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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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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 interested in delivering ribavirin via the pulmonary system in select patients and the severely ill. The vibrating mesh nebulizer was previously demonstrated to be an effective alternative to the small-particle aerosol generator in particle size, chemical makeup, and concentrations of the ribavirin before and after nebulization. However, the antiviral activity of ribavirin has never been examined. We sought to study ribavirin's activity before and after nebulization via vibrating mesh nebulizer. METHODS: We grew and infected human epithelial type 2 cells 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 vibrating mesh nebulizer did not affect the antiviral properties of nebulized ribavirin when compared to non-nebulized drug. Our findings add supporting evidence for the use of the vibrating mesh nebulizer in the administration of inhaled ribavirin. *Key words: ribavirin; inhaled ribavirin; RSV; nebulization; nebulized; activity assay; airway epithelial cells.* [Respir Care 0;0(0):1–●. © 0 Daedalus Enterprises]

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 1986.[2] Ribavirin was initially promising but failed to have any significant impact on the mortality or hospital length of stay 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 inhalation[8-11] 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 h 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 h of continuous therapy.[12] The shorter intermittent duration administration of aerosol has become the standard inhalation schedule as it has been

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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 device, which was initially designed for administration to small children via tent and later during mechanical ventilation.[15] Since the introduction of the small-particle aerosol generator-2, there have been significant advances in aerosol technology. The most recent advancement has been the vibrating mesh nebulizer 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 vibrating mesh nebulizer is commercially available and has been shown to deliver more aerosolized drug, such as albuterol, than traditional jet nebulizers in neonatal, pediatric, and adult models.[27]

Since the vibrating mesh nebulizer is more commonly used than the small-particle aerosol generator-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 small-particle aerosol generator-2. We previously published on the device performance, chemical makeup, and concentration of ribavirin before and after nebulization between the small-particle aerosol generator-2 and vibrating mesh nebulizer in 2016.[28] One question that remains was whether the activity of ribavirin following nebulization by vibrating mesh nebulizer remained the same. Therefore, the goal of this study was to determine if the vibrating mesh nebulizer affects the activity of ribavirin on RSV-infected immortalized human epithelial and primary airway epithelial cells.

QUICK LOOK

Current knowledge

Inhaled ribavirin has been conventionally delivered via the small-particle aerosol generator nebulizer (SPAG). 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 vibrating mesh nebulizer 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 vibrating mesh nebulizer produces similar performance to the small-particle aerosol generator.

What this paper contributes to our knowledge

The vibrating mesh nebulizer did not affect the antiviral properties of ribavirin when compared to non-nebulized drug, adding to the evidence that the vibrating mesh nebulizer is an effective alternative to the (SPAG) in the delivery of inhaled ribavirin. Since the vibrating mesh nebulizer is commonly 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.

Methods

Ribavirin (Sigma-Aldrich, Cleveland, Ohio) was dissolved in normal saline at 3 mg/mL and aerosolized using a vibrating mesh nebulizer driven by an electronic control module (Aerogen Solo and Aerogen Pro-X 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 human epithelial type 2 (HEp-2) cell line (ATCC, Manassas, Virginia), the cells were seeded onto 6-well plates at 2 x 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 h of incubation, and cells were cultured in 3 mL of DMEM per well with 2% fetal bovine serum with or without ribavirin (3 μg/mL, which is the ED50 according to previous in vitro studies). Since we have previously shown that vibrating mesh nebulizer 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 was compared in quadruplicate wells. Culture medium was removed daily (frozen 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 (PBECs, ATCC), the cells were seeded onto 12-well plates at 1.7 x 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 h, the inoculum was removed and replaced with 1 mL of complete medium with or without ribavirin (3 μg/mL). Nebulized and non-nebulized control ribavirin as well as untreated cells was 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 min to remove cell debris. After dilution in serum-free DMEM, 100 µL of medium sample diluted with 400 µL of DMEM was used to inoculate

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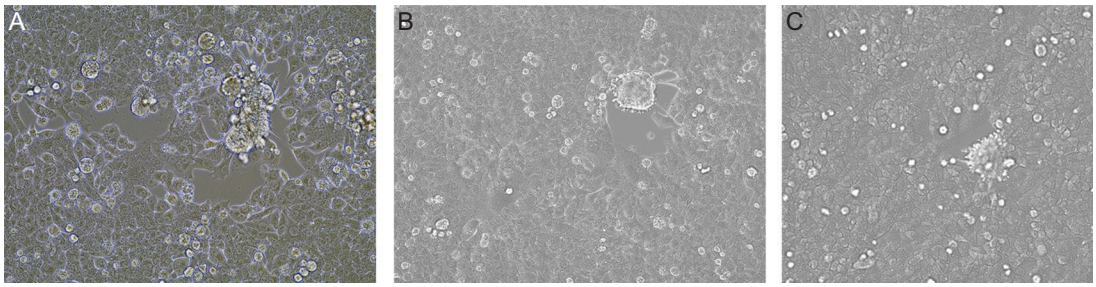

Fig. 1. Effect of non-nebulized and nebulized ribavirin in protection against cytopathic effects of respiratory syncytial virus on human epithelial type 2 cells. Cells were infected at multiplicity of infection of 0.05 for 4 days. Images taken at 200x. A: No ribavirin, B: non-nebulized, C: nebulized.

confluent HEp-2 cells in 12-well plates. After 4 h of incubation, inoculum was removed and overlaid with 0.3% low melting point agarose (Invitrogen, Waltham, Massachusetts) in serum-free DMEM. After 6–7 d of incubation at 37°C and 5% carbon dioxide, cells were fixed with 1% formalin 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 (CPE) 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 Maryland). Reverse transcription was carried out with the GoScript Reverse Transcription System (Promega, Durham, North Carolina). Primers for reverse transcription included the oligo(dT) primer (0.25 μg/reaction), random primers (0.25 μg/reaction), and an RSV-specific primer (nucleotides 1,140–1,157 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 1,431–1,452. Final concentration of both primers was 300 nM. The gene for tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ was used as the internal reference with published primers.[31] Using a LightCycler 96 System (Roche, Basel Switzerland), the amplification program was 95°C for 2 min, 45 cycles of 95°C for 15 s, 57°C for 15 s, 72°C for 30 s, and 78°C for 5 s, Plaque numbers and intracellular viral RNA levels of quadruplicate samples were compared using the Mann-Whitney U test. Statistical significance was called at $P < .05$.

Results

We first examined the protective potency of nebulized ribavirin against RSV-induced CPE 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 (Fig. 1). Development of cell fusion and plaques was 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 PBECs. At MOI of 0.2, almost all cells fused into giant syncytia in 3 d, which was partially prevented by either non-nebulized or nebulized ribavirin at 3 μg/mL (Fig. 2A). At MOI of 0.02, almost all untreated cells developed CPE in the form of fusion, rounding, or detachment within 5 d (Fig. 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 d (Fig. 4A, $P < .05$), 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 d (Fig. 4B, $P < .05$), 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 ($P < .05$ for both HEp-2 and PBEC cultures). In both cell types, he virustatic effect of nebulized and non-nebulized ribavirin was not statistically significant as determined by measurements of intracellular viral RNA.

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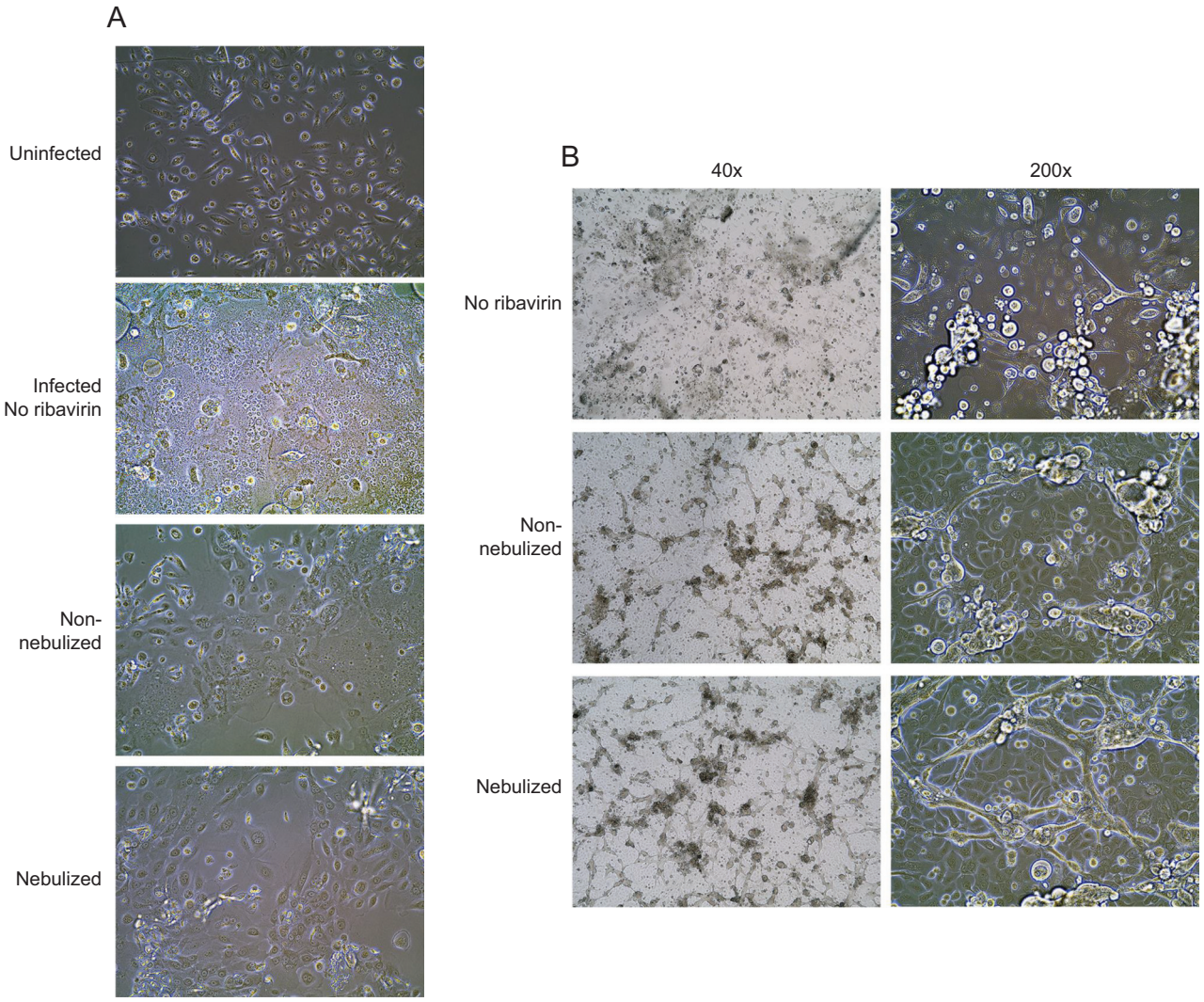

Fig. 2. Effect of non-nebulized and nebulized ribavirin in protection against cytopathic effects of respiratory syncytial virus on primary human bronchial/tracheal epithelial cells. A: Cells were infected at multiplicity of infection (MOI) of 0.2 for 3 days. Images were taken at 200x. B: Cells were infected at MOI of 0.02 for 5 days. Images were taken at 40x and 200x.

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 in nebulizer output. We have previously compared the device performance and chemical makeup before and after nebulization between the FDA-approved nebulization delivery device (small-particle aerosol generator-2) and the vibrating mesh nebulizer, 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 vibrating mesh nebulizer 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

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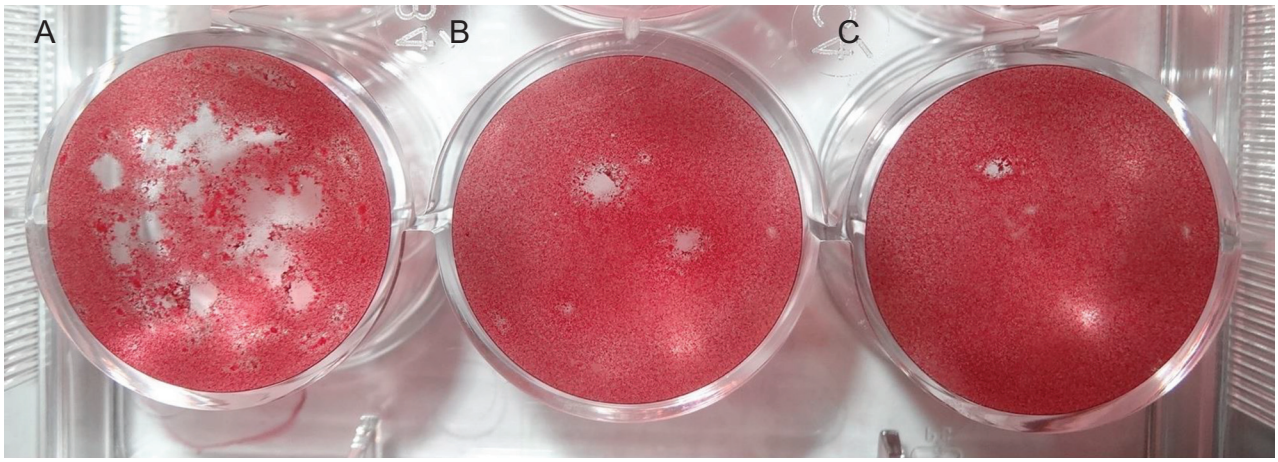

Fig. 3. Effect of non-nebulized and nebulized ribavirin on respiratory syncytial virus (RSV) replication. Representative plaque assays of human epithelial type 2 (HEp2) cell supernatant after 4 days of RSV infection at multiplicity of infection of 0.05. Plaque assay was conducted using HEp2 cells. Each well received 0.1 mL of supernatant at 100-fold dilution. A: untreated, B: nebulized, C: non-nebulized.

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 PBECs than in HEp-2. To assess the therapeutic potency of nebulized ribavirin in the treatment of bronchiolitis, the drug was applied to RSV-infected PBECs.

CPE of RSV in PBECs 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 PBECs.[34,35] We found that the long strain is strongly cytopathic in PBECs, produces high titers of progeny viruses in the culture medium, and the protective effect of ribavirin in these cells is not altered by vibrating mesh nebulizer aerosolization.

Since we used only one laboratory strain of RSV, we do not yet know the effect of vibrating mesh nebulizer 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

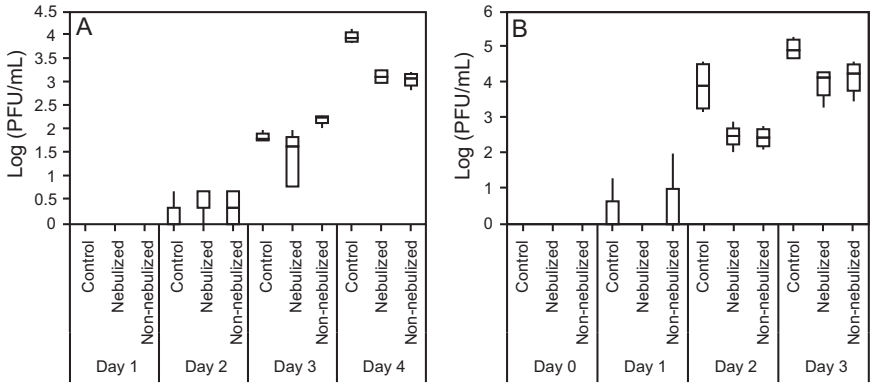

Fig. 4. Growth kinetics of respiratory syncytial virus. A: Human epithelial type 2 cells infected at multiplicity of infection (MOI) of 0.05 for 4 days. B: Primary human bronchial/tracheal epithelial cells infected at MOI of 0.2 for 3 days. Quadruplet samples are presented as box plot. Boxes encompass the interquartile range with the median at the center lines. Whiskers encompass minimum and maximum values.

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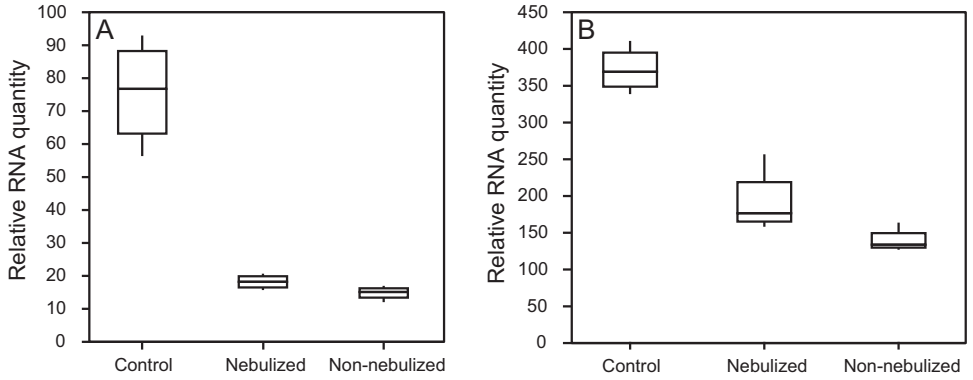

Fig. 5. Quantification of intracellular respiratory syncytial virus (RSV) RNA. A: RSV RNA in human epithelial type 2 cells after 3 days of infection at multiplicity of infection (MOI) of 0.01. B: RSV RNA in primary human bronchial/tracheal epithelial cells after 3 days of infection at MOI of 0.2. Quadruplet samples are presented as box plot. Boxes encompass the interquartile range with the median at the center lines. Whiskers encompass minimum and maximum values.

product, and one should exercise caution in extrapolating the results to other nebulizer technologies.

Conclusions

The vibrating mesh 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 vibrating mesh nebulizer in the administration of inhaled ribavirin. These data conclude our evaluation for the vibrating mesh nebulizer nebulization of ribavirin and provide an approach to future evaluation of nebulizers and their effects on the medication they are delivering.

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