The Laryngoscope
Lippincott Williams & Wilkins

Effects of a Low Concentration Hypochlorous Acid Nasal Irrigation Solution on Bacteria, Fungi, and Virus

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Objectives/Hypothesis: Saline irrigation would be more effective for chronic sinusitis patients if it had bactericidal effects. Low concentrations of hypochlorous acid may be used as a nasal irrigation solution. First, we developed a 0.85% NaCl solution by adding NaCl to tap water (pH 7.0 and 8.4) and measuring the concentration of free chlorine and hypochlorous acid after giving the solution a short electrical impulse of 20 seconds. Then we investigated whether the derived hypochlorous acid had a toxic effect on human primary nasal epithelial cells, if and what effect it had on the expression of mucin genes, and, finally, if it had bactericidal, fungicidal, or virucidal effects.

Study Design: In vitro biochemical experiment.

Methods: We treated human primary nasal epithelial cells with 3.5 ppm of hypochlorous acid and then examined the cells for cytotoxicity. We also investigated the bactericidal, fungicidal, and virucidal effects by challenging the cells with the following microorganisms *Aspergillus fumigatus, Haemophilus influenzae, Klebsiella pneumoniae, Rhizopus oryzae, Candida albicans,* Methicillin-resistant *Staphylococcus aureus, Streptococcus pneumoniae,* and *Streptococcus pyogenes.* To study the virucidal effects of HOCl, we used the human influenza A virus to challenge the cells.

Results: In the cytotoxicity assay and in the morphological examination, the cells did not show any toxicity at 30 minute or 2 hours after treatment with HOCl. More than 99% of bactericidal or fungicidal activity was noted for all species, except for *Candida albicans*, in tap water at either pH 7.0 or 8.4. In addition, a 3.2-$\log_{10}$ reduction was achieved in cells challenged with the human influenza A virus.

Conclusions: A low concentration HOCl solution can be used as an effective nasal irrigation solution.

Key Words: Hypochlorous acid, nasal irrigation.

***Laryngoscope,* 118:1862–1867, 2008**

INTRODUCTION

Mucociliary clearance is one of the most important host defense mechanisms and plays a critical role in protecting the human airways against external stimuli and infections.[1,2] Mucociliary clearance is primarily regulated by the amount and rheologic properties of the secreted mucin and by ciliary activity,[3,4] and defects in mucociliary clearance can result in the development or aggravation of various nasal diseases.

Saline nasal irrigation increases mucociliary clearance by facilitating the removal of nasal discharge, and also reduces mucosal inflammation by decreasing the number of bacteria, allergen, or fungi.[5] However, saline irrigation shows limited effectiveness in removing viscous mucus and bacteria, because mucin is hydrophobic and negatively charged. Therefore, we thought that saline irrigation would be more beneficial to chronic sinusitis patients if it were to have bactericidal effects.

Hypochlorous acid (HOCl) is a weak acid that forms when chlorine dissolves in water. In the human body, hypochlorous acid is generated in activated neutrophils by myeloperoxidase-mediated peroxidation of chloride ions, and contributes to the destruction of bacteria.[6] Therefore, we focused on the fact that low concentrations of hypochlorous acid can be produced by the electrolysis of NaCl dissolved in tap water. First, we developed a 0.85% NaCl solution by adding NaCl to tap water (pH 7.0 and 8.4) and measured the concentration of free chlorine and hypochlorous acid after giving the solution a short electrical impulse of 20 seconds. Then we investigated whether the derived hypochlorous acid

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Editor's Note: This Manuscript was accepted for publication May 8, 2008.

Supported by a KOSEF SRC grant funded by the Korea government (MOST) (R11-2007-040-02001-0).

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DOI: 10.1097/MLG.0b013e31817f4d34

had a toxic effect on human primary nasal epithelial cells, if and what effect it had on the expression of secretory mucin genes and finally, whether it had bactericidal, fungicidal, or virucidal effects.

MATERIALS AND METHODS

Production of Free Chlorine and HOCl Using the Salicid Device (Dolki Ltd., Korea)

Test material was generated in situ using the Salicid devices in conjunction with the Salicid Packets containing 315 mg NaCl. The three devices were randomly used for each of the eight challenge microorganisms and overall, each of the three Salicid devices was tested with each of the challenge microorganisms. For each test, one Salicid packet was added to the test device containing 35 mL of sterile tap water (pH 7.0 or 8.4). The device was shaken 10 times and then the generation button was activated and once the green indicator light was on, the device was used for 20 seconds. Once the light was activated, the device was shaken two to three times and the product was immediately dispensed for testing.

Cell Culture

Passage-2 normal human nasal epithelial (NHNE) cells (1×10^5 cells/culture) were seeded in 0.5 mL of culture medium onto 24.5-mm, 0.45-μm pore Transwell clear culture inserts (Costar, Cambridge, MA). Cells were cultured using a 1:1 mixture of bronchial epithelial cell growth medium and Dulbecco's modified Eagle's medium containing all supplements. Cultures were grown submerged for the first 9 days, during which time the culture media was changed on the first day and every other day thereafter. An air-liquid interface was created on the ninth day by removing the apical medium and feeding the cultures only from the basal compartment. The culture media was changed daily after creating the air-liquid interface.[7] Two weeks after confluence, the NHNE cells were treated with the test material for 30 minutes or 2 hours.

Cytotoxicity Test in Cultured Human Primary Nasal Epithelial Cells

Cytotoxicity was evaluated by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded at a density of 9.6×10^3 cells in 100 μL media into each well of 96-well plate, and incubated in a humidified atmosphere at 37°C, 5% CO_2 for 24 hours. Two microliters of 3.5 ppm HOCl solution dissolved in dimethyl sulfoxide was added to each well and incubated for 24 hour to allow the drug to take effect. Ten microliters of a MTT solution was added to each well and incubation was continued at 37°C for 4 hour to allow the MTT to be metabolized. Thereafter, the media was removed and the cells were lysed in 200 μL dimethyl sulfoxide per each well to dissolve the formazan by shaking. The optical density (OD) of each well was determined using an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm to obtain sample signal (OD570–OD630).

Morphological Examination

To examine changes in cell morphology, 30 minute after HOCl treatment and 2 hours after HOCl treatment, the NHNE cells were fixed in 10% buffered neutral formalin, embedded in paraffin and sectioned. The cells were then stained with hematoxylin-eosin. For scanning electron microscopic studies, the treated cells on the porous membrane were fixed with chilled 2.5% glutaraldehyde for 4 to 6 hour and washed with 0.1 mol/L phosphate buffer saline. The cells were postfixed with 1% osmium tetroxide for 2 hours. Specimens were observed using a scanning electron microscope (H-800; Hitachi, Japan).

Reverse Transcriptase-Polymerase Chain Reaction for Secretory Mucins

Oligonucleotide primers for secretory mucins were designed based on the sequences for the mucin genes (Table I). Oligonucleotide amplimers for β2-microglobulin (β2M; Clontech Laboratories, Palo Alto, CA) were used as a control and generated a 335-bp polymerase chain reaction (PCR) fragment. Reverse transcriptase-PCR reactions were performed using a Cetus DNA thermal cycler (Perkin-Elmer Biosystems, Foster City, CA) according to the manufacturer's recommendations. Annealing was performed for 1 minute at 58°C for *MUC2,* 60°C for *MUC5AC* and β2-microglobulin, and 55°C for *MUC5B, MUC6, MUC7,* and *MUC8*. We used comparative kinetic analysis to compare the mRNA levels for each gene under each set of culture conditions, as described previously. PCR products (*MUC2,* 438 bp; *MUC5AC*, 337 bp; *MUC5B*, 415 bp; *MUC6*, 293 bp; *MUC7*, 209 bp; *MUC8*, 239 bp) were separated by electrophoresis on a 2% Seakem agarose gel (FMC, Rockland, ME) containing 50 ng/mL ethidium bromide and were photographed with Polaroid type 55 film. Each experiment was repeated three times.

TABLE I.
Primers Used for the Experiments.

Gene Name	Primer Sequences (5′–3′)	Amplicon (bp)	Accession Number
MUC2	Forward: TGC CTG GCC CTG TCT TTG	438	NM_002457
	Reverse: CAG CTC CAG CAT GAG TGC		
MUC5AC	Forward: CGA CAA CTA CTT CTG CGG TGC	337	AJ001402
	Reverse: GCA CTC ATC CTT CCT GTC GTT		
MUC5B	Forward: CTGCGAGACCGAGGTCAACATC	415	NM_002458
	Reverse: TGGGCAGCAGGAGCACGGAG		
MUC6	Forward: TCA CCT ATC ACC ACA CAA C	293	NM_005961
	Reverse: GGA GAA GAA GGA AAA AGA G		
MUC7	Forward: CCA CAC CTA ATT CTT CCC	209	NM_152291
	Reverse: CTA TTG CTC CAC CAT GTC		
MUC8	Forward: ACA GGG TTT CTC CTC ATT G	239	U14383
	Reverse: CGT TTA TTC CAG CAC TGT TC		

Bactericidal, Fungicidal, and Virucidal Test

The results of the time kill test were obtained by request from MICROBIOTEST (Sterling, VA). This test was inspected by the quality assurance unit of MICROBIOTEST in compliance with current Good Laboratory Practice regulations. Test conditions included the following challenge microorganisms *Aspergillus fumigatus* (ATCC 36607), *Haemophilus influenzae* (ATCC 19418), *Klebsiella pneumoniae* (ATCC 11296), *Rhizopus oryzae* (ATCC 34965), *Candida albicans* (ATCC 10231), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), *Streptococcus pneumoniae* (ATCC 6304), and *Streptococcus pyogenes* (ATCC 19615). Each challenge organism was confirmed by gram stain or wet mount procedure and colony morphology. All sterility controls exhibited no growth. The active ingredient in the generated test material was hypochlorous acid and the neutralizer used was 0.85% NaCl containing 0.5% $Na_2S_2O_3$. Contact time was 30 seconds and temperature was 21°C. Percent reduction was calculated using the following equation.

$$\text{Percent reduction (\%)} = \frac{\text{Average initial counts control} - \text{test results}}{\text{Average initial counts control}} \times 100$$

Concerning the virucidal test, the challenge microorganism was human influenza A virus (A/Hong Kong 8/68-H3N2). Embryonated chicken eggs were used as the host. The stock virus was prepared in RPMI 1640 containing 10% fetal bovine serum. The stock virus was diluted 1:100 in sterile phosphate-buffered saline to achieve ≤0.1% serum. We checked the virus infected embryos using the hemagglutination reaction. The active ingredient in the generated test material was hypochlorous acid and the neutralizer was fetal bovine serum containing 0.5% $Na_2S_2O_3$. Contact time was 30 seconds and temperature was 25°C. The 50% embryo infectious/lethal dose per milliliter was determined using the Spearman-Karber method using the following formulas:

$$m = x_k + (d/2) - d\sum p_i$$

m = the logarithm of the titer relative to the test volume; x_k = the logarithm of the smallest dosage which induces infection in all cultures; d = the logarithm of the dilution factor; p_i = the proportion of positive result at dilution i.

The values were converted to 50% embryo infectious/lethal dose per milliliter using sample inoculums of 0.20 mL.

RESULTS

Generation of Free Chlorine and Hypochlorous Acid

Applying the methods described earlier, HOCl solutions were generated from two sources of sterile tap water with a pH of 7.0 or 8.4. Concentrations of free chlorine (HOCl + sodium chloride) and HOCl were measured immediately after the experiment and then 5, 10, 20, 30, and 40 minutes later. Experiments were repeated three times for each pH and the average value and SD of the results were calculated. The concentration values of free chlorine in sterile tap water of pH 7.0 immediately after the experiment and at 5, 10, 20,30, and 40 minutes later were 3.23 ± 0.12, 3.13 ± 0.06, 2.9 ± 0.26, 2.8 ± 0, 2.17 ± 0.06, and 1.63 ± 0.25 (unit: ppm), respectively. As for the concentrations of HOCl, the results were 2.26 ± 0.12, 2.2 ± 0, 2.03 ± 0.21, 2 ± 0, 1.5 ± 0, and 1.13 ± 0.15 (unit: ppm), respectively (Fig. 1A).

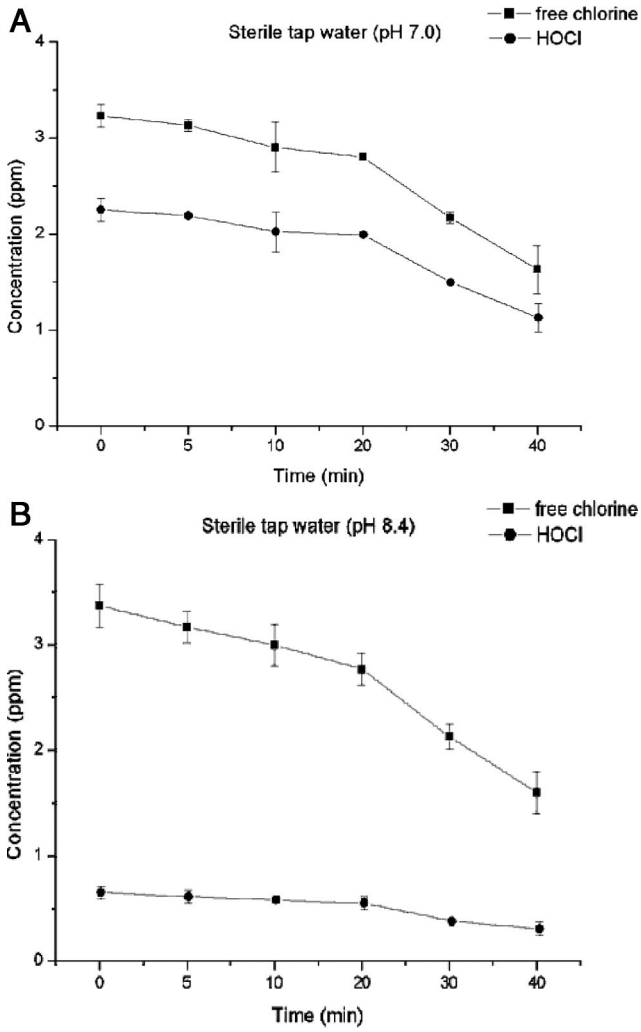

Fig. 1. Generation of free chlorine and HOCl in solution with pH 7.0 tap water (A) and pH 8.4 tap water (B).

Next, for the pH 8.4 sterile tap water, the free chlorine values were 3.37 ± 0.21, 3.17 ± 0.15, 3 ± 0.2, 2.77 ± 0.15, 2.13 ± 0.12, and 1.6 ± 0.2 (unit: ppm), respectively, and the HOCl values were 0.67 ± 0.06, 0.63 ± 0.06, 0.6 ± 0, 0.57 ± 0.06, 0.4 ± 0, and 0.33 ± 0.06 (unit: ppm), respectively (Fig. 1B).

An approximate amount of less than 3.3 mg/L of free chlorine was produced immediately after the experiment. In addition, the concentration of HOCl was found to be relatively lower in alkaline pH and the concentration decreased with time. Additionally, the minimal amounts of ozone and hydrogen peroxide that should be produced were not detected in this experiment because the amount of hypochlorous acid produced was relatively higher than the amounts of ozone and hydrogen peroxide.

Cytotoxicity and Morphological Changes in NHNE Cells After Treatment With HOCl

In cytotoxicity assay, cells did not show any toxicity at 30 minute or 2 hours after treatment with HOCl (Fig. 2). In addition, we used scanning electron and light mi-

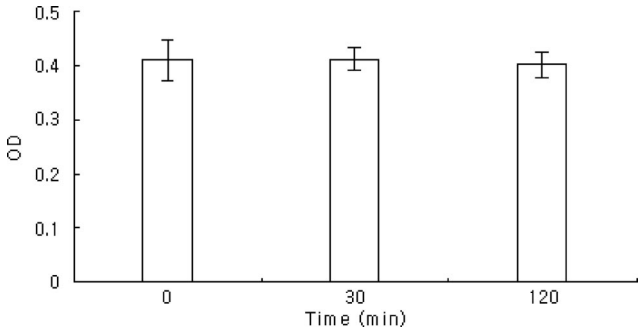

Fig. 2. Cytotoxicity assay in human primary nasal epithelial cells did not show any differences when compared with control.

croscopy to examine morphological changes in NHNE cells after treatment with 3.5 ppm of HOCl. In the control study (before treatment), NHNE cells were healthy, had well-differentiated cilia, and the cell-to-cell integrity was maintained (Fig. 3A). Thirty minutes after treatment, we noted well-differentiated cilia and well-maintained cell-to-cell integrity (Fig. 3B). Furthermore, even at 2 hours after treatment no morphological changes were observed (Fig. 3C) in comparison with the control (Fig. 3A).

Effect of HOCl on Expression of Secretory Mucin Genes in Normal Human Nasal Epithelial Cells

In a comparison of the expression of the *Muc2, Muc5B, Muc5AC, Muc6, Muc7, Muc8,* and *β2 microglobulin* genes before treatment and 30 minutes and 2 hours after treatment, we saw no difference in gene expression when we used 3.5 ppm of HOCl. From this observation, we speculate that the mRNA levels of the secretory mucin genes are not affected by HOCl treatment (Fig. 4).

Results of the Bactericidal, Fungicidal, and Virucidal Tests

As a result of bactericidal testing against *A. fumigatus* (ATCC 36607), *H. influenzae* (ATCC 19418), *K. pneumoniae* (ATCC 11296), *R. oryzae* (ATCC 34965), *C. albicans* (ATCC 10231), MRSA (ATCC 33591), *S. pneumoniae*

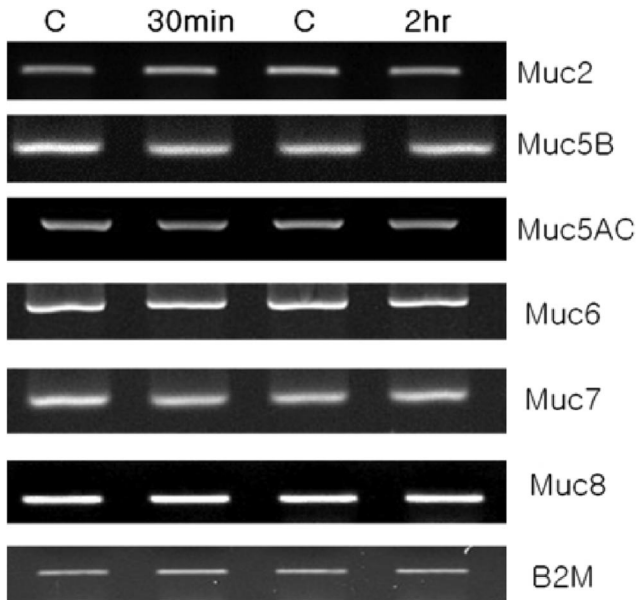

Fig. 4. mRNA expression levels of secretory mucin genes at 30 minute and 120 minute after exposure to 3.5 ppm of HOCl solution.

(ATCC 6304), and *S. pyogenes* (ATCC 19615), we found more than 99% of bactericidal activity in all species except *C. albicans* in both pH 7.0 and 8.4 environment (Table II and III). In particular, a 6-$\log_{10}$ reduction was observed for *K. pneumoniae*. An approximately 3.2-$\log_{10}$ reduction was achieved with the influenza virus.

DISCUSSION

HOCl is a major component of bleaching agents or detergents and is also commonly used to disinfect tap or swimming pool water because of its strong sterilizing power.[8,9] In vivo, it is produced by the action of H_2O_2 and neutrophil-derived myeloperoxidase and exhibits a strong antibacterial activity as a potent toxic or oxidizing agent.[6,10,11]

HOCl can be produced in many ways, and in this experiment we produced HOCl with the Salicid device,

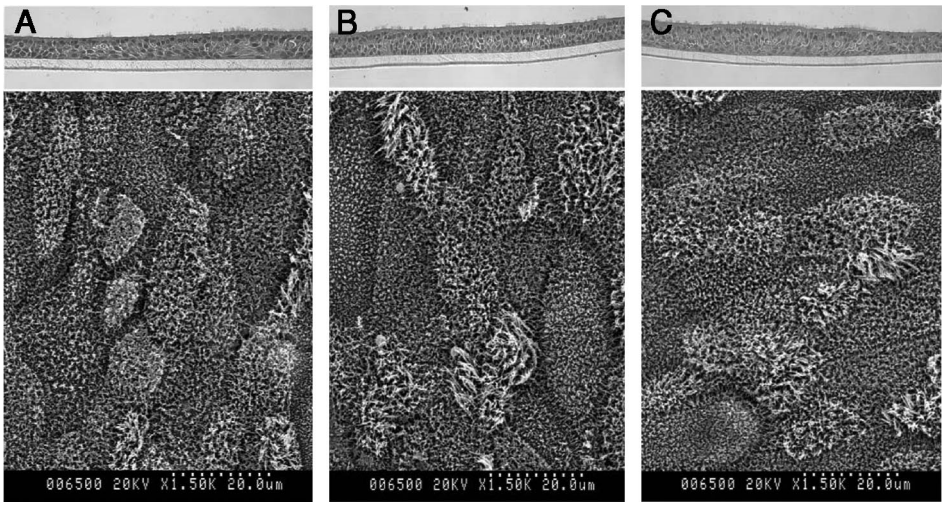

Fig. 3. Microscopic and electromicroscopic features before exposure (A), 30 minute after exposure (B), and 120 minute after exposure (C); Normal human nasal epithelial cells were healthy with many well-differentiated cilia. No differences were seen between groups.

TABLE II.
Results of Bactericidal Test in pH 7.0 Sterile Tap Water.

Microorganism	Initial CFU/mL	Replicate 1		Replicate 2	
		CFU/mL Recovered	Percent Reduction	CFU/mL Recovered	Percent Reduction
Staphylococcus aureus (MRSA)	5.2×10^7	2.0×10^4	99.96	1.8×10^4	99.97
Streptococcus pneumoniae	8.9×10^5	4.0×10^3	99.55	3.9×10^3	99.56
Streptococcus pyogenes	9.6×10^5	4.1×10^3	99.57	4.3×10^3	99.55
Haemophilus influenzae	8.0×10^5	4.0×10^3	99.5	4.4×10^3	99.45
Klebsiella pneumoniae	7.5×10^7	1.0×10^1	99.99	2.0×10^1	99.99
Aspergillus fumigatus	1.1×10^7	3.4×10^4	99.69	3.3×10^4	99.7
Rhizopus oryzae	9.2×10^7	3.2×10^4	99.97	3.2×10^4	99.97
Candida albicans	1.7×10^6	6.2×10^4	96.35	5.3×10^4	96.88

which produces HOCl through the electrolysis of a salt solution as shown in the following reaction.[11]

$$\text{a) } 2Cl^- + 2e^- \leftrightarrow Cl_2$$

$$\text{b) } Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^-$$

HOCl is expressed in different forms under different pH conditions, such as Cl_2(aq), Cl_2(gas), and OCl^-. HOCl is most stable in its highest amount under pH 3 to 6. Under a lower pH environment, the amount of Cl_2(aq) increases, and in the more alkaline pH environment, the amount of OCl^- increases so that under pH 7.5, the amount of HOCl and OCl^- becomes 50:50.[11] According to the results of previous experimental studies, OCl^- showed relatively lower bactericidal activity than HOCl, and so to maintain stronger bactericidal activity, the HOCl solution was used under an acidic pH environment.[9] However, strongly acidic HOCl gradually loses its antibacterial effect over long-term preservation and therefore problems of long-term storage and safety measures due to its highly corrosive character have been noted.

First, we knew that free chlorine is produced with sterile tap water of pH 7.0 or 8.4 and NaCl, and the result is approximately 3.3 ppm of HOCl in solution. This concentration is within the World Health Organization safety standard for drinking water of 5 mg/L.[12] In addition, we found out that the concentration of HOCl was lower in alkaline pH than in neutral pH.

Additionally, using light and electron microscopy, we did not observe any morphological changes in HOCl-treated human primary nasal epithelial cell and our analysis of mucin gene expression revealed that mucin gene RNA levels are also not affected by HOCl treatment. As a result, we confirmed that the HOCl solution does not have cytotoxic or gene-altering effects. In an earlier studies, HOCl or sodium hypochlorite were reported to have no teratogenic or carcinogenic effects, so the concentration of HOCl used in this study is relatively safe for the human body.[8,12]

As the pH of tap water varies according to region, we used two sources of tap water with a pH of either 7.0 or 8.4 to produce the HOCl solution used in our experiments. In our analysis of bactericidal effects against *A. fumigatus* (ATCC 36607), *H. influenzae* (ATCC 19418), *K. pneumoniae* (ATCC 11296), *R. oryzae* (ATCC 34965), *C. albicans* (ATCC 10231), MRSA (ATCC 33591), *S. pneumoniae* (ATCC 6304), and *S. pyogenes* (ATCC 19615), we found that more than 99% of bactericidal activity was noted in all species, except for *C. albicans*, in both pH 7.0 and 8.4 water. In particular, a 6-$\log_{10}$ reduction was observed for *Klebsiella pneumonia* (Table II and III). In addition, an

TABLE III.
Results of Bactericidal Test in pH 8.4 Sterile Tap Water.

Microorganism	Initial CFU/mL	Replicate 1		Replicate 2	
		CFU/mL Recovered	Percent Reduction	CFU/mL Recovered	Percent Reduction
Staphylococcus aureus (MRSA)	5.0×10^7	3.0×10^4	99.94	3.0×10^4	99.94
Streptococcus pneumoniae	8.6×10^5	4.3×10^3	99.5	5.1×10^3	99.4
Streptococcus pyogenes	9.8×10^5	5.2×10^3	99.47	5.7×10^3	99.42
Haemophilus influenzae	7.8×10^5	5.6×10^3	99.28	4.5×10^3	99.42
Klebsiella pneumoniae	7.4×10^7	3.5×10^1	99.99	1.0×10^1	99.99
Aspergillus fumigatus	1.0×10^7	3.0×10^4	99.7	3.0×10^4	99.7
Rhizopus oryzae	9.1×10^7	3.0×10^4	99.97	3.0×10^4	99.97
Candida albicans	1.6×10^6	7.2×10^4	95.5	6.3×10^4	96.06

approximately 3.2-$\log_{10}$ reduction was achieved when the cells were challenged with Influenza virus, thereby proving the virucidal activity of HOCl.

Landa-Solis et al. used a super-oxidized water (SOW) solution containing 20 ppm of HOCl under neutral pH that was produced from the electrolysis of NaCl solution using purified water and they reported that the SOW shows over 99.99% bactericidal and fungicidal activity after 30 second contact with *E. coli, S. aureus, P. aeruginosa, S. typhi,* and *C. albicans*. In addition, they observed complete inactivation of HIV-1, and a 3-$\log_{10}$ reduction in adenovirus after 5 minutes of contact and complete inactivation after 10 minutes.[9] Accordingly, they suggested that SOW could be an effective and safe disinfectant in hospital practice. However, even though it was found to be effective as a disinfectant, the concentration of HOCl is SOW was too high, making it impossible to use as a nasal irrigation solution in humans.

Recently, Chiu et al.[13] reported that 1% baby shampoo in normal saline improved symptoms of thickened nasal discharge and postnasal drip in 60% of postoperative endoscopic sinus surgery patients through the inhibition of *Pseudomonas* biofilm formation, but not by eradication of preformed *Pseudomonas* biofilms.

In conclusion, we can suggest that a nasal irrigation solution containing HOCl at ppm less than 3.5 has no toxic effect on nasal mucosa, is safe to drink, and has over a 99% bactericidal effect on numerous pathogens, making it a beneficial irrigation solution for patients with various nasal diseases. However, we did find that the concentration of HOCl and free chlorine deteriorates with time, which means it may be critical to maintain the appropriate application time for maximal effect. In addition, we used tap water in our experiment and therefore, additional evaluation is necessary to determine whether small minute compounds were produced during the electrolysis process that might have mediated the observed effects.

On the other hand, Feldman et al.[14] have demonstrated a statistically significant slowing of the ciliary movement and ciliary dyskinesia when the concentration of HOCl is above 100 μM (approximately 5.25 ppm). Morris et al.[15] measured the change in airway resistance of a mouse after exposure to chlorine gas and sodium hypochlorite aerosol (approximately 0.8 ppm), and found a significant increase in airway resistance.

Therefore, even though we use a low concentration of HOCl in our nasal irrigation solution, further studies are necessary to study the effects of HOCl on ciliary dysfunction and nasal obstruction.

CONCLUSION

In this study, we demonstrate that a low concentration HOCl solution has bactericidal effects without any cytotoxicity. Therefore, rather than using the traditional normal saline irrigation, bactericidal HOCl solution should be used for nasal irrigation and is expected to produce better clinical outcomes. However, further studies are required to study the toxicity and other effects of HOCl solution in the human body.

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