Efficacy of a seawater solution enriched with copper, hyaluronic acid and eucalyptus against nasal pathogens*

Song Huang¹, Samuel Constant¹, Barbara De Servi², Marisa Meloni², Josip Culig³, Amina Saaid⁴

¹ Epithelix, 18 chemin des Aulx CH-1228 Plan-Les-Ouates, Geneva, Switzerland
² Vitroscreen, Via Mosè Bianchi, 103 20149, Milan, Italy
³ University of Applied Health Sciences, Minamsa cesta 38, 10000, Zagreb, Croatia
⁴ Laboratoire Fumouze, 110-114 rue Victor Hugo, 92686, Levallois-Perret, France

Abstract
Background: Common cold is the most common disease which mainly affects the upper respiratory system. It is caused by viral or, in a small percentage, by bacterial infections. Current therapy options focus on symptomatic relief of the disease such as nasal saline irrigation, an easy-to-apply, comfortable, non-toxic method. In this study, a novel hypertonic seawater solution enriched with hyaluronic acids, eucalyptus oil, copper and manganese salts, Stérimar Stop & Protect Cold and Flu (SSPCF), has been investigated with respect to efficacy against viral and microbial infections.

Methodology: An in vitro 3D reconstituted human nasal epithelium tissue model, MucilAir™, has been used in this study. Pretreatment and post-treatment anti-viral effects of SSPCF was measured through HRV-A16 viral load assays in order to evaluate the preventive and therapeutic efficacy of SSPCF, respectively. Anti-bacterial effects of SSPCF was assessed via Staphylococcus (S.) aureus growth inhibition and fluorescence bead-based phagocytosis assays.

Results: One-hour SSPCF treatment pre- or post-viral infection inhibited the viral replication up to 99.78 and 59.91%, respectively. S. aureus growth was completely eliminated (100%) in SSPCF treated tissues after 1 hour of treatment. Phagocytosis rate was 3.28 folds higher in SSPCF treated tissue as compared to saline treated controls.

Conclusions: Under the conditions of this in vitro study, SSPCF appears effective against some species of rhinoviruses (as in common cold) and S. aureus in vitro.

Key words: hypertonic seawater, nasal irrigation, Staphylococcus aureus, common cold, rhinovirus, HRV-A16

Introduction
Common cold (viral acute rhinosinusitis) is an upper respiratory tract infection which primarily affects the nasal respiratory mucosa and mainly causes nasal blockage/obstruction/congestion for a duration of less than 10 days (1). Children and adults are estimated to suffer from up to 5 and 3 colds per year, respectively (2), which also causes time-off from school or work, translating to an economic and social burden (3). Rhinovirus infections are the main cause of common cold cases and lead to epithelial barrier dysfunction without causing cell death and enhance the invasion of bacteria which may cause bacterial superinfections (4-6). When common cold symptoms worsen after 5 days or persist for more than ten days up to 12 weeks, the disease is called post-viral acute rhinosinusitis. A small subgroup of the post-viral rhinosinusitis (estimated around 0.5-2.0%) (7) is caused by bacteria (mainly S. pneumoniae, H. influenzae, M. catarrhalis and S. aureus) to develop acute bacterial rhinosinusitis (8).

Treatment options for common cold concentrate around symptomatic relief (8), among which, nasal irrigation (NI) with

Poster Presentation: The outcomes of this work have been presented as a poster at the “27th Congress of the European Rhinologic Society” in London (United Kingdom), on April 2018.
saline solution is recommended as a safe, easy-to-use, comfortable and not-expensive method. In addition, NI has been shown to, at least partially, remove viruses and inflammatory mediators, and inhibit viral replication in the nasal cavities in upper respiratory tract infections. Saline NI applications also have been shown to decrease sinus medicine use in 35% of patients. Ragab et al. showed that there are no differences in nasal and total symptom scores of children with common cold who received saline NI combined with amoxicillin or placebo treatment. In the placebo group, children presented less adverse effects, suggesting that the saline NI alone (no antibiotic) is not only sufficient but also safer. Thus, improving the NI formulations may improve the symptomatic relief outcomes with a better safety profile and contribute to the decrease in the irrational use of medicinal products.

Hypertonic solutions are frequently used for NI and, due to their high salt concentration, were claimed to reduce oedema by draining water from surrounding tissues through diffusion of osmolar gradients, and have been shown to be effective in the elimination of nasal congestion in chronic rhinosinusitis. In addition, in vitro studies have shown that hypertonic saline solutions are safe to use on nasal tissues as they do not compromise nasal epithelial integrity.

In this study, anti-viral and anti-bacterial properties of a hypertonic seawater formulation (2.3% NaCl) enriched with hyaluronic acids, eucalyptus oil, copper and manganese salts, Stérimar Stop & Protect Cold and Flu (SSPCF), were tested in vitro. Additionally, the effect of the formulation on phagocytosis was evaluated.

**Materials and Methods**

Biological model (test system) used for the in vitro studies

The in vitro assays were performed in a 3D reconstituted human nasal epithelium model, MucilAir™ (Epithelix Sàrl, Geneva, Switzerland) for its great potential as a model to test respiratory sensitizers. For maintenance, inserts were incubated in 500µl of MucilAir™ culture medium in a CO2 incubator (37°C, 5% CO2, 100% humidity, Heracell).

**Viral load assays**

Replication of HRV-A16 has been previously shown to be successful in the MucilAir™ model in which the viral RNA load reaches a maximum in 24-48 hours after infection. In order to evaluate the effect of SSPCF on viral load, both pre- and post-treatment approaches were followed.

**Effect of treatment prior to HRV-A16 infection**

HRV-A16 virus stocks were prepared in HeLa Ohio and Vero cells as previously described. Prior to viral inoculation, tissues were treated 1 hour with saline (0.9%) (in triplicate) or SSPCF (n=3) or 5mM Rupintrivir (n=2, Santa Cruz Biotechnology Inc., Dallas, Texas, United States), a potent and irreversible inhibitor of human rhinovirus 3C protease. At 0h, tissues were apically inoculated with 100µl HRV-A16 (2.8x10^4 RNA copies /ml). After 3.5 hours of incubation at 34°C, supernatants were aspirated, and tissues were rinsed. 200µl of MucilAir™ culture medium was added for 20 minutes and the virus were harvested. The epithelia were further cultured at the air-liquid interface. At 24h, viral load was collected the same way. The viral load was quantified with Entero/Glu/08 one-step real-time PCR as previously described.

**Effect of treatment after HRV-A16 infection**

At 0h, tissues were inoculated with 100µl of HRV-A16 viral suspension (2.8x10^4 RNA copies /ml). Virus were rinsed out by 200µl of MucilAir™ culture media for 20 minutes. Tissues were treated with SSPCF (n=3) and 5000nM Rupintrivir (n=3) or saline (0.9%) (n=3) for 24h, and further incubated for 24 hours. Supernatants were lysed, and viral load was quantified. RNA was extracted with the QIAamp® (Qiagen, Hilden, Germany) Viral RNA Mini Kit. The extracted RNA was then quantified with the QuantiTect RT-PCR kit (Qiagen, Hilden, Germany) by TaqMan ABI 7000 instrument (Applied Biosystems, Waltham, MA, USA).

**Bacterial assay**

*S. aureus* (ATCC 6538) was thawed out and subcultured in TCS agar plates and then grown in suspension at 30-35°C up to 3.2x10^6 CFUs (calculated by OD_620). SSPCF was added to the suspension to a final concentration of 1%, to ensure minimal changes in growth characteristics. Incubations were then performed under rotary agitation (180 rotations/min) at 35°C. Monitoring was carried out at 0, 1, 3 and 24h of incubation by measuring the bacterial density (calculated as CFU/ml). As negative control, untreated samples (growth medium) was used.

**Phagocytosis assay**

To evaluate whether SSPCF can induce phagocytic activity, tissues were treated for 1 hour with control (saline solution) or SSPCF, containing-FITC coupled latex beads. A volume of 100µl of rabbit IgG-FITC conjugated-coated latex beads (1:10 saline dilution) (Phagocytosis assay kit, Cayman Chemical Company, Ann Arbor, MI, USA) were added to tissues and incubated in 30µl of saline or SSPCF in 24-well plates. Tissues were incubated for 1 hour for 37°C. 50µl of Trypan Blue solution were added and plates were incubated at RT for 2 min to quench FITC fluorescence of non-internalized beads. Excess Trypan Blue was aspirated, and fluorescence intensity of the cells was measured (485nm/535nm). Cells were also analysed in an inverted fluorescent microscope (Leica DM IL LED FLUO, Wetzlar, Germany) equipped with filters (485nm/535nm) after supernatant aspiration.
Results

Effect on viral replication
Results of 1-hour SSPCF pre-treatment before HRV-A16 infection showed that viral replication was almost completely inhibited in this sample as compared to saline-treated control (99.78% inhibition, 2.05x10^7 vs 4.50x10^4, respectively (Figure 1A). The effect of SSPCF was similar to the effect of rupintrivir. Results of the post-infection treatment (24 hours after infection) showed that 24 hours of treatment (48 hours after infection) decreased the viral load compared to saline-treated control, reaching up to 60% inhibition (6.73x10^8 vs. 2.70x10^8 copies RNA/ml) (Figure 1B).

Effect on bacterial growth
The efficacy of SSPCF against S. aureus was tested by monitoring bacterial growth at 1, 3 and 24 hours after treatment. As shown in Figure 2, treatment with SSPCF has an anti-bacterial effect compared to untreated control (growth medium). Starting from as early as one hour after treatment the S. aureus colony forming unit counts decreased from over 1.0x10^6 to 0 and is maintained throughout the course of the experiment (24h).

Effect on the phagocytic activity of nasal epithelial cells
Figure 3A presents fluorescent micrographs of cells derived from SSPCF or saline pre-treated tissues incubated with fluorescent beads. Tissues treated with SSPCF engulfed more beads indicating an increased phagocytotic activity compared to saline control. The quantification of fluorescence intensity revealed that the increase was more than 3-fold (Figure 3B, p<0.001).

Discussion
The present study aimed to test the efficacy of a hypertonic seawater solution enriched with hyaluronic acids, eucalyptus oil as well as copper and manganese salts against pathogens such as human rhinovirus which are known to cause common cold (4,5) and S. aureus which is an important pathogen in respiratory infections (22), in an in vitro model of reconstituted human nasal epithelium. Manganese has been shown to inhibit the anaphylactic histamine release (23) and reduces inflammatory response (24). Hyaluronic acid increases tissue hydration and shortens healing time (25,26).

The model used in this study, MucilAir™ is composed of basal cells, ciliated cells and mucus cells in a proportion similar to what one observes in vivo (27). Moreover, MucilAir™ is functionally differentiated, conserves tissue integrity (TEER>200 Wcm^-2) and also secretes mucus. The activity of the main epithelial ionic channels, such as CFTR, EnaC, Na/K ATPase, is preserved and the epithelia is shown to respond in a regulated and vectorial manner to the pro-inflammatory stimulus, TNF-α (27). A large panel of
cytokines, chemokines and metalloproteinases has been detected in MucilAir™ tissues (e.g. IL-8, IL-6, GM-CSF, MMP-9, GRO-a). Most importantly, MucilAir™ replicates the main function of the airway epithelial cells, the mucociliary clearance driven by synchronized cilia-beating, and has been successfully used for acute, long-term and chronic in vitro studies. Given that MucilAir™ is functionally robust and successfully mimics human nasal epithelium, it is important to conduct a clinical trial with SSPCF in order to demonstrate the safety and efficacy of it in vivo settings.

At the performance level, treatment with SSPCF prior to HRV-A16 infection reduced viral load compared to saline-treated cells (almost 100%), as measured by viral RNA copy numbers, indicating the inhibition of rhinovirus replication. The reduction was similar to the one caused by treatment with rupintrivir, a well-known viral protease inhibitor. 60% viral replication inhibition was also observed when treatment was performed after viral infection. Therefore, SSPCF has a prophylactic effect against HRV-A16 and is also effective when used post-infection (~100% and 60% inhibition, respectively).

In addition, efficacy of SSPCF in control of bacterial growth was also assessed. The results in Figure 1 show that, as early as 1 hour after treatment, SSPCF exerted anti-bacterial effect on S. aureus (complete inhibition), compared to untreated control cultures.

Respiratory epithelial cells orchestrate the immune defense response against incoming toxins and pathogens. Pathogen recognition pathways in epithelial cells can stimulate phagocytosis. An increase in phagocytic activity was also observed upon treatment with SSPCF compared to treatment with saline solution. The performed assay employed IgG-FITC conjugated latex beads. It would be interesting to investigate whether there is an increase in phagocytosis of HRV-A16 or S. aureus via live cell imaging to confirm that the increase in phagocytic activity shown with fluorescent beads is contributing to an antimicrobial effect. Others have previously shown that primary nasal epithelial cells cultured in vitro in an air-liquid interface similar to the MucilAir™ model, are able to phagocyte other pathogens such as Aspergillus fumigatus. Upon colonizing the nose, S. aureus competes with the bacteria of the nasal microflora and has been shown to form a biofilm enabling other bacteria species to grow. Biofilms provide bacteria with a protective environment against host defense which may lead to chronic diseases such as chronic rhinosinusitis. In this regard, topical irrigation treatments, including nasal saline irrigation, have been shown to be effective in the elimination of biofilms both in animal and human chronic rhinosinusitis models as reviewed recently. Therefore, the prevention of bacterial growth demonstrated in this in vitro study may be due to the partial or complete elimination of biofilm, or the prevention of its formation.

Conclusions

Overall, results of the present study support that in vitro, SSPCF is able to inhibit HRV-A16 replication and S. aureus growth, and SSPCF is effective in protecting the nasal epithelial tissues from viral and bacterial pathogens. In vitro tests presented in this study suggest that SSPCF has (i) an inhibitory effect on viral replication when applied pre- or post-infection by HRV A16, (ii) anti-bacterial effect on S. aureus; and (iii) phagocytosis-enhancing effect that could be useful against viruses and bacteria involved in common cold. Safety and efficacy evaluation of SSPCF in the clinical settings is needed to prove the beneficial effects of the formulation at the clinical level.
Acknowledgement
Authors would like to thank Dr. Philippe Contencin and Dr. Annahita Ghassemi for their valuable contribution to the elaboration and revision of the manuscript.

Authorship contribution
SC, MM and AS established the study conception and design. SH and BDS developed the experimental methodology. SH and BDS acquired, analysed and interpreted data. JC and AS wrote, reviewed and revised the manuscript. SC, MM and AS supervised the study.

Conflict of interest
AS works as EU Technology & Innovation Manager at Church & Dwight, Co., Inc. SH, SC, BDS, MM and JC declare no conflict of interest in this work.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
Not applicable.

Funding
This study has been sponsored by Church & Dwight, Co., Inc.

References
32. Peacock SJ, de Silva J, Lowy FD What deter-
Hypertonic seawater against nasal pathogens