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A Minimally Invasive Method for Intratracheal Instillation of Drugs in Neonatal Rodents to Treat Lung Disease

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June 4, 2020

Lyndsay Troyer, Ph.D.

Senior Science Editor JoVE

1 Alewife Center Suite 200

Cambridge, MA. 02140

Re: Invited article regarding intratracheal instillation of drugs in neonatal rat pups

Dear Dr. Troyer,

Thank you for inviting us to submit a manuscript to your prestigious journal. Attached is the initial version of the manuscript titled ‘A simple, non-invasive method for intratracheal instillation of drugs in rat pups as a treatment strategy for neonatal lung disease’. The manuscript has a description of the procedure, a figure and a table attached. We have not had access to the animal lab for the last 2 months and in addition have moved this month to the Case Western Reserve University, Cleveland from the University of Illinois, Chicago. This has resulted in our inability to take more pictures to be uploaded as figures 2 and 3. We expect to have the animal protocol approved and access to animal facilities by August 2020.The video recording and addition of more figures could be completed at that stage.

Thanking you,

Sincerely,

Anantha Harijith, MD, MRCP(UK)

Associate Professor of Pediatrics,

Case Western Reserve University.

Cleveland, OH. 44106.

TITLE:

A Minimally Invasive Method for Intratracheal Instillation of Drugs in Neonatal Rodents to Treat Lung Disease

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KEYWORDS:

neonate, intratracheal instillation, intubation, transoral, otoscope

SUMMARY:

This technique of instilling drugs directly into the trachea of neonatal rodents is important in studying the impact of locally administered drugs or biologicals on neonatal lung diseases. Additionally, this method can also be used for inducing lung injury in animal models.

ABSTRACT:

Treatment of neonatal rodent with drugs instilled directly into the trachea could serve as a valuable tool to study the impact of a locally administered drug. This has direct translational impact because surfactant and drugs are administered locally into the lungs. Though the literature has many publications describing minimally invasive transoral intubation of adult mice and rats in therapeutic experiments, this approach in neonatal rat pups is lacking. The small size of orotracheal region/pharynx in the pups makes visualization of laryngeal lumen (vocal cords) difficult, contributing to the variable success rate of intratracheal drug delivery. We hereby demonstrate effective oral intubation of neonatal rat pup – a technique that is non-traumatic and minimally-invasive, so that it can be used for serial administration of drugs. We used an operating otoscope with an illumination system and a magnifying lens to visualize the tracheal opening of the rat neonates. The drug is then instilled using a 1 mL syringe connected to a pipette tip. The accuracy of the delivery method was demonstrated using Evans blue dye administration. This method is easy to get trained in and could serve as an effective way to instill drugs into trachea. This method could also be used for administration of inoculum or agents to simulate disease conditions in animals and, also, for cell-based treatment strategies for various lung diseases.

INTRODUCTION:

Neonates born prematurely have poorly developed lungs requiring many interventional therapies such as long-term ventilation. These interventions place the surviving neonates at a high risk of subsequent sequelae[1]. Experimental animal models serve as an important tool in simulating various disease conditions, studying the pathobiology of diseases, and evaluating therapeutic interventions. Even though a broad range of animal models from mice, rat, and rabbit to pre-term lambs and pigs are available, mice and rat are the most used.

The primary advantage of using mice and rats are the relatively short gestation period and reduced cost. They are also readily available, easy to maintain in disease-free environments, genetically homogeneous and have relatively less ethical concern[2,3]. Another major advantage of the rodent model is that at birth the neonatal pup is at late canalicular/early saccular stage of lung development which is morphologically equivalent to the lung of a 24-week preterm neonatal human infant going on to develop bronchopulmonary dysplasia[4]. In addition, as their lung development rapidly progresses to completion within the first 4 weeks of life, it is feasible to study the post-natal lung maturation in a reasonable time frame[4]. Despite these advantages, the small size of the mice and rat pups is a source of concern for various interventions, which compels most researchers to use adult animals rather than pups[5]. Neonatal lungs are in a developmental stage and the response of a neonate to an inciting agent differs from that of an adult. This makes it appropriate to use neonatal animal models to study human neonatal disease conditions.

There are different methods to administer drugs/ biological agents to the lung. This includes intranasal[6,7] or intratracheal[8,9,10] instillation as well as aerosol inhalation[11,12]. Each approach has its own technical challenges, advantages, as well as limitations[13]. Intratracheal route of administration of therapeutic agents is preferred to study the direct therapeutic impact in the organ bypassing the systemic effects. This route could also be used to study lung pathology caused by inciting agents. There are both invasive and minimally invasive techniques to do this and is easy to perform in adults. However, in pups, because of the small size of the animal, there are technical challenges associated with the intubation process. The current study presents a simple, consistent, non-surgical intratracheal instillation (ITI) method in rat pups that could be used to study the efficacy of various neonatal therapeutic interventions as well as to generate animal models simulating neonatal respiratory diseases.

PROTOCOL:

All experiments were approved by the Institutional Animal Care and Use Committee (protocol # 2020-0035) at the Case Western Reserve University. All animals were treated in accordance with the NIH guidelines for the care and use of laboratory animals.

1. Animals

1.1. Commercially obtain pregnant Sprague Dawley rats.

1.2. Maintain animals at an approved veterinary facility with 14 h/10 h light-dark cycle and 45-60% relative humidity.

2. Preparation of test compound

2.1. Use Evans blue dye as the test compound to assess the efficacy of the intratracheal instillation procedure.

2.2. Prepare a 0.25% (w/v) solution of the dye in phosphate-buffered saline (pH 7.2) and filter sterilize using a 0.45 µm syringe filter.

3. Administration of anesthesia

3.1. Anesthetize rat pups using gas anesthesia (3% isoflurane in 100% oxygen), using a modified delivery system adapted for small rat neonates.

3.2. Check for the loss of tail and pedal reflexes and shallow breathing to ensure the proper depth of anesthesia for carrying out the procedure.

4. Intratracheal instillation (ITI)

4.1. Use rat pups at post-natal day 5 (PN 5) for the ITI. Average weight of a PN 5 rat pup is 12 grams.

4.2. Restrain the anesthetized rat pup on an inclined flat platform using laboratory labelling tape. The pup is restrained at an angle of about 45° in the supine position.

4.3. Open the mouth of the neonate, and gently pull the tongue out to one side using a blunt forceps.

4.4. Use a small otoscope speculum of 2 mm diameter connected to the otoscope to hold the tongue gently and for proper visualization of the larynx.

4.5. Use the throat illuminator system i.e., the Operating Otoscope, and the magnifying lens for proper visualization of vocal cords (**Figure 1**).

4.6. Positioning the animals at an angle of 45˚ in an inclined plane. The wired bar lids of mouse cages are used (**Figure 2**).

NOTE: Positioning the animal at an angle of 45˚ provides better visualization of tracheal opening without the interference of the epiglottis.

4.7. Take a long-angled pipette tip which is used for loading western blot gels. Cut the base of the pipette tip using a surgical blade so that it fits well into the tip of 1 cc syringe.

4.8. Use the sterile 1 mL syringe fitted into a long-angled pipette tip to deliver 30-50 µL of the substance into the lung. Invert the syringe and aspirate nearly 0.9 cc air into the 1 mL syringe

connected to the pipette tip followed by the dye or the substance to be delivered. This allows the air behind the dye to be pushed into the trachea after the dye is administered as shown in **Figure 3**. The intratracheal administration is achieved by visualizing the laryngeal lumen (vocal cords) and inserting the pipette tip fitted to a syringe into the tracheal lumen.

4.9. Use the speculum of the otoscope to hold the tongue and expose the vocal cords. Speculum serves the role of the blade of a laryngoscope. Bend the pipette tip to an angle of 30° to facilitate easy introduction of the agent through the cone-shaped speculum into the tracheal opening.

4.10. Introduce the pipette tip into the tracheal opening to the point of about 2 mm beyond the vocal cords. Push the piston of the syringe to administer the dye or the drug through the speculum of the operating otoscope as shown in **Figure 3**. The introduction of air into the lung soon after the administration of the agent prevents the substance from coming back to the laryngeal cavity.

4.11. After administering the pup with the dye or normal saline, place the pups on an integrated circulating fluid heating pad (38 ˚C) until their respiratory movements are regular. After complete recovery from anesthesia, reunite the pups with the dam.

5. Characterization of ITI delivery

5.1. After ITI, euthanize the rat pups by giving excessive anesthesia (Ketamine 100 mg/kg and Xylazine 10 mg/kg) / thiopentone followed by exsanguination at an appropriate time post-administration. Euthanasia was performed as part of the experiment to collect lung tissue to demonstrate the efficacy.

5.2. Secure the euthanized rat pup on a dissection board and wipe the chest and abdomen with 70% ethyl alcohol.

5.3. For evaluating the distribution of the dye throughout the lung, remove the lungs from the animal using sterile technique and display the lungs as appropriate for imaging (**Figure 4A,B**).

REPRESENTATIVE RESULTS:

The instillation of Evans blue revealed multifocal distribution of the dye involving all pulmonary lobes (**Figure 4A,B**). Our result as shown in **Figure 4** demonstrates efficacy of distribution to all lobes. The picture is taken immediately after ITI of the dye into the trachea. 100% efficacy was achieved in instilling the dye into the trachea followed by its spread into all the lobes on both sides. It is expected that the dye would spread further within the lobule of the lung. With repeated administration, we have been able to ensure 100% success in delivering this to the lung to both lobes and all lobules. We have ensured that no dye reaches stomach or outside of lungs. This testifies the efficacy of technique as 100% administration into the lungs. The isoflurane anesthesia allowed faster recovery of the pups after the procedure.

Rat pups from day 5 tolerated this procedure and took less than 5 minutes to carry out following anesthesia. Some animals though developed transient apnea, regained the normal respiratory pattern in a few minutes.

FIGURE LEGENDS:

Figure 1: Otoscope components. (**A**) power source 2.5 V (**B**) magnifying lens (**C**) transilluminator (**D**) speculum.

Figure 2: The positioning of the animal. The positioning of the animals at an angle of 45˚ provided better visualization of tracheal opening without the interference of the epiglottis.

Figure 3: Intratracheal instillation. Visualization of the tracheal opening using otoscope/ throat illuminator system to achieve direct delivery to lungs.

Figure 4: ITI instillation and Evans blue staining. (**A**) ITI instillation delivers the dye throughout the lungs. The dye can be seen distributed to both lobes of the lung as indicated by the black arrow. Absence of dye in the stomach confirms the success of the technique (red arrow). (**B**) Lungs from rat pups instilled with 50 µL of 0.25% Evans blue dye.

DISCUSSION:

Intratracheal instillation is an excellent method that offers several advantages over the existing methods for respiratory disease interventions as well as disease model development. It is a quick method and with experience, can be performed with an average speed of 2-3 minutes per animal. The key considerations for a successful intubation are proper sedation of the animal, it's correct positioning, especially the head, as well as accurate depth of placement/ size of the specula in the oropharynx. Proper sedation would allow sufficient working time for the operators, especially beginners. Positioning of the animal at a 45˚ angle is important for proper visualization of vocal cords. Placement of speculum at the right depth helps in retraction of the tongue throughout the procedure which again allows good visualization of the vocal cord. A team of two people can easily coordinate this work. One could coordinate the anesthesia and caging of animals while the other could deal with the Instillation. The most technically challenging part of ITI is the correct intubation into the trachea. Success of the technique is confirmed by administration of dye following intubation. It is very important to confirm the initial step of correct intubation, as there is a good chance for the tubing to slip into esophagus resulting in the delivery of the substance into the stomach, rather than the lung.

The only part that one must be careful is the potential trauma associated with misintubation. One also must be very gentle and careful in order to avoid penetrating through the trachea or the tissue surrounding the vocal cords. It is also recommended not to conduct ITI if there have been 2 or 3 misses[2].

There are different routes for administration of drugs/biological agents with each one having its own inherent advantages and disadvantages. Selection of a method is based principally on study objectives and nature of the intervention. Both intranasal instillation and aerosolization

techniques deliver agents to the upper respiratory tract as well as the lungs. This benefits studies involving upper respiratory tract[13,21] however, the delivery of a substance to lungs is unreliable. In addition, swallowing, sneezing and the varying breathing rates may lead to inconsistencies in the doses delivered. However, the physicochemical properties of some substances affect their efficient aerosolization[15]. Researchers use intratracheal inoculation to get around this problem, which regardless of particle size and viscosity, delivers inoculum/drugs directly into the lungs[23]. The two main intratracheal delivery methods include transoral intratracheal[14,15] and transtracheal instillation with or without tracheotomy[16,17]. ITI is a procedure where a wide range of treatment doses can be administered to a large number of animals quickly, once trained[18]. While transoral intratracheal instillation is routinely used in adult rats, the more invasive technique such as surgical incision was required in neonates[16,19,20]. Researchers still avoid the use of this transoral ITI technique in pups because of several reasons. The small size of the neonatal rodent makes the visualization of the laryngeal lumen difficult along with poor success in intubation. Also, the traditional metal laryngoscope used for ITI in adults cannot be used in neonate because of the small size of the oral cavity and the fragile mucosal tissues[16,18,10]. Smaller speculum and catheters are required to view the laryngeal cavity and deliver the therapeutics/ agents into the lung. The operator must be highly skilled to achieve this. Finally, recovery from anesthesia, hypothermia, maternal rejection, and cannibalism create additional problems for rat neonate recovery and survival[21,22]. Our study employed the use of gas anesthesia followed by recovery in heating pads and reuniting with lactating dams. This avoids problems associated with hypothermia, maternal rejection, or cannibalism. Many of the non-surgical intervention studies involve a blind intubation of the trachea through the oral cavity. This is especially not acceptable in the case of drug where the effect may be missed if it is wrongly instilled into esophagus. In this study, the tracheal opening is visualized using an otoscope and a slightly bent pipette tip is inserted directly into the trachea to deliver the substance, the dye in this case. Our technique demonstrates an effective way of administering the drug into the trachea of a small rat pup.

The process of ITI, is a reliable method when performed following meticulous training. Once trained it can be done rapidly and effectively as in adult rodents[13,24,25]. The correct endotracheal instillation can be confirmed by several methods including the dye or liquid movement in a tubing or syringe[26,27,28]. As it is possible to visualize the tracheal opening in this method, the misses are very less. Apnea was observed in a few pups immediately after ITI which was recovered spontaneously[18,29]. Using otoscope along with the smallest speculum served as a perfect fit for the small oral cavity of the neonatal rat[18]. The results of this study indicated that the substance can be consistently delivered to all the lobes of the lung as confirmed by the dye localization. This method would be of great significance in experimental studies in which neonatal rats are required to reliably mimic neonatal lung conditions[30,31,32]. This technique could also be used to carry out lung function studies[33] as well as cell/ stem cell transplantation studies[34,35,36] which currently employs surgical interventions and could be distressing to pups.

This technique also contributes to the principles of Refinement and Reduction in animal research. This method serves as an alternative to direct intratracheal injection with a needle which is a blind technique and is invasive as it pierces the trachea causing pain and bleeding. In complete contrast this technique serves to reduce pain while refining the introduction of a drug into the

trachea, achieving immediate reduction of pain and suffering, and improvement of welfare of animals involved in research[37]. In addition, the administration of drug into trachea is directly visualized ensuring efficacy. Though the instillation of drug into trachea is widely practiced in larger animal our refinement to use this in a 5-day old rat pup is the innovation we would like to stress here.

This article offers a simple, minimally invasive, and reproducible method which could be used for administration of injurious agents in order to simulate pathological conditions as well as for local administration of drugs, antioxidants, cells/ stem cells for neonatal therapies.

ACKNOWLEDGMENTS:

This work was supported in part by R01HD090887-01A1 from NICHD to AH. The authors also acknowledge the facilities provided by Dr. Peter Mc Farlane's lab such as inhalation anesthesia/ heating pad system. Ms. Catherine Mayer's valuable assistance in setting up of the system is appreciated. No role was played by the funding body in the design of the study, collection, analysis and interpretation of data or in writing the manuscript.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

Figure 3

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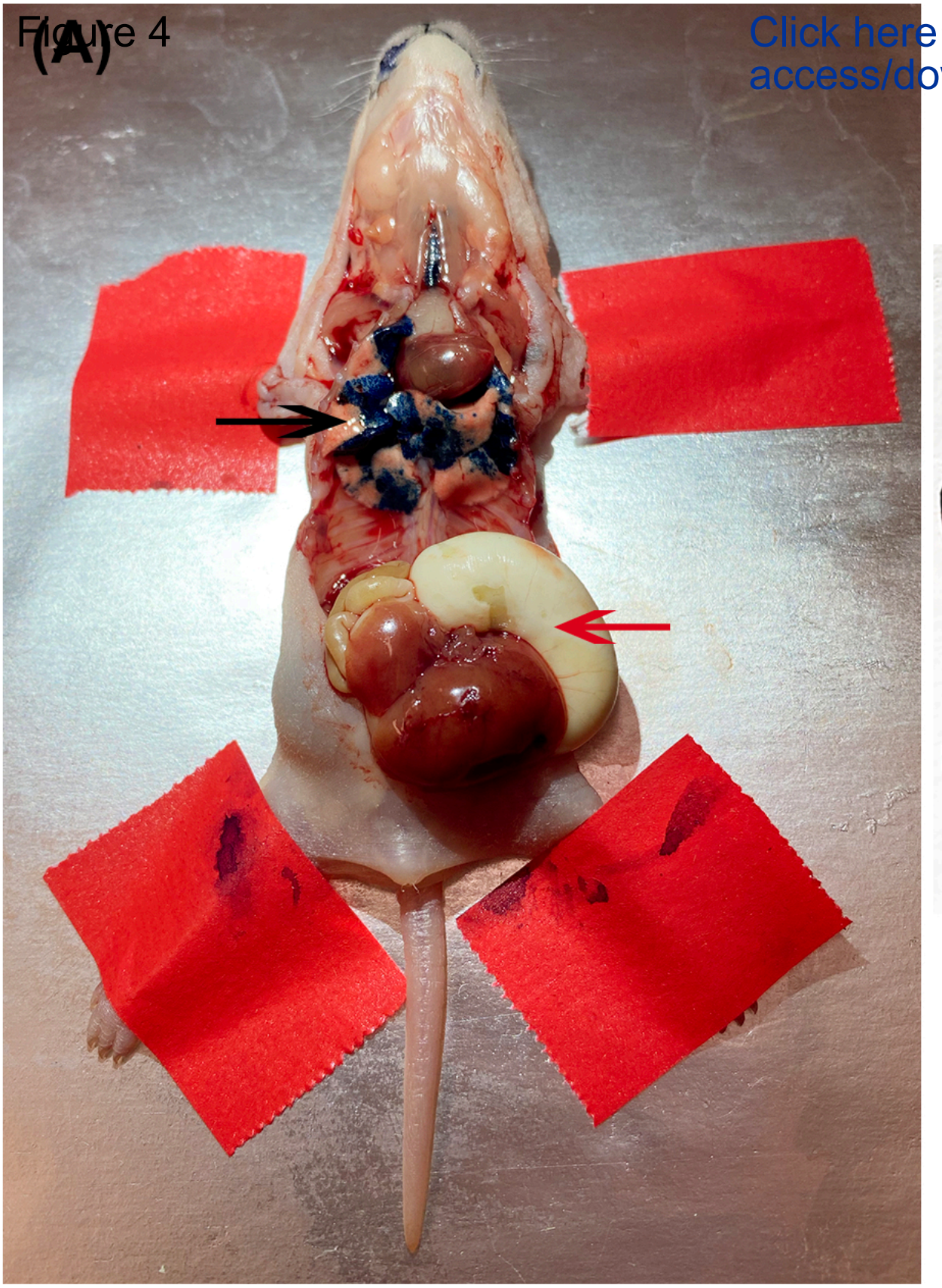
Figure 4



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Evans Blue dye	Sigma-Aldrich, St Louis, MO, USA	314-13-6	Confirmation of drug admin
Ketamine Hydrochloride	Hospira. Inc, Lake Forest, IL, USA	Dispensed from Animal care facility	For sedation
Operating Otoscope	Welch Allyn, Hillrom, Chicago, IL, USA	21770- 3.5V	For visualization of vocal cc
Otoscope Rechargeable Handle	Welch Allyn, Hillrom, Chicago, IL, USA	71050-C	
Pipette tip (Gel loading)	Fisherbrand	02-707-139	Administering the drug
Platform for restraining (inclined plane)	Animal care facility	Dispensed from Animal care facility	Wired roof of mice cage ca
3M Micropore Surgical White Paper (sticking tape)	3M, St. Paul, MN, USA	1530-2	
Luer Lock SyringeSyringes (1 ml)	BD Franklin Lakes, NJ , USA	NBD2515	Administering the drug
Xylazine	Hospira. Inc, Lake Forest, IL, USA		For sedation

istration into lungs
ords
n be used

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have fully proofread the manuscript and have to the best of our ability ensured that there are no spelling or grammar mistakes.

2. Please provide an email address for each author.

Response: The email addresses of all authors have been provided.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

Response: This is a very important comment and we have changed the language of the manuscript to the imperative tense in the protocol section.

4. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Response: More details have been added to the protocol steps as suggested.

5. Please include one liner space between each step and substep of the protocol and highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We have done the needful as suggested.

6. Line 74: Please remove the details regarding the product used and include them in the table of materials.

Response: This has been done.

7. Line 87: Please mention the position in which the rat pup was restricted.

Response: The position has been explained.

8. Line 92: Please move the details of the instrument used to the table of materials

Response: The details have been moved to the table of materials as suggested.

9. Line 101: Please mention the dose of the anesthesia used.

Response: This is done as suggested.

10. Please reference all of the figures in the text. For the representative result section, please ensure that a table or Figure is included and referenced to show the efficacy of the technique.

Response: The figures have been referenced in the text. The description of figure showing the high efficacy of the technique as seen in the figure is explained. Following repeated ITI the efficacy improved to about 100% success with distribution to all 4 lobules of the right and the single lobe of the left lung.

11. Please revise the table of materials in alphabetical order.

Response: This is done.

Reviewers' comments:

We are extremely grateful to all the three reviewers who have provided a thorough and insightful review of our manuscript. We hope to significantly improve the quality of the manuscript by implementing all the suggestions

Reviewer #1:

Manuscript Summary:

The authors present an innovative technique to deliver intratracheal substances to neonatal

rats. The major contribution of the technique described by the authors is that is allows for a non-surgical, minimally invasive technique to deliver intrapulmonary substances to in a well-described model of neonatal lung disease. The technique has translational potential in that it will allow the investigation of drugs to prevent or treat experimental neonatal lung disease, or could be used to invoke lung injury to model neonatal lung injury.

The authors have made revisions to improve the original manuscript however there remains potential to improve the manuscript. The main concern is the lack of results demonstrating the effectiveness of the technique.

Major Concerns:

1. In performing this technique the tracheal is intubated, as such the technique is not non-invasive as the title and text suggests. Perhaps a more suitable term would be minimally invasive or less invasive. The authors describe the technique well in line 60-61 as a "simple, consistent, non-surgical intratracheal instillation (ITI) method"

Response: We fully agree with the comment of the reviewer and have accordingly modified the title as suggested to minimally invasive.

2. Methodology: The authors have improved the description of the methodology used to intubate the trachea, however this is presented in the representative results section (lines 110-116) and would be better off in the methodology section.

Response: We have moved the description to the methodology section as suggested and the representative results section is shortened to avoid repetition.

3. The lack of results demonstrating the successfulness of the method is concerning. No data beyond a single image of Evans blue in a representative lung is presented. There is no data, which quantifies the proportion of ITI substance that reaches the lung or the distribution within the lung. Do the authors have any data examining the distribution of the injected substance or the variability of injections in multiple animals?

Response: We had significant variation to begin with. However, with repeated administration we have been able to ensure 100% success in delivering this to the lung to both lobes and all lobules. We have ensured that no dye reaches stomach or outside of lungs. This testifies the efficacy of technique as 100% lung administration. As shown in the picture the dye, though it reached all the lobules, did not go uniformly to all the regions within the lobule. That degree of spread is difficult to achieve without flooding the lungs which would compromise the survival and defeat the purpose. It is expected that if the drug reaches all the lobules that would have the desired effect. There was no dye in the stomach which proves that 100% of the dye administered reached the lung. The percentage of drug reaching the lung improved to 100% following practice over a period of 2-3 months involving about 30- 40 animals. We have eliminated the variation by repeated practice.

Minor Concerns:

1. Given the precision required to intubate the trachea of a neonatal rat the steps these steps could do with a clearer description. It remains unclear whether the pipette used to intubate the trachea was inserted through the lumen of the otoscope speculum or alongside it? Was the speculum used to hold the tongue or to displace the tongue laterally (Line 90-91 step 4.4)?

Response: We agree that this step requires significant degree of precision and practice. Trial and error with many types of otoscopes and rodent specific laryngoscope (Harvard) were done before arriving at the equipment described. The pipette was inserted through the speculum of the otoscope while visualizing the vocal cords through the magnifying lens of the otoscope

designed for intervention. Speculum was used to hold the tongue and expose the vocal cords. In other word the speculum served the role of the blade of a laryngoscope. We have explained this in the manuscript.

2. What was the time period between ITI and euthanasia? Did the authors examine multiple time points as the time between injection and examination could affect the pulmonary distribution of the injected substance.

Response: Euthanasia was performed soon after the ITI instillation. The aim of this study was to standardize the technique and prove that the drug could be delivered to a rat pup as young as 5 days of age with no leakage into the esophagus or stomach. Delivering the drug reliably into the lungs avoiding instillation into the esophagus is the first milestone to be achieved followed by administering at the vocal cord level so that the drug goes into both lobes. The technique has been explained further stressing this aspect. Spread of dye within the lobule of lung over a period of time was not tested. In this manuscript we intended only to study and demonstrate the technique of ITI of the dye. Spread of the dye within the lung is an interesting phenomenon and would vary from one compound to another depending on the physical property of the compound injected. The dye injected need not represent the properties of the drugs that could be used. This is not under the control of the person who does ITI. We would like to clarify that our animals survived for at least 14 days after instillation of dye or saline and were sacrificed thereafter.

3. Without quantifying the effectiveness of pulmonary substance delivery the authors should avoid statements comparing the technique described to other techniques in the literature or alluding to their technique being superior. For example line 28- "less effective nasal…"

Response: We agree with this suggestion and have removed the comparison.

4. Several of the references are incomplete and require revision

Response: References have been thoroughly reviewed. In addition to the setting of using the pattern for JoVE in the reference management software, Zotero for arranging the reference pattern, we compared with the pattern used in other published manuscripts in JoVE. The way we have the references quoted is fully in compliance with the requirements of JoVE.

Reviewer #2:

Manuscript Summary:

The authors described a non-invasive method to intratracheally instilled Evans blue (30-50 µl) in neonatal rodents on postnatal day 5.

General concerns:

1. PROTOCOL: Please describe how to fit a sterile 1ml syringe into a long-angled pipette tip in detail.

Response: The long-angled pipette tip used for loading the western blot gel was used for this purpose. The pipette tip was cut 0.5 cm away from it's base so that the pipette tip fits well into the tip of a 1ml syringe.

2. REPRESENTATIVE RESULTS, lines 116-117: The introduction of air into the lung soon after administration of the agent prevented the substance from coming back to the laryngeal cavity. Does this study introduce air into the lungs soon after administration of Evans blue? Please describe the detailed methods of introduction of air into the air. How to disconnect the fitted 1 ml syringe and pipette tip and introduce air (volume?) into the lungs?

Response: Thank you for indicating the need to clarify this point.

We aspirated about 0.9 ml of air first into the syringe followed by about 30 microliters of the dye. Then the piston of the syringe was advanced so that the dye moved forward to reach the tip of

the pipette tip. The pipette tip was introduced into the trachea between the vocal cords, dye was injected first followed by the 0.9 ml of air which was between the dye and the syringe.
3. PROTOCOL: Please describe how to measure the depth of the pipette tip that was inserted into the desired position because the small orotracheal region in neonatal rats.
Response: We introduced about 2 mm of the pipette tip beyond the vocal cords. The dye is dark in color and it is difficult to see any marking in the pipette tip. If giving any clear solution it is possible to mark the point of 2 mm away from the pipette tip.
Reviewer #3:
A simple, non-invasive method for intratracheal instillation of drugs in neonatal rodents as a treatment strategy for lung disease by Tara Sudhadevi et al.
This paper shows an invasive method of administration of drugs and components into larynx/trachea. It has direct translational impact considering the fact that surfactant and drugs are administered locally into the lungs of preterm/term newborns. Conversely this procedure is used to be performed in an open local surgery to see and to puncture the pups' larynx/trachea (Porzionato A., et al. 2019). The method here reported sounds effective in the instillation of drugs and compounds, as cellular products.
Response: The average weight of a 5-day old rat pup is about 12 grams.
b) Effective implementation of Refinement and Reduction requires both concepts to be considered at an early stage to improve the general research strategy of a project. A staged approach before embarking on large or complex experiments is useful, starting with thorough background research of the published literature and considering the possibility of conducting a small pilot study (a careful pubmed research on this topic is needed to be sure that no data are available for pups). Refinement is probably the most effective of the Three Rs in achieving immediate reduction of pain and suffering, and improvement of welfare of animals involved in research. The approach is of great relevance since reducing pain, suffering and distress is a crucial aspect of the moral debate about animal research, and a legal requirement. That is why I suggest to specify the used doses also for euthanasia.
Did the Authors conceptualize or hypothesize whether or not this new no-surgical approach to the pup larynx/trachea will have improvement in reduction or refinement in their project?
Response: Thank you for bringing in the crucial topic of Refinement and Reduction. This method serves as an alternative to intratracheal injection with a needle which is blind technique and is invasive as it pierces the trachea causes pain, bleeding and is blind in nature with questionable efficacy. In complete contrast this technique serves to reduce pain while refining the introduction of a drug into the trachea. In addition, the administration of drug into trachea is directly visualized ensuring efficacy. Though the instillation of drug into trachea is widely practiced in larger animal our refinement to use this in a 5-day old rat pup is the innovation we would like to stress here. We have added this aspect to the discussion.

Also, as we are100% sure about the efficacy of delivery relative to blind methods, this will definitely contribute to implementation of reduction.

We used the standard dose of drugs for anesthesia such as Ketamine at 100mg/kg and Xylazine at 10 mg/kg followed by exsanguination. Euthanasia was performed as part of the experiment to collect lung tissue to demonstrate the efficacy. This is added to the revised version.
c) Abstract lines 22-23: The small size of orotracheal region/pharynx in the pups makes visualization of trachea difficult, contributing to the variable success rate of intratracheal drug delivery.

The Authors are assumed not to try to inspect necessarily the "trachea (as in the abstract)", but instead the larynx, accordingly they rightly state in the representative results:
Representative Results
The intratracheal administration was achieved by visualizing the laryngeal lumen (vocal cords) and inserting the pipette tip fitted to a syringe into the tracheal lumen. The positioning of the animals at an angle of 45° provided better visualization of tracheal opening without the interference of the epiglottis.
Some useful insights for the Authors can be obtained from:
"A Comparison of Rodent and Nonrodent Laryngeal and Tracheal Bifurcation Sensitivities in Inhalation Toxicity Studies and Their Relevance for Human Exposure" by Vasanthi Mowat, et al. 2017.
Response: We are grateful for the comments and the useful reference so kindly provided. We have made the appropriate changes as suggested. Abstract has been re-worded.
d) The positioning of the animals at an angle of 45 degrees provided better visualization of tracheal opening without the interference of the epiglottis.
The figure could be more representative for the audience, by showing (arrow) the 45° angle (head vs. body ??).
Response: We have added a figure to demonstrate the animal placed in 45 degrees. The angle is marked as requested in the figure (Figure. 2).

Tara Sudhadevi, PhD. is a Postdoctoral Research Scholar at the Department of Pediatrics, Case Western Reserve University, Cleveland, OH. She holds a PhD in Biological Sciences from Sree Chitra Tirunal Institute for Medical Sciences and Technology, India. Her research interests include studying the effect of various therapeutics on neonatal lung disease management using hyperoxia models, mechanisms involved neonatal lung development under normal and hyperoxic conditions. Her research is also focused on understanding the aberrant mitochondrial dynamics linked with the neonatal lung diseases. As a first author she has played a critical role in developing techniques related to the procedure described in this manuscript.

Alison W Ha, BS. Is currently a graduate student from University of Illinois, Chicago pursuing her research at Case Western Reserve University, Cleveland, OH. She completed her BS in Biology from University of Illinois, Chicago, IL and is pursuing PhD in Biochemistry investigating the mechanisms of lung injury leading to bronchopulmonary dysplasia in preterm neonates. She has gained over 5 years of experience in acquiring skills related to neonatal rodent experiments and has played a significant role in the development of technique described in this manuscript.

Anantha Harijith, MD. The senior and corresponding author is a physician scientist and is an Associate Professor of Pediatrics at Case Western Reserve University, Cleveland, OH. He completed his residency in Pediatrics from the Bronx Lebanon Hospital of the Albert Einstein College of Medicine New York and fellowship in Neonatology from the Columbia University Medical Center, New York. As a clinical neonatologist pursing research in lung diseases seen in preterm neonates, he has developed techniques that could help in direct administration of drugs into the lungs in animal models thus avoiding the systemic administration and the associated side effects. His main area of research is related to lysophospholipid signaling in bronchopulmonary dysplasia (BPD) and oxidant induced lung injury. He is currently funded by the National Institutes of Health, American Heart Association and the Chiesi Foundation.