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17 June 2020

Dear Editors of *Journal of Visualized Experiments*,

Thank you for considering our manuscript titled '*Intratracheal administration of dry powder formulation in mice*'. We appreciate the valuable comments from the editor and reviewers. We have improved the manuscript in response to the suggestions. We highlighted the changes in the manuscript. Our responses to the comments are shown below:

Response to Editorial Comments

1. The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.

   Thanks for the comments, we have revised the manuscript to improve the language of the manuscript.

2. Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
   1) 1.1: Mention all relevant specifications about the powder. What is the chemical composition? What is the particle size?
   2) 3.1: mention animal strain

   Thanks for the comments, we have revised the manuscript to include more specific details.

3. Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs):
   1) modifications and troubleshooting,
   2) limitations of the technique,
   3) significance with respect to existing methods,
   4) future applications and
   5) critical steps within the protocol.

   Thanks for the comments, we have revised the discussion of manuscript to address the comments above.

4. References: Please spell out journal names.

   We have made the changes accordingly.

5. Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company

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brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are MicroSprayer®, (PennCentury™)

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

We have made the changes accordingly.

6. Table of Materials:
   1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as powder, animal strain.
   2) Sort items in alphabetical order.

We have made the changes accordingly.

7. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

The re-print permission is uploaded in the supplementary files section as suggested, and the figure is cited accordingly.

Response to Reviewer 1

1. The authors stated in the introduction that "Dry powder formulation could be delivered to the deep lung region of the mouse intratracheally by proper intubation". I do not think this viewpoint is correct. intubation is only one of the factors that may influence the delivery of dry powders into the deep lung. Pls revise.

   Thanks for the comment. We have now revised the manuscript to clarify that intubation is only one of the factors that influence the delivery of dry powders into the deep lung.

2. As the powder-loaded tip is upstanding before insufflation, I am curious if the powders could be still kept in the tip without flowing out.

   For fine powders engineered to be inhalable in human, *i.e.* particles with aerodynamic diameters in the range of 1 – 5 µm, they are generally sufficiently cohesive (particularly after gentle tapping) to

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remain inside the tip until a pressure of dispersion has been applied. In our experience, the powder could be kept in the tip without flowing out.

3. The authors create a bevel at the tip of the guiding cannula. However, this dramatically increases the risk to damage the trachea during intubation.

   We appreciate the comment. There may be an increased risk (moderate risk at most) of damaging the trachea during intubation. Therefore, we recommend not to create a sharp angle at the tip, but a relatively blunt bevel to reduce the risk of injury while increasing the chance of successful intubation. We have added this information in the revised manuscript.

4. Pls perform more experiments to validate the device.

   We have published papers to demonstrate the administration of powder formulation using the device (Liao et al 2019; Qiu et al 2019;). Similar device has also been reported by other groups (Ihara et al 2015; Ito et al 2019; Miwata et al 2018). Please refer to those papers for details of experiments

   - Ihara, D. et al. Histological Quantification of Gene Silencing by Intratracheal Administration of Dry Powdered Small-Interfering RNA/Chitosan Complexes in the Murine Lung. Pharmaceutical Research. 32 (12), 3877-3885, (2015).
   - Ito, T., Okuda, T., Takashima, Y. & Okamoto, H. Naked pDNA Inhalation Powder Composed of Hyaluronic Acid Exhibits High Gene Expression in the Lungs. Molecular Pharmaceutics. 16 (2), 489-497, (2019).
   - Liao, Q. et al. Porous and highly dispersible voriconazole dry powders produced by spray freeze drying for pulmonary delivery with efficient lung deposition. International Journal of Pharmaceutics. 560 144-154, (2019).
   - Miwata, K. et al. Intratracheal Administration of siRNA Dry Powder Targeting Vascular Endothelial Growth Factor Inhibits Lung Tumor Growth in Mice. Molecular Therapy - Nucleic Acids. 12 698-706, (2018).
   - Qiu, Y. et al. Effective mRNA pulmonary delivery by dry powder formulation of PEGylated synthetic KL4 peptide. Journal of Controlled Release. 314 102-115, (2019).

5. Please mention the reconstituted SFD powder was delivered by using PennCentury.

   We have included this information in the revised manuscript.

6. Birendra et al. recently reported a similar device in EJPS (https://doi.org/10.1016/j.ejps.2018.08.010), pls cite and comment.

   Thanks for the comment. We have cited this article, and made a brief comparison between the two devices. Birendra et al. described the method that uses a 20 G cannula tube for intubation as well as powder loading. A syringe is connected to the cannula tube after intubation for powder dispersion. All the materials used (e.g. otoscope, cannula and syringe) are standardized. In our method, intubation is performed with a separate guiding canula followed by powder administration through the syringe connected gel-loading tip. While the two approaches appear to be similar, ours has

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offered several distinct advantages. Firstly, it allows confirmation of correct intubation (to the trachea but not the oesophagus) before drug administration. This step is particularly helpful for less experienced user. Secondly, the guiding cannula can act as a protecting shield to prevent any secretion or moisture in the trachea from contaminating the gel-loading tip, allowing a more accurate emitted dose measurement by weighing. Lastly, the more flexible guiding cannula together with the optical fibre may enable easier intubation. We have included these information in the revised manuscript.

Response to Reviewer 2

1. Is the gel-loading tip length adjustable? How the operator can determine the right length?

   The length of the gel-loading tip is not adjusted and is used as it is. This would be more user-friendly as the powder can be loaded into the tip without the need for length adjustment each time. The depth of intubation is determined by the dimension of the guiding cannula, which also serves as a holder to the gel-loading tip. When the gel-loading tip was fully inserted into the guiding cannula, the end of the tip should be only slightly protruded.

2. It seems from Figure 6 that the volume of air is affecting the animal body weight, how many ml of air do the author suggest to administer to mice or rat? Is 1 ml the maximum volume suggested?

   The volume of air used to disperse the powder depends on a number of factors: (i) the species and strain of animals; (ii) the age of animals; (iii) the properties of the powder (e.g. particle size distribution, cohesiveness and density); and (iv) the mass of powder to be administered. Operators should first determine the minimal volume required to disperse the powder in vitro, and then test for tolerance in animal. We have included these information in the revised manuscript.

3. In order to increase the volume of air, is the syringe capacity changeable ?

   Yes. The size of syringe can be changed for different volume of air used to disperse the powder. We have included this information in the revised manuscript.

4. Could be the system adaptable to administer liquid pulmonary formulation?

   Yes, the incubation procedure can be adopted to administer liquid formulation for pulmonary delivery. We have included this information in the revised manuscript.

5. Can you weight the gel loading tip before and after the administration to measure the emitted powder dose?

   Yes. Users can weigh the gel-loading tip before and after the administration to measure the emitted powder dose with a highly sensitive analytical balance. Since the gel loading tip is inserted into the guiding cannula rather than being directly exposed to the trachea of the animal, there is a minimal risk of contaminating the gel loading tip with moisture/ secretion in the trachea. We have included this information in the revised manuscript.

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Response to Reviewer 3

1. Numerous English language errors that can be easily remedied.

   We have improved the language of the revised manuscript.

2. In the abstract and discussion, the authors mention that the dry powder formulations can be "delivered to multiple mice at the same time". This need to be changed, as it suggests that a single person can deliver a formulation to many mice all at once (as could occur with a nose-only chamber that had multiple chambers for multiple mice), which is simply not the case. The sentence must be rewritten.

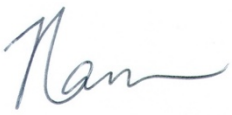
   We appreciate the comment. The sentence is now rephrased in the revised manuscript to read 'Because the pipette tips are disposable and inexpensive, different dry powder formulations can be loaded into different tips in advance. Various formulations can be evaluated in the same animal experiment without the need of device cleaning and dose refilling, thereby saving time and eliminating the risk of cross-contamination from residual powder.'

3. Figure 7 - I'm bothered by the lack of a control in both the lung images and the quantified data. A non-mRNA (or naked mRNA) containing powder delivered to mice and imaged should be included

   We appreciate the comment. We have included a control group in Figure 7 of the revised manuscript.

We hope the changes will make the manuscript suitable for publication in the *Journal of Visualized Experiments*.

Yours sincerely,



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