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Note

Non-invasive pulmonary aerosol delivery in mice by the endotracheal route

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Abstract

In this report we present in detail a non-invasive pulmonary application method that can be a useful tool in studying drug and vaccine delivery to the lower airways. In this method the formulation is sprayed directly into the lungs of mice via the endotracheal route using a MicroSprayer™ aerolizer. Mean droplet size produced was 8 μm, appropriate for deposition in the large airways. Endotracheal application of suspension of fluorescent nanospheres, 200 nm in size, by this method resulted in nanoparticle deposition in the smaller airways (bronchi and bronchioles). Mice showed full recovery one day after administration of 50 μl of formulation. Furthermore, no mortality was observed as a result of the technique. We conclude that this endotracheal application is a useful tool for studying pulmonary drug delivery in mice. The technique is especially useful for the pulmonary application of vaccines, since it enables multiple administrations without a need for analgesics.

Keywords: Pulmonary delivery; Nanoparticles; Aerosol application; Mice; Endotracheal administration

1. Introduction

The pulmonary route for local and systemic drug delivery is constantly being investigated for the purpose of targeting drugs to specific lung cell populations and for non-parenteral systemic delivery of macromolecular drugs. In addition, research exploring the possibility of using the pulmonary route to establish a mucosal as well as systemic immune response against airborne pathogens is on the rise [1–3].

Animal testing plays a very important role in the assessment of aerosol delivery with respect to efficiency, reproducibility, and safety of drugs and vaccines. Even though pulmonary application in small laboratory animals such as mice is considered complicated and often discouraging, it appears to be mandatory in pulmonary disease models and in vaccination studies [4]. The most common method of pulmonary drug application is the intratracheal instillation. This method has been used for the application of subunit vaccines, cationic lipoplexes and polymeric microspheres into mice lungs [3,5–8]. The advantages of intratracheal instillation are direct application of the drug into the lungs accompanied by minimal drug loss in the nose, throat and upper airways, the dose given is quantifiable, the application itself takes a short time and costs are relatively low. Nevertheless, this method results in a poor distribution of the formulation in the lung, mice can tolerate only small volumes, and the most important disadvantage is that it requires invasive surgery. The latter represents a problem when multiple administrations over longer experimental periods of time have to be performed, as is the case in vaccination studies. It is accompanied by pain and discomfort to the animal and requires treatment with analgesics to comply with the guidelines of most national ethical committees. Such analgesic treatment is bound to influence immunologic readout, and therefore, could be problematic in vaccination studies. Another method for aerosol application is the use of inhalation chambers. These chambers are used for whole body or nose

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only exposure and are designed for restrained, unrestrained or anesthetized animals. The aerosol is generated by a nebuliser connected to the exposure chamber and the animal is exposed for a period of time according to the dose required [9–12]. This method is non-invasive and results in good peripheral distribution of the agent in the lungs [9]. However, it is difficult to determine the exact dose that reaches the lungs since there is the possibility of drug loss in the chamber, on the animal's skin as well as in the nose and throat. This represents a drawback when investigating dose dependent effects of drugs and vaccines. Furthermore, using a nebuliser requires a lot of time, especially when large groups of animals are used for the study.

The mouse is the animal model of choice for testing vaccines against several diseases such as tuberculosis, mainly because of its well-characterized immune system and the possibility to create strains showing specific immunologic characteristics (e.g. knock-out mice, different HLA-type mice). The methods for pulmonary delivery in mice described in literature (as discussed above) are not optimal for vaccination experiments where multiple administrations of specified doses of the vaccine are needed.

Here we describe in details an improved non-invasive method for pulmonary delivery in mice. It is our intention to share our experience with this technique to guide and to foster work in this particular interesting area of drug and vaccine delivery.

2. Materials and methods

2.1. Animals

Female Balb/c and C57BL/6 mice (6–12 weeks old; Charles River, Sulzfeld, Germany) were used for in vivo pulmonary administration. Animal experiments were performed according to the 'Principles of Laboratory Animal Care' as defined by NIH and were permitted by the Ethical Committee of Leiden University (Leiden, The Netherlands).

2.2. Aerosolisation of solutions and suspensions

Aerosolisation was performed using a MicroSprayer™ aerosoliser (IA-1C; Penn-Century, Philadelphia, PA, USA) suitable for mice, attached to a high-pressure syringe (FMJ-250; Penn-Century). This device is an aerosol generator consisting of a sub-miniaturized atomizer located in the tip of a 1.25′ stainless steel tube, which is attached to a hand-operated, high-pressure syringe. This device previously showed improved delivery efficiency compared to aerosol inhalation [13] and did not cause adverse effects [14].

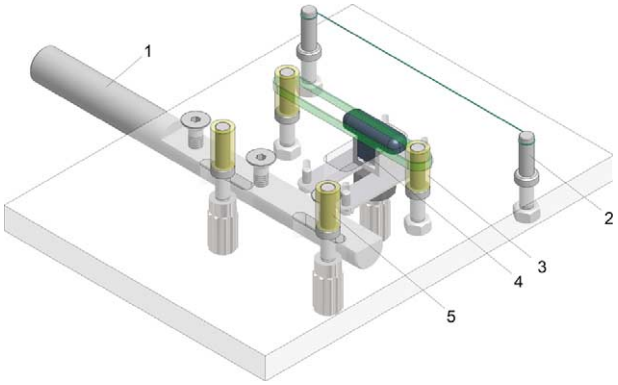

Fig. 1. Custom made mouse support for endotracheal application in mice: (1) stainless steel handle allowing angle adjustment; (2) a nylon wire for suspending the mice on their front teeth; (3) chest support using a rubber band to allow opening of the jaw while keeping the mouse static; (4) special neck support to enable trachea angle change; (5) adjustable barriers to prevent lateral mouse movement during intubation.

2.3. Aerosol droplet size measurements

Aerosol size measurements based on the time-of-flight principle were performed in an Aerosizer (TSI, St Paul, MN, USA) with PBS [15]. Experiments were performed in triplicate and at ambient temperature. Mean mass aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the aerosol droplets were determined by the standard system software.

2.4. Support for endotracheal application

A special support was custom-made from Perspex and attached to a stainless steal handle that allowed adjustment at different angles (Fig. 1). The support was equipped with a nylon wire, which was used to suspend the mice by their upper teeth and adjustable barriers to avoid lateral movement of the mouse during endotracheal application.

2.5. Endotracheal administration

The method of endotracheal administration was a modification of the procedure described by Brown et al. [16]. Mice were anaesthetized by an intraperitoneal injection of 100 mg/kg body weight ketamine (Nimatek; Eurovet, Bladel, The Netherlands) and 10 mg/kg body weight xylazine (Rompun; Bayer, Mijdrecht, The Netherlands). Mice were suspended at a 45° angle by the upper teeth. The light source's (lamp type FLQ85E; Helmut Hund, Germany) flexible fiber-optics arm was adjusted to provide optimal illumination of the trachea. A small spatula was used to open the lower jaw of the mouse and blunted forceps were used to help displace the tongue for maximal oropharyngeal exposure. After a clear view of the trachea was obtained, the MicroSprayer tip was endotracheally

Box 1

A guide for pulmonary endotracheal administration

1. The mouse is anaesthetized using ketamine/xylazine mixture. Injection can be applied intraperitoneally or subcutaneously.
2. The working space is pre-arranged: light source, a small spatula, blunt forceps and the MicroSprayer™ loaded with the formulation should be closely accessible.
3. The Perspex support is adjusted to 45–60° angle (Fig. 2a). The mouse footpad reflex is checked to verify it is asleep. The mouse is suspended by hanging it on its upper teeth and its back leaning on the Perspex support. To better immobilize the mouse, additional band on the stomach will prevent the mouse from falling and will pin the mouse on the support (Fig. 2b). The person applying the formulation is positioned behind the mouse, opposite to the light source.
4. The mouth is opened using a small spatula (Fig. 2c). The tongue is grabbed gently with the blunted forceps and is moved to the left. The tongue is quite long so it should be pulled out of the mouth in order to get a clear view at the trachea (Fig. 2d). While extending the tongue with the forceps, the spatula is used again to open the mouth and at the same time immobilize the tongue at its position outside the mouth (Fig. 2e).
5. While one hand keeps the mouth open with the spatula, the forceps are put aside and the light source is adjusted so the trachea can be visualized. This is the most important stage in the intubation: the trachea is visualized by the differences in colors in the mouth cavity. While the mouth cavity is lightened with shades of orange–red, the trachea will appear as a white light spot (Fig. 2e). It is small but can be easily visualized. In order to see it better, the Perspex support and the light source could be further adjusted until the bright white spot can be seen. The neck support can also be adjusted. It is very important to keep the tongue out and push the jaw to the front to get the best view of the trachea.
6. The MicroSprayer™ tip is aimed at the trachea (the volume stop of the high-pressure syringe should be taken off before intubation. This way it is possible to immediately spray the formulation once intubation is accomplished). The tip is inserted until it bends (1.25 in.), which is the distance from the mouth to the carina (the first bifurcation) of a mouse (Fig. 2f). Once in the trachea, the tip would slide in easily and there is no need to use any power to push the tip down. A small tilt of the tip to the front (parallel to the skin) will help it slide in. If it fails, the tip should be taken out so the mouse does not choke. Repeating the procedure should start from the fixation of the tongue and visualization of the trachea.
7. Once the tip is in the trachea, the formulation is sprayed. The tip is left inside the trachea for a few second before it is withdrawn from the mouth.
8. The mouse is taken off the support and laid on its back. Its body temperature is kept by a heating source (lamp, electric mat etc.) until it awakes from the anaesthesia.

inserted and 50 μl of solution or suspension was sprayed. The tip was immediately withdrawn and the mouse was taken off the support (see Box 1 and Fig. 2 for step by step instructions).

2.6. Method validation and aerosol distribution

Saline containing 5% India ink (Pelikan, Hannover, Germany) was used to verify successful application into the lung [17]. The mice were sacrificed by cervical dislocation 10 min after administration and the lungs were dissected and photographed. A 0.2% (w/v) suspension of fluorescent nanoparticles (Fluoresbrite plain YG, 200 nm; Polysciences, Warrington, USA) in water of pH 6.0 was used to assess aerosol distribution. The mice were sacrificed by cervical dislocation 30 min after administration, the lungs were dissected and frozen using cold *iso*-pentane. Cryosections of 10–30 μm were cut, collected on slides and visualized by a Zeiss IM-35 inverted fluorescent microscope (Zeiss, Oberkochen, Germany), attached to a CCD series 200 camera system (Photometrics, Tucson, AZ, USA).

3. Results

3.1. Aerosol droplet size

Measurements of droplets sizes obtained by spraying from the Penn-century Microsprayer™ were performed by using the time-of-flight principle. Mean mass aerodynamic diameter (MMAD) of the droplets was 8.01 ± 0.57 μm with a geometric standard deviation (GSD) of 1.1.

3.2. Method validation

Pulmonary delivery of India ink solution as an aerosol confirmed that the aerosol was applied through the trachea, avoiding drug loss by intragastric application via the esophagus (Fig. 3). India ink was clearly visualized in

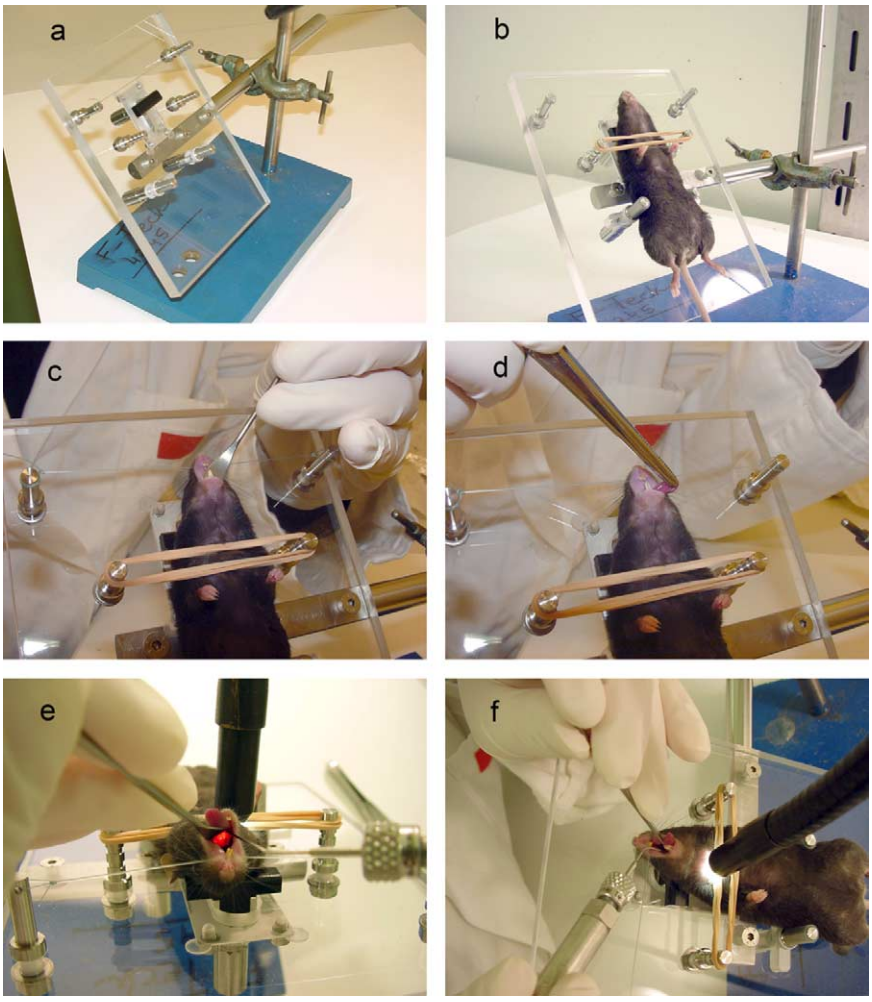

Fig. 2. Step-by-step guidance for endotracheal administration in mice. See Box 1 for detailed explanation.

the airways while no color was visualized in the stomach after this application (not shown).

3.3. Aerosol distribution using fluorescent nanoparticles

Suspension of fluorescent nanobeads (200 nm) was sprayed into the trachea in order to visualize the distribution of particulate delivery systems in this size range.

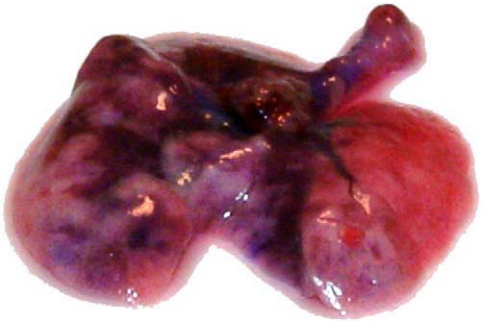

Fig. 3. India ink distribution in the airways of C57BL/6 mouse after endotracheal administration of aerosol.

Fluorescence associated with the nanobeads was detected at different levels of the pulmonary tree, suggesting a wide distribution in the lungs. As shown in Fig. 4, the fluorescent beads were not only found in the larger airways, but were

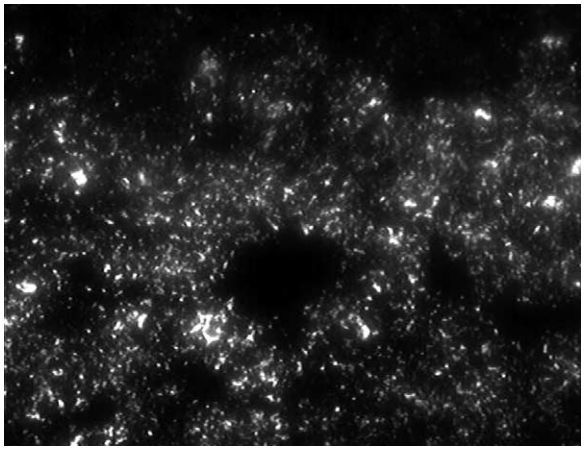

Fig. 4. Image of a 10 μm transverse lung section, at ×20 magnification showing the distribution of fluorescent nanoparticles after endotracheal administration.

distributed over the whole lung cross section. This suggests that the nanobeads applied by endotracheal application had access to the smaller airways of the lung.

3.4. Practical advantages in in vivo vaccination studies

This application was also used in our laboratory for the administration of DNA vaccines against *Mycobacterium tuberculosis* in 6–12 weeks old mice [18]. The vaccination regimen of this study demanded three pulmonary vaccinations at three weeks intervals. Once a mouse was anaesthetized, the entire procedure took about 3 min from the moment the mouse was taken from the cage, given the required aerosol dose and put back into the cage. The mice used in this study showed full recovery after 1 day and were kept alive until the time for immune response analysis.

4. Discussion and conclusions

The pulmonary application method described here offers the opportunity of multiple administrations of drugs or vaccines in mice avoiding any surgical procedure. The main challenge of endotracheal application in small animals such as mice is the visualization of the trachea, which is essential for reliable lung administration. Our experimental set-up facilitated tracheal visualization and enabled easy intubation regardless of the applicator used. The experiments using India ink solution verified successful intubation of the trachea and ruled out application to the esophagus. The droplet size produced was in the size range for deposition in the large airways, however, this does not exclude the possibility of aerosolized materials in the smaller size range to continue and move into smaller airways, as was shown for fluorescent nanobeads.

The experimental application of aerosols to mice lungs is made difficult due to the small size of the animal. Intratracheal instillation, though not correctly reflecting the natural inhalation mechanism, is therefore resorted to. With this report, we have demonstrated that the direct aerosolization of solutions and suspensions by endotracheal administration is feasible, and can easily and routinely be applied. The advantage of this method lie furthermore in minimal drug loss during application with an effective distribution in the lungs and offers fast processing of dozens of animals in a short period of time with relatively little suffering of the animals.

In conclusion, we believe this manuscript will assist researchers in implementing this endotracheal application that is suitable for different drugs, vaccines and challenge studies with pathogens that transmit diseases through inhaled aerosols.

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