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Research Article

Use of a Kappa-carrageenan Nasal Solution in the Prophylaxis Treatment of Respiratory Infection Against Viruses Influenza (H1N1), Coronavirus (SARS-CoV-2) and Chronic Obstructive Pulmonary Diseases (COPD)

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ABSTRACT

Purpose: The nasal mucosa is the primary site of infection and replication for most cold-causing viruses including influenza (H1N1) and SARS-CoV-2. It was hypothesized, carrageenan's would block viral entry at respiratory mucosa and interfere with viral replication propagation locally. The goal of this experiment was to test the antiviral effects of kappa carrageenan and Iota carrageenan in fully differentiated human airway epithelial cells cultivated at the air-liquid interface against influenza (H1N1) and SARS-CoV-2 virus infection.

Methods: To assess the efficacy of test product on human airway epithelial cells, apical viral replication (genome copy number) was quantified, tissue integrity was measured. Epithelia (MucilAir™-Pool) cells were used for the study. The experiment was designed to test the antiviral effects of kappa carrageenan in fully differentiated human airway epithelial cells cultivated at the air-liquid interface against influenza (H1N1) and SARS-CoV-2 infection.

Results: Study results show the reference drug oseltamivir inhibits apical H1N1genome copies by >3 log10, Kappa carrageenan by >1 log10 units and Iota carrageenan by 2 units. About SARS-CoV-2 infection study results indicate; SARS-CoV-2 apical replication at both time points, by 3.2; 2.3 log10 and 2.1 and 1.1 log10 respectively. Drug Remdesivir inhibits apical SARS-CoV-2 genome copies at 48 and 72 hours, by 3.4 and 4.2 log10, respectively. Kappa carrageenan and Iota carrageenan effectively inhibits SARS-CoV-2 apical replication at both time points, by 3.2, 2.3 log10 and 2.1 and 1.1 log10 respectively.

Conclusion: The study demonstrates that antiviral effect of developed Kappa Carrageenan nasal spray product has better efficacy than the Iota carrageenan comparator product.

Keywords: Kappa-carrageenan; H1N1; SARS-CoV-2; TEER; Epithelia; COPD

Abbreviations: SARS-CoV-2: Severe-Acute-Respiratory-Syndrome-Related Coronavirus (SARSr-CoV), COPD: Chronic Obstructive Pulmonary Disease, TEER: Trans-Epithelial Electrical Resistance, CFTR: Cystic Fibrosis Transmembrane Conductance Regulator, TNF: Tissue Necrosis Factor, RT-PCR: Reverse Transcription Polymerase Chain Reaction

INTRODUCTION

The most common infectious disease in humans is acute viral upper respiratory tract infection, generally known as the common cold [1]. Colds are a common ailment that affects both children and adults [2]. Respiratory viruses such as rhinovirus, coronavirus, parainfluenza, influenza, respiratory syncytial virus, adenovirus, enterovirus, and metapneumovirus are responsible for most common colds [3,4]. Chronic Obstructive Pulmonary Disease (COPD) is an inflammatory disease of the lower airways which is rare symptomatic below the age of 40. COPD is characterized by a severe limitation of airflow that is not fully reversible, over treatment with a bronchodilator. Viral infections will worsen the situation in COPD patients [5].

Influenza is a viral infection of the lungs and airways with one of the strains of influenza viruses. It causes a runny nose, sore throat, fever, cough, headache, muscle aches and general feeling of illness. In 2009-2010, there was an epidemic of the H1N1 strain of influenza virus that became widespread and was considered a pandemic. This strain had a combination of genes from pig (swine), bird, and human influenza viruses [6].

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SARS was first reported in Asia in February 2003. In the recent years SRAC-CoV-2 was SARS is an airborne virus and can spread through small droplets of saliva in a similar way to the cold and influenza. SARS-CoV-2 was the severe and readily transmissible disease to emerge in the 21st century and showed a clear capacity to spread along the routes. With this recent outbreak in 2019 of SARS CoV-2 epidemic, there is a need of the alternate and prophylaxis treatment of the disease [7].

There are several viruses causes a rhinitis and respiratory infections and successful treatment material should have a broad antiviral capability and should not cause resistance. Carrageenan's could be one such alternative. Since the nasal mucosa is the primary site of infection and replication for most cold-causing viruses, it was hypothesized that early and targeted treatment of the nasal mucosa with carrageenan's would block viral entry at the level of the respiratory mucosa and interfere with viral replication locally [8]. Studies *in vitro* and *in vivo* have shown the effectiveness of carrageenan against several viruses such as human Rhinovirus (hRV), Influenza A and Respiratory Syncytial Virus (RSV). Administration of carrageenan in humans has been demonstrated to be safe. Carrageenan is regarded as a safe in the United States and is approved as a direct food addition under FDA standards.

Carrageenan's are a type of linear, sulfated polysaccharide that may be extracted from a variety of red seaweed species. carrageenan's are divided into three commercially relevant families based on the amount and position of sulphate moieties on the hexose scaffold structure: Iota, kappa, and lambda. Their solubility and gelling qualities differ from one another [9]. The formulation was used to test the inhibitory effect on apical exposure affected influenza (H1N1) and coronavirus (SARS-CoV-2). The goal of this experiment was to test the antiviral effects of Abbott's medicines in fully differentiated human airway epithelial cells cultivated at the air-liquid interface against virus infection. Epithelia (MucilAir™-Pool) were put together with a mixture of cells isolated from fourteen different normal nasal donors. MucilAir™ is a reconstituted human 3D tissue from airways and lung surgical pieces, fully differentiated, pseudostratified *in vitro* epithelium. Epithelial cell line "air-liquid interface" model presents high trans-epithelial electrical resistance, cilia beating as well as mucus secretion, demonstrating overall functionality of the epithelial tissue.

MATERIALS AND METHODS

Kappa carrageenan nasal spray preparation

A nasal spray containing 0.9% w/v saline and 0.12% w/v kappacarrageenan has been developed as a medical device. Carrageenan nasal spray medical device is a non-pressurized dispenser that deliver a spray containing a defined formulation i.e., kappa-carrageenan (1.2 mg/ml) in liquid dosage form. This is further evaluated for efficacy study and *In-Vitro* characterization.

Evaluation of nasal spray formulations

A nasal spray formulation was evaluated for the various physical properties like appearance, pH, density, osmolality, viscosity, and film formation.

Assay system: The assay technique used for this study was Epithelix' proprietary technology MucilAir™. MucilAir™ is a pseudostratified and ready-to-use 3D model of human airway epithelium, constituted with primary human epithelial cells freshly isolated from nasal, tracheal or bronchial biopsies. When switched at the air-liquid interface, the progenitor cells undergo a progressive differentiation and polarization to a fully ciliated epithelium. The mature MucilAir™ is comprised of basal cells, ciliated cells and mucus cells. The proportion of these various cell types is preserved compared to what one observes *in vivo* [10].

Moreover, MucilAir™ is functionally differentiated, secretes mucus and are electrically tight (TEER>200 Ωcm^2). The activity of the main epithelial ionic channels, such as Cystic Fibrosis Transmembrane Conductance Regulator (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 [11]. A large panel of cytokines, chemokines and metalloproteinases has been detected in MucilAir™ (e.g., IL-8, IL-6, GM-CSF, MMP-9, GRO-α). Most importantly, MucilAir™ exhibits the major function of the airway epithelial cells, the mucociliary clearance driven by synchronized cilia-beating.

Evaluation of formulation barrier effect on Influenza (H1N1) infection using MucilAir™-Pool

At t=0h, Abbott's Carrageenan formulation and Iota carrageenan were applied on the apical side of MucilAir™-Pool. After 1 hour, 10µl of inoculum (e.g., H1N1=1.1E7 genome copy number/ml) were applied on the apical side of MucilAir™-Pool for 3h at 34°C. Epithelia were then washed thrice with MucilAir™ culture medium to clean the inoculum.

Apical washes were performed with 200µl of MucilAir™ culture medium during 20 min at 34°C for 3 and 24-hours time points. 20 µl treatment was renewed apically after the washes 3 hours post inoculation. Supernatants were lysed and viral RNA was extracted with the QIAamp® Viral RNA kit (Qiagen). Viral RNA was then quantified by RT-PCR (QuantiTect Probe RT-PCR, Qiagen) with the qTOWER3 detection system. Ct data were reported to the standard curve and presented as genome copy number/ml on the graphs. Statistical analysis was performed by two-way ANOVA with Dunnett's multiple comparison post-tests using Prism 6 GraphPad software (La Jolla, USA). The values $P<0.05$ were considered statistically significant. TEER value measurement was done to observe the effect of Carrageenan solution on non-infected and infected epithelial cells as well.

Evaluation of kappa carrageenan formulation barrier effect on SARS-CoV-2 infection using MucilAir™-Pool

Virus inoculation: The SARS-CoV-2 strain used in the study was isolated by precisely inoculating VeroE6 cell monolayers

with a nasal swab sample obtained from Bichat Claude Bernard Hospital, Paris. Once characteristic cytopathic effect was observable in more than 50% of the cell monolayer, supernatants were collected and immediately stored at -80°C. The complete viral genome sequence was obtained using Illumina MiSeq sequencing technology and was deposited under the name BetaCoV/France/IDF0571/2020. Viral stocks were titrated by tissue culture infectious dose 50% (TCID 50/ml) in VeroE6 cells, using the Reed and Muench statistical method.

Inoculations were performed with 150 µl at a theoretical Multiplicity of Infection (MOI) of 0.1 (50 000 Texas Center for Infectious Disease (TCID50) for an average of 500 000 cells in MucilAir™), applied to the apical side of the cultures for 1 hour at 37°C, 5% CO_2. Non-infected vehicle control was exposed also to 150 µl of culture medium on the apical side for 1 hour. Unbound viruses were removed after one hour of incubation period. New viral particles were collected by 10 min after apical washes at 48 and 72-hours post-inoculation and quantified by RT-PCR.

Reference antiviral drug remdesivir was purchased from MedChemExpress, (HY-104077) and was diluted in Dimethyl Sulfoxide (DMSO) and used at 5 µM (final concentration of DMSO was 0.05%) in the basolateral medium. Reference antiviral was added after one hour of viral inoculation and altered drug every day.

Testing strategy: Apical exposure was performed by liquid pipetting. Endpoints were measured at 48 and 72 hours from the apical side. Apical one-hour pre-infection exposure and repeated exposure once a day post-infection of test compound on MucilAir™-Pool. Viral genome copy number and Trans-Epithelial Electrical Resistance (TEER) were measured at Day 2 and 3.

Real-time Taqman probe RT-PCR

From the 200 µl apical washes, 140 µl was used for viral RNA extraction with the QIAamp® Viral RNA kit (Qiagen), obtaining 60 µl of eluted RNA. Viral RNA was quantified by quantitative RT-PCR (EXPRESS One-Step Superscript™ RT-PCR Kit, Invitrogen, 1178101K) using 2 µl of viral RNA with Mastermix and two ORF1b-nsp14 specific primers (5'- TGGGGYTTTACRGGTAACCT-3'; 5'-AACRCGCTTAACAAAGCACTC-3') and probe (5'-FAM-TAGTTGTGATGCWATCATGACTAG-TAMRA-3') of SARS-CoV-2 designed by the School of Public Health/University of Hong Kong. Samples were analyzed on StepOnePlus™ Real-Time Polymerase Chain Reaction (PCR) System (Applied Biosystems). Ct data were determined and relative changes in gene expression were calculated using the 2-ΔCt method and reported as the fold reduction relative to the mean of vehicle treated infected inserts.

Tissue Integrity (TEER)

TEER is a dynamic parameter that reflects the state of epithelia and is typically between 200 to 600 Ω.cm^2. An increase in the (TEER) value indicates a blockage of the ion channel activities. A notable decrease of the TEER values (but >100 Ω.cm^2) could be observed in certain cases, reflecting an activation of the ion channels. Disruption of cellular junction or holes in the epithelia result in TEER values below 100 Ω.cm^2. When an epithelium is damaged, a decrease of TEER would be associated with an increase of Lactate Dehydrogenase (LDH) release or a decrease of the cell viability.

After addition of 200 µl of culture medium to the apical compartment of MucilAir™ cultures, resistance was measured with an EVOMX volt-ohm-meter (World Precision Instruments UK, Stevenage) for each condition. Membrane had a 100 Ω resistance of the membrane and 0.33 cm^2 is of the complete surface of the epithelium.

Statistical analysis

Data were expressed as mean ± standard error of mean and assumed to have normal distribution. Differences between three or more groups were tested by one-way or two-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison post-tests using Prism 6 GraphPad software (La Jolla, USA). Differences among two groups were tested by Student's t test. The values $P<0.05$ were considered statistically significant.

RESULTS

Kappa carrageenan nasal spray preparation

Nasal spray formulation was prepared and evaluated for different physical properties. The results of the study are reported in Table1.

Test	Iota carrageenan product	Kappa carrageenan product
Appearance	Clear viscous liquid	Clear viscous liquid
pH	6.68-7.02	6.76-7.01
Density (g/ml)	1.0079	1.0047
Osmolality (mOsMol/kg)	352	350
Viscosity (cps)	2.5-10	2.40-10.1

Table 1: Physical properties of nasal spray preparation.

Aqueous solution of kappa carrageenan solution is used allowed to form the films over the time (Figure 1), this film formation indicates mucus protecting properties of the kappa carrageenan provides a barrier on mucus and protecting it.

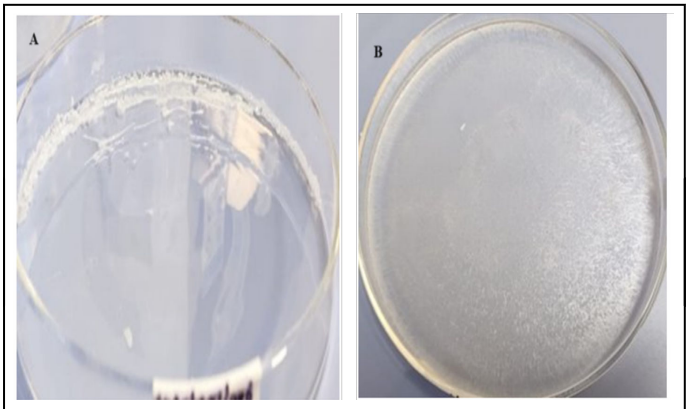

Figure 1: (A): Placebo solution of Kappa Carrageenan; (B): Kappa Carrageenan film after drying.

Evaluation of formulation barrier effect on Influenza (H1N1) infection using MucilAir™-Pool

Effect of Carrageenan on TEER: Non-infected mock infected non-treated controls were in the normal range of TEER (200-800 Ω.cm^2). TEER was preserved after Oseltamivir treatment. Non-infected and infected epithelia treated with carrageenan displayed a decrease of TEER, but the integrity of epithelia was preserved. TEER was preserved after exposure to Iota carrageenan (Figure 2 and 3).

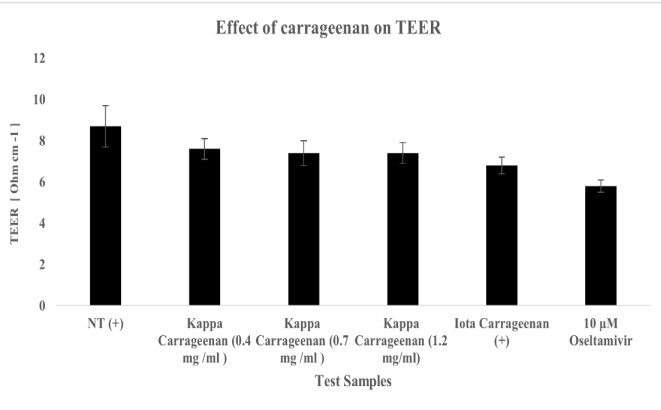

Figure 2: Effect of one-hour pre-treatment and repeated apical exposure to kappa carrageenan on tissue integrity in MucilAir™-Pool upon H1N1 infection. TEER was measured (n=3 cultures). Threshold limit is 100 Ω.cm^2.

Effect of Carrageenan on H1N1 apical replication

No viral genome was detected in non-infected epithelia H1N1 (input was 1.1E7 gc/ml). Non-treated control showed a good replication of H1N1 24 hours (5.4E8 gc/ml), Oseltamivir efficiently inhibited H1N1 replication (3 logs: 6.5E5 gc/ml). Carrageenan reduced H1N1 replication at all concentrations (>1 log), Iota carrageenan reduced H1N1 replication (>2 logs) (Figure 4).

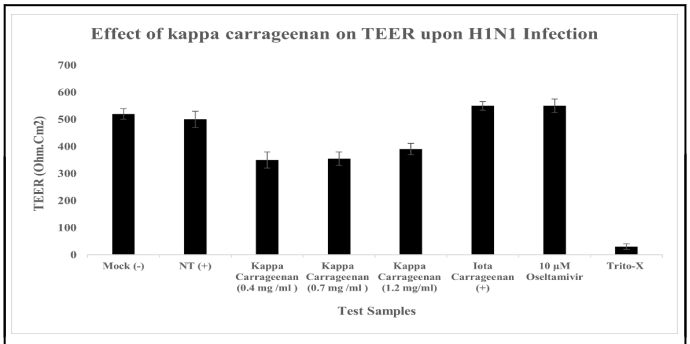

Figure 3: Effect of one-hour pre-treatment and repeated apical exposure to kappa carrageenan, Iota carrageenan, Ostelmivir and Triton X on tissue integrity in MucilAir™-Pool upon H1N1 infection. TEER was measured (n=3 cultures). Threshold limit is 100 Ω.cm^2.

Assay system and testing strategy: Vehicle control (20 µl 0.9% NaCl (-)) showed TEER values in the normal range of MucilAir™-Pool (200-800 Ω.cm^2). TEER values are interpreted as an ON-OFF parameter using the threshold limit of 100 Ω.cm^2. SARS-CoV-2 infection reduced TEER at 72 hours, but all values stayed above 100 Ω.cm^2. Exposure to remdesivir prevented the virus induced decrease of TEER at 72 hours. Exposure to Kappa Carrageenan reduced TEER at both time points, but the tissue integrity was maintained (>100 Ω.cm^2) (Figures 5).

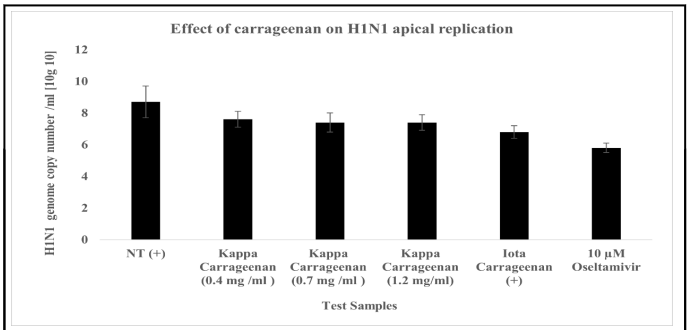

Figure 4: Relative H1N1 genome copy number on the apical side of MucilAir™-Pool with one hour pre-treatment and repeated apical exposure to Iota Carrageenan and Oseltamivir at 10 µM was added in basolateral medium.

Effect of kappa carrageenan on tissue integrity upon SARS-CoV-2 infection

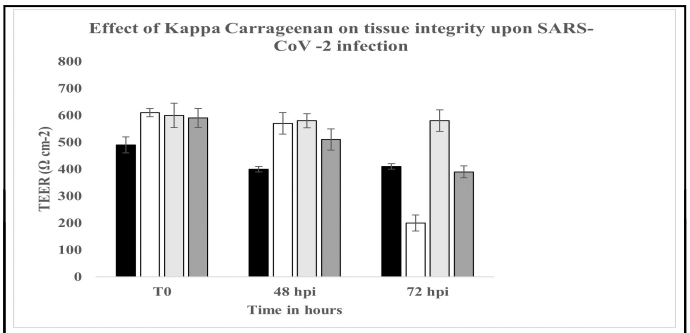

Figure 5: Effect of one-hour pre-treatment and repeated apical exposure to kappa carrageenan on tissue integrity in MucilAir™-Pool upon SARS-CoV-2 infection. TEER was measured before inoculation, 2 and 3-days post-infection (n=3 cultures). Threshold limit is 100 Ω.cm^2. **Note:** (■) 0.9% NaCl (-); (▫) 0.9% NaCl (+); (▫) Remdesivir 5µ (+); (▫) Kappa Carragenan.

Effect of kappa carrageenan on SARS-CoV-2 apical replication

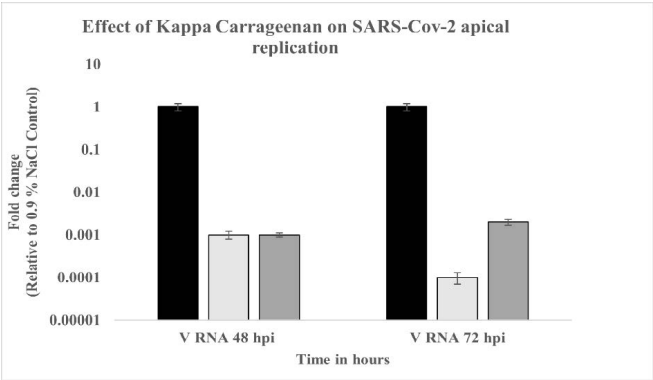

Figure 6: Relative SARS-CoV-2 genome copy number on the apical side of MucilAir™-Pool with one hour pre-treatment and repeated apical exposure to kappa carrageenan. Antiviral remdesivir at 5 µM was added in basolateral medium. **Note:** (■) 0.9% NaCl; (▫) Remdesivir 5µM; (▫) Kappa Carrageenam.

Antiviral control remdesivir significantly reduced apical SARS-CoV-2 genome copies at both time points. The magnitude of inhibition was 3.4 log10 at 48 hours and 4.2 log10 at 72 hours (*vs.* vehicle control). Exposure to Kappa Carrageenan significantly reduced apical SARS-CoV-2 genome copies at both time points. The magnitude of inhibition was 3.2 log10 at 48 hours and 2.3 log10 at 72 hours (*vs.* vehicle control) (Figure 7).

Effect of Iota Carrageenan on tissue integrity upon SARS-CoV-2 infection

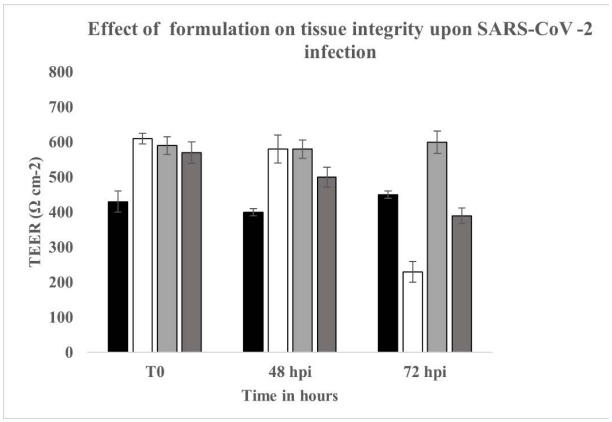

Figure 7: Effect of one-hour pre-treatment and repeated apical exposure to formulations on tissue integrity in MucilAir™-Pool upon SARS-CoV-2 infection. TEER was measured before inoculation, 2-days and 3-days post-infection (n=3 cultures). Threshold limit is 100 Ω.cm^2. **Note:** (■) 0.9% NaCl (-); (▫) 0.9% NaCl (+); (▫) Remdesivir 5 µ(+); (■) Kappa Carragenan(+).

Vehicle control (20 µl 0.9% NaCl (-)) showed TEER values in the normal range of MucilAir™-Pool (200-800 Ohm.cm^2). TEER values are interpreted as an ON-OFF parameter using the threshold limit of 100 Ω.cm^2. SARS-CoV-2 infection reduced TEER at 72 hours, but all values stayed above 100 Ω.cm^2.

Exposure to remdesivir prevented the virus induced decrease of TEER at 72 hours. Exposure to Iota Carrageenan prevented the virus induced decrease of TEER at 72 hours (Figure 8).

Effect of Iota Carrageenan on SARS-CoV-2 apical replication

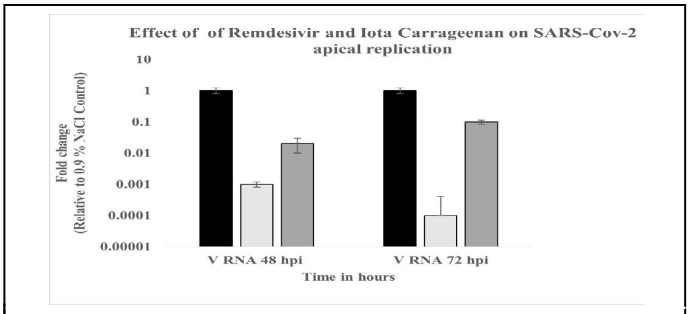

Figure 8: Relative SARS-CoV-2 genome copy number on the apical side of MucilAir™-Pool with one hour pre-treatment and repeated apical exposure to Iota Carrageenan. Antiviral remdesivir at 5 µM was added in basolateral medium. Statistical comparison was performed comparing all conditions to vehicle using two-way ANOVA with Dunnett's multiple comparison post-tests (Prism 6.0 GraphPad, *p<0.05, **p<0.01***p<0.001, ****p<0.0001). **Note:** (■) 0.9% NaCl; (▫) Remdesivir 5 µM; (▫) Iota Carrageenan.

Antiviral control remdesivir significantly reduced apical SARS-CoV-2 genome copies at both time points. The magnitude of inhibition was 3.4 log10 at 48 hours and 4.2 log10 at 72 hours (*vs.* vehicle control). Exposure to Iota Carrageenan significantly reduced apical SARS-CoV-2 genome copies at both time points. The magnitude of inhibition was 2.1 log10 at 48 hours and 1.1 log10 at 72 hours (*vs.* vehicle control).

DISCUSSION

In this study, it was reported that the kappa carrageenan could inhibit the reproduction of virus by changing the binding of High Availability (HA) to Madin-Darby Canine Kidney (MDCK) cells, suppressing messenger Ribonucleic Acid (mRNA) and protein expression after internalization *in vitro*, This indicates that kappa carrageenan suitable against the H1N1 and other viruses [11].

Study results shows the reference drug Oseltamivir inhibits apical H1N1 genome copies by >3 log10, kappa carrageenan by >1 log10 units and Iota carrageenan by 2 units.

SARS-CoV-2 decreases TEER at 72 hours, but the tissue integrity is preserved. Reference antiviral drug Remdesivir inhibits apical SARS-CoV-2 genome copies at 48 and 72 hours, by 3.4 and 4.2 log10, respectively. Kappa carrageenan efficiently inhibits SARS-CoV-2 apical replication at both time points, by 3.2 and 2.3 log10, respectively. However, no effect is observed on the virus-induced decrease in TEER value. Iota carrageenan effectively inhibits the apical replication of SARS-CoV-2 at both time points, by 2.1 and 1.1 log10, respectively. It seems to prevent the decrease in TEER value induced by the virus.

CONCLUSION

In conclusion, the study demonstrates the antiviral effect of kappa carrageenan on genome copies on the apical side of MucilAir™-Pool cultures, when applied pre and repeated dosing post infection of H1N1 and SARS-CoV-2. The comparator compound, Iota carrageenan has a less potent antiviral effect on genome copies of H1N1 and SARS-CoV-2 but shows a beneficial effect on TEER of the epithelium. Therefore, carrageenan nasal spray medical device is intended for "Spraying of nasal cavity in acute and chronic diseases of the upper respiratory tract and bronchi, such as rhinitis, sinusitis, laryngitis, pharyngitis, tonsillitis, tracheitis, bronchitis, etc. In summary kappa carrageenan can be utilized for the prophylaxis treatment of viral infections.

DECLARATION

Availability of data and materials: The datasets generated during this study are available from the corresponding author upon request.

Competing interest: The authors have no competing interest, funding, or financial relationships.

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Author contributions: All authors contributed to the study's conception and design. Data evaluation was performed by Dr, Sagir Syed and H Srinivasa. Dr. Sagir Syed wrote the first draft of the manuscript, and the final corrections were made under the supervision of Dr Padmanabha RV Reddy and Craig Newby. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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