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Respiratory System Evaluation

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1 OBJECTIVE

This document describes the methodology for performing an evaluation of the respiratory system in adult C57 normal and *mdx* mice.

2 SCOPE AND APPLICABILITY

Evaluation of the respiratory system dysfunction in *mdx* mice (Gosselin et al. 2003; Matecki et al. 2003; Mosqueira et al. 2013), caused in part due to diaphragmatic damage (Stedman et al. 1991) is an important parameter to consider in the design and evaluation of potential therapeutics for muscular dystrophy. There are significant advantages to evaluating the respiratory system of *mdx* mice during pre-clinical studies: a) it is a clinically relevant deficit and, b) spirometric or plethysmographic measurements are non-invasive and can be repeated in a longitudinal manner during the trial and/or performed as end-point for evaluating the efficacy of various therapies for muscular dystrophy.

Respiratory function can be evaluated in *mdx* mice by measurement of ventilatory parameters [respiratory rate (f_R), tidal volume (V_T) and minute ventilation ($\dot{V}_e$)] as well as integrity of hypoxic and hypercapnic-ventilatory reflexes (HVR and HCVR, respectively). Other parameters might also be evaluated such as inspiratory time (Ti), expiratory time (TE) and duration (total time). Although it is also possible to obtain peak inspiratory flow (PIF), peak expiratory flow (PEF), forced vital capacity (FVC) and force expiratory volume in 1 second (FEV_1), these parameters should not be used in animal because they depend on the voluntary maximal inspiration and expiration. These evaluations can be assessed spirometrically with a pneumotachometer using a cylindrical chamber to contain the animal or a nose pneumotachograph (*in vivo*) or using intratracheal measurements (as an end-point measurement). Evaluation can be also made using a plethysmograph (*in vivo*). While the clinically useful parameters maximal expiratory flow-volume and maximal inspiratory pressures have been measured in mice and rats (Lai & Chou 2000; Barreiro et al. 2010), these methods have not as yet been applied to *mdx* mice and hence reference values are not as yet available.

The age, sex and strain are factors that have to be considered while designing the trial. These factors are known to influence respiratory parameters (Tatsumi et al, 1991; Tankersley et al., 1994; Han et al., 2001).

3 CAUTIONS

Evaluation of respiratory system requires careful trial design and experimental technique. The age, sex and strain are factors that have to be considered while designing the trial. Mice need to adequately acclimate to the chamber and environmental conditions

standardized (dark/light conditions; sound) as the evaluation is a sensitive *in vivo* measurement. The apparatus used should have adequate (microliter) sensitivity and be calibrated before and after use to determine accuracy. Values obtained with a pneumotachometer using the whole body cylindrical chamber may need to be corrected using a correction factor obtained by using a pneumotachometer attached to the rodent nose via a conical nosepiece.

4 MATERIALS

4.1 Animals

Twelve to 20 week old mice (C57BL/10ScSn and C57BL/10ScSn-DMD^{mdx}/J) from Jackson Laboratory were used.

4.2 Apparatus used to evaluate respiratory parameter

4.2.1 Equipment

For all techniques, it is necessary a computer connected with an analog-digital (AD) converter and installed a software for data recording and analysis. Here, it is suggested the analog-digital converter PowerLab and the software LabChart, both from ADInstruments. The AD converter is connected to a spirometer pod (FE141, ADInstruments) and this in turn to pneumotachometer (Respiratory Flow Head MLT1L, ADInstruments). For calibration it is necessary a calibrated micropipette P200.

The three different devices used to connect the animal to the pneumotachometer are:

1) whole body cylindrical chamber

Apparatus used is a custom made, cylindrical chamber (12 cm long and 6.4 cm in diameter) made of clear plastic (Fig. 1). Internal dimensions are 10.2 cm long and 5.0 cm in diameter. The end discs are fitted with air-tight gaskets and removed for access to the interior of the chamber (i.e. placing mouse inside). The chamber contains a removable flat plastic platform in one-section cylinder shape with the same longitudinal of the chamber in order to support the mouse and reduce dead space. The chamber's final volume with the platform is 150 mL. The chamber has two inlets, of which one is used to inlet a gas mixture (N_2, O_2 and/or CO_2), and the second is used as outlet and is connected to the pneumotachometer. The chamber is placed in a cardboard box to avoid distraction and mouse stress during the recordings. The doors of the box are opened to ensure the mouse is awake prior to recording.

2) Nose pneumotachometer (NP):

The apparatus is used to obtain a correction factor for thorax expansion during inspiration, which reduces the observed V_T value when using the whole body cylindrical chamber. The NP was constructed from a 15 mL cylindrical tube (USA Scientific, Ocala, Fl; Cat no. 1485-0810) with two ports (Fig. 2). To obtain an air-tight seal, parafilm "M" (American National

Can, Greenwich, CT, Cat no. PM-996) is used to form an air-tight seal between mouse nose and tube.

3) Intratracheal (IT):

Mice are anesthetized with a mixture of ketamine (100mg/kg) and xylasine (20mg/kg) and kept on a temperature controlled heating plate. The neck is exposed and the trachea is cannulated. A 1.0 cm long and 0.1 cm in diameter tube of polyethylene is used to connect the trachea to the pneumotachometer. Recording electrodes can also be placed under the phrenic nerve and into the intercostals/diaphragm muscle for additional monitoring/synchronization.

4.2.2 Pneumotachometer

The ventilatory parameters are recorded using a Spirometer Pod (FE141 ADInstruments, Colorado Springs, CO) with P1 channel end connected to the animal through 3 different devices. The pneumotachometer (Respiratory Flow Head; MLT1L, ADInstruments) is connected to the spirometer pod. The other end is open for differential measurements of the spirometer. The analog output channel signal is connected to an analog-digital converter (PowerLab/8SP, ADInstruments). The output (digital) signal obtained is acquired by PC using Chart version 5 or above. The whole set up (except for the PC) is placed inside of a Faraday cage to avoid electromagnetic field interference on the recordings. The apparatus sensitivity is calculated and apparatus calibrated using graded injections of room air. For mice apparatus should be verified as being able to measure c. 2.5 µL or less.

4.2.3 Whole body plethysmography

Ventilatory parameter can be measured, based on Drorbaugh and Fenn's principle (Epstein et al, 1978). According to this principle, the pressure in the chamber increases during inspiration because addition of water vapor to the inspired gas and to warming of the inspired gas from the temperature in the chamber to that in the alveoli; conversely, pressure decreases during expiration because of condensation of the water vapor and cooling of the expired gas. Measurements of these pressure changes in comparison to a referent chamber (Fig. 3) can be used to calculate T_{tot}, V_t and $\dot{V}_e$. A whole-body Plethysmograph is available from Buxco (www.datasci.com/products/buxco-respiratory-products/finepointe-whole-body-plethysmography).

The Plethysmograph is composed by two superimposed Plexiglas chamber, with capacity of 450 ml and 100 ml. The smaller one serve as a reference for pressure measurements, and the bigger one contain the animal.

4.3 Gas mixture

A commercially available gas mixer is used (Columbus, Pegas 4000 MF) to mix 100% N_2 and 100% O_2 tanks to obtain different oxygen levels (100, 21, 18, 15, 12, 10, 8, 4 and 0%). For CO_2 pre-mixed gas is used in order to expose the animal to the desired levels (10% CO_2 in

90% O_2 or air and 5% CO_2 in 90% O_2 or air). The levels of O_2 and N_2 were monitored using a micro-sensor oxygen meter (World Precision Instruments), which is calibrated using 100% O_2 and 100% N_2.

5 METHODS

5.1 With a Pneumotachometer and whole body cylindrical chamber

Calibration of Apparatus:

1. Bring the cage with mouse from the animal facility to the laboratory two to three hours before the start of the training session to recover from the transportation and new environment stresses. Water and food is provided *ad libitum.*
2. Avoiding excessive noise and manipulation to weight the mouse, and depending on the experimental procedure, insert it into the training chamber for at least 30 minutes breathing O_2 21% or anesthetize it for IT recordings.
3. The calibration protocol is essentially as described in the transducer manufacturers' manual (ADInstruments). Briefly, zero the Spirometer clicking the ZERO button on the SPIROMETER input amplifier dialog box from the channel function pop-up menu and select 10Hz low pass filter. The minimum data acquisition rate is 400 B/s, but it is recommended to use 1KB/s.
4. Click RECORD button and inject in about 1 to 2 seconds 100 µL using a calibrated micropipette into the pneumotachometer end which is connected to the spirometer P1 channel. The sensitivity of the pneumotachometer is described on fig. 5.
5. Select the time range that contains the inflow of 100 µL injected and recorded on the flow channel. Select from the SPIROMETRY FLOW dialog of the channel flow the CUSTOM option from FLOW HEAD and type 0.1 on INJECTED VOLUME dialog box. See note below.
6. Open a new channel and select the DIGITAL FILTER from the channel function pop-down menu. Select the FLOW channel on the CHANNEL SOURCE dialog box, HIGH PASS from FILTER TYPE and type 1 on the CUTOFF FREQUENCY dialogs boxes. Alternatively, it is possible to select the option DRIFT CORRECTION on the SPIROMETRY FLOW menu. These two possibilities will avoid the drift of the flow signal out of the zero line.
7. Select from the volume channel function pop-down menu the SPIROMETER VOLUME option and then the digitally filtered flow channel on the SPIROMETRY FLOW DATA dialog box.
8. Insert the mouse into the chamber and repeat step 3 only.

SOFTWARE NOTE:

The spirometer extension has limited significance values (decimal points), hence it is not possible to input the INJECTED VOLUME dialog box in µL. For this reason, 100 µL injected

volume has to be input as liters (100 L). Therefore, the values of V_T and $\dot{V}_e$ obtained from the Spirometer extension's REPORT must be read in µL.

Whole body cylindrical chamber recording:

1. Place the mouse for 30 minutes into the training chamber breathing room air for acclimatization purpose.
2. Switch the mouse to the recording chamber and ZXSEWQ21start to record while flushing O_2 21%.
3. During the experimental sessions expose the mouse to different levels of O_2 (100, 21, 18, 15, 12, 10 and 8%) until a stable response is obtained without leading to distress. For O_2 18 % and 15% this can be done in 15 to 20 minutes and below 12% O_2 no more than 5 minutes (as that level of hypoxia is stressful). Each hypoxic maneuver is interspaced by 10 minutes of air (21%). The same protocol is used for hypercapnia studies. Additional caution has to be taken at 8% O_2 to avoid prolonged apneas and possible death, returning to 21% O_2 as soon as possible after recording the HVR.

Determination of Correction factor for cylindrical chamber using NP:

1. Anesthetize the mouse with ketamine & xylazine mix as described above.
2. Record the cylindrical chamber and the NP during normoxia of the same mouse.
3. Select five different segments of cylindrical chamber and NP recordings during normoxia exposure.
4. From Spirometer extension's REPORT, obtain the V_T values of the five WBP and NP samples.
5. Normalize the V_T by the body weight.
6. Average the normalized V_T for cylindrical chamber and NP.
7. Divide the V_T normalized values from NP by V_T normalized values from WBP to obtain correction factor (CF).
8. Multiply the V_T and the $\dot{V}_e$ from cylindrical chamber by the calculated CF.

IT recording:

1. Calibrate as above.
2. Place anaesthetized mouse in supine position, make a small incision on the midline of the neck.
3. Remove the sternohyoideus and sternothyreoideus muscles and proceed to perform a small incision over the distal third of the trachea, enough to insert the intratracheal tube.
4. Insert the polyethylene tube to connect the trachea to the pneumotachometer

5. The gas mixture is positioned on the open end of pneumotachometer for 15 seconds of stable and constant response. However, exposures lower than 8% O_2 has to be controlled to avoid apneas, distress and possible death.

5.2 Whole body Plethysmography

Calibration of the Plethysmograph is essentially as described in the manufacturers' manual. Procedure is very similar to what have been described in the previous paragraphs. A known volume (150 µl) of air is injected into the animal chamber with a syringe to correlate the injected volume with the differential pressure measured between the two chambers. The pressure difference between the reference and animal chamber is measured using a pressure transducer, range ± 0.1 mb.

A 700 ml/min flow of dry air through the admission chamber and reference chamber should be constantly delivered to avoid CO_2 and water accumulation and to maintain a constant temperature. During the measurement, temperature and humidity should be constantly monitored.

Place the mouse into the animal chamber until the mouse is motion less. Then start the measurement. Protocols for ventilatory stimulation with gasses are as described in a previous chapter.

6 EVALUATION AND INTERPRETATION OF RESULTS

1. For data analysis, use only the digital filtered flow and volume channel.
2. Select from the 21% O_2 a 10 seconds fragment with frequency between 3 and 4 Hz and stable volume curves (fig. 4). Click the REPORT option from the SPIROMETER tab and write down the values for frequency (f_R), V_T and $\dot{V}_e$.
3. Repeat steps 1 and 2 five times for each hypoxic or hypercapnic level.

   Normalize all the V_T and $\dot{V}_e$ values by the body weight.
4. Average five measurements for each gas level for each animal.
5. Plot each parameter independently and calculate the effect of hypoxia or hypercapnia in each group.

Advantages/Disadvantages:

The advantage of the method and the technique described here are the low cost, flexibility of the evaluations (gases and drugs), adaptability of the equipment and accuracy of the measurement.
The disadvantage of this method is the impossibility to measure forced inspiratory and expiratory volumes and voluntary hyperventilation due to these parameters require active

collaboration of the subject. This disadvantage is also present in other plethysmograph chambers available.

The advantages of cylindrical chamber over IT recording methods:

1. Cylindrical chamber is performed on awake (non-anesthetized) animals. (Anesthetics are known to suppress/modulate respiratory responses).
2. Cylindrical chamber affords the possibility of performing repeat evaluations during the course of a pre-clinical trial in addition to being used as an end-point measurement.

The advantages of IT over cylindrical chamber recording:

1. Time course of gas changes much faster than using cylindrical chamber.
2. Provides the chance to record phrenic nerve output and/or electromyogram from the intercostals or diaphragm for accurate timing synchronization information.

Note:

1. The main disadvantage of the cylindrical chamber is that it is time consuming. Even with the training and acclimatization procedures, there is a possibility that the mice are providing a stressed response due to being enclosed in a small space.
2. The main disadvantage of the IT recording is the use of anesthesia, known to modulate respiration.

The advantage of whole body Plethysmograph:

The volume of the chamber is 450ml which have a large advantage the fact that the mouse is not restrained as for the smaller cylindrical chamber. Indeed, the mouse can freely move in this chamber, and it is known that baseline breathing patterns can be modified in restraint conditions (Dauger et al, 1998).

The disadvantage of whole body Plethysmograph:

The disadvantage is that whole body Plethysmograph is very time consuming. It is advisable up to 3 days acclimating period for the mice towards to the chamber, otherwise it would be necessary ca. 60 to 80 minutes until the mouse is perfect quiet and motionless.

Citation

Until May 2019, this SOP has been cited by:

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8 APPENDIX

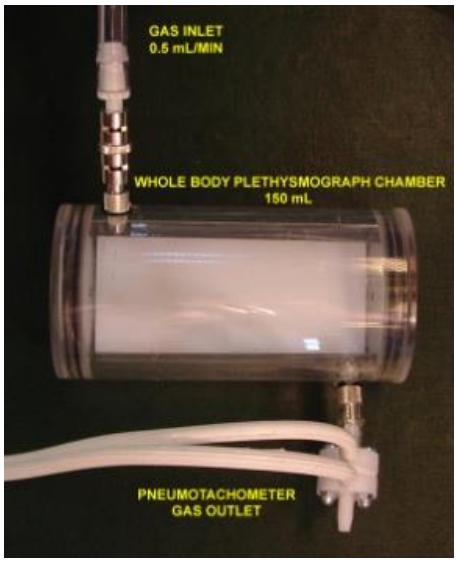

Figure 1. Custom made chamber for cylindrical chamber. By using the flat plastic insert, the final volume or dead space of the chamber is reduced to c. 150ml. The chamber has two ports: one for the gas inlet and one for the pneumotachometer (Hickey MM et al. 2010, Mosqueira et al 2013).

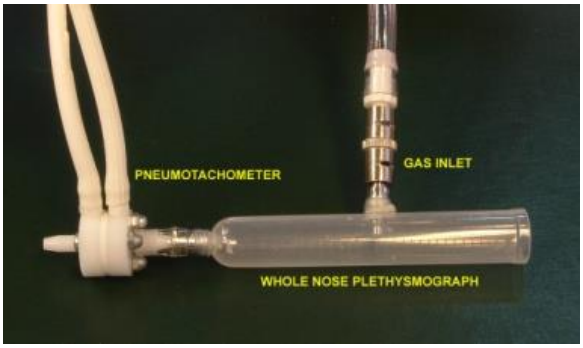

Figure 2. Custom made chamber for NP. The chamber consists of a 15 mL polypropylene tube, with two ports to support gas inlet and outlet. The pneumotachometer is placed on the outlet port.

Figure 3: Whole body plethysmograph (Commercial chamber from Buxco, www.datasci.com/products/buxco-respiratory-products/finepointe-whole-body-plethysmography)

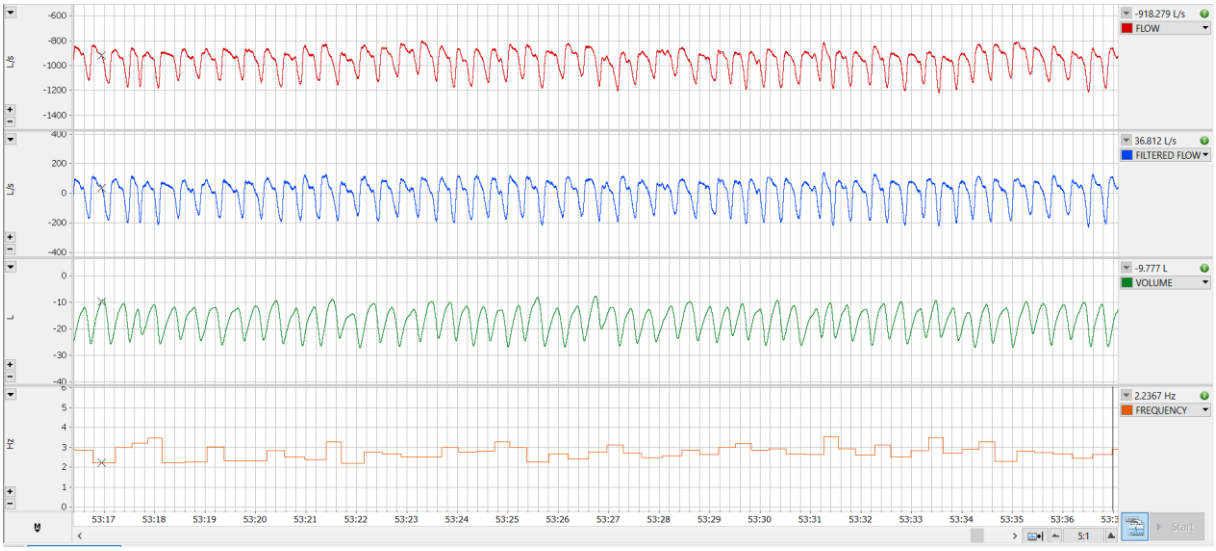

Figure 4. Ventilatory recording using whole body cylindrical chamber with pneumo tachometer. Traces show a representative 20 second recording from an awake mouse breathing 21% O_2 (equivalent of room air). Channel 1 represents raw flow data, Channel 2 is high-pass filtered flow signal from Channel 1, Channel 3 is the volume obtained from channel 1 (or channel 2) and Channel 4 represents ventilatory frequency calculated in Hz from Channel 1.

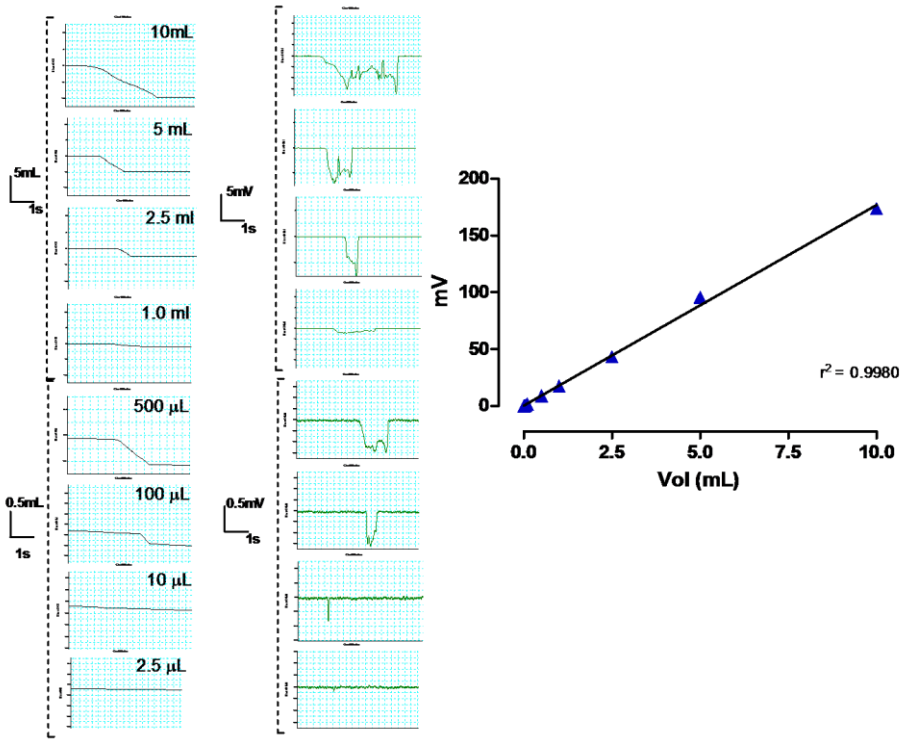

Figure 5. Calibration curve and verification of apparatus sensitivity of the whole body cylindrical chamber with pneumotachometer. Recording were made during injection of air from a syringe (1.0 to 10 mL) and a micropipette (1 to 1.000 µL). Displacements of injected volume (x-axis) and output voltage (y-axis) are linear and provide a calibration curve.