



A 3D “*In Vitro*” Model to Study Hyaluronan Effect in Nasal Epithelial Cell Line Exposed to Double-Stranded RNA Poly(I:C)

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Abstract

Environmental agents, including viral and bacterial infectious agents, are involved in the alteration of physicochemical and biological parameters in the nasal epithelium. Hyaluronan (HA) has an important role in the regulation of tissue healing properties. High molecular weight HA (HMW-HA) shows greater anti-inflammatory responses than medium molecular weight HA (MMW-HA) and low molecular weight HA (LMW-HA). We investigated the effect of HMW-HA, MMW-HA and LMW-HA on the regulation of physicochemical and biological parameters in an “*in vitro*” model that might mimic viral infections of the nasal epithelium. Human nasal epithelial cell line RPMI2650 was stimulated with double-stranded RNA (dsRNA) Poly(I:C) for 5 days in air-liquid-interface (ALI) culture (3D model of airway tissue). dsRNA Poly(I:C) treatment significantly decreased transepithelial electrical resistance (TEER) in the stratified nasal epithelium of RPMI2650 and increased pH values, rheological parameters (elastic G' and viscous G''), and Muc5AC and Muc5B production in the apical wash of ALI culture of RPMI2650 in comparison to untreated cells. RPMI2650 treated with dsRNA Poly(I:C) in the presence of HMW-HA showed lower pH values, Muc5AC and Muc5B production, and rheological parameters, as well as increased TEER values in ALI culture, compared to cells treated with Poly(I:C) alone or pretreated with LMW-HA and MMW-HA. Our 3D “*in vitro*” model of epithelium suggests that HMW-HA might be a coadjuvant in the pharmacological treatment of viral infections, allowing for the control of some physicochemical and biological properties affecting the epithelial barrier of the nose during infection.

Key Words: Hyaluronan, (dsRNA) Poly(I:C), Anti-inflammatory responses, Rheological properties, Mucins, Epithelial integrity

INTRODUCTION

Nasal epithelium acts as a barrier to protect the upper airways from environmental agents, including viral and bacterial infectious agents. Inflammatory stress induces injury and, consequently, nasal epithelium damage. This process can result in generalized epithelial hyperplasia, goblet-cell metaplasia, cilia loss, fibrosis, and basement membrane thickening (Holgate, 2000; Pawankar *et al.*, 2012).

Airway bacterial or viral infections increase mucus production and secretion in the epithelium of the airways. Mucin5AC (Muc5AC) and Mucin5B (Muc5B) are the main mucins involved in mucus production. Goblet cells and submucosal glands secrete mucins in the human respiratory tract (Hovenberg *et al.*,

1996; Wickström *et al.*, 1998). Mucin production affects the rheological properties of air liquids (Lafforgue *et al.*, 2018). Changes of rheological parameters cause defective mucociliary clearance, promoting airway mucus accumulation, altering ciliary movements, and increasing the depth of the liquid layer (Rogers, 2007; Fahy and Dickey, 2010). The pH acidification of biological fluids affects electrostatic interactions of mucins, increases liquid viscosity of the airway surface, and promotes defective mucociliary clearance mechanisms in nasal mucosa (Seagrave *et al.*, 2012; Tang *et al.*, 2016). The diffusion barrier of ions and solutes is regulated by tight junction (TJ) and adherent junction (AJ) complex proteins in the adjacent epithelial cells of the respiratory tract. TJ disruption can cause lung disease (Xiao *et al.*, 2011). Transepithelial electrical resistance

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(TEER) is considered a marker of barrier integrity in polarized epithelium and is well correlated with TJ disruption in the air liquid interface (ALI) cultures of epithelial cells (Ducheyne *et al.*, 2011; Kürti *et al.*, 2013; Zhao *et al.*, 2018).

Hyaluronan (HA) influences cell function, modifying the extracellular micro and macro environments, and through its viscoelastic nature, it may alter the penetration of viruses and bacteria. HA is involved in mucociliary clearance and in the healing and repair mechanisms of the nasal epithelium. HA acts in a size-dependent manner. The breakdown of high molecular weight HA (HMW-HA ~1,600 kDa) or *de novo* synthesis by the action of hyaluronan synthases during the inflammatory process, produced by low molecular weight HA (LMW-HA, ~500 kDa) and medium molecular weight (MMW-HA ~900 kDa) (Noble, 2002; Lennon and Singleton, 2011). Many lung disorders including allergy, asthma, pulmonary fibrosis and COPD showed higher levels of LMW-HA molecules, involved in control gene expression, protein synthesis (such as cytokines, chemokines), and mechanisms of oxidative stress, playing a crucial role in inflammation (Petrey and de la Motte, 2014). In contrast to the effect of LMW-HA, HMW-HA display anti-inflammatory and immunosuppressive properties (Laurent *et al.*, 1995; Noble, 2002; Liu *et al.*, 2008; Dahiya and Kamal, 2013; Gelardi *et al.*, 2013; Albano *et al.*, 2016). In medical areas, due to its physicochemical and biological tissue healing properties, HMW-HA might be useful in the treatment of the inflammatory processes as a device to assist the prevention and the treatment of nasal viral infections and of related processes (inflammation, angiogenesis, fibrosis, and cancer).

In this study, we aim to study the physicochemical and biological properties of the nasal epithelium using a 3D *in vitro* model of nasal epithelial cell line RPMI 2650, cultured in ALI and stimulated with double-stranded RNA (dsRNA) Poly(I:C) to mimic the potential action of a viral infection in the damage and repair mechanism of the nasal epithelial barrier. For this purpose, we studied the pH values, rheological properties (Elastic modulus (G') and Viscous modulus (G'')), and Muc5AC and Muc5B synthesis in apical wash, as well as the TEER in stratified nasal epithelium obtained by RPMI 2650 cell line cultured in ALI. Finally, we evaluated the potential

therapeutic application of HMW-HA as a co-adjuvant in pharmacological treatments for alleviating the negative effects of nasal viral infections on the integrity of the nasal epithelium by the control of some of the physicochemical and biological properties of the tissue.

MATERIALS AND METHODS

Cell culture

RPMI 2650 cell line (ATCC-CCL-30) was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and supplied at Passage 26. RPMI 2650 cells, isolated from a squamous cell carcinoma of the nasal septum in 1962, are the only commercially available cell line simulating the human nasal epithelium (Wengst and Reichl, 2010). The RPMI 2650 cells were routinely maintained in polystyrene tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂. Cells were cultured in complete culture Minimum Essential Media (MEM) containing 10% FCS, L-glutamine 2 mM, gentamicin 50 mg/mL, MEM NEAA 0.5%, and sodium pyruvate 1 nM. The mycoplasma infections were routinely tested using a MycoAlert Plus Mycoplasma Detection kit (Lonza, Rockland, ME, USA).

ALI culture

RPMI 2650 cell line cultured in Air Liquid Interface (ALI) represent an "*in vitro*" system biology of nasal epithelial cells, able to grow with morphological and functional features resembling human polarized barrier formation and the functions of epithelial cells in the nasal epithelium layer (Wengst and Reichl, 2010). Different cell culture conditions can have a significant effect on cell ultrastructure, barrier integrity, and gene expression in the ALI culture of RPMI 2650 (Karp *et al.*, 2002; Kreft *et al.*, 2015). RPMI 2650 cells were plated and grown on collagen-coated filters with a 0.4-micron pore size (Transwell, Costar, Cambridge, MA, USA) at a density of 6×10^5 cells/filter, and inserted into 12-well culture plates in medium (MEM containing 10% FCS). When the cells achieved confluence, ALI conditions were created (day 0) removing the medium

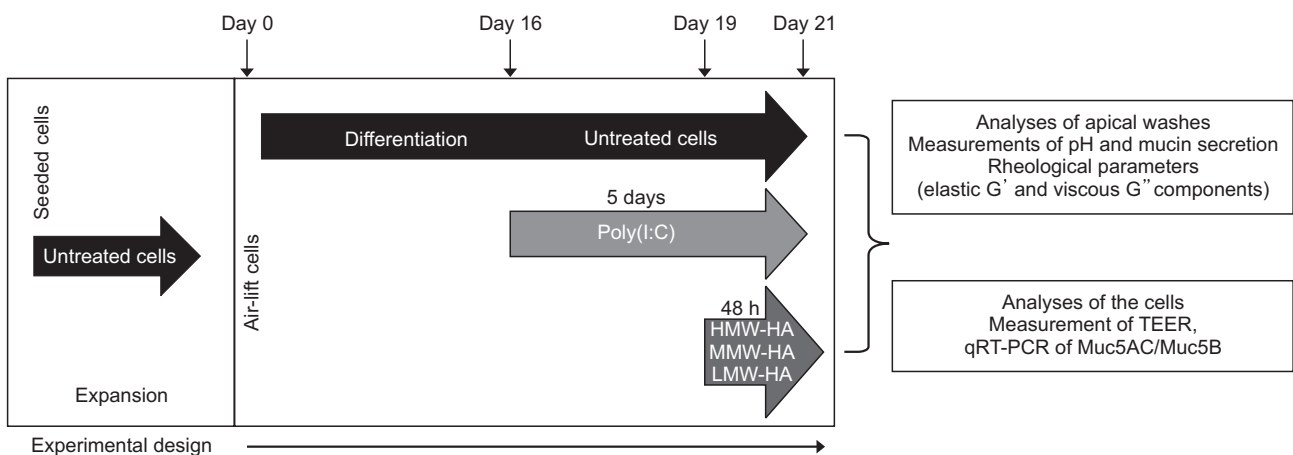


Fig. 1. Schematic overview of RPMI 2650 differentiation in the presence of Poly I:C, HMW-HA, MMW-HA and LMW-HA. RPMI 2650 were seeded in transwell inserts and expanded until confluency. Cells were subsequently airlifted and media was exchanged to medium with or without Poly I:C and finally the cells were stimulated with or without HMW-HA, MMW-HA and LMW-HA for 48 h. The medium was renewed every 2-3 days and samples were taken for analysis after 21 days of differentiation.

from the apical chambers and replacing ALI culture complete medium in the basolateral chambers. Growth supplements, bovine pituitary extracts, bovine serum albumin, and retinoic acid were added to MEM containing 10% FCS to obtain the complete medium for ALI culture.

Hematoxylin histochemical staining

After 21 days of culturing, ALI culture inserts were fixed with 4% paraformaldehyde and dehydrated in gradient alcohol series before they were embedded in paraffin. Sections of 4- μ m thickness were employed for hematoxylin staining, as previously described (Wu *et al.*, 2017). The morphology of cells was observed under the Leica microscope (Leica, Wetzlar, Germany) and acquired using a Leica Application Suite V3.3 (LAS) software (Leica) for image analysis at 40X magnification with a scale bar of 50 μ m.

ALI stimulation

After day 0, fresh medium (50 μ L in the apical chamber and 1.0 mL in the basal chamber) was provided every 48 h, and the ALI culture were allowed to remain for 16 days. On day 16, ALI culture of RPMI 2650 cells were exposed to (dsRNA) Poly(I:C) (InvivoGen, San Diego, CA, USA) to mimic viral infections as previously described (Ambrus *et al.*, 2006). Cells cultured in complete culture medium (MEM containing 10% FCS) were stimulated with RNA (dsRNA) Poly(I:C) (0-12.5-25-50 μ g/mL) as previously described (Ball *et al.*, 2015; Heijink *et al.*, 2016). The medium was added to the cells during the last 5 days of the 21 days in the ALI culture. (dsRNA) Poly(I:C) was added in the apical and basolateral compartment to reproduce the interaction between nasal epithelium and nasal mucosa during viral infections. After 3 days of stimulation with (dsRNA) Poly(I:C) (50 μ g/mL) (day 19), ALI culture of RPMI 2650 were treated with HA for 48 h (until 21 days) (added to the cells with a fresh solution). We used HMW-HA (1600 kDa, IALUCLENY, 0.12%, Chiesi Farmaceutici S.p.A., Parma, Italy) (100 μ g/mL) or MMW-HA (900 kDa, YABRO, 0.3%, Ibsa Farmaceutici Italia srl, Lodi, Italy) (100 μ g/mL) or LMW-HA (370 kDa, cod. H7630, Sigma Chemical Co., St. Louis, MO, USA) (100 μ g/mL) as previously described (Albano *et al.*, 2016) (Fig. 1).

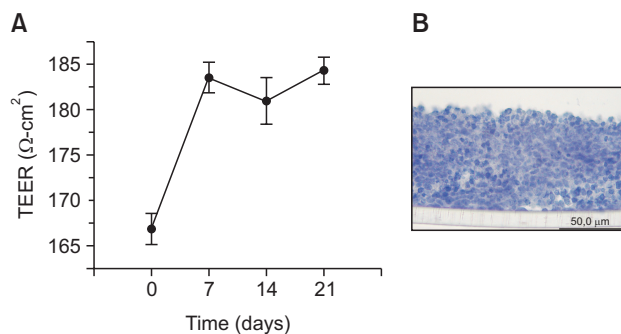


Fig. 2. Trans Epithelial Electric Resistance (TEER) (Ohms (Ω)), as quantitative measure of the integrity of airway epithelium barrier. (A) TEER values measured in RPMI 2650 grown in ALI culture at different time (0-7-14-21 days) measured to monitor the integrity of nasal epithelium before the stimulation with Poly(I:C); (B) a representative image of epithelial cell layer of RPMI 2650 cultured in ALI obtained by hematoxylin staining. Image analysis at 40X magnification, scale bar 50 μ m.

At the end of stimulation, the apical surface of stratified epithelium was washed once rapidly with warmed PBS (500 μ L), and the solution was removed (apical wash). The “apical wash” was recovered and stored at -80°C until an analysis of the proteins, pH values, and rheological properties (elastic and viscous components) was conducted, as described below. After apical wash collection, membranes of transwell inserts (0.4-micron pore size) from each experimental condition were cut out and processed for RNA isolation, as described below (Fig. 1).

Measurement of TJ integrity by TEER

After the confluence of the cells cultured in ALI (day 0), the TEER values were measured at 7, 14, and 21 days to control the TJ integrity of stratified epithelium in the absence of stimulation. Furthermore, TEER values were measured after 5 days of stimulation to study the TJ integrity under the experimental conditions of the ALI culture. Briefly, 500 μ L of pre-warmed PBS were added to the apical compartment for 10 min at 5% CO_2 , 37°C before the TEER measurements were taken. After TEER measurements were recorded, the apical solution was removed to restore ALI culture conditions. TEER values were measured using the EVOM[®] resistance meter and Endohm[®] chamber (World Precision Instruments, Sarasota, FL, USA).

Measurement of pH

pH was measured in the apical wash of RPMI2650 cells

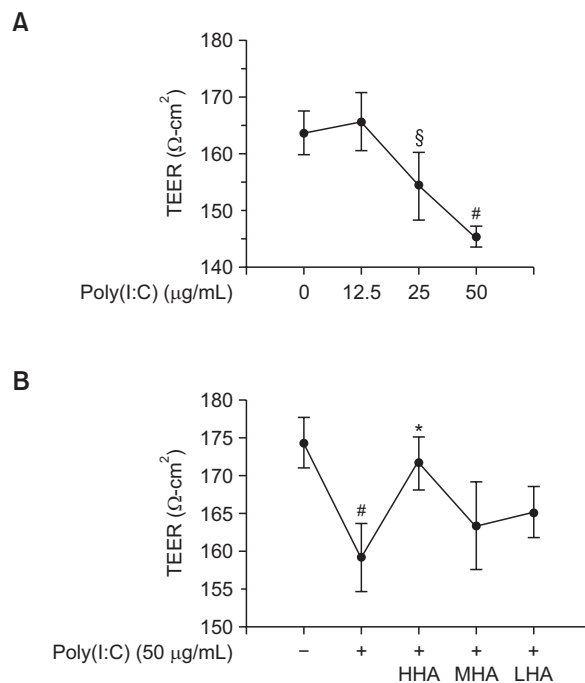


Fig. 3. Poly(I:C) and HMW-HA affect TEER values. (A) TEER values of ALI culture of RPMI 2650 stimulated with different concentration of Poly(I:C) (0 to 50 μ g/mL) for 5 days (n=3); and (B) effect of HMW-HA, MMW-HA, and LMW-HA on the TEER values of the cells stimulated with Poly(I:C) (50 μ g/mL) (n=6). Data are expressed as mean \pm standard error. Statistical analysis was performed by ANOVA followed by Fisher’s PLSD correction. # p <0.05 vs. untreated cells, § p <0.05 vs. Poly(I:C) (12.5 mg/mL)-treated cells, and * p <0.05 vs. Poly(I:C)-treated cells.

cultured in ALI. pH was measured using a pH-meter (Corning 240, Science Products Division, NY, USA) with a 0-14.00 pH range.

Measurement of mucin secretion in apical washes

Mucin concentrations were assessed in the apical wash as previously described (Burgel *et al.*, 2001). Briefly, 96-well plates (Nunc MaxiSorp, Thermo Fisher Scientific, Roskilde, Denmark) were coated with samples/standard diluted in Carbonate/Bicarbonate buffer at 37°C until dry. Porcine stomach mucin (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard. Plates were washed three times with high-salt PBS (HSPBS; PBS, 0.5 mol/L NaCl, and 0.1% Tween 20) and blocked with 2% BSA, fraction V (Sigma-Aldrich) for 1 h at room temperature. After three washes, plates were incubated with a mouse monoclonal anti-Muc5AC Ab (45M1, cod SC-21701, Santa Cruz Biotechnology, Inc., CA, USA) or a mouse monoclonal anti-Muc5B Ab (5B#19-2E, cod SC-21768, Santa Cruz Biotechnology, Inc.). After washing with HSPBS, goat anti-mouse IgG HRP conjugate Ab (Sigma-Aldrich) was added to each well for 1 h at room temperature. Finally, plates were washed with HSPBS, and peroxidase substrate (3,3',5,5'-Tetramethylbenzidine –TMB- Liquid Substrate System) was added to each well at room temperature, followed by 1 M H₂SO₄ to stop the reaction. The absorbance was measured at 450 nm using a Wallac 1420 Victor2 multilabel counter (Perkin-Elmer Life Sciences, Turku, Finland).

Quantitative Reverse Transcription (qRT-PCR) for Muc5AC and Muc5B

Total RNA was extracted from RPMI 2650 cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Then, it was reverse-transcribed into complementary DNA (cDNA), using M-MLV-RT and oligo (dT)12-18 primer (Invitrogen). qRT-PCR of Muc5AC and Muc5B transcript was carried out in a StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM labeled probe and primers (pre-validated TaqMan Gene expression assay for Muc5AC (Hs01365616 m1) and Muc5B (Hs00861588), Assays on Demand, Applied Biosystems). Muc5AC and Muc5B gene expression was normalized to the GAPDH endogenous control gene. The relative quantitation of gene expression was performed with the comparative CT method (2^{-ΔΔCt}), plotted as a fold-change and compared with untreated cells as a reference sample.

Rheology

The rheological properties, both Elastic modulus (G') and Viscous modulus (G''), were studied in the apical wash of ALI culture of RPMI2650 cells. Rheological properties of all experiments were obtained by performing oscillatory stress sweeps in the range of 0.025-50 Pa at a frequency of 1 Hz (frequency between tidal breathing and mucociliary clearance). The analysis was performed with a rotational-controlled stress rheometer (AR-1000, TA Instruments, Leatherhead, UK) using an acrylic cone-plate geometry (angle <0.0174 rad, radius 20 mm, truncation 26 μm) at a temperature of 20°C to avoid loss of water (Seagrave *et al.*, 2012).

Statistical analysis

A Kolmogorov–Smirnov Normality test was performed to assess normal data distribution. Analysis of variance (ANOVA)

corrected with a Fisher's test and a *t*-test was used for comparison. Data are expressed as mean ± standard deviation (SD). *p*<0.05 was accepted as statistically significant.

RESULTS

TEER values in ALI culture of RPMI 2650

The RPMI 2650 were seeded on a transwell insert, and TEER values were measured at different times (0, 7, 14, 21 days). The TEER increased, reaching a value >180 Ohms*cm² over 7 day and maintaining these levels for 21 days of ALI culture differentiation (Fig. 2A). The TEER values suggest a good degree of stratification occurred from day 7 until 21 days in the ALI culture in the RPMI 2650 cell line of the nasal epithelium. Morphological analysis revealed the formation of an intact epithelial cell layer on the membrane of RPMI 2650 cultured in ALI obtained after hematoxylin staining (Fig. 2B). TEER values significantly decreased in the cells stimulated with (dsRNA) Poly(I:C) (50 μg/mL) compared to untreated cells or to the cells stimulated with (dsRNA) Poly(I:C) 12.5 μg/mL (*p*<0.03 and *p*<0.02, respectively) (Fig. 3A). 50 μg/mL of (dsRNA) Poly(I:C) concentration was used in the experimental procedures with HA. HMW-HA significantly increased the TEER val-

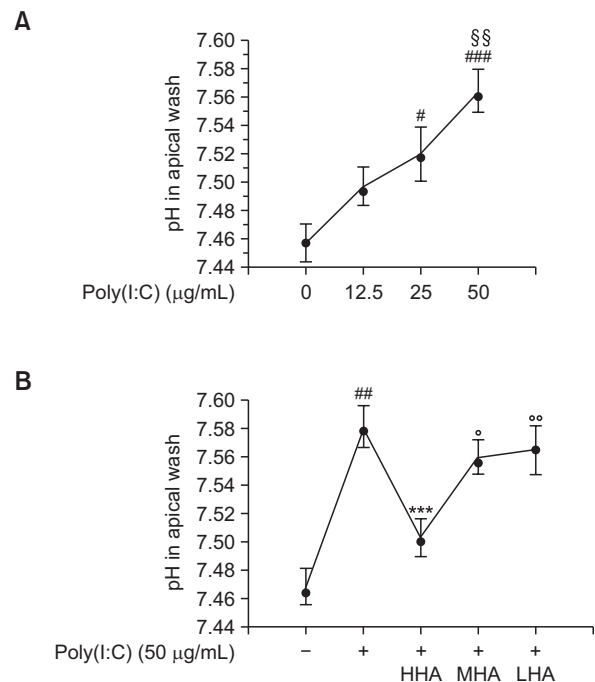


Fig. 4. Effect of Poly(I:C) on the pH values in the Apical Wash from RPMI 2650 stimulated with Poly(I:C). RPMI 2650 were grown in ALI culture for 21 days and stimulated with Poly(I:C) for 5 days: (A) dose-response (n=4) Poly(I:C) (0 to 50 μg/mL) and (B) effect of different MW of HA (HMW-HA, MMW-HA, and LMW-HA) on pH in RPMI 2650 cells stimulated with Poly(I:C) (50 μg/mL) (n=6). pH measures were performed by pH meter. Data are expressed as mean ± SD. Statistical analysis was performed by ANOVA followed by Fisher's PLSD correction. #*p*<0.05, ##*p*<0.01 and ###*p*<0.001 vs. untreated cells, §§*p*<0.01 vs. Poly(I:C) (12.5 mg/mL)-treated cells, ****p*<0.001 vs. Poly(I:C) (50 mg/mL)-treated cells, and °*p*<0.05 and °°*p*<0.01 vs. Poly(I:C) (50 mg/mL)+HHA-treated cells.

ues of RPMI 2650 stimulated with (dsRNA) Poly(I:C) 50 µg/mL compared to the cells stimulated with (dsRNA) Poly(I:C) 50 µg/mL alone ($p<0.05$). Furthermore, MMW-HA and LMW-HA did not statistically affect TEER levels in RPMI2650 stimulated with (dsRNA) Poly(I:C) in comparison to the cells stimulated with (dsRNA) Poly(I:C) 50 µg/mL alone (Fig. 3B).

pH levels in ALI culture of RPMI 2650

pH levels significantly increased in the apical wash of RPMI 2650 stimulated with (dsRNA) Poly(I:C) 50 and 25 µg/mL in comparison to untreated cells ($p<0.02$, $p<0.0004$, respectively) (Fig. 4A). Furthermore, HMW-HA significantly decreased the pH values in apical wash of RPMI 2650 stimulated with Poly(I:C) 50 µg/mL, in comparison to the cells stimulated with (dsRNA) Poly(I:C) 50 µg/mL alone ($p<0.0009$). Finally, we observed that MMW-HA and LMW-HA showed increased pH levels in an apical wash of RPMI 2650 cultured in ALI and stimulated with (dsRNA) Poly(I:C) 50 µg/mL in comparison to the cells stimulated with HMW-HA and (dsRNA) Poly(I:C) 50 µg/mL ($p<0.02$, $p<0.006$, respectively) (Fig. 4B).

Mucin proteins

(dsRNA) Poly(I:C) 50, 25 and 12.5 µg/mL significantly increased Muc5AC secretions in a dose-dependent manner in apical washes of RPMI 2650 cell culture in ALI compared with untreated cells ($p<0.0001$, 0.003 and $p<0.006$, respectively) (Fig. 5A). (dsRNA) Poly(I:C) 50 and 25 µg/mL significantly increased Muc5B secretions in apical washes of RPMI 2650 cells compared to untreated cells ($p<0.0001$ and $p<0.003$, re-

spectively) (Fig. 5B). HMW-HA significantly inhibited Muc5AC and Muc5B secretions in RPMI 2650 treated with (dsRNA) Poly(I:C) compared to the cells treated with Poly(I:C) alone ($p<0.0007$ and $p<0.0008$, respectively). Furthermore, MMW-HA significantly reduced the Muc5AC and Muc5B in RPMI 2650 stimulated with (dsRNA) Poly(I:C) in comparison to Poly(I:C) alone ($p<0.03$ and $p<0.006$, respectively). Finally, LMW-HA did not affect mucin secretions in RPMI 2650 stimulated with (dsRNA) Poly(I:C) (Fig. 5C, 5D).

Mucin mRNA

Although treatment with (dsRNA) Poly(I:C) 12.5, 25 and 50 µg/mL for 5 days affected the production of Muc5AC protein in ALI culture of RPMI 2650, we did not observe increased levels of Muc5AC mRNA (Fig. 6A). HA did not modify the basal values of Muc5AC mRNA in RPMI 2650 (Fig. 6B). However, the levels of Muc5B mRNA significantly increased in the cells stimulated with (dsRNA) Poly(I:C) 50 and 25 µg/mL in comparison to untreated cells ($p<0.0008$ and $p<0.05$, respectively) (Fig. 6C). Furthermore, HMW-HA significantly decreased the levels of Muc5B mRNA in RPMI 2650 stimulated with (dsRNA) Poly(I:C) 50 µg/mL in comparison to the cells stimulated with (dsRNA) Poly(I:C) 50 µg/mL alone ($p<0.002$). Finally, MMW-HA and LMW-HA showed considerably increased Muc5B mRNA expression ($p<0.04$ and $p<0.0001$, respectively) in the cells stimulated with (dsRNA) Poly(I:C) 50 µg/mL in comparison to the cells HMW-HA pretreated and stimulated with (dsRNA) Poly(I:C) 50 µg/mL (Fig. 6D).

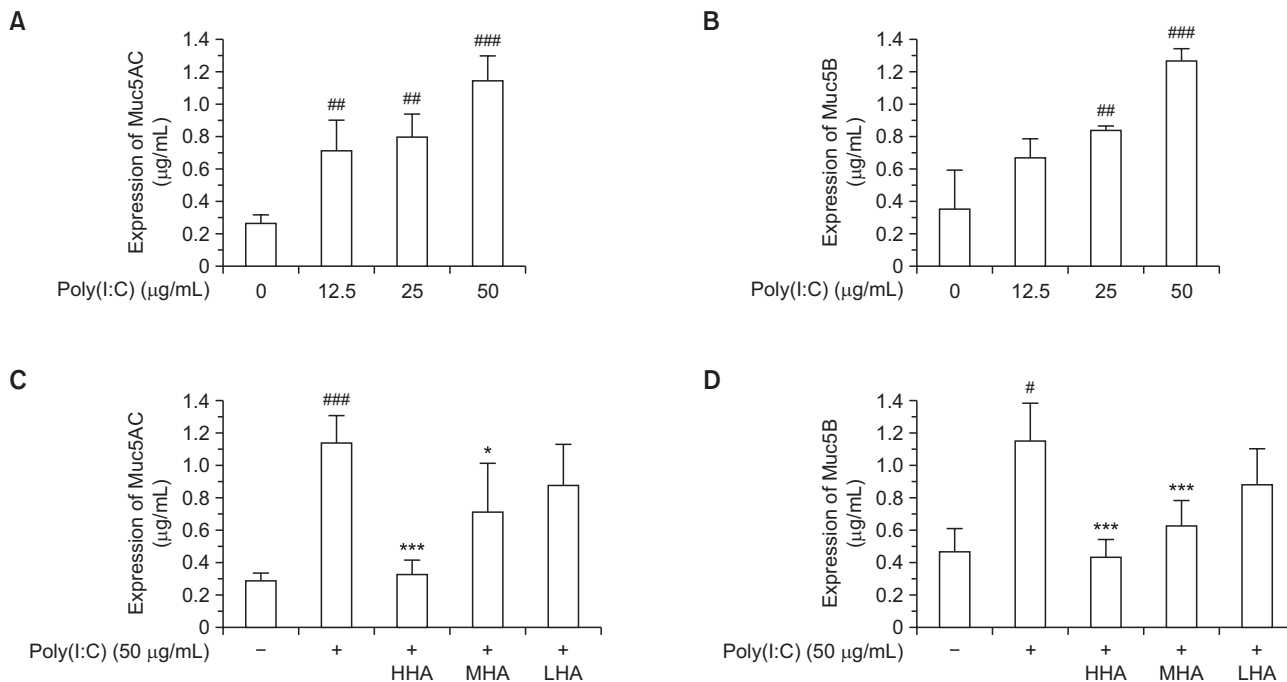


Fig. 5. Effect of Poly(I:C) stimulation on Muc5AC and Muc5B mucin secretion in the Apical wash of ALI culture of RPMI 2650 cells. RPMI 2650 were grown in ALI culture for 21 days and stimulated with Poly(I:C) (0 to 50 µg/mL) for 5 days: (A) dose-response of Muc5AC (n=3), and (B) Muc5B (n=3) secretion. Effect of different MW of HA (HMW-HA, MMW-HA, and LMW-HA) on (C) Muc5AC (n=3), and (D) Muc5B secretion (n=3) in RPMI 2650 cells stimulated with Poly(I:C) (50 µg/mL). Muc5AC and Muc5B concentration was assessed in the apical wash samples by using ELISA method (as described in Materials and Methods). Data are expressed as mean ± SD of the concentration expressed as µg/mL. Statistical analysis was performed by ANOVA followed by Fisher's PLSD correction. # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ vs. untreated cells, and * $p<0.05$ *** $p<0.001$ vs. Poly(I:C) (50 mg/mL)-treated cells.

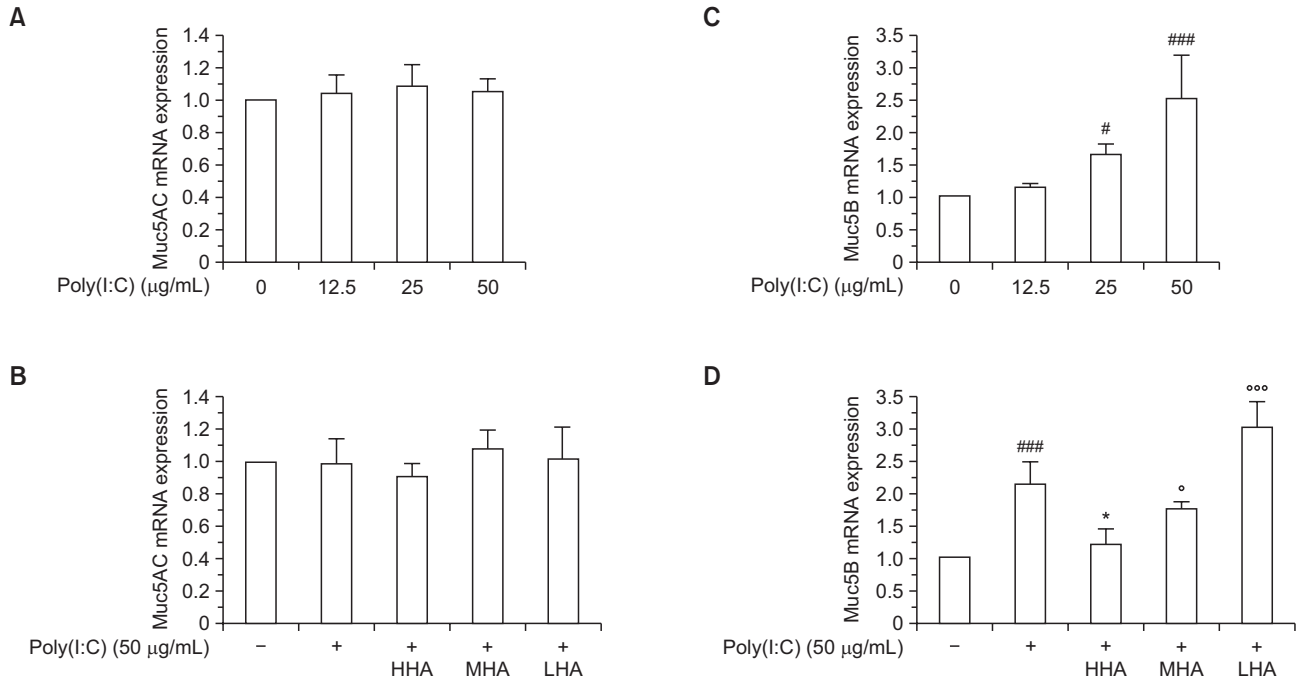


Fig. 6. Effect of Poly(I:C) stimulation on Muc5AC and Muc5B mRNA expression in the cells from ALI culture of RPMI 2650 cells. RPMI 2650 were grown in ALI culture for 21 days and stimulated with Poly(I:C) for 5 days (A) dose-response of Poly(I:C) (0 to 50 µg/mL) on Muc5AC (n=3), and (B) Muc5B (n=3) mRNA. Effect of different MW of HA (HMW-HA, MMW-HA, and LMW-HA) on (C) Muc5AC (n=3), and (D) Muc5B mRNA (n=3) in RPMI 2650 cells stimulated with Poly(I:C) (50 µg/mL). Total RNA was extracted and Muc5B mRNA expression was assessed by real-time PCR (see the Materials and methods section for details). Relative quantitation of mRNA was carried out with the comparative CT method ($2^{-\Delta\Delta Ct}$) and was plotted as fold-change compared to untreated cells chosen as the reference sample. Data are expressed as mean \pm SD of fold change over untreated cells. Statistical analysis was performed by ANOVA followed by Fisher's PLSD correction. # $p < 0.05$ and ### $p < 0.001$ vs. untreated cells, * $p < 0.05$ vs. Poly(I:C) (50 mg/mL)-treated cells, ° $p < 0.05$ and °°° $p < 0.01$ vs. Poly(I:C) (50 mg/mL)+HHA-treated cells.

Rheology data

Rheological parameters, both the elastic (G') and viscous (G'') moduli, significantly increased in RPMI 2650 stimulated with (dsRNA) Poly(I:C) 50 µg/mL in comparison to the cells treated with (dsRNA) Poly(I:C) 12,5 µg/mL, 25 µg/mL and untreated cells ($p < 0.0001$, $p < 0.0001$ and $p < 0.001$ respectively) (Fig. 7A). Furthermore, we observed that elastic (G') and viscous (G'') moduli appreciably diminished in RPMI 2650 pretreated with HMW-HA and stimulated with (dsRNA) Poly(I:C) 50 µg/mL (both $p < 0.0001$, $p < 0.0001$ and $p < 0.001$ respectively) in comparison to the cells stimulated with (dsRNA) Poly(I:C) 50 µg/mL alone (Fig. 7A). Additionally, we observed that elastic (G') and viscous (G'') moduli were significantly reduced in RPMI 2650 pretreated with MMW-HA or LMW-HA and stimulated with (dsRNA) Poly(I:C) 50 µg/mL (for both $p < 0.0001$, $p < 0.0001$ and $p < 0.001$ respectively) in comparison to the cells stimulated with (dsRNA) Poly(I:C) 50 µg/mL alone (Fig. 7B).

DISCUSSION

In our study, a 3D "in vitro" model of nasal epithelium supported the role of HMW-HA in the control of some physico-chemical and biological properties often involved in the damage of barrier integrity during viral infection in the upper airways. In particular, we studied the ability of HMW-HA to control the rheological properties (G' and G'') modulus, pH

measurements, Muc5AC and Muc5B proteins in the apical wash, and TEER values in ALI culture of RPMI 2650 cell line (nasal epithelial cell line) exposed to (dsRNA) Poly(I:C) (used extensively in the cellular *in vitro* models of viral infections) (Ambrus *et al.*, 2006). We provide evidence supporting the use of HMW-HA, rather than MMW-HA or LMW-HA, as a co-adjuvant of pharmacological treatment to alleviate the harmful effects of viral infections in the epithelial barrier of the nose (Fig. 8: Graphical abstract).

The airway epithelium represents an active interface against respiratory viruses through its immunological defenses, and acting as a physical barrier of airways, controls rapid virus clearance through its mucociliary apparatus. HA has physiological structural functions involved in homeostatic integrity, lubrication, and maintaining the structure of tissues (Dahiya and Kamal, 2013). Respiratory viruses have cytotoxic effects and enact elaborate strategies to evade the antiviral mechanisms of the immune responses. Therefore, viruses disrupt epithelial integrity, increasing the paracellular permeability and damaging the epithelial repair mechanisms (Vareille *et al.*, 2011). TJ and AJ contribute to the barrier function and to paracellular permeability through exclusive cooperation in the apicolateral membranes of the airway epithelium (Ganesan *et al.*, 2013). The intercellular junctions provide protection from inhaled pathogens, environmental insults, and external injury, promoting the regulation of gene expression, cell proliferation, and differentiation in the airway epitheliums of healthy sub-

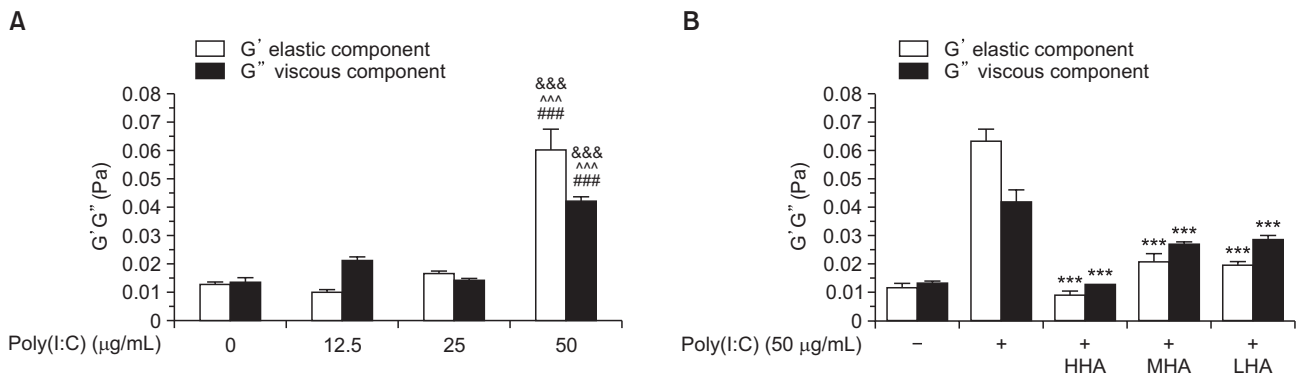


Fig. 7. Effect of Poly(I:C) on rheological parameters of viscoelasticity in the Apical Wash in RPMI 2650 cells. RPMI 2650 were grown in ALI culture for 21 days and stimulated with Poly(I:C) for 5 days: (A) dose-response of Poly(I:C) (0 to 50 μg/mL) (n=3), and (B) effect of different MW of HA (HMW-HA, MMW-HA, and LMW-HA) in RPMI 2650 cells stimulated with Poly(I:C) (50 μg/mL) on Elastic modulus (G') and Viscous modulus (G'') (n=3). Data are expressed as mean ± SD of G' or G'' (Pa). ###p<0.001 vs. untreated cells, ^^^p<0.001 vs. Poly(I:C) (25 mg/mL)-treated cells, &&&p<0.001 vs. Poly(I:C) (25 mg/mL)-treated cells, ***p<0.001 vs. Poly(I:C) (50 mg/mL)-treated cells.

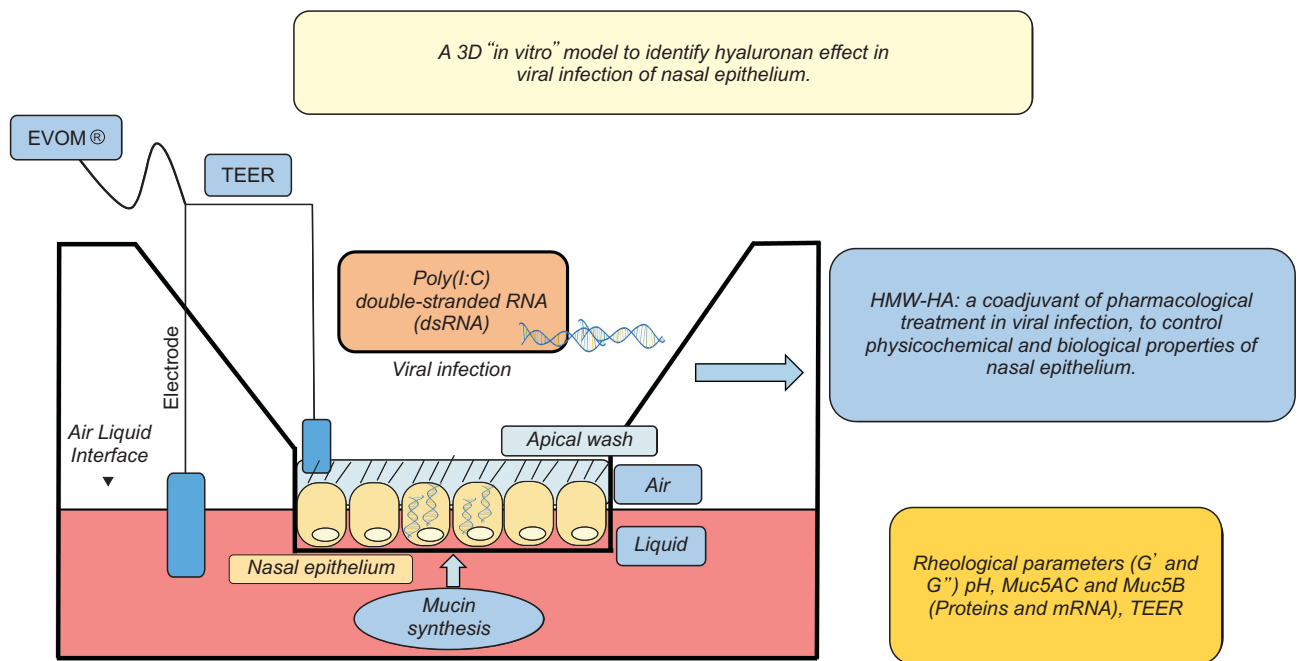


Fig. 8. Graphical abstract: Effect of HMW-HA on TEER measurements, pH values, rheological properties, and Muc5AC and Muc5B production in an "in vitro" model of nasal viral infection.

jects (Balda and Matter, 2009).

Cell culture systems are indispensable tools in "in vitro" research studies. The classical model of epithelial cell culture is a two-dimensional monolayer system (2D in vitro model) of adherent cells. However, a 2D in vitro model of epithelial cells does not reflect the in vivo situation, where cells develop within a complex three-dimensional (3D) microenvironment. Thus, 3D cell cultures were introduced to improve nasal and bronchial epithelial layer simulation in "in vitro" studies. The ALI culture system is a useful 3D tool to mimic the in vitro physiological processes of epithelial cells (Rayner et al., 2019). In fact, the 3D in vitro model of ALI culture of RPMI 2650 nasal cells develops a differentiated human nasal epithelium that can be used to study drug deposition and the permeation of

products (Pozzoli et al., 2016). This model represent an appropriate epithelial permeation barrier of the nose with sufficient TEER and TJ protein expression (Lee et al., 2005; Bai et al., 2009; Wengst and Reichl, 2010). Our previous studies showed that HMW-HA, rather than MMW-HA or LMW-HA, is involved in the treatment of nasal epithelium, acting as a classic anti-inflammatory/antioxidative drug in IL-17A-mediated nasal inflammation (Albano et al., 2016). In this scenario, the use of RPMI 2650 cell line cultured in ALI might be of interest to study the potential effect of HMW-HA on the physicochemical and biological properties of epithelium altered during viral infections. TEER value is a widely accepted method to measure the dynamics and integrity of TJ in 3D "in vitro" models of epithelial cell culture (Srinivasan et al., 2015). dsRNAs are a

molecular pattern produced by most viruses during their replication in viral infections and are universal viral molecules. dsRNA Poly(I:C) is a well-known ligand for TLR3, identified as the first antiviral TLR and involved in the recognition of dsRNA (Alexopoulou *et al.*, 2001). A synthetic analog of viral dsRNA Poly(I:C) induces airway inflammation by TLR3 (Ieki *et al.*, 2004). However, TLR3 is a crucial “danger” signaling receptor. It is involved in controlling the delicate balance between tolerance and inflammation, on the one hand, and inflammation and disease, on the other hand (Vercammen *et al.*, 2008). Since then, it has been observed that the long-term activation of TLR3 by Poly(I:C) induces inflammation and impairs lung function in mice (Stowell, 2009). For these reasons, to better understand the negative consequences of TLR3 activation in the nasal epithelial barrier used in our experimental procedures, we chose a longer exposure time (5 days) of RPMI 2650 to poly I:C. We found that Poly(I:C) reduced the values of TEER, and these values were restored by the pretreatment of RPMI 2650 cells with HMW-HA, rather than MMW-HA or LMW-HA. These findings might support that dsRNA Poly(I:C) mimic viral infections in ALI culture of RPMI2650 by TLR3 activation, generating epithelial barrier damage. The pre-treatment of the cells with HMW-HA promotes healing of tissues in ALI culture exposed to dsRNA Poly(I:C).

The pH measurement is a repeatable and practical method to obtain an objective parameter to assess the intact state of the nasal mucosa in the clinical setting (England *et al.*, 1999). A more alkaline nasal mucosa is of greater clinical interest, and some common allergens are instrumental in the alkalization of pH values in allergic rhinitis (Marsh *et al.*, 1981). Clinical symptoms of viral rhinitis (watery, rhinorrhea, nasal congestion) are similar to the symptoms of allergic rhinitis (Tantilipikorn, 2014). Both pathological conditions can affect the upper airways of a considerable numbers of patients. Since it has previously been shown that the pH increases in allergic rhinitis, a logical step is to study mucosal pH and its alteration in our “*in vitro*” model of nasal viral infection. We show that the stimulation of ALI culture of RPMI 2650 cell line with a synthetic analog of viral dsRNA Poly(I:C) shifts the pH of apical wash toward alkaline values. The pretreatment of the cells with HMW-HA, rather than MMW-HA or LMW-HA, restored the pH values in our “*in vitro*” model. Interestingly, our results might support the concept that the treatment of nasal epithelium with HMW-HA might control the status of nasal mucosa, stabilizing the process of alkalization of the air liquid surface during the rhinorrhea and congestion involved in nasal viral infections.

The RPMI 2650 cell line closely reflects the epithelium of human upper airways (karyotype, cytokeratin expression, secretion of mucoid materials), and it was previously used to study the interactions of cytokines and allergens with airway epithelial cells (Salib *et al.*, 2005; Reichl and Becker, 2012; Kreft *et al.*, 2015). Viral infections can increase mucin secretion and synthesis, promoting rheological changes (elastic G' and viscous G'' moduli) of the fluids. A defective accumulation of airway mucus alters the ciliary beating or uncoupling movement of the cilia due to changes in the liquid layer depth of the epithelium (Rogers, 2007). In subjects with rhinorrhea, these factors may lead to discomfort and coughing, and in extreme cases, complete blockage with mucus secretions can occur in both the upper and lower airways (Srinivasan *et al.*, 2015). There is therefore a need to improve therapeutic agents for

the treatment of mucociliary function under these pathophysiological conditions. New products and formulations for nasal delivery can be tested using 3D *in vitro* preclinical models (Srinivasan *et al.*, 2015). Accordingly, we demonstrated that Poly(I:C) increases the synthesis of glycoprotein Muc5AC/Muc5B, and rheological parameters (elastic G' and viscous G''), restored by HMW-HA, rather than MMW-HA or LMW-HA, in apical wash of ALI culture of the RPMI 2650 cell line. Our results suggest that HMW-HA might promote the blockage of mucus secretions and control the rheological changes of the liquid layer in 3D *in vitro* nasal epithelium. In this scenario, we might support the concept that HMW-HA treatment represents a coadjuvant to therapeutic agents to improve airway mucus accumulation and fluidity under viral infections of the nose. However, although the analysis of Muc5B mRNA obtained similar findings to that of Muc5B proteins, the Muc5AC mRNA does not appear to be modulate like its protein. The differences observed in Muc5AC and Muc5B mRNA profiles might be explained by the different timings of the posttranslational or posttranscriptional mechanisms of mucins. Therefore, further study might be necessary to clarify the different times of synthesis and transcription regulation of mucins in ALI culture of RPMI 2650 cell line stimulated with Poly(I:C).

pH values of nasal mucosa can affect the mucin networks (Bansil and Turner, 2006; Thornton *et al.*, 2008; Lai *et al.*, 2009; Ambort *et al.*, 2012). The pH of airway surface liquid is comparably alkaline in chronic bronchitis and rhinitis or more acidic in the cystic fibrosis or pneumonia, suggesting frequent breakdowns in the pH regulatory mechanisms in airway diseases (Fischer and Widdicombe, 2006). Muc5AC and Muc5B are acidic glycoproteins with a low isoelectric point (Fischer and Widdicombe, 2006), which should support the acidification of the airway surface liquid. However, in our “*in vitro* model”, despite the increased production of mucins, we observed the alkalization of apical wash of the ALI culture of RPMI 2650 stimulated with dsRNA Poly(I:C) in comparison to untreated cells. These conflicting results might suggest the existence of cellular mechanisms that reflect selective regulation of specific transporters of the cellular membrane and regulate nasal pH apical surface liquid (our apical wash), independently of acid mucin secretions. HA-HMW is able to control these mechanisms by playing a protective role in the alkalization of the epithelium that might be involved in nasal viral infections. However, further study might be necessary to better clarify these mechanisms.

In conclusion, we show the ability of HMW-HA to control physicochemical and biological properties, such as the TEER measurements of stratified cells, pH values, rheological properties, and Muc5AC and Muc5B production, in the apical wash of a 3D “*in vitro*” model of a nasal epithelial cell line (RPMI2650 ALI cultured) stimulated with dsRNA Poly(I:C) (Fig. 8). Altogether, our findings might provide evidence of the beneficial effects of HMW-HA (1,600 kDa) as a coadjuvant to classic pharmacological treatments of viral infections, supporting the control of some physicochemical and biological properties of the nasal epithelium involved in damaging the barrier. However, we suggest further studies be conducted to assess our observations in clinical practice.

CONFLICT OF INTEREST

Three co-authors of the paper – Luca Cavalieri, Martina Sammarco and Eleonora Ingrassia – are employees of Chiesi Farmaceutici S.p.A., Parma, Italy. There are no other competing interests for this study.

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