TREAT-NMD Activity A07: Accelerate preclinical phase of new therapeutic treatment development

Work package 7.4: Develop standardised protocols and procedures for harmonising and accelerating pre-clinical studies (including standardised data analysis)

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1.   OBJECTIVE

The objective of this SOP is to describe how treadmill or wheel running exercise can be used to aggravate the dystrophic phenotype in *mdx* mice to better determine the benefits, if any, of a specific treatment. *Mdx* mice have a less severe phenotype than humans, and therefore, specific exercise can be used to worsen the phenotype. For the treadmill, mice are subjected to an enforced running paradigm where speed, duration and elevation (typically down hill running) are used to increase muscle injury. For the wheel running, mice voluntarily run on a wheel in their cage. However, the frequency, intensity and duration of the wheel running can also be modified. Thus, exercise can be used to intensify the mechanical stress on muscle fibers to prolong the "degenerative phase" spontaneously observed around 3-4 weeks of age, and thereby determine if a treatment is effective in reducing this aggravated disease state. While the purpose of this SOP is to describe an approach to aggravate the dystrophic phenotype, specific exercise protocols or experimental conditions can also be used as a potential therapeutic approach to muscular dystrophy. These are not described in this SOP.

2.   SCOPE AND APPLICABILITY

Running exercise on either a treadmill or a wheel can be used to worsen the *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), or gene or cellular strategies (Denti et al., 2006). Both treadmill and wheel running can be used as early as weaning age. 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. 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. These include housing conditions (light, humidity, background noise …), feeding state, schedule of the experiments (morning / afternoon), strain, and sex and age of the mice.

As there is evidence that exercise can modify the progression of the disease, it is essential to use standardized protocols and the proper control mice. It is also crucial to

maintain standard conditions throughout the protocol, especially when exercise is performed chronically. This is particularly relevant when using the treadmill, since voluntary running activity is generally performed by mice at night, while it is generally more convenient and feasible to perform treadmill running during normal working hours.

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 required while the mice run. This exercise can be initiated during early maturation (although usually at or after weaning) and continued for set periods of time (e.g., one to many weeks) or indefinitely throughout the mouse's life, according to the experimental purposes.

Disadvantages

There can be wide variation in the amount of running between individual mice, requiring a large number of animals to reach statistical significance. 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 mechanical stimulus. Control of the parameters facilitates comparison of results from different labs. 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 individual mice. As a consequence, the time required to conduct the test will be substantial. Finally, treadmills are expensive.

4.   MATERIALS

A variety of methods can be used for exercise:

4.1   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   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 from two different treatment groups could be matched as follows. Wt and *mdx* will be randomly paired and initially staggered by one night, with the *mdx* but not wt mouse of each pair running the first night. When the running dose (i.e., distance per night) for the *mdx* runner is known, this dose will be used to set the distance the paired wt runner will run the 2nd night. Thus, 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.

5.2 Treadmill exercise

One mouse is placed in each lane and is enforced to run at a certain speed. Downhill and uphill running are generally more exhausting and require very short sessions to avoid injury and test failure (Vilquin *et al*., 1998).

Chronic exercise

When horizontal running is used for chronic exercise, a fixed protocol should be used, such as running at 12 m/min for 30 min twice a week (Granchelli *et al*., 2000; De Luca *et al*., 2003, 2005). Lower speeds can be used according to specific purposes, while higher speeds may easily induce exhaustion. In all cases, the mice have to finish the established protocol, even if a certain avoiding behavior is observed. To prevent this problem, the mice can be stimulated by a gentle touch of the tail, the presence of a smooth barrier at the end of the lane, or a sound stimulus. The use of an electric shock is an alternative and offers the advantage of less involvement by the operator; however, this method should be avoided for ethical reasons especially when treadmill is used for chronic exercise. Sometimes the mouse has to stop for a few minutes before starting again with the protocol. As the protocol is not exhaustive, this can be due to both light fatigue and/or influence of the circadian rhythm.

Please note, this chronic exercise protocol is used specifically to worsen the dystrophic phenotype to better assess a given drug treatment. For example, a drug treatment could be used in parallel to evaluate its effectiveness to limit pathology (spontaneous or exercise-aggravated). Other parameters (in vivo and mostly ex vivo) can then be used to evaluate the worsening of the disease as well as the effect of a drug therapy. 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. Other specific treadmill protocols (i.e. acute exhaustion tests) can also be used to assess disease severity and/or drug effects (Brunelli et al., 2007: Denti et al., 2006: Minetti et al., 2006; Burdi et al., 2009). Details about these protocols are not provided in the present SOP.

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 influence progression of the dystrophic pathology the descriptive data obtained are used to determine,

   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 the pathology

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 3-5 stops per each treadmill trial session; no wheel exercise)

   -    Abrupt changes 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.

When exercise is used as modifier of pathology, *in vivo* (grip strength, resistance to exercise; open field, etc.) and *ex vivo* (muscle functional, contractile histological and biochemical analyses) readout parameters should be used to validate the efficacy of the protocol, as extensively described in the literature. Details about these readout parameters are beyond the scope of this SOP and can be instead found in other specific SOPs.

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