

The lack of effect of benzalkonium chloride on the cilia of the nasal mucosa in patients with perennial allergic rhinitis: a combined functional, light, scanning and transmission electron microscopy study

J. P. M. BRAAT, G. AINGE*, J. A. K. BOWLES*, D. H. RICHARDS*, D. VAN RIESSEN*, W. J. VISSER† and E. RIJNTJES

Department of Ear, Nose and Throat Surgery, Leyenburg Hospital, The Hague, the Netherlands, *Glaxo Research and Development Limited, UK and the Netherlands and †Department of Cellbiology and Genetics, Erasmus University, Rotterdam, the Netherlands

Summary

Background Controversy still exists about the effect of 0.02% benzalkonium chloride (BKC), a preservative in many nasal sprays, on human nasal epithelium *in vivo*.

Objective To determine the safety of BKC by assessing its effect on the function and morphology of cilia of human nasal epithelium.

Methods A single-centre, double-blind nasal biopsy study in 22 patients with perennial allergic rhinitis, receiving fluticasone propionate aqueous nasal spray (FPANS) containing BKC, BKC plus placebo or placebo alone for 6 weeks. Before, at two weekly intervals during treatment and 2 weeks after treatment ceased an indigocarmine saccharine transport time (ICST) was performed.

Results ICST results did not significantly vary between the groups. There was no statistical relationship between the number of ciliated cells present and the treatment the patients received. Scanning and transmission electron microscopy examination showed no effects of BKC.

Conclusion Despite reports of its ciliostatic effects *in vitro*, BKC did not have such an effect when it was applied for 6 weeks (with/without fluticasone propionate) to the nasal mucosa of perennial allergic rhinitis patients *in vivo*.

Keywords: benzalkonium chloride, perennial allergic rhinitis, fluticasone propionate, *in vivo* study, indigocarmine saccharine transport time, light microscopy, scanning microscopy, transmission microscopy

Clinical and Experimental Allergy, Vol. 25, pp. 957–965. Submitted 21 October 1994; revised 14 December 1994; accepted 26 January 1995.

Introduction

Mucociliary clearance is considered to be the main defence mechanism of the respiratory tract. The rheological properties of mucus as a 'carrier band', the ciliary beats and periciliary fluid are all fundamental for the transport of mucus along the airway epithelium [1,2].

The lining of the nasal cavity consists of olfactory and pseudostratified ciliated respiratory epithelium except for the nasal valve area and the anterior parts of turbinates [3]. With the exception of the anterior and posterior half of the epiglottis and the vocal cords, the lining from the larynx to the main bronchi contains cuboidal ciliated epithelium [4]. Thus, the upper divisions of respiratory tract are lined by cilia actively moving mucus with pathogens towards the oropharynx where it is disposed of by swallowing. This defence

Correspondence: Dr J. P. M. Braat, ENT Department, Leyenburg Hospital Leyweg 275, 2545 CH The Hague, The Netherlands.

mechanism should not be altered or damaged by using intranasal medication.

The effects of additives such as the preservative benzalkonium chloride (BKC), widely used in nasal preparations and eyedrops, have been studied extensively and data on possible side-effects of BKC seem inconclusive [5–13], with some *in vitro* studies indicating a ciliostatic effect of the preservative.

A recent study on a human nasal respiratory mucosa culture model reported toxic effects of BKC on nasal mucosa [14]. However, *in vitro* studies on ciliated epithelium have several disadvantages, perhaps because the mucus and periciliary fluid is more or less depleted depending on different experimental conditions [6,7,12] and such models may have little relevance to the conditions *in vivo*. Substances applied to the nasal mucosa *in vivo* may be diluted by the mucus [5] or eliminated by mucociliary clearance. In addition there is a continual and natural replacement of ciliated epithelial cells occurring in the nose [16,17]. In order to determine the clinical relevance of *in vitro* studies, *in vivo* topical treatment of animals' nasal mucosa with high doses of BKC containing corticosteroid sprays was evaluated and no depletion of ciliated cells or deleterious ultrastructural changes in cells were reported [18].

The present investigations were undertaken to elucidate the effects of BKC on the human nasal mucosa in clinical circumstances using a single-centre, randomized, double-blind, placebo-controlled study of patients suffering from non-seasonal allergic rhinitis. This is the first report of a combined functional and microscopical investigation of patients treated with BKC.

Materials and methods

Patients

Non-smoking patients between 16 and 65 years of age with a 3+ result on skin-prick test for house dust mite (ALK Soluprick extract of 13 common allergens, [19]) were considered for the study. Only patients with symptoms of non-seasonal allergic rhinitis warranting regular therapy for at least 1 year were admitted to the study. Patients with signs of nasal hyperreactivity, nasal polyps, or with a history of treatment of nasal polyps, nasal airway infection or who had undergone nasal surgery within the 6 weeks prior to the study were excluded. Furthermore, patients with a significant deviation of the septum were excluded. Patients who had taken systemic or inhaled corticosteroids within 4 weeks, intranasal corticosteroids within 4 weeks, inhaled or intranasal sodium cromoglycate, nedocromil or astemizole within 6 weeks and xylometazoline hydrochloride within 2

weeks of the start of the study were excluded. No medication, other than the test substances described and 60 mg tablets of terfenadine, twice daily maximum as rescue medication, was allowed.

Thirty patients were selected for the study, and 24 patients entered the study after an initial run-in period of 2 weeks during which patients were unaware of receiving only placebo. Full physical examination was carried out and biochemistry/haematology parameters and urinalysis were measured. Twenty-two patients completed the study. The study was approved by the local ethics committee and each patient voluntarily gave informed consent prior to entering the study. All subjects were selected from the ENT outpatients population at Leyenburg Hospital, the Hague, the Netherlands.

Treatment

During the 2 week run-in period patients received intranasally, two actuations, twice daily into each nostril of placebo from bottles fitted with metering/atomizing pumps. Subsequently, patients were randomly assigned to receive for 6 weeks one of the following treatments: 200 µg fluticasone propionate, formulated as an aqueous nasal spray containing 0.02% BKC (FPANS; $n = 8$), 0.02% BKC in placebo aqueous nasal spray ($n = 8$) or placebo aqueous nasal spray ($n = 6$) twice daily.

All treatment was administered, using similar bottles to those used in the run-in period and consisted of two actuations twice daily into each nostril. Each bottle of spray was used for only 1 week before being replaced by a fresh bottle. Residual volume in each bottle was afterwards checked for patient compliance.

Assessment of patients

Before, at 2 weekly intervals during treatment and 2 weeks after treatment ceased, anterior rhinoscopy was performed. Features assessed were turbinate swelling, crusting, bleeding, mucosa colour and nasal secretions. These were scored for right and left nostril separately and on a nominal scale: normal = 0 or abnormal = 1. If abnormal the mucosa colour was specified: red = 1, white = 2 and blue = 3. Nasal secretions were specified: clear = 1, opaque = 2 and purulent = 3. An indigo carmine saccharine transport time (ICST) was determined to assess mucociliary clearance [20,21]: a droplet of indigocarmine (blue) in solution with saccharine is placed 2 cm behind the tip of the inferior turbinate. The time (in min) required to perceive these substances (sweetness as sensed by the patient; blue line noted on the posterior pharyngeal wall by investigator)

approximates the ciliary transport time or mucociliary clearance.

Before treatment and after 6 weeks of treatment two nasal biopsies were taken from each patient under local anaesthesia using Gerritsma forceps [22]. Biopsies were taken 2 cm behind the anterior tip of the inferior turbinate according to a randomized scheme ensuring that for each patient the same nostril was not biopsied both before and after the treatment period. The specimens were fixed in buffered paraformaldehyde/glutaraldehyde after a 15 min rinse in 0.9% saline.

One of the biopsies from each patient was critical-point-dried and processed for scanning electron microscopy (SEM). A subjective assessment of the mucosal surface was made by two microscopists separately and a measure of the ratio of ciliated cells to non-ciliated cells was made on 10 randomly selected surface areas of $30 \times 50 \mu\text{m}$ using a magnification of $2000\times$. The other biopsy was processed after post-fixation in osmium tetroxide/potassium ferricyanide into epon resin for light and electron microscopy. Sections ($1 \mu\text{m}$) were stained with toluidine blue for light microscopy and ultrathin sections were stained with uranyl acetate and lead citrate before ultrastructural examination using a Philips CM10 transmission electron microscope (TEM).

For light microscopy (LM) the length of mucosa present in each $1 \mu\text{m}$ section was measured using a Quantimet 970 image analyser and the number of ciliated epithelial cells present was counted. In addition an assessment of the histopathological features of rhinitis was made. In particular the extent of oedema and inflammatory cell infiltration was subjectively scored for all available biopsies.

For TEM all aspects of the ultrastructure of ciliated epithelial cells were examined independently by two electron-microscopists and at least 15 photomicrographs at magnifications up to $38\,750\times$ were prepared from each biopsy. The biopsy specimens processed for SEM were examined using a Philips SE5103 electron microscope at magnifications up to $5000\times$. All microscopy specimens were coded and the microscopists were not aware of the treatment the patients had received.

Results

Patient compliance was very good with doubtful compliance noted for only one patient.

Anterior rhinoscopy

There were few changes in the appearance of the nasal mucosa of individual patients although an improvement in nasal colour and secretions was noted, particularly in

the FPANS group. Influence of treatment (FPANS-placebo; BKC-placebo), however, on the parameters scored was not found. The results were analysed with the Exact Trend Test or Fisher's Exact Test ($P < 0.054$).

ICST

The results of the ICST test were analysed using repeated measurement analysis of variance and no statistically significant difference was found between the different treatment groups at any of the measurement points (rm-ANOVA, $P = 0.27$). The 2-weekly-results per treatment group are shown in Fig. 1. The mean values and standard errors for each group separately are as follows: FPANS 10.8 (SE 0.5; $n = 8$), BKC/placebo 12.9 (SE 2.1; $n = 8$) and placebo 13.1 (SE 1.1; $n = 6$) min.

Microscopy

Examination of biopsies by LM, TEM and SEM showed that intact epithelium was present at 38 of the 44 biopsy time points. Ciliated epithelium was present in 33 occasions.

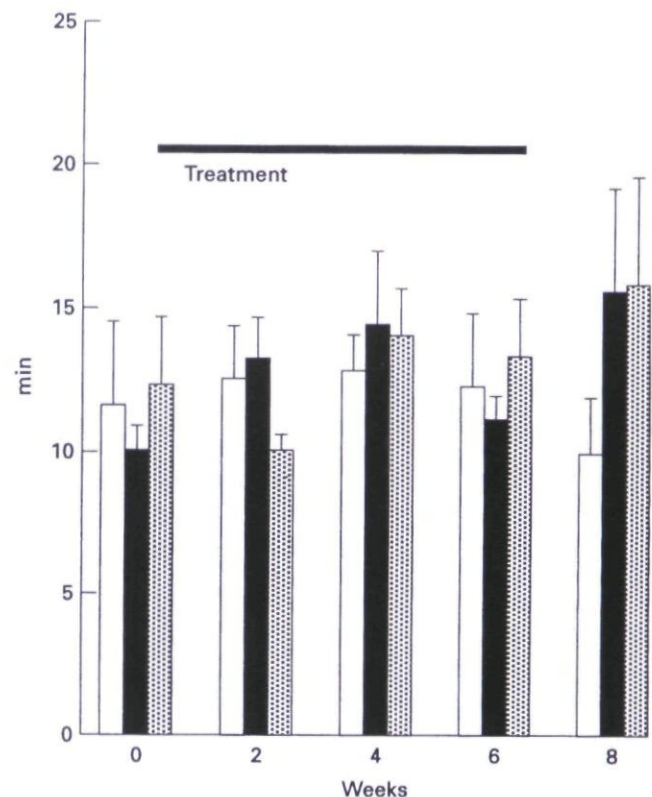


Fig. 1. Ciliary Transport Times (CTT) CTT were measured with the Indigocarmine/saccharine test (ICST). □. FPANS ($n = 8$). ■. BKC/placebo ($n = 8$). ▨. Placebo ($n = 6$).

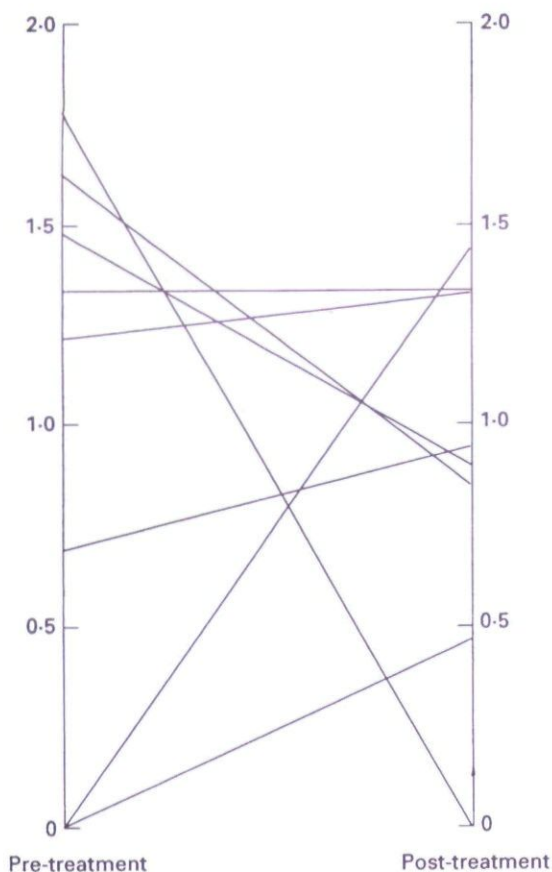


Fig. 2. $10\log$ of ciliated cell counts of BKC treated biopsy specimens pre- and post-treatment. No statistically significant changes could be detected (paired *t*-test and Wilcoxon ranked sum test).

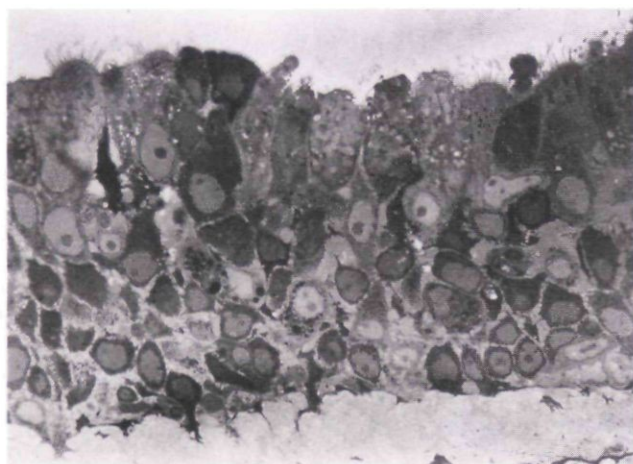


Fig. 3. Patient E.R., LM-photograph pre-treatment ($\times 1000$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.

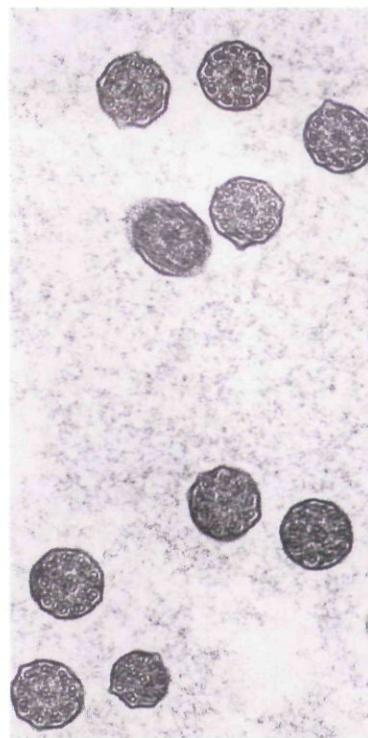


Fig. 4. Patient Y.S., TEM-photograph pre-treatment ($\times 38\,750$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.

Light microscopy The number of ciliated cells present in each specimen varied greatly (0–59/mm before treatment and 1–56/mm after treatment) but the number of ciliated cells was not related to the treatment the patients received (Wilcoxon ranked sum test, $P = 0.49$). In BKC treated patients alone (Fig. 2) no statistically significant trend could be found between pre- and post-treatment ciliary cell counts: paired *t*-test after square root transformation, $P = 0.368$. Differences between groups were analysed by the Wilcoxon ranked sum test and again were not statistically significant ($P > 0.33$). Figures 3 to 6 illustrate the typical LM appearance of ciliated epithelium.

Histopathology Although there was much variation in the extent of oedema and inflammatory cell infiltration in the biopsies, after FPANS, four of the eight patients showed an improvement in these features. When analysing the data with the Exact Trend Test (comparing a three-level ordinal outcome score between both the BKC-containing treatment groups and the placebo group) no significant differences were seen, $P = 0.720$ and $P = 0.735$.

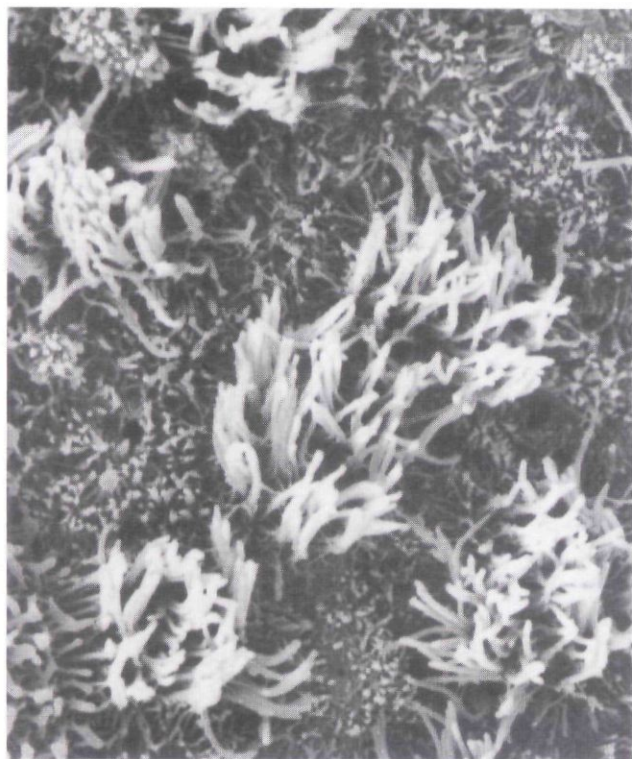


Fig. 5. Patient Y.S., SEM-photograph pre-treatment ($\times 2980$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.

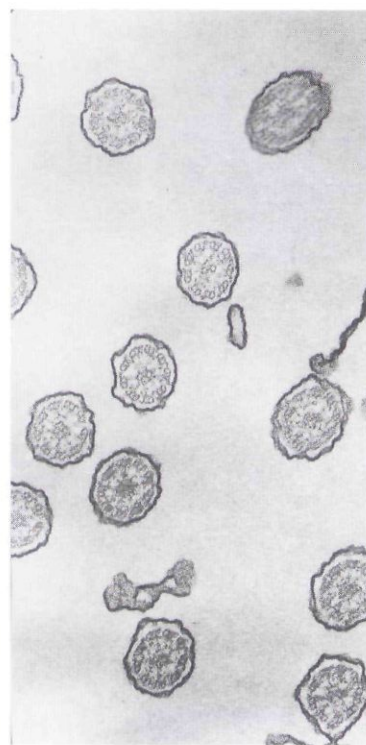


Fig. 7. Patient Y.S., TEM-photograph after 6 weeks treatment FPANS ($\times 38\,750$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.



Fig. 6. Patient E.R., LM-photograph after 6 weeks treatment FPANS ($\times 1000$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.

When comparing the two BKC-containing treatment groups together with placebo, again no significant difference was observed: $P = 0.627$.

Transmission Electron Microscopy Occasionally (six out of 44 biopsies) in patients both before and after treatment, although well preserved epithelium was present, there were no ciliated cells. Many features such as nuclei and rough endoplasmic reticulum showed little variation between patients, although there was considerable variation in several other aspects of the ultrastructure of ciliated epithelial cells of biopsies. Some cells showed swollen mitochondria. Some had very electron dense mitochondria and some showed numerous cytoplasmic vacuoles of varying size. On occasions the vacuoles appeared to be derived from swollen smooth endoplasmic reticulum (SER). Golgi apparatus was almost invariably a predominant feature of the cytoplasm. The cilia appeared similar in most biopsy specimens and their outer membranes were intact. It was possible to observe the classic 9 + 2 configuration of microtubules. Dynein areas and radial spokes were observed in cilia from patients' specimens both before



Fig. 8. Patient Y.S., SEM-photograph after 6 weeks treatment FPANS ($\times 2980$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.

and after treatment in all groups. However, on very few occasions the microtubules of a few cilia were arranged in a 9 + 3, 9 + 4, 8 + 2 and 9 + 0 configuration and compound cilia were present. In all aspects of the ultrastructure the differences seen were not related to the treatment the patients had received. Typical results are illustrated in Figs 4, 7, 9 and 10.

Scanning Electron Microscopy On a number of specimens (17/44) a layer of mucus was still present despite the 15 min saline rinse they had received. As a result 23 out of 44 specimens showed epithelium with varying numbers of ciliated cells. On a few occasions (4/44) no epithelium was present due to trauma occurring during processing of the biopsies. When ciliated cells were seen no abnormalities could be detected in their cilia no matter what the treatment.

In five cases (four pre- and one post-treatment) it was possible to make an estimate of surface area of the specimens covered by ciliated cells. A median value of 28% ciliated surface was found. In the one post treatment biopsy (FPANS treated) 92% of the surface was covered by ciliated cells. Figures 5 and 8 illustrate typical SEM appearance.



Fig. 9. Patient E.R., TEM-photograph pre-treatment ($\times 5500$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.

Discussion

The variation in the morphological and, to a lesser extent, the functional (ICST) results for patients, irrespective of treatment received is considered to reflect variation which naturally will be observed in a small population of patients. However, the biopsies were taken from the inferior turbinates, areas which are known to receive significant deposition of inhaled substances [23] and it is to be expected that if damage to ciliated cells had occurred as a result of FPANS or BKC it would have been detected. No evidence of damage was seen. It is considered unlikely that the use of patients suffering from allergic rhinitis would have masked deleterious effects of BKC since 28 days intranasal treatment of

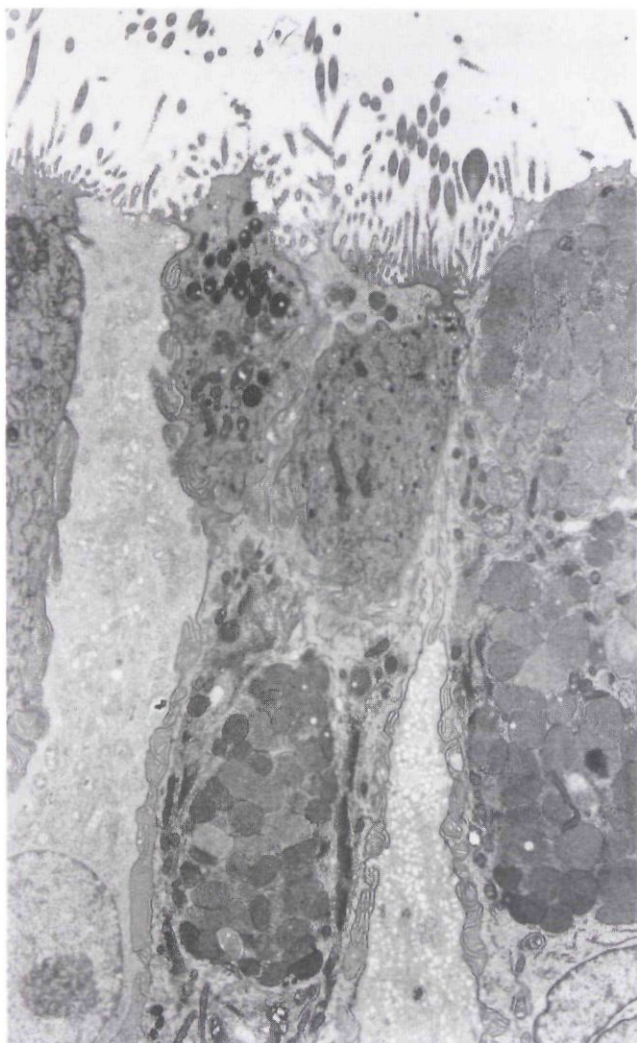


Fig. 10. Patient E.R., TEM-photograph after 6 weeks treatment BKC + placebo ($\times 5500$). This patient received twice daily two actuations in both nostrils of a nasal spray containing 0.02% benzalkonium chloride (BKC) for 6 weeks.

normal laboratory animals with either beclomethasone dipropionate aqueous nasal spray (BDPANS) or FPANS also showed no ultrastructural changes in ciliated cells [18].

The variation in the number of ciliated cells present in the biopsies irrespective of treatment is related partly to the natural differences which occur in the cell types present in different areas of the turbinate epithelium [24]. In addition, Mygind and Bretlau [25] reported that only 20% of the anterior part of the turbinates in both normal and perennial rhinitis patients are covered with ciliated cells.

In allergic rhinitis it must be expected that the state of the nasal passages will show considerable variation according to the intensity of allergen challenge around

the time of the patient's visit to the clinic. Consequently, there will be fluctuations in the extent of oedema and cellular infiltration which are unrelated to treatment. Bearing this in mind it is not surprising that the results of the histopathological examination were not absolute. However, it is encouraging that there was evidence of a reduction in inflammation in four of the eight FPANS-treated patients.

The results confirm the functional study of Scadding *et al.* [26] who treated 60 perennial rhinitis patients for 1 year with FPANS and found all nasal rhinitis symptoms were improved. The nasal mucociliary clearance time was significantly improved, olfactory function improved, however, there was no significant change in mean peak airflow and mean airways resistance of the patients.

There is a clear difference between *in vivo* results such as those presented here and the results of previous workers' functional studies of ciliated epithelial cells *in vitro* where the cells are relatively mucus depleted. Van de Donk [5] using chicken embryo trachea *in vitro*, for example, reported that 0.01% BKC irreversibly decreased the ciliary beat frequency by almost 30% after a 20 min exposure. Later work [20,27] with human adenoid ciliated epithelial cells *in vitro* gave similar findings although *in vivo* mucociliary clearance time was not significantly affected when 0.01% BKC was applied to the nasal mucosa of volunteers. Using an *in vitro* human adenoid cell culture, after exposure to glucocorticosteroids containing BKC, it was reported that ciliated cells were replaced by non-ciliated cells [14]. In the present study when FPANS containing BKC or BKC alone was given *in vivo* by metered nasal spray no depletion or damage to ciliated cells could be detected by LM or EM. Braga *et al.* [6] used an *ex vivo* frog palate preparation to measure mucociliary transport velocity (MTV). They also examined the preparations with SEM and reported that there was no effect on MTV or on the appearance of cilia after treatment with 0.01% BKC. They considered the frog palate preparation to resemble more closely the situation of the nasal mucosa of patients *in vivo*, not least because it retains an intact mucus layer. Stanley *et al.* [12] drew attention to the importance of the mucus layer. They showed in patients treated *in vivo* with betamethasone nose drops, containing 0.02% BKC, for up to 4 weeks, there was no effect on ciliary beat frequency or mucociliary clearance. However, when similar nose drops were tested *in vitro* ciliostasis occurred within 5 min. They suggested that, *in vivo*, cilia are protected by the dilutional effects of nasal mucus and perhaps the mucus also forms a barrier. In the clinical situation, the non-rhinitic nose will contain approximately 0.4 mL mucous [12] and the rhinitic nose probably contains much more. Thus, a dose of nasal spray will be diluted

at least fourfold and it may be as a result of this that BKC *in vivo* does not damage the nasal mucosa. In addition, protein and other organic materials rapidly inactivate quaternary ammonium compounds such as BKC [28] and we suggest it is possible that BKC is rendered inactive by the protein present in nasal secretions. The available evidence including the results of the present study suggest therefore that the results of *in vitro* work have little relevance to the situation which pertains *in vivo* and should be interpreted with extreme caution. In this *in vivo* study no deleterious effects of BKC could be detected after it was repeatedly applied to nasal ciliated epithelium for 6 weeks.

Conclusion

Although the number of patients studied was small, it is concluded that 0.02% benzalkonium chloride, despite reports of its ciliostatic effect *in vitro*, did not have such an effect when it was repeatedly applied for 6 weeks (with or without fluticasone propionate) directly to the nasal mucosa of perennial allergic rhinitic patients *in vivo*.

Acknowledgments

The authors wish to express their gratitude to GLAXO plc. for financial support, to Mr P. Mulder and Mr R. Lyons for statistical assistance, to Professors C. D. A. Verwoerd and J. F. Jongkind and Dr W. Fokkens for valuable comments on the manuscript and Dr D. Poynter for the histopathological evaluation. Miss E. van Schaik's help in patient care and administrative affairs was also very much appreciated.

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