

Systemic and pulmonary effects of fluticasone administered through a metered-dose inhaler in rats

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Background: Metered-dose inhalers (MDIs) are convenient, simple, inexpensive, and reproducible devices for administering aerosolized drugs through the pulmonary route, but methods have not been available for use of these devices in small animals.

Objective: We sought to test the efficacy of delivery of fluticasone through an MDI to rats with a rodent-adapted spacer chamber and to compare this treatment with systemic dexamethasone for the acute pulmonary allergic inflammatory response.

Methods: Changes in body and thymus weights were used as indicators for systemic steroid effects. Rats were sensitized to ragweed pollen extract 2 weeks before the experiment, and pulmonary allergic responses were evaluated 48 hours after a single aerosolized antigen challenge on the basis of bronchoalveolar leukocytes, lung tissue sections, total lung capacity, and forced expiratory volumes.

Results: Inhaled fluticasone caused dose-related systemic effects, indicating successful pulmonary drug delivery. Inhaled fluticasone was more effective than placebo but less effective than systemic dexamethasone in attenuating the increase in lung eosinophils and inflammatory infiltrates and the decrease in total lung capacity associated with the allergic inflammatory response. Inhaled fluticasone prevented airway obstruction and proximal inflammation, as did dexamethasone, but it appeared to have less effect in areas of lung served by the most distal airways.

Conclusion: This is an effective method for use of MDIs to deliver inhaled drugs to small laboratory animals, and it should be valuable for investigations of treatment effects, as well as for *in vivo* testing of delivery devices. (J Allergy Clin Immunol 2004;114:1027-32.)

Key words: Asthma, animal models, aerosols

Abbreviations used

BAL: Bronchoalveolar lavage
FEV_{0.2}: Forced expiratory volume in 0.2 seconds
FVC: Forced vital capacity
MDI: Metered-dose inhaler
TLC: Total lung capacity

Inhaled drugs are the mainstay of therapy for obstructive pulmonary diseases and frequently are used in animal models to investigate disease mechanisms and to test agents in a therapeutic context.¹ Because the biophysics of aerosol generation and pulmonary drug delivery are complex, the deposition of drug in the airways is highly dependent on the manner in which the aerosol is generated and inhaled, and dose-response relationships might not be reproducible among different delivery systems.² Metered-dose inhaler (MDI) products are designed to produce aerosolized drug in a consistent manner, allowing predictability of effects when inhaled with optimal technique. However, MDI devices do not adapt readily for inhaled drug administration to small animals, and thus animal models lack a simple standardized method for aerosol drug delivery, and MDI systems have not had a suitable small animal method for *in vivo* investigations. The goals of this study were to determine whether MDI-administered fluticasone could be delivered effectively to the lungs of a small animal by using a rodent-adapted spacer chamber and to compare the efficacy of this treatment with systemic dexamethasone for suppressing an acute allergic pulmonary inflammatory response.

METHODS

Animals

Male BN/SsN HSD (Brown Norway) rats were purchased (Harlan, Indianapolis, Ind) and housed in an American Association for Accreditation of Laboratory Animal Care-accredited facility. All procedures were approved by the University of Wisconsin Animal Care and Use Committee and conformed to "the Guide for the Care and Use of Laboratory Animals" (1996). All rats were sensitized to short ragweed pollen extract (Hollister-Stier Labs, Spokane, Wash), with a single subcutaneous injection of extract (1 mg of protein)

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FIG 1. Administration of aerosolized drug to a rat with the MDI and modified spacer chamber.

combined with adjuvant (Imject Alum; Pierce Biotechnology, Rockford, Ill) injected about 2 weeks before the beginning of the study.

Aerosol treatments

An infant MDI spacer chamber (AeroChamber; Monaghan Medical, Plattsburgh, NY) was modified by the investigators to allow delivery of an MDI aerosol to an awake rat. The face mask and valve were removed from the spacer chamber and replaced with a fenestrated latex membrane that served as a nose cone for the rat. The rat was positioned with only its nose and mouth within the chamber opposite the MDI. The MDI was actuated in the standard horizontal position, and then the chamber was rotated into a vertical position, with the rat's nose at the bottom (Fig 1). The rat was allowed to breathe from the chamber for 1 minute after the actuation, and the treatments were conducted once daily in the morning. Placebo and fluticasone propionate (44, 110, and 220 μ g per actuation) MDIs were provided by GlaxoSmithKline (Research Triangle, NC) for the study. These chlorofluorocarbon-propelled MDIs deliver particles having a mean mass aerodynamic diameter of approximately 3 μ m.³

Study design

There were 6 treatment groups, each with 6 rats (Table I). For antigen challenge, rats were placed in a plastic box into which an aerosolized solution of 2% short ragweed pollen extract or placebo (PBS) aerosol was delivered from an ultrasonic nebulizer (Ultraneb 99; DeVilbiss, Somerset, Pa) for 20 minutes. The challenges were performed after the seventh daily MDI treatment or the second daily dexamethasone treatment, and the rats were studied 2 days after the challenge (24 hours after the last treatment). In rats high-dose systemic dexamethasone for 3 days effectively suppresses the pulmonary allergic inflammatory response, causes a marked catabolic effect with weight loss, and causes involution of the thymus gland.⁴⁻⁶ Systemic dexamethasone and inhaled placebo were used as positive and negative treatment controls with which to compare inhaled fluticasone treatments. Inhaled drug treatments were started 7 days before allergen challenge to detect more subtle effects on body weight and thymus weight.

Outcome variables

The rats were assessed both for systemic effects of glucocorticoid and for local suppression of the pulmonary allergic inflammatory response to evaluate the efficacy of drug delivery through the pulmonary route. We measured body weights daily during the treatments and thymus weights at the end of the experiment as indicators of systemic glucocorticoid effects. In preliminary experiments we established that the ragweed pollen extract allergen challenge produces a moderately severe eosinophilic pulmonary inflammatory response that peaks in 2 days after the challenge and is detectable as increased numbers of bronchoalveolar lavage (BAL) fluid leukocytes, inflammatory infiltrates in lung tissue sections, reduced total lung capacity (TLC), and altered airway physiology. These variables were used to assess the pulmonary allergic response among treatment groups.

Physiology studies

Measurements were conducted in a pulmonary maneuvers apparatus (Buxco Electronics, Sharon, Conn), as described in detail previously.⁷ Rats were sedated with 40 mg/kg pentobarbital intraperitoneally, and an orotracheal tube (PE240, 5 cm) was placed atraumatically. Rats were positioned in a total-body plethysmograph that was equipped with software-controlled valves to positive and negative pressure reservoirs that allowed lung inflations and forced expirations analogous to spirometry in human subjects and measurement of thoracic gas volume (Boyle's Law method). A reduction in TLC caused by a loss of airspace, airway occlusion, and decreased lung compliance was used as an indicator of pulmonary inflammation. The forced expiratory volume in 0.2 seconds (FEV_{0.2}; analogous to FEV₁ in human subjects) and the forced vital capacity (FVC) were measured during a forced expiration from TLC and were expressed as fractions of TLC volume to evaluate airway function relative to available lung volume.

Pulmonary inflammation

After physiologic measurements, the rats were anesthetized with urethane (Sigma, St Louis, Mo) and killed by means of exsanguination. The chest was opened, and a clamp was placed on the left mainstem bronchus to allow BAL of the right lung. The right lung was filled with PBS to TLC by means of gravity and drained 5 times, the lavage fluid was centrifuged, and the cell pellet was resuspended in 1 mL of buffer. The total number of BAL leukocytes was determined with a cell counter (Beckman Coulter, Hialeah, Fla), and slides were prepared for a differential leukocyte count on the basis of 200 cells. Lungs were fixed by filling with formalin, and paraffin sections of the left lung were stained (Giemsa) and evaluated for inflammation.

Quantitative assessment of inflammatory infiltrates was conducted with SigmaScan Pro 5 image analysis software (Statistical Solutions, Saugus, Mass). Lung sections from the 220- μ g fluticasone propionate (F220), dexamethasone, and placebo treatment groups were evaluated in a blinded manner. For each section, 10 digital images were captured by using the 20 $\times$ objective of the microscope, the first placed randomly at a tangent to the pleural edge, followed by 4 additional fields placed 2 fields apart along the pleural edge of the section and 5 fields in the central area of the section also spaced 2 fields apart. When a field included a lumen of a large airway or blood vessel, the field was moved slightly until it was tangent to the luminal space. Inflammatory infiltrates were stained blue, reflecting large numbers of cell nuclei, and the intensity of infiltrate was quantified as the proportion of blue color in each digital image.

Data analysis

All variables were analyzed by using ANOVA with a general linear hypothesis model, and planned pairwise comparisons were

TABLE I. Study groups

Group	Treatment	Challenge
Ctrl	Placebo MDI daily $\times 8$ d	Placebo
Plac	Placebo MDI daily $\times 8$ d	Ragweed pollen extract
F44	Fluticasone propionate 44 μg MDI daily $\times 8$ d	Ragweed pollen extract
F110	Fluticasone propionate 110 μg MDI daily $\times 8$ d	Ragweed pollen extract
F220	Fluticasone propionate 220 μg MDI daily $\times 8$ d	Ragweed pollen extract
Dex	Dexamethasone 500 $\mu\text{g}/\text{kg}$ subcutaneously daily $\times 3$ d	Ragweed pollen extract

done with the Fisher least-significant-difference test. An orthogonal contrast was used to test whether all fluticasone treatments combined differed from placebo treatment with regard to BAL fluid eosinophils. Cell-count data and image-analysis data were log-transformed to conform with parametric assumptions. SYSTAT statistical software (version 10; SPSS Inc, Chicago, Ill) was used for all analyses.

RESULTS

Systemic effects

Fig 2 shows the change in body weight during the treatment period in the 6 study groups. There was a dose-related effect of fluticasone MDI treatments on body weight, with the weight changes being significantly different among each of the 3 doses ($P < .01$). Weight gain in the 44 μg fluticasone propionate (F44) group was not significantly different compared with that seen in the placebo group, but weight gain was significantly reduced in the 110- μg fluticasone propionate (F110) group compared with in the placebo group ($P < .0001$), and the F220 group lost weight during the 8 days of treatment ($P < .0001$). However, 3 days of systemic dexamethasone treatment caused a more dramatic loss of body weight than 8 days of the highest dose of inhaled fluticasone ($P < .0001$). Similarly, thymus weight was reduced after fluticasone MDI treatments in a dose-related manner, with significant differences among each of the 3 doses ($P < .02$; Fig 2). The thymus weights of the F44 group were not significantly different from those of the placebo group, and the thymus weights of the F220 group were not significantly different from those of the dexamethasone group (Fig 2). These results demonstrate that systemic absorption of the inhaled fluticasone occurred in a reproducible and dose-related manner and confirm the efficacy of the MDI aerosol for pulmonary drug delivery to rats.

Pulmonary effects

Both MDI-administered fluticasone and systemic dexamethasone treatments significantly reduced the numbers of BAL leukocytes compared with placebo ($P \leq .01$ for each treatment vs the placebo group), but there were no significant differences in BAL leukocytes among the various glucocorticoid treatment groups (Fig 3). Similarly, glucocorticoid treatments reduced the numbers of BAL fluid eosinophils, with the dexamethasone group having the greatest reduction compared with the other allergen-challenged groups ($P < .004$). The fluticasone

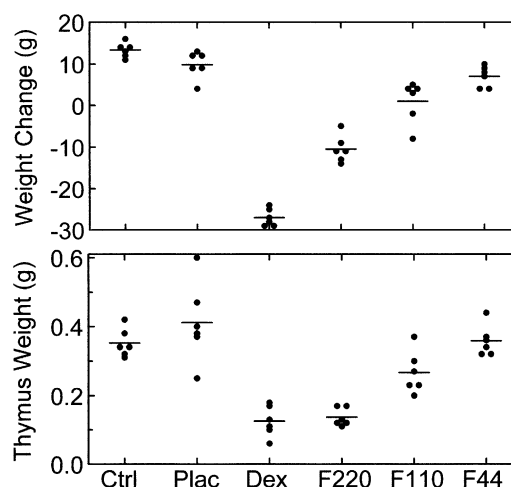


FIG 2. Change in body weight during the 8 days of MDI treatments or the 3 days of systemic dexamethasone treatment and thymus weights at the end of the studies. Each dot represents one rat, and bars indicate the mean of each study group of 6 rats. Study groups are defined in Table I.

groups were not significantly different from one another with regard to BAL eosinophil numbers but together had fewer eosinophils compared with the placebo group ($P = .007$; Fig 3). Fig 4 shows reduced TLC in the placebo group compared with the unchallenged control group ($P < .001$), which is associated with the pulmonary allergic inflammatory response. Systemic dexamethasone effectively protected the rats from this TLC reduction ($P < .001$), but among the fluticasone-treated groups, only the highest dose had a significant effect ($P = .02$) compared with placebo, and this treatment was less effective compared with systemic dexamethasone ($P = .001$). Both inhaled fluticasone and systemic dexamethasone treatments protected the rats from inflammation-associated airway obstruction, as indicated by a significantly higher $\text{FEV}_{0.2}$ and FVC values in the dexamethasone, F44, and F220 groups compared with values seen in the placebo group ($P < .03$; Fig 4). The F110 group did not achieve statistically significant protection from airway obstruction compared with the placebo group because of one outlier in the F110 group that had reduced $\text{FEV}_{0.2}$ and FVC values; this rat was similar to the others in the F110 group with regard to the indicators of systemic effects and pulmonary inflammation, however. The F220 group had $\text{FEV}_{0.2}$ and FVC values comparable with those of the dexamethasone group ($P > .13$; Fig 4). Left lung tissue

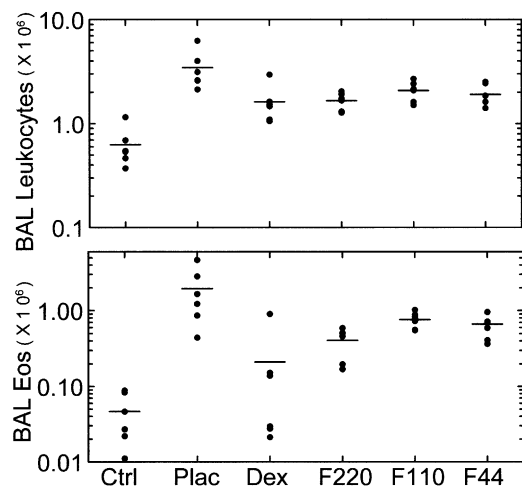


FIG 3. Numbers of leukocytes and eosinophils obtained from right lung BAL fluid 2 days after challenge with aerosolized allergen. Each *dot* represents one rat, and *bars* indicate the geometric mean of each study group of 6 rats. Study groups are defined in Table I.

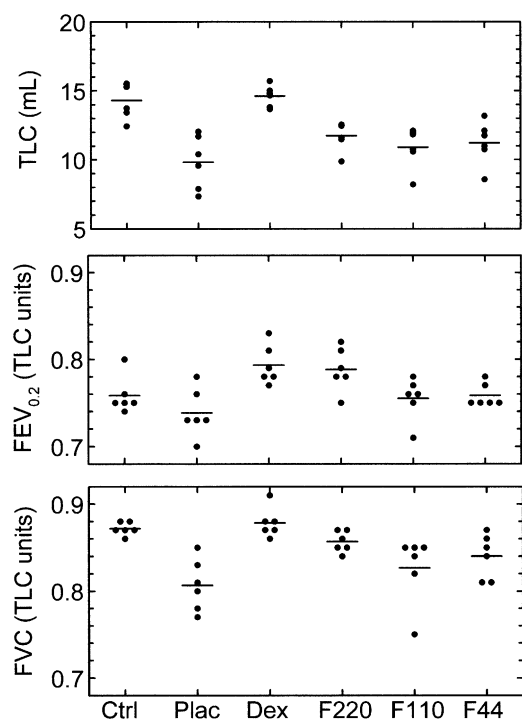


FIG 4. TLC, FEV_{0.2}, and FVC measured 2 days after challenge with aerosolized allergen. FEV_{0.2} and FVC are expressed as a fraction of TLC. Each *dot* represents one rat, and *bars* indicate the mean of each study group of 6 rats. Study groups are defined in Table I.

sections revealed extensive inflammatory infiltrates in the placebo group (Fig 5, A and D) and minimal inflammation in the dexamethasone group (Fig 5, C). Quantitative assessment of inflammatory infiltrates (Table II) revealed that the F220 group had more inflammation compared with the dexamethasone group ($P < .0001$) and less

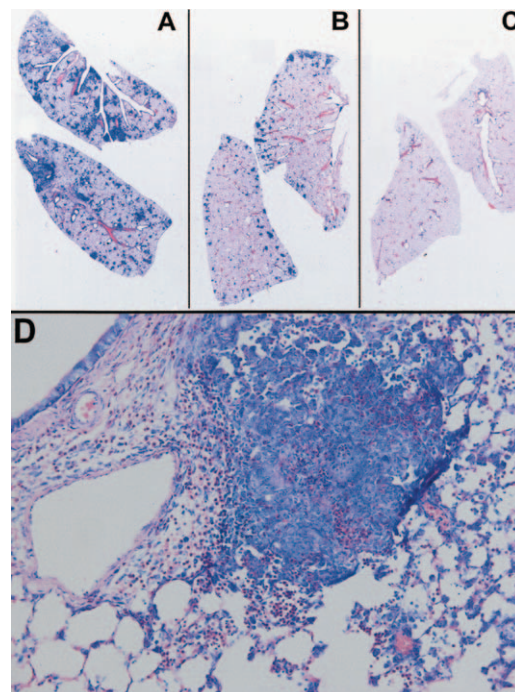


FIG 5. Giemsa-stained mid-sagittal sections of left lungs 2 days after allergen challenge. Areas of inflammatory infiltrates (*blue color*) at low magnification in lungs from the placebo (A), F220 (B), and dexamethasone (C) groups are shown. D, Higher magnification of Fig 5, A, showing eosinophilic inflammation in the wall of a large airway, surrounding an adjacent blood vessel, and in a portion of the adjacent alveoli.

inflammation compared with the placebo group ($P = .0001$). In fluticasone-treated rats the inflammatory infiltrates were more prominent near the pleural surface compared with the central area ($P = .03$), suggesting less-efficient drug delivery to the areas served by the airway branches having the greatest distance from the airway opening (Fig 5, B, and Table II). In contrast, lung sections from the dexamethasone and placebo groups showed no difference in the intensity of inflammation in the sub-pleural versus central areas ($P > .5$; Fig 5 and Table II).

DISCUSSION

Pulmonary drug delivery to a rat is effective with an MDI and a modified spacer chamber, as demonstrated by dose-related systemic glucocorticoid effects and protection from acute pulmonary allergic inflammation. The advantages of this method are the simplicity of the technique, the ability to use standardized dosage forms, and the limitation of external surface drug exposure to just the areas around the nose and mouth. The technique should be readily adaptable to other small animals that are used for laboratory experiments because the aerosol drug dose would be scaled to the animal's ventilation volume per minute.

The daily MDI treatment was not as effective as systemic high-dose dexamethasone for preventing the

loss of TLC caused by acute pulmonary allergic inflammation. There are several potential explanations for this. First, this was a high-dose allergen challenge that produced moderate-to-severe pulmonary inflammation in the placebo-treated group, so that any treatment that did not achieve profound suppression of the immune response likely would be overwhelmed. Second, it might be necessary to achieve high systemic concentrations of glucocorticoid (as was done with the dexamethasone treatment) to suppress the extrapulmonary components of the immune response to allergen challenge more completely.⁸ Third, once daily dosing of inhaled fluticasone might not have had sufficient duration between doses to suppress a vigorous acute inflammatory response. Fourth, the subpleural pattern of inflammation in the fluticasone groups and the lack of a distinct dose response for the anti-inflammatory effect of the inhaled therapies suggest that distribution of aerosolized drug within the airways, rather than drug potency, might be an important determinant of efficacy. This might explain the failure of inhaled drug to prevent inflammation, as did the systemic drug, even though the MDI treatment appeared to protect the more central areas of the lung nearly as well as dexamethasone, as shown in lung tissue sections and as measured on the basis of FEV_{0.2}. This observation is consistent with the growing recognition of distal airway involvement in human asthma and the concern that conventional inhaled steroid therapy might not reach these areas effectively.⁹

Studies in human subjects with radiotracer techniques have identified aerosol particle size and inhalation technique as important variables affecting total pulmonary deposition and regional deposition of inhaled drugs.¹⁰⁻¹⁷ Comparison of 2 formulations of beclomethasone MDIs revealed that the one having a median particle diameter of 1.1 μm deposited a larger total amount of drug in the lungs, as well as a larger proportion in the peripheral regions of the lungs, compared with the MDI that generated particles with a median diameter of 3.5 μm .¹² Aerosols of 5- μm monodisperse particles and 7.3- μm median diameter particles were found to have reduced alveolar deposition with smaller inhalation volumes in subjects with preexisting airway obstruction.^{14,16} In contrast, the peripheral versus central distribution pattern was minimally affected by changes in inhalation volume, inhalation flow rate, or breath holding, with aerosols having median particle sizes of 2 to 3.2 μm ,^{11,13,14} although inhalation techniques and the distance of an MDI from the mouth did affect the total amount of drug reaching the lungs.^{11,13,15} The peripheral versus central distribution of aerosol from a nebulizer was not different when inhaled with normal tidal breathing than when intermittent deep breaths were included.^{14,17} Although the peripheral-central distribution patterns noted with radiotracer studies are consistent with relative deposition in large central airways versus distal airways and alveoli, the detectors could not discern whether alveoli in the central regions of the lung received aerosol similarly to alveoli in the outer regions of the lung. The current study in rats

TABLE II. Quantitative histologic assessment of inflammation in central versus subpleural regions of lung sections

Treatment group	Central region	Subpleural region
Dexamethasone	3.24	3.02
F220	6.31	9.55*
Placebo	12.59	14.45

Numbers are geometric least-squares means (pooled GSE, 1.1) of blue pixels $\times 10^5$ obtained from digital images.

* $P = .03$ versus central region.

revealed that the subpleural region of the lungs was less protected by inhaled fluticasone from allergic inflammation than was the central region of the lung, presumably because of inadequate penetration of aerosolized drug to the distal airways. However, the alveoli in the central regions of the lung also had less inflammation, despite being connected to conducting airways of a similar caliber, suggesting that the total linear distance of aerosol travel might be an important factor in addition to airway size. Although tidal breathing of an aerosol without a breath hold did not cause altered relative distribution to distal areas of the lung in adult human subjects,^{14,17} it is possible that tidal breathing might favor a more central deposition pattern in smaller lungs and higher breathing frequencies. If so, peripheral lung deposition might be inadequate in human infants receiving aerosolized drugs through tidal breathing from an MDI-spacer chamber or a nebulizer. The ability to use histologic evaluation of treatment effects makes an animal model a valuable tool for investigating ways to optimize pulmonary drug delivery.

The results of this study can be compared with those of a previous work by Vanacker et al¹⁸ that demonstrated the efficacy of nebulizer-aerosolized fluticasone for allergen-induced pulmonary inflammation in a rat model. Fluticasone, 0.1, 1, or 10 mg doses, in 30 mL of vehicle was nebulized into a plastic box containing the rats immediately before nebulizing an aerosol of 1% ovalbumin allergen into the same box for 30 minutes; the treatments and ovalbumin challenges were conducted every 2 days for 2 weeks.¹⁸ Although the repeated allergen exposure and the 2-day intervals between treatments preclude direct comparisons with our current study, which used daily treatments and a single allergen challenge, it appears that the 44- and 220- μg fluticasone MDI treatments used in the current study had systemic and pulmonary effects analogous to the 1-mg and 10-mg nebulized doses used in the Vanacker et al studies.¹⁸ This suggests a marked improvement in efficiency of pulmonary drug delivery with the MDI-plus-spacer method compared with nebulizing the drug into a much larger box, although even with the MDI-spacer method it is likely that only a small fraction of the drug introduced into the spacer chamber by the MDI was actually inhaled by the animal. Rats are exquisitely sensitive to the catabolic and thymus effects of systemic glucocorticoids, and thus changes in

body weight and thymus weight are useful indicators of the amount of drug inhaled. Our study and the study of Vanacker et al¹⁸ demonstrate further that within the dose range of inhaled fluticasone that produces undetectable-to-unequivocal effects on body and thymus weights in rats, there is also dose-related protection from pulmonary allergic effects.

In conclusion, an MDI used with a rodent-adapted spacer chamber is an effective way to administer aerosolized drugs to small laboratory animals, and the commercially available strengths of fluticasone provide an overlapping range of pulmonary anti-inflammatory and systemic glucocorticoid effects when administered once daily. This technique should be useful as a simple, inexpensive, and standardized method for testing the effects of drug treatments in animal models of pulmonary disease and also as a method for using animal models to investigate how MDI characteristics might affect drug delivery and therapeutic efficacy.

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