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Pulmonary delivery of dry powders to rats: Tolerability limits of an intra-tracheal administration model

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ABSTRACT

The inhaled route is increasingly developed to deliver locally acting or systemic therapies, and rodent models are used to assess tolerance before clinical studies. Endotracheal intubation of rats with a probe which generates powder aerosols enables controlled administration of drug directly into the respiratory tract. However, preliminary observations of intratracheal powder administration procedures have raised concerns with regard to pulmonary safety. The aim of the present work was to evaluate the safety of intra-tracheal administration of dry powder in a rat model.

Sixty animals were administered various volumes of air alone, lactose or magnesium stearate through a Microsprayer® (Pencentury, USA). The mass of powder actually delivered to each animal was calculated. Rats were sacrificed immediately after administration, and the lungs, trachea and larynx were removed and examined for gross pathology.

The mass of powder delivered varied, the full dose being rarely delivered. About one third of the administration procedures resulted in respiratory failure, and macroscopic pulmonary lesions were observed in about 55% of animals. Lung damages were observed with air alone, lactose and magnesium stearate. In conclusion, artifacts observed with this technique may limit the relevance of the model. These observations are particularly important in the context of regulatory toxicity studies.

1. Introduction

The systemic delivery of macromolecules by inhalation is attracting considerable attention, as a number of peptides or proteins are more efficiently absorbed from the lung than from the oral, nasal, or transdermal routes. Efficient systemic absorption results from the lung's unique physiological features: a large absorptive surface area, a very thin diffusion path to the bloodstream, high blood flow, relatively low local metabolic activity and avoidance of first-pass hepatic metabolism (Barnes, 1993). There are three types of inhalation systems: dry powder inhalers, metered dose inhalers and nebulizers. Dry powder inhalers are propellant-free and environmentally safe, easy to use, and the stability of the formulation is guaranteed by the dry state. The value of dry-powder inhalation has been recognized both for local administration of candidate drugs to the respiratory tract to treat asthma, chronic obstructive pulmonary disease (Kawashima et al., 1998; Chapman et al., 2007) or tuberculosis (Sharma et al., 2001), and for systemic administration of numerous compounds such as hormones (Bosquillon et al., 2004), neurotransmitters (Bartus et al., 2004), chelating agents (Gervelas et al., 2007), hypoglycemic or immunomodulating drugs (Gao et al., 2009), and vaccine candidates (de Swart et al., 2007; Morello et al., 2009). Dry powder formulations comprise the drug itself and the excipients used to improve its physico-chemical stability and/or aerosolization properties. For instance, insulin has been formulated with mannitol, glycine and sodium citrate in dry powder aerosols which have been tested in clinical trials (Skyler et al., 2001).

Because drug and vaccine candidates require extensive testing in animal models prior to their approval for use in humans,

Abbreviations: Syr, syringe; AP, Air-Pump; Ex1, excipient 1 (magnesium stearate); Ex2, excipient 2 (lactose).

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experiments must mimic closely the intended formulation and delivery method of the final product. However, most studies are performed using models of acute administration in rodents, assessing lung deposition, efficacy and pharmacokinetics, and very few have focused on lung tolerability or safety.

Some authors have used tracheal intubation with insufflations to administer a powder aerosol *via* the respiratory route to mice or rats. This allows the drug to be deposited directly into the lungs, thereby ensuring accuracy of dosing and overcoming the potential pharyngeal and upper airway losses inherent in intranasal delivery. Published protocols for intubation of the mouse/rat trachea often involve blind intubation and invasive surgery, or require the investigator to fashion the necessary equipment (*e.g.* custom-designed fiber-optic light guide (Rivera et al., 2005) or light-carrying laryngoscopic blade (Molthen, 2006)). These procedures require extensive skill and time and increase the risk of harming the animal. In addition, the recovery time and risks to the animal following surgical procedures limit studies that require multiple dosing *via* the respiratory route. Although homemade equipment may prove cost-effective, commercially available equipment adapted to the intubation procedure allows greater accuracy and reproducibility of the procedure itself, and avoids loss of time and materials when making the equipment (Morello et al., 2009). A large number of studies use the commercial PennCentury DP-4 insufflation device connected to a syringe, which puffs powder from a reservoir through a delivery tube. However, unpublished personal observations of this procedure have revealed methodological artifacts affecting precise quantification of administered powder and lung safety/tolerability which may limit its relevance, particularly for toxicity studies. The aim of the present work was to evaluate lung tolerability when administering dry powders into the respiratory tract of rats using the DP-4 insufflator.

2. Methods

2.1. Materials

The test articles were dry powder formulations: magnesium stearate (Sigma–Aldrich Chimie S.a.r.l., St. Quentin Fallavier, France) and α-lactose monohydrate (Sigma–Aldrich Chimie S.a.r.l., St. Quentin Fallavier, France). Magnesium stearate (excipient 1 [Ex1]) has a promising potential to improve efficiency of dry powder aerosols (Ganderton, 1992). Lactose (excipient 2 [Ex2]) is routinely used as carrier in dry powder aerosols (Pilcer et al., 2011).

2.2. Conditions for powder administration

Powders were administered *via* the intratracheal route using a Microsprayer® consisting of an endotracheal metal tube (delivery tube) connected to an aerosol generator (Model DP-4 Dry Powder Insufflator – DPI, PennCentury, USA). Air puffs were administered using either a plastic syringe (5 mL) or the AP-1 Air Pump (capacity: 0–5 mL – PennCentury, USA). For both Ex1 and Ex2, a maximum of 10 mg could be loaded in the DP-4 reservoir. To evaluate the mass of powder delivered by the DPI, the device was weighed before and after each air puff of 2 mL (PRECISA, Poissy, France). The minimum numbers of air puffs required to fully empty the DPI were determinate (see Fig. 1 for experimental set-up). The median (min/max) mass of delivered powder was then calculated as a percentage. Using the DP-4 loaded with maximum amounts of excipient (*i.e.* 10 mg), 2 air puffs of 2 mL were needed to deliver 96% (88–99%) of Ex1, while 5 air puffs of 2 mL were needed to deliver 97% (95–100%) of Ex2 (Fig. 2).

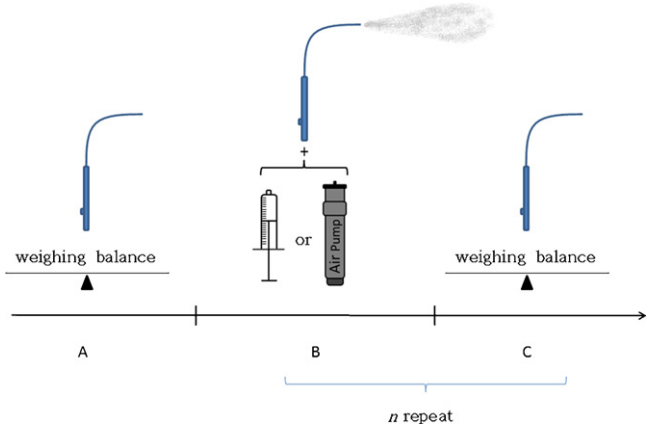

Fig. 1. Experimental *in vitro* setup used to determine the number of air puff required to empty the DPI. (A) The chamber of the insufflator device was loaded with Ex1 or Ex2, then the DPI was weighed; (B) 2 mL of air dispensed by a syringe or by the Air Pump forced the powder to be aerosolized; (C) the DPI was reweighted. The weight (A) and (C) were compared to determine the mass of powder administered. (B) and (C) were repeated until the DPI was fully empty. Ex1: excipient 1 (magnesium stearate), Ex2: excipient 2 (lactose).

2.3. Particle size

The volume median particle diameter (min/max) of each powder sprayed by the DP-4 was measured by laser diffraction (Mastersizer-X, Malvern Instruments, UK) following the standard experimental set-up (Majoral et al., 2007; Pilcer et al., 2008). Briefly, the aerosol passed through the laser beam and was directed toward an extraction pump (40 L/min). The measurement was automatically performed during the aerosol generation by the activation of the data acquisition for an obscuration higher than 5%. The dispersion code was "polydisperse" and the optical presentation was the 2RHA suitable code for dry particle: the real refractive index was 1.45 for the powder, the imaginary refractive index was 0.1 for the powder and the real refractive index was 1 for the air. The volume median diameter for the Ex1 and Ex2 aerosols was 19 ± 3 μm and 59 ± 7 μm respectively (6 experiments per excipient, results in mean ± standard deviation).

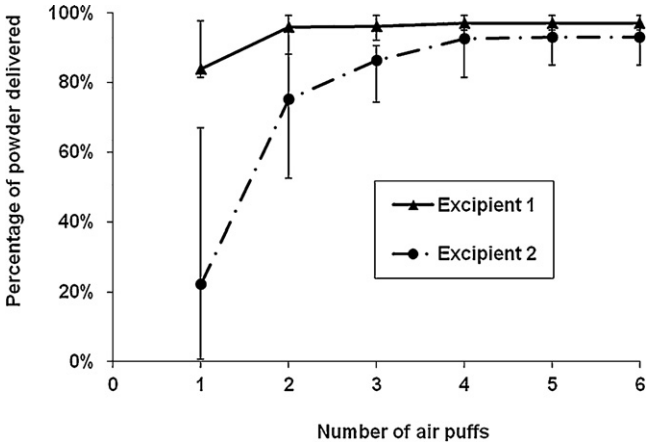

Fig. 2. Powder delivered with the DP-4 in the *in vitro* study. Number of 2 mL air puffs needed to empty the DP-4 Microsprayer® (air puffs were administered using a plastic syringe, the same results were obtained with the AP-1 Air Pump). Results are expressed as the median percentage (Min/Max) of initial mass of powder loaded in the DP-4 device (6 experiments for each number of air puffs and for each excipient).

Table 1
Percentages (median and range) of delivered powder using a DP-4 Microsprayer® connected to a syringe (Syr) or to an AP-1 Air Pump (AP).

Compound	Excipient 1 (magnesium stearate)		Excipient 2 (lactose)	
Groups	10 mg Ex1-Syr	10 mg Ex1-AP	10 mg Ex2-Syr	10 mg Ex2-AP
Median value (%)	92	71	35	16
Range of %	7–100	14–85	2–71	2–97
Global variation	$p = 0.05$ (KW exact test – Monte Carlo 99% confidence interval: 0.047–0.059)			

2.4. Animals and housing conditions

All animal experiments were conducted in compliance with Council Directive No. 86/609/EEC of 24th November 1986 on the harmonization of laws, regulations and administrative provisions regarding the protection of animals used for experimental or other scientific purposes and decree no. 2001-486 of 6th June, 2001 (Official Journal of French Republic of 8th June, 2001). Accordingly, animal facilities and experimental areas are listed under number C37-261-3 by the "Direction Départementale de la Protection des Populations d'Indre et Loire", and the study director is entitled to supervise and to practice experimental studies by authorization no. 37 075. Female Sprague Dawley rats (8 weeks of age; body weight range 200–224 g) were obtained from the Etablissements JANVIER (Le Genest-Saint-Isle, France). All animals were housed in pairs (cage type III, 800 cm^2) in a temperature-controlled room ($22 \pm 2\,^{\circ}C$) with a 12-h light/12-h dark cycle. They had free access to water and food (diet 20-16, Harlan, France). The experimental protocol was conducted according to NIH guidelines for the Care and Use of laboratory animals.

2.5. Experimental design

Sixty rats were divided into 2 groups with 5 sub-groups each (6 rats per sub-group). For one group, a 5 mL plastic syringe was used to administer air or powder, and for the other, the AP-1 Air Pump was used. The 5 sub-groups in each group were administered air alone (2 mL, 2×2 mL or 5×2 mL), Ex1 (10 mg/rat) or Ex2 (10 mg/rat) (Fig. 3). Before administration, rats were placed in a chamber and anesthetized for 4 min with isoflurane at a 4% vaporizer output concentration (Aerrane®, Centravet, France). Animals were then rapidly placed in a supine position at a 45° angle (suspended by their incisors) on a work stand (T.E.M., France). The tongue was gently pulled out of the mouth with a spatula to obtain a clear view of the trachea. An otoscope (T.E.M., France) was used to keep the mouth open and to immobilize the tongue outside the mouth. A trained operator inserted the Microsprayer® into the trachea *via* the larynx, and air or powder was administered into the respiratory tract. The actual mass of powder delivered to each animal was measured by weighing the device before and after administration.

2.6. Observations and examinations

Rats were weighed before administration. Respiratory arrest and deaths occurring during the administration procedure were recorded. Immediately after administration, rats were killed by exsanguination *via* the abdominal aorta. The lungs, trachea and larynx were removed, photographed and examined for gross pathology by an observer blinded to the protocol, based on observations of ventilatory-induced lung injury by Dreyfuss and Saumon (1998). Lungs were classified according to the type and severity of external pulmonary damage: category 1 "no lung damage", category 2 "zones of atelectasis", and category 3 "enlarged and congested lungs". Lung, trachea and larynx were then harvested, weighed, dried in an oven at 60 °C for 72 h and reweighed in order to assess the organs edema. The lung wet to dry weight ratio was calculated as follows: $(\text{weight}_{wet} - \text{weight}_{dry})/\text{weight}_{wet} \times 100$.

2.7. Statistical analysis

Non-parametric exact procedures were performed using the StatXact-3 software (Version 3.0.2, CYTEL Software Corporation); medians, quartiles (Q1 and Q3) and/or minimum and maximum values were used as descriptive statistics. Overall group differences in weight, lung wet to dry *ratio* and percentage of delivered powder were assessed using the Exact Kruskal–Wallis Test (KW). When overall differences were statistically significant, between-group comparisons were performed using an Exact singly ordered Kruskal–Wallis Test (single ordered KW) and an Exact Permutation Test with General Scores (PTGS).

Differences in pulmonary damage (qualitative data) were investigated using the Exact Pearson's Chi-square Test ($PChi^2$). A $p \leq 0.05$ (α) was considered as statistically significant.

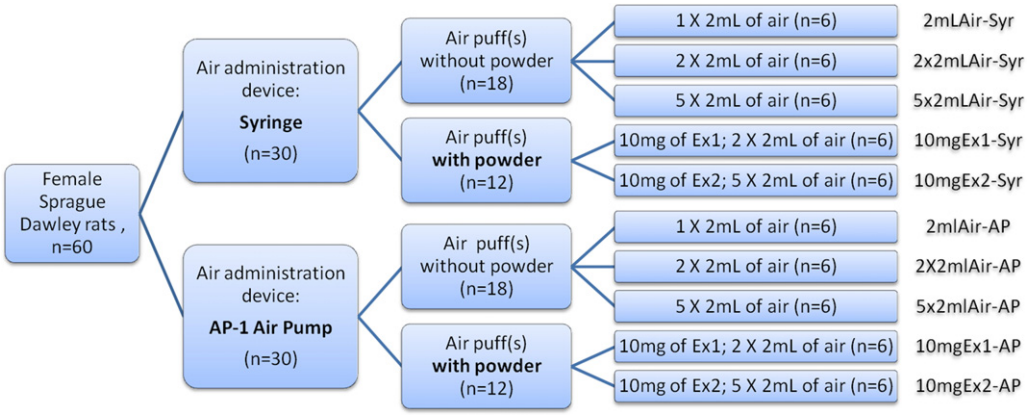

Fig. 3. Summary of the experimental design.

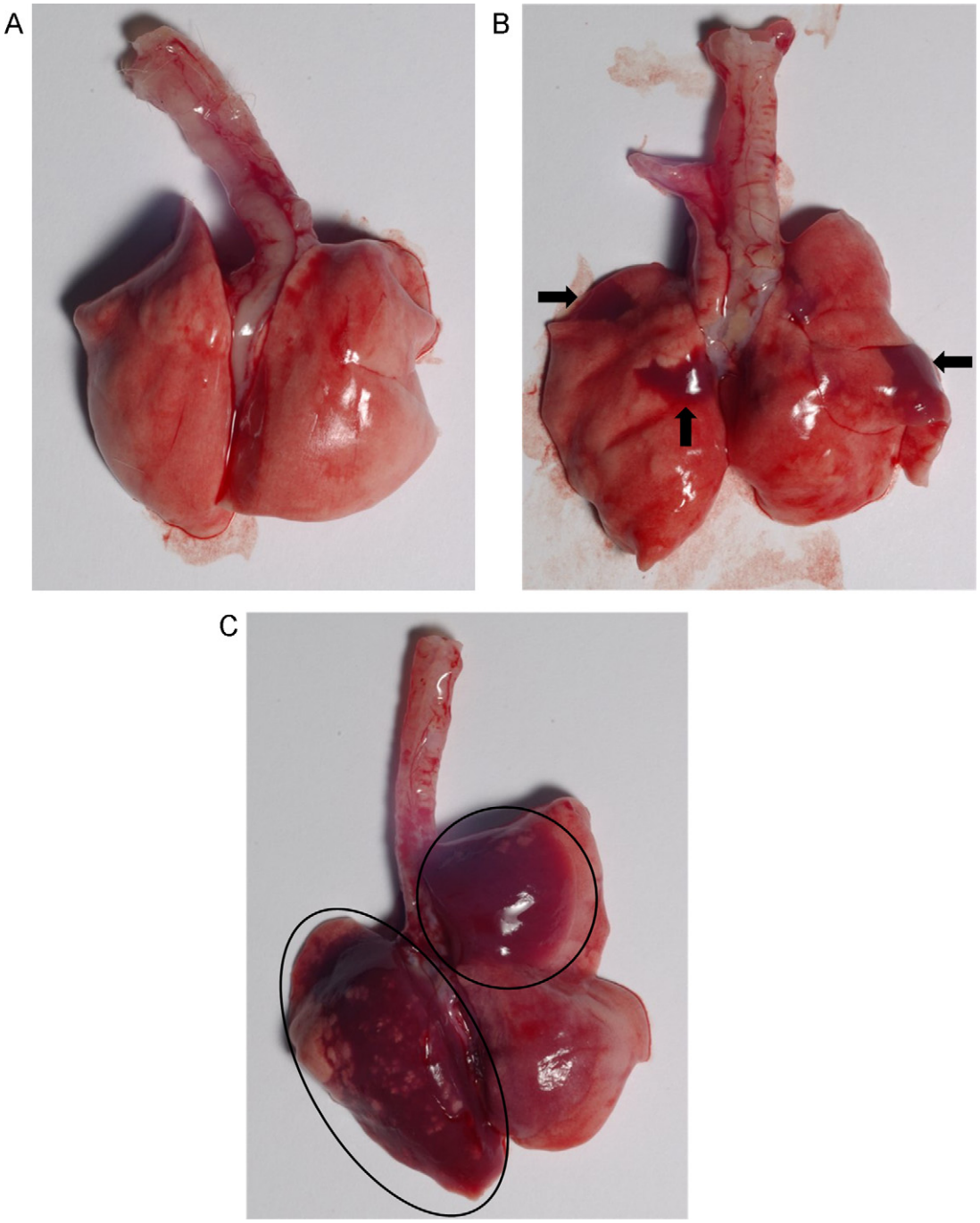

Fig. 4. Gross pathology of rat lungs. After air and/or dry powder administration, sacrifice and removal of lungs, trachea and larynx, an observer blinded to the protocol classified lungs into category 1: "no lung damage" (A); category 2: "zones of atelectasis" (B); or category 3: "enlarged and congested lungs" (C).

3. Results

3.1. Administered doses

The actual mass of powder delivered to each animal varied according to the compound and dose. As shown in Table 1, the total mass of powder was rarely delivered. The data also show very high within-group variability, with a wide range in the percentage of actually delivered loads. No difference was observed between the syringe (Syr) and AP-1 Air Pump (AP) conditions (Syr *versus* AP: $p = 0.87$ for Ex1 and $p = 0.55$ for Ex2, PTGS). However, Ex1 was administered more efficiently than Ex2, but due to high within-group and between-group variability, differences were not significant ($p = 0.062$, KW).

3.2. Clinical results

The initial median weight of rats in the groups was similar ($p = 0.75$, KW), ranging from 212 to 256 g. Respiratory arrests of short duration (~4–5 s) were observed during 9 of the 60 administration procedures, all occurring in powder administered animals. Air alone never induced respiratory failure. Thus, approximately one third of the powder-administration procedures (9/24) resulted

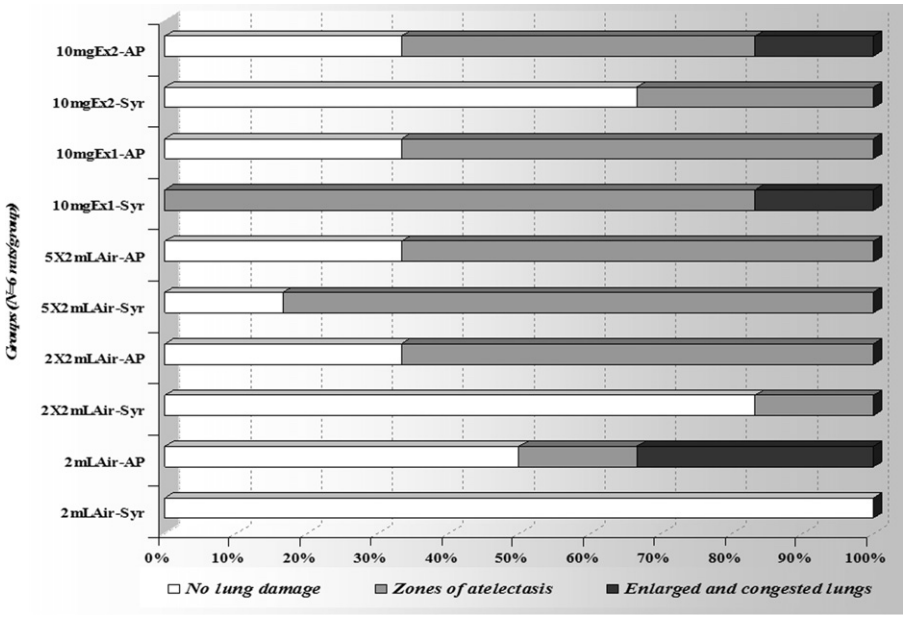

Fig. 5. Percentages of lungs with no damage, with one or more zones of atelectasis, or which were enlarged and congested, per group. Animals were administered air puffs (1× 2 mL, 2× 2 mL and 5× 2 mL with a syringe or with an Air Pump) or dry powders (10 mg of Ex1 or 2) pushed out of the Microsprayer reservoir by air puffs (2× 2 mL and 5× 2 mL). Syr: syringe, AP: Air-Pump, Ex1: excipient 1 (magnesium stearate), Ex2: excipient 2 (lactose).

in respiratory failure, but only one in death. Eight of the 9 arrests occurred with Ex1.

3.3. Gross pathology

Macroscopic pulmonary lesions were detected in 33 of the 60 rats. Lesions consisted of atelectasis, congestion, or edema (Fig. 4A–C). As shown in Fig. 5, lesions were observed under nearly all conditions, but varied in severity according to groups (unordered $PChi^2$, $p = 0.02$). Lung damage (category 2 and category 3) was observed in 66% (16/24) of the powder administered animals, and in 47% (17/36) of air administered animals. Only the group of rats receiving a volume of 2 mL air using a syringe (group 2 mL Air-Syr) showed no macroscopic lesions. Due to the small sample size and to the high within-group variability, between-group differences were not significant (singly ordered KW, $p = 0.17$). However, the number of rats with lung damage increased with the number of insufflations and the volume (singly ordered KW tendency, $p = 0.0009$). The most severe lung damages (congestion and edema) were observed during administration of 2 mL of air alone *via* the AP-1 Air Pump or during administration of 10 mg powder.

3.4. Lung wet to dry ratio

There was no difference in lung wet to dry *ratio* between the experimental groups ($p = 0.34$, KW). Median percentages of humidity in the lungs varied slightly from 77.7 to 79.6% according to groups

4. Discussion

We evaluated the lung tolerability of pulmonary delivery of excipient in dry powder (lactose and magnesium stearate) to rats using a specific device. Respiratory arrest of short duration and macroscopic pulmonary lesions were recorded; lesions could be observed after administering both a single small volume of air and different dry powders. Furthermore, we observed an important variability in terms of the mass of powder actually delivered by the DP-4 device.

Mortality or safety data are rarely reported in studies using dry powder inhalers in rodent models. We observed 3% mortality in the rats in our experiment, and 9% has previously been reported in mice (Morello et al., 2009). This mortality rate could be acceptable but the rate of respiratory failure and lung damage observed in the present study could be a problem, particularly for experiments designed for pharmacokinetic studies or lung absorption measurements. Lung damage was observed in all groups, including the 2 mL Air-AP group in which rats were administered 2 mL of air alone *via* the AP-1 Air Pump. Pulmonary lesions secondary to high tidal volume ventilation have been widely reported. Several studies have examined the role of lung distension in the genesis of pulmonary edema (Dreyfuss and Saumon, 1998), revealing that excessive distension, or "stretch", with large tidal volumes causes the disruption of pulmonary epithelium and endothelium, increases permeability and is associated with lung edema. In our study, rats were administered 2 mL, 2× 2 mL or 5× 2 mL of air with or without powder. Morello et al. (2009) also observed that an air puff greater than 0.25 mL sometimes resulted in the immediate death of mice. In a control group of Wistar rats administered with 3 mL of air bolus without dry powder, the total protein concentration in broncho-alveolar lavages was greater than in that of untreated rats (Codrons et al., 2003). A high protein concentration in broncho-alveolar fluid strongly suggests permeability-type edema. Some of the deaths or respiratory arrest might have been due to laryngeal spasm. If animals are not perfectly anesthetized, endotracheal stimulation with the Microsprayer® could lead to laryngeal spasm (reflex adduction of the vocal cord) which could be fatal. The upper airway obstruction, secondary to the laryngeal spasm, is then responsible for a negative-pressure pulmonary edema, consistent with the lung damage observed in our study. Negative-pressure pulmonary edema following laryngeal spasm during general anesthesia is a well-recognized but rare complication secondary to upper airway obstruction in human general anesthesia (Alalami et al., 2008). However, in the present study, direct laryngoscopy revealed no adduction of the vocal cords while rats were

making inspiratory efforts; therefore no laryngeal spasm was diagnosed. Thus, there is little evidence to support this hypothesis, although laryngeal spasm could be misdiagnosed in small animal models.

Particle size of powder aerosol obtained with the DP device ranges between 1 and 14 μm according to the compound (Alcock et al., 2002; Grainger et al., 2004; Koushik et al., 2004; Qian et al., 2009; Ungaro et al., 2009). However, large particle sizes, comparable with those obtained in our study (47–59 μm for lactose, about 20 μm for magnesium stearate), have also been reported: powder aerosol containing insulin mannitol (5–21 μm) (Todo et al., 2003), lipid-based microparticles (4–20 μm) (Dellamary et al., 2004), chitosan microparticles (20–150 μm) (Huang et al., 2005) and azithromycin loading powders (4.7–52.1 μm) (Zhang et al., 2010). Lung deposition of these large particles has been measured in macaques and rats with liquid aerosols (Beck et al., 2002; Montharu et al., 2010), but there are no studies reporting the distribution of aerosol deposition in the lungs of rats or macaques for powder aerosols.

Lactose and magnesium stearate are crystalline and thus have an intrinsic tendency to take up moisture from their surroundings (a hygroscopic property), resulting in increased particle size when passing through the humid environment of the airways. The humidity differences between rat airways and the conditions of *in vitro* experiments could result in more proximal lung deposition than expected, and could also contribute to relative tracheal obstruction. The high rate of respiratory failure observed during powder administration could thus be secondary to a resistive respiratory load generated by proximal aerosol deposition. In an anesthetized rat model with inspiratory resistive load (75% of the peak tracheal pressure developed during a prior 30-s occlusion), bradypnea was observed seconds after the obstruction; respiratory pump failure occurred in all rats when the inspiratory resistive load was maintained, and most of the rats died within 5 min (Simpson and Iscoe, 2007). The mechanism responsible for the bradypnea was unclear but may have been part of a central reflex mechanism designed to reduce diaphragmatic activity, thereby avoiding fatigue and pump failure. Besides inspiratory resistive load, aerosol administration also induces expiratory occlusion and elevated end-expiratory lung volume, well-known causes of cardiovascular failure (Michard et al., 1999). Cardiovascular failure secondary to expiratory load has previously been reported in a spontaneously breathing anesthetized rat model (Simpson et al., 2009). Our observations are consistent with the cardio-respiratory consequences of respiratory load, as previously described (Simpson and Iscoe, 2007; Simpson et al., 2009), which could be a plausible explanation.

5. Conclusion

Study of the pulmonary route for local and systemic drug delivery is on-going, and animal testing plays a very important role in the assessment of aerosol delivery. The development of devices for dry-powder pulmonary insufflation in animals is particularly relevant for investigations in this field as it closely mimics clinical use in humans. However, the rat model should be used with caution. We observed that lung damage in the rat was probably due to the procedure itself as insufflation of air alone could result in pulmonary congestion. The consequences appear to be minor because the mortality rate was low, but this outcome should be taken into account in pharmacokinetic studies. Indeed, the probable disruption of the alveolar-capillary barrier in the rat model could limit the relevance of systemic bioavailability. In order to improve this method of pulmonary delivery, we strongly recommend that mortality rates and other safety data should be reported systematically; a control group with insufflation of air alone also appears to be essential.

Contributions

All authors participated in the research and/or article preparation.

A. Guillon: conception and design of the study, acquisition of data, interpretation of data, drafting the article.

J. Montharu: conception and design of the study, *in vivo* research, acquisition of data.

L. Vecellio: in vitro tests, drafting the article content.

V. Schubnel: *in vivo* research technician.

G. Roseau: *in vivo* research technician.

J. Guillemain: revising the article critically for important intellectual content.

P. Diot: revising the article critically for important intellectual content.

M. de Monte: conception and design of the study, *in vivo* research, acquisition of data, analysis and interpretation of data, drafting the article.

Disclosure statement

Guillon A., Montharu J., Vecellio L., Schubnel V., Guillemain J., Diot P., and de Monte M. declare that they have no conflict of interest.

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