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)

SOP Title	**Behavioral Phenotyping for Neonates: Hind Limb Suspension Test (a.k.a. Tube Test)**
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1. OBJECTIVE

The procedure provides a detailed guideline on how to perform the hind-limb suspension test (a.k.a. Tube Test) on neonatal mice. The tube test evaluates the proximal hind limb muscle strength, weakness and fatigue in the animal. It also assesses general neuromuscular function, body muscle strength and posture.

2. SCOPE AND APPLICABILITY

The ideal animal age range for the tube test is from postnatal (P) 2 to 12 (P0 is defined as the day of birth). Older animals may develop enough strength to simply escape the tube. Modification of the tube test to accommodate older animals is possible; however, other factors that affect motor function and performance such as level of anxiety and motivation begin to play a more prominent role. The tube test is generally performed in 2 consecutive trials; however, additional trials may be performed to assess the development of muscle fatigue in the animals. Since the state of alertness of neonates affects motor performance, it is recommended that negative geotaxis test or other motor tests are administered immediately before the tube test. Note: evaluation of motor function in neonates should not last more than 3-4 minutes at any given time since neonates (younger than P10) lack the ability to thermo-regulate their body temperature and thus are vulnerable to hypothermia once separated from the dam.

3. CAUTIONS

The tube test is better applied in conjunction with other tests to assist in the interpretation of outcomes. We propose conducting righting reflex evaluations and/or geotaxis tests along with the tube test to evaluate motor function in a high-throughput drug efficacy screening platform alongside body weight and survival. Body temperature is perhaps the single most important factor that has significant effects on muscle activity and neural function in infants (Kreider and Blumberg, 2005). Testing infant rodents at room temperature can result in hypothermia and consequently impact neuromuscular function and ultimately behavior (El-Khodor et al., 2008).

4. MATERIALS

- Timer
- 50mL Conical Tube
- Cotton ball(s)

- 50mLTube Holder
- Heating Pad
- Weigh boats (neonates are placed in weigh boats on heating pad)
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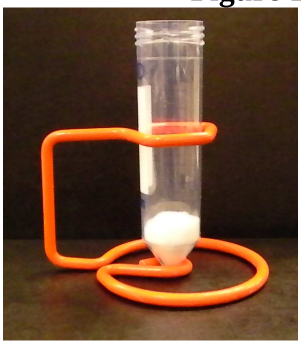
5. METHODS

5.1. Procedure

a. Tube test is performed last in the selected battery of motor tests.
b. The tube test is conducted over 2 consecutive trials. There is no time limit. The session ends when the animal falls into the tube.
c. Tube test consists of a 50mL conical tube held straight up with the tube holder. The conical tube must sit squarely in the holder and the holder must sit directly on a level lab bench (Figure 1).

Figure 1:



d. The conical tube contains at least 2 cotton balls at the bottom, which serves a cushion for the neonates. Make sure that the cotton is pushed down to the bottom of the tube and that the cotton will not interfere with the test - there should be ample space between the cotton balls and the neonate's body. If your cotton balls are large in size, only 1 may be needed.
e. Plug in your heating pad and adjust the setting to be around 37°C. If placing a towel drape on the heating pad, make sure that the towel drape is flush against the pad.
f. Select your neonate for testing. Pups should only be removed from the litter immediately prior to testing. 1 pup is tested at a time.
g. Gently pick up the neonate by the tail and place it inside the tube face down, with its hind legs over the rim of the tube so that it is hanging off the rim.
    - Make sure to position the animal so his hind legs are facing towards you. At the same time with your free hand start the timer.

- Make sure to step away from the table so that you do not accidentally bump the table, interfering with the animals’ testing. You want to make sure that your eye sight is level with the rim of the conical tube to accurately count all pulls.

h. As soon as the neonate falls from the rim and into the conical tube, stop the timer and record the scores on the run sheet.

i. Once data is recorded, carefully take the conical tube from the holder with one hand and tilt it at an incline to retrieve the neonate safely into the other hand. Once the neonate is retrieved insert the conical tube back into the holder and replace the neonate hanging on the inside of the tube (for the second trial). Start the timer once again and follow the same procedure for a second trial.

j. Once the animal has completed all testing, it is placed in a weigh boat on the warmed heating pad. Once phenotyping is complete for the entire litter, the neonates may be returned to the mom in their home cage.

k. Cotton balls are tossed daily. Conical tubes and holder are cleaned thoroughly with disinfectant at the end of the day. Conical tubes are replaced weekly.

5.2. Parameters to measure

a. Time spent hanging (seconds). This is the time the neonate hangs on the rim of the tube prior falling.

b. Number of Pulls. A pull is when a neonate attempts to lift its body using its hind limb muscles while suspended from the rim of the tube.

c. Hind Limb Suspension (HLS) Score: HLS score relates to the position of the legs and tail of the animal. The HLS score is an overall evaluation of the hind-limb spread during the first 10-15 seconds of hanging onto the lip of the tube, after which the observer does not change the score even if the animal shows a lower or higher score at a later time during the test. If the animal falls within 5 seconds, then the HLS score is evaluated based on this time period. The effect of fatigue on the HLS score is normally captured in subsequent trials. The inter-rater reliability for the number of pulls and HLS score was above 95% (El-Khodor et al. 2008). The animal is scored on a scale of 0 to 4 (see Figure 2).

Figure 2

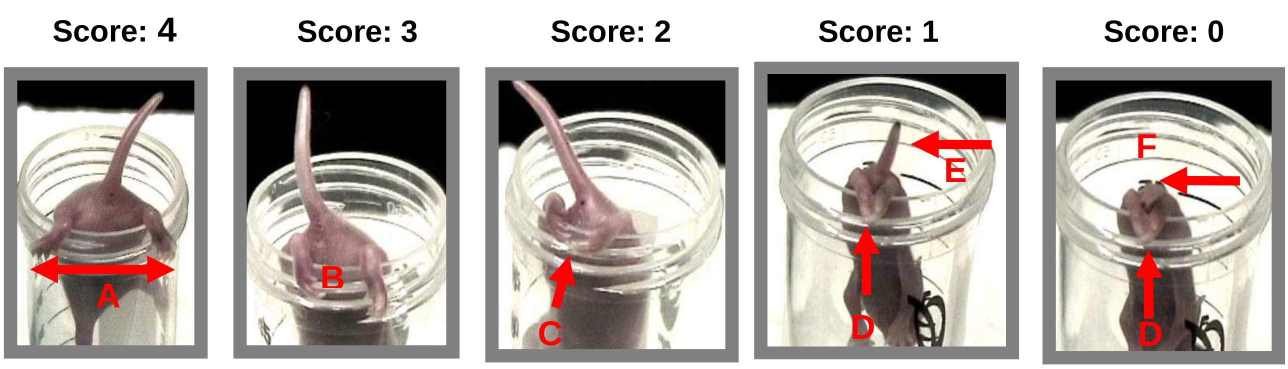


Fig.2. The tube test and the scoring criteria for the hind-limb score (HLS). Mice were suspended by their hind limbs from the lip of a standard 50 ml plastic centrifuge tube. The posture adopted was scored according to the following criteria: score of 4 indicates normal hind-limb separation with tail raised (A); score of 3, weakness is apparent and hind limbs are closer together but they seldom touch each other (B); score of 2, hind limbs are close to each other and often touching (C); score of 1, weakness apparent and the hind limbs are almost always in a clasped position (D) with the tail raised (E); a score of 0 indicates constant clasping of the hind limbs (D) with the tail lowered (F) or failure to hold onto the tube.

5.3. Notes

a. If the neonate is unable to suspend from the tube and falls instantly, a default score will be assigned.
   Hang time = 0
   Pulls = 0
   HLS = 1
b. In cases where the neonate jumps or climbs into the tube, the animal can be retested after a resting period (at least 1 hour) or the following day. **If the mouse is not re-tested then no scoring parameters are entered (cells are left blank) and a comment is written. Do not enter the default score or zeros in this case.**

6. EVALUATION AND INTERPRETATION OF RESULTS

The tube test is a novel motor function test that has only been recently introduced to the scientific community and is still under validation process. We expect that better understanding of the physiological and neuromuscular systems mediating the response in this test would become available in the near future. Since the publication of the tube test in early 2008 (El-Khodor et al., 2008), several published studies demonstrated the predictive value of this test in the evaluation of neuromuscular function and therapeutics in neonatal mouse models of neuromuscular disorders (Kreider and Blumberg, 2005; Gogliotti et al., 2010; Heier and DiDonato, 2009; Sumner et al., 2009; Takahashi et al., 2010; Nizzardo et al., 2011; Riessland et al., 2011). Statistical analysis for the tube test parameters as well as power analysis is detailed in the published manuscript by El-Khodor et al. 2008.

7. REFERENCES

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8. APPENDIX

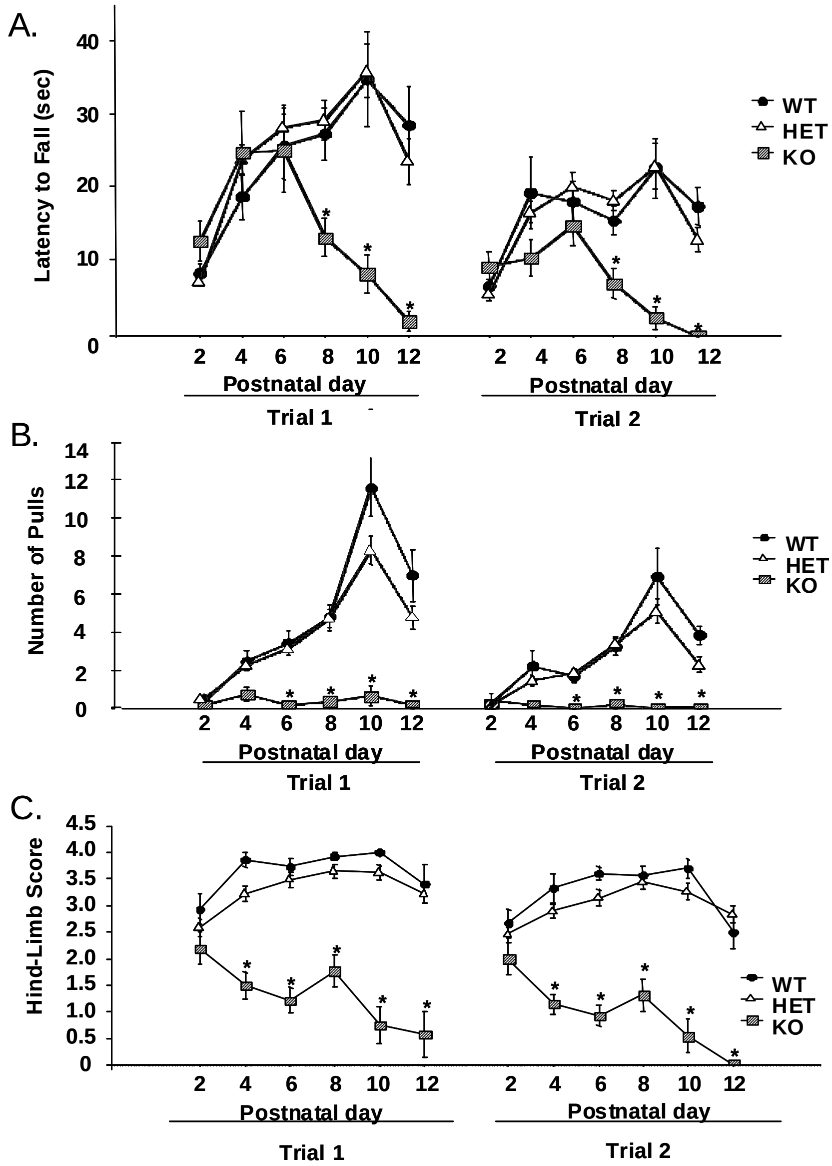

Fig.3. Hind-limb suspension test (tube test). (A) Time spent hanging onto the edge of the tube, (B) number of pulls and (C) hind-limb scores (HLS). The tube test was administered in two consecutive trials. Values presented are mean $\pm$ SEM; * $p < 0.01$. Empirical observations with neonatal pups exposed to the tube test showed that WT mice readily held onto the lip of the tube with their hind limbs from postnatal day (P) 2 until P12. Beyond day 12, WT mice became sufficiently strong and coordinated to escape. By inspection, it was evident that the movements of neonates to pull themselves upwards involved largely the proximal hind -limb muscles, which parallels an important group of muscles affected in SMA. This novel, sensitive and clinically relevant test most likely evaluates muscle strength and fatigue in proximal hind-limb muscles, however, there is also

involvement of distal muscles of the feet as well as a possible 'motivation' component to their efforts. Although WT and HET mice progressively increased the number of pulls and the length of time they held on to the lip of the tube, the KO mice made few, if any, pulling attempts from neonatal day 2 onwards (Fig. 3B). Although the WT and HET mice showed consistent improvement in motor function after P2 as observed in better performance in the geotaxis test, the tube test and righting reflex, the KO mice lagged behind and never developed the ability to perform these tests. This observation of inability to develop muscle strength is similar to the reported clinical observation in patients with severe form of SMA: infants do not develop the ability to straighten themselves as opposed to losing such ability as the disease progresses.

Finally, we have previously shown that the performance of P8 mice on the tube test is influenced by pharmacological manipulation, fatigue, intrinsic state of the pup at time of testing (arousal level) and hypothermia but not by time of day when the test is administered (El-Khodor et al., 2008).