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

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Author	Maaike van Putten Leiden University Medical Center, Department of Human Genetics, Leiden, the Netherlands
Working group members	Annemieke Aartsma-Rus (Leiden University Medical Center, Department of Human Genetics, Leiden, the Netherlands) Olivier Dorchies (Pharmacology Laboratory, University of Geneva, Switzerland) Kanneboyina Nagaraju (Children's National Medical Center, Washington DC, USA) George Carlson (Department of Physiology, Midwestern University Glendale, AZ, USA)
SOP responsible	Maaike van Putten
Official reviewer	Olivier Dorchies

TABLE OF CONTENTS

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 and their allelic variants in the C57BL/6J and DBA/2J genetic backgrounds. The test is based on the latency of a mouse to fall off a metal wire and allows assessment of the natural course of the disease or the efficacy of genetic or pharmacologic treatment strategies. Although DMD mouse models generally have a less severe phenotype than DMD patients, differences in hanging performance between wild type (C57BL/10ScSnJ, C57BL/6J or DBA/2J) and dystrophic (*mdx, mdx*^{5Cv} or D2-*mdx)* 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* and its allelic variants in diverse genetic backgrounds). 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. Most mice comply with the exercise in order not to fall off the wire. However, 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 for those parameters that can be controlled by the operator: age, sex and weight of control and test animals, 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. Note that this test also has a learning component, as such measuring hanging performance only once will not be reliable.

During the experiment, constant supervision is needed, to allow recording of the correct hanging time (Fig 1) 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 (Fig 2). In case this happens, the animal should be put 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 wild type mice. However, most mice comply with the test and do not show inappropriate behavior.

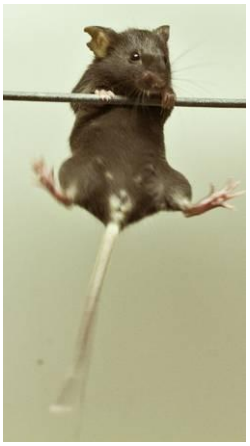
A  B 

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.*

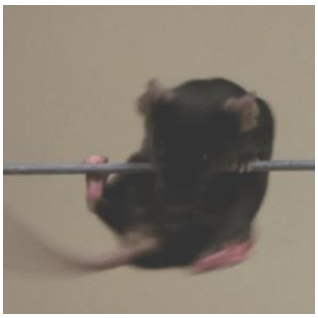
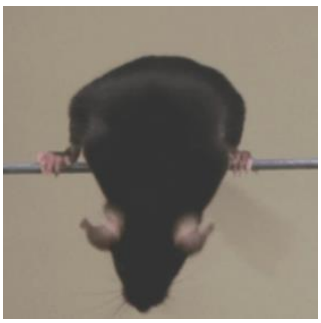
 

Figure 2: *Inappropriate behavior; balancing on the wire.*

Investigators should be familiar with handling mice. Ideally, the same investigator should perform the tests for all cohorts throughout the study to prevent variation. 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 their physical condition.

Disadvantages

Constant supervision is necessary, so testing of two or more mice in parallel is not recommended. 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 affect the test by influencing anxiety, force, compliance etc. of the mouse. However, as noted before, this happens primarily

with strong wild type mice, which do not face any difficulties finalizing the test, and the frequency tends to decline in the following test sessions. Please note that high individual variability is possible, which makes relatively large groups of mice (n > 6), essential.

4 MATERIAL

A 55 cm wide 2-mm (± 0.2mm) thick metallic wire (can be either uncoated plain wire or a multi-stranded twisted wire) 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: only males should be used, except when conducting experiments on the roles of sex hormones. Mice should be randomized over treatment and placebo groups based on body weight. We advise to take along wild type animals as controls in intervention studies. 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 show poor compliance. In order to limit the stress to the animals, it is preferred that the mice have been regularly manipulated by the investigator who will perform the test. However, no acclimatization to this test is needed. One should only keep in mind that the first test session is less reliable than the subsequent sessions due to learning effects. Since the test implies behavioral response, strains from different genetic backgrounds may not respond equally (e.g. DBA/2J differ from C57BL/10ScSnJ), making the use of wild type mice of matching background important.

5 METHODS

To assess hanging performance, the following steps should be followed:

1. The mouse, handled by the tail, is allowed to grasp the middle of the wire with its fore limbs only (Fig 1A).

2. The tail is released while the mouse is still grasping the wire with its fore paws. Upon release, a timer is started. Strong mice will try to catch the wire with the hind limbs and tail as well, which is allowed (Fig 1B).

3. Improper behavior like balancing or jumping off should be addressed by returning the mouse on the wire without stopping the timer (Fig 2).

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

5. When a mouse is able to hang for the maximum duration of the test (600 sec), it is gently removed from the wire and returned to its cage and the hanging time is recorded.

6. Mice that fall off before the 600 sec. limit, are given a maximum of two more tries to do so, getting a recovery period of 1 min between trials.

7. The longest hanging time of the trials is used for further analysis.

To reduce time spend performing this test, a maximum hanging time of 600 sec is set. The majority of (young) adult wild type mice can hang for 600 sec easily, while dystrophic mice cannot. Mice that fall off the wire before 600 sec are given up to two more tries. This is done to reinsure that mice are really unable to hang due to physical inabilities, and do not fall due to clumsiness. A shorter maximum hanging duration could be considered when testing older mice (i.e >1 year). It is advisable to perform a pilot study including dystrophic and wild type mice to identify a suitable time limit that still allows for a large phenotypic/therapeutic window.

Body weight must be recorded before the experiment. For mice that are given an unlimited hanging time, or if all mice tested did not reach the time limit, it is advised to correct for the effect of body weight by expressing the Holding impulse as an outcome measure:

Holding Impulse (s*g) = Body mass (grams) x Hang Time (sec).

This reflects the tension (impulse) that the animal develops for maintaining itself on the wire against gravity (Fig 6B). The Holding impulse cannot be used when some of the mice reached the time limit, since it remains unknown what the maximum hanging time of the mice would be when no fixed time limit was used.

Using the two fore limbs start position, a clear distinction can be made between mice that manage to grasp the wire with their hind limbs and those who are incapable to do so. However, especially older mice of >1 year of age can have difficulties with this starting position and fall shortly after release. In studies focusing on old mice, investigators could consider applying a start position in which all four limbs grasp the wire. Hereto, after the mouse grasps the wire with the fore limbs, it is gently lowered so that its hind paws grasp the wire a few cm apart from the fore paws. The mouse is then gently accompanied while it turns upside-down along the axis of the wire. Using the four limbs as start position will allow old mice to hang for longer, allowing more accurate data collection.

It is recommended to repeat this test multiple times throughout the study to obtain a robust data set on functional performance. However, to avoid familiarization at least 1-week intervals should be used between consecutive sessions.

Benchmark data

Data obtained using a fixed hanging time of 600 sec in C57BL/10ScSnJ and *mdx* males (8, 9) are shown in Fig 5. Since some mice reached the maximum hanging time, the holding impulse could not be used as outcome measure.

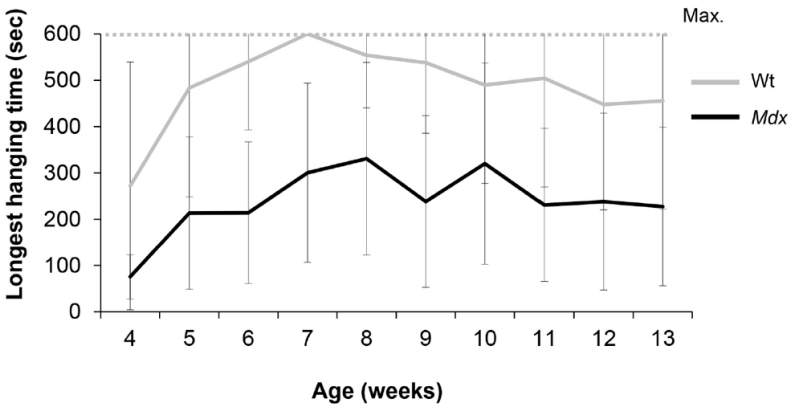


Figure 5: *Hanging performance of male C57BL/10ScSnJ (n=6) and mdx (n=18, 4-10 weeks, n=13, 11 and 12 weeks, n=10, 13 weeks) mice over time, obtained with a start position with the fore limbs. The maximum hanging time was set at 600 sec, and mice were given three tries to reach this time limit. The performance of mdx mice is consistently significantly lower compared to that of wild type mice. Maximum hanging time allowed is indicated by the dotted line. Data are represented as mean±st.dev (from (9)).*

The period of time for which 16-month-old *mdx*^{5Cv} mice maintained their grasp was lower than two minutes and about 2.5 times shorter than that for age-matched C57BL/6J wild types (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 6). Here, the average time of the three hanging trials was used. Although tamoxifen treated *mdx*^{5Cv} mice managed to hang for a similar duration as wild types, their holding impulse was lower, due to the smaller body weight of the tamoxifen treated mice. This highlights the usefulness of the holding impulse as additional outcome measure.

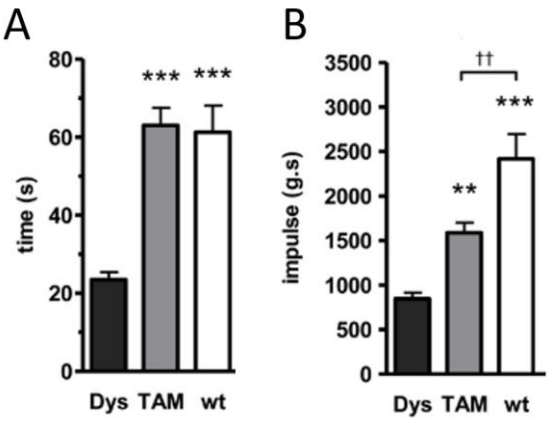


Figure 6: *Performances of 16-months-old treated and non-treated mdx*^{5Cv} *and wild type mice, utilizing a start position with four paws (from (10)). Dys: untreated dystrophic mice, TAM: treated dystrophic mice, wt: untreated wild type mice.*

For the D2-*mdx* model, hanging performance is significantly impaired from the age of four weeks onwards and deteriorates with age (Fig 7). Female mice were included in the study as a reference. Notably, female D2-*mdx* mice outperform males, highlighting the importance of including mice of a single gender in preclinical studies. We highly recommend to solely use males for experiments.

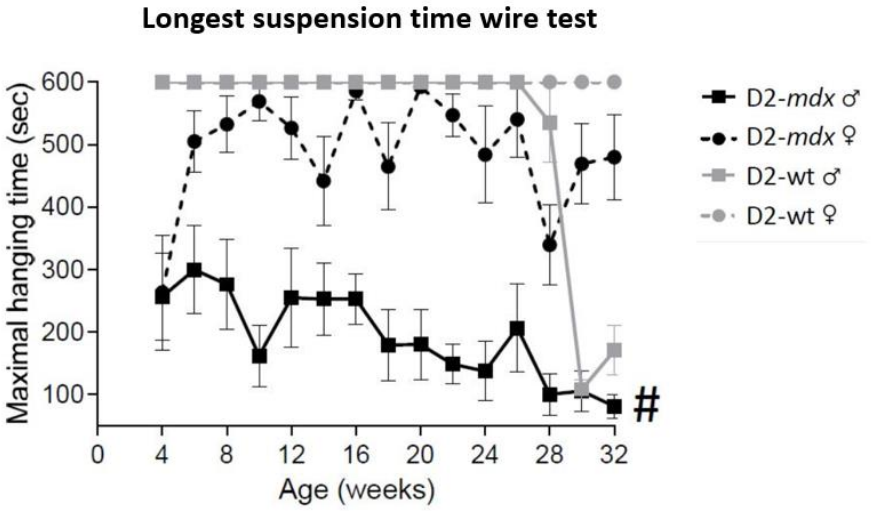


Figure 7: *Hanging wire performance of male and female D2-mdx mice and gender matched DBA/2J wild types. The start position was with the fore limbs. A maximum hanging time of 600 sec was used, and mice were given three tries to reach this time limit. Female D2-mdx mice outperformed D2-mdx males. Data are represented as mean ± s.e.m., n=10 D2-mdx males and females, n=6 DBA/2J males and females (from (11)).*

6 EVALUATION AND INTERPRETATION OF RESULTS

The hanging test described in this SOP is relatively simple, reliable and applicable to young and old *mdx* mice and their allelic variants in diverse genetic backgrounds.

Several statistical methods can be used to analyze the data. The longest suspension time can be analyzed as a single continuous variable using methods to compare means between treatment groups at single time points or over time. If interested in comparing longest suspension time at a single time point, cross-sectional statistical methods, such as student's t-tests or analysis of variance if the assessment is normally distributed, and Wilcoxon rank sums (Mann-Whitney U tests) or Kruskal-Wallis tests if the assessment is not normally distributed are appropriate. Here the null hypothesis that the central value of the assessment between two or more groups is equal is being tested. Assessments collected over time require more complex statistical models to adequately test the hypothesis of interest, the most common being a mixed effects linear regression model, sometimes referred to as a mixed model for repeated measures. These models allow to account for the intra-mouse variability present from repeated assessments on the same mouse and allow for missing data on an individual mouse. Like an analysis of variance model, they can test for the main effects of time (max. hanging time differs over time) and treatment (max. hanging time differs between mice groups). They can also test the hypothesis that the change over time is different between mice groups. These longitudinal models are preferred over other more

simple comparisons, such as the change from baseline, as they utilize all of the collected data, account for the variability observed within each mouse, and allow the inclusion of covariates where necessary.

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Previous versions of this SOP included the “falls and reaches” method. Given that this method has not been used over the last decade, and experts of the method cannot be reached for updates and guidance, it was decided to remove it from the SOP. To allow reference to the method, we provide the original text in the appendix.

Appendix 1.

The "falls and reaches"-method

In this method mice are subjected to a 180 sec (or longer) lasting hanging test, during which a "falling" and "reaching" score is recorded. When a mouse falls or reaches one of the ends of the wire, the "falling" score or "reaching" score are diminished or increased by 1, respectively. For this test only a 55 cm long wire can be used as described in the material section. It is of great importance for the outcome of this method that the length of the wire remains constant between different labs, since this influences the reach outcome.

1. Timer is set to the time limit of the test (i.e. 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.

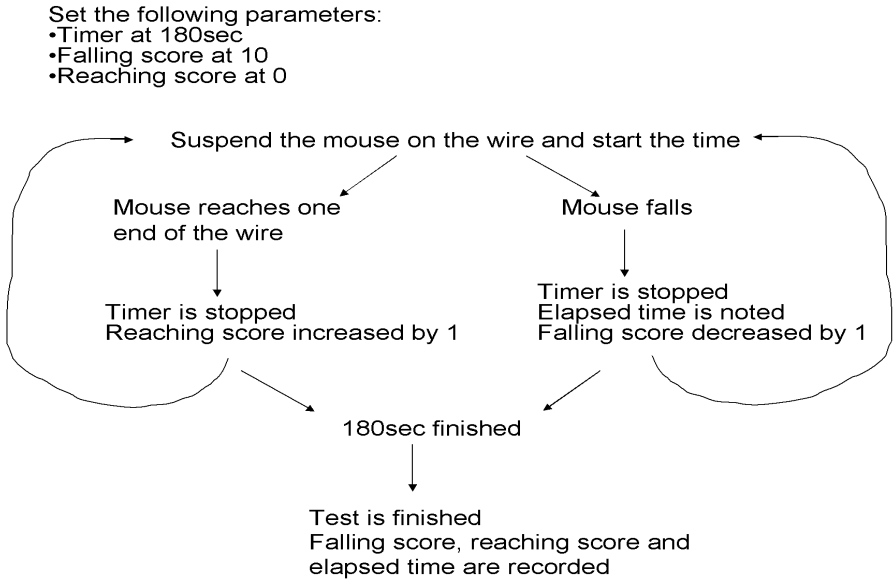

Figure 3: *Flow chart of the "falls and reaches" method.*

The protocol allows to draw a “Kaplan-Meier-like” curve of the falls (8) (Fig 4).

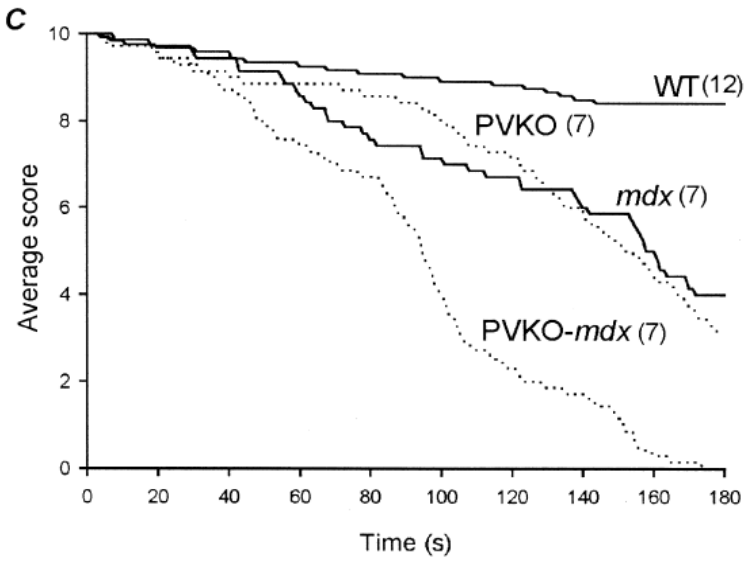

Figure 4: *Average “falls” score from four strains of mice during a 180-sec wire test protocol (from (7)).*