ORIGINAL ARTICLE

Effects of Nasal Saline Spray on Human Neutrophils

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Background: Nasal saline spray (NSS) used in the treatment of rhinitis and sinusitis often contains the preservative benzalkonium chloride (BKC). Previous studies have shown that corticosteroid nasal sprays and topical decongestants containing BKC damage respiratory mucosa, decrease mucociliary activity, and inhibit neutrophil functions in vitro.

Objective: To evaluate the effects of NSS with BKC on human neutrophils.

Design: Prospective, basic science observations.

Methods: Human neutrophils were exposed to NSS with BKC or to phosphate-buffered saline (PBS) at varying times and concentrations. The cells were examined for morphologic changes by light microscopy and for viability as determined by trypan blue exclusion. Lactate dehydrogenase levels were measured to quantify neutrophil cell lysis. In vivo morphologic changes were studied in neutrophils obtained from the oral mucosa in human volunteers who rinsed their mouths with NSS or PBS.

Results: Neutrophils exposed to NSS concentrations as low as 15% showed near-total cell lysis, and neutrophils exposed to 20% NSS demonstrated no cell viability by trypan blue staining. Phosphate-buffered saline–exposed cells were unaffected. The release of lactate dehydrogenase from lysed neutrophils increased sharply at NSS concentrations higher than 10% but remained stable in PBS-exposed cells. All neutrophils isolated from NSS oral rinses were lysed, while a mean of 78% of neutrophils from PBS rinses showed normal morphologic structure.

Conclusions: Nasal saline spray with BKC is toxic to human neutrophils even at concentrations far lower than those found in commercially available preparations. Saline solutions without BKC appear to be safer alternatives, and additional studies are needed to determine the clinical significance of these findings.

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ALL MULTIUSE aqueous nasal sprays, including corticosteroid nasal sprays, topical decongestants, and nasal saline solutions, contain buffering agents and preservatives. The role that these additional agents might play in causing or exacerbating rhinitis symptoms has been debated for some time.

Nasal saline spray (NSS) is often used for relief of nasal dryness and to debride the nose and restore ciliary function in the treatment of sinusitis and rhinitis. Nasal saline spray is available as an over-the-counter preparation and is typically considered to be without harmful adverse effects. However, in addition to buffered saline, most NSS preparations contain the preservative benzalkonium chloride (BKC). Benzalkonium chloride is a quaternary ammonium antimicrobial agent found in numerous medications, including nasal sprays, ophthalmic drops, and bronchial inhalers. Studies[1-3] of topical decongestants containing oxymetazoline hydrochloride or xylometazoline hydrochloride, as well as BKC or thiomersal as preservatives, have shown inhibition of neutrophil functions in vitro. It was also concluded that BKC was the most toxic component in the nasal preparations studied.[3] Corticosteroid nasal compounds preserved with BKC have also been shown to reduce mucociliary activity, damage human respiratory mucosa, and decrease neutrophil functions in vitro.[4,5] In addition, Graf and Hallen[6] showed that decongestant sprays containing BKC produced more rebound swelling than decongestant sprays without BKC in healthy volunteers.

Other investigators, however, have found no deleterious effects associated with BKC. Ainge et al[7] and Braat et al[8] demonstrated no changes in the nasal ciliary function of monkeys or humans exposed to topical preparations containing BKC. Similarly, McMahon et al[9] and Storaas et al[10] failed to

show any significant effects on human nasal mucosa exposed in vivo to BKC-containing nasal sprays.

Although numerous studies have examined the potential benefits of saline nasal irrigation, to date no one, to our knowledge, has specifically addressed the potential harmful adverse effects of NSS.[11] In this study, the morphologic changes, cell viability, and lactate dehydrogenase (LDH) activity of human neutrophils exposed to NSS or buffered saline without preservatives were examined.

METHODS

REAGENTS AND CHEMICALS

Dextran solution (average molecular weight approximately 500 000), Ficoll, sodium chloride, Triton X-100, and LDH optimized bioassay were purchased from Sigma-Aldrich Corp (St Louis, Mo). Hanks balanced salt solution (HBSS) and phosphate-buffered saline (PBS) were purchased from BioWhittaker, Inc (Walkersville, Md). Hypaque 76 was purchased from Sanofi Winthrop Pharmaceuticals (New York, NY), and heparin sodium was purchased from Elfing-Sinn (Cherry Hill, NJ). Nasal saline sprays used included Deep Sea from Major Pharmaceuticals (Livonia, Mich) and Ayr from B.F. Ascher & Co Inc (Lenexa, Kan).

PURIFICATION OF NEUTROPHILS

Heparinized venous blood samples were obtained from healthy adult volunteers with approval of our institutional review board. The blood was separated by Hypaque-Ficoll step-gradient centrifugation, dextran sedimentation, and hypotonic lysis. The cells were then resuspended in HBSS at a concentration of 1×10^6/mL. These steps provide a cell preparation that is typically more than 95% neutrophils as measured by modified Wright-Giemsa staining of morphologic structure. All cells were used the same day they were obtained. Each experiment was performed with neutrophils isolated from a different donor to ensure that the observed effects were not donor-specific.

PREPARATION OF CHEMICALS

Neutrophils were exposed to 1 of 2 commercially available NSS containing BKC or to 1 of 2 control solutions containing PBS without BKC or to a "homemade" saline solution (HSS) without BKC. Homemade saline solution was prepared by mixing 650 mg of sodium chloride with 100 mL of deionized water. All the solutions had similar pH levels (6.5), although the HSS was slightly more acidic (range, 5.5-6.0). Nasal saline spray, HSS, and PBS were diluted in HBSS and incubated with 50 µL of neutrophil suspension (0.5×10^6 cells) to obtain final dilutions of 1% to 20% and a cell concentration of 5×10^6/mL. The NSS used in the experiments contains BKC at a concentration of 0.01%. The final concentration of BKC in the experimental conditions ranges therefore from 0.0001% to 0.002%. After incubation at the desired concentration and exposure time, 400 µL of HBSS was added to terminate the incubation, bringing the final concentration of cells to 1×10^6/mL and the concentration of NSS, HSS, or PBS from 0.2% to 4% during analysis. Freshly prepared buffers were used for each experiment, and all incubations were performed at room temperature (24°C) in covered cuvettes.

IN VITRO EXPOSURE OF NEUTROPHILS AND ANALYSIS

Morphologic changes were studied by exposing neutrophils to NSS or PBS at concentrations of 1%, 5%, 10%, 15%, or 20% for 3 minutes. Five exposures at each saline concentration were performed. Cells were analyzed for morphologic changes by Wright-Giemsa staining and light microscopy. Those demonstrating abnormal cell membrane borders, loss of characteristic staining pattern, or atypical nuclei were considered morphologically abnormal. Trypan blue exclusion was used to assess neutrophil viability. Equal volumes of cell suspension and trypan blue were mixed on a microscope slide and then examined by light microscopy by 2 independent observers (M.B. and E.J.D.). Living cells with intact cell membranes do not take up the dye, while nonviable cells are unable to exclude the dye and stain dark blue.

A quantitative determination of neutrophil cell lysis was performed using an optimized bioassay to detect LDH released from lysed cells. Neutrophils were exposed to NSS or PBS at concentrations of 1%, 5%, 10%, 15%, or 20% for 3 minutes. Seven exposures at each saline concentration were performed. Neutrophils were also exposed to a constant concentration (15%) of NSS or PBS for 3, 10, and 30 minutes; 5 exposures were performed at each time interval in this experiment. Samples were centrifuged to remove cellular debris. Standardized aliquots of the suspension were added to the LDH kit reagents, and the LDH released by disrupted neutrophils into solution was measured by spectrophotometry. Maximal LDH level was determined by exposing 0.5×10^6 neutrophils to 20% Triton for 5 minutes to lyse all the cells.

IN VIVO EXPOSURE OF NEUTROPHILS AND ANALYSIS

In vivo effects of NSS were studied in healthy volunteers who used a mucosal rinse of NSS or PBS. Oral rinses were used to simplify the exposure and isolation of neutrophils. Volunteers were asked to refrain from eating, drinking, smoking, or brushing their teeth for 1 hour before the mouth rinse. Volunteers first rinsed with 15 mL of distilled water for 5 seconds to clear loose debris from their mouths. They then vigorously rinsed with 15 mL of PBS or NSS for 30 seconds and expectorated into a 50-mL tube. Hanks balanced salt solution was added to the tube to bring the total volume to 50 mL, and the tube was centrifuged at 1500 rpm for 5 minutes at 25°C. The supernatant was removed, and the pellet was washed with 50 mL of HBSS. The tube was centrifuged again, the supernatant was removed, and the pellet was resuspended in 15 mL of HBSS. The PBS and NSS rinses were performed in the mornings on separate days.

For analysis, a 100 µL sample of the washed cell suspension was plated with a cytospin, stained, and examined by light microscopy. There were various cells in these preparations, including bacteria, mucosal epithelial cells, and neutrophils. One hundred neutrophils were counted under high power (at ×100) and were classified as having normal or abnormal morphologic structure. The counts were performed independently by 2 observers (M.B. and E.J.D.), and the mean of the 2 counts was recorded.

STATISTICAL ANALYSIS

Differences in LDH activity were examined using the Wilcoxon rank sum test. Differences were considered significant at $\alpha < .05$. The medians and ranges of the data are presented.

RESULTS

IN VITRO NEUTROPHIL MORPHOLOGIC STRUCTURE AND VIABILITY

Neutrophil morphologic structure was dramatically altered by exposure to NSS (**Figure 1**). Cells exposed to 20% PBS solution for 3 minutes exhibited normal cellu-

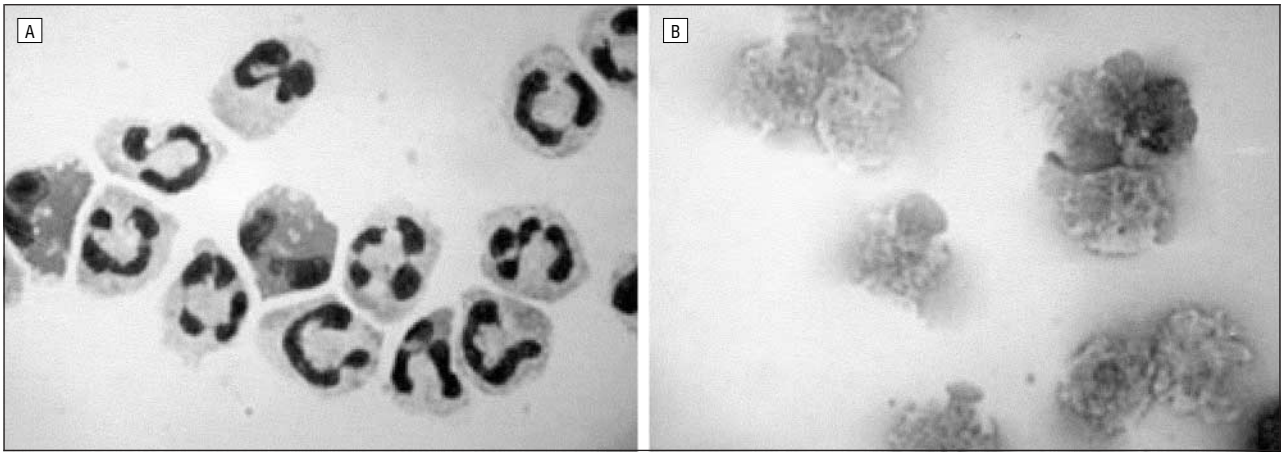

Figure 1. Wright-Giemsa staining of neutrophils exposed to 20% phosphate-buffered saline (PBS) (A) or 20% nasal saline spray (NSS) (benzalkonium chloride concentration, 0.002%) (B). The cells exposed to PBS appear normal, while the cells exposed to NSS show severe alteration of their cell membranes and intracellular structures (original magnification ×1000).

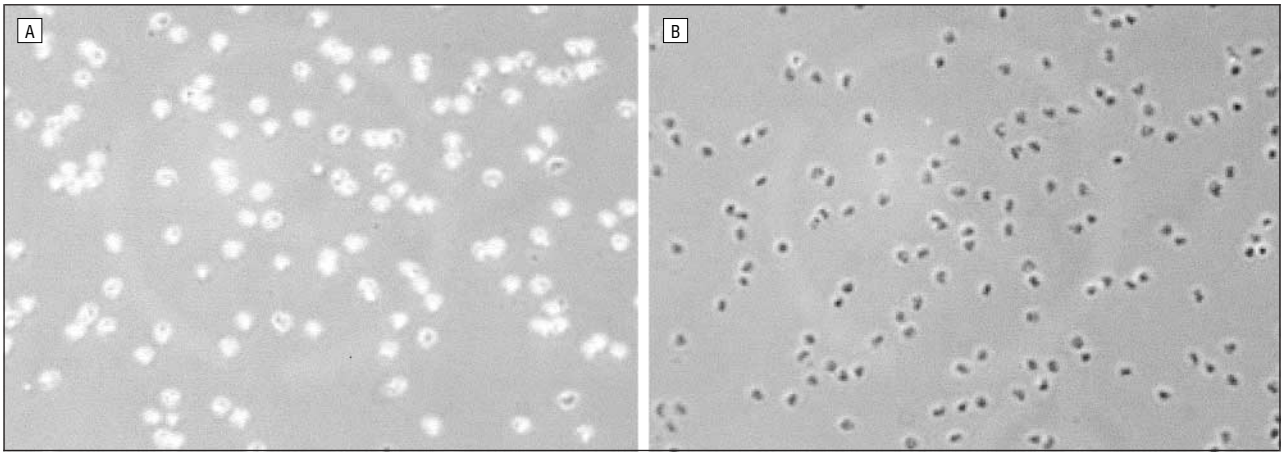

Figure 2. Trypan blue staining of neutrophils exposed to 20% phosphate-buffered saline (PBS) (A) or 20% nasal saline spray (NSS) (B). The neutrophils exposed to PBS have intact cell membranes, and the dye is not able to penetrate the cells. The cells exposed to NSS stained dark with the trypan blue, indicating disruption of the cell membranes (original magnification ×40).

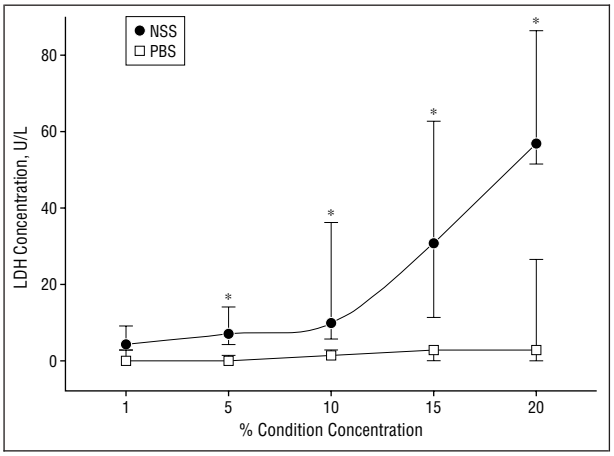

Figure 3. Lactate dehydrogenase (LDH) concentration in solution from neutrophils exposed to varying concentrations of one brand of nasal saline spray (NSS) or phosphate-buffered saline (PBS) for 3 minutes. Exposure to concentrations of NSS of 5% or greater (benzalkonium chloride concentration, 0.0005%) caused a statistically significant increase in LDH concentration over exposure to PBS. Maximal LDH concentration was 80 to 120 U/L as measured by neutrophils lysed in Triton. Data are expressed as median and range. Asterisk indicates significant difference (95% confidence interval).

lar architecture, with sharp, distinct cell membrane borders, characteristic staining patterns, and typical multilobulated nuclei (Figure 1A). Neutrophils exposed to 20% NSS for 3 minutes demonstrated severe disruption of cell membranes and intracellular structures (Figure 1B). Similar effects were seen at lower concentrations of NSS, although more intact cells survived.

Neutrophils exposed to 20% PBS remained viable and did not stain with trypan blue (**Figure 2**A), while cells exposed to 20% NSS stained dark blue (Figure 2B), indicating disruption of their membranes. Similar effects were seen at lower concentrations of NSS, but there was less extensive staining at lower concentrations.

QUANTIFICATION OF NEUTROPHIL CELL DEATH

Mean LDH activity resulting from neutrophil lysis was graphed as a function of increasing concentration of NSS and PBS, with a constant incubation time of 3 minutes (**Figure 3**). Neutrophils exposed to NSS at increasing concentrations demonstrated a significant increase in LDH activity at concentrations above 5% ($P<.05$). The cells

exposed to PBS showed small amounts of LDH activity and little increase in activity with increasing concentrations of PBS.

Figure 4 shows mean neutrophil LDH activity as a function of increasing exposure time to 15% NSS and 15% PBS. Neutrophils that were exposed to 15% NSS demonstrated an increase in LDH activity with increasing incubation time. Cells exposed to 15% PBS showed minimal LDH activity and no increase over time.

In both experiments, the neutrophils exposed to 20% NSS for 3 minutes or 15% NSS for 30 minutes demonstrated LDH activity approaching maximal LDH levels (80-120 U/L) for the experiment as determined by Triton cell lysis. Experiments performed with HSS showed levels of LDH activity similar to those obtained with PBS (data not shown). The data presented are from one brand of NSS only. The results from the second brand are similar but are not presented.

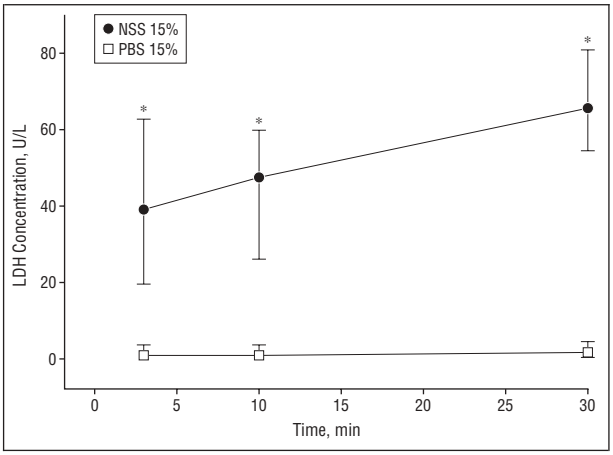

Figure 4. Lactate dehydrogenase (LDH) concentration in solution from neutrophils exposed to 15% nasal saline spray (NSS) or 15% phosphate-buffered saline (PBS) over time. Lactate dehydrogenase values were measured at 3, 5, and 30 minutes after exposure. Neutrophils exposed to NSS showed a gradual increase in LDH activity over time, whereas cells exposed to PBS did not. Data are expressed as median and range. Asterisk indicates significant difference (95% confidence interval).

IN VIVO NEUTROPHIL MORPHOLOGIC STRUCTURE

Neutrophil morphologic structure was significantly altered in the cells isolated from the NSS oral rinses compared with the cells isolated from the PBS oral rinses. Cell counts from the 2 oral mucosal rinses are shown in **Figure 5**. Neutrophils isolated from the NSS rinse exhibited 100% abnormal morphologic structure, while a mean of only 22% of those isolated from the PBS rinse were considered abnormal cells.

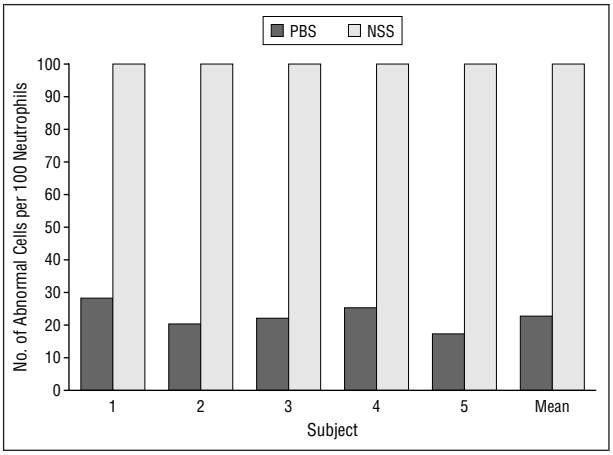

Figure 5. Bar graph illustrates the mean number of abnormal neutrophils per 100 neutrophils counted from oral rinses with nasal saline spray (NSS) or phosphate-buffered saline (PBS). Nearly all neutrophils from the NSS rinses were abnormal in morphologic structure, whereas a mean of 22% of neutrophils from the PBS rinses were abnormal. Data are shown for each individual and for the mean.

COMMENT

Nasal saline spray is available without a prescription and is frequently used in the medical treatment of sinonasal disease and following sinus or nasal surgery. Potential benefits of NSS include moistening of dry nasal mucosa, clearance of debris from the nasal passageways, and improved mucociliary function. Nasal saline spray is conveniently packaged in multiuse bottles that make administration simple and relatively inexpensive. However, multiuse containers are subject to bacterial growth over time unless antimicrobial preservatives are used in the solutions. The most frequently used preservative in NSS and other aqueous nasal sprays is BKC. Previous studies[1-5] have documented the cytotoxic effects of BKC on neutrophils and nasal mucosa.

The present study demonstrates that NSS containing BKC, even at concentrations far lower than those in commercially available preparations, alters neutrophil morphologic structure, decreases cell viability, and increases neutrophil LDH activity in a concentration- and time-dependent manner. We also found that NSS is toxic to neutrophils in an in vivo model by exposure to oral mucosa. None of these effects were seen with PBS, which is similar in composition to NSS but without the preservative BKC. In vitro experiments were repeated with an unbuffered HSS, not containing preservative, which produced results similar to those obtained with PBS.

Neutrophils are the primary cellular mediators of acute inflammation and are found abundantly on the mucosal surfaces of the nose and oral cavity. These cells provide a front line of defense against invading pathogens through their functions of phagocytosis and degranulation. Functional neutrophils must respond in adequate numbers if the body is to successfully combat infectious processes, including sinusitis. Benzalkonium chloride appears to inhibit this process. Furthermore, the release of inflammatory mediators from lysed neutrophils into surrounding tissues may result in increased vascular permeability and recruitment of other neutrophils and immune cells to the area. Therefore, it is possible that the lysis of mucosal surface neutrophils can result in a local inflammatory response in the absence of pathogenic microorganisms. Because NSS is often used 4 or more times a day, the clinical significance of neutrophil cell lysis may be greater than that associated with nasal sprays used only once or twice a day.

Eosinophils also play a crucial role in the pathogenesis of rhinitis, especially in patients with allergy. Neutrophils, however, outnumber eosinophils several-fold and

are abundantly found on the mucosal surfaces lining the mouth, nose, and paranasal sinuses. The lysis of neutrophils would, therefore, be expected to result in the release of a greater amount of inflammatory mediators and possibly result in a clinical effect. Our observations of the few eosinophils and basophils that remained after neutrophil purification showed that NSS severely disrupted their cellular structure as well.

Initially, we attempted to study neutrophil function by examining stimulated calcium ion release and aggregation of neutrophils following exposure to NSS. It quickly became evident, however, that the NSS-exposed cells were already lysed and the data from these initial experiments were uninterpretable. It is not known if the few intact remaining neutrophils following NSS exposure continue to function normally, as we are unable to isolate these cells in the laboratory.

This study does not specifically address the clinical significance of long-term NSS use, and we are unaware of any other study that has examined the possible link between neutrophil lysis and clinical symptoms in the nose. Most patients derive significant benefit from the use of NSS, despite the presence of BKC. One possible reason for this disparity may be that the mechanical washing effect of the saline spray removes the lysed cells along with other debris. Another possible explanation is that mucosal proteins in the nose may bind to BKC and inactivate it. It can be argued that evaluating the effects of NSS on neutrophils from oral rinses is not the same as examining nasal mucosa neutrophils. We concede this point, and we used oral rinses in this study because this technique provides an abundance of neutrophils, which makes qualitative evaluation in our study design easier. This is the first study of which we are aware, however, that examines the effects of BKC on human neutrophils in vivo and that specifically evaluates the potential harmful adverse effects of NSS.

The widespread use of NSS and the common assumption that NSS is harmless necessitate a closer evaluation of its use and potential adverse effects. While there are few preservative-free corticosteroid or topical decongestant preparations available, saline for nasal irrigation can easily be made at home without potentially harmful additives. Patients should be counseled regarding the need to periodically make fresh saline preparations and methods to avoid contamination. Previous investigations have called for the elimination of BKC from medicinal preparations, and the need for alternative preservatives has been stated.[5] Recently, Cho et al[12] showed that corticosteroid preparations containing BKC or potassium sorbate, a common food preservative, caused inflammatory changes in rat nasal mucosa, although the potassium sorbate–induced changes were not as severe and improved with continued exposures. Interestingly, we have found that corticosteroid nasal spray preserved with potassium sorbate did not cause nearly as much neutrophil disruption as did corticosteroid sprays containing BKC (M.B., unpublished observations, 2000).

CONCLUSIONS

We conclude that multiuse preparations of NSS are toxic to human neutrophils and that substances used to preserve NSS, particularly BKC, are responsible for the toxicity. We believe that the lysis of neutrophils in the nasal mucosa may impair the cellular response to pathogenic organisms and lead to the release of inflammatory mediators, thus exacerbating the symptoms of rhinitis that NSS is intended to relieve. Homemade saline solutions and solutions without BKC appear to be safer alternatives. Alternative preservatives and novel methods of preservative-free packaging of aqueous nasal sprays should be sought. Finally, further studies are needed to assess the clinical significance of these laboratory findings.

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