Using smaller quantities of the polymers may result in shorter residence times and as such be better tolerated. Since CMC is not toxic nasally, it is promising for further investigation especially with respect to the effect of quantity of the polymer administered. For use in non-chronic diseases, CMC could already be used.

4. Conclusions

The effect of CMC on CBF was concentration- and time-dependent. In vivo, a mild-to-moderate nasal mucosal inflammation was observed. CMC is thus promising as an intranasal mucoadhesive platform for use in non-chronic and sub-chronic disease conditions. Its use in chronic diseases requires further investigations.

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