Original Research *Highlight article*

Respiratory mechanics evaluation of mice submitted to intravenous methacholine: Bolus vs. continuous infusion

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Impact statement

Respiratory mechanics studies are associated with fundamental research and translational studies; the present work thus investigates this particular matter. Our current research describes differences and similarities between two different ways of administrating a very prevalent bronchoconstrictor (methacholine) in an aging process scenario. The core issue of our work is related with troubles we find with the bolus protocol and the application of the mathematical model used to assess the respiratory mechanics. Our findings reveal the continuous infusion as an alternative to these problems and we hope to provide the proper foundations to a more reliable assessment in the respiratory field.

Abstract

The application of a bronchoconstrictor, usually Methacholine (MCh), in respiratory mechanics studies is usually accompanied by the assessment of respiratory mechanics in a dose–response curve. The MCh used in the dose–response curve can be inhaled (i.h.) and intravenous (i.v.) and there are studies comparing i.v. bolus and i.h. MCh in both mice and rats. However, MCh i.v. can be injected at short time interval (bolus) or in continuous infusion. This comparison is relevant since the way MCh is applied influences the mathematical model. We chose an aging process scenario to compare both protocols. This study aims to compare respiratory mechanics of 3-, 6-, and 10-month SAMR1 mice and how both administration methods (continuous infusion and bolus) impact respiratory mechanics evaluation. Both protocols were capable of assessing the difference among ages and doses in: peak or plateau; and area under the curve analysis. The respiratory mechanics parameters were Rn, G, and H (two-way analysis of variance: groups and doses with a $P < 0.05$ for all). Also, the infusion protocol presented a higher sensitivity to dose increment. In conclusion, both protocols were able to discriminate intragroup and intergroup differences. In the bolus protocol, the highest value of each curve dose may not correspond to the highest real value, and the loss of this point may be a problematic factor in the sample size. These factors are not present in the infusion protocol. Additionally, at this lineage and age screening, the infusion protocol appeared to be more sensitive to differences among ages when compared to the bolus protocol.

Keywords: Pulmonary, age, engineering, lung, pharmacology, respiratory mechanics

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Introduction

In the current literature, there are a large number of studies that assess respiratory mechanics in small animals mechanically ventilated.[1–5] These studies aim to describe the physiological mechanisms associated with the changes of lung function as, for example, observed in the aging process.[6]

The constant phase model (CPM) is the mathematical model, a frequency domain one, used herein and is widely applied in the literature.[7] Respiratory mechanics studies may be carried out with a mathematical model as the response of a dose–response curve, which is plotting the drug effects as a function of its concentration. These curves present important parameters, such as maximum effect; the modeling of drug response[8]; assessment of responsiveness[9]; reactivity and sensitivity[10] and many others.

Nevertheless, parameters of respiratory mechanics, such as elastance and airway resistance, obtained through mathematical modeling may vary during the dose–response curve according to different factors. Among these factors are the selection of the animal lineage;

the pathophysiological model and, as expected, the concentration of the delivered drug reaching the target organ.

The cholinergic agent methacholine (MCh) is a bronchoconstrictor usually applied in respiratory mechanics studies or in order to assess the reactivity in asthma[11] both in *in vitro* and *in vivo* experiments. The route of administration *in vivo* affects how the drug reaches the target organ and the respiratory mechanics evaluation. Indeed, there are studies that compare inhaled (i.h.) and intravenous (i.v.) routes in mice[12] and in rats.[13]

Most of the studies of respiratory mechanics employing i.v. MCh use it through a bolus protocol, i.e. a rapid injection of the drug. Yet, there are some works that apply a continuous infusion protocol.[14–17] The way the MCh is injected intravenously, bolus or infusion, may alters the dose–response curve (presence of a peak or plateau) and may influence the respiratory mechanics evaluation.

As the world longevity increases[18] and the lung diseases are associated with the aging process,[19] this particular subject becomes more prevalent in the literature. Hence, a description of the respiratory mechanics aging process should provide a proper scenario to study the two protocols of i.v. MCh. Moreover, it is relevant to describe how each protocol is able to describe respiratory mechanics in the aging process.

In order to compare the protocols, we chose a screening of age with a health-aging resistant strain (SAMR1)[20,21] generally used in cohort studies as a control over senescence-accelerated mouse, once the SAMR strain presents a normal aging process.

Therefore, this study aims to compare respiratory mechanics of 3-, 6-, and 10-month SAMR1 mice submitted to MCh administration and to compare continuous infusion and bolus protocols. Additionally, this work aims to determine how both protocols impact the respiratory mechanics evaluation.

Methods

Animals

All the experiments involving laboratory animals were evaluated and approved by the "Ethics Committee for Animal Use" (protocol number 59/2016) from the Institute of Biomedical Sciences—University of São Paulo. The procedures abide by Brazilian National Law number 11794 from 08/10/2008, which regulates all the research activities involving animal use in the country (Table 1).

The mice (infusion animals—3 months: 10, 6 months: 13, 10 months: 7; bolus animals—3 months: 15, 6 months: 13, 10 months: 11) were anesthetized with an intraperitoneal (i.p.) injection of ketamine (120 mg/kg) and xylazine (12 mg/kg). After the proper anesthesia, tracheostomy and cannulation were performed with a metal cannula 18 G (BD Company, USA), and the animal was ventilated (flexiVent, SCIREQ, Canada) with a tidal volume of 10 mL/kg, PEEP of 3 cmH_2O and 150 breaths per minute. Then, a needle attached to a flexible PVC tube (Critchley Electrical Products PTY, Australia) was inserted through the right jugular vein.

The respiratory muscles were blocked with pancuronium bromide (1 mg/kg i.p.). After 7 min, two alveolar recruitment (volume ramp up to the value of 30 cmH_2O) maneuvers were performed.

Bolus protocol

After the recruitments, phosphate buffered saline (PBS) was injected through the right jugular vein. Then, 15 perturbations of 3 s each were performed, in apnea, with a 5-s programmed interval between each assessment. The CPM parameter peak values were the measurements associated with the highest values of airway resistance after the bolus injection. Usually, the maximum value occurred at the second measurement (second perturbation).

The perturbation consisted in a sum of 13 sinusoids (Hz): 1, 1.5, 2.5, 3.5, 5.5, 6.5, 8.5, 9.5, 11.5, 14.5, 15.5, 18.5, and 20.5. After the measurements, the animals were ventilated for 2 min until the next injection (Figure 1).

The same perturbations were performed for all doses of MCh: 0.03, 0.1, 0.3, 1 mg/kg. Automation routines of the ventilator control program were used to perform the perturbations.

Continuous infusion protocol

The animals of the infusion protocol were anesthetized, tracheostomized, blocked with pancuronium bromide and recruited in the same way as those in the bolus protocol. After the recruitments, PBS was injected, also through the right jugular vein, for 5 min with a continuous infusion pump (11 Plus, Harvard Apparatus, USA). Then, the MCh solution was injected for 2 min ("wash out") before the measurement setup.

In the continuous infusion protocol, the dose was increased by incrementing the volume, rather than the solution concentration. The concentration of the MCh solution was 320 μg/mL and the injected MCh doses were: 48, 96,

Table 1. Characteristics of groups.

	Infusion			Bolus		
Age	n	Weight (g)	Bal ($\times 10^5$ cel/cm^3)	n	Weight (g)	Bal ($\times 10^5$ cel/cm^3)
3 Months	10	38.6 ± 4.0	1.1 ± 0.7	15	44.1 ± 3.6	2.3 ± 0.6
6 Months	13	40.5 ± 2.8	1.0 ± 0.7	13	44.8 ± 5.0	2.8 ± 1.1
10 Months	7	43.1 ± 4.6	0.9 ± 0.6	11	45.7 ± 4.7	1.2 ± 0.6

Weight in grams; Bal: bronchoalveolar lavage; mean and standard deviation.

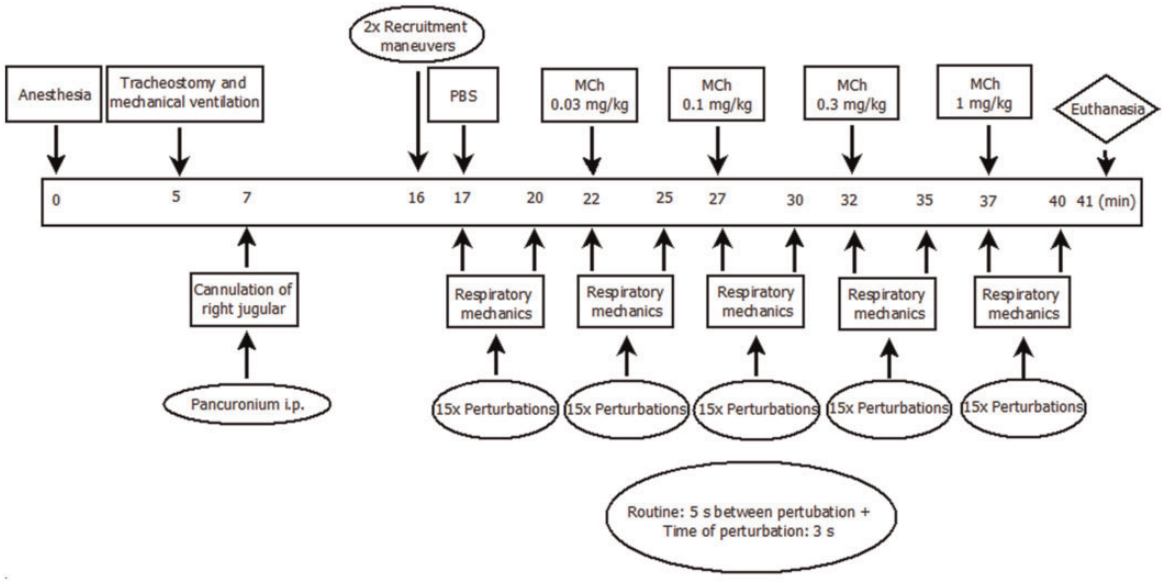

Figure 1. Diagram of *bolus* protocol.

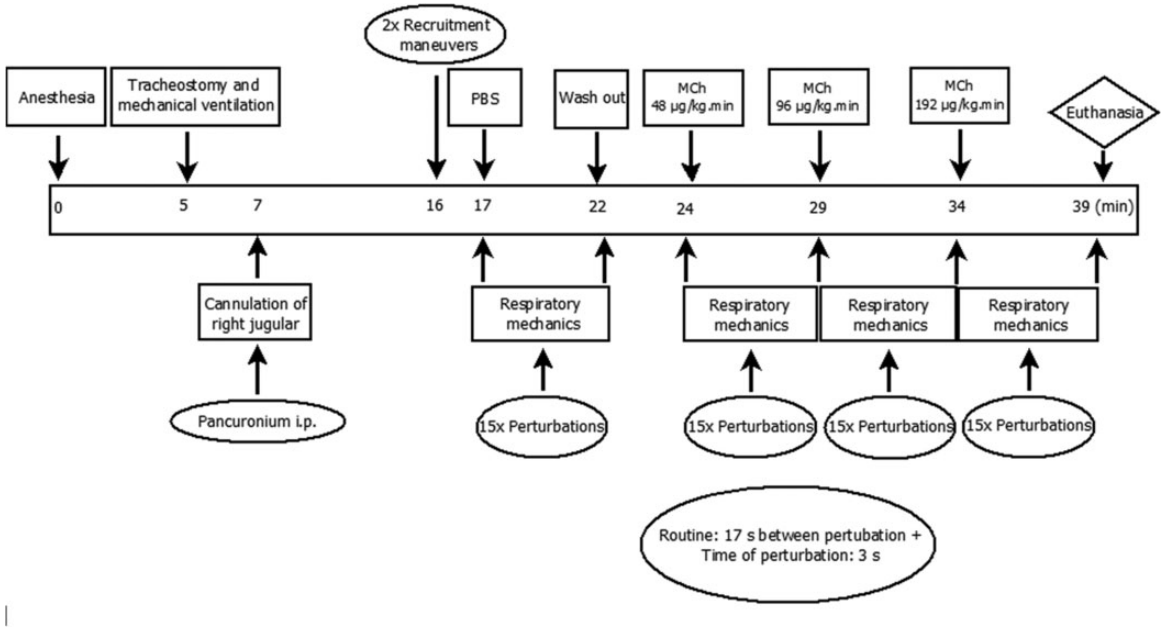

Figure 2. Diagram of infusion protocol.

and $192\,\mu g\,.\,kg^{-1}\,.\,min^{-1}$ and the infusion time was 5 min and CPM parameters used as plateaus were assessed at the final of the each dose infusion (5 min). Thus, at each dose increment, the flow rate was doubled and the flow calculation was performed based on the weight of each animal.

The same 15 perturbations performed in the bolus protocol were executed in the continuous infusion protocol. However, the inter-perturbation time was increased to 17 s. As in the bolus protocol, automation routines were used in the ventilator control program (Figure 2).

The CPM. The CPM is a frequency domain model with parameters endowed with physiological meaning. This model has been widely applied to studies on the assessment of respiratory mechanics equations (1) and (2)

$$Z_{rs}(f) = R_n + j \cdot 2 \cdot \pi \cdot f \cdot I + \frac{G - j \cdot H}{(2 \cdot \pi \cdot f)^{\alpha}} \quad (1)$$

$$\alpha = \frac{2}{\pi} \tan^{-1} \frac{H}{G} \quad (2)$$

where Z_{rs} is the respiratory system input impedance, R_n is the Newtonian Resistance, I is the airway inertance, j is the imaginary unity, and f is the frequency. Parameter G is associated with tissue energy dissipation and H is

associated with the potential elastic energy storage in lung tissue.[1]

This model is adjusted to the estimated impedance values.[22] The model fit was accepted when the coefficient of determination (COD) was higher than 0.9.[23] Therefore, this threshold was used as an exclusion criterion and, particularly for the bolus group, in case of low COD value at the highest value of a particular dose, this dose could not be used as it would jeopardize the experiment. This exclusion process is less adverse in the infusion protocol, since the analysis was performed using three scenarios: using the last value of each dose; the mean of the last two values and the mean of the last three values.

Statistical analysis. The main variables of this study were the CPM parameters. The peak and plateau values were selected from the bolus and infusion protocols, respectively. In addition, for both protocols, the area under the curve (AUC) was calculated for all the doses using trapezoidal integration (Matlab, The MathWorks, USA).

In order to assess the capacity of discriminating doses of MCh and the different ages of both protocols (bolus and infusion), the two-way analysis of variance (two-way ANOVA) was used as a statistical test. Additionally, the Bonferroni was selected as post-test.

In the case of increment response, when the doses were doubled or quadrupled, we used two-way ANOVA and Sidak's multiple comparisons test. Since the doses of the bolus protocol were not in a scale of 2, we used a Piecewise Cubic Hermite Interpolating Polynomial to find the corresponding response to the interpolated doses of 100, 200, and 400 μg/kg. The same interpolation was applied to find the doses required to achieve 150, 200, and 300% of R_n baseline response in bolus and infusion protocols. The statistical significance was $P < 0.05$.

Results

Dose–response curves

Figure 3 shows the characteristics of R_n, G, and H from bolus and infusion protocols, respectively. As expected, in the bolus protocol, a rapid increase of R_n and G is perceptible, followed by a sharp decrease. In turn, in the infusion protocol, an increase of the same parameters was observed with a plateau instead of the bolus characteristic decrease. H remains practically constant in both protocols responding to MCh only at the last dose.

Area under the curve

The graphs of Figure 4 present the behavior of the AUC of R_n, G, and H from the bolus and infusion protocols, respectively. Visually, there is congruence between the peak or plateau and the AUC behavior.

Bolus vs. infusion

In order to compare both protocol behaviors, we assessed the increment (ratio between the responses) of the CPM model parameters (R_n, G, and H) when the doses where doubled and quadrupled. This analysis should provide a proper understanding of sensitivity to drug variation in each protocol. Infusion (last measure of each dose): 48 to 96 and 48 to 192 $\mu g \,.\, kg^{-1}.min^{-1}$. Bolus: interpolation of the peak values of each bolus protocol dose. Figure 5 presents the interpolation of one animal per group

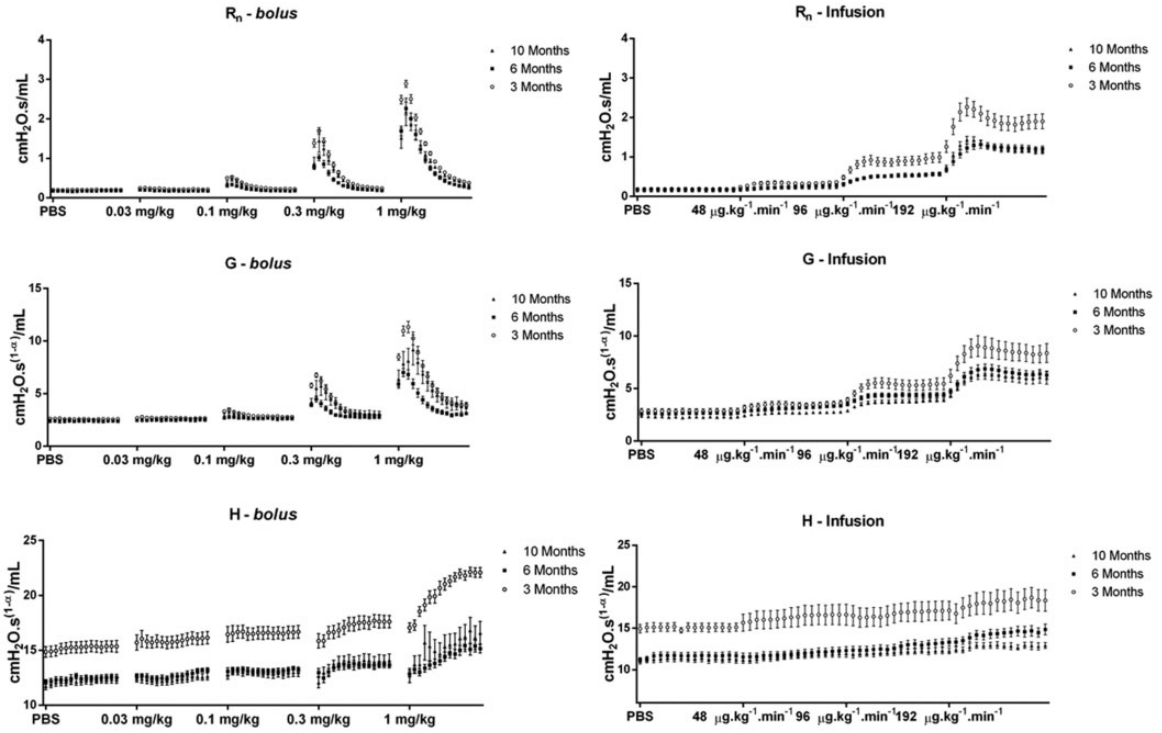

Figure 3. The *bolus* (left) and infusion (right) dose–response curves of all ages and doses. Mean and error.

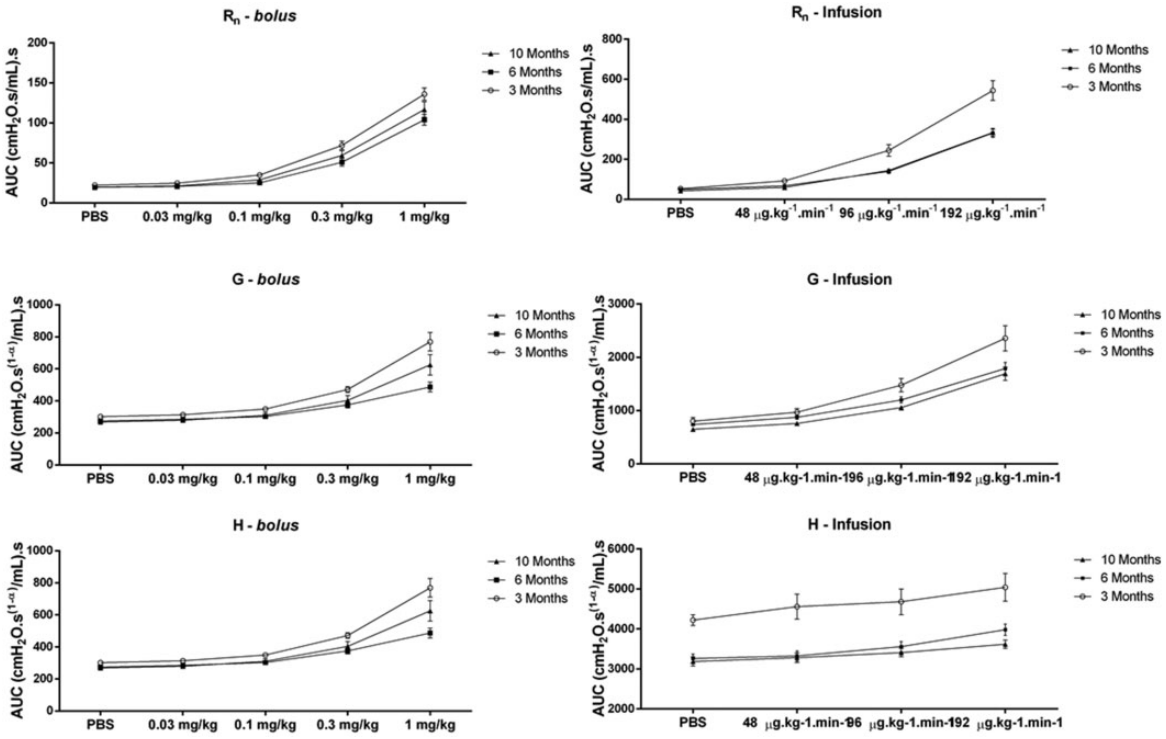

Figure 4. Comparison of the area under the curve (AUC) of the bolus (left) and infusion (right) protocols. Mean and error.

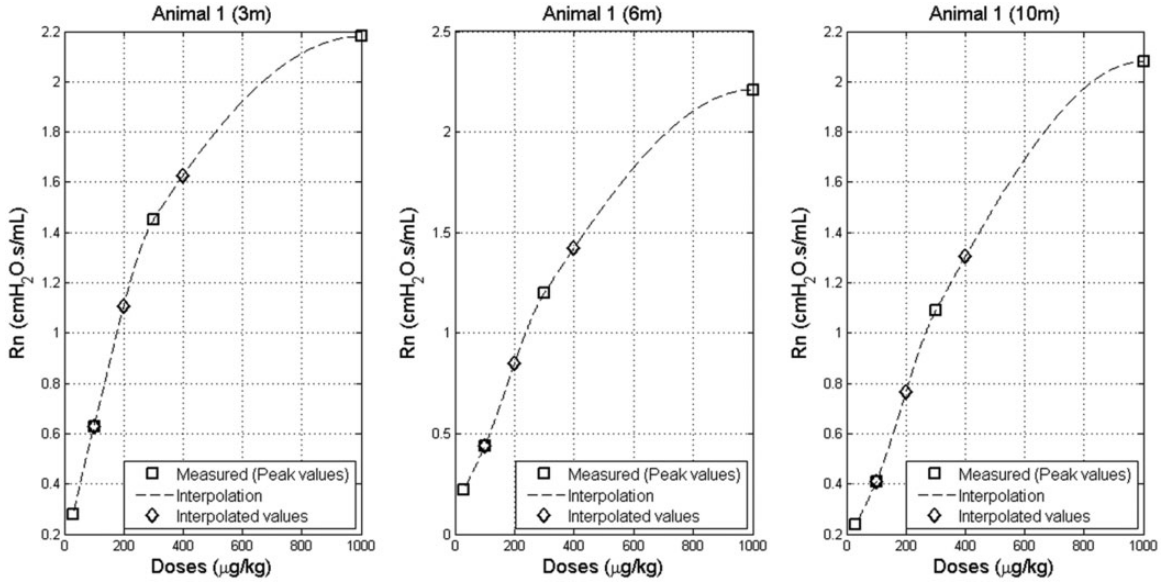

Figure 5. Interpolation of one animal per group (each age) in the bolus protocol in order to obtain the doses of 100, 200, and 400 μg/kg. Along with the interpolation, the measured and the interpolated values are shown.

(each age) in the bolus protocol in order to obtain the doses of 100, 200 and 400 μg/kg. Finally, Figure 6 presents the graphs of the increment of each parameter along with the statistical analysis.

Additionally, Table 2 presents the doses required, median and interquartile range, to achieve 150, 200, and 300% of R_n baseline response in bolus and infusion protocols.

Statistical analysis

The *P* value relative to doses and age in the two-way ANOVA was lower than 0.05 to all parameters and protocols, except for *P*, value relative to age in peak comparison of R_n in the bolus protocol. Therefore, Tables 3 and 4 show all the statistical analyses relative to post-test.

Table 3 presents the statistics of the comparison relative to the bolus protocol. The first part summarizes the

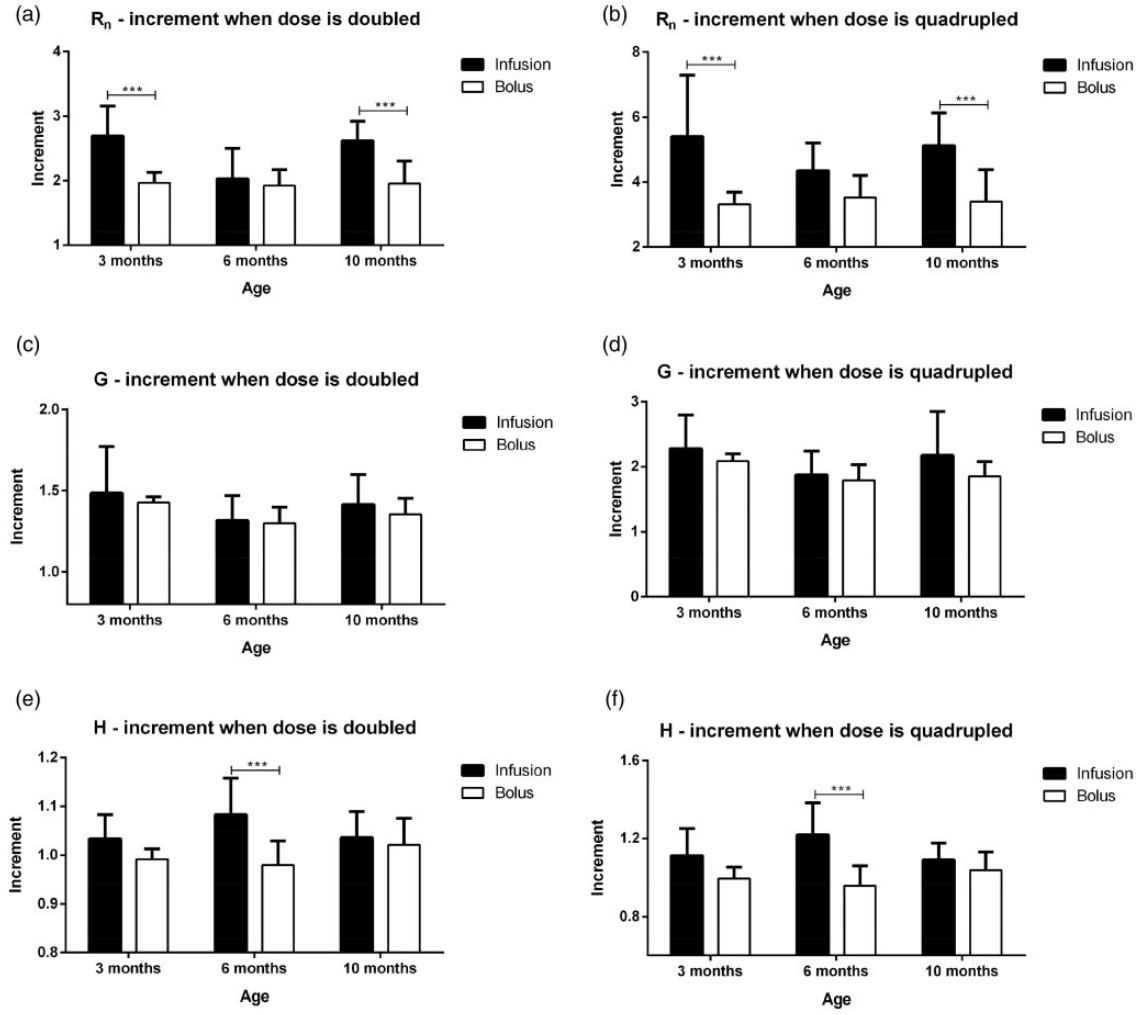

Figure 6. The increment of each parameter when the doses are doubled (a,c,e) ($P < 0.03$ for ages in R_n and *G*; $P < 0.001$ for protocols in R_n and *H*) and quadrupled (b,d,f) ($P < 0.05$ for ages in *G*; $P < 0.0001$ for protocols in R_n and *H*). Infusion: 48 to 96 and 48 to 192 μg . kg^{-1}.min^{-1}. Bolus: 100 to 200 and 100 to 400 μg/kg. ***Corresponds to $P < 0.001$.

Table 2. Doses required to achieve 150, 200, and 300% of R_n baseline response in bolus and infusion protocols.

R_n		Bolus (μg/kg)		Infusion (μg/kg/min)	
	Dose required to achieve	Median	(IQ)	Median	(IQ)
3 months	150% of baseline response	44.8	(40.0 to 49.7)	33.0	(26.0 to 39.8)
	200% of baseline response	68.7	(59.1 to 77.8)	51.6	(40.5 to 56.6)
	300% of baseline response	111.1	(89.3 to 127.9)	69.9	(59.1 to 75.0)
6 months	150% of baseline response	62.9	(54.9 to 83.2)	47.0	(38.0 to 53.8)
	200% of baseline response	95.4	(87.9 to 119.3)	66.9	(60.6 to 74.9)
	300% of baseline response	164.4	(140.4 to 186.1)	95.0	(79.1 118.5)
10 months	150% of baseline response	59.9	(44.5 to 70.7)	40.6	(34.5 to 51.2)
	200% of baseline response	95.8	(72.8 to 116.7)	56.6	(53.5 to 63.8)
	300% of baseline response	150.1	(108.9 to 194.2)	76.1	(69.4 to 80.7)

IQ: interquartile range.

comparison associated to peak values, whereas the second depicts the comparison relative to AUC. The post-test values presented in Table 3 were only presented when the source of variation, for example age, was statistically significant. There was one *P* value (R_n in the peak comparison, $P = 0.0597$) that was considerate in Table 3. However, since this value is not strictly below 0.05, it needs to be mentioned.

Table 4 describes the statistics of the comparison regarding the infusion protocol. The first part summarizes the comparison associated to plateau values, whereas the

Table 3. Post-test statistical values of CPM parameters in the *bolus* protocol.

		3 vs. 6 months	6 vs. 10 months	3 vs. 10 months
(a) Comparison of peak values				
			Bolus—peak	
R_n	PBS	NS	NS	NS
	0.03 mg/kg	NS	NS	NS
	0.1 mg/kg	NS	NS	NS
	0.3 mg/kg	<0.05	NS	NS
	1 mg/kg	<0.05	NS	NS
G	PBS	NS	NS	NS
	0.03 mg/kg	NS	NS	NS
	0.1 mg/kg	NS	NS	NS
	0.3 mg/kg	<0.01	NS	NS
	1 mg/kg	<0.001	NS	<0.001
H	PBS	<0.01	NS	<0.01
	0.03 mg/kg	<0.001	NS	<0.001
	0.1 mg/kg	<0.001	NS	<0.001
	0.3 mg/kg	<0.001	NS	<0.01
	1 mg/kg	<0.001	NS	<0.001
b) Comparison of AUC				
			Bolus—AUC	
R_n	PBS	NS	NS	NS
	0.03 mg/kg	NS	NS	NS
	0.1 mg/kg	NS	NS	NS
	0.3 mg/kg	<0.01	NS	NS
	1 mg/kg	<0.001	NS	<0.05
G	PBS	NS	NS	NS
	0.03 mg/kg	NS	NS	NS
	0.1 mg/kg	NS	NS	NS
	0.3 mg/kg	<0.05	NS	NS
	1 mg/kg	<0.001	NS	<0.01
H	PBS	<0.01	NS	<0.01
	0.03 mg/kg	<0.001	NS	<0.001
	0.1 mg/kg	<0.001	NS	<0.001
	0.3 mg/kg	<0.001	NS	<0.001
	1 mg/kg	<0.001	NS	<0.001

PBS: phosphate buffered saline.

second depicts the comparison concerning the AUC of the same protocol.

Data exclusion

Animal exclusion from the bolus protocol is presented in Table 5. The exclusion criterion was, mainly, the COD <0.9 at the peak response in any dose of MCh.

No animal needed to be excluded from the analysis of the continuous infusion protocol, even with a few low COD values.

Discussion

In order to compare bolus and infusion protocols in an aging process scenario, we used the SAMR1 strain ("*accelerated senescence-resistant*").[20,21] This lineage is an aging-resistant strain and presents a few respiratory system characteristics described by the literature, such as pressure-volume (PV) curve behavior and morphological analyses.[20] The aging process is an appropriate scenario since the world's population is presenting a higher life expectation and, with that, the age-associated diseases become more prevalent.[18,19]

We used a dose–response curve to MCh and this drug is usually applied to challenge the respiratory system in respiratory mechanics. There are two studies that compare i.h. and i.v. MCh.[12,13] Essentially, in mice,[12] the i.v. MCh leads to a more homogeneous bronchoconstriction and, in rats,[13] the i.v. MCh is responsible for the predominance of airway contraction rather than parenchyma.

Peták *et al*.[13] compared i.h with i.v. in rats using an infusion protocol with a wavetube device to assess the respiratory mechanics. Jonasson *et al*.[12] compared the i.h. in mice with a bolus protocol and they used the same small animal ventilator we used in this work.

One study compared wavetube and flexiVent[24]; its authors found that the CPM parameters obtained through wavetube and flexiVent are not comparable in an experimental protocol or without correction. Therefore, we used the modeled parameters obtained *via* flexiVent for both protocols.

There is one study previous to Peták's *et al*. and Jonasson's *et al*. works that compares the route

Table 4. Post-test statistical values of CPM parameters in the infusion protocol.

		3 vs. 6 months	6 vs. 10 months	3 vs. 10 months
(a) Comparison of plateau values - last value; mean of two last values and mean of three fast values of each dose				
		Infusion—plateau		
R_n	PBS	NS	NS	NS
	48 mg.kg^{-1}.min^{-1}	NS	NS	NS
	96 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
	192 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
G	PBS	NS	NS	NS
	48 mg.kg^{-1}.min^{-1}	NS	NS	NS
	96 mg.kg^{-1}.min^{-1}	NS	NS	<0.05
	192 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
H	PBS	<0.01	NS	<0.001
	48 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
	96 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
	192 mg.kg^{-1}.min^{-1}	<0.01	NS	<0.001
(b) Comparison of AUC				
		Infusion—AUC		
R_n	PBS	NS	NS	NS
	48 mg.kg^{-1}.min^{-1}	NS	NS	NS
	96 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.01
	192 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
G	PBS	NS	NS	NS
	48 mg.kg^{-1}.min^{-1}	NS	NS	NS
	96 mg.kg^{-1}.min^{-1}	NS	NS	<0.05
	192 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
H	PBS	<0.01	NS	<0.001
	48 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
	96 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001
	192 mg.kg^{-1}.min^{-1}	<0.001	NS	<0.001

Note: The statistics was the same for the three scenarios. PBS: phosphate buffered saline.

Table 5. Excluded and non-excluded animals from bolus protocol.

	3 Months	6 Months	10 Months
Excluded animals	7	2	2
Non-excluded animals	8	11	9

Note: The exclusion criteria was COD < 0.9 in a peak response. Number of animals excluded due to low signal to noise ratio or COD <0.9.

(inhaled, i.v.—bolus and i.v.—infusion) of MCh in sham rats.[25] In this work, Salerno and Ludwig modeled the resistance and elastance with the equation of motion and without a commercial ventilator.

They found that the overall incidence of highly constricted airways was higher in the aerosol group compared with the continuous infusion group. In addition, the overall incidence of local dynamic hyperinflation was higher in the i.v. groups vs. the aerosol group.

Finally, in an imaging protocol study,[26] it was found that the degree of bronchoconstriction and number of ventilation defects were consistently less in infusion of MCh than for similar doses delivered as a bolus. Moreover, ventilation defects recovered more quickly following infusion than after bolus injection.

The characteristics of the dose–response curves are described in Figure 3. In the bolus protocol, the rapid increase[27] and decrease of R_n and G was present. In the infusion protocol, an increase of these parameters with a plateau was observed.

H remains practically constant in both protocols responding to MCh only at the last dose, meaning that the tissue elastance only increases with a high dose of MCh.

There was a notable difference in CPM parameters among ages in both protocols. The tissue elastance must be highlighted since there is a decrease of the elastic recoil associated with the aging process.[20] The airways enlargement associated with aging process should be related with lower resistance. Thus, both protocols should be able to discriminate the parameters of CPM.

Indeed, both protocols managed to assess the difference among doses and ages. The infusion protocol appeared to be more sensitive to differences between ages and doses, as seen in the statistical analysis of Table 4 as compared with Table 3. However, further analyses with different scenarios, e.g. with a lung inflammation, should be carried out.

So far, we can realize that in an aging process screening, both protocols performed according to expectations. However, once the pharmacokinetics of MCh is different between bolus and infusion, due to absorption and degradation, it may not be feasible to directly compare the doses in both protocols. In future work, it may be relevant to assess the required concentration of MCh in each protocol for a given response.

In the current literature, we may perceive that comparisons of intermittent vs. infusion of, for example, an antibiotic delivery to humans has been published.[28,29]

Additionally, in these studies the comparisons were carried out in a parallel analysis, thus comparing the outcomes of one protocol vs. the other.

In our present work, we proposed a direct comparison of both methods (not of doses but the pattern of the curve). We studied the increment of the parameters when the doses are doubled and quadrupled, so that we could compare the behavior of the dose–response itself. Figure 6 allows verifying that the infusion protocol presented, overall, a higher increment in the elastance and airway resistance when compared with bolus. Complementarily, Table 2 presents the doses required to a same set of increments in both protocol. This table shows that one could not simply calculate the amount of MCh delivered in each protocol in order to find a dose correspondence.

This phenomenon can be associated with the fast absorption and degradation of the drug,[8] especially in the bolus protocol. Since the MCh is continuously injected in the infusion protocol, the response increment observed with the increment of the drug may be higher in this protocol due to the balance between degradation and infusion. For the bolus protocol, the effect is rapidly observed and the MCh is soon degraded. Unfortunately, these analyses do not provide a dose correspondence, which would require the pharmacokinetics coefficients to fully model the degradation, but this is not the scope of this work.

Hence, there was difference in the increments of the dose–response curves of both protocols and the protocols were capable of discriminating groups. However, those findings are not the only relevant issues regarding respiratory mechanics. For example, the perturbation used herein lasted 3 s; however, the CPM can be applied to longer perturbations[1] and this is important to the bolus protocol discussion.

A major error would be the non-matching of the maximum value of a parameter to the true response peak simply because the evaluation is not performed with a negligible time interval between measurements. Furthermore, if the maximum measurement presents a COD <0.9 or low signal to noise ratio, the entire experiment may be compromised, as can be seen from the number of animals excluded (Table 5).

The continuous infusion protocol does not present these errors, since the values used are associated to the "steady state" condition after 5 min.[17] For example, in case the last measurement presents an exclusion criterion, it is possible to use the penultimate or to standardize the use of the average of the last points, since the statistical analysis was not different using the last point or the mean of the two or three last points as we can see in legend of Table 4a.

Consequently, the exclusion of one measurement would not jeopardize the analysis. However, the implementation of continuous infusion presents some milestones that should be taken into consideration when designing the study.

In conclusion, both protocols were capable of evaluating intragroup and intergroup differences. Nonetheless, in the bolus protocol, the highest value of each curve dose may not correspond to the highest real value, and the loss of this point may be a problematic factor in the sample size. These factors are not present in the infusion protocol. Additionally, at this lineage and age screening, the infusion protocol presented a higher sensitivity to dose increment and it appeared to be more sensitive to differences among ages when compared to the bolus protocol.

Authors' contributions: All the authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; MAO and RLV conducted the experiments; RLV conducted the analysis and wrote the manuscript; WTL and HTM contributed with infrastructure and study design.

DECLARATION OF CONFLICTING INTERESTS

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