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Effects of Benzalkonium Chloride and Potassium Sorbate on Airway Ciliary Activity

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Key Words
Preservatives · Ciliary beat frequency · Benzalkonium chloride · Potassium sorbate

Abstract
Objectives: Preservatives are indispensable components of aqueous multidose topical formulations. The purpose of our study was to investigate the effects of two representative preservatives, benzalkonium chloride (BKC) and potassium sorbate (PS), on rabbit tracheal ciliary beat frequency (CBF). ***Methods:*** Rabbit tracheal ciliated cell culture was established and CBF was determined using high-speed digital imaging methods. The effects of preservatives at different concentrations on CBF were observed over a 10-min exposure period. ***Results:*** BKC induced inhibition of CBF in a concentration-dependent manner. Ciliary beating was stopped by 0.01% BKC after 5 min of exposure. A low concentration of PS (0.12%) only resulted in a mild decrease in CBF during a 10-min exposure. The CBF decreased by 13.0% from baseline after 10 min. However, there was no statistically significant difference compared with the corresponding control condition. Application of 0.24, 0.48 and 0.96% of PS to rabbit tracheal cells resulted in an increase in CBF, with an increase of 105 ± 9.8, 107.6 ± 4.0, and 117.1 ± 9.5% relative to baseline CBF after 10 min of exposure, respectively. ***Conclusions:*** PS could be considered as a safer and more promising preservative than BKC for use in topical formulations.

Introduction

The administration of drugs via the upper airway (nasal) or lower airway (tracheal and bronchial) has become a common method of medication in recent years. In particular, nasal sprays or drops have been widely used for respiratory tract disorders of the nasal cavity and paranasal sinuses in the field of otorhinolaryngology. A prerequisite of nasally applied preparations is that drugs and additives in the dosage form do not interfere with normal nasal functioning, such as the nasal mucociliary clearance system [1]. Mucociliary clearance is one of the most important local defense mechanisms of the respiratory tract. The coordinated beating of cilia plays an essential role in efficient mucociliary clearance, and ciliary beat frequency (CBF) is one of the basic functional ciliary pa-

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rameters [2]. Hence, it is important to investigate the influence of active drugs and additives in the dosage form on CBF to evaluate the safety of nasally administered drugs and various additives such as preservatives [1].

Preservatives are indispensable components of traditional multidose nasal formulations due to the necessity for repeated administration and their aqueous nature, which is susceptible to microorganism infestation. Benzalkonium chloride (BKC) is a quaternary ammonium compound, which is by far the most commonly used preservative for the prevention of bacterial contamination in nasal sprays or drops. Although it has been recognized that excipients in nasal formulations should be harmless to nasal tissues, several studies have demonstrated the impairment of mucociliary clearance by BKC [3–5]. Potassium sorbate (PS) is a white crystalline powder which is used in foods, cosmetics and drug preparations to inhibit mold, yeast and bacterial growth. Recent research data suggest that PS may be a safer preservative for nasal ciliated epithelium [6, 7]. The purpose of the present study was to determine the effects of BKC and PS on CBF in rabbit tracheal mucosa cultures and to compare their ciliotoxicities.

Materials and Methods

Chemicals and Materials

Dulbecco's modified Eagle medium (DMEM) and sterile Hanks' balanced salt solution (sHBSS) were purchased from Sigma Co. Ltd. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid and flavin mononucleotide were purchased from Merck. Streptomycin sulfate was purchased from Amresco, penicillin G sodium salt and amphotericin B solubilized from Sigma. BKC and PS were purchased from Sigma.

Tissue Isolation and Culture

The establishment of rabbit tracheal epithelium primary cultures has been described in detail previously [8]. Briefly, tracheas were removed aseptically from 2-month-old New Zealand rabbits. The tracheal epithelium was separated from the underlying cartilage and cut into 1-mm^2 explants. As needed, explants were placed onto slides precoated with a thin film of rat tail collagen, which were placed into four-well dishes in advance (one or two explants per slide). Petri dishes were then added to a minimal amount of DMEM/$NaHCO_3$ culture medium supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 U/ml; streptomycin, 100 U/ml, and amphotericin B, 0.25 μg/ml). After a few minutes, explant adherence had occurred and 0.5 ml culture medium was added to each culture dish. Cultures were incubated in humidified 95% air/5% CO_2 at 37°C, and the culture medium was changed every 2 days.

Measurement of CBF

Six- to eight-day tissue cultures were used for measurements of CBF. Fields of view with well-grown rabbit tracheal cilia which swung regularly were selected using an inverted microscope (Olympus IX 71, Tokyo, Japan) at a magnification of ×400. The images of cilia were captured by a high-speed digital camera capture system (PULNiX High-Speed Digital) at a frame rate of 240 frames per second with a sampling interval of 3 s and results were transmitted to a computer workstation. A region of interest was selected and analyzed with IPLab4.0 software. CBF was calculated by determination of fluctuations in light intensity [8, 9]. All experiments were performed at a constant temperature of 25 ± 1°C.

The effect of preservatives on CBF was determined over a 10-min period of exposure. The four-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 ambient temperature. The cell culture medium was replaced by 1 ml of sHBSS and the culture left to stabilize for at least 10 min, after which CBF was recorded as baseline values of CBF (prior to exposure). The sHBSS was then replaced by 1 ml of a solution of the preservative to be tested and CBF was determined at 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 min after exposure. The test concentrations were 0.005, 0.0075 and 0.01% for BKC and 0.12, 0.24, 0.48 and 0.96% for PS. All concentrations of preservatives were prepared in sHBSS (pH 7.4).

Data Presentation and Statistical Analysis

All data were expressed as percentages relative to the baseline values, which were considered as 100% and presented as mean percentage ± SD. The differences of data between and within groups were statistically analyzed by analysis of variance followed by Student's t test using Microsoft Excel 2002. A p value <0.05 was considered as significant.

Results

Effect of BKC on CBF

Figure 1 shows the effects of BKC (0.0, 0.005, 0.0075 and 0.01%) on the CBF of rabbit tracheal cilia. The absolute value of CBF in test conditions ranged from ~8 to ~14 Hz and the value of controls remained constant over the 10 min of the experiments. The CBF was significantly increased by up to 42.2% at 3 min after exposure to 0.005% BKC, which was statistically significant compared with the corresponding control condition ($p < 0.01$). However, CBF gradually decreased and the difference between 0.005% BKC and the control group was reduced to 13.4% at 10 min after exposure ($p < 0.05$). At a concentration of 0.0075% BKC, CBF dramatically decreased over time. Mean CBF decreased by up to 90.3% of the baseline level after 10 min of exposure, in which 76.9% of cilia were observed to be in stasis. At a concentration of 0.01% BKC, there were 2 out of 13 ciliated cells (15.4%) in ciliostasis after a 3-min exposure period, and all ciliary beat activity had completely ceased at 6 min after exposure. Overall, BKC demonstrated significant concentration-dependent inhibitory effects on CBF (table 1).

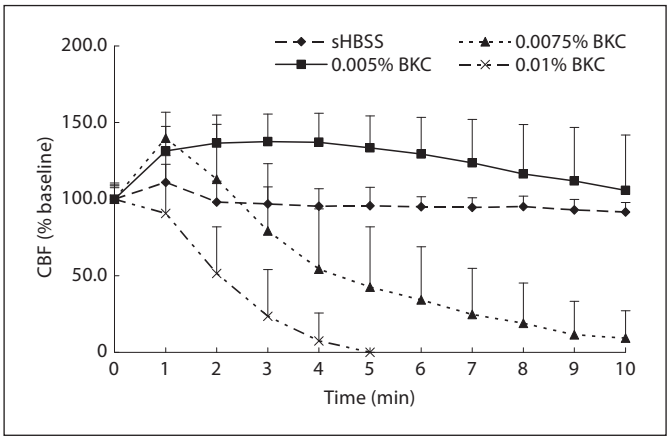

Fig. 1. Effect of BKC at different concentrations on the CBF of rabbit tracheal cilia. CBF values were calculated as percentages of CBF values at baseline (immediately before exposure). The exposure period was 10 min. BKC showed strongly concentration-dependent inhibitory effects on the CBF. In the 0.01% group, mucociliary activities were completely inhibited by 0.01% BKC by 5 min after exposure.

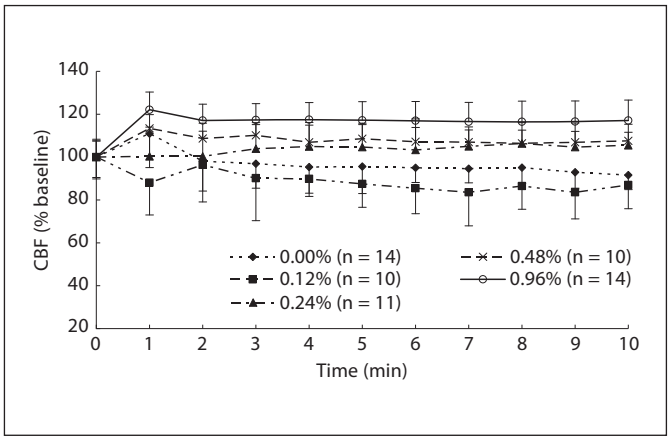

Fig. 2. Effect of PS at different concentrations on the CBF of rabbit tracheal cilia. CBF values were calculated as percentages of CBF values at baseline (immediately before exposure). The exposure period was 10 min. A trend of increase in CBF as a function of concentration was observed. There were significant differences compared with the corresponding control condition at 10 min after exposure at concentrations of 0.24, 0.48 and 0.96% ($p < 0.01$).

Table 1. Changes in CBF measurements for BKC at different concentrations tested over 10 min in rabbit trachea in vitro

Concentration	CBF, %										
	0 min	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min
sHBSS (n = 14)	100 ± 10.2	110.9 ± 15.8	98.1 ± 13.9	96.9 ± 11.4	95.3 ± 12.1	95.6 ± 12.6	95.0 ± 6.8	94.6 ± 6.6	95.1 ± 7.3	92.9 ± 7.4	91.6 ± 6.8
BKC											
0.005% (n = 13)	100 ± 10.5	131.5 ± 16.0	136.6 ± 18.3	137.5 ± 18.0*	137.2 ± 18.9*	133.5 ± 20.9	129.5 ± 23.9	123.7 ± 28.3	116.5 ± 32.2	111.8 ± 35.0	105.8 ± 36.1
0.0075% (n = 13)	100 ± 9.3	139.8 ± 16.9	112.9 ± 35.9	79.2 ± 44.0	54.3 ± 39.3	42.6 ± 39.3	34.3 ± 34.6	24.7 ± 30.1	18.9 ± 26.3	11.4 ± 21.9	9.3 ± 17.9
0.01% (n = 12)	100 ± 8.7	90.6 ± 32.1	51.5 ± 30.4	23.5 ± 30.5	7.4 ± 18.3	0.0 ± 0.0	–	–	–	–	–

All data are expressed as percentages relative to baseline values (immediately before exposure, i.e. 0 min) which were considered as 100% and are presented as mean percentage ± SD. * $p < 0.01$, compared with the sHBSS group (control).

Table 2. Changes in CBF measurements for PS at different concentrations tested over 10 min in rabbit trachea in vitro

Concentration	CBF, %										
	0 min	1 min	2 min	3 min	4 min	5 min	6 min	7 min	8 min	9 min	10 min
sHBSS (n = 14)	100 ± 10.2	110.9 ± 15.8	98.1 ± 13.9	96.9 ± 11.4	95.3 ± 12.1	95.6 ± 12.6	95.0 ± 6.8	94.6 ± 6.6	95.1 ± 7.3	92.9 ± 7.4	91.6 ± 6.8
PS											
0.12% (n = 10)	100 ± 9.5	88.1 ± 15.1	96.3 ± 17.2	90.2 ± 19.9	89.8 ± 8.1	87.5 ± 10.9	85.5 ± 11.9	83.6 ± 15.7	86.5 ± 10.8	83.6 ± 12.4	87.0 ± 11.1
0.24% (n = 11)	100 ± 7.6	100.3 ± 13.7	100.3 ± 15.5	103.9 ± 11.3	104.9 ± 11.3	104.7 ± 11.1	103.3 ± 14.5	105 ± 9.1	106.3 ± 9.8	104.7 ± 11.2	105.5 ± 9.8*
0.48% (n = 10)	100 ± 7.5	113.4 ± 6.4	108.7 ± 3.4	110.2 ± 5.9	106.9 ± 8.0	108.6 ± 6.6	107.2 ± 6.6	107.0 ± 5.7	106.4 ± 6.2	106.9 ± 5.1	107.6 ± 4.0*
0.96% (n = 14)	100 ± 8.3	122.1 ± 8.2	117.1 ± 7.6	117.3 ± 7.6	117.5 ± 7.9	117.2 ± 8.6	117.0 ± 8.9	116.5 ± 9.0	116.5 ± 9.6	116.6 ± 9.6	117.1 ± 9.5*

All data are expressed as percentages relative to baseline values (immediately before exposure, i.e. 0 min) which were considered as 100% and are presented as mean percentage ± SD. * $p < 0.01$, compared with the sHBSS group (control).

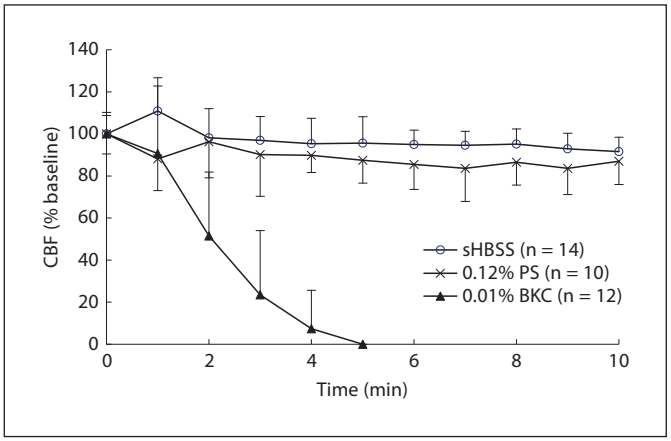

Fig. 3. Comparison of effects of BKC and PS at the concentration used in nasal formulations (0.01 and 0.12%, respectively) on CBF. As shown in the graph, mucociliary activities were completely inhibited by 0.01% BKC at 5 min, while there was no notable effect of PS at 0.12% during 10 min of exposure compared with the corresponding control condition.

Effect of PS on CBF

Generally, there was no notable effect of PS on CBF. As shown in figure 2, a trend of CBF increase with PS concentration increase from 0.12 to 0.96% was observed. A low concentration of PS (0.12%) only resulted in a mild decrease in CBF during the 10 min of exposure. The CBF decreased by 13.0% from baseline after 10 min, although this was not statistically significant compared with the corresponding control condition. Application of 0.24, 0.48 and 0.96% PS to rabbit tracheal cilia cells resulted in an increase in CBF, with increases of 105 ± 9.8, 107.6 ± 4.0, and 117.1 ± 9.5% relative to baseline CBF after 10 min of exposure, respectively (all $p < 0.01$ compared with the corresponding control condition, table 2).

Discussion

BKC is a preservative widely used in topical multidose formulations to prevent contamination. Its effect on nasal mucociliary clearance has not been clearly established. Some studies, including both in vitro and in vivo studies [3–5], have suggested that BKC does produce adverse effects on mucociliary activity. Other data, however, suggest BKC lacks deleterious effects [10–13]. The discrepancy in these results, particularly between in vitro and in vivo findings, could be explained by the possibility of problems in the experimental design and methodology as well as the difference between in vitro and in vivo conditions [13, 14]. Particularly, there was a lack of a mucus layer in vitro, which protects the ciliated epithelium of the nasal cavity. It is worth noting that most results showing no deleterious effects on CBF were observed in in vivo studies. Given the healthy human nose has about 0.4 ml mucus [15] and generally spray dose volumes are of the order of 0.1 ml in clinical practice, a fivefold dilution of nasal preparations may, in fact, be more realistic when considering ciliotoxic potential. Thus, in establishing the actual toxicity of preservatives, measuring CBF in vitro is probably too sensitive an approach. In our study, CBF demonstrated extreme sensitivity to changes in the concentration of BKC, as shown by changes from a strong inhibition of mucociliary activity by 0.01% BKC to a slight and positive stimulation by 0.005%, which was only half the original level. Given that the most commonly used concentration of BKC as a preservative in clinical practice is 0.01%, the actual concentration of BKC in the nasal cavity after administration of nasal drops or sprays is expected to be below 0.005% due to dilution. This could therefore explain the fact that the deleterious effects on nasal ciliated epithelium in vitro are not reflected in the findings in vivo or in the clinic.

On the other hand, PS concentrations in the range of 0.12–0.96% showed no significant effects on CBF in this study. As discussed above, although in vitro experiments could not precisely duplicate in vivo conditions due to the lack of a protective mucus layer, in vitro experiments on CBF are a very useful tool for studying the ciliotoxicity of various compounds due to their controllable properties without other interfering factors such as stress, hormone secretion or inflammatory mediators. If there is an effect on CBF observed in vitro under standardized conditions and the exclusion of cofactors, it can be assumed that such an effect does exist in vivo. As our study shows, PS was a safer preservative than BKC on CBF at the commonly used concentrations (0.01% for BKC and 0.12% for PS, fig. 3). There was only a mild increase in CBF even when the concentration of PS tested was up to 0.96% (about 5 times the commonly used concentration).

In conclusion, the potential ciliotoxicity of BKC at the concentration commonly used in nasal formulations (0.01%) was proven. However, a slight, positive effect on CBF was observed for BKC when the concentration was diluted to half of the original value, i.e. 0.005%, implying that BKC may be a cilia-friendly preservative in the human nasal cavity. By contrast, PS showed no effect on mucociliary activity and is thus considered as a safer and more promising preservative.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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