The effect of a low concentration of hypochlorous acid on rhinovirus infection of nasal epithelial cells

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ABSTRACT

Background: Low concentrations of hypochlorous acid (HOCl) have been shown to exhibit both antibacterial and anti-influenza virus activity, but HOCl still has not been used to kill human rhinovirus (HRV). To model the antiviral effect of nasal irrigation with low-level HOCl in patients with the common cold, we tested the effects of a low concentration of HOCl on HRV infection of primary human nasal epithelial cells (HNECs).

Methods: Cells were infected with HRV for 24 hours and treated with HOCl three times, for 5 minutes each time, at 12-hour intervals. The effects of HOCl on rhinovirus-induced secretion of IL-6 and IL-8 were assessed by ELISA and HRV replication was determined by viral titration.

Results: HOCl treatment significantly inhibited HRV-induced secretion of IL-6 and IL-8 and significantly reduced viral titer. The effects of HOCl peaked at 1 minute after HOCl generation and decreased thereafter.

Conclusion: These in vitro findings indicate that nasal irrigation with low-level HOCl solution may improve clinical symptoms in patients with the common cold.

Keywords: Hypochlorous acid, nasal irrigation, human rhinovirus, primary human nasal epithelial cells

Methods

Production of Low-Concentration HOCl

A low-concentration HOCl solution was generated in situ as previously described, using Salicid devices (Dolki, Ltd., Seoul, Korea) in conjunction with Salicid packets containing 315 mg of NaCl. One Salicid packet was added to the test device, which contained 35 mL of tap water at pH 7.0, yielding a 0.85% (w/v) NaCl solution. The concentration of HOCl in solution was approximately 3.5 ppm immediately after production. The osmolarity of the solution was 3.54 Osm/L.

Cell Culture

Cell cultures of mucosal specimens and infection of cells with HRV were performed as previously described. To obtain primary HNEC, the inferior turbinates of 12 patients were removed during turbinoplasty and digested with 0.1% (w/v) dispase for 24 hours at 37°C in airway epithelial cell growth medium. The cells were centrifuged twice at 1200 rpm for 5 minutes. Subsequently, cells were washed with PBS and seeded at a density of 1.5 × 10⁴ cells/mL into 24-well dishes. Cells were grown in fully humidified air containing 5% (v/v) CO₂ at 37°C. This study was approved by the Institutional Review Board of the Asan Medical Center.

Test for Cytotoxicity of HOCl

Cytotoxicity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Cells were seeded at 1 × 10⁵ cells/well in 96-well flat-bottom plates (Corning, Inc., New York, NY). The cells were precultured for 24 hours before the addition of the HOCl. The HOCl was added to give a final volume of 100 µL/well. Cells were treated with 1 mL of HOCl solution at 1, 5, or 20 minutes after production of HOCl three times for 5 minutes each every 12 hours. The concentration of HOCl presented in the solution at 1, 5, and 20 minutes after production were 3.5, 3.3, and 2.9 ppm, respectively. The MTS assay was developed after 48 hours of incubation. The optical density of the samples was measured at 570 nm using an automatic microplate reader (Model 550; Bio-Rad, Hercules, CA). Cells treated with medium alone served as a control.

Measurement of Transepithelial Resistance (TER)

To evaluate the effect of HOCl treatment on barrier function of nasal epithelium, functional changes in the integrity of junctional proteins of nasal epithelium were determined by measuring TER in primary human differentiated mucociliary nasal epithelial cells after a 2-day HOCl treatment. TER is a widely used indicator of permeability in vitro. Cells were cultured to confluence on the inner chambers of 12-mm Transwell inserts with 0.4-µm (pore size) filters (Corning Inc., New York, NY). TER was measured using an EVOM voltmeter with an ENDOHM-12 electrode (World Precision Instruments, New Haven, CT). The values for cell-covered filters are expressed in standard units of ohms per square centimeter after sub-

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tracting the resistance of blank fillers and are presented as the mean ± SD. Cells treated with medium alone served as a control.

**Saccharine Test**
To evaluate the effect of HOCl treatment on nasal mucociliary clearance, a saccharine test was performed in 12 healthy volunteers (aged 21–34 years). This population had normal nasal endoscopic findings, no history of allergic rhinitis, or recent upper respiratory tract infection. A saccharine test was performed on two occasions for each volunteer, one before the treatment with HOCl solutions 1 minute after HOCl production and the other 2 hours after the HOCl treatment. The saccharine test was done by placing a 1.0-mm particle of a commercially available saccharine tablet approximately 1 cm behind the anterior border of the inferior turbinate. The time elapsing until the first experience of a sweet taste at the posterior nasopharynx is recorded as the saccharine transit time (STT) in minutes.11

**HRV-16 Infection and HOCl Treatment**
HRV-16 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Viral stocks were prepared by infection of susceptible cell monolayers (Ohio Hep cells), as described previously.12 To assess the direct effects of HOCl on HRV, 1 × 10^6 HRV was mixed with 1 mL of medium or 1 mL of HOCl solution at 1, 5, or 20 minutes after production of HOCl, and viral titers were measured. The 1, 5, and 20 time points were set up to check the influence of elapsed time after initial HOCl generation, because the actual concentration of HOCl would decrease gradually after production.

To infect HNecs with HRV, HNecs (2 × 10^5 cells/well) were plated in 12-well plastic tissue culture plates (Costar, Cambridge, MA) in F-12K Nutrient Mixture ( Gibco, Carlsbad, CA) supplemented with 10% (v/v) FBS and infected with HRV-16 at a multiplicity of infection of 1 for 4 hours at 33°C. The cells were subsequently washed with PBS and incubated at 33°C. Twenty-four hours after infection, aliquots of cells were incubated with HOCl solutions at 1, 5, or 20 minutes after HOCl production, three times, for 5 minutes each time, every 12 hours, to simulate nasal irritation in a real clinical setting, and washed with PBS immediately after each HOCl treatment. As a control group, HRV-infected cells were cultured at 33°C for 2 days without HOCl treatment. Viral titers were measured in the PBS wash supernatants. Culture media were decanted at the end of each experiment for measurement of IL-6 and IL-8.

**Measurement of Viral Titer**
Medical Research Council-5 human lung fibroblasts were cultured at 2 × 10^5 cells/well in 96-well microplates (Falcon Labware, Oxnard, CA) in minimum essential medium (GIBCO). On an experimental day, 125 µL of medium was removed from each well, 25-µL aliquots of serial 10-fold dilutions of each viral suspension were added, and the plates were incubated at 33°C for 7 days. Viral cytopathic effects were examined using an inverted microscope. The amount of suspension required to infect 50% of Medical Research Council-5 cells (50% tissue culture infection dose [TCID50]) was determined, and each viral titer was expressed as TCID50/mL.

**Quantification of IL-6 and IL-8**
Immunoreactive IL-6 and IL-8 were quantitated using dual-antibody ELISA kits (Biosource, Nivelles, Belgium) according to the manufacturer’s protocol. The sensitivity limit of each kit was 10 pg/mL. Data were expressed in picograms per milliliter and were derived by extrapolation from a standard curve generated in parallel with each assay.

**Statistical Analysis**
Differences between groups were analyzed using the paired Student's t-test and Mann-Whitney U test. All data are reported as means ± SDs. A value of p < 0.05 was considered statistically significant.

**RESULTS**

**Cytotoxicity and TER in HNecs after Treatment with HOCl**
In cytotoxicity assay, cells did not show any toxicity at 1, 5, and 20 minutes after treatment with HOCl (Fig. 1). In addition, we used TER to evaluate the effect of HOCl solution on barrier function in HNecs. The mean TER of the control group was 249.8 ± 17.6 Ω/cm², whereas mean TER in HNecs with HOCl solution at 1, 5, and 20 minutes after HOCl production were 259.8 ± 26.1, 251.5 ± 20.5, and 244.8 ± 24.5 Ω/cm², respectively. There was no significance difference between control group and HOCl treatment groups (p > 0.05; Fig. 2).

**Saccharine Test**
The mean STTs in before and after HOCl treatment were 9.8 ± 2.3 and 10.3 ± 1.7 minutes, respectively. The mean STT of our volunteers was not significantly different between before and after treatment with HOCl solution (p > 0.05; Fig. 3).

**Viral Titer**
The mean viral titer in the HRV suspension in media alone was 5.0 log TCID50/mL, whereas mean viral titers in viral suspension treated
with HOCl solution at 1, 5, and 20 minutes after HOCl production were 1.0, 2.3, and 4.0 log TCID₅₀/mL, respectively (Fig. 4). The mean viral titers in the 1- and 5-minute HOCl groups were significantly lower than that of the HRV group ($p < 0.05$), but the difference between the 20-minute HOCl group and the HRV control group was not significant. Moreover, antiviral effects were significantly lower in the HOCl 5- and 20-minute groups than in the HOCl 1-minute group.

The mean viral titers in supernatants of primary HNECs infected with HRV was 3.58 log TCID₅₀/mL. The mean viral titers in supernatants of cells incubated with HOCl solution at 1, 5, and 20 minutes after HOCl production were 1.45, 1.70, and 2.14 log TCID₅₀/mL, respectively (Fig. 5), all significantly lower than in the control HRV group ($p < 0.05$). Moreover, HOCl the 1-minute group showed the biggest antiviral effect in the HOCl treatment groups.

**Secretion of IL-6 and IL-8**

The mean concentration of IL-6 was significantly higher in the supernatant of HRV-infected cells than in control cells (339.9 ± 25.4 pg/mL versus 155.2 ± 13.9 pg/mL, $p < 0.05$; Fig. 6). However, treatment of infected cells with HOCl solution 1 and 5 minutes after HOCl generation significantly reduced the concentration of IL-6 compared with control HRV-infected cells (210.7 ± 21.6 pg/mL versus 339.9 ± 25.4 pg/mL, $p < 0.05$; 288.0 ± 23.7 pg/mL versus 339.9 ± 25.4 pg/mL, $p < 0.05$, respectively). The IL-6 concentration in the supernatant of infected cells treated with HOCl 20 minutes after HOCl production was somewhat lower than that in the supernatant of untreated infected cells (308.5 ± 20.9 pg/mL versus 339.9 ± 25.4 pg/mL), but the difference was not significant.
HRV infection has been shown to increase the expression and secretion of various cytokines, including IL-1β, IL-6, IL-8, IL-9, IL-11, and tumor necrosis factor α.20–22 The effects of these cytokines, either individually or in combination, likely underlie the clinical manifestations of HRV infection.23 IL-6 plays an important role in the development of common cold symptoms, by inducing pyrexia.24 IL-8 is a strong chemoattractant for neutrophils and can also activate recruited neutrophils, resulting in the release of cytotoxic granule contents.24 HRV is a potent stimulator of both IL-6 and IL-8 production, and there is substantial evidence for a direct correlation between IL-6 and IL-8 levels and symptom severity in experimental HRV infections.25 Our finding that HOCl inhibits virally induced IL-6 and IL-8 production suggests that HOCl treatment can relieve the symptoms of HRV infection.

To our knowledge, there have not been any in vitro human or animal safety data using HOCl solution as a nasal irrigation. Kim et al. reported that a low concentration HOCl solution did not show any cytotoxicity or morphological changes in normal HNECs in vitro experimental setting.26 To evaluate the effect of HOCl solution on mucociliary clearance and barrier function of nasal epithelium, we performed saccharine test and measured TER. Our results showed that low-concentration HOCl was expected to have no significant effect on these functions of nasal epithelium.

Our study had several limitations. First, because all experiments were performed in vitro, additional work is required to evaluate the effects of HOCl in the human respiratory epithelium. Second, our use of tap water to generate HOCl may have introduced impurities during electrolysis, which, although low in concentration, could conceivably have affected our results. Third, growing the cells in submersion would force dedifferentiation of the cells. A better model would have been the air-liquid interface cultures. Finally, there has not been any research on the effect of HOCl nasal irrigation on olfaction. Despite these limitations, however, our findings suggest that HOCl may have beneficial effects when used to treat the HRV-induced common cold, findings that should be further substantiated in well-designed clinical trials.

CONCLUSION

We have shown that low-concentration HOCl inhibited the HRV-induced secretion of IL-6 and IL-8 from, and viral replication in, HNECs. These effects decreased over time after initial production of HOCl solution. The findings suggest that nasal irrigation with HOCl solution may relieve symptoms of the common cold.

REFERENCES


