Optimized Aerosol Delivery to a Mechanically Ventilated Rodent

Ronan J. MacLoughlin, H.Dip, B.Sc.,1,2 Brendan D. Higgins, Ph.D.,1,3 John G. Laffey, M.D.,1,3 and Timothy O’Brien, M.D., Ph.D.1,2

Abstract

Background: Aerosol delivery through an endotracheal tube during mechanical ventilation of small animals, simulating neonates and small infants, has shown to be influenced by a variety of factors including aerosol generator type, droplet/particle size, ventilator circuitry and ventilation regime. A review of the literature indicates that reported aerosol deposition rates in rodents are quite low, with lung deposition in anesthetized, mechanically ventilated rats reported to be ~3.9 and ~8% in anesthetized, spontaneously breathing rats. The optimization of aerosol delivery to both in vitro and in vivo models of anesthetized mechanically ventilated rodents is described in this study.

Methods: Characterization and optimization of the in vitro system performance relied on gravimetric analysis, laser diffraction droplet sizing, and spectrophotometric analysis of drug mass on inspiratory filters. The optimized setup was subsequently employed in vivo to determine deposition of a tracer aerosol in the rat lung.

Results: In vitro testing confirmed that droplet size, ventilation regimen, breath actuation setting, and the inclusion of a drug recycling step had the greatest effect on inhaled mass. During testing, improvements of up to 41% were seen in inhaled mass values between runs with the addition of a recycling step. The negative effects of the aerosolization process on albuterol sulphate were minimal. In vitro deposition rates of 29.95 ± 1.54% of the original dose were recorded (n = 3). In vivo deposition rates of Evans blue were highly comparable (30.88 ± 5.73%) (n = 6). Intratracheal instillation of the tracer dye resulted in deposition of 87.34 ± 6.23% of the original dose.

Conclusions: This optimized experimental setup allows for greater inhaled mass than previously reported. The addition of a recycling step may prove to be a significant improvement in achieving higher deposition in mechanically ventilated lungs; however, the suitability of the test agent for repeated nebulization needs assessment.

Key words: aerosol, vibrating mesh, rodent, mechanical ventilation, deposition, inhaled efficiency, nebulizer, albuterol sulphate, droplet size, Evans blue

Introduction

Small animals are increasingly being used as highly relevant models for the investigation of clinical pulmonary disease and the therapeutic utility of various agents.1–4 The route of treatment delivery to the lungs is typically intratracheal, and delivery can be achieved through a choice of techniques, including instillation and aerosolization. The delivery technique employed will have a direct bearing on the deposition profile in the lung, and is therefore a very important factor in the potential effectiveness of the therapy. The location of deposition of an aerosol in the lung may have significant implications on the absorption profile and the resulting pharmacokinetics with deposition in the respiratory airways, typically resulting in greater absorption than that seen in the conducting airways.5–8 Direct liquid instillation has been shown to deposit liquid formulations in a heterogeneous pattern, localizing predominantly in the larger airways, while aerosolization results in a more diffuse, homogeneous pattern with deposition in the gas exchanging smaller airways and alveolar regions.9,10 However, it is important to note that aerosolization is a less efficient system of delivery, with lung deposition in anesthetized, mechanically ventilated rats previously reported to be approximately

1Regenerative Medicine Institute (REMEDi), National University of Ireland, Galway, Ireland.
2Department of Medicine, National University of Ireland, Galway, Ireland.
3Department of Anaesthesia, National University of Ireland, Galway, Ireland.
3.9%\(^\text{a}\) and approximately 8% in anesthetized, spontaneously breathing rats.\(^\text{b}\) This inefficiency is a result of inertial impaction of droplets throughout the aerosol delivery system, coupled with exhalation of droplets and the residual unnebulized volume of test agent remaining in the aerosol generator. Direct instillation provides a more precise dose, and so is generally the delivery method of choice in pharmacokinetic studies; however, aerosolization permits noninvasive delivery, a key element in therapies for chronic diseases.

Although considered technically difficult, endotracheal delivery using a mechanical ventilation system may be more efficient than other aerosol delivery methods as it allows for direct delivery of the agent below the vocal cords. In contrast, nose cone and whole-body systems do not supply the aerosol directly to the lungs and so incur losses through nasal deposition.\(^\text{c}\) Furthermore, mechanical ventilation provides a method for maintaining efficient gas exchange, especially important when respiratory depressant anesthetics are used, and facilitates easy monitoring of physiologic metrics during treatment.

Aerosol delivery during mechanical ventilation has been shown to be affected by a number of variables. Type of aerosol generator, aerosol generation pattern, droplet size, and ventilator parameters (tidal volume, inspiratory flows, and I:E ratios) have been shown to impact the efficiency of aerosol drug delivery to the lung during mechanical ventilation in humans.\(^\text{d}\) Delivery of therapeutic agents via aerosol necessitates a system design that allows maximal respirable aerosol through the system into the lungs. A large amount of the test agent that is not deposited in the lung rains out in the ventilator circuit and becomes unavailable for inhalation as an aerosol. The ability to recover and renebulize this material could potentially increase delivery to the lung. This is an especially important point in regard to the evaluation or use of expensive formulations or agents. The option to recycle this material requires an aerosol generator that does not alter the drug even after repeated nebulization. Jet nebulizers can generate shearing forces that can damage complex molecules and tend to increase concentration of formulations, while ultrasonic nebulizers can heat formulations, potentially causing heat damage to susceptible formulations.\(^\text{e}\) Consequently, a protocol was developed to include an evaluation of the impact of “recycling” rained out aerosol from the ventilator circuit on lung delivery. To identify the most efficient way to deliver aerosol to an intubated Sprague-Dawley rat, we constructed an in vitro model of mechanical ventilation, and systematically evaluated the variables that have previously been shown to impact delivery efficiency during mechanical ventilation in other species. Those variables tested included aerosol droplet size, ventilator regimen, breath actuation settings, and the recycling of rainout. The effects of those variables have not been fully described in the literature to date with respect to aerosol delivery in rats. The optimized aerosol delivery system was subsequently applied in vivo to determine deposition of a tracer aerosol in the lungs of Sprague-Dawley rats.

Materials and Methods

Mechanical ventilation

The setup for mechanical ventilation to evaluate aerosol delivery of various agents to the lungs of Sprague-Dawley rats is represented in Figure 1. The ventilation parameters tested were selected to work within the physiologic parameters of adult Sprague-Dawley rats in the 350–500 g range (Table 1).

The SAR-830/6P (CWE Inc., Ardmore, PA) small animal ventilator was used and was operated in a volume control mode with room air. The nebulizers used were breath actuated Aeroneb\(^\text{f}\) Pro nebulizers (Aerogen Ireland Ltd, Galway, Ireland), containing the Aerogen Inc. OnQ\(^\text{g}\) vibrating aerosol generator. The vibrating mesh nebulizer is driven by an electronic controller that is integrated with a microprocessor and flow or pressure sensor that allows for adjustable, timed generation of the aerosol within the breath. Aerosol can be generated at any time point throughout the breath but typically during inspiration. Generation during expiration has also been reported.\(^\text{h}\) The breath actuation controller used with the nebulizer in the series of experiments described below allows for variation of the actuation settings based on pressure changes in the ventilation circuit.

Given the relatively small-bore diameter of the ventilator tubing, compared to that used in clinical settings, and the effect this would have on impaction of droplets, and subsequent deposition efficiencies, the individual nebulizers were chosen based on the manufacturer’s droplet size specification, indicating low droplet sizes [volumetric mean diameter (VMD)] that is, 3.38, 4.29, and 5.21 \(\mu\)m. These VMD results were verified using the Malvern Spraytec Particle analyzer (Malvern Instruments Ltd., Malvern, UK). The test nebulizers selected also had a flow rate of less than 0.2 mL/min measured with albuterol sulphate.

The ventilator circuit was composed of oxygen tubing (4 mm i.d./7 mm o.d) (Venticare Oxygen tube, Flexicare Medical, Glamorgan, UK). A pressure transducer from the

![FIG. 1. Diagram of ventilation and nebulization system used throughout optimization experiments. (1) CWE Sar-830/AP ventilator, (2) inspiratory limb, (3) Aeroneb Pro nebulizer, (4) T-piece, (5) Aeroneb Pro breath-actuated controller, (6) pressure transducer, (7) Y-piece, (8) 14-gauge endotracheal tube, (9) expiratory limb.](image)

### Table 1. Range of Ventilator Regimen Tested during Optimization Experiments

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Breath rate per minute</th>
<th>I:E ratio</th>
<th>Tidal volume per breath (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>90</td>
<td>2:1</td>
<td>2.7</td>
</tr>
<tr>
<td>b.</td>
<td>90</td>
<td>1:2</td>
<td>1.5</td>
</tr>
<tr>
<td>c.</td>
<td>90</td>
<td>1:1</td>
<td>2.0</td>
</tr>
<tr>
<td>d.</td>
<td>45</td>
<td>2:1</td>
<td>6.0</td>
</tr>
<tr>
<td>e.</td>
<td>45</td>
<td>1:2</td>
<td>2.0</td>
</tr>
<tr>
<td>f.</td>
<td>45</td>
<td>1:1</td>
<td>4.0</td>
</tr>
</tbody>
</table>
nebulizer controller attached to a y-piece (CWE Inc), registered the pressure increase on inhalation (positive pressure ventilation) and the drop in pressure on expiration. Positive end expiratory pressure (PEEP) was set at 2.0 cmH2O. A 14-gauge catheter was used as an endotracheal tube (BD Inspyle™ catheter, BD Becton-Dickinson, Oxford, UK) and was connected to the y-piece. This size endotracheal tube was chosen in order to minimize turbulent flow and maximize aerosol release from the system. An endotracheal tube of this size is at the upper limit of size for intubation past the vocal cords of Sprague-Dawley rats in the 350–500 g range; however, in our experience, rats within this weight range can be intubated with a 14-gauge endotracheal tube without damage to the cords.

The nebulizer was interposed in the inspiratory limb of the ventilator circuitry, proximal to the y-piece and endotracheal tube. Initially, two configurations of a T-piece were tested for suitability. T-piece #1 was a custom modified neonatal T-piece with an internal volume of 1.9 mL. T-piece #2 was a custom modified neonatal T-piece with double the internal volume of T-piece #1, that is, 3.8 mL. The nebulizer was filled with 0.5 mL of 1 mg mL⁻¹ albuterol sulphate.

Breath actuation settings were dictated by the nebulizer controller. Using these actuation settings, aerosol generation was initiated by predefined changes in airway pressures.

The test lung consisted of a 20-mL round-bottom glass collection tube. It was placed at a level higher than the nebulizer and T-piece to avoid drug condensate flowing down the ventilator tubing and endotracheal tube and entering the test lung. This size collection tube provided sufficient compressible air volume to allow ventilation, similar in operation to the larger sealed test lungs used in pediatric setups. This in vitro model of a rat lung employs rapid respiratory rates, small volumes, and small droplet sizes and so depends on sedimentation of aerosol in the tube over time. It is especially suited to systems incorporating low velocity aerosols and no additional gas flow. Commercial test filters were deemed unsuitable as they had dead space volumes greater than the volume between the endotracheal tube and the lungs of rats in the 350 to 500 g range.

Residual volume

Residual volume was described as the volume of albuterol sulphate remaining in the nebulizer drug reservoir after nebulization was completed. The outside surfaces of the nebulizer were dried thoroughly with a lint-free cloth and the weight of the nebulizer measured using an analytical balance (Mettler AE200, Leicester, UK). The nebulizers were washed with distilled water and dried fully between nebulization runs.

Residual Volume was calculated as;

\[
\text{Mass of nebulizer after nebulization} - \text{Mass of dry nebulizer.}
\]

For this and all subsequent gravimetric measurements, it was assumed that the density of the albuterol sulphate solution is equal to 1.0 kg m⁻³, that is, 1 g of water is equal to 1 mL of water.

Effect of nebulization on albuterol sulphate

Due to the potential adverse effect of nebulization on therapy formulations the effect of nebulization using the Aereoneb® Pro on concentration, pH, and temperature were investigated.

Concentration

Albuterol Sulphate (3 mL of 1 mg mL⁻¹) (Norton IVAX, Waterford, Ireland) was nebulized into a 20-mL round-bottom glass collection tube. The tube was centrifuged at \(1500 \times g\) for 2 min in order to pool the drug (Sigma, Model 3-16K, Österode am Harz, Germany). The concentration of the nebulized drug in a 1-mL aliquot was determined using UV spectrophotometry at a wavelength of 275 nm and compared to the nonnebulized control \((n = 3)\). The mass of recovered albuterol sulphate was calculated by interpolation on a standard curve of albuterol sulphate concentrations.

In order to test the effect of repeat nebulization on the concentration of the test drug, 3 mL was nebulized into a glass collection tube, pooled, and placed back into the nebulizer drug reservoir for a second round of nebulization. The concentration of this twice nebulized solution was determined using UV spectrophotometry as described.

\[\text{pH}\]

The pH of the test drug was measured using a pH meter (Mettler Delta 340; Leicester, UK). The pH of the once nebulized and twice nebulized samples were compared to the nonnebulized control.

Temperature change

The temperature change recorded in 3 mL of albuterol sulphate solution in the nebulizer drug reservoir was measured using a thermocouple attached to a data logger (Agilent Technologies, Dublin, Ireland) \((n = 3)\). Readings were taken in the nebulizer drug reservoir every 2 min throughout the entire nebulization period until the end of dose as indicated by the nebulizer controller.

Droplet size characterization by laser diffraction

Droplet size distributions described by VMD were measured by a Malvern Spraytec particle size analyzer (Malvern Instruments Ltd.) with RT Sizer software. The horizontal distance between the distal end of the endotracheal tube and detector lens was 2 cm. This measure prevents evaporation of droplets and thus an artificial reduction in droplet size before measurement. The ventilator circuitry was orientated horizontally, in line with the Spraytec analyzer so as to mimic the orientation of the proposed system in use in vitro.

A 5-L min⁻¹ vacuum flow was implemented through the system ensuring laminar flow and reducing droplet size growth through collision with other droplets. The vacuum also ensured that the droplets only passed through the laser beam only once. The center of the emitted aerosol plume was directed through the center of the laser beam to increase the accuracy of data acquisition.

Data acquisition began when beam obscuration exceeded 3% and continued for 1 s. The data acquisition rate was set to 500 Hz, that is, 500 individual readings per second were taken characterizing the droplet size distribution. The data reported for each individual measurement is an average of the 500 individual readings. In order to verify the accuracy of the generated data, the Spraytec analyzer’s laser diffraction apparatus was tested with a reference reticle (Malvern Instruments Ltd.). The reference reticle consists of titanium particles of defined sizes embedded in a glass bar. The size of the reticle
droplets was measured by the Spraytec analyzer and the results compared with the nominal values given for the reference reticle supplied by the manufacturer.

Droplet size was measured using 1 mg mL\(^{-1}\) albuterol sulphate and is described by volumetric mean diameter (Dv50). Geometric standard deviation (GSD) and fine particle fraction (FPF) (percentage of droplets between 1.50 and 4.50 \(\mu\)m in size) were also recorded.

**Droplet size characterization by cascade impaction**

Droplet size distributions described by mass median aerodynamic diameter (MMAD) were measured by using a Marple Miller Series 290 Cascade Impactor (Copley Scientific, Nottingham, UK). This is a low flow cascade impactor operating at 2 L min\(^{-1}\) flow. Droplet size (MMAD) was measured using 1 mg mL\(^{-1}\) albuterol sulphate. The droplet size cutoff points were set at 21.30, 14.80, 9.80, 6.00, 3.50, 1.55, 0.93, 0.52, and 0.00 \(\mu\)m, respectively, for each of the nine stages of the cascade impactor. Albuterol sulphate was recovered from the filters by incubating with an extraction solution of ethanol and water. Recovery of albuterol sulphate from the filters was shown to be 99.13 \(\pm\) 1.99%.

The amount of albuterol sulphate recovered from each stage filter was determined with the use of a spectrophotometer (SpectraMax 384 Plus, Molecular Devices, Sunnyvale, CA) by measuring the absorbance at 275 nm and interpolation on a standard curve of albuterol sulphate concentrations. The masses of albuterol sulphate recovered at each stage were represented on a log-probability plot and the MMAD was determined from this plot.

**Rainout**

For the purposes of this experiment, rainout was described as the volume of aerosolized albuterol sulphate droplets that impacted distal to the nebulizer and proximal to the endotracheal tube. Rainout values were recorded after delivery of the entire dose as indicated by the nebulizer controller. The nebulizer and all ventilator components exposed to the aerosol were weighed before and after nebulization using an analytical balance. The nebulizer and all components were washed with distilled water and dried fully between nebulization runs.

Rainout (mL) was calculated as:

\[
\text{Weight of exposed components after nebulization} - \text{dry weight of exposed components before nebulization}
\]

**Recycling**

At the end of the nebulization run the volume of drug that had rained out in the T-piece was collected using a pipette by disconnecting the nebulizer for a short period. This rainout was transferred to the nebulizer drug reservoir in order for it to be renebulized. Only one round of recycling was performed.

**Inhaled efficiencies**

The albuterol sulphate delivered to the end of the endotracheal tube was captured in the test lung. The mass of drug in the tube, determined as described above, was expressed as a percentage of the original dose. Recovery of albuterol sulphate from the glass tubes was shown to be 99.87 \(\pm\) 0.49%. Normalization of the inhaled dose to the original dose placed in the nebulizer drug reservoir, takes into account losses due to rainout, that is, droplet deposition, throughout the ventilator system as well as unnebulized residual volumes.

**Animal care**

Six male Sprague-Dawley rats, 350–500 g (Harlan Laboratories, Bicester, UK), were used in each group for the lung distribution study in vivo. The study was approved under the animal licence granted by the Department for Health and Children, Ireland, and the Animal Ethics Committee of the National University of Ireland, Galway. Upon arrival, the rats were allowed acclimatized for at least 1 week under standardized temperature (21–22°C), humidity (50–60%), and light (12:12 light:dark) conditions until used. Animals had access to food and tap water ad libitum.

**Instillation of Evans blue**

The animals were anesthetized with intraperitoneal 80 mg kg\(^{-1}\) Ketamine (Ketalar\textsuperscript{TM}, Pfizer Healthcare, Dublin, Ireland) and 2% Isoflurane\textsuperscript{®} (Abbott Laboratories Ireland Ltd., Dublin, Ireland). After confirming depth of anesthesia by absence of response to paw compression, intravenous access was gained via the dorsal penile vein, and anesthesia was maintained with repeated intravenous boluses of Saffan\textsuperscript{™} (0.9% alfaxalone and 0.3% alfadolone acetate; Schering Plough, Bray, Ireland) as required. Laryngoscopy was performed (Welch Allyn Otoscope\textsuperscript{®}, Buckinghamshire, UK), and the animals were intubated with a 14-gauge intravenous catheter (BD Insyte\textsuperscript{®}, Braun, Melsungen, Germany) attached to deliver 300 \(\mu\)L of 3 mg mL\(^{-1}\) Evans blue (Sigma-Aldrich Ireland Ltd. Dublin, Ireland) in distilled water. Following instillation, the animal was allowed to breathe spontaneously for approximately five breaths. More Saffan\textsuperscript{™} was administered and the animal was then euthanized by administration of \(\sim\)0.4 mL of the muscle relaxant, Nimbex\textsuperscript{®} (2 mg mL\(^{-1}\) cisatracurium besylate; GlaxoSmithKline (Ireland) Ltd., Dublin, Ireland).

**Aerosolization of Evans blue**

Once anesthetized and intubated, animals were connected to the ventilator circuit and immobilized by administration of \(\sim\)0.3 mL Nimbex. The lungs were mechanically ventilated (SAR-830/P, CWE, Ardmore, PA) at a respiratory rate of 45 min\(^{-1}\), with a tidal volume of \(\sim\)6 mL kg\(^{-1}\), 2 cm\(\text{H}_2\text{O}\) PEEP and 2:1 inhalation:exhalation ratio. The nebulizer actuation was set to deliver for the first 25% of inhalation only. To minimize lung derecruitment, a recruitment manoeuvre consisting of 10 cm\(\text{H}_2\text{O}\) PEEP for 20 breaths was applied before aerosolization begun.

Airway pressures were monitored throughout aerosolization using an MP30 BIOPAC Student Lab\textsuperscript{TM} data acquisition module (BIOPAC Systems Inc., Goleta, CA). Anesthetic and muscle relaxant were administered as required. The animal was ventilated for a further 2 min after recruitment to ensure the airway pressures were stable, then 0.5 mL of 3 mg mL\(^{-1}\) Evans blue solution was nebulized into the lung. A typical breath profile for a mechanically ventilated rat during
aerosolization using this setup is shown in Figure 8. After completion of the first cycle of aerosolization, the rainout was collected from the T-piece and recycled into the nebulizer medication cup as described above. Following completion of aerosolization, the animal was ventilated for a further 2 min before being euthanized.

Determination of Evans blue content in the lungs

After euthanasia, the trachea and lungs were excised, with care taken to minimize the amount of connective tissue attached. A 20-gauge cannula was sited in the pulmonary artery, a small incision made in the left atrium with a spring scissors, and the pulmonary vasculature was then perfused with heparinized saline (2 IU heparin mL⁻¹). Samples were subsequently prepared for homogenization by dicing finely with a scalpel blade. Samples were homogenized (PT 1300D Homogenizer, Polytron, Kinematica Inc., NY) in 5 mL formamide at 27,000 rpm. Samples were placed in an incubation shaker at 60°C for ~14 to 18h. After incubation, samples were centrifuged at 5000 g for 30 min at room temperature. A 200-μL sample of each homogenate supernatant was taken for spectrophotometric analysis. The amount of Evans blue recovered was determined by measuring the absorbance at 620 nm with a spectrophotometer (SpectraMax 384 Plus, Molecular Devices) and interpolation on a standard curve of Evans blue concentrations (0.046 mg mL⁻¹ to 0.0015 mg mL⁻¹). The Evans blue standards were made up in homogenate supernatant isolated from untreated control lungs.

By adding a range of known amounts of Evans blue to untreated lung homogenate, processed using the above protocol, recovery was shown to be 100.20 ± 1.85% (n = 5).

Data analysis

All results are presented as means ± standard deviation unless otherwise stated.

Results

Effect of nebulization on albuterol sulphate

Both the concentration and pH of the test samples were not significantly changed after nebulization. The results suggest that nebulization has little effect on both concentration and pH of the 1 mg mL⁻¹ albuterol sulphate solution even after two rounds of nebulization (Table 2).

Temperature change of albuterol sulphate solution

Temperature change was measured taking care not to touch the vibrational element of the nebulizer (n = 3). The largest temperature rise recorded over ambient conditions (18.5°C) was 7.2°C for the three, 24-min nebulization runs (Fig. 2).

Residual volume

Traditional aerosol generators such as ultrasonic and jet nebulizers, typically have a large residual volume of non-nebulized test agent. In order to characterize the residual volume, three nebulizers were tested using albuterol sulphate (n = 3). For each of the runs, the nebulizers were weighed before and after nebulization and the difference in weights recorded as residual. The mean recorded was 0.056 ± 0.013 mL (Table 3).

Droplet size characterization

The droplet size produced by 18 individual test nebulizers was initially measured using both laser diffraction and cascade impaction as described. We recorded a correlation of 95% between the VMD and MMAD readings (Fig. 3). Given the excellent correlation with, and the speed and ease of use of, the laser diffraction method over the cascade impaction method, we chose to subsequently characterize droplet sizes using laser diffraction only. The similarity between the laser diffraction and cascade impaction methods observed is in agreement with that seen by Vecellio et al. (2001). (21)

Effect of ventilator setup configuration on droplet size emitted at the end of the endotracheal tube

Using continuous nebulization, three nebulizers were tested for droplet size (VMD) at the end of the endotracheal tube in the two separate setup configurations, that is, T-pieces #1 and #2. The VMD reading (n = 3) for each of the three nebulizers were verified as being in agreement with the manufacturer’s droplet size specification (see Table 4).

The T-piece configurations designed for testing were expected to allow the maximum amount of aerosol to enter into the inspiratory tubing, whilst selecting for the smallest droplets generated, theoretically allowing deeper penetration into the lung. Rainout, generated by the larger droplets with greater inertia occurred predominately in the T-piece, thus facilitating ease of recycling and renebulization of the albuterol sulphate. It was noted that the T-piece with the larger internal volume (3.8 mL) consistently emitted a smaller droplet size for all three nebulizers and would be expected to allow a greater amount of aerosol to be delivered to the end of the endotracheal tube. We chose to continue our optimization experiments with this modified T-piece #2.

Effect of ventilator regimen on droplet size

Given the wide variety of clinically relevant ventilatory regimen employed with rats in the 350–500 g range, we chose to investigate the effect, if any, on the droplet size at the end of the endotracheal tube. The ventilator circuit was assembled and placed in line with the Spraytec analyzer. Using continuous nebulization, three nebulizers were tested. The regimen tested varied in their breath rates and inspiratory times. The variables tested are indicated in Table 1.

As can be seen in Figure 4, the effect on droplet size across the range of regimen tested was minimal. The changes recorded were within the measurement accuracy of the Spraytec analyzer (±0.50 μm, personal observation), and it was

---

**Table 2. Effect of Nebulization on the Concentration and pH of 1 mg mL⁻¹ Albuterol Sulphate through Two Successive Rounds of Nebulization**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nebulized once</th>
<th>Nebulized twice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (mg mL⁻¹)</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.01</td>
<td>0.99 ± 0.01</td>
</tr>
<tr>
<td>pH</td>
<td>3.99 ± 0.02</td>
<td>3.98 ± 0.02</td>
<td>4.00 ± 0.00</td>
</tr>
</tbody>
</table>

*aData represent mean ± SD; n = 3.*
concluded that the range of ventilator regimen tested had minimal bearing on droplet size. This confirmed the validity of the chosen regimen in subsequent investigations.

**Rainout**

The rainout produced across a range of breath actuation settings is set out in Table 5. It was noted that the majority of the rainout occurred within the T-piece. This allowed for easy recovery of rainout, and thus, the introduction of a recycling step. It is clear from the results that the recycling step reduced the rainout volume at the end of dose.

**Effect of ventilation regimen on in vitro inhaled efficiency**

Three continuously operating nebulizers were tested under the same ventilator regimen as above and deposition in the test lung quantified ($n = 3$). All configurations tested with all three nebulizers outperformed previously reported in vivo data for deposition in the rat lung. Overall, the 2:1 inhalation:exhalation (I:E) ratio regimen performed best, providing more time for the aerosol to be generated and delivered down the inspiratory limb. The 45 breaths per minute, 2:1 ratio (13.19 ± 0.21% inhaled efficiency) outperformed the 90 breaths per minute 2:1 ratio (11.75 ± 0.91% inhaled efficiency) when tested with the smallest droplet size nebulizer (nebulizer 2, VMD 3.38 μm) (Fig. 5). These results validate the choice of T-piece #2 and suggest that the lower droplet size nebulizer, coupled with a low breath rate and high I:E ratio would be most suitable for higher deposition in this small bore system.

**Effect of breath actuation on in vitro inhaled efficiency**

The final metric to be characterized in the in vitro optimization experiment was the effect of the breath actuation settings on the inhaled mass. These were tested with the optimized system that had evolved over the successive rounds of testing described above. In summary, the optimized system consisted of a low droplet size nebulizer, 45 breaths per minute ventilation rate, 2:1 inhalation:exhalation ratio and T-piece #2 with an internal volume of 3.8 mL.

The breath actuated settings on the nebulizer controller were seen to improve inhaled mass over continuous nebulization in all cases except for the 75% of inhalation actuation setting (Fig. 6). The recycling step, as expected, also lead to an increase in inhaled mass. This is in line with the rainout results (Table 5) where less rainout was measured in the system after a recycling step. This renebulized aerosol would have been expected to have exited the endotracheal tube or been carried down the expiratory limb. The most efficient actuation setting proved to be the 10% preload plus 10% of inhalation setting with a recycling step (33.15 ± 3.15% inhaled efficiency). This setting began to generate a bolus of aerosol before inhalation

| TABLE 3. RESIDUAL VOLUME IN NEBULIZER DRUG RESERVOIR POSTNEBULIZATION |
|--------------------------|------------------|
| Residual Volume (mL) | Standard deviation (mL) |
| Nebulizer 1 | 0.069 ± 0.003 |
| Nebulizer 2 | 0.054 ± 0.015 |
| Nebulizer 3 | 0.044 ± 0.003 |

*a* $n = 3$.

![FIG. 2. Change in temperature of albuterol sulphate solution recorded in the nebulizer drug reservoir over the course of the entire dose ($n = 3$).](image)

![FIG. 3. Correlation between the volumetric mean diameter (VMD) and the mass median aerodynamic diameter (MMAD) values recorded for vibrating mesh test nebulizers ($n = 18$). There was a significant correlation between VMD and MMAD readings ($p = 0.95$).](image)
FIG. 4. Change in droplet size recorded over a range different of ventilator regimen. All changes recorded were within the measurement accuracy of the Malvern Spraytec and so ventilator regimen was deemed to have little effect on droplet size ($n = 3$). BR = breathing rate, I:E = inspiratory:expiratory ratio.

FIG. 5. Effect of ventilator regimen on inhaled efficiency ($n = 3$). Values shown are mean ± SD. BR = breathing rate, I:E = inspiratory:expiratory ratio.

Table 4. Droplet Size Characterization for Three Nebulizers Measured in Various Ventilator Setup Configurations

<table>
<thead>
<tr>
<th>Manufacturer’s specification (µm)</th>
<th>T-piece #1 (µm)</th>
<th>T-piece #2 (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizer 1 5.21 ± 0.03</td>
<td>3.24 ± 0.11</td>
<td>3.10 ± 0.05</td>
</tr>
<tr>
<td>Nebulizer 2 3.38 ± 0.02</td>
<td>2.64 ± 0.04</td>
<td>2.35 ± 0.04</td>
</tr>
<tr>
<td>Nebulizer 3 4.29 ± 0.05</td>
<td>3.17 ± 0.14</td>
<td>2.79 ± 0.05</td>
</tr>
</tbody>
</table>

aData represent mean ± SD; $n = 3$. T-piece #1 had an internal volume of 1.9 mL, T-piece #2 had an internal volume of 3.8 mL.

began, and for the first 10% of inhalation, thus loading the inspiratory limb with aerosol and maximizing aerosol delivered down past the endotracheal tube.

Time to delivery of 0.5-mL albuterol with this breath actuation setting was approximately 42 min. As this was deemed too long, we chose to proceed with the next best performing breath actuation setting, that is, aerosol generation for the first 25% of inhalation plus recycling. Time to delivery using this setting was approximately 22 min and the inhaled mass was 29.95 ± 1.54% of the original dose. The trade off was ~3% of deposition in the test lung, as determined in vitro, and given the gain with respect to time to delivery of dose, we chose to proceed to the in vivo experiments with these actuation settings.

In vivo delivery of Evans blue

In vivo instillation of the Evans blue tracer aerosol saw 87.34 ± 6.23% of the original dose being detected in the lungs of the Sprague-Dawley rats ($n = 6$). Losses were accounted for by residual Evans blue remaining in the syringe and the endotracheal tube. (Fig. 7).

Utilizing the 25% of inhalation plus recycling breath actuation setting, inhaled efficiencies of 30.88 ± 5.73% were recorded ($n = 6$) (Fig. 7). This inhaled efficiency was not significantly different to the inhaled efficiencies recorded for these settings in vitro ($p = 0.71$). Time to delivery of 0.5 mL was approximately 24 min. A typical breath signal recorded during aerosol delivery for a tracheal intubated Sprague-Dawley rat, mechanically ventilated on the optimized system described above, is illustrated in Figure 8. The breathing pattern was not influenced by the introduction of aerosol into the system and remained consistent throughout the duration of ventilation.

Discussion

To date, the factors affecting aerosol deposition in mechanically ventilated rats have not been described in the literature. In the present study we detail the development of an optimized aerosol delivery system, capable of delivering approximately 30.88 ± 5.73% of the original dose of Evans blue to the lungs in an in vivo model of a tracheally intubated, mechanically ventilated rat. This optimization was brought about through an application of knowledge of basic aerosol characteristics to the system design and systematic testing.

Aerosol characteristics and behavior dictate that in a small-bore diameter system, such as described above, droplet size becomes an extremely important factor in the system's...
Breath actuation was also identified as a method for decreasing droplet impaction and is a feature seen in an increasing number of aerosol generator control systems. Generating aerosol during the inspiratory cycle has been shown to increase aerosol delivery efficiency both on and off the ventilator. In the rat, low tidal volumes and high respiratory rates combine to result in very short inspiratory times. The time for an aerosol generator to begin generating a steady stream of aerosol varies with different technologies. Use of a jet nebulizer requiring 80 ms or an ultrasonic nebulizer requiring 150 ms is impractical for use with such a breath profile. The ability of the Aeroneb vibrating mesh technology to generate aerosol within 2 ms allowed us to maximize breath actuated aerosol generation within these short inspiratory times.

During aerosolization, the volume of impacted aerosol droplets is described as rainout. The generation of aerosol during the breath cycle, initiated by a pressure stimulus, allowed for the timed introduction of aerosol during inspiration only. As a result, less rainout was observed when using breath-actuated over continuous nebulization settings. The volume of aerosolized albuterol sulphate that did impact and rainout within the system was quantified gravimetrically and shown to vary with actuation setting. During testing, it was noted that the majority of rainout occurred in the T-piece. As this fluid was easily accessible without major disruption to ventilation, a recycling step was introduced where rainout in the T-piece was extracted using a pipette and renebulized. The setting delivering the maximal amount of aerosol proximal to the endotracheal tube in vitro was the 10% preload plus 10% of inflation plus recycling setting (0.055 ± 0.02 mL rainout volume). Subject to the suitability of the test agent, as determined by appropriate stability tests, this recycling step represents a simple modification to the nebulization strategy that greatly increases the amount of aerosol released by the delivery system past the endotracheal tube into the lungs. During the testing of the various breath actuation settings above, increases of between ~26 and ~41% were seen in inhaled mass values after the addition of a recycling step. This simple modification may prove to be a significant factor in the feasibility of use for aerosolized high value test agents; however, the issue of sterility of this recycled fluid would need to be addressed in an in vitro delivery system.

Albuterol sulphate was tested for its suitability for re-nebulization, and this showed little effect on its concentration (1.00 ± 0.00 mg mL⁻¹ vs. 0.99 ± 0.01 mg mL⁻¹) and pH (3.99 ± 0.02 vs. 4.00 ± 0.00) after two rounds of nebulization. Heat generation, often a characteristic of aerosol generation, was characterized over the course of three nebulization runs. The largest increase in temperature over ambient in the albuterol sulphate solution was recorded as 7.2°C. The observed steady rise in temperature can be explained by the reducing volume of solution in the nebulizer drug reservoir, which even at its peak was still below 37°C. This result confirms the suitability of the vibrational mesh technology for delivery of a wide variety of agents harboring therapeutic potential as it does not exceed 37°C, after which point, temperature-sensitive agents such as viral vectors, plasmid DNA, and proteins could be denatured or otherwise adversely affected. It is important to note that one would expect little effect on a solution, although this may not apply to suspensions, due to the sieving effect of nebulization when...
the drug particles cannot physically get through the apertures on the vibrating mesh. However, other agents may be susceptible to shear forces and temperature changes generated when the liquid passes through the apertures and this underlines the importance of measuring the effect of nebulization on the test agent of choice.\(^{(26)}\) The effect of shear force generated on nebulization was not tested here.

After nebulization, the volume of aerosol that does not constitute rainout is classified as either residual volume or as inhaled mass. Residual volumes in jet and ultrasonic nebulizers are considered high, constituting a large percentage of inhaled mass. Residual volumes in jet and ultrasonic nebulizers recorded for the nebulizers tested here were relatively low. The largest residual volume recorded for the three nebulizers was 0.069 ± 0.003 mL representing approximately 13.8% of the original 0.5-mL dose. The lowest recorded residual volume represents approximately 8.8% of the original 0.5-mL dose (Table 3).

Inhaled masses, expressed as a percentage of the original dose, were tested across the range of ventilator regimen and breath-actuation settings. The effect of ventilator regimen coupled with continuous nebulization was recorded for all three test nebulizers. The configuration of delivery system that produced the highest inhaled mass was a combination of a T-piece with an internal volume of 3.8 mL, a nebulizer producing a low droplet size at the end of the endotracheal tube (2.35 ± 0.04 μm VMD) and a low breath rate with high I:E ratio (45 breaths per minute, 2:1). We recorded inhaled masses up to 13.19 ± 0.21%, which represent a marked increase on those previously reported in vivo for anesthetized, mechanically ventilated rats.\(^{(51)}\)

Taking this evolving system design forward and testing with the various actuation settings, we recorded inhaled efficiencies up to 33.15 ± 3.15% in vitro. Again, as expected given the rainout values, the best performing actuation setting was the 10% preload plus 10% of inhalation plus recycling setting. Other values recorded ranged from 12.71 to 29.95%, highlighting the significant influence these actuation settings have on system performance. When taking into account the time to delivery of dose for these settings, we chose to proceed to the in vivo experiments with the next best performing breath actuation settings, that is, 25% of inhalation plus recycling. The loss of ~3% of inhaled mass was counterbalanced by the gains in delivery times. The inhaled mass results recorded for the 25% of inhalation plus recycling settings tested in vivo (30.88 ± 5.73%) were not significantly different to the in vitro results (29.95 ± 1.54%) (\(p = 0.71\)). This correlation validates the design of the in vitro lung model indicating that such a model, relying on sedimentation of aerosol over time, provides an accurate predictor of in vivo results.

Although not as effective at delivering a high percentage of the original dose to the lung as intratracheal instillation (87.34 ± 6.23%), the results above indicate that a greater inhaled mass than previously reported, can be achieved by aerosol delivery in a mechanically ventilated rodent model. Furthermore, given the advantages of aerosol delivery with respect to location of deposition and subsequent therapeutic response, aerosols remain an attractive method of delivery of active agents to the lung.

The testing and results presented in this study signify the development of a standardized ventilator system delivering a high percentage of the original dose to the lungs in both in vitro and in vivo models of a mechanically ventilated rat. This has been achieved through the use of a combination of a low flow rate, low residual volume breath-actuated vibrating mesh nebulizer, generating a small droplet size and capable of generating an aerosol within 2 ms of actuation, coupled with appropriate ventilation regimen and the introduction of a single rainout recycling step.

Acknowledgments

This research was funded by Science Foundation Ireland (SFI) and Health Research Board (HRB) Ireland. We would like to thank Jim Fink and Aerogen Ireland Limited (Galway Business Park, Galway, Ireland) for the use of the Aeroneb® Pro nebulizer systems in this study.

Author Disclosure Statement

No conflicts of interest exist.

References


Received on August 12, 2008
in final form, March 1, 2009

Reviewed by:
Rajiv Dhand
Theresa Sweeney

Address correspondence to:
Professor Timothy O’Brien
Regenerative Medicine Institute (REMEDI)
University Department of Medicine
Clinical Science Institute
National University of Ireland, Galway
Galway, Ireland

E-mail: timothy.obrien@nuigalway.ie