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**Effect of Vibrating Mesh Nebulizer Aerosol Technology on the In Vitro Activity of Ribavirin
Against Respiratory Syncytial Virus**

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Running head: Aerosolized Ribavirin for RSV

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Abstract

Background: Ribavirin is an antiviral drug that for many years has been administered to the lungs by aerosolization. Despite advancements in oral delivery routes, there has been a renewed interest in delivering ribavirin via the pulmonary system in select and the severely ill. The vibrating mesh nebulizer (VMN) was previously demonstrated to be an effective alternative to the small particle aerosol generator (SPAG) in particle size, chemical makeup, and concentrations of the ribavirin pre and post nebulization. However, the antiviral activity of ribavirin has never been examined. We sought to study ribavirin's activity pre and post nebulization via VMN. **Methods:** We grew and infected human epithelial type 2 cells (HEp2) and primary airway epithelial cells with respiratory syncytial virus (RSV). We then compared the antiviral effect of non-nebulized (control) and aerosolized ribavirin to untreated controls. We used traditional plaque assay and real-time polymerase chain reaction to determine the quantity of virus. **Results:** Both non-nebulized (control) and nebulized ribavirin reduced the size of RSV plaques compared to untreated controls. Additionally, the non-nebulized and nebulized ribavirin equally inhibited RSV replication. There were no statistically significant differences between the non-nebulized and nebulized ribavirin across all time points. **Conclusions:** The VMN nebulizer does not affect the antiviral properties of nebulized ribavirin when compared to non-nebulized drug. Our findings add supporting evidence for the use of the VMN in the administration of inhaled ribavirin.

Key Words: *Ribavirin, inhaled ribavirin, RSV, nebulization, nebulized, activity assay, airway epithelial cells*

Quick Look**Current knowledge**

Inhaled ribavirin has been conventionally delivered via the small particle aerosol generator (SPAG) nebulizer. However, the SPAG is infrequently used and considered cumbersome as it has multiple reusable parts that require cleaning, requires 50 psi outlet to operate, and has multiple configurations depending on application. In contrast, the newer vibrating mesh nebulizer (VMN) aerosol technology has advantages of being easy to use, does not require flow to operate and can be placed within multiple oxygen delivery or mechanical support applications. It has been reported previously that the VMN nebulizer produces similar performance to the SPAG. However, activity of ribavirin following nebulization has never been explored.

What this paper contributes to our knowledge

The VMN nebulizer does not affect the antiviral properties of ribavirin when compared to non-nebulized drug, adding to the evidence that the VMN is an effective alternative to the SPAG in the delivery of inhaled ribavirin. Since the VMN is frequently used, disposable, and does not require flow to operate, it may be a safer option particularly in the application of noninvasive or invasive mechanical ventilation.

Introduction

Ribavirin is a nucleoside analog that has had good in vitro activity against respiratory syncytial virus (RSV).¹ Ribavirin was developed for the treatment of RSV via inhalation and was approved by the FDA in 1986². Ribavirin was initially promising but failed to have any significant impact on the mortality or hospital duration in infants and young children.³ Despite the disappointing results of earlier studies, ribavirin has been shown to be an important treatment option in the management of RSV infections in patients with immunocompromised conditions associated with solid-organ and hematopoietic stem cell transplantation.⁴⁻⁷ Ribavirin has been shown to be effective at halting the progression of infection from the upper to the lower respiratory tract and reducing the morbidity and mortality associated with lower respiratory tract infections.² Ribavirin has been administered orally, intravenously, and by inhalation⁸⁻¹¹ and in combinations with intravenous immunoglobulin, palivizumab and/or glucocorticoids.^{5, 6} The latest strategy of a short duration, high dose, aerosolized ribavirin therapy (60 mg/mL), administered for 2 hours 3 times daily has been shown to be well tolerated by patients while achieving the same ribavirin blood and secretion levels as the previously recommended 20 hours of continuous therapy.¹² The shorter intermittent duration administration of aerosol has become the standard inhalation schedule as it has been proven to be equally effective as continuous delivery while reducing environmental contamination.^{13, 14}

Ribavirin was approved for inhalation with the pneumatic Small Particle Aerosol Generator (SPAG-2, Valeant Pharmaceuticals, Rochester, NY) device, which was initially designed for administration to small children via tent and later mechanical ventilation.¹⁵ Since the

introduction of the SPAG-2, there have been significant advances in aerosol technology. The most recent advancement has been the vibrating mesh nebulizer (VMN) aerosol generator that has been reported with a variety of interfaces common to acute and critical care applications (eg, mask, mouthpiece, high-flow nasal cannula, and both invasive and noninvasive ventilation).¹⁶⁻²⁶ The VMN is commercially available (Aerogen Solo, Galway, Ireland) and has been shown to deliver more aerosolized drug, such as albuterol, than traditional jet nebulizers in both neonatal, pediatric, and adult models.²⁷

Since the VMN nebulizer is more commonly used than the SPAG-2, we felt from an efficiency perspective that it would improve the care of these fragile patients if it can be proven to be equivalent to the SPAG-2. We previously published on the device performance, chemical makeup, and concentration of ribavirin pre and post nebulization between the SPAG-2 and VMN in 2016.²⁸ One question that remains was whether the activity of ribavirin following nebulization by VMN remained the same. Therefore, the goal of this study was to determine if the VMN affects the activity of ribavirin on RSV-infected immortalized human epithelial and primary airway epithelial cells.

Methods

Ribavirin (Sigma-Aldrich, Cleveland, OH) was dissolved in normal saline at 3 mg/mL and aerosolized using a VMN nebulizer driven by an electronic control module (Aerogen Solo and Aerogen ProX controller, Aerogen, Galway, Ireland). Condensation of aerosol was collected aseptically in a 15-mL centrifuge tube, into which the nebulizer was placed. For RSV cultures in

the HEP-2 cell line (obtained from American Type Culture Collection (ATCC), Manassas, VA), the cells were seeded onto 6-well plates at 2×10^6 cells per well in 2 mL of Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum. When wells were 70-80% confluent, RSV (Long strain, ATCC) was inoculated at a multiplicity of infection (MOI) of 0.01 or 0.05.

Inoculum was removed after 4 hours of incubation, and cells were cultured in 3 mL of DMEM per well with 2% fetal bovine serum with or without ribavirin (3 $\mu\text{g}/\text{mL}$ which is the ED50 according to previous in vitro studies). Since we have previously shown that VMN does not affect the concentration of ribavirin as determined chemically, the same volume of nebulized and non-nebulized ribavirin stock was used.^{29, 30} The effect of the non-nebulized ribavirin solution, aerosol collection, or the same volume of normal saline were compared in quadruplicate wells. Culture medium was removed daily (freeze for plaque assay) and replaced with 3 mL of fresh medium containing ribavirin at the same concentration.

For RSV cultures in primary human bronchial/tracheal epithelial cells (PBEC, obtained from ATCC), the cells were seeded onto 12-well plates at 1.7×10^5 cells per well in 1 mL of complete Airway Epithelial Cell Medium (ATCC). When wells were 70-80% confluent, cells were infected with RSV at an MOI of 0.02 or 0.2. After 4 hours, the inoculum was removed and replaced with 1 mL of complete medium with or without ribavirin (3 $\mu\text{g}/\text{mL}$). Nebulized and non-nebulized control ribavirin as well as untreated cells were compared in quadruplicate wells. Medium from each well was removed daily for plaque assay and replaced with 1 mL of fresh medium containing ribavirin at the same concentration.

For viral plaque assay, medium samples were centrifuged at 3,000 g for 15 minutes to remove cell debris. After dilution in serum-free DMEM, 100 μ L of medium sample diluted with 400 μ L of DMEM was used to inoculate confluent HEp-2 cells in 12-well plates. After four hours of incubation, inoculum was removed and overlaid with 0.3% low-melting-point agarose (Invitrogen, Waltham, MA) in serum-free DMEM. After 6-7 days of incubation at 37°C and 5% carbon dioxide, cells were fixed with 1% formaldehyde in normal saline, stained with 0.05% neutral red, and fusogenic plaques were counted macroscopically. Dubious plaques were confirmed by microscopic examination.

For quantitative polymerase chain reaction, when cytopathic effect was widespread in control wells and microscopic plaques started to appear in ribavirin-treated wells, culture medium was completely removed and total cell RNA was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD). Reverse transcription was carried out with the GoScript Reverse Transcription System (Promega, Durham, NC). Primers for reverse transcription included the oligodT primer (0.25 μ g/reaction), random primers (0.25 μ g/reaction), and an RSV-specific primer (nucleotides 1140-1157 of the long strain at 700 nM). $MgCl_2$ concentration was 1.5 mM. Chain extension was carried out at 42°C for 60 min. Polymerase chain reaction was performed using the GoTaq qPCR Master Mix (Promega). Forward primer was the same as the reverse transcription primer. Reverse primer was nucleotides 1431-1452. Final concentration of both primers was 300 nM. The gene for tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta (YWHAZ) was used as the internal reference with published primers.³¹

Using a LightCycler 96 System (Roche), the amplification program was 95°C 2', 45 cycles of 95°C 15", 57°C 15", 72° 30", 78 5". Plaque numbers and intracellular viral RNA levels of quadruplicate samples were compared using the Mann-Whitney U test. Statistical significance was called at $p < 0.05$.

Results

We first examined the protective potency of nebulized ribavirin against RSV-induced cytopathic effect on the HEp-2 line. Both non-nebulized and nebulized ribavirin (3 µg/mL) reduced the size of RSV plaques in HEp-2 cells compared to untreated control cultures (Figure 1). Development of cell fusion and plaques were delayed in the presence of ribavirin. There was no obvious difference between treatments with non-nebulized ribavirin and nebulized ribavirin.

We found the long strain of RSV strongly cytopathic in PBEC. At MOI of 0.2, almost all cells fused into giant syncytia in 3 days, which was partially prevented by either non-nebulized or nebulized ribavirin at 3 µg/mL (Figure 2A). At MOI of 0.02, almost all untreated cells developed CPE in the form of fusion, rounding, or detachment within 5 days (Figure 2B). In the presence of non-nebulized or nebulized ribavirin, syncytia of infected cells formed a meshwork surrounding healthy cells or cells in early stages of fusion. There was no obvious morphological difference in infected cultures treated with non-nebulized or nebulized ribavirin.

We analyzed the growth kinetics of RSV in HEp-2 and PBEC cultures using a plaque assay in HEp-2 cells. As seen in Figures 3 and 4, non-nebulized and nebulized ribavirin equally reduced the number of plaques in the viral cultures. In the case of HEp-2 cultures at MOI of 0.05, plaque

numbers from untreated and treated cells became significantly different after 4 days (Figure 4A, $P < 0.05$), and there was no statistically significant difference between non-nebulized and nebulized ribavirin at all time points. In the case of PBECs at MOI of 0.2, plaque numbers from untreated and treated cells became significantly different after 2 days (Figure 4B, $P < 0.05$), and there was no significant difference between non-nebulized and nebulized ribavirin at all time points.

At the end of the infections, we quantified intracellular RSV RNA using real-time reverse-transcription polymerase chain reaction. As shown in Figure 5, intracellular viral RNA was significantly more abundant in untreated cultures ($P < 0.05$ for both HEP-2 and PBEC cultures). In both cell types, the virustatic effect of nebulized and non-nebulized ribavirin was not statistically significant as determined by measurements of intracellular viral RNA.

Discussion

Generic nebulizers are used indiscriminately with inhaled medication based on cost and perceived benefits, often without regard to the nebulizer used in the FDA drug approval process of a medication. As new technologies come along, we must be careful in our assumptions and not only look at aerosol generation features, but what it could possibly do to the medication administered. It is well known that concentration, air flow or pressure, volume and temperature play a role on output of nebulizers. We have previously compared the device performance and chemical makeup pre and post nebulization between the FDA approved

nebulization delivery device (SPAG-2) and the VMN, and here we explored the intended activity (therapeutic aim) of the medication post nebulization. This step is often not taken as it is assumed. In the case of ribavirin, we felt it is only proper to explore whether the activity of this expensive drug is affected by the nebulizer. Such data would allow us to confidently conclude whether ribavirin can be effectively nebulized with VMN without negatively affecting the drug itself.

Ribavirin may inhibit multiple viral and cellular enzymes involved in viral replication, and the spectrum of ribavirin targets may differ in various host cells.³² Moreover, as a nucleoside analog, the virustatic effect of ribavirin depends on phosphorylation by cellular enzymes.³³ If the drug is altered during nebulization in any way that affects recognition by target enzymes or phosphorylation enzymes, its virustatic activity will vary depending on the cell type to which it is applied. In the case of RSV, the human epithelial cell line, HEP-2, has been the standard tool for its propagation and titration. Consequently, we first studied the virustatic effect of nebulized and non-nebulized ribavirin in this cell line and showed that nebulization does not alter its potency. However, the bronchial epithelial tissue is the natural site of infection, and it is known that RSV replicates differently in primary bronchial epithelial cells than in HEP-2. To assess the therapeutic potency of nebulized ribavirin in the treatment of bronchiolitis, the drug was applied to RSV-infected primary human bronchial/tracheal epithelial cells (PBEC). Cytopathic effect (CPE) of RSV in PBEC is not as well documented as in HEP-2 cells. Clinical isolates of RSV tend to induce minimal morphological changes in airway epithelium *in vitro*, producing low titers of progeny viruses, although the prototypic RSV A2 strain is strongly

cytopathic and more productive in PBEC.^{34, 35} We found that the Long strain is strongly cytopathic in PBEC, produced high titers of progeny viruses in the culture medium, and the protective effect of ribavirin in these cells is not altered by VMN aerosolization.

Since we used only one laboratory strain of RSV, we do not yet know the effect of VMN on the potency of ribavirin against clinical strains, although a correlation can be assumed based on previous studies of ribavirin against various RSV strains.^{29, 30} In addition, we compared the effect of nebulized and non-nebulized ribavirin on RSV replication by continuous exposure *in vitro*, so we do not yet know if nebulization would affect the pharmacokinetics of ribavirin in the human body when the drug is inhaled intermittently. Finally, our study was solely based on the Aerogen product, and one should exercise caution in extrapolating the results to other nebulizer technologies.

Conclusions

The VMN nebulizer did not affect the antiviral properties of nebulized ribavirin when compared to the non-nebulized ribavirin. Our findings add supporting evidence of the utility of the VMN nebulizer in the administration of inhaled ribavirin. These data conclude our evaluation for the VMN nebulization of ribavirin and provides an approach to future evaluation of nebulizers and their effects on the medication they are delivering.

Figure 1. Effect of non-nebulized and nebulized ribavirin in protection against cytopathic effects of RSV on HEp-2. Cells were infected at MOI of 0.05 for 4 days. Images taken at 200x.

Figure 2: Effect of non-nebulized and nebulized ribavirin in protection against cytopathic effects of RSV on PBECs. A: Cells were infected at MOI of 0.2 for 3 days. Images were taken at 200x. B: Cells were infected at MOI of 0.02 for 5 days. Images were taken at 40x and 200x.

Figure 3. Effect of non-nebulized and nebulized ribavirin on RSV replication. Representative plaque assays of HEp-2 cell supernatant after 4 days of RSV infection at MOI of 0.05. Plaque assay was conducted using HEp2 cells. Each well received 0.1 mL of supernatant at 100-fold dilution.

Figure 4. Growth kinetics of RSV. A: HEp-2 cells infected at MOI of 0.05 for 4 days. B: PBECs infected at MOI of 0.2 for 3 days. Quadruplet samples are presented as box plot. Boxes encompass the interquartile range with the median at the junction of orange and grey areas. Whiskers encompass minimum and maximum values.

Figure 5. Quantification of intracellular RSV RNA. A: RSV RNA in HEp-2 cells after 3 days of infection at MOI of 0.01. B: RSV RNA in PBECs after 3 days of infection at MOI of 0.2. Quadruplet samples are presented as box plot. Boxes encompass the interquartile range with the median at the junction of orange and grey areas. Whiskers encompass minimum and maximum values.

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Figure 1.

Figure 2A

Uninfected

Infected
No ribavirin

Non-
nebulized

Nebulized

Figure 2B

Figure 3.

Figure 4.

Figure 5.