Journal of Visualized Experiments

Simplifying Transcutaneous Intratracheal Drug Delivery in the Newborn Preterm Rabbit

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61982R2
Full Title:	Simplifying Transcutaneous Intratracheal Drug Delivery in the Newborn Preterm Rabbit
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TITLE:

Simplifying Transcutaneous Intratracheal Drug Delivery in the Newborn Preterm Rabbit

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KEYWORDS:

neonate, bronchopulmonary dysplasia, intubation, intratracheal, respiratory therapy, pulmonary disease, animal experimentation, preterm birth

SUMMARY:

Transcutaneous intratracheal injection allows for effective intrapulmonary drug delivery during spontaneous respiration. Single and multiple injections are well tolerated with no effect on survival. The technique is simple to perform and can examine the effect of substances on lung development and the prevention of lung injury in newborn rabbits.

ABSTRACT:

Intratracheal (IT) drug delivery allows the direct delivery of pharmaceutical substances to the lung, maximizing potential pulmonary benefit and minimizing systemic drug exposure. The transcutaneous technique is simple and allows for the IT delivery of substances to the lung of prematurely born rabbits shortly after birth. Newborn pups are anesthetized with inhaled Isoflurane before being placed in a supine position with the neck extended. The larynx is identified and stabilized before transcutaneous placement of a 26-gauge (G) catheter into the trachea. Following catheterization of the trachea, a 30 G blunt needle attached to a Hamilton syringe is introduced into the IT catheter and is used for delivering a precise volume into the trachea during spontaneous respiration. After the IT injection is completed, the needle and catheter are withdrawn, and the pup is allowed to recover from anesthesia. Transcutaneous IT injection delivers a large proportion of the injected substance to the lung, with the majority remaining in the lung 3 hours after the intervention. The injections are well tolerated from the day of birth and can be repeated for multiple consecutive days without influencing survival. This technique can be used to investigate the effect of pharmaceutical agents on lung development and in the prevention of neonatal lung injury in preterm rabbits.

INTRODUCTION:

Chronic neonatal lung disease (CNLD) following premature birth continues to occur in a significant number of infants[1]. Improved modern neonatal care has significantly increased survival and decreased the majority of significant complications following preterm birth. While neurological, gastrointestinal, and ophthalmological complications have decreased, respiratory complications remain largely unchanged over the past 2 decades with nearly one in two infants born before 28-week gestation developing lung disease.

Prematurity, inflammation, oxidative damage, and ventilator-associated injury all play a role in the pathophysiology of CNLD and poor respiratory outcomes following preterm birth[2,3,4]. Despite the significant advancements of modern neonatal care, limited effective therapy is available to treat or prevent the development of CNLD[5,6].

New approaches and interventions are required to develop therapy to prevent and treat CNLD. Intrapulmonary drug delivery is an attractive intervention to deliver drugs to the lung and could alter the course of respiratory disease in neonates. Intrapulmonary drug therapy has the benefit of direct delivery of active agents to the lung, thereby minimizing accumulation of the drug in off-target organs[7,8], potentially limiting systemic side effects. Despite over 2 decades of intrapulmonary surfactant replacement, no additional intrapulmonary drugs have been validated to improve neonatal respiratory outcomes. Recently budesonide-surfactant combination therapy has been described to improve pulmonary outcomes following preterm birth in mechanically ventilated infants[9,10]. However, much remains unknown on the functional and structural effects of IT drug therapy, few new therapies have been identified, and the value of intratracheal drug delivery in the neonatal period remains uncertain. Animal models are required to identify potential drugs and aid the development of much needed therapy for CNLD.

Animal studies examining newborn lung disease are most commonly performed in small animal models such as rats and mice[11,12,13]. The rabbit has the additional advantage of preterm delivery to more closely mimic the structure and function of the immature human lung[14]. A limitation of the preterm rabbit is the difficulty of accessing the airway to allow the delivery of intrapulmonary interventions. While adult rabbit and rodent models allow trans-oral endotracheal intubation, these techniques are difficult in newborn pups due to their small size and the unique anatomy of the upper airway[15,16]. Alternative approaches are required to allow access to the trachea for the delivery of drugs in newborn rabbit pups.

In this manuscript, we describe the use of a transcutaneous needle tracheostomy to allow tracheal intubation and drug delivery.

PROTOCOL:

For all experiments involving IT injection, permission has been sought from the Animal Ethics Committee of KU Leuven, and all guidelines of animal welfare and care of KU Leuven were adhered to.

1. Preparation

1.1. Collect all required materials to complete the IT injection (**Table 1**).

1.2. Ensure that the exhaust of the anesthetic chamber is open and connected to a scavenger to prevent exposing the researcher to Isoflurane.

2. Delivery of pups

NOTE: Rabbit pups (New Zealand white-Flemish giant hybrid) were delivered via hysterotomy on day 28 gestation (term 31 days) during the saccular phase of lung development as previously described by our group[17]. Pups can be placed in either normoxia to study lung development following preterm birth, or hyperoxia to study acute lung injury.

2.1. Sedate dam with an intramuscular injection of 1 mL ketamine (100 mg/mL) and 1 mL xylazine (2%) delivered to the quadriceps with a 2 mL syringe and 26 G needle. Once deeply sedated, place the dam in a supine position on the operating table. Adequate anesthesia is confirmed by deep slow respiration, decreased jaw tone, and the lack of response to an ear pinch.

2.2. Shave the central abdomen with an electric shaver and sterilize the surgical field with iodine-based solution.

2.3. Euthanize the dam with an intravenous bolus of 1 mL of T61 delivered in the lateral vein of the ear.

2.4. Immediately perform a midline abdominal incision through the skin, muscle sheath, and abdominal muscles into the abdominal cavity.

2.5. Extend the incision to expose the bicornate uterus.

2.6. Immediately make an incision in the uterus using a pair of scissors and deliver each of the pups via the hysterotomy. Rapid delivery of the pups is essential to ensure survival.

2.7. On delivery, dry each pup using a paper towel, this both dries and stimulates the pup.

2.8. Place the dried pup in the warmed (36 ˚C), humidified (50% relative humidity) incubator.

2.9. Allow 1 h for the recovery and transition to the extra-uterine environment. All the surviving pups are randomized to the predetermined treatment groups.

3. Anesthesia

3.1. Flood the induction chamber with Isoflurane (2.5%, 2.5 minimum alveolar concentration (MAC), 2 L/min)

3.2. Place pups in the induction chamber until adequate level of anesthesia is achieved (decreased spontaneous movement, diminished foot reflex to painful stimuli, decreased respiratory rate).

NOTE: Eyes of the rabbit pup are not open on day 28 gestational age (as in this experiment) and, therefore, no topical eye ointment is required to prevent dryness of the cornea. Should IT injections be performed later in life, topic ointment should be applied to the open eyes.

4. Positioning for intratracheal injection

4.1. Position the pup on the mounting stage, supine with neck extended, and nose inserted into the nosecone to provide continued anesthesia.

4.2. Restrain the paws of the pup using non-stretch adhesive tape to tape the paws to the mounting stage.

4.3. Identify the tracheal and laryngeal cartilage rings, which are visible as dark subcutaneous midline structure in the upper trachea superior to the thoracic inlet.

4.4. Sterilize the skin over the trachea with alcohol solution (80% ethanol solution)

4.5. Grasp and stabilize the larynx with Allis forceps with the non-dominant hand of the operator.

5. Performing the intratracheal injection (Figure 1)

5.1. Prepare to cannulate the trachea with the 26 G intravenous cannula (best done with the dominant hand) while stabilizing the trachea with the Allis forceps.

NOTE: To ensure sterility of the procedure, use a new sterile cannula for each pup.

5.2. Penetrate the skin with the cannula at a 45˚ angle to the skin at the level of the thyroid cartilage.

5.3. Slowly advance the cannula and stylet until the trachea is cannulated, a subtle “give” is felt as the needle penetrates the trachea and enters the airway lumen.

5.4. Once the cannula with the stylet is in the lumen of the trachea, stop advancing the stylet and advance the plastic cannula over the stylet into the trachea while holding the stylet stationary. Do not advance the plastic cannula >10 mm or selectively intubating either the left or right main bronchus is risked.

5.5. Withdraw the stylet from the cannula leaving the plastic cannula sheath in the trachea.

5.6. Confirm the IT position of the cannula by injecting a small amount of normal saline (0.9% NaCl, 5 µL) into the cannula using a Hamilton syringe and 30 G blunt needle. Once the saline has been injected into the cannula, remove the Hamilton syringe and needle. Observe the air-fluid level within the plastic cannula; movement of the air-water level in the cannula with spontaneous respiration confirms placement of the cannula within the airway.

5.7. Draw up the required amount of substance into the Hamilton syringe using the 30 G blunt tip needle.

5.8. Introduce the 30 G blunt tipped needle (attached to the Hamilton syringe) into the plastic cannula (in the trachea), and slowly inject the substance into the trachea over 5–10 s.

5.9. Remove the Hamilton syringe needle from the plastic cannula and the plastic cannula from the trachea.

6. Recovery from procedure

6.1. Free the pup from the mounting stage and stimulate respiration with tactile stimulation of the pup.

6.2. Return the pup to a separate cage from the unanesthetized pups cage in the warmed (36 ˚C), humidified (50% relative humidity) incubator; place in a 30° head up position until sufficiently recovered from anesthesia and able to maintain sternal recumbency. Do not leave the pups unattended until they recover from the anesthetic.

REPRESENTATIVE RESULTS:

Representative results of the technique of single and repeated daily transcutaneous IT injections have been published and demonstrate that survival was not influenced by IT injection (single or multiple injections), nor did IT injection with placebo (saline) alter the lung function or lung structure compared to controls[18].

Additionally, we have validated the technique in a series of experiments that investigated pulmonary delivery of IT delivered normal saline and surfactant using a radioactive tracer (2-deoxy-2-[18F]fluoro-D-glucose (FDG)) and positron emission tomography/computed tomography (PET-CT) scan at two time points (10 min and 150 min following injection) to quantify the distribution and pulmonary delivery of substances injected using the transcutaneous IT injection[18]. The transcutaneous IT injection described in this paper delivered the majority (>82%) of the total injected tracer to the lung. Furthermore the majority of the tracer remained in the lung 3 h after delivery both when delivered on day 1 and day 7 following delivery at day 28 gestation (**Figure 2**)[18].

FIGURE LEGENDS:

Figure 1: Transcutaneous intratracheal drug delivery in the newborn preterm rabbit. Illustration of transcutaneous intratracheal injection technique. Adapted with permission from Salaets et al.[18].

Figure 2: Pulmonary distribution of substances following transcutaneous IT injection in the preterm rabbit. (**A**) Study design, IT injections, and PET-CT performed on days 1 and 7 following delivery at day 28 gestation (term 31). (**B**) Representative PET-CT images of pulmonary distribution of FDG activity 10 min after transcutaneous intratracheal injection. (**C**) Representative full body PET-CT demonstrating the majority of FDG activity is intrapulmonary with minimal extra-pulmonary activity in the stomach and upper airway. (**D**)

Distribution of FDG-activity 10 min after injection in all groups (n = 12). (**E**) Intrapulmonary distribution, the graph represents the minimum relative lung volume in which 90% of the activity was present. n = 3 for each group. Statistical analysis performed using Kruskal-Wallis test with post-hoc Dunn correction for multiple comparisons for organ distribution (**D**), distribution activity within the lung normally distributed and analyzed using paired *t*-test and ANOVA with a Bonferroni-Sidak multiple-comparisons test. Adapted with permission from Salaets et al.[18].

DISCUSSION:

Several critical steps should be followed to successfully perform IT injection. When performed correctly, the transcutaneous IT injection method allows for effective and reliable intrapulmonary drug delivery in the preterm rabbit. Temperature control is important as the newborn pups easily become hypothermic, which can negatively influence survival. Prior to placing the pups in the induction chamber, temperature control should be ensured to maintain normothermic conditions. A heating matt placed under the induction chamber ensures that the pups remain warm. The heating pad was turned on well before the pups were placed in the induction chamber to allow the chamber to slowly heat up. Due to the low conductivity of the Perspex used in the induction chamber, high temperature heating lamps should be avoided as they can melt the induction chamber walls before the interior of the chamber reaches the required temperature (36 °C).

Adequate anesthesia is required to allow restraint of the animals and a pain-free injection. It is important to avoid excessively deep anesthesia and hypoventilation or apnea. An adequate level of anesthesia for the IT injection is reached once animals are sedated but maintain spontaneous respiration and respond to stimulation of the periphery (foot pinch).

The correct positioning of the pup allows for successful IT injection on the first attempt in the majority of cases (**Figure 1**). Key steps to aid IT injection are to adequately extend the neck while avoiding any rotation, identification, and stabilization of the larynx (appears as a darker, raised cartilage structure in the midline of the airway). Rotation of the neck does not allow the catheter to be advanced in the same coronal axis as the trachea, which minimizes the chance of success.

When catheterizing the trachea, a subtle “give” is felt when the needle punctures the trachea. Once this is felt, the needle should no longer be advanced. Rather, the plastic sheath of the catheter should be carefully advanced into the airway while the needle is held stationary. Care should be taken not to advance the catheter through the posterior tracheal wall. The catheter should be advanced (over the stylet) 5 mm into the tracheal lumen. If introduced further than 10 mm there is a risk of selectively intubating either the left or right main bronchus. The stylet and catheter can be used for performing several transcutaneous IT injects; however, it is best to perform no more than five injects per cannula/stylet as too many injections leave the stylet blunt and increase the difficulty of successfully performing the intervention.

The most effective method to confirm the correct IT placement of the plastic cannula is to observe movement of an air-fluid interface in the cannula during spontaneous respiration. Following placement of the cannula, 5 µL of saline is introduced into the cannula, using a

Hamilton syringe with a 30 G blunt needle. The saline forms a visible air-fluid interface in the translucent cannula and when correctly placed, the air-fluid interface moves up and down in the catheter during spontaneous respiration, confirming the IT placement. IT injection should be performed slowly over 5–10 s while the pup is breathing spontaneously. Importantly, no air bolus should be used to flush the injected substance into the lung. Previous experiments in our laboratory to refine the transcutaneous IT injection technique investigated the use of an air bolus, finding that it led to air trapping, over-distension of the lungs and increased mortality. Following the completion of the IT injection, it is important to observe the pups to ensure that adequate spontaneous respiration continues until the pups have recovered from anesthesia. Should pups become apneic or breathe irregularly, stimulation by pinching the paw restores respiration.

The technique has many advantages, including its simplicity, the short exposure to anesthesia, and reliable pulmonary drug delivery. Compared to nebulization, intratracheal injection has superior pulmonary delivery[19]. Additionally, the intratracheal delivery of drugs such as corticosteroids potentially minimizes off-target drug effects and systemic side-effects seen due to the systemic absorption of inhaled steroids[20].

Limitations include potential trauma and injury to the skin and trachea that is avoided by techniques such as transoral intubation and nebulization. Though rare, drugs can be injected into the subcutaneous skin or extra pulmonary into the intrapleural space. The anesthesia used for the technique has additional risks and requires careful monitoring to ensure the correct depth of anesthesia is reached. If overly anesthetized, pups can become hypoxic due to respiratory depression while inadequate anesthesia makes the technique difficult to perform and will lead to pain. An additional limitation of our study is that we were unable to compare intra-tracheal injection to endotracheal intubation and delivery of drugs via endotracheal tube, which is considered as the gold standard of intra-pulmonary drug delivery.

While trans-oral intubation is possible in adult rabbits and rodents, the long snout of the preterm rabbit greatly increases the difficulty of successfully orally intubating the preterm rabbit. We are able to demonstrate that transcutaneous injection delivers a large proportion of the injected substance to the lung and remains in the lung for 3 h following IT injection. Additionally, daily injections are well tolerated and the technique does not influence survival, lung function, or alveolar structure. While this technique has been developed for use in the preterm rabbit, it can be adapted to other rodent models of lung disease.

The transcutaneous IT injection described in this paper allows for the investigation of potential therapy to prevent neonatal lung injury in a model with great translational value. The intervention provides a simple, effective technique to access the airway and deliver pharmaceutically active substances to the lung. The technique is well tolerated by newborn animals and can be used to investigate the influence of intrapulmonary medication on lung development and the prevention of neonatal lung injury[21]. Research to identify therapy to prevent neonatal lung injury and the development of chronic neonatal lung disease is urgently required as no current effective prophylaxis or therapy is available.

ACKNOWLEDGMENTS:
This research was supported by a C2 grant from KU Leuven (C24/18/101) and a research grant

from the Research Foundation – Flanders (FWO G0C4419N). A.G. is supported by the Erasmus+ Programme of the European Commission (2013–0040). Y.R. is holder of an FWO-SB fellowship (Research Foundation – Flanders, 1S71619N). None of the funding bodies were involved in the design of the study and in the collection, analysis, and interpretation of data.

DISCLOSURES:

The authors have no conflicts of interest to declare.

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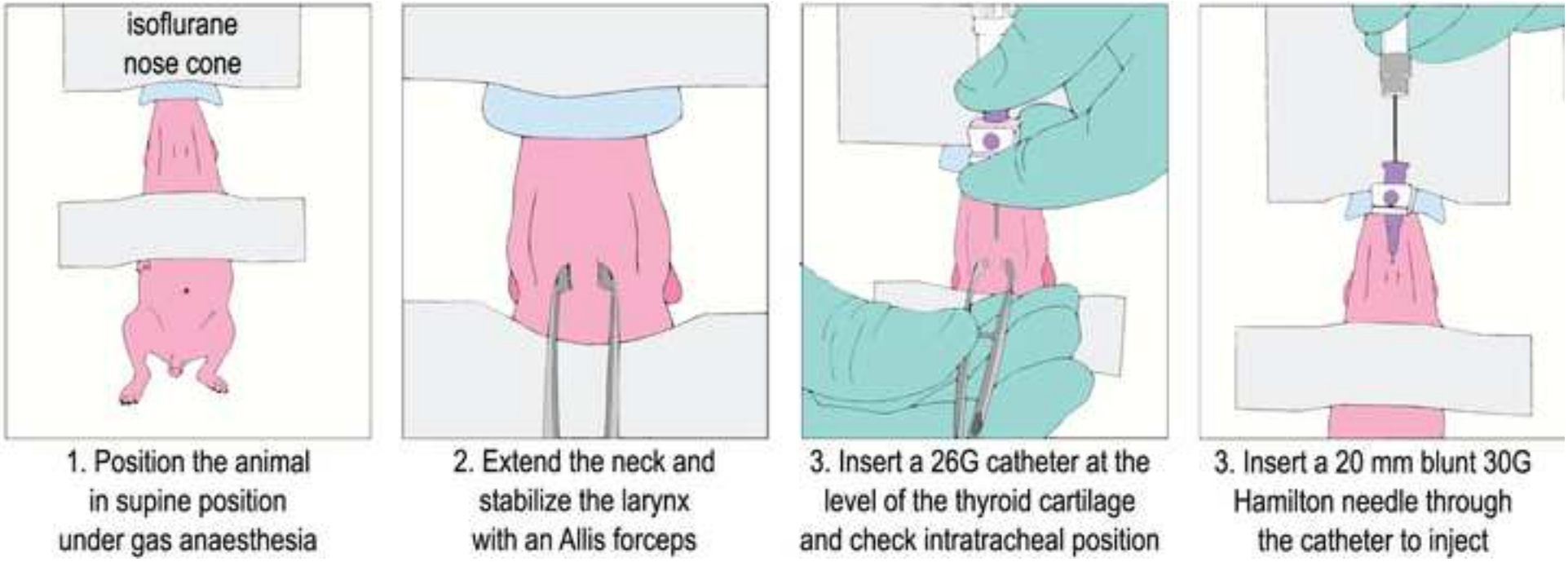
Figure



Figure

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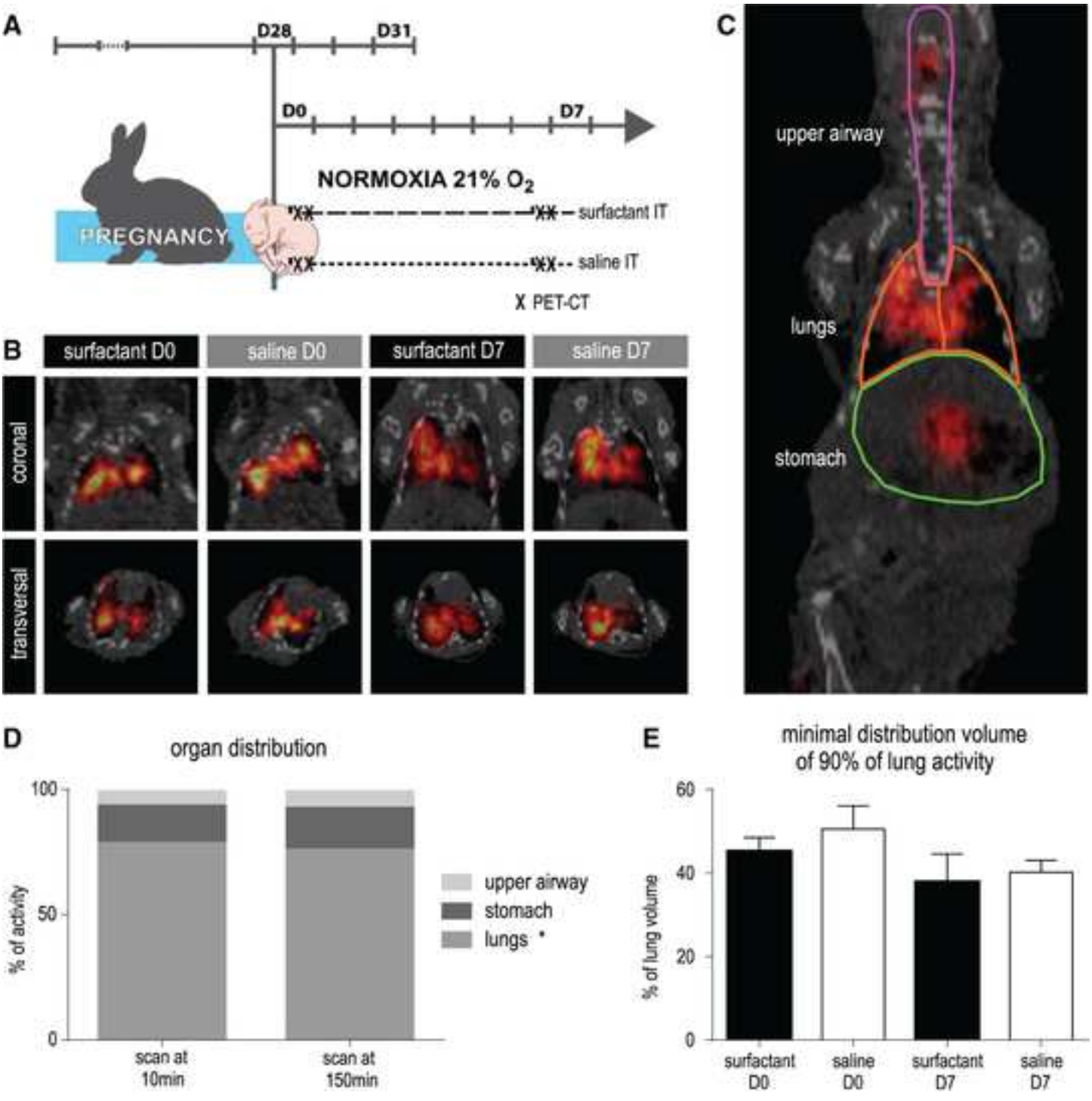

Material

Anesthesia

Heating matt to prevent cooling during anesthesia

Isoflurane vaporizer with oxygen supply

Isoflurane (Iso-Vet; 1000 mg/g)

Plexiglas induction chamber with exhaust and scavenger

Positioning for injection

Mounting stage

Nose cone connected to anesthetic circuit

Scavenger system

Tape to restrain limbs

Intratracheal injection

Allis tissue forceps

19-mm-long 26-gauge catheter

Hamilton syringe (10µl with 20 mm blunt 30-gauge needle

Pharmaceutical substance of choice

Saline (0.9% NaCl)

Animal housing

Humidity- and temperature-controlled incubator

Supplier

Dechra Veterinary Products NV, Belgium
In house built

In-house built (made out of styrofoam to allow flexible positioning

Any

BD Biosciences
Hamilton Company

Okolab Srl. Custom built cage incubator. Alternatively, in-house built cage incubators can be used

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7638-01		1
	as per protocol	
	5 µl per animal	

Dear Dr. Bajaj,

Thank you for the opportunity to respond to the comments of the editorial staff and the reviewers on our paper "**Simplifying Transcutaneous Intratracheal Drug delivery in the Newborn Preterm Rabbit".**

We have responded to all the editorial comments in the text as requested and have detailed our response to the reviewers' comments below. We hope that these changes satisfy both you as the editor and the reviewers and will lead to publication of our manuscript.

Yours sincerely,
Yannick Regin (on behalf of the co-authors)

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

Revisions have been made on the attached version in the journals style.

2. Please address specific comments marked in the manuscript.

We have responded to the specific comments and highlighted the changes made to the text.

3. Please provide the number of pups studied, and the statistical analysis performed. Please also include what was the control in such a case?

We have expanded the figure legends of the representative results, figures 1 and 2, to include both the number of pups studied and the statistical analysis used to compare the groups.

4. Please address all the reviewers' comments as well.

We have carefully revised the manuscript and responded to all the reviewers' comments.

5. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The figure we have adapted is from the article Local pulmonary drug delivery in the

preterm rabbit: feasibility and efficacy of daily intratracheal injections Salaets T *et al.* American Journal of Physiology-Lung Cellular and Molecular Physiology 2019 316:4, L589-L597 **Volume 316Issue 4** April 2019 and thus according to the journal's editorial policy as authors we can republish part of the published work. https://journals.physiology.org/author-info.permissions

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The revised submission is somewhat better but problems persist that the authors failed to address.

Major Concerns:

1. The Introduction's paragraph 3 clearly leads the reader down the path that better approach is necessary for preterm infants with CNLD. This is contrary to the authors assertion that they are not leading readers.

With little effective therapy or prophylaxis for CNLD or prophylaxis, we the authors are of the opinion that improved therapies/interventions are required to limit the development and severity of CNLD. We have rewritten Paragraph 3 of the introduction to emphasize that the value of intratracheal drug therapy is uncertain and not to lead the reader to the conclusion that intratracheal drug delivery is the best or only therapy but is rather a treatment option that requires further investigation.

2. ***The study lacks the gold-standard of endotracheal intubation to deliver the tracer. This is an expectation when a new method is proposed. This limitation should be explicitly added to the limitation paragraph of the Discussion***.

We have included the lack of comparison to endotracheal intubation and drug delivery via endotracheal tube as a limitation of our method. (lines 318-321)

3. ***The figures do not provide the number of rabbit pups, contrary to the authors' response.***

We have expanded the figure legends to include the number of pups included in all the representative results in Figures 1 and 2.

Reviewer #2:

Manuscript Summary:

The Authors have addressed my concerns and the concerns of the other reviewers

Reviewer #4:

Manuscript Summary:

The authors clearly and meticulously describe the intratracheal drug administration technique to efficiently deliver pharmaceutical substances directly to preterm rabbits' lungs.

Minor Concerns:

Extra refs may be added (line 67 and 299).

Extra references have been added to line 67. Line 67 has been reformulated. Concerning line 299: No references are available as this is unpublished data from our laboratory. To clarify this, line 300 has been reformulated

"Figure 1" should be indicated in the corresponding paragraph (5.Performing the intratracheal injection)

Figure 1 has been indicated as suggested. Line 165