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Repeated Orotracheal Intubation in Mice

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TITLE:

Repeated Orotracheal Intubation in Mice

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KEYWORDS:

airway resistance, mouse, pulmonary function, tracheal intubation, refinement, repeat intubation, respiratory system, respiratory tract disease

SUMMARY:

The goal of this article is to describe a refined method of intubation of the laboratory mouse. The method is noninvasive and, therefore, ideal for studies that require serial monitoring of respiratory function and/or instillation of treatments into the lung.

ABSTRACT:

The literature describes several methods for mouse intubation that either require visualization of the glottis through the oral cavity or incision in the ventral neck for direct confirmation of cannula placement in the trachea. The relative difficulty or the tissue trauma induced to the subject by such procedures can be an impediment to an investigator's ability to perform longitudinal studies. This article illustrates a technique in which physical manipulation of the mouse following the use of a depilatory to remove hair from the ventral neck permits transcutaneous visualization of the trachea for orotracheal intubation regardless of degree of skin pigmentation. This method is innocuous to the subject and easily achieved with a limited understanding of murine anatomy. This refined approach facilitates repeated intubation, which may be necessary for monitoring progression of disease or instillation of treatments. Using this method may result in a reduction of the number of animals and technical skill required to measure lung function in mouse models of respiratory disease.

INTRODUCTION:

The laboratory mouse is a common animal model for human respiratory disease. Thus, there are several published methods for mouse intubation for the purpose of both instillation of treatments and measurement of respiratory mechanics. Most of the described procedures require visualization of the glottis through the oral cavity with specialized equipment such as a laryngoscope or fiber-optic light source[1-7]. However, this can be difficult when a relatively large

cannula is required, as it can obscure the view of the researcher. Limjunyawong et al.[8] have addressed this concern with a method of intubation in which a small cutaneous incision is made along the midline of the ventral neck allowing for visualization of the trachea. Following the procedure, the incision is closed with tissue adhesive.

For studies requiring frequent repeated intubations, successive incising and closure of this site requires debridement of the skin margins and tissue trauma to the ventral neck. The purpose of the transcutaneous tracheal visualization approach to oral intubation is to provide a refined, noninvasive technique specifically suitable for repeated intubation studies as well as single intubation events in mice.

PROTOCOL:

All animal activities described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of The Ohio State University and were conducted in AAALAC-accredited facilities.

1. Procedure preparation

1.1. Construct the intubation platform. To achieve the appropriate platform slope, use a three-inch (7.6 cm) 3-ring binder. Fold a 15−20 cm length of 3-0 silk or other thread material in half and adhere the ends of the thread to the top of the inclined platform with tape to create a suspension loop (**Figure 1**).

1.2. Select a cannula of the appropriate size and length.

NOTE: For a 20−30 g mouse, a 1−1.5 inch (2.5−3.8 cm) long catheter up to 18 G can be used. For this study, 18-week-old female BALB/c and 10-week-old C57BL/6 mice (n = 3 of each strain) were used. An opaque white catheter sheath provides the best transcutaneous visualization.

1.3. Cut a bevel at the distal tip of the catheter and smooth the cut surface with abrasive paper to create a rounded bevel tip. Gently create a slight bend in the cannula approximately 1 cm from the bevel (**Figure 2**).

NOTE: A new catheter should be used for each mouse.

1.4. Anesthetize the mouse with ketamine (5.4 mg/g body weight) and xylazine (16 µg/g body weight) administered intraperitoneally.

NOTE: Proper anesthetic depth is achieved by lack of response of the mouse to a firm toe pinch.

1.5. Suspend the mouse in a supine position on the intubation platform by hooking the upper incisors around the silk thread at the top of the angled surface (**Figure 3**). Once the mouse is

squarely positioned in dorsal recumbency, gently grasp the base of the tail and retract the tail towards the table. Place a piece of tape over the base of tail to secure the mouse.

1.6. Apply depilatory cream (**Table of Materials**) to the ventral cervical region for 30−45 s then remove all depilatory cream from the cervical region using a dry gauze. Repeat application process if needed. Thoroughly rinse the skin with saline or distilled water to remove any residue then wipe dry.

2. Intubation procedure

2.1. Use straight, flat forceps in the nondominant hand to gently retract the tongue in a manner that sufficiently opens the mouth for introduction of the cannula.

NOTE: Rat tooth forceps should not be used as this will damage the tongue.

2.2. With the dominant hand, advance the cannula into the mouth such that the end that is distal to the slight bend is against the roof of the subject's mouth.

2.3. Release the tongue and slide the flat edge of the closed forceps caudally along the ventral neck until the manubrium is reached. This motion laterally displaces the salivary glands and flattens the muscle covering the trachea. The trachea appears transcutaneously as a white line (**Figure 3A**). If necessary, rotate the forceps in a craniodorsal direction while maintaining tension on the skin in a caudal direction to cause the laterally displaced salivary glands to peak. This maneuver creates more contrast around the trachea (**Figure 3B**).

NOTE: Avoid excessive force on the ventral neck as it can collapse the trachea and impair breathing.

2.4. Advance the cannula while simultaneously angling the distal tip of the cannula ventrally by supination of the dominant hand with simultaneous flexion of the wrist.

2.5. The proper placement of the cannula is indicated by visualization of the opaque cannula in the trachea (**Figure 4B,D**). If the cannula has been advanced past the level of the origin of the masseter muscle and visualization of the cannula in the trachea has not been confirmed, retract the cannula and reattempt the maneuver.

2.6. Confirm proper cannula placement by connecting a lung inflation bulb to the cannula and observing thoracic expansion with concurrent depression of the device.

2.7. Without displacing the cannula, carefully unhook the incisors of the mouse from the intubation platform. Move the mouse to a horizontal platform (**Table of Materials**) and insert the cannula to the adaptor on the ventilator. Following the deep inflation, ventilate the mouse for 60 s then measure respiratory resistance.

3. Recovery

3.1. Once the procedure is complete, move the mouse to a warmed platform. Provide constant stimulation via light toe or tail pinches to encourage spontaneous respiration.

3.2. Extubation can occur when the mouse just begins to chew. Grasp the cannula at the level of the hub and gently pull the tube cranially and away from the mouse until the cannula is completely removed from the subject's mouth.

NOTE: It is preferable to provide airway support with the rigid cannula for as long as possible during the recovery process.

3.3. Once extubated, transfer the mouse to a clean recovery cage with heat support. Continuously monitor the mouse until it is fully ambulatory, and recovery is complete.

REPRESENTATIVE RESULTS:

Serial monitoring of baseline pulmonary function

Eighteen-week-old female BALB/c and 10-week-old C57BL/6 mice (n = 3 of each strain) were intubated using the described method on day 0, 3, 10, and 17. Following intubation on each day, the subject was connected to a mechanical ventilator supplied with 100% oxygen (**Table of Materials**). Respiratory resistance (Rrs) was measured using the forced oscillation technique for 60 s following a deep inflation to 25 cm H_2O held for 5 s. No software errors associated with this sustained breath hold along with Rrs values within physiological range provide additional support for proper placement of the cannula. Data revealed no significant differences of measured Rrs observed between time points within each strain (**Figure 5**). It is assumed that the absence of an increase in Rrs over time indicates lack to trauma-associated inflammation in the respiratory system over four successive time points.

Statistical analysis

Descriptive statistics (mean and standard error) were calculated using statistical analysis software (**Table of Materials**). The Kolmogorov-Smirnov method was used to verify the Gaussian data distribution. Statistical analyses of datasets were made by unpaired ANOVA, with a *post hoc* Tukey-Kramer multiple comparison post-test. All data are presented as mean ± SEM. $P < 0.05$ was considered statistically significant.

FIGURE LEGENDS:

Figure 1: Intubation platform. The intubation platform consists of a three-ring binder with a loop of silk thread adhered to the top of the binder to create a suspension loop.

Figure 2: Cannula preparation. (**A**) Lateral view of the prepared cannula. Note the gentle angle created approximately 1 cm from the rounded bevel at the distal end of the catheter and the orientation of the cannula angle in relation to the bevel. (**B**) Dorsoventral view of the prepared

cannula. Note the rounded and smoothed edge of the bevel.

Figure 3: Tracheal visualization. (**A**) Forceps are placed on the ventral neck and the skin is gently retracted caudally to laterally displace the salivary glands and provide visualization of the trachea as a white structure on ventral midline (black arrow). (**B**) Craniodorsal rotation of the forceps on the ventral neck creates a protrusion of the salivary glands (*). The trachea is visualized as the white linear structure on ventral midline between the salivary glands (black arrow).

Figure 4: Proper cannula placement. (**A**) C57BL/6 mouse positioned on the intubation platform with the cannula introduced into the proximal oral cavity. (**B**) C57BL/6 mouse with the cannula properly placed in the trachea. Note the cannula can be easily visualized as the white structure within the trachea (white arrow). (**C**) BALB/c mouse positioned on the intubation board with the cannula introduced into the proximal oral cavity. (**D**) BALB/c mouse with the cannula properly placed in the trachea. The white cannula can be easily visualized within the trachea (black arrow).

Figure 5: Serial measurement of resistance. No significant differences of measured Rrs observed between time points within each strain.

DISCUSSION:

Intubation using the transcutaneous tracheal visualization technique offers a refined approach to the standard skin incision method. With special attention to several key steps, intubation can be easily and quickly achieved. The animal must be placed squarely in dorsal recumbency on the intubation platform with the mouse secured in gentle retraction. This will extend the animal into vertical alignment and proper positioning for intubation. In addition, the depilatory cream should not remain in contact with the animal's skin for longer than 30−45 s and should be thoroughly rinsed to removal all residue. Extended skin contact with the depilatory cream will cause unnecessary pain for the animal and ulcerations can obstruct the view of the trachea[9]. It is imperative to use the proper wrist motion as the dominant hand introduces the catheter into the glottis. The dominant wrist should flex while the hand moves in a supination motion. It is also critical to monitor the subject closely as the flat edge of the forceps are pressed on the ventral neck to visualize the trachea. Pressure from the forceps will occlude the trachea and cause hypoxia if maintained for a prolonged duration. If the patient appears cyanotic, allow a brief pause for mucus membranes to return to a pink color and for respiration to stabilize before repeating attempts.

Extensive mouse intubation experience was not necessary to perform this technique. The most common complications in inexperienced individuals include laryngeal trauma and upper airway inflammation due to multiple intubation attempts. Close monitoring is necessary during the recovery of these patients as medical intervention with nonsteroidal anti-inflammatories may be indicated. Repeated unsuccessful intubation attempts may result in tissue trauma and inflammation of the distal oral cavity, which could result in upper respiratory noise, dyspnea, hypoxemia, prolonged recovery, inability to perform repeated intubation or death.

Several modifications are recommended in the event that intubation is not successful. First,

ensure the bevel of the cannula is smooth, rounded and cut to the appropriate length for the animal's size. The bevel edge may be smoothed using abrasive paper to minimize tissue trauma and facilitate intubation[7]. In addition, check that the cannula exhibits a slight curve of approximately 15° at one-third distance away from the bevel and the tip of the cannula is beveled at a 45° angle as described in Brown et al.[6]. Always check that the catheter is in the proper orientation before and while performing this procedure.

For this study, mice were intubated for repeated lung function tests using a mechanical ventilation system to record lung function measurements. A large, 18 G cannula was used to create a tight seal. To perform repeat lung function studies on mice with a smaller tracheal diameter due to age or strain, it may be challenging to place a larger cannula. If a smaller cannula is elected for use, ensure that a proper seal can still be achieved, and that the resistance of the cannula is not higher than resistance of the test subject's airway[10]. A successful deep inflation perturbation is adequate confirmation of an appropriate seal. Note that such a seal is unnecessary if only installation of treatments into the lung is desired.

Although the described method has made modifications that prevent external tissue damage, the upper limit of frequency of intubation is still a function of cumulative trauma to the glottis and trachea due to excessive introduction of the cannula. Concurrent monitoring of a control group for significant increases in airway resistance during a study is recommended since tissue trauma is accompanied by inflammation that will result in decreased luminal diameter of the trachea. Significant increases in airway resistance over the course of repeated intubation procedures were not observed in the current study. Mice remained clinically normal for the study duration and gross necropsy of upper airway structures was unremarkable at study conclusion in all animals.

In summary, the described intubation technique offers a noninvasive method for placing endotracheal cannulas with minimal equipment including an inclined surface, forceps, a polypropylene cannula and depilatory supplies. This refined method enables repeated intubation events without recurrent tissue trauma and pain associated with a cutaneous incision site on the ventral neck or a tracheotomy procedure. In addition, this method reduces the number of mice required as individual mice may be repeatedly intubated throughout the course of a study. It also eliminates the need for specially designed intubation restraint devices, scopes or transilluminating equipment for airway visualization. BALB/c and C57BL/6 strains were used in this study to demonstrate technique success in both light and dark pigmented strains and animals of a relatively young age and small size (10−20-week-old mice). This refined technique is suitable for single or repeated intratracheal instillation of compounds, bronchoalveolar lavage, imaging or lung function testing. This minimally invasive, versatile method can be implemented for virtually any procedure that requires access to the lower respiratory tract.

ACKNOWLEDGMENTS:

The authors thank Lucia Rosas, Lauren Doolittle, Lisa Joseph and Lindsey Ferguson for their technical assistance and the University Laboratory Animal Resources for their animal care support. This work is funded by NIH T35OD010977 and R01-HL102469.

DISCLOSURES:

The authors have nothing to disclose.

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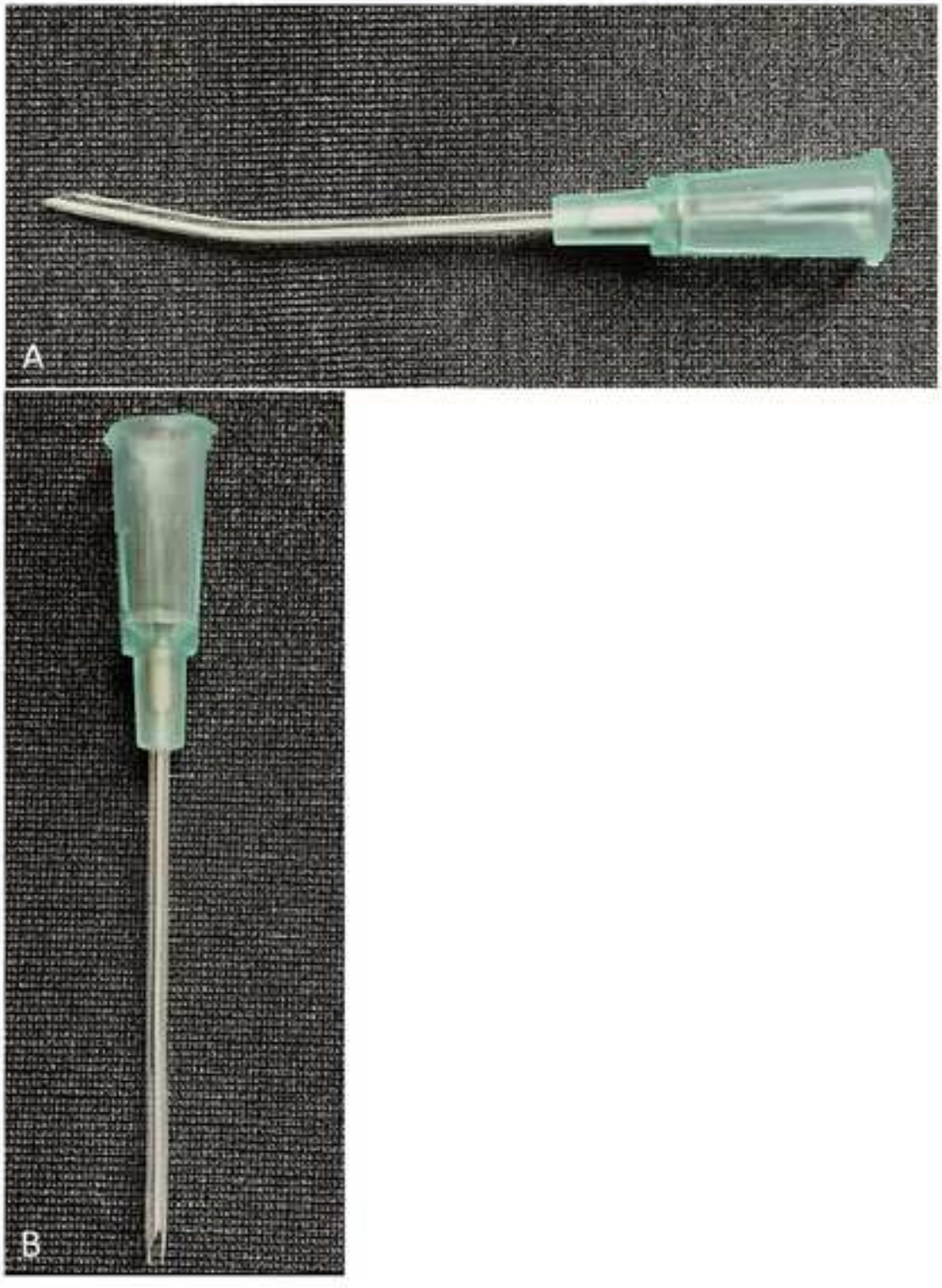

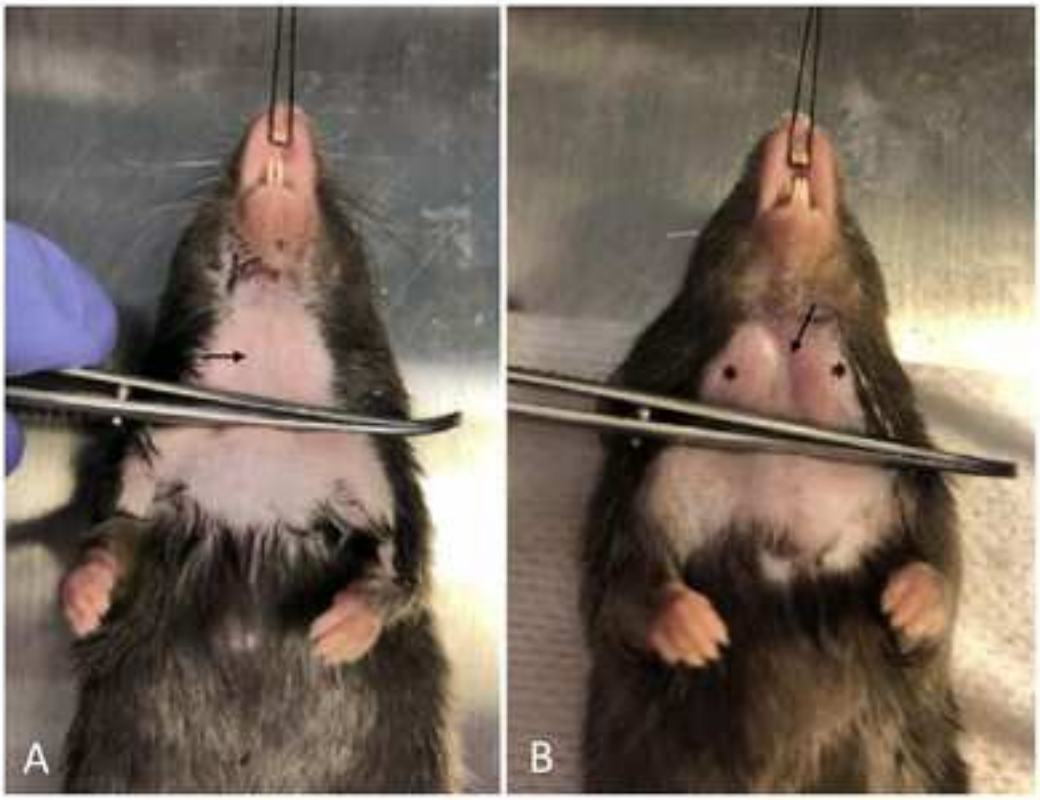

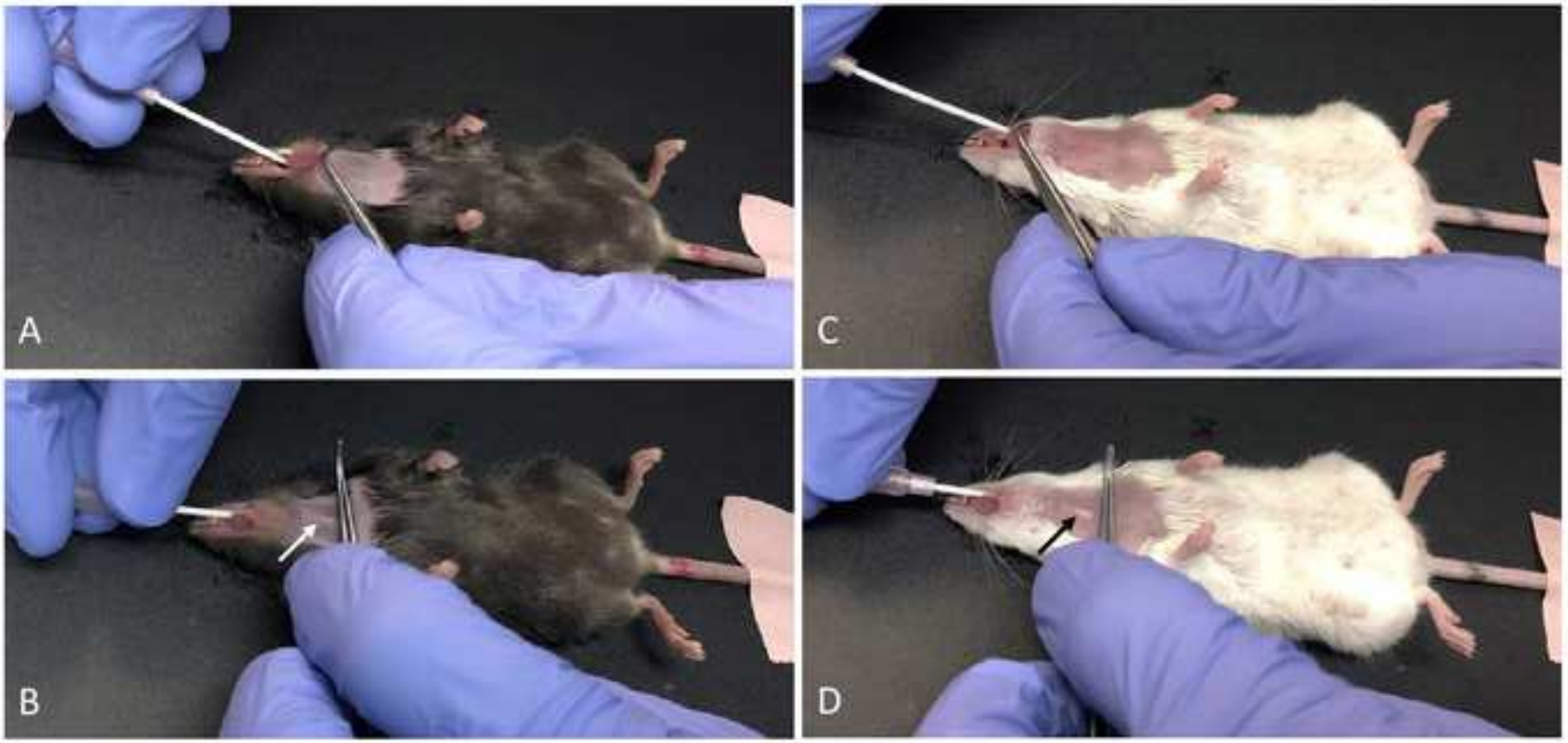

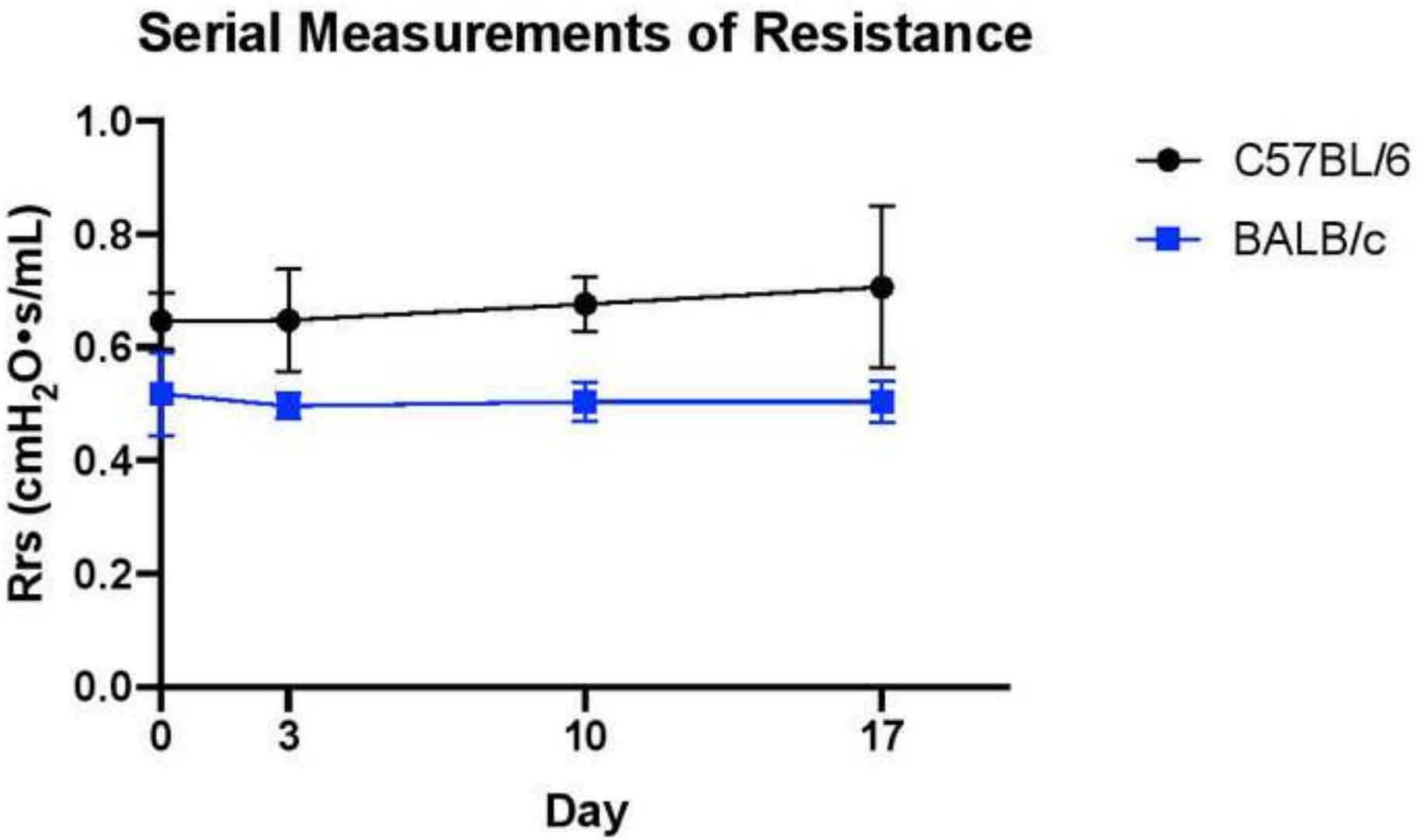

Name of Material/ Equipment	Company	Catalog Number
18Gx1 1/4" intravenous catheter, Safelet	Fisher Scientific	#14-841-14
70% ethanol, 10L	Fisher Scientific	25467025
Abrasive paper (sandpaper)	Porter-Cable	74001201
AnaSed (xylazine sterile solution) injection (100 mg/ml)	Akorn Animal Health	NDC# 59399-111-50
Blue labeling tape (0.5 in x 14 yds)	Fisher Scientific	15966
Braided silk suture without needle, nonsterile, (3-0)	Henry Schein	Item #1007842
Deltaphase Isothermal Pad	Braintree Scientific	39DP
Deltaphase Operating Board	Braintree Scientific	39OP
Distilled water	ThermoFisher	15230253
Eye Scissors, angled, sharp/sharp	Harvard Apparatus	72-8437
FlexiVent (FX Module 2)	Scireq	N/A
Gauze sponges	Fisher scientific	13-761-52
Heavy-Duty 3" 3-Ring View Binders	Staples	24690CT
Instat Software	Graphpad	N/A
Insulin syringe (0.5 cc, U100)	Fisher Scientific	329461
Ketamine HCl Injection, USP (100 mg/ml)	Llyod Laoratories	List No. 4871
Lung inflation bulb	Harvard Apparatus	72-9083
Micro Forceps, Curved, Smooth	Harvard Apparatus	72-0445
Nair (hair removal lotion), 9 oz bottle	Church & Dwight	42010440
Sterile saline (0.9%), 10 ml	Fisher Scientific	NC9054335

Comments/Description
Cannula for intubation
Cleaning cannula
Cannula preparation
Anesthesia
Restraint on intubation platform
Intubation platform
Mouse thermoregulation and recovery
Mouse recovery (prior to extubation)
Cleaning mouse following depilation
Cannula preparation
Record lung function data (not required to perform procedure, used in this study to validate procedure)
Hair removal
Intubation platform
Statistical analysis software
Anesthesia administration
Anesthesia
Confirm cannula placement
Retract tongue and create tension on neck for cannula visualization
Hair removal
Anesthesia, cleaning skin following hair removal

Rebuttal Document
Refined Method for Repeat Orotracheal Intubation in Mice
Authors: AM Nelson, KE Nolan, IC Davis

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly reviewed the manuscript for spelling and grammatical errors.

2. Please revise lines 159-163 to avoid textual overlap with previously published work.

The phrase referring to previously published studies has been removed from the statistical analysis section and this section has been reworded to avoid textual overlap (lines 191-195 in revision document).

3. Line 52: Please use a superscript arabic numeral to cite Limjunyawong et al. (2015).

Superscript Arabic numeral has been added for citation at line 55 in the revision document. A superscript Arabic numeral has also been added at line 278 in the revision document to cite Brown et al. (1999).

4. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

The entire manuscript has been revised to remove all personal pronouns.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Flexivent, Scireq, Nair®, Instat , GraphPad, etc.

All information regarding commercial products has been removed from the text. These products have been replaced with generic terms followed by (Table of Materials).

6. 1.3: Please specify the age, gender and strain of mouse used.

The mouse strain, age and gender information was included in the original manuscript in the representative results section (line 178 in revision document) and has also been added to the

procedure preparation section of the protocol (subsection 1.2, lines 80-81 in revision document).

7. 3.2: Please describe how extubation is done.

Details regarding mouse extubation have been added to the recovery section of the protocol (subsection 3.2, lines 164-166 in revision document).

8. Lines 147-150: Please include such methodological details in the protocol section.

Information describing the use of the ventilator system has been added to the protocol under subsection 2.7. (lines 140-157 in revision document).

9. References: Please do not abbreviate journal titles; use full journal name.

The full journal titles for all references materials have been added to the reference section.

10. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.

All relevant information has been added to the materials list and sorted alphabetically by material name.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In this manuscript "Refined method for repeat orotracheal intubation in mice" by Nelson et al., the authors describe a non-invasive intubation method for the use in experiments with laboratory mice that allow monitoring respiratory function and/or instillation of treatments into the lungs at multiple times through the same study. Therefore, this protocol allows to reduce the number of mice required for an experiment since the same mice can be intubated multiple times during the course of the experiment. Importantly, the authors demonstrate the feasibility of using this intubation protocol in BABL/c and C57BL/6 young and small size (10-20 weeks old). Overall, the manuscript is well written and clear, with the important steps nicely explained. Importantly, it looks like this protocol can be used for researcher without previous experience.

Major Concerns:
None.

Minor Concerns:
Pictures in the figures are very clear although since this protocol will be filmed, it is unclear their relevance. The authors should consider the possibility of including in the discussion a

couple of examples where investigators could use this intubation method in their research.

Although this procedure will be filmed, the authors consider the inclusion of labelled images to provide a valuable component to the paper. These images demonstrate specific key features of the technique including visualization of the cannula in the trachea and proper cannula construction. This manuscript is intended to serve as a training document to allow researchers to view the tutorial video and have a printable version of the manuscript with reference images to refer to while performing this technique in their laboratory. Therefore, the authors have elected to keep the figures included in the revised document.

The discussion section includes a brief list of examples in which this method could be used for various research purposes including single or repeated intratracheal instillation of compounds, bronchoalveolar lavage, imaging or lung function testing. The authors have added a statement to highlight the versatility of this technique (lines 320-321 in revision document).

Reviewer #2:

Manuscript Summary:

It is a very well written very interesting manuscript and it will be worthwhile to be published

Major Concerns:

None

Minor Concerns:

I am proposing to use both American and European metric systems (for example page 3 line 76 "inch"). Page 7 Line 232 please correct the word gauge

To remain consistent with the format of the journal, the document has been revised to include the European metric system for all measurement. American metrics were only included for cases in which the product was specifically labelled with such metric (Ex: three inch 3 ring binder, 1-1.5 inch cannula). The term gauge has been corrected.

Reviewer #3:

Manuscript Summary:

"Refined Method for Repeat Orotracheal Intubation in Mice"

The authors describe as mentioned in the title and in this manuscript a method for a non-invasive orotracheal intubation in mice.

The manuscript is easy to follow, all steps are clearly explained and all the materials and equipment needed is listed. All critical steps are highlighted and additional information/notes are included. Following the steps listed in the procedure would lead for an experienced researcher to the described outcome without inducing tissue damage and on the other hand for a less experienced researcher easy to learn.

The method described in this manuscript represents an improvement of the previously described orotracheal intubation methods. By visualizing the trachea during oral intubation, better control is possible. The authors mentioned in their summary that this method is suitable

for multiple applications.

Minor Concerns:
I would recommend to insert an image with the intubation platform into the manuscript and also histological images of the intact trachea and epiglottis after repeated intubation in order to demonstrate that this method does not cause tissue trauma as mentioned.
I have some concerns about depilation and "copiously rinse the skin" of the ventral neck area. I would like to address the question if the authors recognized any kind of skin irritations.

Conclusion:
This manuscript describes an easy to follow method for the orotracheal intubation in mice.

An image of the intubation platform has been included in the revised manuscript.

While histological images of the trachea and larynx would allow direct visualization of the presence of inflammatory cells, the authors believe this extends beyond the scope of this study as a methods paper. Instead, authors focused more heavily on demonstrating the lack of functionally significant inflammation by performing airway resistance measurements since previous studies have shown that any intubation in any species may result in irritation to the tracheal tissues. To provide more support, additional information was added to emphasize the lack of clinical abnormalities and gross necropsy findings in mice throughout this study (lines 295-298 in revision document).

The authors have extensive experience using depilation cream and therefore did not identify any skin irritations. The protocol and discussion sections (lines 104 and 246 in revision document) includes strict guidelines for maximum contact time of this product to animal skin and proper cleaning methods of the depilated area. Stringent adherence to these instructions throughout this study ensured that skin irritations did not occur.

Reviewer #4:
General comments:
The authors describe a simple, refined, and non-invasive technique suitable for studies of respiratory function with repeated intubation procedures on laboratory mice. The measurement of respiratory mechanics in mice models is one of the essential techniques for investigating human respiratory diseases including respiratory infections. However, it is often difficult to perform these experiments requiring special equipments such as a laryngoscope or fiber-optic light source in the containment laboratory specialized for handling infectious materials. The described method herein by the authors is non-invasive and easily achieved without special equipments. Moreover, the method allows researchers to measure serially lung functions with repeated intubation on an animal, which would contribute to more accurate evaluation of the animal experiments due to negligible inter-individual difference. This paper will be of interest to researchers in the respiratory disease field, and the detailed procedure in this paper is worth visualizing. The manuscript is well written and presented. I have a few minor comments and suggestions, listed below.

Minor Concerns:

(1) The authors should describe the air leakage out of the lung with mechanical ventilation. The selection of appropriate size of cannula is the determinant of sealing around the cannula in the trachea. The authors used 18 gauge cannula for 20-30 gram mouse. The authors should validate that the trachea and vocal cords provide a seal around the cannula in such case.

The authors validated that the trachea provided an appropriate seal around the cannula based on fact that the deep inflation maneuver on the ventilation system (Flexivent) was maintained for each mouse at a pressure of 25 cm H_2O for 5 seconds; a sudden drop in this pressure would have been detected by the software. This means that the cannula was an appropriate diameter for the size of the trachea for the measurements of respiratory function performed in this study. This verification process is addressed in the discussion section (lines 281-288 in revision document).

(2) The description on the intubation platform is obscure. The photographs or illustrations of the platform would help the readers to understand.

An image of the intubation platform was added to the revised manuscript.