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Effect of preservatives on ciliary beat frequency in human nasal epithelial cell culture: Single versus multiple exposure

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Abstract

As preservatives may impair mucociliary clearance, we wanted to systematically study their time-dependent effect on the ciliary beat frequency (CBF) in human nasal epithelial cells (HNEC). CBF was determined using a high-speed digital imaging method. Five preservatives were selected including benzalkonium chloride, phenylethyl alcohol, methylparaben, propylparaben and chlorbutol. We were interested in the effect of these preservatives on CBF after single and repetitive exposure. Methylparaben (0.0033%), propylparaben (0.0017%) and chlorbutol (0.005%) did not impair CBF, neither after a single short-term exposure period, nor after a single long-term exposure period. Long-term exposure to benzalkonium chloride (0.001%), phenylethyl alcohol (0.125%) and a combination of methyl- and propylparaben (0.0033 and 0.0017%) significantly decreased CBF. After a short-term exposure period, CBF recovered for phenylethyl alcohol and the combination of methyl- and propylparaben. Benzalkonium chloride decreased CBF non-reversibly. For two compounds, the effect on CBF was evaluated after repetitive exposure during 15 min for 5 consecutive days. Benzalkonium chloride resulted in ciliostasis for all concentrations tested after 5 days. Phenylethyl alcohol revealed a concentration-dependent effect on CBF, but no ciliostasis was observed. In conclusion, methylparaben, propylparaben and chlorbutol can be considered as cilio-friendly. Repetitive exposure revealed a cumulative effect on CBF for benzalkonium chloride and phenylethyl alcohol.

Keywords: Preservatives; Ciliotoxicity; Ciliary beat frequency; Nasal epithelial cells

1. Introduction

The nasal route is widely used for the administration of drugs for both topical and systemic action (Kublik and Vidgren, 1998). Nasal drug delivery also holds promise for vaccination, while recent reports related to olfactory transport have created interesting perspectives for brain delivery (Westin et al., 2005). A prerequisite for nasally applied formulations is that drugs and additives in the dosage forms do not interfere with normal nasal functioning (Merkus et al., 1991). One of the most important local defense mechanisms of the respiratory tract is the mucociliary clearance (Jorissen et al., 2000), whose efficiency depends on the physiological control of the ciliated cells and on the rheological properties of the mucus blanket (Jorissen, 1998). The ciliary beat frequency (CBF) is one of the basic functional ciliary parameters determining the mucociliary clearance (Boek et al., 1999; Jorissen et al., 2000). Several factors influencing the ciliary beat frequency have been described, including temperature, pH and osmolarity (Ingels et al., 1991).

Traditional multidose nasal sprays or drops require preservatives to prevent microbial contamination (Gibson Mark, 2001). However, the use of preservatives in nasal formulations remains controversial. Although benzalkonium chloride is by far the most used preservative in aqueous nasal formulations, several studies have revealed the impairment of the mucociliary clearance by benzalkonium chloride and other preservatives in vitro (Batts et al., 1990; Joki et al., 1996; Boek et al., 1999; Riechelmann et al., 2004; Hofmann et al., 2004; Arnitz et al., 2006). Some authors observed morphological changes in the nasal respiratory mucosa by application of benzalkonium chloride in vivo (Berg et al., 1997; Cho et al., 2000; Cureoglu et al., 2002; Lebe et al., 2004), illustrating its potentially toxic effect. Others state that the toxic effect of benzalkonium chlo-

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ride is inactivated in vivo by proteins present in nasal secretions (Richards, 2000; Riechelmann et al., 2004). Taken together, caution is recommended, when using benzalkonium chloride (Graf, 1999; Bernstein, 2000).

The purpose of this study was to determine the time-dependent effect of selected preservatives on CBF in human nasal epithelial cell cultures (HNEC). We tested a variety of frequently used preservatives in aqueous nasal formulations, such as benzalkonium chloride, phenylethyl alcohol, methylparaben, propylparaben and chlorbutol (Gibson Mark, 2001). In contrast to most studies described in the literature, in which the effect of preservatives on the CBF was explored after a single exposure period, we were also interested in the effect of repetitive exposure of HNEC to preservatives on CBF. Two compounds were selected: benzalkonium chloride and phenylethyl alcohol. This test was designed to simulate the clinical use of nasal drug application. Once daily, during 5 consecutive days, the HNEC monolayer was exposed to a solution of the preservative tested for 15 min. CBF was measured daily before and after exposure to various concentrations.

2. Materials and methods

2.1. Chemicals and materials

Protease Type XIV and penicillin–streptomycin solution (10.000 IU/ml and 10.000 μg/ml, respectively) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). DMEM-Ham's F12 1:1 medium, Ultroser G and NU-serum were obtained from Life Technologies Ltd. (Paisley, UK). Benzalkonium chloride and chlorbutol were purchased from Certa (Braine-l'Alleud, Belgium), phenylethyl alcohol and methylparaben from Federa (Brussels, Belgium) and propylparaben from Merck (Darmstadt, Germany).

2.2. Cell isolation and culture

Human nasal epithelial cells were isolated from nasal biopsies according to the procedure described by Jorissen et al. (1989). Briefly, the human nasal epithelial tissues were enzymatically dissociated using 0.1% protease solution in DMEM-Ham's F12 1:1 medium, supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin for a period of 16–24 h at 4 °C after rinsing the tissues three times in saline solution (0.9% NaCl). At the end of the protease incubation, the large pieces of tissue were removed, and the protease activity was inhibited by adding 10% NU-serum. The cells were washed three times in DMEM-Ham's F12 1:1 medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin and 2% Ultroser G by centrifugation (800 rpm, 5 min, 4 °C). After the last centrifugation, the cell pellets were resuspended in 10 ml of the medium and incubated for 1 h in a 25 cm^2 plastic tissue culture flask in a CO_2 incubator (5% CO_2–95% air, 37 °C) to allow selective attachment of the contaminating fibroblasts and macrophages. The cell number was determined with a Coulter Multisizer counter (Northwell, UK). The cells were plated in 0.2% rat-tail collagen pre-coated six-well plates at a density of 5×10^5 cells/well in a final volume of 3 ml medium. The medium was changed 24 h after plating and subsequently every other day. Human nasal epithelial cell cultures formed microscopically confluent layers, consisting of ciliated and non-ciliated cells 5 days after plating. Experiments with CBF measurements were performed on days 6–10 after plating.

2.3. Data acquisition

All experiments were performed at a constant temperature of 24 °C. An inverted microscope (Olympus IX70) was used at a magnification of 600 times. A MotionScope high-speed digital camera and PCI application software, running in a Windows 2000 environment (Redlake MASD Inc., San Diego, CA) were used for image acquisition. The images were captured at a frame rate of 512 frames per second with a sampling interval of 2 ms. A sequence of 1024 images was recorded for each area. Each sequence of frame-by-frame images was stored in a file folder containing 1024 TIF format files for later analysis.

2.4. Ciliary beat frequency calculation

CBF was calculated as described before (Dimova et al., 2005). Briefly, a CBF value is computed locally for each pixel separately by spectral analysis of the variation of the pixel intensity over time. First, a region of interest (ROI) is selected, which is defined as all pixels for which the standard deviation of the intensity variation over time exceeds a threshold value of 5 dB. For each pixel in the ROI, the influence of noise on the CBF computation is reduced by spatial averaging of the intensity signal at each time point within a 3×3 pixel region centered around that pixel. Fast Fourier Transformation (FFT) analysis is then applied to the smoothed intensity signal. The CBF is then computed as the frequency corresponding to the maximal FFT amplitude value in the range 0–20 Hz. The analysis method was implemented in Matlab (The Mathworks Inc., Natick, MA) and a graphical user interface was developed for CBF measurement and histogram analysis, which allows deriving overall statistics (mean, standard deviation and median) for the CBF of all beating cilia in the image.

2.5. Determination of the time-dependent effect

The time-dependent effect was determined up to 360 min. The six-well culture plates were removed from the incubator at least 1 h before the start of the experiment in order to allow the medium to adapt to the environmental temperature. The culture plates were stored in an air-conditioned room at 24 °C, where the CBF measurements were performed. The HNEC monolayer was exposed to a solution of the preservative tested for the whole time period studied (i.e. 360 min) or for a short-time period of 15 min, after which the test solution was replaced by cell culture medium. The exposure period was set at 15 min as this is described as the normal residence time in the nasal cavity (Marttin et al., 1998). However, we also wanted to determine the effect after a long-term exposure period (360 min) in order to simulate the longer

nasal residence time due to decreased or inhibited ciliary activity. CBF was determined at defined time points, being 0, 10, 20, 30, 45, 60, 120, 180, 240, 300 and 360 min. All solutions of the preservatives were made in isotonic sodium chloride solution (pH 7.4). The effect of isotonic sodium chloride solution on CBF remains unclear. Boek et al. (1999) observed a mild but significant decrease in CBF after 1 h of exposure. Other authors did not observe any effect of isotonic sodium chloride solutions on CBF. Min et al. examined the effect of different concentrations of sodium chloride on CBF. They concluded that isotonic and hypotonic solutions did not result in ciliary slowing. However, hypertonic solutions decreased CBF significantly (Min et al., 2001). Wabnitz and Wormald (2005) found that exposure to isotonic sodium chloride solution did not result in any difference compared to the baseline CBF. Similarly, previous experiments performed at our laboratory did not reveal any influence of isotonic sodium chloride solution on the ciliary activity compared to cell culture medium.

2.6. Repetitive exposure test

In order to simulate the clinical use of nasal drops or sprays, we exposed the HNEC monolayer to a solution of benzalkonium chloride or phenylethyl alcohol for 5 consecutive days during 15 min each day. CBF was measured every day just before and 6 h after the 15 min exposure period, resulting in following time points: 0, 6, 24, 30, 48, 54, 72, 78, 96 and 102 h. All solutions of the preservatives were made in isotonic sodium chloride, adjusted to pH 7.4. The six-well culture plates were removed from the incubator at least 1 h before the start of the CBF measurement, in order to allow the medium to adapt to the environmental temperature. Afterwards, the HNEC were exposed to a solution of the preservative tested and the test solutions were replaced by cell culture medium after 15 min. Then the six-well plates were incubated at 37 °C for 5 h.

2.7. Data presentation and statistical analysis

All data were calculated as percentages of the average CBF value of the corresponding control condition. Absolute CBF values of the control condition ranged from 5 to 8 Hz. Test conditions are presented as mean ± S.D. of 6 (methylparaben, propylparaben and chlorbutol) or 18 (benzalkonium chloride and phenylethyl alcohol) CBF determinations. Two-sided 95% confidence intervals based on a *t*-distribution were calculated for each of the test conditions. Test conditions were considered to differ statistically significantly from control conditions if the calculated confidence interval did not include 100% ($p < 0.05$). Various test conditions were compared using a *t*-test and were considered to be significantly different if $p < 0.05$.

3. Results and discussion

3.1. Time-dependency experiments

The results of the time-dependency experiments are summarized in Table 1. At concentrations used in formulations for nasal application, long-term exposure of the monolayer to benzalkonium chloride (0.01%), phenylethyl alcohol (0.5%), propylparaben (0.017%) and a combination of methyl- and propylparaben (0.033 and 0.017%) resulted in ciliostasis after 180, 120, 300 and 120 min, respectively. When the solution of the preservative was replaced by culture medium after a 15 min exposure period, the CBF values observed after 360 min amounted to 62 ± 17, 66 ± 12, 94 ± 18 and 73 ± 10% of the CBF values of the corresponding control condition for benzalkonium chloride, phenylethyl alcohol, propylparaben and a combination of methyl- and propylparaben, respectively. Exposure of the monolayer for 360 min to methylparaben (0.033%) and chlorbutol (0.05%) decreased CBF statistically significantly to circa 44 ± 13 and 66 ± 8% of the CBF values of the control condi-

Table 1
CBF measurements after 20 and 360 min in time dependency experiments for different preservatives tested

Preservative tested	Concentration tested (%)	%CBF of the control condition			
		20 min		360 min	
		Long-term exposure	Short-term exposure	Long-term exposure	Short-term exposure
Methylparaben	0.033	62 ± 10*	71 ± 9*	44 ± 13*	88 ± 13
	0.0033	125 ± 25	113 ± 15	103 ± 17	104 ± 12
Propylparaben	0.017	46 ± 6*	50 ± 12*	Stasis*	94 ± 18
	0.0017	113 ± 28	111 ± 14	89 ± 23	96 ± 14
Methyl- and propylparaben	0.033 and 0.017	27 ± 3*	47 ± 6*	Stasis*	73 ± 10*
	0.003 and 0.0017	86 ± 8*	115 ± 15	80 ± 10*	98 ± 14
Chlorbutol	0.05	71 ± 11*	79 ± 14*	66 ± 8*	105 ± 12
	0.005	94 ± 22	104 ± 16	91 ± 14	108 ± 17
Phenylethyl alcohol	0.5	11 ± 9*	52 ± 17*	Stasis*	66 ± 12*
	0.125	69 ± 23*	56 ± 15*	89 ± 23	93 ± 15
Benzalkonium chloride	0.01	81 ± 24	80 ± 29	Stasis*	62 ± 17*
	0.001	120 ± 26	92 ± 23	29 ± 16*	76 ± 20*

CBF values are expressed as percentage of the CBF of the corresponding control condition. Values presented are means ± S.D. of $n = 6$ data.
* Significantly different from the control condition ($p < 0.05$).

tion, respectively. However, for the latter preservatives, rinsing the monolayer after a short exposure period resulted in a complete recovery of the CBF to the same extent as the CBF values of the corresponding control condition.

Since in physiological conditions all drugs locally administered in the nostrils are diluted by the presence of mucus, we have repeated these experiments with more diluted concentrations of the preservatives. A dilution of 10 times by mucus is described as clinically relevant (Lebe et al., 2004). The diluted concentrations of methylparaben (0.0033%), propylparaben (0.0017%) and chlorbutol (0.005%) did not impair the ciliary activity. However, CBF was decreased statistically significantly when the HNEC monolayer was exposed for 360 min to benzalkonium chloride (0.001%), phenylethyl alcohol (0.125%) and a combination of methyl- and propylparaben (0.0033 and 0.0017%). The CBF values amounted to 29 ± 16, 56 ± 15 and 80 ± 10% of the CBF values of the control condition, respectively. After a short 15 min exposure period, CBF recovered for phenylethyl alcohol and the combination of methyl- and propylparaben. However, for benzalkonium chloride, a statistically significant decrease in CBF was observed, even after rinsing the monolayer. The CBF values amounted to 76 ± 20% of the CBF values in the control condition. In Fig. 1, the time-dependent effect of 0.01% benzalkonium chloride on the CBF is presented. For the long-term exposure period, ciliostasis occurred after 180 min. When the test solution was replaced by culture medium after a short-term exposure period of 15 min, CBF continuously decreased to 62 ± 17% of the CBF values of the corresponding control condition. An example of the time-dependent effect of a preservative which appeared to be safe, is presented in Fig. 2. The presence of 0.0033% methylparaben did not result in a statistically significant decrease in CBF, not after a short-term exposure period, nor after a long-term exposure period.

Benzalkonium chloride appeared to be the only preservative tested that statistically significantly impaired ciliary activity even in the more diluted concentration and after a single short-time exposure period. Methylparaben (0.0033%), propylparaben (0.0017%) and chlorbutol (0.005%) appeared to be safe preservatives for nasal application, since no effect on the CBF could be observed using these preservatives after correction of the concentrations tested for dilution by mucus.

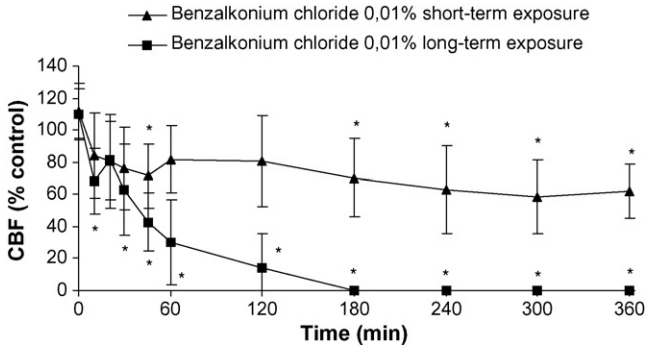

Fig. 1. Time-dependent effect of benzalkonium chloride (0.01%) on the ciliary beat frequency of human nasal epithelial cells. CBF values were calculated as percentages of the CBF values of the corresponding control condition. CBF measurements were performed at defined time points, when the monolayer was either exposed for a short-time (15 min) or a long-time (360 min) period to 0.01% benzalkonium chloride. A gradual decline of the CBF as function of time was observed. Both test conditions resulted in a statistically significant difference compared with the corresponding control condition ($^{*}p < 0.05$).

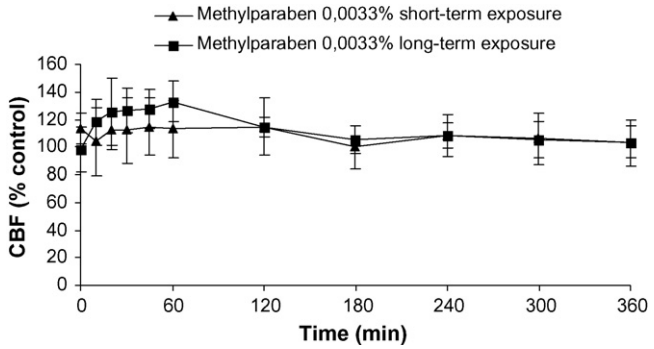

Fig. 2. Time-dependent effect of methylparaben (0.0033%) on the ciliary beat frequency of human nasal epithelial cells. CBF values were calculated as percentages of the CBF values of the corresponding control condition. CBF measurements were performed at defined time points, when the monolayer was either exposed for a short-time (15 min) or a long-time (360 min) period to 0.0033% methylparaben. Both test conditions (short-term or long-term exposure to the solution of methylparaben) did not show any difference compared with the corresponding control condition.

3.2. Repetitive exposure tests

Since nasal formulations are mostly used for longer time periods, we investigated the effect of repetitive exposure of the HNEC monolayer to a solution of a preservative. We wanted to explore whether repetitive exposure resulted in a cumulative effect on the CBF. Two preservatives were selected: one which appeared to be safe after single exposure (phenylethyl alcohol) and one which impaired ciliary activity (benzalkonium chloride). A 15 min exposure period on 5 consecutive days was selected. CBF was measured two times every day: the first time before the 15 min exposure period to the solution of the preservative tested and the second time 6 h after the exposure period.

Repetitive exposure of the HNEC monolayer to different concentrations of phenylethyl alcohol revealed a cumulative time- and concentration-dependent effect of phenylethyl alcohol on the CBF (Fig. 3). No ciliostasis was observed at any of the exam-

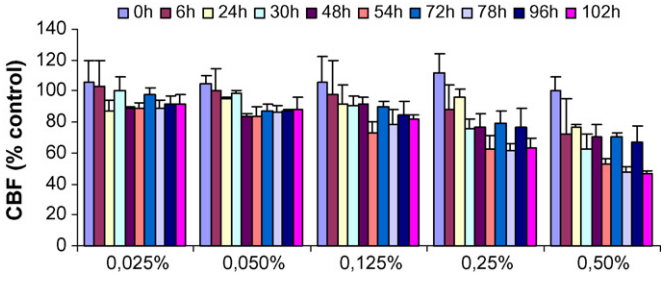

Fig. 3. Repetitive exposure test for different concentrations of phenylethyl alcohol. CBF values were calculated as percentages of the CBF values of the corresponding control condition. None of the concentrations phenylethyl alcohol tested resulted in ciliostasis. The lowest concentrations (0.025 and 0.05%) resulted in a small decrease in CBF. Higher concentrations of phenylethyl alcohol (0.125, 0.25 and 0.5%) resulted in a decrease in CBF which recovered overnight.

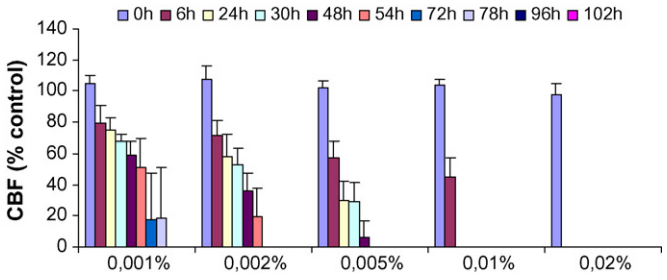

Fig. 4. Repetitive exposure test for different concentrations of benzalkonium chloride. CBF values were calculated as percentages of the CBF values of the corresponding control condition. The highest concentration (0.02%) resulted in a non-reversible immediate ciliostasis. At the fifth day even the lowest concentration of benzalkonium chloride (0.001%) resulted in ciliostasis.

ined time points. For the lowest concentrations of phenylethyl alcohol tested (0.025 and 0.050%), CBF was decreased to 92 ± 6 and $88 \pm 8\%$ of the CBF values of the corresponding control, respectively, at the fifth day. At higher concentrations of phenylethyl alcohol (0.125, 0.25 and 0.5%), CBF amounted to 82 ± 3, 63 ± 6 and $47 \pm 2\%$ of the CBF values of the corresponding control condition 6 h after the incubation period. Before the 15 min exposure period, CBF increased to 85 ± 9, 77 ± 12 and $67 \pm 11\%$, respectively, indicating an overnight recovery of the CBF.

As was observed in the short-term single exposure experiments, the effect of phenylethyl alcohol on CBF appeared to be characterized by a concentration-dependent fast decrease followed by recovery. In contrast to the results obtained with phenylethyl alcohol, a longer incubation period was required to observe a decrease in CBF after exposure to benzalkonium chloride. Repititive exposure of the HNEC monolayer to benzalkonium chloride resulted in a cumulative and non-reversible decrease in CBF (Fig. 4). The highest concentration of benzalkonium chloride tested (0.02%) showed immediate and non-reversible ciliostasis after the first exposure period. Lower concentrations of benzalkonium chloride (0.01, 0.005, 0.002 and 0.001%) showed ciliostasis at later time points, being 24, 54, 72 and 96 h, respectively. Even the lowest concentration tested (0.001%) eventually resulted in ciliostasis after five single exposures of 15 min on 5 consecutive days.

In order to determine whether a preservative is cilio-friendly enough to be used in nasal formulations, these data indicate that recovery of CBF after exposure to a solution of the corresponding preservative, is an important parameter that has to be taken into account. As repetitive exposure of the nasal mucosa appeared to result in a cumulative effect on CBF, it can be assumed that these cumulative effects will be more severe for compounds that induce a non-reversible decrease in CBF, compared to those which result in recovery after the exposure period. Since, for most conditions an effect could be observed after a single daily exposure, one may expect that multiple daily exposure will result in a more severe effect on the CBF. Therefore, the reversibility of the decrease in CBF caused by a compound can be considered to be an important parameter, determining the safety for nasal application.

The present system is characterized by the absence of mucus. For this reason, we have used more diluted concentrations of the preservatives compared to the concentrations used in nasal formulations in order to obtain clinically more relevant concentrations. Therefore, we can conclude that, if no effect can be observed in the used system, the compounds will be safe to use with respect to the ciliary beating. However, if a negative effect on the ciliary beat frequency is observed, further research is required. Also the presence of specific interactions as reported for benzalkonium chloride and mucus remains to be investigated.

4. Conclusion

At concentrations used in nasal formulations, the possible ciliotoxicity of preservatives was confirmed. However, when more diluted concentrations were used in order to correct for the absence of mucus in the experimental test system, methylparaben, propylparaben and chlorbutol appeared to have no effect on the CBF. Therefore, these preservatives can be considered as cilio-friendly. For the other preservatives tested, especially benzalkonium chloride, caution is recommended when used for nasal application. For the first time, the effect of repetitive exposure was assessed. In these tests, more diluted concentrations of phenylethyl alcohol did not result in an ongoing decrease in CBF as was observed for benzalkonium chloride, but appeared to be rather safe with respect to the ciliary activity. For benzalkonium chloride, a cumulative effect on the ciliary activity was observed. The results of this study illustrate the possible cumulative effect on CBF after repetitive exposure of the nasal epithelium to preservatives. The methodology used in this study can easily be adapted to explore the possible cumulative effect after multiple daily exposure.

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