Please quote this SOP in your Methods.

Use of treadmill and wheel exercise to assess dystrophic state

SOP (ID) Number	DMD_M.2.1.003
Version	1.0
Issued	November 12th , 2008
Last reviewed	May 23rd, 2015
Author	Robert W. Grange Virginia Tech, Department of Human Nutrition, Foods and Exercise, Blacksburg, VA, USA
Working group members	Annamaria De Luca (Sezione di Farmacologia, Dipartimento Farmacobiologico, Facoltà di Farmacia, Università di Bari, Italy) Annemieke Aartsma-Rus and Maaike van Putten (Leiden University Medical Center, Department of Human Genetics, Leiden, The Netherlands) Jean-Marc Raymackers (Université Catholique de Louvain, Louvain-la-Neuve, Belgique) Kanneboyina Nagaraju (Children's National Medical Center, Washington DC, USA) Markus Rüegg (Biozentrum, University of Basel, Switzerland)
SOP responsible	Robert W. Grange
Official reviewer	Markus Rüegg

TABLE OF CONTENTS

1. OBJECTIVE 3
2. SCOPE AND APPLICABILITY 3
3. CAUTIONS 3
   - 3.1. Wheel test 4
   - 3.2. Treadmill 4
4. MATERIALS 5
   - 4.1. Voluntary wheel running 5
   - 4.2. Enforced treadmill exercise 6
5. METHODS 6
   - 5.1. Wheel running 6
   - 5.2. Treadmill exercise 8
6. EVALUATION AND INTERPRETATION OF RESULTS 9
7. REFERENCES 11

1. OBJECTIVE

The objective of this SOP is to describe how treadmill or wheel running exercise can be used to evaluate the disease-related impairment of neuromuscular function in *mdx* mice to better determine the benefits of a specific treatment. The ultimate aim of a treatment should be to improve neuromuscular function. Because the dystrophic process affects limb skeletal muscle, the diaphragm and the heart, improvements in these muscles should be ideally reflected in better physiologic performance. Physiologic performance can be assessed by the mouse running on a treadmill or on a wheel. For the treadmill, mice are subjected to a running paradigm to determine the time to exhaustion and the total distance run. For the wheel running, mice voluntarily run on a wheel in their home cage, and the improvement in distance run per night, per week, per month etc. is assessed. These approaches can be used to quantify the disease-related impairment in either sedentary and/or chronically exercised (i.e., exercise trained) *mdx* mice. While the purpose of this SOP is to describe an approach to evaluate the dystrophic phenotype with or without treatment, and in a sedentary or trained state, specific exercise protocols or experimental conditions can also be used to aggravate the dystrophic process. This approach is described in a separate SOP (DMD_M.2.1.001, Use of treadmill and wheel exercise for impact on mdx mice phenotype).

2. SCOPE AND APPLICABILITY

Running exercise on either a treadmill or a wheel can be used to assess *mdx* phenotype and/or to evaluate the efficacy of therapeutic interventions with pharmacotherapies (Granchelli et al., 2000; De Luca et al., 2003; Radley and Grounds, 2006; Brunelli et al., 2007; Minetti et al., 2006), gene or cellular strategies (Denti et al., 2006), or nutritional interventions (Call et al., 2008; Dorchies et al., 2006). Both treadmill and wheel running can be used as early as weaning age, although mice with spinal muscle atrophy have been trained on a running wheel as early as 10 days old (Grondard et al., 2005). As described below, it is mandatory that the treadmill controls provide for setting and maintaining a specific speed and for setting and maintaining a horizontal, downhill or uphill orientation. Downhill running is used to generate eccentric contractions *in vivo*. However, *mdx* mice can barely tolerate this type of running, and thus it can only be used for short-term proof-of-concept approaches. However, this may be a valuable acute test to discriminate the efficacy of a treatment if the dystrophic mice show only minor muscle damage similar to wild type mice, whereas the untreated dystrophic mice demonstrate severe muscle damage. All the conditions described below need to be followed carefully to allow comparison between laboratories. Although less stringent, these recommendations also apply to wheel running.

3. CAUTIONS

As is true for any behavioral tests, variables concerning the environment and the animals used must be kept constant throughout the study. These include housing conditions

(light, humidity, background noise, etc.), feeding state, schedule of the experiments (morning / afternoon), strain, sex and age of the mice.

Exercise can modify the progression of the disease, therefore it is essential to use standardized protocols and the proper control mice (e.g., Call et al., 2008; Carter et al., 1995).

3.1 Wheel test

Advantages

The wheel test reveals each individual animal's voluntary capacity for running. There is no need for further acclimatization after the first session and minimal human intervention is required while the mice run. This exercise can be initiated during early maturation (usually at or after weaning) and continued for set periods of time (e.g., one to several weeks) or indefinitely throughout the mouse's life, according to the experimental purposes. Noninvasive *in vivo* testing offers the great advantage that it can be readministered, so that each animal can become its own control (provided that the mice are examined during a steady-state phase of neuromuscular development, i.e. 4-6 months of age). Obviously, this implies making the same examinations in untreated animals, to differentiate the effects of exercise from those due to the treatment.

Disadvantages

There can be wide variation in the amount of running between individual mice, requiring a large number of animals to reach statistical significance. To determine the number of animals required, a Power analysis should be performed. There are also differences between male and female runners; females run further each night. Other disadvantages, which mainly relate to the use of wheel running for chronic exercise, are that the mice are caged individually, with a possible impact on their behavior, and that a change in wheel properties as a result of dirt accumulation can potentially increase the effort required by the animal to perform the running test. Differences in the design of particular wheels may also account for the different results obtained by different laboratories: Indeed, some designs allow the wheel resistance to be set to increase the workload. Although generally affordable, a complete setup can also be somewhat expensive.

3.2 Treadmill

Advantages

Exercise parameters such as speed, duration and angle of the treadmill can be strictly controlled to fulfill a variety of experimental protocols and allow a precise exercise load to be set. All of the mice belonging to an experimental group undergo the same protocol and this allows precise monitoring of the neuromuscular performance of each mouse in and between

experimental groups. Control of the parameters facilitates comparison of results from different laboratories. Ideally, if the environmental and exercise conditions are the same, this will limit bias.

Disadvantages

Unlike wheel running, treadmill experiments cannot be conducted without an acclimatizing period of 3 to 10 days, during which animals become familiar with the apparatus and can then train for longer periods of time and at higher speeds. Running can induce a certain level of stress in the animal. Continuous supervision by one or more experimenters is required. Although anecdotal evidence indicates that the concomitant running of 5-6 mice ensures better adherence (emulation phenomenon?), experimenters may choose to reduce the number of mice being tested at the same time to more closely observe each individual mouse. As a consequence, the time required to conduct the test will be substantial. Finally, treadmills are expensive. As described above, there is great variability in the performances achieved by different animals, even from the same litter. The response to a given drug itself is certainly different from one mouse to another. There are three possibilities for dealing with this variability: (1) monitor each mouse individually, before, during and after treatment, and express the efficacy of a drug as the post/pre ratio ("mean of the differences", rather than "differences in the means"); (2) adapt the experimental condition to their initial performances; this implies determining for example, the maximal speed at which the animal can run, and measuring time to exhaustion at 80% of the maximal speed; and (3) use only mice that demonstrate a comparable performance, as determined by a preliminary test. Optimally, the 3 possibilities can be used together. This approach will increase the experiment time, but should lower the variability so that an effect has a greater chance to be observed.

4. MATERIALS

4.1 Voluntary wheel running

A metal mouse wheel can be placed on the cage floor, suspended from the cage top or attached to the cage side. Exercise data can be obtained from the wheel via self-built detection systems or by automated commercial systems. Two examples of self-built systems are the following; (1) a small magnet can be attached to the wheel and a sensor from a bicycle pedometer attached to the back of the cage (Radley and Grounds, 2006); (2) a metal tab attached to the rear of the wheel can be used to interrupt a light signal to a photo electric gate. Each signal interrupt can be recorded on a laptop computer using a digital data acquisition card (National Instruments USB-6501, Part # 779205-01) and a custom Labview program (Call et al. 2008). In both cases, the sensor records single wheel revolutions. Daily and total distance (km), as well as maximal and average speed can be recorded each day (e.g., Radley and Grounds, 2006). A more sophisticated computerized monitoring system can be used to collect precise data on the patterns of running and stopping (e.g., Lafayette Instruments, Inc.; MiniMitter with Vital View software, Minimitter Inc.).

4.2 Enforced treadmill exercise

A variety of treadmills are commercially available and allow the mouse to perform horizontal, uphill, or downhill running at a fixed speed. Each lane of the treadmill is physically separated on the same belt, so that up to six mice can be exercised simultaneously at the same speed and angle. Some treadmills have computerized systems to record exercise time, and a grid for delivering a low-intensity electric shock to the mouse's paws when the mouse stops. Other treadmills are built in a closed environment that allows the collection of respiratory gases for detailed analysis of gas exchange. One of the most widely used treadmill models is from Columbus Instruments (illustrated below).

5. METHODS

5.1 Wheel running

Individually caged mice are allowed to freely move on the wheel for exercise. According to the circadian rhythm of rodents, mice generally run at night. The detection system records and saves the active running time for each mouse in each cage. Every day the experimenter can obtain data of the total distance run, the time of major activity and/or of rest, and the intervals of active time, such as shortest and longest lag interval time. Single animal data can be then compared with those of other animals and eventually pooled if responses are uniform. (Hara *et al*., 2002; Radley and Grounds; 2006; Brunelli *et al*., 2007). Note that accumulation of dust and other materials may increase rotational resistance of the wheel. Free movement of the wheel and the correct recording of revolutions should be regularly checked and corrected as necessary. In addition, the experimenter should also be aware that mice may turn the wheel during normal movement in the cage without actually running in it.

Frequency, intensity and duration of wheel running. Frequency is the number of times the animals are allowed to run per week. This could be set for an experiment from 1 to 7 days per week. For non-computerized wheel systems, the wheel would have to be locked by the experimenter to prevent running on non-exercise days. This could be done by using a wire hook from the wheel to the wire cage top for example. Intensity is the load against which the mouse works on the wheel. In most cases, the mice will only run against the resistance of the wheel

itself. However, some computerized systems (e.g., Lafayette Instruments, Inc.) have an electronic brake on the wheel that can increase the resistance through computer control.

Duration is the time the mouse is allowed to run on the wheel for any given exercise session. Normally the mice would be allowed to run all night, but duration of 1 or more hours could also be used. Ideally, with a computerized system, the brake could be programmed to come on after a certain duration. However, if duration is to be manipulated with a manual running system, an experimenter would have to be present to limit the duration by manually locking the wheel.

Matched workloads. Exercise induces both positive and negative adaptations. To avoid differences between treatment groups due to the amount of exercise performed, the dose of exercise should be matched. For example**,** exercise doses for wt and *mdx* runners could be matched as follows: (1) mice from two different treatment groups are randomly paired (e.g., *mdx* and wt mice). *Mdx* running dose (i.e., distance per night) for each pair will be recorded, then this data will be used to ensure a match of the distance run per night by the paired wt runner. Running by wt and *mdx* mice will be initially staggered by one night. *Mdx* but not wt mice of each pair will run the first night, to record the running dose. On the 2nd night through the end of the training period, both *mdx* and wt mice of each pair will run, with wt mice restricted to the dose of running performed by the *mdx* mice the previous night. Wt mice will complete the training period one day later than *mdx* mice. Ideally, computer controlled running wheels for each wt mouse would stop the wheel automatically when the appropriate distance from the respective *mdx* mouse for each pair is reached. If a computerized system is not available, this would have to be done manually.

Example Study: Two groups of *mdx* mice (n=10 per group), one control and one treated are compared for distance run per night, week, month, etc. for a given training period at a given frequency, intensity and duration.
Frequency = 7 times per week
Intensity = wheel resistance only
Duration = self selected
Training period = 3, 6, 12 weeks (e.g., Call et al., 2008; Hayes et al, 1996).
Additional groups of sedentary *mdx* mice without and with treatment could also be included in the study. This would help to distinguish between the effects of the treatment alone and the treatment plus exercise.

Another approach would be to have two groups of sedentary *mdx* mice, one treated with a given drug and one that is not. After a set treatment period (e.g., 6 weeks), both groups of mice are assessed for their capacity to run on the wheel over some set period (e.g., 3 weeks).

More sophisticated analyses could also be conducted while the mice run on wheels in an enclosed chamber so gas exchange can be evaluated (e.g., TSE Systems Inc., LabMaster/Calorimetry system; Columbus Instruments, Inc. Oxymax/CLAMS system).

Voluntary wheel running distances by C57BL/10 and *mdx* mice. Maximum values during the training period are reported below.

Carter et al.,1995: 4 weeks training; young *mdx* mice (gender not reported) aged 4-8 weeks - ~6000 m/night; young C57BL/10 mice - ~9000 m/night; old *mdx* mice aged 6-7months - ~3000 m/night; old C57BL/10 mice aged 6-7 months - ~9500 m/night.
Hayes and Williams, 1996: young male *mdx* mice aged 4-20 weeks - ~5400 m/night; young C57BL/10 - ~8500 m/night.
Call et al., 2008: 3 weeks training; male *mdx* mice aged 3-6 weeks - ~2000 m/night; male C57BL/10 mice aged 3-6 weeks - ~6000 m/night.

Treadmill exercise

One mouse is placed in each lane and is enforced to run at a certain speed with the treadmill orientation held at 0°. The mice are run to exhaustion and the time of running is recorded.

Chronic exercise. When treadmill running is used for chronic exercise, a fixed protocol should be used, according to protocols and procedures described in detail in the SOP M.2.1_001, Use of treadmill and wheel exercise for impact on mdx mice phenotype. (Granchelli *et al*., 2000; De Luca *et al*., 2003, 2005).

In this SOP, the exercise protocols described are used to assess the impact of treatment on neuromuscular function. For example, the effectiveness of a drug treatment could be evaluated by assessing how far the mouse can run before exhaustion. Other parameters (*in vivo* and mostly *ex vivo*) can then be used to evaluate the disease state. Again, these approaches are described in other SOPs. However, information (e.g., the number of stops/mouse; the duration of the stop) obtained during each exercise session are also useful in assessing the disease severity. For example, mice with a more severe disease state stop more frequently than less severely diseased mice. To obtain this "stop" information, the number of stops and the duration (e.g., a stopwatch) of each would have to be recorded. However, to obtain this detailed information, a second experimenter would be necessary or fewer mice would have to be run if there is only one experimenter. Consistency of this latter measurement is more difficult to obtain vs. overall running resistance and therefore less used as readout parameter.

Example exercise training (for evaluation of number of stops during a fixed exercise duration): Exercise compared to a sedentary group of *mdx* mice with and without treatment: (1) 12 m/min for 30 min twice a week for some specified period (e.g., 3, 6 and 8 weeks; Granchelli *et al*., 2000; De Luca *et al*., 2003, 2005). (2) 9m/min for 30 min, twice a week for some specified period (e.g., 3, 6 and 8 weeks; Kaczor et al., 2007; Hudecki et al., 1993).

Example run to exhaustion: The speed is initially set at 5 m/min for 5 min, and then the speed is increased 1 m/min every minute until exhaustion. A specific criterion for exhaustion should be pre-determined. For example, the mouse will not continue running on the treadmill for 20 s despite repeated gentle nudges to make it do so (e.g., Brunelli et al., 2007; Denti et al., 2006).

For this exhaustion test, typical distance values for sedentary wild-type male mice (C57/BL10) aged 8-12 weeks are approximately 250-300 meters. Values for sedentary *mdx* mice of the same age are lower (~180-200 m) with distances even less when the pathology is worse or subsequent to chronic forced exercise protocols (70-100 m).

Treadmill Maintenance. Most commercial treadmills are designed to collect wastes; however this is not sufficient for the belt. This has to be properly and gently cleaned after each exercise session to avoid dust and organic waste from reducing adherence of the mouse paws, as well as change the speed belt efficiently. Periodically, the speed of the belt has to be verified by measuring the time required by a small fixed object on the belt to cover the distance between two points (beginning and end of the lane at a known distance) and verifying that it corresponds with the speed value chosen by the experimenter.

6. EVALUATION AND INTERPRETATION OF RESULTS

When exercise is used to evaluate the progression of the dystrophic pathology and or the effect of a therapy the objective data obtained are used to determine,

1) the total distance run by each mouse until exhaustion (treadmill)
2) the change in the total distance run by each mouse until exhaustion over time (longitudinal studies for chronic treatment)
3) the total distance run by each mouse in acute tests and over time (wheel exercise) and relative speed
4) the duration of each bout of voluntary running session (wheel exercise)
5) change in pattern of running over time (duration and number of shortest and longest running session) (wheel exercise)

Importantly, the collection of the data should include additional information:

a) that all the mice performed the required exercise correctly (treadmill)
b) the total amount/pattern of exercise performed by each mouse
c) inter-animal variability
d) possible correlations with between therapy and pathology course

All observational data are extremely important to understand the consistency of the protocol and to account for concomitant disturbing events, such as stress. These observations include:

- Body weight (to be monitored weekly)
- Food and water consumption (monitored weekly)
- Change in mood (too nervous or too sleepy (monitored daily))

- Marked change in the ability to perform the exercise (i.e., more than 3 stops per each treadmill trial session; no wheel exercise)
- Change in fore limb strength (as assessed by grip strength)
- Change in social habits (for treadmill)

All these data should be consistently recorded in a lab book to provide immediate or subsequent analysis.

7. REFERENCES

Bouchentouf M, Benabdallah BF, Mills P, Tremblay JP. (2006). Exercise improves the success of myoblast transplantation in *mdx* mice. Neuromuscul Disord. 8:518-29.

Brunelli S, Sciorati C, D'Antona G, Innocenzi A, Covarello D, Galvez BG, Perrotta C, Monopoli A, Sanvito F, Bottinelli R, Ongini E, Cossu G, Clementi E. (2007). Nitric oxide release combined with nonsteroidal antiinflammatory activity prevents muscular dystrophy pathology and enhances stem cell therapy. Proc. Natl. Acad. Sci. USA. 104:264-269.

Call, JA, Voelker KA, Wolff AV, Macmillan RP, Evans NP, Hulver MW, Talmadge RJ Grange RW. (2008). Endurance capacity in maturing *mdx* mice is markedly enhanced by combined voluntary wheel running and green tea extract. J Appl Physiol. [Epub ahead of print; doi:101152]

Carter GT, Wineinger MA, Walsh SA, Horasek SJ, Abresch RT, Fowler WM Jr. (1995). Effect of voluntary wheel-running exercise on muscles of the *mdx* mouse. Neuromuscul Disord 5:323–332.

De Luca A, Pierno S, Liantonio A, *et al.* (2003). Enhanced dystrophic progression in *mdx* mice by exercise and beneficial effects of taurine and insulin-like growth factor-1. J. Pharmacol. Exper. Ther. 304:453-463.

De Luca A, Nico B, Liantonio A, *et al.* (2005). A multidisciplinary evaluation of the effectiveness of cyclosporine a in dystrophic *mdx* mice. Am. J. Pathol. 166:477-489.

Denti MA, Rosa A, D'Antona G, Sthandier O, De Angelis FG, Nicoletti C, Allocca M, Pansarasa O, Parente V, Musarò A, Auricchio A, Bottinelli R, Bozzoni I. (2006). Body-wide gene therapy of Duchenne muscular dystrophy in the *mdx* mouse model. Proc. Natl. Acad. Sci. USA. 103:3758-3763.

Dorchies OM, WagnerS, VuadensO, WaldhauserK, BuetlerTM, KuceraP, RueggUT. (2006). Green tea extract and its major polyphenol(-)- epigallocatechingallate improve muscle function in a mouse model for Duchenne muscular dystrophy. Am J Physiol Cell Physiol 290:C616–C625.

Grondard C, Biondi O, Armand, AS, Lecolle S, Della Gaspera B, Pariset C, Li H, Gallien CL, Vidal PP, Chanoine C, Charbonnier F. (2005). Regular exercise prolongs survival in a type 2 spinal muscular atrophy model mouse. J Neurosci. 25(33):7615-22.

Granchelli JA, Pollina C and Hudecki MS. (2000). Pre-clinical screening of drugs using the *mdx* mouse. Neuromuscul Disord. 10(4-5):235-9.

Hara H, Nolan PM, Scott MO, Bucan M, Wakayama Y, Fischbeck KH. (2002). Running endurance abnormality in *mdx* mice. Muscle Nerve 25:207-211.

Hayes A, and Williams DA. (1996). Beneficial effects of voluntary wheel running on the properties of dystrophic mouse muscle. J Appl Physiol 80: 670-679.

Hayes A, Williams DA. (1998). Contractile function and low-intensity exercise effects of old dystrophic (*mdx*) mice. Am. J. Physiol.274 (4 Pt 1):C1138-44.