In vitro effects of preservatives in nasal sprays on human nasal epithelial cells

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ABSTRACT

Background: *The preservatives benzalkonium chloride and potassium sorbate are widely used in nasal drops and sprays. Recently, side effects resulting from mucosal damage caused by benzalkonium chloride and potassium sorbate were reported.*

Methods: *We investigated the toxicity of benzalkonium chloride and potassium sorbate on human nasal epithelial cells* in vitro. *Using primary human nasal epithelial cells, different concentrations of benzalkonium chloride, potassium sorbate, or phosphate-buffered saline (PBS; control group) solutions were cocultured with nasal epithelial cells for 15 minutes. Then, the viability of the cells and the cell morphology were assessed.*

Results: *Nasal epithelial cells were more severely damaged with use of clinical preparations or higher concentrations of benzalkonium chloride than in the control group. In addition, nasal epithelial cell membrane lysis was seen on electronic microscopy in the benzalkonium chloride groups. In contrast, there was no significant cell damage seen in the potassium sorbate groups compared with the control group, even with higher concentrations than clinically used.*

Conclusion: *Potassium sorbate appears to be a relatively safer preservative than benzalkonium chloride for use in nasal sprays and drops* in vitro study.

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Key words: Adverse effect, benzalkonium chloride, cell viability, nasal epithelial cell, potassium sorbate, preservatives, primary cell culture

Local administration of medicine has been adapted to medical treatment for disease in the nasal cavity and paranasal sinuses, including allergic rhinitis and sinusitis. Various preservatives are used for preventing bacterial contamination of nasal spray preparations. The preservatives should not alter the main pharmacologic actions of the drug in the preparation and should not cause side effects in the body. Among the preservatives, benzalkonium chloride and potassium sorbate are the ones we often find in preparations that we use daily in nasal drops, eye drops, cosmetics, and food preparations. Several studies showed that benzalkonium chloride induced alteration of the nasal mucosa *in vitro* and *in vivo*.[1–7] Some of these studies reported that benzalkonium chloride did not induce nasal cell damage.[8,9] The effects of benzalkonium chloride on the nasal passages are controversial. Studies of the effects of potassium sorbate on nasal mucosa are lacking. In 2004, Hofmann *et al.* found that the isolated potassium sorbate did not have negative influence on ciliary beat frequency *in vitro*.[3] But *in vivo*, some studies indicated that even a low concentration of potassium sorbate can lead to nasal lesions, including intraepithelial glandular formation, inflammatory cell infiltration, vascular hyperplasia, and edematous change.[10] Thus, the effects on the nasal mucosa of preservatives used in nasal sprays remain unclear. We investigated the effects of different concentrations of benzalkonium chloride and potassium sorbate on primary human nasal epithelial cells *via* measurement of cell viability and cell morphology.

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METHODS

For the benzalkonium chloride group, a 0.01% benzalkonium chloride solution that is commonly used as a preservative in nasal drops and 0.1, 0.001, and 0.0001% solutions (Sigma Chemical Co., St. Louis, MO) were used for assessing dose dependence. For the potassium sorbate group, a 0.1% potassium sorbate solution commonly used as a preservative in nasal drops and 1, 0.01, and 0.001% potassium sorbate solutions were used for assessing dose dependence (Sigma Chemical Co.). Phosphate-buffered saline (PBS) was used for the control group.

Primary Human Nasal Epithelial Culture with Air–Liquid Interface Method

This study was approved by the Ethics Review Board of the hospital and with informed consent from the patients. The primary human nasal epithelial cell culture with air– liquid interface procedure was modified from methods previously described.[11,12] Human inferior turbinate cells were obtained from 18 patients receiving submucosal resection of inferior turbinates due to chronic hypertrophic rhinitis. Each specimen was rinsed several times with Leibovitz's L-15 medium containing penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). The tissue was then cut into 1- to 2-mm^2 pieces, and three or four pieces of tissue were planted with the epithelium side facing down onto 6-well culture inserts (Falcon, Franklin Lake, NJ), in which the mem-

brane growth area was 4.2 cm^2, and with a pore size of 0.4 μm. Cells were cultured with Airway Epithelial Cell Growth Medium (PromoCell Bioscience Alive, Germany) containing bovine pituitary extract (0.004 mL/mL), human epidermoid growth factor (0.5 ng/mL), insulin (5 μg/mL), transferrin (10 μg/mL), hydrocortisone (0.5 g/mL), epinephrine (0.5 μg/mL), triiodo-L-thronine (6.7 ng/mL), retinoic acid (0.1 ng/mL), and phenol red (0.62 ng/mL). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Media were changed three times weekly. The airway epithelial cells were grown on a porous membrane, on which they formed a continuous epithelial sheet, with the basal aspect exposed to the medium and the apical surface exposed to air. Cells grown on the inserts were confluent after 10 days of incubation. Cells were then detached with 0.1% trypsin-EDTA and seeded into 96- or 24-well culture plates.

Assay of Cell Viability and Morphological Observation

The viability of the cells treated with PBS, benzalkonium chloride, or potassium sorbate was quantified by the trypan blue exclusion method and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and electron microscopy. Trypan blue (Sigma Chemical Co.) chromophore is negatively charged and does not interact with cells unless the membranes are damaged. Therefore, cells that exclude the dye are viable. The cells were washed twice in PBS and detached by swirling with 0.01% trypsin-EDTA. The cells were collected by centrifugation at 1500 rpm for 6 minutes at 4°C and the supernatant was decanted. The cell pellet was resuspended in growth medium. An aliquot of 0.4% trypan blue was mixed with an equal volume of cell suspension for 5 minutes and viable cells were counted using a hemacytometer.

MTT

Cell viability was determined on day 13 using the MTT test (SigmaChemical Co.). MTT is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. The MTT assay is based on reduction of MTT by actively growing cells to produce a blue formazan product with absorbance at 570 nm. This process requires active mitochondria, and freshly dead cells do not cleave significant amounts of MTT. A low MTT absorbance indicates cell death. The colorimetric assay was used for proliferation in 96-well flat-bottom tissue culture plates of good optical quality (Falcon). The final volume of serum-free culture medium in each well was 0.1 mL. At the end of the assay, 0.01 mL of MTT solution added to each well and the cultured cells were incubated at 37°C for another 4 hours. Subsequently, 100 μL of isopropanol/0.04 N hydrochloric acid solution was added to each well and mixed thoroughly. The absorbance was measured in an enzyme-linked immunosorbent assay (ELISA) plate reader at a test wavelength of 570 nm within 1 hour.

Transmission Electron Microscopy

Cells were prefixed with 1.5% glutaraldehyde in 0.2 M of phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated through absolute alcohol, transferred to propylene oxide, and impregnated overnight in Epon resin. This preparation was finally embedded in Epon resin and polymerized at 50°C. Sections were cut with an ultramicrotome to a thickness of 60 nm and stained with uranyl acetate and lead citrate. The samples of nasal epithelial cells were observed using a transmission electron microscope (JEOL 2300; JEOL Tokyo, Japan)

Statistical Analysis

Results are presented as means ± SE. Statistical comparisons of multigroup data were analyzed using analysis of variance followed by the Duncan post test. A value of $p < 0.05$ was considered significant.

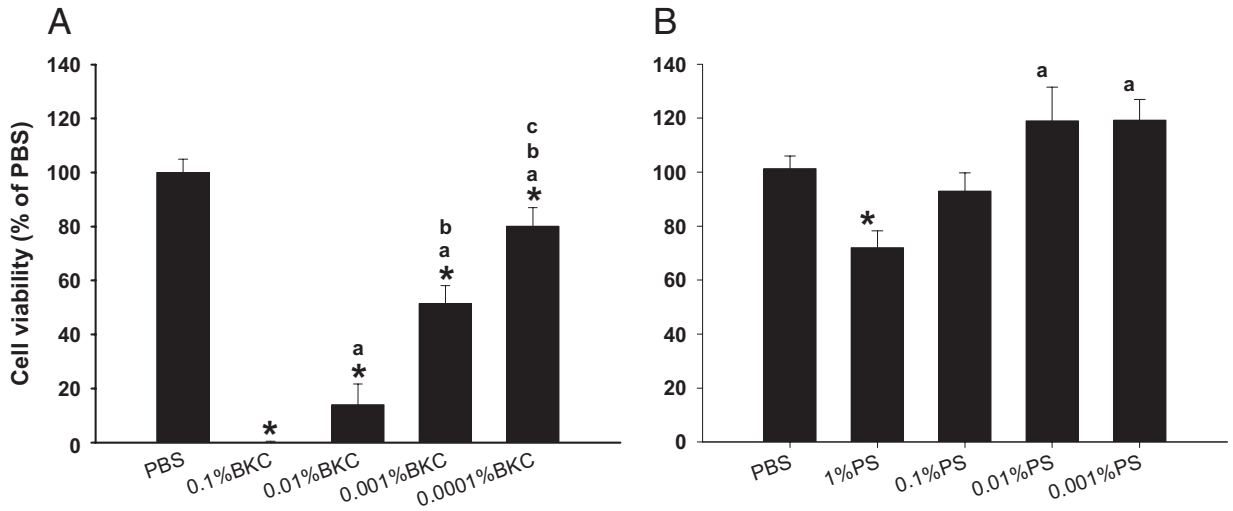

Figure 1. *Trypan blue method for cytotoxicity of benzalkonium chloride (BKC) and potassium sorbate (PS). Primary human nasal epithelial cells were incubated with various concentrations of PBS, BKC, or PS for 15 minutes. Cell viability was evaluated by trypan blue method. Data are means ± SE from three independent experiments. *$p < 0.05$ compared with PBS; a, $p < 0.05$ compared with (A) 0.1% BKC and (B) 1% PS; b, $p < 0.05$ compared with 0.01% BKC; c, $p < 0.05$ compared with 0.001% BKC.*

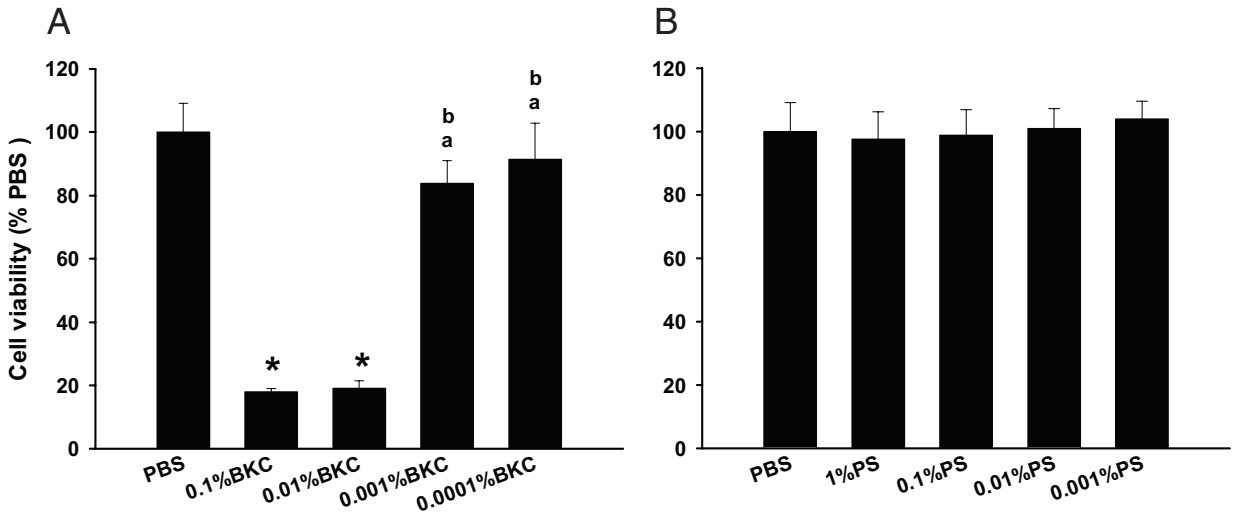

*Figure 2. MTT for cytotoxicity of benzalkonium chloride (BKC) and potassium sorbate (PS). Primary human nasal epithelial cells were incubated with various concentration of PBS, BKC, or PS for 15 minutes. Cell viability was evaluated by MTT assay. Data are means ± SE from three independent experiments. *p < 0.05 compared with PBS; a, p < 0.05 compared with (A) 0.1% BKC and (B) 1% PS; b, p < 0.05 compared with 0.01% BKC.*

RESULTS

Cell Viability

The higher the concentration of benzalkonium chloride or potassium sorbate used, the lesser the cell viability *via* the trypan blue and MTT methods (Figs. 1 and 2). The clinically used benzalkonium chloride concentration of 0.01% significantly reduced cell viability compared with the control group. Only 14 and 19% of nasal epithelial cells survived treatment with 0.01% benzalkonium chloride *via* the trypan blue and MTT methods, respectively. In contrast, the clinically used concentration of potassium sorbate (0.1%) did not induce cell toxicity.

Cell Morphology

After 15 minutes of incubation, 0.01% benzalkonium chloride induced cell swelling (Fig. 3) and loss of cell contact. In the trypan blue exclusion test, nucleoli were stained in almost 90% cells after incubation with 0.01% benzalkonium chloride for 15 minutes. There were no morphological changes in the potassium sorbate and control groups, and nucleoli were stained in only a few cells.

Electron microscopic analysis of the structural features of nasal epithelial cells in the control group and the 0.1% potassium sorbate–treated group and the 0.01% benzalkonium chloride-treated group are shown in Fig. 4. The cells of the control and 0.1% potassium sorbate–treated group showed microvilli (arrows) and keratin intermediate filaments, both of which are specific characters of respiratory epithelial cells; the cells are also tightly bound and showed no destruction of the cell membrane and were well aligned. Nasal epithelial cells incubated with 0.01% benzalkonium chloride for 15 minutes showed the loss of microvilli, destruction of cell membranes, and poor cytoskeletal alignment.

DISCUSSION

Benzalkonium chloride and potassium sorbate usually are added to nasal sprays for prevention of bacterial contamination. In our study, the benzalkonium chloride–induced nasal epithelial cell damage occurred in a dose-dependent manner. Even at the clinically used concentration of benzalkonium chloride, cell viability was reduced. In contrast, the clinically used concentration of potassium sorbate in nasal spray did not induce human nasal epithelial cell damage.

Preparations of various medicines, such as steroids, anti-

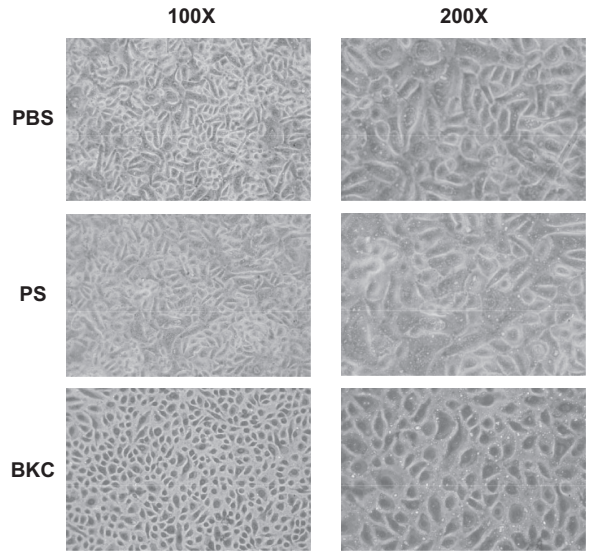

Figure 3. Morphological appearances of primary nasal epithelial cells after incubation with various concentration of PBS, benzalkonium chloride (BKC), or potassium sorbate (PS) for 15 minutes. (Upper Panel) Treated with PBS. The cells are intact with good cell–cell contact. (Middle Panel) Treated with 0.1% PS for 15 minutes. The cell morphology did not change compared with PBS-treated cells. (Lower Panel) Treated with 0.01% BKC for 15 minutes. The cell was rounding and loss cell–cell contact.

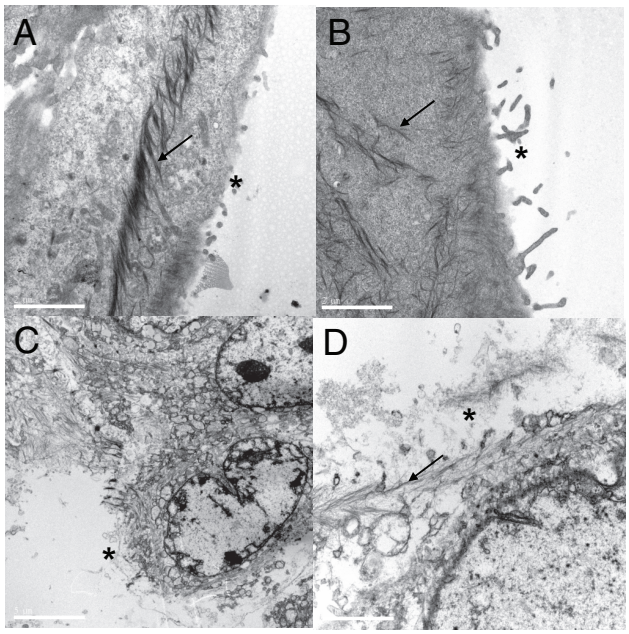

Figure 4. *Morphologic appearances of primary nasal epithelial cells measured by transmission electron microscopy. Cells of control group (A) and 0.1% PS-treated group (B) showed microvilli (*) and keratin intermediate filaments (arrow) both of which are specific characters of respiratory epithelial cells. Nasal epithelial cells incubated with 0.01% BKC (C and D) for 15 minutes demonstrated the losing of microvilli, the lysis of cell membrane (*) and destruction of keratin intermediate filaments (arrow). Scale bar as figure showing 2 μm in A, B and D; 5 μm in C).*

histamines, and decongestants, are administered into the nasal cavity and paranasal sinuses for allergic rhinitis, sinusitis, and chronic hypertrophic rhinitis. In addition to the main drug, the other ingredients of these sprays include various additives, such as osmoregulators, viscosity regulators, preservatives, and pH regulators, which are recognized as harmless to humans. The adverse effects that are reported are mostly for preservatives.[13,14] Benzalkonium chloride and potassium sorbate are the most commonly used preservatives in nasal sprays. Several studies showed that benzalkonium chloride induced alteration of the nasal mucosa *in vitro* and *in vivo*.[1–7] In 2004, Hofmann *et al.*[3] reported that benzalkonium chloride could cause a decrease in cilia beat frequency of nasal mucosa and the change was irreversible if the concentration of benzalkonium chloride was high. In 2000, Storaas *et al.*[4] found that benzalkonium chloride in dosages commonly used as preservatives in nasal decongestant sprays produced short-term glandular secretion and nasal pain *in vivo*. In histological studies, benzalkonium chloride induced squamous metaplasia, edematous change, glandular hyperplasia, and inflammatory cell infiltration in nasal mucosa.[10,15,16] In contrast, some studies reported that benzalkonium chloride–containing nasal sprays did not induce nasal mucosa damage or ciliotoxic effects.[17,18] Other studies showed that benzalkonium chloride–containing nasal sprays had a marked ciliotoxic effect, but did not change the biomarker in nasal secretions or inflammatory cell recruitment.[1] This probably is because the mucosa is well protected by a layer of mucus and by the continual ciliary beating, which quickly moves the benzalkonium chloride from one site to another. This probably explains the discrepancy in results between *in vitro* and *in vivo* studies. In our *in vitro* study, primary human nasal epithelial cells treated with the clinically used concentration of benzalkonium chloride, or even less than the clinically used concentration, for 15 minutes induced severe nasal epithelial damage, including decreased cell viability; loss of cilia; destruction of the cell membrane; and loss of cytoskeleton alignment. Benzalkonium chloride induced great cytotoxicity in human nasal epithelial cells *in vitro*.

Previous studies of the influence of potassium sorbate on nasal mucosa are inconclusive. In 2000, Cho *et al.* showed that preservatives in nasal sprays, including benzalkonium chloride and potassium sorbate, induce nasal histological change, including intraepithelial glandular formation, inflammatory cell infiltration, vascular hyperplasia, and edematous change.[10] The histological changes were pronounced with prolonged duration of administration. In 2004, Hofmann found that potassium sorbate did not influence the ciliary beat frequency of human nasal mucosa *in vitro*.[3] Our results showed that the clinically used concentration of potassium sorbate did not reduce cell viability of human nasal epithelial cells, and it did not destroy the nasal epithelial cell morphology.

CONCLUSIONS

Benzalkonium chloride in nasal sprays reduced the viability of human nasal epithelial cells and induced cell lysis *in vitro*. In contrast, potassium sorbate did not show a significant influence on cell viability and cell morphology *in vitro*. Potassium sorbate appears to be a safer preservative than benzalkonium chloride in topical nasal sprays. However, additional *in vivo* studies are needed to clear up the side effects of preservatives in the human nasal mucosa.

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