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Design and Evaluation of a Restraint-Free Small Animal Inhalation Dosing Chamber

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ABSTRACT The aim of research was to design a small, restraint free, low stress animal dosing chamber for inhalation studies, and to investigate distribution of a model drug within the chamber. A small animal dosing chamber was designed that consisted of a polymethylmethacrylate (PMMA) airtight box ($40.6 \times 11.4 \times 21.6$ cm) with a hinged top, having a nominal wall thickness of 1.25 cm. The chamber was designed to hold up to 14 mice, each having a floor area of approximately 63 cm^2, in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. A "rodent proof" distribution fan was attached to the center of the hinged closure lid. The chamber was divided into 1 $inch^2$ zones (120 in total) to enable a profile of drug distribution within the chamber to be obtained. Small holes were drilled into the side of the chamber and sealed using Parafilm® to allow access to the sampling zones. Syringes (5 mL) with appropriate length polytetrafluoroethylene (PTFE) tubing were inserted into the holes to reach the sampling zones (eight on either side of the chamber giving a total of 16 zones). An aqueous caffeine solution (2% w/v) in glycerol (25% w/v) was prepared and nebulized into the chamber using an Aeroneb Pro® nebulizer. Caffeine containing droplets were circulated into the chamber at a flow rate of 1.5 L/min^{-1}, and the air was recirculated in a closed system for a total of 20 minutes to ensure a high concentration of caffeine droplets throughout. Following nebulization, air samples (5 mL) were withdrawn from the 16 sampling zones of the sealed chamber. The process was repeated in quadruplet until a total of 64 sampling zones had been sampled. The entire experiment was also repeated with the absence of the "rodent-proof" distribution fan. Drug concentrations were calculated from a calibration curve of caffeine using UV absorbance at 272 nm. An average mass of caffeine (Standard Deviation; S.D.) of 5.0 (4.2) mg was detected throughout the chamber when the distribution fan was fitted, and caffeine 12.6 (9.7) mg was detected without the fan. This indicated that presence of the fan caused impingement of the drug on both the chamber walls and fan components; effectively removing nebulized drug from circulation within the chamber. The distribution of drug was plotted using a 3D graph; this revealed a lower concentration at the periphery and a higher concentration in the center of the chamber both with and without the distribution fan in place. In conclusion, a humane, nonrestraint rodent dosing chamber was designed for the efficient

delivery of nebulized drugs for up to 14 mice simultaneously. The highest levels of the model drug caffeine were detectable throughout the small animal dosing chamber without the distribution fan. A circulation flow rate of 1.5 L/min^{-1} was found to be adequate to distribute drug in the chamber. Surprisingly, the results demonstrate that avoiding the use of a distribution fan altogether maximizes the drug concentration within the chamber by reducing impingement of the nebulized drug. The small animal, restraint-free dosing chamber represents an advancement in reproducible dosing via the pulmonary route in the small animal model. The dosing chamber may be adapted to present the lung with an almost unlimited array of compounds, encompassing drugs, toxic compounds, and even pathogens, while still maintaining a relatively stress-free microenvironment for the test subject and furthermore, total safety for the operator.

KEYWORDS Whole body exposure, Mouse lung, Drug distribution, Restraint-free dosing, Animal stress

INTRODUCTION

The mouse has previously been demonstrated to be a good model for small-scale inhalation studies, as the optimal particle size for deep lung exposure in both humans and mice is approximately 3 μm (Miller et al., 1993). There are several methods of delivering a respirable dosage form to a small animal model; these include: intratracheal instillation (Ben-Jebria et al., 1999), nose/head-only exposure (Brown & Pickrell, 1994; Walsh et al., 1980; Warheit et al., 1995), or whole body exposure (Blagoeva et al., 1997; Fukayama et al., 1999; Negishi & Nishimura, 1993; Raeburn et al., 1992). Intratracheal administration of dry powders has been studied in rats with a device such as the Penn-Century insufflator (Codrons et al., 2003). This method is often stressful for the animal and requires extreme precision and expertise by the experimenter to ensure the correct dose administration and accurate anesthetization of the animal (van Zutphen et al., 1993). Additionally, the instillation technique may lead to more central and considerably less uniform lung deposition than other inhalation techniques (Pritchard et al., 1985). Nose-or head-only systems require the use of restraint to ensure an appropriate exposure and therefore subject the animal to a high degree of stress (Karwowski et al., 2001). These restraint systems generally consist of a central chamber with docking ports on the side. Restrainers can be attached to the side of these to enable the exposure of multiple animals to the aerosols (Warheit et al., 2003). For example, the Cannon nose-only chamber for inhalation exposure to small laboratory animals (Sumner et al., 2003a) can be coupled with a respirator (Sumner et al., 2003b) to enable a continuous exposure.

Stress has been linked to physiological changes that could affect the reliability of data within a given animal model. For example, chronic stress has been shown to affect the temperature regulation mechanism in rats (Matuszewich & Yamamoto, 2003), this may have a clinical significance during exposure to drugs. Environmental stresses can compromise experimental data by altering the immune response of an animal (Phalen et al., 1984), making it desirable to eliminate as much unintentional stress as possible. In addition, and directly linked to a pulmonary application, restraint stress has been shown to initiate physiological changes that affect the inflammatory response in the lung during an influenza viral infection; this type of restraint stress is also responsible for a depression of a cell-mediated immune response (Sheridan et al., 1991). Additionally, changes in respiratory patterns have been linked to stress as a cause for asthmatic attacks in humans (Nagata et al., 1999). Furthermore, there is evidence of poor

efficiency of dose deposition in the mouse model using a nose-only inhalation chamber (Nadithe et al., 2003), and this may be due to changes in respiratory patterns induced by stress. It has been indicated that stress may be minimized in animals by adopting a whole body exposure technique (Phalen et al., 1984), unless the whole-body exposure consists of a apparatus that provides a degree of restraint (Phillip et al., 1997). Poor experimental design using nose-only exposure, which may not be housed within a sealed system (Sharma et al., 2001), also has the potential to contaminate the work environment and expose the operator to clinically untested drugs and dosage forms. In this case, a sealed unit can remove the operator from such potential hazards.

The small animal inhalation dosing apparatus (SAIDA) described in this study is designed to present a reproducible and uniform dose of an active agent to up to 14 mice simultaneously. The design encompasses some basic features, such as a specific floor space per animal as determined by the IACUC committee, to minimize stress of overcrowding. Also, distribution fans were incorporated into the design in a way similar to a recently published aerosol infection chamber (Bhaskar & Upadhyay, 2003). Additionally, the apparatus was designed to be a completely sealed unit to allow for mobility and protection of the operator conducting the study from potentially harmful exposure.

Several considerations must be addressed when considering a chamber dosing device (Negishi & Nishimura, 1993; Phalen et al., 1984), including: surface contamination, amount of test material needed, the air cleaning system, cost, potential of excreta to interact with pollutants, losses by impingement, necessity of exhaust air treatment, reliability, and portability. In addition to this, it is of paramount importance that the uniformity of dose distribution within a chamber is investigated (Macfarland, 1983). The experimental design carried out in this study allowed real deposition studies to be conducted within the dosing chamber rather than theoretical flow studies. Air sampling procedures were conducted in the approximate breathing zone of the mice within the exposure chamber previously shown to be appropriate for a rat studies (Ulrich et al., 1992).

The aim of this research was to design an efficient and inexpensive whole-body exposure dosing apparatus, which minimizes stress to the small animal, and to investigate the distribution of the model drug caffeine to determine the device suitability for future inhalation exposure studies.

MATERIALS AND METHODS

Materials

Caffeine, anhydrous U.S. Pharmacopoeia (USP) grade and synthetic glycerol (F.C.C. grade) were purchased from Spectrum Chemicals (Gardena, CA). An Aeroneb Pro® nebulizer system was generously provided by Aerogen, Inc. (Mountain View, CA). Polymethylmethacrylate (PMMA) and polytetrafluroethylene (PTFE) tubing were purchased from Professional Plastics (Austin, TX). Disposable, 5 mL plastic syringes were purchased from Beckton-Dickinson and Co. (Franklin Lakes, NJ). Cooling fans (12VDC; Model KDE1208PTB1-6) were purchased from Sunon, Inc. (Brea, CA). Polyvinylchloride (PVC) connecting tubing (Inner Diameter; I.D.; 1.25 cm) and Parafilm® were purchased from VWR International (West Chester, PA). Silicone adaptor tubing (I.D. 1.59 cm) was purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Caffeine Solution

Caffeine (1.0 g) was dispersed in glycerol (12.5 g). The coarse dispersion was ultrasonicated to disperse the caffeine particles (10 minutes). The caffeine dispersion was then transferred to a volumetric flask of 50 mL and deionized water was added. The aqueous caffeine dispersion was further ultrasonicated (15 minutes) until the caffeine particles had fully dissolved to form a caffeine (2% w/v) and glycerol (25% w/v) solution.

Caffeine Calibration Curve

A caffeine calibration curve was prepared with the following solution concentrations: 10, 20, 40, 60, 80, 100, 120, 140, 160, and 200 µg/L. Absorbance was measured using a DU Series 60 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA), at 272 nm.

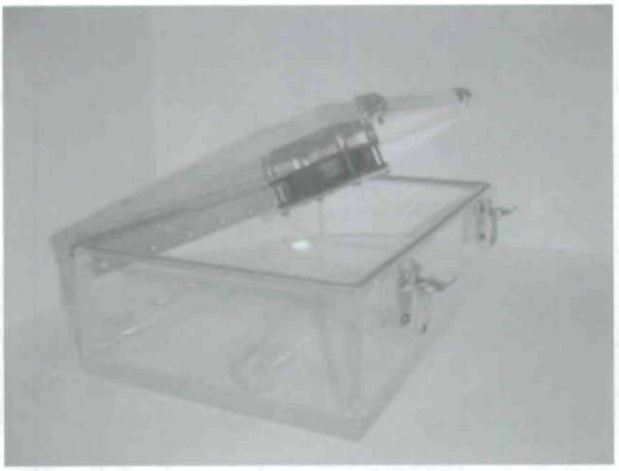

FIGURE 1 Dosing Chamber with Hinged Lid, Sampling Ports Along Each Side and Air-inlet and Outlet Ports Nearside.

Design of a Small Animal Inhalation Dosing Apparatus (SAIDA)

A dosing chamber consisted of a sealed polymethylmethacrylate (PMMA) rectangular box (Fig. 1). The dimensions of the chamber were ($40.6 \times 21.6 \times 11.4$ cm) with a hinged top, having a nominal wall thickness of 1.25 cm. Located at one end of the chamber was an air-inlet adaptor (I.D. 1.25 cm) and an air-outlet adaptor (I.D. 1.25 cm).

The dosing chamber was designed to hold up to 14 mice, each having a floor area of approximately 63 cm^2 (in accordance with IACUC guidelines). A "rodent proof" distribution fan was attached to the center of the hinged closure lid. The chamber was divided into 1 $inch^2$ zones (120 in total) (Fig. 2) to enable a profile of drug distribution to be obtained. Small holes (1 mm I.D.) were drilled into each of the longest sides of the chamber at 2.54 cm intervals to

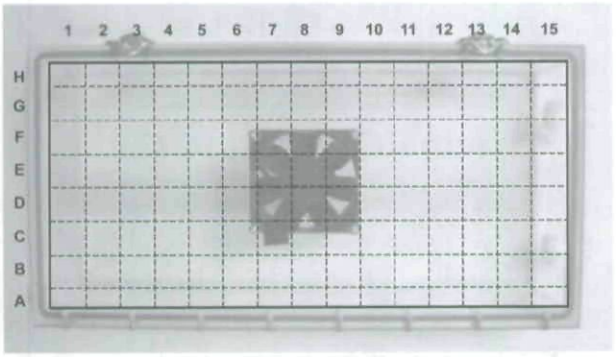

FIGURE 2 Division of Dosing Chamber Into 120 Sampling Zones, Air-inlet and Outlet Ports on the Right.

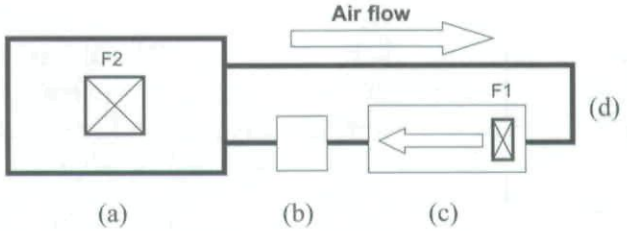

FIGURE 3 Assembled Restraint Free Small Animal Inhalation Dosing Apparatus: (a) Dosing Chamber; (b) Aeroneb Pro® Nebulizer; (c) Circulating Fan Housing; (d) Connecting Tubing; (F1) Circulating Fan; (F2) Drug Distribution Fan.

allow access to the sampling zones. These holes were sealed using Parafilm® manufactured by Pechiney Plastic Packaging (Menasha, WI).

The air-inlet adapter of the dosing chamber was connected directly to the Aeroneb Pro® nebulizer which was, in turn connected to the circulating fan (F1) housing, using the PVC connecting tubing and silicone adaptor tubing. The opposite end of the circulating fan housing was attached to the air-outlet adaptor using the PVC and silicone tubing, to form a closed, airtight system (Fig. 3). Additionally, a side air-inlet arm attached to the F1 housing may also be included, allowing a controlled concentration of nebulized droplets to be introduced into the dosing chamber (not used in this study). Such exposure control has previously been used in another modified chamber apparatus (Murin et al., 2004) for exposure to cigarette smoke in the murine model.

Caffeine Sampling

Syringes were positioned at rows 2, 4, 6, 7, 9, 11, 12, and 14 of columns A and H (Fig. 2). These specific zones were reached by the introduction of a thin plastic tube (of an appropriate length) attached to the top of the disposable, 5 mL plastic syringes. A Parafilm® seal around each hole effectively prevented the escape of the aerosolized caffeine droplets during and directly following nebulization. This configuration of the syringes enabled sampling of 16 sample zones simultaneously. The prepared caffeine solution (5 mL) was added to the reservoir of the Aeroneb Pro® nebulizer. The fan(s) were activated (3200 rpm), which was immediately followed by activation of the nebulizer. The caffeine solution was nebulized into the dosing chamber for 20 minutes. Sampling was then done very rapidly, following the aerosolization time to minimize settling time for the drug containing

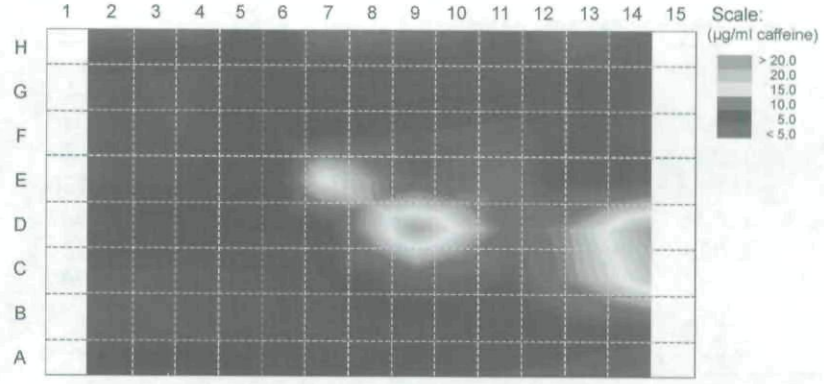

FIGURE 4 Caffeine Distribution within the Dosing Chamber of the SAIDA, in the Presence of the "Rodent-proof" Distribution Fan.

droplets. This was achieved by withdrawing 5 mL of the drug-laden air into each syringe. Following this procedure the chamber was cleaned and dried. Fresh syringes were then placed in rows 2, 4, 6, 7, 9, 11, 12, and 14 of columns B and G, and the sampling process was repeated. This dual column sampling procedure was performed until all column pairs A/H, B/G, C/F, and D/E had been sampled both with and without the drug distribution fan (F2) (Fig. 3). The drug was extracted by rinsing each syringe separately with 2.0 mL deionized water. This was repeatedly expelled into and withdrawn from a 20 mL scintillation vial 4 times to ensure adequate rinsing. The caffeine concentration was determined for each sample zone using the caffeine calibration curve (based on µg/mL caffeine, relative to the absolute concentration from a 5 mL atmospheric sample from the chamber), and the data was displayed using a 3-D contour plot.

Statistical Analysis

The data sets of rows and columns both with and without the drug distribution fan (F2) were compared using a single tailed t-test assuming unequal variances. The significance level ($\alpha = 0.05$) was based on the 95% probability value. P-values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Distribution of caffeine in the chamber of the SAIDA is shown when the internal drug distribution fan is fitted (Fig. 4). An average caffeine concentration of 5.0 (4.2) µg/mL was determined for the 5 mL atmospheric chamber samples. The nebulizer input was at position B15–C15 and the air outlet was positioned at G15–F15. It was observed that between B14–C14 and G14–F14 there were substantially elevated drug concentrations (10–>20 µg/mL drug), and it is likely that a circulatory airflow pattern would have been initiated between the inlet and the outlet, which may have served to concentrate drug levels between these two opposing air streams. In addition, elevated concentrations are also seen around the position of the distribution fan (zones E6–E10

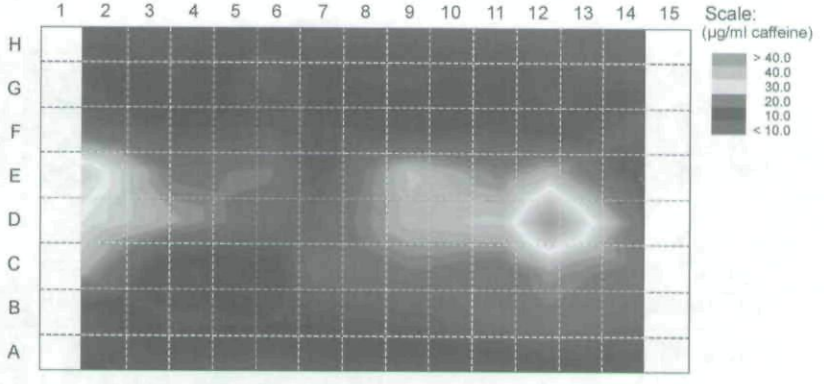

FIGURE 5 Caffeine Distribution within the Dosing Chamber of the SAIDA without the Distribution Fan.

TABLE 1 Comparison of Mean Column Values Using a One-Sided Student's t-Test

P-values	A	B	C	D	E	F	G	H
A	0.341	***0.001***	0.309	0.062	0.331	***0.034***	***0.001***	0.077
B	***0.002***	***0.000***	0.075	***0.018***	0.060	0.139	0.484	***0.000***
C	***0.001***	0.102	***0.004***	0.149	0.457	0.136	0.076	0.499
D	***0.000***	***0.004***	***0.030***	***0.002***	0.119	***0.027***	***0.018***	0.110
E	***0.001***	***0.020***	0.134	0.198	***0.001***	0.124	0.061	0.437
F	***0.001***	0.133	***0.022***	***0.002***	***0.007***	***0.000***	0.144	***0.005***
G	***0.013***	***0.044***	***0.009***	***0.001***	***0.003***	0.174	***0.001***	***0.000***
H	0.152	***0.001***	***0.001***	***0.000***	***0.001***	***0.000***	***0.003***	***0.006***

From left to right columns (without distribution fan). From top to bottom (with fan). *Italicized* numbers indicate a statistical significance ($p<0.05$).

through zones D6–D10), which may also be attributed to opposing airflow streams and a point at which the chamber air is forced to accelerate by the dispersion fan. It is also apparent that there are large regions where the concentration of the caffeine is less than 5.0 μg/mL, particularly in zones G2–G6 through B1–B6; here it should be considered that the inclusion of the distribution fan may actually be inhibiting flow to the back third of the chamber by creating an overall circulatory flow pattern directly from the inlet to the outlet but only as far as the center of the chamber where the fan was positioned.

Distribution of caffeine within the chamber without the internal distribution fan (F2) is shown (Fig. 5). Quite a different pattern of distribution is observed when compared to the distribution of caffeine when the distribution fan is fitted. Again, a circulatory flow was observed immediately between the air inlet and outlet to the chamber, but this time the flow pattern was not disrupted and extended from F14–B14 through F8–B7. This extends high concentration circulatory flow pattern provides a large area of coverage within the chamber that has a concentration greater than 20 μg/mL. Additionally, a high concentration of caffeine is observed at the rear of the chamber (F6–D6 through F2–C2). This provides evidence that the airflow provided by the circulatory fan to drive the droplets from the nebulizer into the chamber is sufficient to allow the drug to be distributed far within the chamber. The peripheries of the chamber walls were still affected by low concentrations of caffeine (<10.0 μg/mL), and a small dead space (a point at which poor air-flow circulation occurs) may be present close to the air outlet (G12–H14). It is evident from the average concentration of caffeine of 12.6 (9.7) μg/mL with this arrangement, that the use of the distribution fan causes impingement of droplets either onto the walls of the chamber or into the mechanism of the fan itself, restricting the amount of drug that remains entrained to be available for inhalation. A common problem associated with chambers intended for toxicological exposure is uneven distribution of the toxin within the chamber. In this paper an average exposure to elevated levels of an active pharmaceutical ingredient is envisioned for a small animal model to initiate a therapeutic response.

TABLE 2 Comparison of Mean Row Values Using a One-Sided Student's t-Test

P-values	2	4	6	7	9	11	12	14
2	***0.016***	0.377	0.418	0.148	0.073	***0.033***	***0.018***	0.052
4	0.144	***0.017***	0.416	0.117	0.059	***0.025***	***0.014***	***0.042***
6	0.161	0.427	***0.005***	0.118	0.061	***0.007***	***0.006***	***0.045***
7	0.123	0.474	0.382	***0.038***	0.293	0.500	0.386	0.182
9	0.354	0.201	0.228	0.165	0.051	0.256	0.343	0.337
11	0.293	0.251	0.288	0.208	0.423	***0.023***	0.306	0.154
12	0.468	0.147	0.163	0.128	0.333	0.279	***0.042***	0.205
14	0.122	0.460	0.372	0.482	0.163	0.206	0.127	0.307

From left to right columns (without distribution fan). From top to bottom (with fan). *Italicized* numbers indicate a statistical significance ($p<0.05$).

Additionally, there are only small areas of the chamber that contain <10.0 μg/mL of the model compound caffeine, which limits the area available for animals to congregate in order to avoid exposure. Low levels of drug (<10.0 μg/mL) are still observed at the periphery of the chamber walls, this is probably due to frictional airflow at these points, but it should be noted that the overall drug concentration is twice that of the instance where the distribution fan was fitted. It is clear that exposure is maximized, and areas of low drug concentration are reduced when the distribution fan is not used.

Using statistical analysis, there is very little significant difference between any of the rows either with or without the fan (Table 1). When the distribution fan is used however, significant differences are observed with rows 11, 12, and 14 compared to rows 2, 4, and 6. This corresponds to the poor availability of drug in the rear portion of the chamber due to turbulence created by the fan. There is no significant difference observed when comparing sample columns (A–H) with the fitted fan (Table 1).

Comparing sample rows (2–14), there is no significant difference in the absence of the fan (Table 2). It is apparent that the highest variation in concentration is seen in the presence of the fan, and low concentrations (<5.0 μg/mL) are found throughout. Without the fan, although there is some variation within the chamber there is an overall increase in drug concentration (>10.0 μg/mL throughout), which would serve to increase the total exposure to the mouse. Therefore, the use of the chamber without the fan is better, as a higher average drug concentration is observed. In the complete assembled small animal inhalation dosing apparatus, the fan (F1) in the premixer spacer is sufficient to distribute the drug throughout the entire chamber. When sampling columns are compared, a significant difference is seen between all of the like columns except A (Table 1). Additionally, there is a clear significant difference apparent when like rows are compared. Only where rows 9 and 14 are compared with like rows (with and without the distribution fan) are there discrepancies (significance is just not observed with row 9; $P = 0.051$). This indicates that when the same rows and the same columns from each sampling experiment are compared, there is a significant difference between both.

When using a fan (F2), columns 7 and 9 and the rows C and E are not affected by the airflow generated inside the chamber. With the absence of the fan (F2), the data show there is no significant difference in the columns, but an increased variance is observed between the rows.

In summary, a higher average concentration of 12.6 (9.7) mg/mL was found without the distribution fan (F2) compared to 5.0 (4.2) mg/5mL when the fan was present. This indicated that a lower degree of variability is observed by omitting the use of this fan.

CONCLUSION

A restraint-free, small animal inhalation dosing apparatus capable of uniform dosing up to 14 mice has been developed. This represents an important advancement over previously reported techniques in reproducible dosing via the pulmonary route in the small animal model. The dosing apparatus requires no restraint to the animals, which has previously been shown to cause undue stress on the model, which may have a detrimental effect of data. Not only is this a more humane approach but also it eliminates uncertainties about experimental variability due to heightened animal stress. A completely enclosed system is presented, which greatly reduces operator exposure and improves operator safety. Additionally, the chamber could also be used as an infection model, allowing the user to safely administer potential pathogens to the lung. Thus, the dosing chamber may be adapted to present the lung with an almost unlimited array of compounds; encompassing drugs, toxic compounds, and even pathogens, while still maintaining a relatively stress-free microenvironment for the test subject and furthermore, complete safety for the operator.

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