Effect of conditioning on rat lung function measurements and implications for exposure and dose estimation of inhaled drugs

Measurement of minute volume may provide a tool for refining inhalation exposure procedures and understanding dosing anomalies or a test material's effect on dose, such as respiratory pharmacology or irritancy

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Introduction

Inhaled administration of substances to animals is technically challenging for quantitative dosimetry. Animals are not "dosed" *per se*, but are exposed to an atmosphere containing a test substance mixed with air. Non-clinical "doses" reported for animals inhaling such atmospheres are calculated using the following equation:

$$eID = \frac{C \times RMV \times T}{BW}$$

………Equation 1

where eID = estimated inhaled dose (mg/kg), C = concentration of a substance in air (mg/L), RMV = respired minute volume (L/min), T = time (minutes; duration of inhalation exposure) and BW = body weight (kg). The minute volume (MV) can be measured or estimated (eRMV) by calculation from body weight using a published algorithm such as that of Alexander, et al.:[1]

$$eRMV = 0.608 \times BW^{0.852}$$

………Equation 2

Although the latter equation is derived from measurements of MV in conscious animals of relevant species (mice, rats, beagle dogs and cynomolgus monkeys) used for non-clinical research, this approach overlooks physiological effects on lung function that can be induced by a substance's properties.[2, 3] Furthermore, MV measurements may vary due to differences in habituation of animals to procedures that also differ between species and protocols used by the 18 laboratories generating data used to derive Equation 2.[1]

Snout-only inhalation exposure chamber with rat restraint tubes

Lung-homogenate concentration data for rats attached to adjacent ports on a snout-only inhalation exposure chamber, and thus breathing from the same aerosol, can vary two- to three-fold (unpublished data). Little is known about the ways environmental factors may affect lung function and therefore inhaled dosimetry of individual animals subjected to "standard procedures" during non-clinical studies. For example, manipulation or disturbance of animals may excite them to varying degrees and the implications of this for lung dose are uncertain. In addition, rats are insensitive to red light[4] and anecdotal data suggested red light may induce a calmer state in restrained rats, which we hypothesized may be ascribed to rats perceiving darkness in an enclosed environment.

Plethysmography is a technique used for measuring changes in the volume of an organ or body, typically due to changes in the volume of blood[5] or air[6] contained therein. A head-out plethysmograph[3, 7] is a vessel that encloses the body of a subject to facilitate

lung function measurements derived from changes in ambient pressure due to changes in body volume as the subject breathes. Since the head of an animal is outside this compartment, its snout or head can be presented simultaneously to an atmosphere for inhaled administration of a test substance, e.g., aerosol, vapor or smoke.

The aims of this study were to measure MV in conscious rats using head-out plethysmography to determine:

• Whether the MV of rats subjected to a two-day acclimatization protocol was similar to that of rats acclimatized over six days;

• Whether there were differences in MV for rats acclimatized and exposed under normal fluorescent ("white") or red-filtered lighting;

• The effect of minimizing restraint on MV and, by inference, confirming requirements for reconditioning rats prior to data acquisition during repeat-dose inhalation studies;

• Whether MV correlated with body-weight-derived estimates (eRMV) and/or changes in body weight with maturation of rats over four weeks.

Results of these investigations were anticipated for refinement of restraint procedures for acclimatization and inhalation exposure of rats, to identify considerations for implementing lung function measurements during repeat-dose studies and to indicate the extent to which eRMV is representative of MV for the small group sizes of animals used in early drug development.

Methods

Measurement of minute volume in rats

Rats were accommodated under standard laboratory conditions[8] for at least five days before undertaking licensed procedures (tube restraint). MV of conscious rats was measured non-invasively using a body plethysmograph similar to that described by Glaab[7] but differing insofar as only the snout of the rat was presented to the test atmosphere. The animal's body was enclosed in the tube, using a rubber diaphragm to form a seal around its neck, and a sensor detected changes in pressure inside the tube as the animal breathed.

Using Boyle's law, lung function parameters derived from changes in pressure inside the body plethysmograph were captured using software that facilitated rejection of anomalous breath-wave signals ascribed to a compromised neck seal, typically during movement of animals in plethysmographs; approximately 700 to 1,000 breaths/hour were recorded for each animal. Plethysmographs were attached to a simple, flow-through design, snout-only, inhalation exposure chamber for aerosol administration.

For data analysis, a mean MV was calculated for each rat for 5-minute periods ending at minus 10 minutes (pre-dose) and/or 5, 15, 30, 45 and 60 minutes of exposure. All animal studies were ethically reviewed

and performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

Estimation of minute volume from body weight

To investigate the influence of body weight on doses estimated using Equation 1, the mean minute volume was measured using head-out plethysmography and compared with an estimate of the respired minute volume calculated from the corresponding pre-dose body weight using Equation 2.[1]

Experimental study designs

A preliminary study was performed to implement methodology and confirm satisfactory data capture by head-out plethysmography. Male Crl:WI(Han) rats were restrained in plethysmographs for data capture concurrent with snout-only exposure to air only. Results (unpublished data) were used to refine the design of two studies. Procedures evolved from study to study and key features are summarized in Figure 1.

A Latin square design was used to investigate the influence of lighting and duration of pre-treatment acclimatization periods on MV during inhalation exposure of rats to a dry powder aerosol;[9] the known pharmacology of the drug ("Compound 1") was not expected to affect lung function. Acclimatization periods of two days (applied to non-clinical studies at our laboratory) and six days (adopted by other laboratories using head-out plethysmography; unpublished communications) were investigated.

Male Crl:WI(Han) rats (4 groups; n = 8 per group; 11 weeks old) were acclimatized to plethysmographs under either normal fluorescent (white) lighting or red-filtered lighting ($\lambda \geq 600$ nm) by progressively extending daily restraint periods over two days (20 and 40 minutes; neck seals present) or six days (20 to 60 minutes over three days without a neck seal, followed by 30 to 60 minutes over three days with a neck seal).
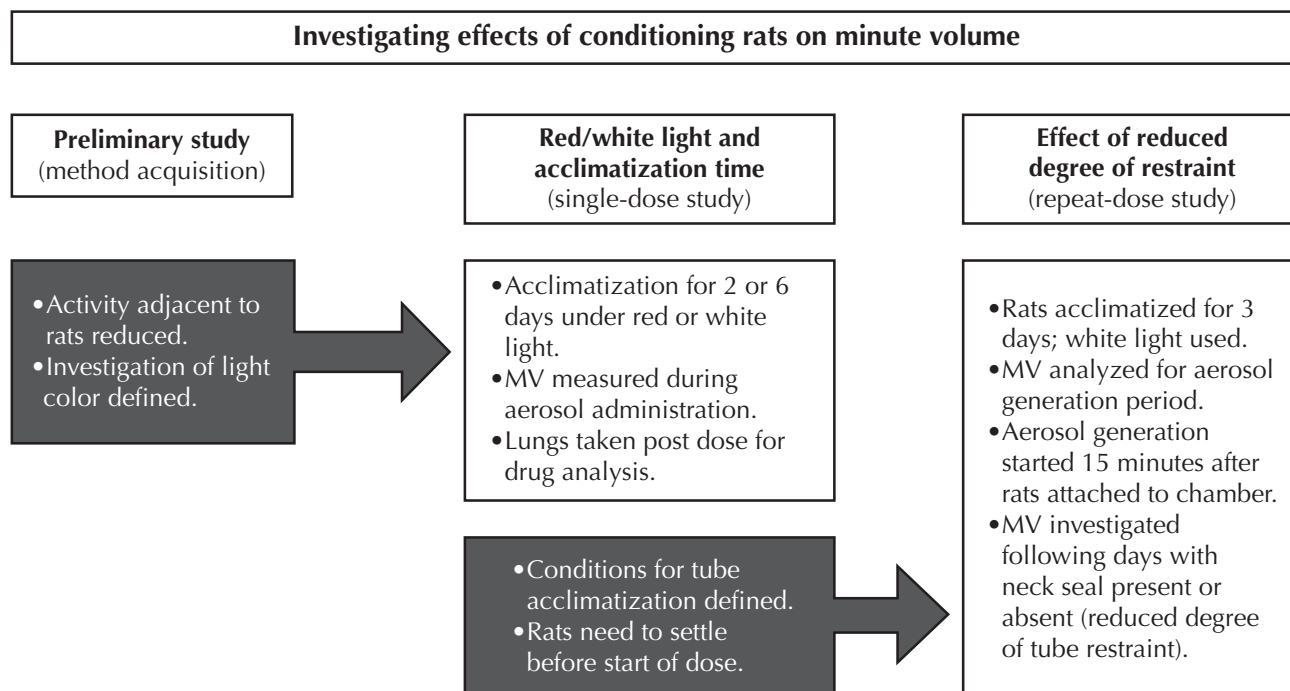
MV was measured during inhaled administration of a dry powder aerosol (600 µg/kg; 60 minutes) under white or red light. The micronized crystalline drug, blended 5% (w/w) in lactose, was dispersed into an inhalation exposure chamber using a Wright dust feed.[10] Rats were euthanized immediately post-exposure and the lungs removed (right and intermediate lobes pooled) and homogenized for drug extraction in a suitable solvent for analysis using a validated HPLC-MS/MS method.

The crystalline form of Compound 1 was known to be of relatively low solubility in simulated lung fluid (unpublished *in vitro* data) and thus minimized drug/lung clearance prior to isolation of lung tissue; unpublished *in vivo* data demonstrated no appreciable decrease in drug/lung-homogenate concentrations up to 24 hours after a single inhaled dose.

Statistical analyses (two-way analysis of variance; ANOVA) was applied to individual drug/lung-homogenate concentrations and the mean MV of

Figure 1

Study design: Investigation of light and duration of acclimatization

Investigating effects of conditioning rats on minute volume

Preliminary study
(method acquisition)

- Activity adjacent to rats reduced.
- Investigation of light color defined.

Red/white light and acclimatization time
(single-dose study)

- Acclimatization for 2 or 6 days under red or white light.
- MV measured during aerosol administration.
- Lungs taken post dose for drug analysis.

Effect of reduced degree of restraint
(repeat-dose study)

- Rats acclimatized for 3 days; white light used.
- MV analyzed for aerosol generation period.
- Aerosol generation started 15 minutes after rats attached to chamber.
- MV investigated following days with neck seal present or absent (reduced degree of tube restraint).

- Conditions for tube acclimatization defined.
- Rats need to settle before start of dose.

animal-specific mean data, which were each pooled for the acclimatization period or lighting conditions (n = 16).

Study design: Investigation of restraint for repeat-dose inhalation studies

There is an ethical desire to minimize the restraint of animals necessary to achieve the scientific objective. In view of the degree of restraint required for head-out plethysmography, a 28-day study was conducted to investigate the effect on MV measurements when neck seals were included or omitted on days of inhalation exposure preceding data capture, with a view to optimizing the degree of neck restraint when incorporating lung function measurements into repeat-dose studies.

For logistical reasons, MV was measured using satellite rats assigned to two toxicology studies, one administering a dry powder aerosol and the other a nebulized solution of the same drug ("Compound 2"). The known pharmacology of the drug (same class as Compound 1) was not expected to affect lung function parameters and no differences in MV were evident for the two aerosol forms.

Male Crl:CD(SD) rats (2 groups; n = 3 per group; 10 weeks old) were acclimatized to plethysmographs with a neck seal for three days by progressively increasing the restraint period each day from 20 to 60 minutes. Rats were exposed under normal fluorescent lighting to an atmosphere containing Compound 2. The dry powder formulation (5% crystalline drug (w/w) in lactose) was dispersed into the inhalation exposure chamber using a Wright dust feed[10] and solutions dispersed using an air jet nebulizer (Pari LC Sprint, Pari Pharma GmbH, Munich, Germany). MV was measured pre-treatment (acclimatization) and Days 1, 4, 14 and 26 to 28 of treatment. On Days 5 to 13 and 15 to 25, rats were restrained without a neck seal and exposed to the test aerosol for 60 minutes.

Results and discussion

Effect on minute volume when rats are allowed to settle in plethysmographs

Mean MV of rats decreased over the first 15 minutes of restraint for all experiments. Please click the link to see Figure 2, which presents representative data. It is common practice during non-clinical studies to perform visual checks on the condition of restrained animals for animal welfare reasons. Transient increases in MV were observed concurrently with this practice in the preliminary experiment or when repositioning a rat that had compromised its neck seal (unpublished data).

Effects of light and acclimatization on minute volume and lung dose

During the preliminary study, rats restrained in plethysmographs under red light appeared calmer (subjective observation) than under white light, with less soiling of fur with excreta. However, these observations were not verified by changes in MV when tested in a study designed to evaluate this.

The MV of rats was similar after illumination of restraint and exposure procedures under red or white light, with no statistical difference in mean values for the 60-minute exposure period (Table 1). Nevertheless, an initial transient elevation in MV was more pronounced for rats acclimatized over two days than six days (Figure 2).

Drug concentrations for lungs taken immediately post-exposure (Table 1) were more variable than MV measured in the same animals and an apparent difference in lung-homogenate concentrations for red versus white lighting was in contrast to trends in MV and therefore "achieved lung dose." Although the variability observed in lung-homogenate concentrations was not unexpected (unpublished data), the apparent differences between these data types suggest the reason for a difference in lung concentrations between rats maintained under red or white light cannot be ascribed to inter-animal differences of MV and therefore achieved lung dose *per se.* Possible explanations for discrepancies or imprecision in drug/lung-homogenate concentrations include processing of lung-homogenate samples for HPLC-UV analysis (e.g., non-uniform drug/lung deposition in conjunction with pooling selected lobes, degree of homogenization and incomplete solvent extraction of the drug).

Effect of restraint on minute volume during repeat-dose inhalation studies
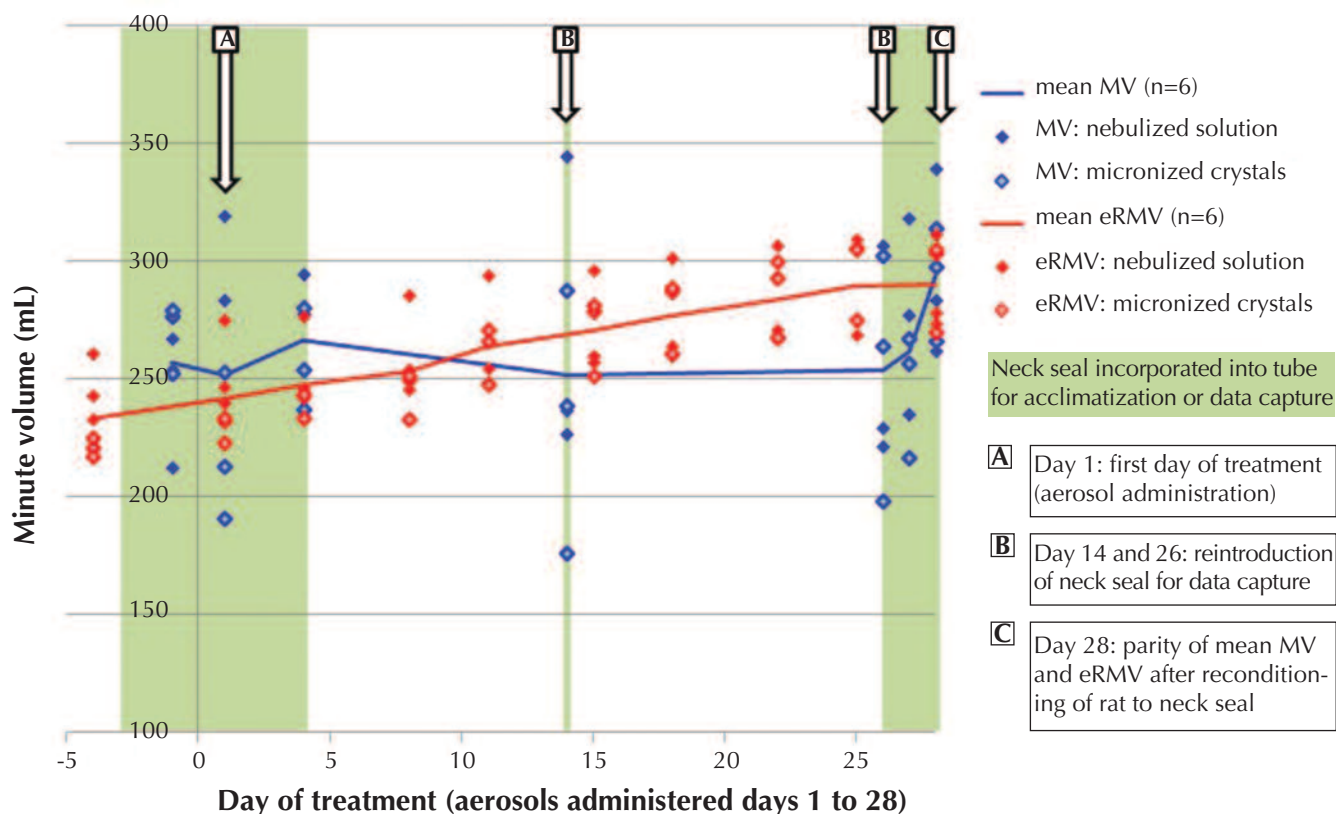
Interventions or activities near rats during inhalation exposure procedures can induce erratic increases in MV (unpublished data) and visual checks on the condition of restrained animals (routine for non-clinical inhalation safety studies) were minimized to avoid excitation of the rats during this study. MV was most variable on Day 1, when rats were first exposed to an aerosol, and on Days 14 and 26 following a treatment period in which the neck seal was omitted from the body plethysmograph for aerosol administration (Figure 3). It is noteworthy that mean MV was similar to or slightly greater than eRMV when the neck seal was used during preceding days of restraint (pretreatment and Days 1, 4 and 28). However, with omission of the neck seal from Day 5 (excluding days of MV measurement), the mean MV decreased with an increase in eRMV (Days 14 and 26). With reintroduction of the neck seal from Day 26, the variability in measured MV progressively reduced and mean MV attained parity with eRMV (Day 28) suggesting rats may require at least two days of reacclimatization to the neck seal before the mean measured MV is representative of eRMV.

Alternatively, this raises the possibility of an observer effect, i.e., that minute volume may change as a consequence of using the neck seal to facilitate measurement of this parameter. Nirogi, et al.[2] published baseline minute volumes for male Wistar rats (body weight 250 to 300 g) of 173 ± 15.7 mL when measured by whole body plethysmography and 228 ± 14.8 mL when measured by head-out plethysmography, suggesting a potential for differences in

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Figure 3

Minute volume (MV) and body-weight-derived estimate of respired minute volume (eRMV) in rats over a 28-day treatment period

Legend:
- mean MV (n=6)
- MV: nebulized solution
- MV: micronized crystals
- mean eRMV (n=6)
- eRMV: nebulized solution
- eRMV: micronized crystals

Neck seal incorporated into tube for acclimatization or data capture

A Day 1: first day of treatment (aerosol administration)

B Day 14 and 26: reintroduction of neck seal for data capture

C Day 28: parity of mean MV and eRMV after reconditioning of rat to neck seal

Measured MV data are shown in blue, with corresponding body-weight-derived eRMV data in red. Mean data points are joined by a blue or red line, respectively. There was no obvious difference in MV or eRMV data for rats administered a crystalline or nebulized aerosol.

lung function measurements between techniques with differing degrees of restraint.

Relationship of measured minute volume and body weight

There was no clear proportional relationship between body weight and measured MV of rats during a preliminary study (unpublished data) and the study examining the effect of light and acclimatization on lung function, when MV was also generally higher and considerably more variable than the body-weight-derived estimate (mean MV ≈ 1.17 x eRMV) (Figure 4). In contrast, when rats were allowed to settle in the plethysmographs for 15 minutes before the start of aerosol generation and had also been acclimatized to the neck seal in days preceding data capture, the MV and eRMV for a given body weight were similar (Figure 5) with a 23% increase in mean MV concurrent with a 30% increase in mean body weight from Days 1 to 28 of treatment.

Conclusions

This work illustrated the importance of considering the experimental design and procedural conduct for acclimatizing animals to restraint procedures and data acquisition by head-out plethysmography, a technique used for assessing lung function and estimating doses in non-clinical inhalation studies.

Key conclusions were:

• Two days of acclimatization of rats (or reacclimatization after a reduced level of restraint) to plethysmographs was sufficient to achieve parity of mean MV (measured) with mean eRMV (body-weight-derived estimate) when rats were allowed to settle for 15 minutes before initiating aerosol generation.

• There was no difference in MV for rats subjected to red or white light during acclimatization and inhalation exposure procedures.

• Minute volume (MV) measurements were more variable than corresponding body-weight-derived estimates (eRMV), especially when animals were exposed to an aerosol for the first time.

Although rats were successfully acclimatized for a 60-minute exposure period over two days, experiments requiring detection of more subtle changes in lung function (e.g., a dose response in respiratory pharmacology) may require a modified and possibly more prolonged approach for acclimatization. An ethical desire to minimize the degree of restraint applied to animals during repeat-dose inhalation studies must be weighed against a potentially deleterious effect on the precision and accuracy of lung function measurements.

These preliminary conclusions are limited by small group sizes that are nevertheless typical of those used during non-clinical studies in early drug development, where there is a desire to minimize the number of animals used. Measurement of MV during non-clinical studies may therefore provide a tool for refining inhalation exposure procedures, understanding anomalies in quantitative lung doses and understanding potential effects on inhaled dosimetry associated with the properties of a test substance, such as respiratory pharmacology or irritancy.

References

1. Alexander DJ, Collins CJ, Coombs DW, Gilkison IS, Hardy CJ, Healey G, Karantabias G, Johnson N, Karlsson A, Kilgour JD, McDonald P: Association of Inhalation Toxicologists (AIT) working party recommendation for standard delivered dose calculation and expression in non-clinical aerosol inhalation toxicology studies with pharmaceuticals. Inhal Tox 2008, 20:1179-1189.

2. Nirogi R, Shanmuganathan D, Jayarajan P, Abraham R, Kancharla B: Comparison of whole body and head out plethysmography using respiratory stimulant and depressant in conscious rats. J Pharmacol Toxicol Methods 2012, 65(1):37-43.

3. Vijayaraghavan R, Schaper M, Thompson R, Stock MF, Alarie Y: Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract. Arch Toxicol 1993, 67:478-490.

4. Szél Á, Röhlich P: Two cone types of rat retina detected by anti-visual pigment antibodies. Exp Eye Res 1992, 55:47-52.

5. Pointel JP, Gin H, Drouin P, Vernhes G, Debry G: Venous plethysmography: Measuring techniques and normal values. Angiology 1981, 32:145-154.

6. Pennock BE, Cox CP, Rogers RM, Cain WA, Wells JH: A noninvasive technique for measurement of changes in specific airway resistance. J Appl Physiol Respir Environ Exerc Physiol. 1979, 46(2):399-406.

7. Glaab T, Daser A, Braun A, Neuhaus-Steinmetz U, Fabel H, Alarie Y, Renz H: Tidal midexpiratory flow as a measure of airway hyperresponsiveness in allergic mice. Am J Physiol Lung Cell Mol Physiol 2001, 280:L565-L573.

8. Home Office: Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes; Presented to Parliament pursuant to Section 21(5) of the Animal (Scientific Procedures) Act 1986. Stationery Office 2014, ISBN 9781474112390.

9. Paul GR, Somers GI, Taylor G: Implications for room lighting and the duration of acclimation protocols on the dosimetry of inhaled drugs in rats. Respiratory Drug Delivery 2016, 2:327-332.

10. Wright BM: A new dust-feed mechanism: J Sci Instrum 1950, 27:12-15.

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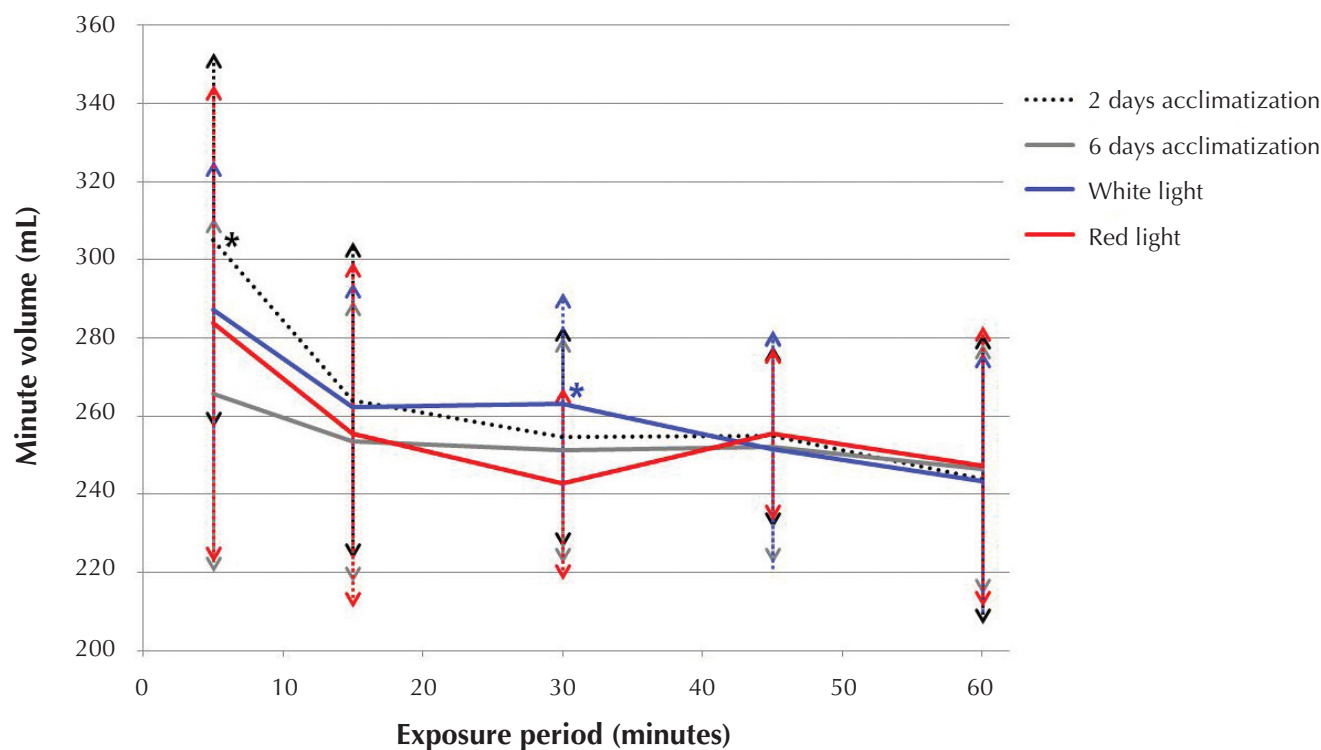
Table 1

Minute volume during a single (60-minute) exposure and drug/lung-homogenate concentration post-exposure for rats acclimatized for two or six days under red or white light

Latin Square Design (data pooled)	Acclimatization [A]		Lighting Conditions [B]	
	2 Days	**6 Days**	**White**	**Red**
Minute volume of rats during inhalation exposure				
Mean (mL)	258.1	253.8	262	254.6
sd	18.4	25.2	22.2	25.2
CV	7%	10%	8%	10%
n	16	16	16	16
Lung-homogenate concentration after a single exposure				
Mean (µg/g)	39	35.2	33.5	40.7**
sd	8.19	4.69	4.75	6.81
CV	21%	13%	4%	17%
n	16	16	16	16

** Red versus white light (drug/lung-homogenate concentration): p < 0.01 (two-way ANOVA).
Rats used for measurement of MV during inhaled administration of a crystalline drug were data-pooled (n = 16) for statistical analysis of lighting (A) and duration of acclimatization (B).
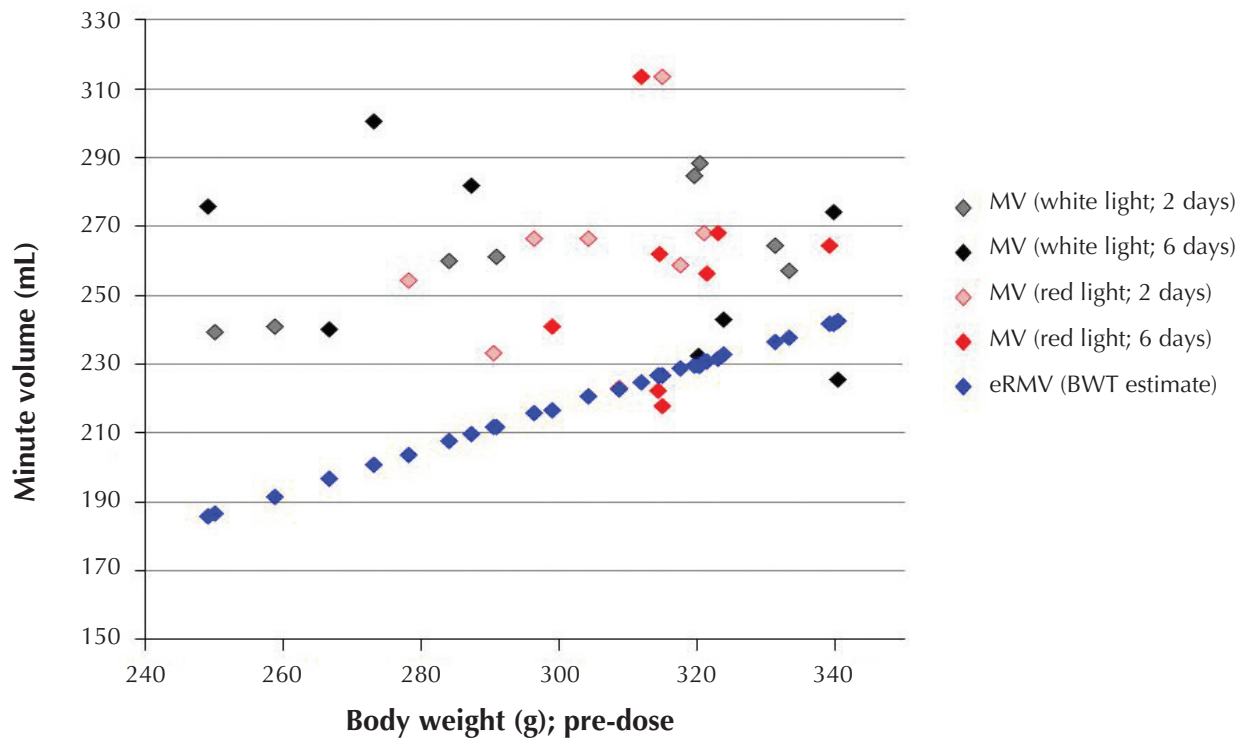
Figure 2

Mean minute volume (with standard deviation) of rats during a single 60-minute inhalation exposure after two or six days of acclimatization under red or white light

p < 0.05 for six-day versus two-day acclimatization at 5 minutes; red versus white light at 30 minutes. Latin square design (n = 16 per variable) with two-way ANOVA of acclimatization period (red and white light data pooled) and lighting (two- and six-day acclimatization protocols pooled).
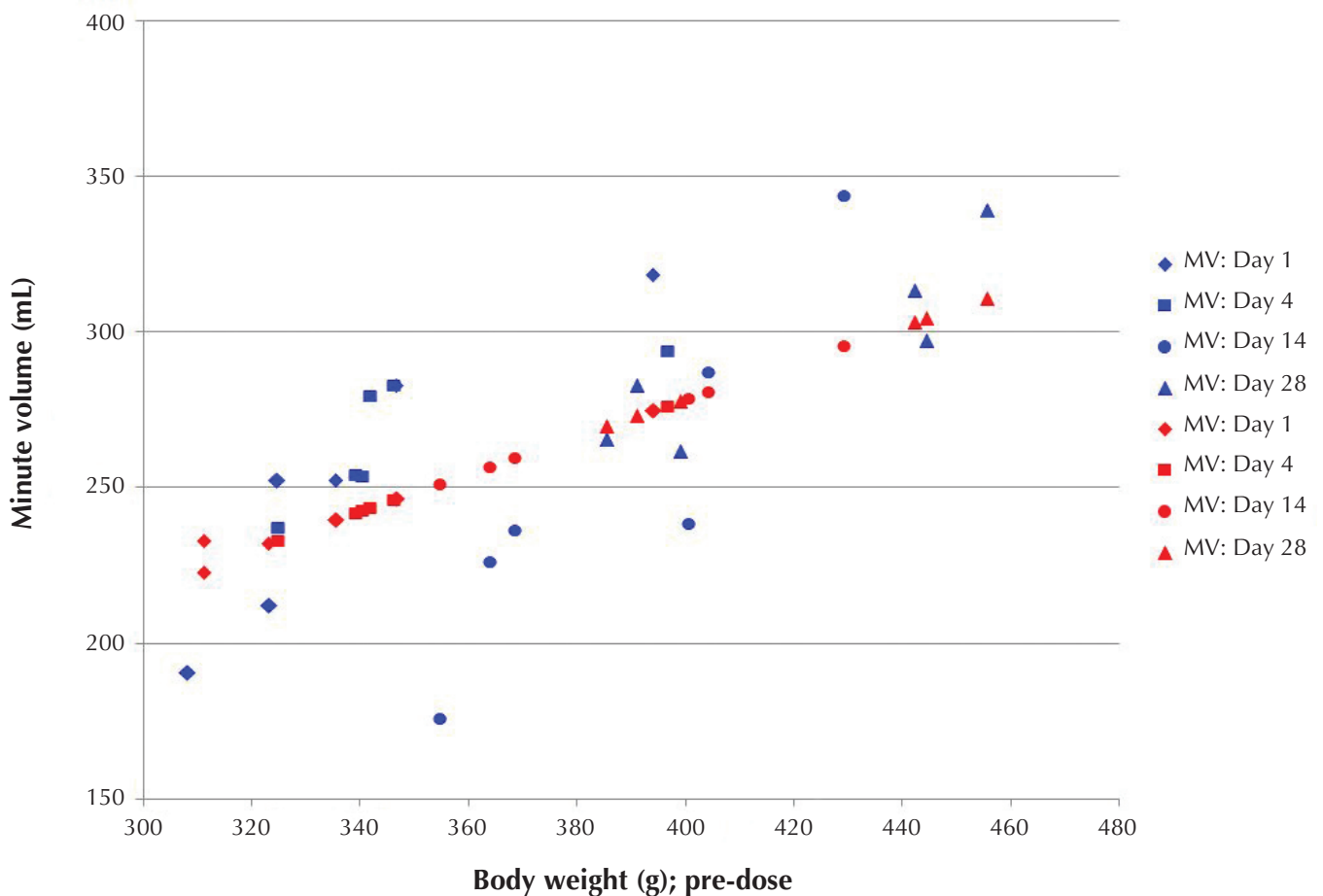
Figure 4

Minute volume (MV) and the body-weight-derived estimate of respired minute volume (eRMV) plotted against body weight; aerosol generation was started immediately after rats were attached to the chamber

Measured MV data were generally higher than the corresponding body-weight-derived estimates (eRMV; blue data points) irrespective of lighting color and days of acclimatization. Overall, mean MV (260 mL; sd = 24.8) was significantly higher (p < 0.01; two-way ANOVA) than eRMV (221 mL; sd = 15.9).

Figure 5

Minute volume (MV) and the body-weight-derived estimate of respired minute volume (eRMV) plotted against body weight; aerosol generation was started 15 minutes after rats were attached to the chamber

Measured MV (blue data points) correlated with the estimated body-weight-derived eRMV (red data points). MV was measured in rats (n = 6) during inhaled administration of a dry powder or nebulized aerosol (60 minutes), started 15 minutes after rats were attached to the inhalation exposure chamber.