PULMONARY PRESSURE-VOLUME CURVES OF ELASTASE-TREATED AND CONTROL RATS

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Abstract: The objective of this experiment is to test if the emphysematous and the control rats can be classified according to the pulmonary pressure-volume curves. Emphysema was simulated by instilling elastase to the rat lungs and leaving them to develop the disease for 6 weeks. The pressure-volume curves were acquired by a custom-designed ventilator. The pressure at the inflection point of the inhalation limb of the curves has been used as a feature to separate the two classes of animals. The extension of emphysema in the rat lungs was assessed post-mortem by measuring the mean surface of the alveoli. This was possible after extracting the lungs, slicing them, photographing them and analysing the pictures. The mean surface of the alveoli distinguishes clearly the control from the emphysematous rats, which verifies the deteriorating effect of elastase over the lung alveoli. However, no clear correlation was found between the pressure-volume curves inflection-points and the animal classes.

1 INTRODUCTION

The pressure-volume (PV) curve of the lungs has been an extensive object of research by many groups for different purposes (Jose G. Venegas, 1998)(R. Peslin, 1996)(R. Scott Harris, 2000). PV curves of the human lungs, as measured by spirometry, are a useful tool to the clinicians as they provide with useful information for the disease state of patients suffering from lung diseases such as chronic obstructive pulmonary disease, acute respiratory distress syndrome, asthma, cystic fibrosis and emphysema (Lumb, 2005)(West, 2008). In the intensive care unit the PV curves analysis of mechanically ventilated patients helps to determine the optimum level of positive end-expiratory pressure to prevent side-effects such as acute lung injury (R. Scott Harris, 2000)(Roy G. Brower, 2004). Based on PV curves measurements on mechanically ventilated rabbits, (R. Peslin, 1996) developed a model to study the mechanical properties of the lungs.

In (R. Scott Harris, 2000) a model for the quantification of PV curve parameters is introduced. In this publication an attempt is made to classify control and emphysematous rats by using data that have been extracted from pulmonary PV curves using the afore mentioned model. The advantages of the used method are the simplicity of the used equipment and the straight-forwardness of both the mathematical and the disease model. The measurements are based on the ventilator as described in (Rigoberto Perez de Alejo, 2005). The ventilator that was actually used is an updated version that has the possibility of PV curves acquisition.

A sensitive method to measure the mechanical properties of the rat lungs would prove useful for the effectiveness evaluation of new drugs tested on rats.

2 METHODS

In this study the experimental results of two experiments are analysed. The first, referred to as experiment 1, was performed in April 2008 and the second, referred to as experiment 2, in June 2008.

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2.1 Animal Preparation

The race of rats is male Wistar, weighting between 250 and 350 g.

In experiment 1 ten animals were used: 5 controls and 5 elastase-induced. At 6 weeks prior to the experiment 5 of the animals were treated with elastase and 5 with saline. The time before the experiment was enough for emphysema to develop in the lungs of the elastase-treated animals. (Joseph P. Dugas, 2004) and (Stefanida Kononov, 2001) reported that emphysema was detectable 2 and 4 weeks respectively after elastase instillation. The control animals were treated with saline to ensure that the difference of behaviour between the two categories is not due to the stress of washing the lungs with the fluid (Stefanida Kononov, 2001).

15 units of fresh elastase SIGMA# E0127 in a final volume of instillation of 0.5 mL was instilled to the rats after they were anaesthetised with isoflurane. The liquid was instilled through a catheter, which was inserted all the way to the lungs with a viewing tool similar to an otoscope.

In experiment 2 ten animals were used: 7 elastase-induced and 3 controls. 6 weeks prior to the experiment 7 of the animals were treated with elastase and the other 3 were treated with saline.

The elastase that was used to instill the animals in June was the same as the one used in April. In the meantime, it was preserved at 4^{o}C. 30 units of elastase were dissolved in a final volume of 0.5 mL. The concentration of elastase used in experiment 2 was higher than in experiment 1 because in experiment 1 elastase was fresh, whereas in experiment 2 it was not. Elastase is known to lose its effectiveness after it is removed from its package. Therefore, a larger concentration of old elastase is necessary to reach the same effect as with fresh elastase.

The instillation was performed by placing the animal with an angle on a specially designed ramp. The liquid was then introduced through the mouth to the lungs. It was ensured that the liquid went to the trachea and not the oesophagus by blocking the rat's nose with a pair of pliers.

2.2 Experimental Procedure

The experimental procedure was the same both in experiment 1 and 2.

The animals were initially anaesthetised first with isoflurane and subsequently peritoneally with a NARCOREN (sodium pentobarbital) dose of 100 μL/100 g. Then they were tracheotomised and a tube was inserted and tied at their trachea. The exterior part of this tube is suitable for the connection of the ventilator (section 2.3). The animal was firstly imaged in a MRI scanner and after 20-30 minutes it was brought to the ventilator for the PV curve acquisition. The acquisition lasted 10-20 minutes depending on the animal's response to the mechanical respiration. Some animals need longer time than others to adapt to the exterior pace of breathing without counteracting. Finally, the rats were scanned by CT and after that their lungs were perfused with saline and subsequently extracted and fixed with a 10% paraformaldehyde solution for 24 h. The fixed lungs were brought to the Fundación Jimenez-Diaz, Madrid, Spain and cut with a microtome in 5 μm slices, which were photographed with a LEICA DFC290 camera. 54 pictures correspond to the lungs of each animal.

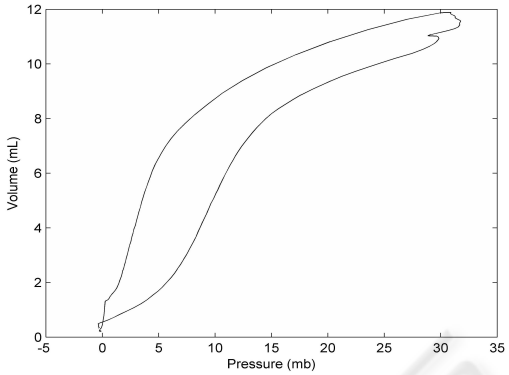

Figure 1: A PV curve of control 3 rat from experiment 1.

In this study the PV curve measurements data as well as the morphometry data from the lung pictures are analysed.(section 2.4.2)

2.3 Ventilator

The device that was used to acquire the pressure-volume curves is a ventilator that has been designed and manufactured in the Universidad Complutense de Madrid (Rigoberto Perez de Alejo, 2005). The version that was actually used is an update of (Rigoberto Perez de Alejo, 2005). An important difference between the first and the second version that was used in the present study is that the now PV curves acquisition is possible. Reliability of the pressure and volume indications of the ventilator has been verified with a water column and a syringe.

Before each measurement the ventilator was calibrated and system tightness and integrity was verified. A slightly positive drift of the volume indication was maintained to ensure proper function of the auto-reset-volume feature of the ventilator. The signals were converted to digital at a frequency rate of

50 Hz and each acquisition lasted 30 s, corresponding to 1500 datapoints (measurements of pressure and volume).

Each animal was connected to the mechanical ventilation initially in normal mode of respiration. The breathing rate was set at 60 breaths per minute, the tidal volume at 3 mL and the ratio of the inspiratory time to total cycle duration ratio was 0.33. Under this volume the recorded pressure inside the lungs was 10 mbars. Correct volume was constantly verified through the sensor indications.

The PV curves were acquired in the acquisition mode of the ventilator. The air was pushed inside the lungs of each animal until it reached either 15 mL or 30 mbars of pressure. In this way it was ensured that maximum lung capacity was reached without risking injuring the animal. Because the time of inspiration is not controlled by the ventilator, this implies that in each acquisition a different number of curves was registered. Just after the inhalation ended, without any apnoea, each animal was allowed to expire without obstruction for 2 s. Subsequently, the new PV curve cycle started by pushing again air inside the lungs. This process was repeated for 30 s before the animal returned to normal mode of respiration. In this way a total of 7 to 8 consecutive, full PV curves were acquired. Such a group of curves consists one set of measurements. Each set of measurements comprises 1500 points, which are the number of measurements that the ventilator performs in 30 s at a sampling rate of 50 Hz. The 1500 points of each acquisition were stored in a different ASCII format file.

For each animal a different number of sets of measurements was acquired. This number as well as the total acquired PV curves for all the sets of measurements appear in tables 1 and 2. The time between consecutive sets of measurements varied from 3 to 10 minutes depending on the response of each animal to the mechanical ventilation and to the stress of inhaling consecutively, many times volumes equal to the total lung capacity.

In experiment 1 more sets of measurements were acquired than in experiment 2. The reason for this is discussed in section 4.1.

Table 1: Experiment 1. C: control animal. E: elastase-treated animal.

C/E	Sets	Total cycles
C1	5	33
C2	4	29
C3	5	37
C4	5	36
C5	2	14
E1	4	27
E2	6	40
E3	5	35
E4	6	42
E5	4	26

Table 2: Experiment 1. C: control animal. E: elastase-treated animal.

C/E	Sets	Total cycles
C1	2	17
C2	2	17
C3	2	17
E1	1	7
E2	2	16
E3	2	16
E4	2	19
E5	2	17
E6	2	16
E7	2	16

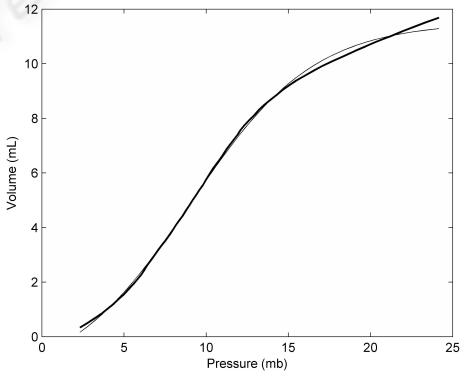

Figure 2: The thick line corresponds to the inflation limb of a PV curve. The thin line represents the fitted sigmoidal function. The pressure of the inflection point is 9 mb.

2.4 Data Processing

2.4.1 PV Curves

After the experiment the data was analysed by fitting a sigmoidal function ((Jose G. Venegas, 1998) and (R. Scott Harris, 2000), equation 1) to the inflation limb of each PV curve. According to (Jose G. Venegas, 1998) the deflation limb does not provide useful information because exhalation was allowed to occur without obstruction.

The model to fit the pulmonary PV curves of (Jose G. Venegas, 1998) and (R. Scott Harris, 2000) was preferred from others (R. Peslin, 1996) for its simplicity and comprehensive physical interpretation of its parameters.

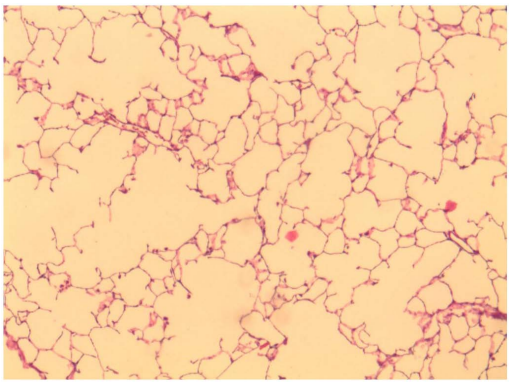

Figure 3: An image of the upper left lobe of the control 4 rat.

$$V = a + \left[\frac{b}{1 + e^{-(P-c)/d}} \right] \quad (1)$$

Parameter c in equation 1 equals the value of the pressure at the inflection point, which is defined as the point where the second derivative changes sign. In the case of the PV curves it corresponds to the maximum compliance point (West, 2008).

The inflation limbs of the PV curves were fitted by equation 1 by using two different error minimisation algorithms: the simplex Nelder-Mead algorithm (Jeffrey C. Lagarias, 1998) and a genetic algorithm.

The function *fminsearch* of MATLAB ® was used with initial conditions $[a,b,c,d] = [1,15,10,3]$ sufficiently close to the values of each parameter. The termination criteria were either a final mean squared error 10^{-4} or 10000 iterations.

For the genetic algorithm 10000 genes have been generated at each step. The fitting process in every case was interrupted after 100 steps. The used application has been implemented in the C programming language.

2.4.2 Morphometry

The pictures of the lungs that have been shot as described in section 2.2 were analysed to assess the mean surface of the alveoli. For this purpose the program MIPAV (Medical Image Processing, Analysis, and Visualisation, http://mipav.cit.nih.gov) was used and the following image analysis strategy was used:

1. entropy minimisation,
2. colour to grey-scale conversion,
3. thresholding (figure 4),
4. object extraction and object size measurement (figure 5).

Entropy minimisation in step 1 is aiming to remove shading from the image (B. Likar, 2000)(Russ, 2000). According to (B. Likar, 2000) most of the shading effects of microscopy images are successfully removed by this technique.

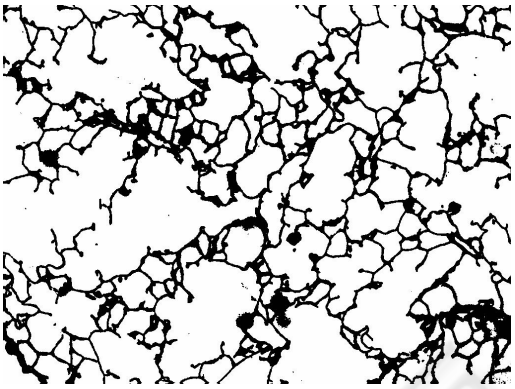

Figure 4: Image 3 thresholded.

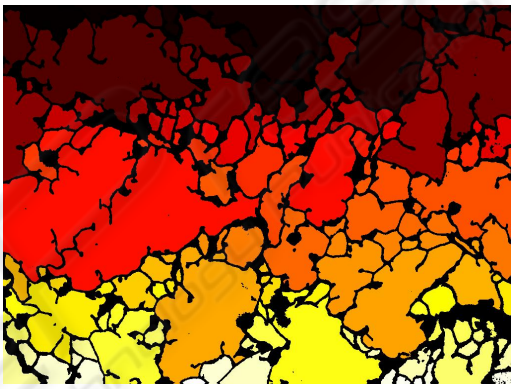

Figure 5: The extracted objects from image 4.

The corrected image was converted from RGB (red-green-blue) format to greyscale. Each channel contributes to the final greyscale image with the same weight, that is $1/3$ (Edward R. Dougherty, 2003).

Until this point all the images were processed identically. The greyscale image was converted to binary (figure 4) with a different threshold for each case. Thresholds have been chosen for each image according to their contrast and brightness. Optimum value has been decided each time by the user. The criterion was to distinguish clearly each alveolus from its neighbours.

To calculate the mean surface of the alveoli, first the surface of each alveolus is measured. For this purpose the function "*ID objects*" of MIPAV was applied to the binary image. Its output appears in figure 5. "*ID objects*" measures the number and number of pixels of enclosed areas in the binary image. These areas correspond to the alveoli. The dimensions of each pixel are $(1.28\mu m)^2$, therefore its surface equals $1.638\ \mu m^2$.

The objects with size less than 130 pixels have been considered that do not correspond to alveoli but to artifacts of the image analysis and therefore excluded from the processed data.

3 RESULTS

The experimental results of both PV curve measurements and morphometry appear in tables 3 and 4.

The mean values $\overline{C}$ and $\overline{C}_{gen}$ include all the PV curves that have been measured for each animal.

$$\overline{C} = \frac{1}{N}\sum_{i}^{N} c_i, \tag{2}$$

where c_i is the c parameter of equation 1 that corresponds to the i PV curve. N is the number of PV curves acquired for each animal. $\overline{C}_{gen}$ corresponds to the results of the genetic algorithm.

$\overline{S}$ is the mean alveoli surface for each animal. The mean alveoli surface for all the control rats is $\overline{S}_c = 3911.4\ \mu\text{m}^2$, $\sigma(\overline{S}_c) = 1192\ \mu\text{m}^2$. The mean alveoli surface for all the elastase rats is $\overline{S}_e = 7438\ \mu\text{m}^2$, $\sigma(\overline{S}_e) = 3044\ \mu\text{m}^2$.

In tables 3 and 4 the first and the third column hold the pressure at which inflection occurs as assessed by the simplex and the genetic algorithm respectively. Units are mbar. Columns 2 and 4 hold the standard deviations of the respective parameters. The last column holds the mean surface of the alveoli in μm^2.

Tables 5 and 6 hold the mean-squared fitting error for each experiment. Specifically, $\overline{\varepsilon}_1$ and $\overline{\varepsilon}_2$ equal

$$\overline{\varepsilon} = \frac{1}{M}\sum_{j}^{M}\frac{1}{N}\sum_{i}^{N}(\hat{x}_i - x_i)^2, \tag{3}$$

where x_i is an experimentally measured value of pressure, $\hat{x}_i$ is the estimated value for x_i, N is the number of measured points and M the number of sets of measurements.

Figures 6 and 7 correspond to experiments 1 and 2 respectively. In both of them the x-axis represents the inflection point pressure as given by the simplex algorithm and the y-axis represents the mean alveoli surface.

4 DISCUSSION

4.1 Number of Sets of Measurements

In experiment 1 more sets of measurements were performed than in experiment 2, therefore more PV curves and more data. These data appear analytically in tables 1 and 2. The reason for this was that in the first experiment it was sought whether the pressure of the inflection point changes over time for the same animal. It was observed that even though the breathing behaviour of the animal may change over time, this does not affect the inflection point. Therefore, in the second experiment the sets of measurement were restricted to 2 only.

Table 3: Experiment 1. C: control animal. E: elastase-treated animal.

C/E	$\overline{C}$	$\sigma(C)$	$\overline{C}_{gen}$	$\sigma(C_{gen})$	$\overline{S}$
C1	8.81	0.21	8.64	0.27	3866.4
C2	8.96	0.18	8.6	0.37	2052.4
C3	9.89	0.33	9.39	0.59	4723.4
C4	8.64	0.55	8.18	0.63	5153.7
C5	8.52	0.09	5.98	2.01	3760.9
E1	8.83	0.17	8.41	0.34	5750.6
E2	7.44	0.26	7.17	0.44	12773.9
E3	9.06	0.29	8.41	0.63	6256.8
E4	8.58	0.48	8.21	0.53	5396.4
E5	8.53	0.36	8.2	0.58	7012.2

Table 4: Experiment 2. C: control animal. E: elastase-treated animal.

C/E	$\overline{C}$	$\sigma(C)$	$\overline{C}_{gen}$	$\sigma(C_{gen})$	$\overline{S}$
C1	10.44	0.7	10.24	0.88	4556.5
C2	8.91	0.47	8.89	0.47	5063.3
C3	9.5	0.72	9.21	0.73	4040.1
E1	9.44	0.07	9.11	0.25	5502.1
E2	7.87	0.37	7.57	0.57	5921
E3	7.51	0.12	7.39	0.24	7647
E4	6.16	0.33	6.15	0.37	8943.1
E5	8.53	0.25	8.49	0.23	7610.3
E6	8.13	0.12	7.93	0.29	7576.8
E7	7.84	0.26	7.79	0.28	6020.2

4.2 Error-minimisation Algorithm

Two minimisation algorithms were used to model the PV curves. The mean-squared error of the simplex algorithm has always been lower than that of the genetic, as it appears in tables 5 and 6.

4.3 Morphometry

The mean alveoli surface of every control rat is less than 5153.7 μm^2 (C4). The same value for the elastase rats is greater than 5396.4 μm^2 (E4). This means that knowing the mean alveoli surface of a rat it can be deduced with certainty whether it is control or treated

Table 5: Experiment 1. C: control animal. E: elastase-treated animal.

C/E	$\bar{\varepsilon}_1$	$\bar{\varepsilon}_2$
C1	$1.49\ 10^{-2}$	$1.66\ 10^{-2}$
C2	$1.76\ 10^{-2}$	$2.38\ 10^{-2}$
C3	$1.4\ 10^{-2}$	$2.99\ 10^{-2}$
C4	$1.9\ 10^{-2}$	$2.53\ 10^{-2}$
C5	$1.02\ 10^{-3}$	$4.86\ 10^{-3}$
E1	$1.06\ 10^{-2}$	$1.41\ 10^{-2}$
E2	$2.57\ 10^{-2}$	$2.69\ 10^{-2}$
E3	$1.5\ 10^{-2}$	$2.37\ 10^{-2}$
E4	$3.41\ 10^{-2}$	$3.66\ 10^{-2}$
E5	$3.48\ 10^{-1}$	$3.5\ 10^{-1}$

Table 6: Experiment 2. C: control animal. E: elastase-treated animal.

C/E	$\bar{\varepsilon}_1$	$\bar{\varepsilon}_2$
C1	$7.46\ 10^{-2}$	$7.72\ 10^{-2}$
C2	$5.13\ 10^{-2}$	$5.14\ 10^{-2}$
C3	$7.38\ 10^{-3}$	$1.17\ 10^{-2}$
E1	$1.74\ 10^{-2}$	$2.37\ 10^{-2}$
E2	$4.17\ 10^{-2}$	$4.32\ 10^{-2}$
E3	$2.37\ 10^{-2}$	$2.41\ 10^{-2}$
E4	$3.47\ 10^{-2}$	$3.52\ 10^{-2}$
E5	$1.61\ 10^{-1}$	$1.62\ 10^{-1}$
E6	$1.43\ 10^{-2}$	$1.5\ 10^{-2}$
E7	$2.3\ 10^{-2}$	$2.32\ 10^{-2}$

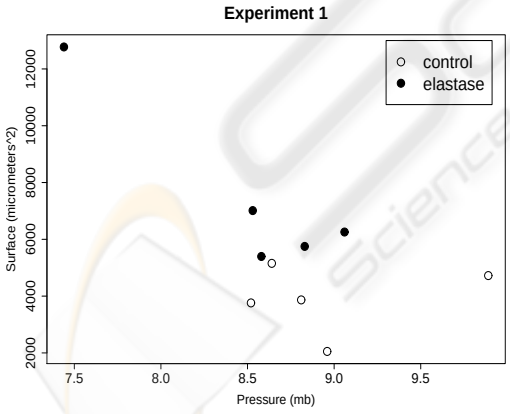

Figure 6: Inflection points pressure over mean alveoli surface for elastase and control rats for experiment 1.

with elastase. The mean values of the two classes of animals ($\bar{S}_c = 3911.4\ \mu\text{m}^2$, $\bar{S}_e = 7438\ \mu\text{m}^2$) show a clear separation as well.

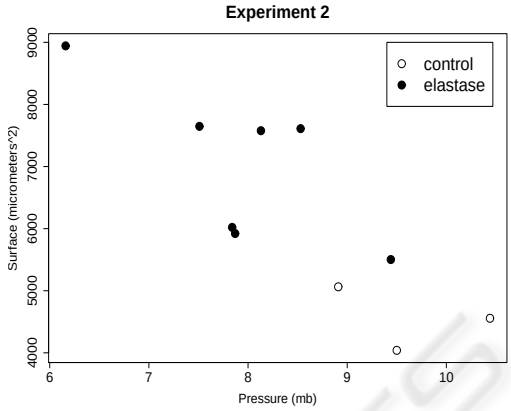

Figure 7: Inflection points pressure over mean alveoli surface for elastase and control rats for experiment 2.

4.4 PV Curve Data

No clear connection between the inflection points and the disease state of the animals appears. It seems that an inflection point with pressure less than 8.5 mb (E2, E4, E5) is an indication of disease, but the results are not statistically significant to draw this conclusion as secure.

4.5 Induction Methodology

In experiment 1 no ramp was used for the induction of the disease, as it was done in experiment 2. The morphometrical results (section 4.3) showed that even in experiment 1 the elastase had an important effect to the rat lungs.

5 CONCLUSIONS

The accuracy of the inflection point pressure measurement is high because the pressure sensors are proven to give accurate estimates. The volume at which the inflection occurs is measured less accurately because the volume indications are less accurate. For this reason the inflection point volume is not mentioned.

The inflection point pressure represents the point of maximum alveoli recruitment. It is the critical pressure at which the lungs totally dilate to reach their total capacity (Jose G. Venegas, 1998). After this point the inflation of the PV curve turns its curvature downwards and enters a relatively flat area. The position of the inflection point is directly affected from the mechanical properties of the lungs and specifically from its compliance. An emphysematous lung is

more compliant than a healthy lung, which means that the same volume is achieved with less pressure (West, 2008). This means that the volume that corresponds to the maximum recruitment point, same for subjects of similar size, should be achieved with less pressure for the elastase-treated animal. However, this is not what was observed.

A reason that no difference is found between the PV curves inflection points, which are related to the viscoelastic properties of the lungs, could be that there is no actual alteration in these properties. It should be considered that either the rats have recovered in 6 weeks after the induction of elastase or that the elastase, even though it destroys the alveolar walls, leaves the elastic properties of the lungs unaffected.

To verify any of these assumptions, a long-term study should be performed. For this purpose, the animals should not be tracheotomised and sacrificed but intubated, so that the same rats can be measured and their lung compliance registered over the course of time. With intubation a pressure of 30 mbar, as was used in this experiment, cannot be reached. In spite of this, the inflection point of the PV curves can be still calculated.

In a future experiment a higher inhalation time should be tested, by increasing the airflow resistance in the inspiration tube. In this way the inflection points may provide clearer classification between normal and elastase-treated rats. For this purpose, changes are already under development in the home-made mechanical ventilation device.

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