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The use of hanging wire tests to monitor muscle strength and condition over time

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1 OBJECTIVE

The hanging wire tests that are described in this SOP can be used to assess global "subacute" muscle function and coordination over time in young and old *mdx* mice. The test is based on the latency of a mouse to fall off a metal wire upon exhaustion. With the use of several described experimental designs, the natural course of the disease or the efficacy of genetic or pharmacologic treatment strategies can be assessed. Despite *mdx* mice having a less severe phenotype than DMD patients, differences in hanging performance between wild type (C57BL/10ScSnJ) and dystrophic (*mdx or mdx*^{5Cv}*)* mice can be seen and experimental interventions can improve hanging performances.

2 SCOPE AND APPLICABILITY

Derived from Gomez's "taut wire test" and Rafael's SHIRPA phenotypic assessment of *mdx* mice (1, 2), the hanging wire test is performed in order to demonstrate a motor neuromuscular impairment and motor coordination in a new strain, or as an *in vivo* preclinical tool, using models of neuromuscular disorders (e.g. *mdx* mouse). Provided its simplicity, the test was also used in pharmacological studies, for evaluating the neuromuscular tone (3).

Because of the nature of the test, it is not possible to relate the outcome to a sole neuromuscular defect (as force or fatigue). In particular, animal weight, balance and behavior can influence the results of the test. Mice need to be randomized for weight. The impulse (weight (in grams) times time (in seconds)) is the final outcome measure (s*g) reflecting the minimal amount of sustained tension (impulse) that is needed to oppose gravity. Nonetheless, since the mice's behavior clearly indicates that they do not want to fall off the wire, the test provides us with a tool in which the animal puts all its effort to cooperate. Unfortunately, some mice find ways to avoid hanging by balancing on the wire or falling off it on purpose, and this should be corrected for. The test can be used as early as weaning age. All the conditions described below need to be followed carefully to allow comparison between laboratories.

3 CAUTIONS

In order to avoid intra- and inter-operator variability, a high degree of standardization should be reached: age, sex and weight of control and test animals, same conditions (type of room, room temperature, room occupancy, time of the day, odors, etc…). Ideally, the test should be performed at multiple occasions (i.e. bi-weekly) throughout the pre-clinical study to get a robust result.

During the experiment, constant supervision is needed, in order to be able to record the correct hanging time and to distinguish mice that did or did not fall on purpose. Mice that let themselves fall off the wire on purpose are easy to distinguish, since these mice manage to first hang face down by their hind limbs only, and then let themselves fall. When this happens, the mouse should be placed back on the wire in the starting position immediately. Some mice manage to balance on the wire, which does not cost as much energy as hanging. This behavior should not be tolerated, and corrected by putting the animal back in starting position (for a movie see: (4)). It is very important to correct inappropriate behavior or take noncompliant mice out of the experiment as otherwise inaccurate recordings are made (5). Note that these kinds of inappropriate behavior occurs primarily in strong (C57BL/10ScSnJ wild type mice). However, most mice comply with the test and do not show inappropriate behavior.

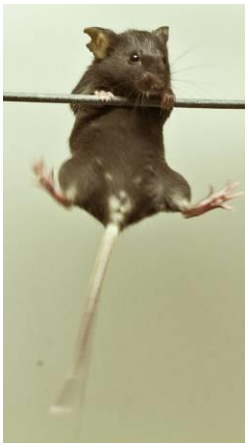
A  B 

Figure 1: A. The start position of the mouse with the fore limbs attached to the wire. **B**. Depending on the physical ability of the mouse, both hind limbs and the tail will also be used during the test.

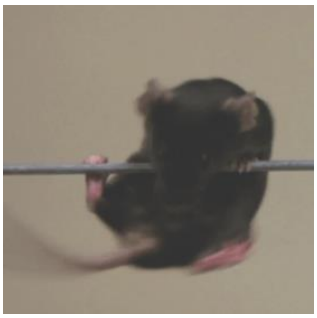
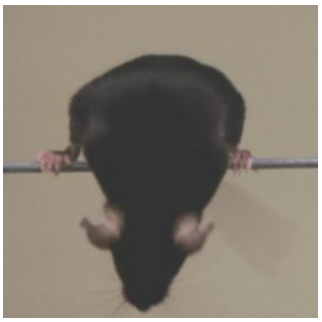
 

Figure 2: Inappropriate behavior; balancing on the wire.

Investigators should be familiar with mice. Soft bedding must be placed underneath the wire to break the fall. The wire should not be set too high from the ground, in order not to harm the mice, however high enough to prevent the mice from dropping themselves on purpose. Practically, a height of 35 centimeters is best.

Advantages

The hanging wire test is a very inexpensive test since the equipment can be self constructed. Most animals are willing to perform the task, despite of their physical condition.

Disadvantages

Constant supervision is necessary, so no more than 2 mice can be tested simultaneously per investigator. In case of inappropriate behavior of the mice forced removal of the mouse by the operator is required. When this happens multiple times, it can influence the test by influencing anxiety, force and compliance etc of the mouse. However, as noted before, this happens primarily with wild type mice, which do not face any difficulties finalizing the test, and the frequency tends to lower in the following test sessions. Also high individual variability occurs, which makes relative large sample sizes essential.

4 MATERIAL

A 55 cm wide 2-mm thick metallic wire (can be either a plain wire or a multi-stranded twisted wire, possibly plastic-coated) is secured to two vertical stands. The wire must be tightly attached to the frame to avoid vibration or unwanted displacement of the wire while the investigator is handling the animals or during the measurements, since these unwanted effects would interfere with the animal's performance. The wire is maintained 35 cm above a layer of bedding material to prevent injury to the animal when it falls down.

Animals: Mice as young as four weeks, and as old as approximately 19 months of age have been reliably evaluated with this test, but the test might also be applicable for older mice (6, 7). Mice younger than four weeks of age may be too excitable to provide reliable values. In order to limit the stress to the animals, it is preferable that the mice have been regularly manipulated by the investigator who will perform the test. However, no acclimatization to this test is needed. Since the test implies behavioral response, different strains may not respond equally (e.g. sv129 differ from C57BL/10ScSnJ), making the use of wild type mice of corresponding backgrounds important.

5 METHODS

5.1 The "falls and reaches"-method

In this method mice are subjected to a 180 sec lasting hanging test, during which a "falling" and "reaching" score is recorded. When a mouse falls or reaches one of the sides of the wire, the "falling" score or "reaching" score are diminished or increased by 1, respectively. A Kaplan-Meier-like curve can be created afterwards. For this test only a 55 cm

long wire can be used described in the material section. It is of great importance for the outcome of this method that the length of the wire is remained constant between different labs, since this influences the reaches outcome.

1. Timer is set to 180 sec. The “falling” score is set to 10, and the ”reaching” score is set to 0.
2. A mouse is handled by the tail and brought near the wire. The operator lets it suspend by the fore limbs only. As soon as the animal is properly suspended, the timer is started. After being released, most animals catch the wire with the four limbs. This is allowed.
3. If the timer reaches 0 sec, go to step 7.
4. If the animal reaches one end of the wire, timer is stopped and “reaching” score is increased by 1. Then, go to step 6.
5. If the animal falls, the timer is stopped, the falling score is diminished by 1 and the elapsed time is noted.
6. Provided “falling” score is >0, the procedure is restarted at step 2. If the time is over, go to step 7.
7. Test is finished. Reaching and falling scores are recorded as well as the elapsed times between falls.

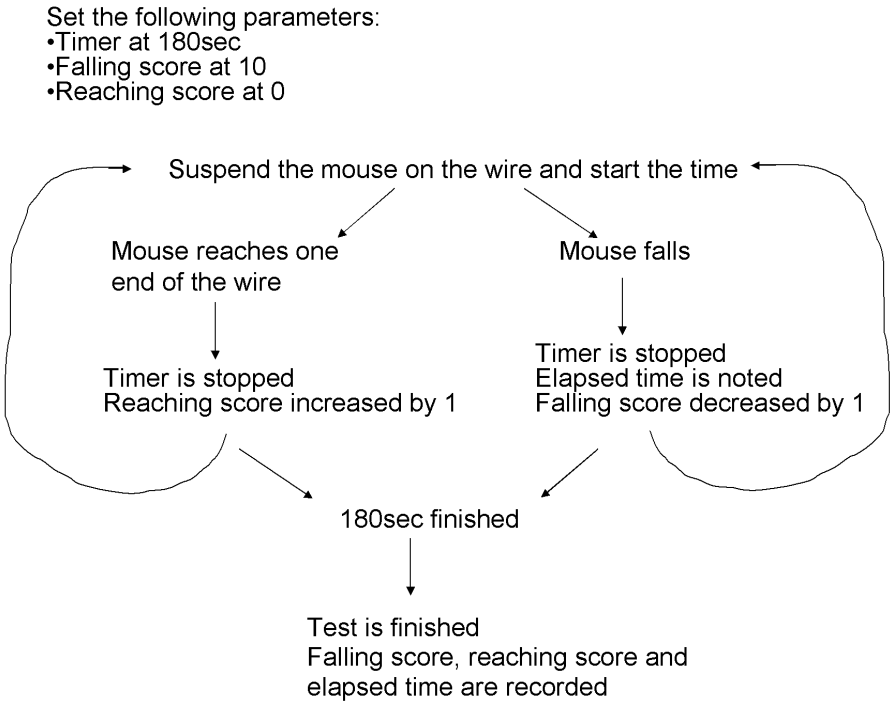


Figure 3: Flow chart of the “falls and reaches” method.

The protocols allows to draw a "Kaplan-Meier-like" curve of the falls (8) (Fig 4).

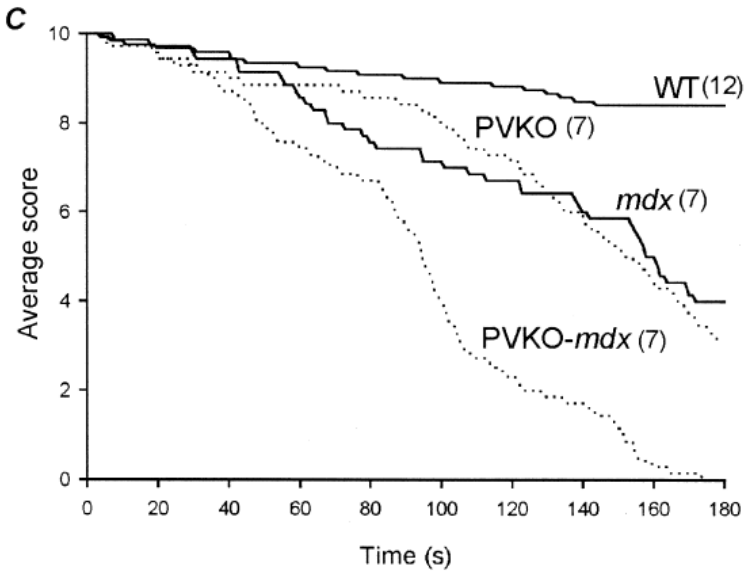


Figure 4: Average "falls" score from four strains of mice during a 180-sec wire test protocol (from [7]).

5.2 The longest suspension time-method

A simpler protocol consists of measuring the longest suspension time in three trials. With this method an unlimited hanging time can be used or one with a fixed limit. When a fixed limit is used a hanging time of 600 seconds is recommended. Start position of the mice can also be varied; with either two or four limbs.

1. The mouse, handled by the tail, is allowed to grasp the middle of the wire with its fore limbs and is gently lowered so that its hind paws will grasp the wire a few cm apart from the fore paws.
2. The mouse is then gently accompanied while it turns upside-down along the axis of the wire.
3. The tail is released while the mouse is still grasping the wire with its four paws. Upon release, a timer is started.

As an alternative to the first three steps in this protocol, where the mouse starts with all the limbs, one could also use a start position with the two fore limbs, as is used in the "falls and reaches" method [8,9]. Using the two fore limbs start position, a clear distinction can be made between mice who manage to grasp the wire with their hind limbs and those who are incapable to do so.

4. The time until the mouse completely releases its grasp and falls down is recorded.

5. The mouse is given three trials per session, with 30-sec recovery period between trials.

When a fixed time of 600 seconds is used, mice that reach this fixed maximum, independent on the trial number, are allowed to stop with the experiment, while others are directly retested for a maximum of three times (9, 10). In this case the maximum hanging time is recorded (9, 10). In experiments where all mice have to hang for three trials, either the maximum hanging time or the average hanging time of the three trials can be used as an outcome measure.

For mice that are given an unlimited hanging time, the effect of body weight can be diminished by using the Holding Impulse (s*g) = Body mass (grams) x Hang Time (sec) as an outcome measure. This reflects the tension (impulse) that the animal develops for maintaining itself on the wire, against gravity for the longest period of time. When a fixed limit is used (600 sec), the Holding impulse cannot be used, since it remains unknown what the maximum hanging time of the mice would be when no fixed time limit was used. In case none of the mice managed to hang for the fixed hanging limit, using the Holding impulse is preferred.

Body weight must be recorded either before or after the experiment. Measures may be repeated over time. However to avoid familiarization at least 1-week intervals should be used between consecutive sessions. It is recommended to repeat this test multiple times throughout the intervention study to obtain a robust data set on functional performance.

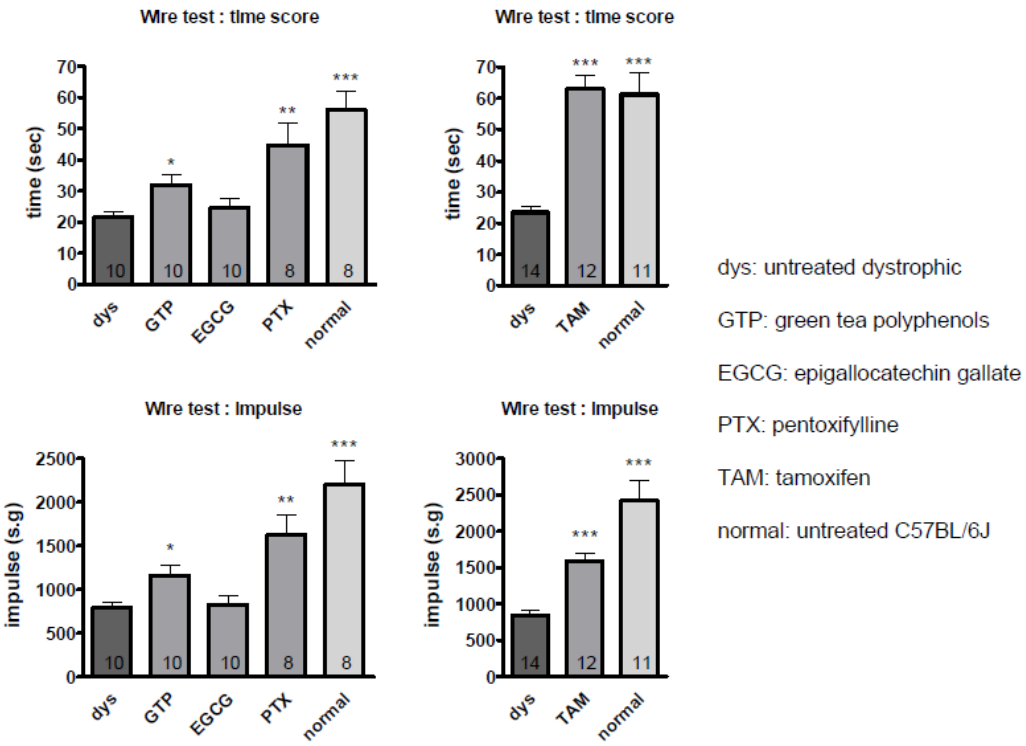


Figure 5: Performances of 16-months-old treated and non-treated dystrophic and wild type mice.

Using the longest suspension time method, the period of time for which 16-month-old dystrophin-deficient mice (Dmd-*mdx*^{5Cv}) maintained their grasp was about 2.5 times shorter than that for age-matched normal mice (C57BL/6J) (mean ± s.e.m., 21.5 ± 1.94 sec, n=10 versus 55.89 ± 5.95 sec, n=8, respectively, p<0.001) (10) (Fig 5). Here the average of the three hanging times was used. Pharmaceutical interventions in Dmd-*mdx*^{5Cv} mice involving administration of green tea polyphenols and pentoxifylline for 15 months significantly improved the wire test performance to 31.82 ± 3.47 sec (p<0.05, n=10) and 44.75 ± 6.83 sec (p<0.01, n=8), respectively (Dorchies et al., unpublished data). In this example the Holding impulse could be used since none of the mice reached the fixed time limit.

Data obtained using a start position with the two fore limbs and a fixed hanging time of 600 seconds in six C57BL/10ScSnJ males and five *mdx* males that conducted a functional test regime consisting of four functional tests [8,9] are shown in Fig 6.

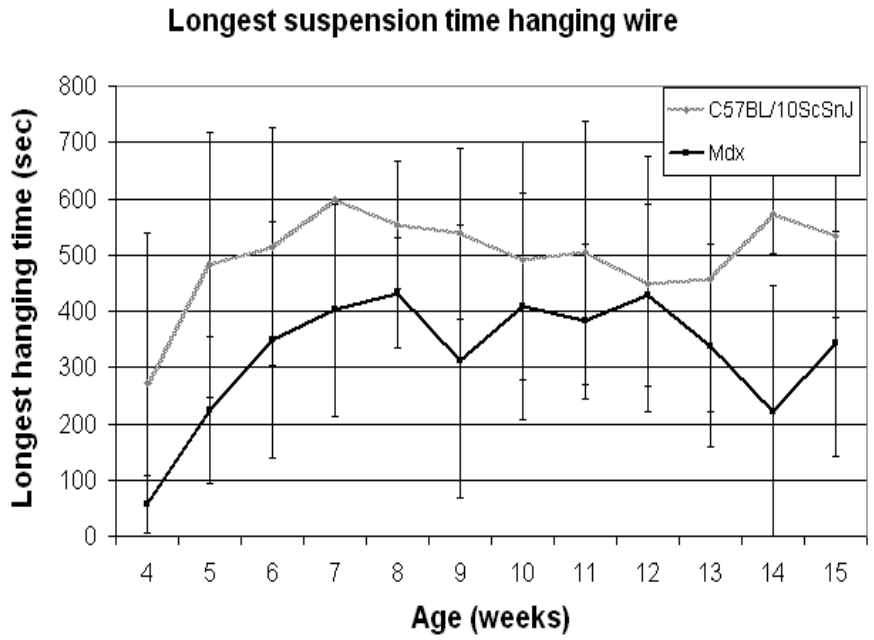


Figure 6: Hanging wire performance of C57BL/10ScSnJ (n=6 males) and *mdx* (n=5 males) mice over time, obtained with the longest suspension time method, in which the start position was with the two fore limbs. The maximum hanging time was set at 600 sec, and mice were given three tries to reach this time limit. Mice that did hang for 600 seconds did not have to complete the three trials. The performance of the *mdx* mice is consistently significantly lower compared to that of wild type mice. Data are represented as the mean and standard deviation [9].

6 EVALUATION AND INTERPRETATION OF RESULTS

Both hanging wire tests described in this SOP are relative simple, reliable and are applicable to young and old *mdx* mice. The "falls and reaches" method is suitable to determine muscle function whereas muscle condition is measured with the longest suspension time method.

When no data is available about the strains to be investigated or when variability is high the "falls and reaches" protocol may be particularly useful. Indeed, the "Kaplan-Meyer" reflects the mean behavior of several animals, each of them being taken as its own control (i.e. starting with a score of 10). While differences can be seen after max. three falls in some strains (i.e. between C57BL/10ScSnJ and *mdx* mice), it is possible that the longest or average suspension time may not adequately differentiate strains of mice more close on a phenotypical point of view. For example, in Fig. 4, the score "7" (reflecting the fact the animals have fallen three times, on average), is reached at about the same time for *mdx* and PVKO-*mdx* (e.g. ~80 and ~110 sec). The average suspension times (~26 sec and ~36 sec, for *mdx* and PVKO-*mdx* mice, respectively) may be too close to be differentiated.

C57BL/10ScSnJ mice usually do not fall more than once in 180 sec. C57sv129 may fall 2-4 times, while *mdx* mice fall more than 5-6 times (3-month-old animals). Older animals fall more often, regardless of their genotype (6, 8).

An advantage of the "falls and reaches" method is that the testing takes less time in comparison to the longest suspension time if the 600 sec time limit is used. Disadvantages include that the "falls and reaches" method is more subjected to behavior making the longest suspension time method a more robust measure. Additionally, this method is more sensitive to assess possible therapeutic effects since the window between *mdx* and wildtype mice is larger.

In control animals, no correlation is seen between animal weight and wire test falling score for the "fall and reaches" methods (Fig. 7A). However, in *mdx,* there is a slight negative association between body weight and score, which is best seen after 90 sec. This supports the idea of matching the groups of animals for weight and for determining the impulse.

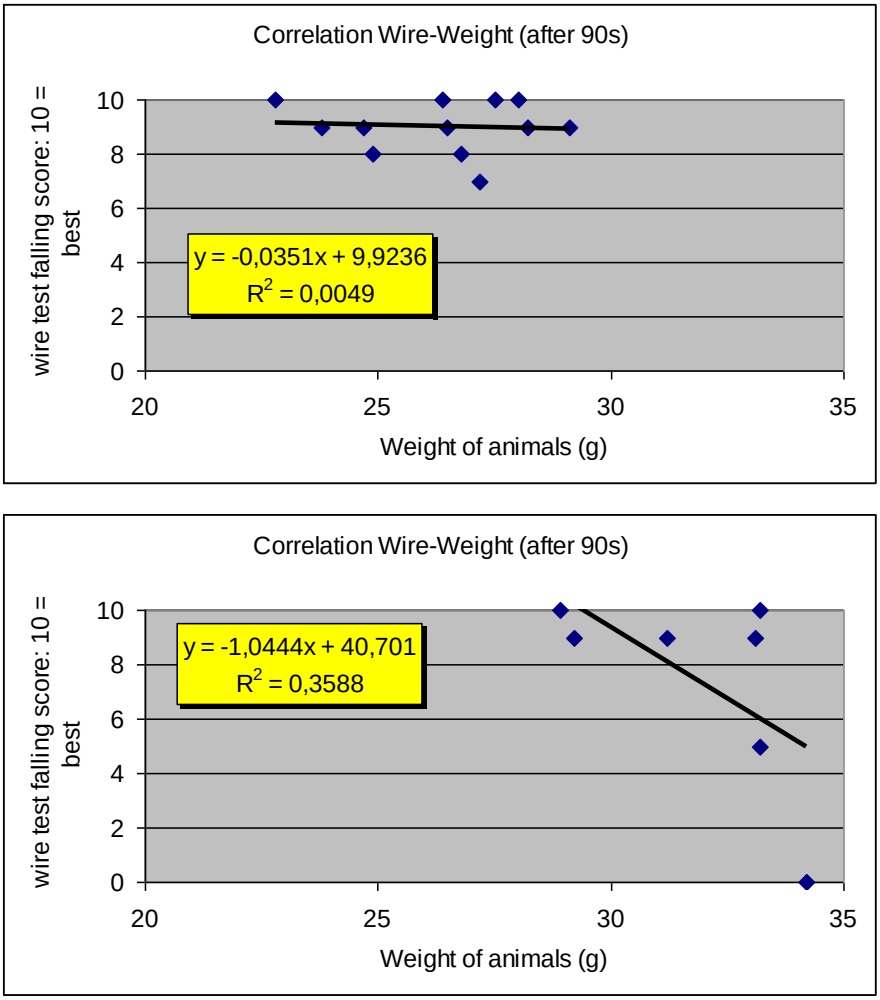


Figure 7. Correlation between individual "falls" scores and body weights in control C57BL/10ScSnJ **(A)** and *mdx* **(B)** mice. (JM Raymackers, unpublished data).

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