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

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

Determination of the total phasic (or acute) forward pulling tension (FPT) exerted by the fore- and hindlimb musculature of mice.

2 SCOPE AND APPLICABILITY

The Whole Body Tension (WBT) procedure is used to determine the ability of mice to exert tension in a forward pulling maneuver that is elicited by stroking the tail. It is thought to reflect the maximal acute phasic force the mouse can achieve to escape a potentially harmful event.

3 CAUTIONS

The investigator responsible for performing the WBT test should be trained in animal handling procedures. The WBT test should be performed at approximately the same time of day for all experiments within a particular treatment study.

Each investigator who performs the WBT procedure should practice the procedure a few times before using the results, in order to optimize conditions for obtaining the maximum forward pulling tension (FPT). The results thus obtained from a group of nondystrophic mice can be compared to the results obtained from several laboratories (see Table 1 below) in order to feel confident that the procedure is being followed in a consistent way between laboratories.

In general, the best practice is for a single examiner to obtain all of the WBT measurements for any one particular treatment study. Inter-trial and inter-examiner tests of reliability (see Appendix Figure 4) can be used to ensure that reliable measurements are taken by different members of the lab.

Since the amount of developed force depends on the weight of the animal, the WBT measurement is obtained by dividing the maximal FPTs by the body weight. Nevertheless, test- and control populations should be appropriately matched for body weight before starting the experiment. It is quite useful to report changes in body weight along with changes in WBT for individual mouse populations. Caution should be taken when interpreting results that are mainly due to changes in body weight.

Ideally, animals should be tested at 2-4 month of age. This is the period of maximal forward pulling tension for both nondystrophic and mdx populations (Appendix Figure 3). A comparison of WBT values obtained over the lifespan of nondystrophic (C57Bl10SnJ) and mdx mice is shown in Appendix Figure 3 (Siegel et al., 2009).

Repeated (i.e. every day or week) evaluation using WBT should be avoided, since mice become accustomed to the protocol, and produce less force for escaping. (see last paragraph).

Although the outcome is a force measurement, the escape response also implies behavioral pathways. Therefore it is necessary to establish a strict criterion for a set number of FPTs over a given interval of time (see number 2 under Notes and Precautions). The results from WBT sessions that do not meet this criterion should not be analyzed.

Advantages: (see Section 6 for definitions)

1. It is a fast, noninvasive, in vivo functional evaluation.

2. The WBT procedure directly measures the total phasic tension developed by both fore- and hindlimbs in a relatively easily elicited voluntary motor movement.

3. The tension measured using the WBT procedure is determined by the action of both proximal and distal extensor muscles for all 4 limbs in conjunction with the corresponding distal flexor muscles that are involved in maintaining traction during the forward pulling maneuver. The procedure therefore measures the tension developed by the primary antigravity muscles along with the distal musculature involved in gripping and climbing.

4. The primary measures obtained (WBT5 and WBT10) represent the maximum phasic tension that can be developed over several attempts. The procedure is analogous to making 20 to 30 measurements of the tension developed by a sprinter at the start of a race, and taking the top 5 and top 10 starts as the best estimate of the sprinter's starting ability.

5. The secondary measures obtained (FR and PDP) represent measures of the proportional weakening that occurs over the top 10 forward pulling maneuvers. In conformance with the sprinter analogy, these measures would indicate the consistency in the amount of tension that was generated by the sprinter over the total of 20 or 30 starts. More reliable sprinters, of course, would consistently generate high tensions at each "start" and therefore exhibit relatively tight distributions of ordered start times over 20 to 30 starts. Reductions in FR and increases in PDP that are characteristic of mdx mice are behavioral indicators of a deficit in the ability to consistently exert force in a forward pulling maneuver and may be the result of use-dependent weakening or pain that occurs when the maneuver is executed (Siegel et al., 2009).

6. The WBT procedure has been used in different laboratories with consistent results (Table 1).

Table 1. Evaluation of the WBT5 indicates the overall reproducibility and consistency of the whole body tension method for assessing the total body strength of adult nondystrophic mice. The results obtained from 6 different laboratories indicate that nondystrophic mice exert WBT5 values of approximately 13 to 16 times body weight over a wide range of ages. N values represent the number of determinations and the number of mice used in each study.

	Age (month)	N	WBT 5 normalized (g Force /g body weight)
Carlson & Makiejus, 1990	1- 2 - 5 months 2.5 - 5 months 5 - 11 months	56,42 24,17 17,7	13.8-14.1
Makiejus et al., 1991	>5 months	7,17	13.2
Hudecki et al., 1993	4 – 5 months 5 – 6 months	15 15	14.6 15.6
Deconinck et al., 1997	3 - 4 months	9	14.5
Raymackers et al., 2003	3 month	9	15.2
Fougerousse et al., 2003	7 month*	10	10.3

* strain not from a C57Bl10 background, but well from a sv129 one.

7. The WBT test provides a robust measure of recovery towards nondystrophic levels with the utrophin overexpressing mdx mouse exhibiting a 52% recovery in WBT (escape test; Deconinck et al., 1997; Gillis, 2002). This value is similar to the recovery in isometric force in diaphragm and extensor digitorum longus from the same group of mice. When the overexpression of utrophin is started later and/or at a lower concentration, muscle functions can be fully restored in vitro, but not in vivo, showing the importance of associating an in vivo experiment to preclinical testings (Squire et al., 2002).

8. The inter-trial reliability for the determination of WBT10 is relatively high (about 0.8) for a behavioral measure (Carlson et al., 2010).

9. In practice, an N of approximately 10 mice in each treatment group is required to achieve significance at $p < 0.05$ for a 30% difference in WBT10 or WBT 5.

10. Treatments or conditions which primarily alter muscle mass without changing the tension developed per unit of muscle mass (or cross sectional area) produce corresponding alterations in FPT that are not associated with changes in either WBT5 or WBT 10 (Carlson et al., 2010a).

Disadvantages:

1. The procedure involves skill in animal handling and new investigators should practice the technique before using the results (usually one or two sets of trials with each trial involving about 10 mice).

2. The procedure is not very high throughput although generally about 10 mice can be sampled in an hour.

3. It is easiest if two individuals work together in obtaining WBT with one individual keeping track of the mice and the other responsible for eliciting the forward pulling maneuvers.

4 MATERIALS

1. Isometric tension transducer adapted to measure tensions in the range of 100 to 500 gms. We use a Grass FT03 transducer with the appropriate spring adjustments to measure tensions in this range.

2. Analogue or digital recording device for measuring tensions generated at the transducer.

3. A wire or metal thread (30 gauge or less) attached at one end to a small alligator clip (2 cm) and at the other end to a hook or other attachment that rapidly and directly fastens into the transducer. Grass instruments provides a standard hook for their transducer. The main requirements for this thread and hook assembly is that it is bendable but not very compliant against forces applied along the wire cross section. We have tested other material such as strong fishing line but have found them to be a bit too compliant. The length of this thread assembly (from the tip of the alligator clip to the hook attachment site) should be about 7 to 9 cm. A 5-7 cm iron chain attached to an alligator clip has also been used.

4. For each mouse, we use 2 small squares of adhesive tape (duct tape is OK) about 1 to 2 cm square that will be used to attach the thread to the tail of the mouse. A piece of cotton compress is also useful for attaching the alligator clip to the tail.

5. A tube assembly that the mice will freely enter and that contains a wire mesh that the mice will firmly grip as they perform forward pulling maneuvers. Siegel et al. (2009) used 2 sizes (1.5 inch and 0.5 inch inner diameters) of solid PVC tube that were purchased at a hardware store. Mice 2 months or older were tested in the larger diameter tube and those at 30 days were usually tested in the smaller diameter tube. The interior of the tube is lined with an aluminum mesh (14 mesh .020 aluminum) that is typically used as a window screen and can be purchased at a hardware store. The tubes are approximately 1 foot long and the aluminum screen is attached to the interior of the tube at various locations with hot glue. The PVC tube serves a solid support for the wire mesh. It is not necessary for the wire mesh to be completely stationary relative to the solid tube as the mice perform forward pulling maneuvers. It is fine if there is a little play to the wire mesh such that the wire mesh moves backward slightly (about 2 mm) as the mouse attempts to pull forward. The tube assembly is held in a horizontal position with a ring clamp and is positioned in front of the tension transducer (see Appendix Fig. 1) which is oriented to measure the tension generated by horizontal forward pulling maneuvers (forward pulling tensions, FPTs). Mesh and tube should be cleaned on a regular basis.

5 METHODS

1. Weigh the mouse to the nearest gram.

2. Attach one of the squares of adhesive tape to the tail of the mouse about 1 to 2 cm from the base of the tail; a preliminary cleaning of the region may facilitate the adherence. At this point make sure that the mouse is on a smooth and slippery surface so that tension cannot be developed in the limbs when the mouse tries to get away.

3. Attach the wire thread assembly to the tail of the mouse by clamping the alligator clip to the adhesive on the tail of the mouse.

4. Attach the second square of adhesive around the small alligator clip in order to strengthen the attachment of the wire thread assembly to the tail.

5. Lead the mouse into the mouth of the tube so that all 4 limbs are within the tube and immediately hook the other end of the wire assembly to the transducer. Usually the mouse is initially situated with the hindlimbs at a point about 1 to 6 cm from the mouth of the tube.

6. Record and identify all spontaneous forward pulling maneuvers as well as those that are evoked by stroking the tail with a serrated forceps. In general, we typically apply individual strokes to the tail at approximately 2 to 4 second intervals. It is important to begin applying strokes to the tail as soon as possible (i.e. within a few seconds) after the mice enter the tube. Applying strokes rapidly (at 2 to 4 second intervals) essentially keeps the mouse's attention towards trying to escape. **Hurting the animal and producing bleeding is not more effective and should be avoided.** If strokes are not applied for several seconds the mice will tend to turn around in the tube and try to escape out the front end. If this happens, just re-orient the tube to coax the mouse to re-enter and immediately resume applying tail strokes (putting some food at the opposite end of the tunnel may help the mouse staying in it and trying to pull in the proper direction). Make sure that all forward pulling maneuvers are identified and that the corresponding forward pulling tensions (FPTs) are identified on the recording. Generally, after acquiring about 20 FPTs, the position of the isometric transducer and tube is adjusted to allow the mouse to enter the tube more deeply so that only the tip of the tail protrudes from the mouth of the tube. This often incites the mouse to "try harder" as the experimenter continues to apply strokes to the tip of the tail. It is best not to stroke the tail at very regular intervals since stroking the tail unexpectedly to surprise the mouse seems to produce stronger escape trials. After obtaining another 5 to 10 FPTs, the mouse is removed from the tube, the wire assembly removed from the tail, and the mouse is replaced in the cage.

Notes and Precautions:

(1) In general, steps 2 through 5 should take less than a minute so that the mouse is minimally excited between the time it is removed from the cage and the time it has entered the tube. It is good to have an assistant help with this procedure to expedite these steps.

(2) It is of course preferable to obtain at least 20 FPTs during the procedure but it is not always possible to do so. If a mouse seems to stop responding to tail strokes, it is useful to let the mouse rest in place for several seconds to 2 minutes before resuming tail strokes. During this time, one can re-orient the tube so that the position of the mouse within the tube changes. If one cannot obtain 15 FPTS within about 5 minutes, it is best to stop the session and try again in a couple of days if possible. Do not use the results unless a minimum number of FPTs have been recorded (e.g. 15 FPTs in about 5 minutes). **Usually, however, this lab obtains 20 or more FPTs during a 3 minute session.** Generally, the FPTs recorded after 120 seconds are not par of the 10% strongest pulls. Applying the stroke in a non foreseeable pattern may prevent habituation and thus allow the animal to develop higher pulls.

(3) It is of course best if the individual responsible for stroking the tail and identifying FPTs is not aware of the mouse identity. The experiments are best done in a blind fashion with an assistant.

6 EVALUATION AND INTERPRETATION OF RESULTS

1. Identify the top 10 FPTs on the tension recording and order them 1 through 10 (i.e., FPT1, FPT2, …..FPT10).

2. Measure the top 10 FPTs in grams of tension and divide the average of the top 10 FPTs by the body weight to determine the WBT10.

3. Divide the average of the top 5 FPTs (gms) by the body weight to determine the WBT5.

4. The WBT1 may also be used in comparing treatment groups. It is obtained by dividing the top FPT (FPT1) by the body weight.

5. Divide each of the top 10 FPTs by the maximum FPT (i.e., FPT1/FPT1, FPT2/FPT1,FPT3/FPT1,….. FPT10/FPT1) in order to normalize each FPT to the maximum FPT (i.e. FPT1).

6. Construct an X-Y plot of ordered FPT: X axis: 1,2 ,3 ,4 5, 6, ….., 10

7. Y axis: FPT1/FPT1, FPT2/FPT1, FPT3/FPT1,…, FPT10/FPT1

8. Perform a linear regression of the X-Y plot in (5) with normalized FPT (Y) as the dependent variable and ordered pull (X) as the independent variable. The negative slope of this relationship is defined as the “Proportional Decline per Pull” (PDP) which is a direct measure of the proportional extent of muscle weakening that occurs following a maximal effort forward pulling maneuver.

9. Determine the ratio of WBT10/WBT5 (“Functional Reserve”) as a measure of the extent of muscle weakening that occurs over the top 10 forward pulling maneuvers. WBT5/WBT1 can also be used (Deconinck et al., 1997).

10. Although WBT is generally used as a correction for body weight, it is also possible to use the maximum FPTs (in gms) particularly in cases where changes in FPT are induced by changes in muscle growth (atrophy or hypertrophy).

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8 APPENDIX

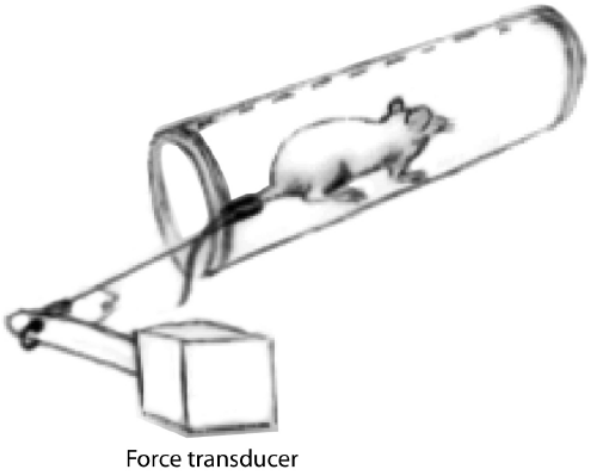

Figure 1. Diagram showing mouse position inside the tube with tail attached to a force transducer. Note that the tube walls are not normally transparent and are shown here as transparent only for purposes of illustration (drawing by Evelyn Carlson).

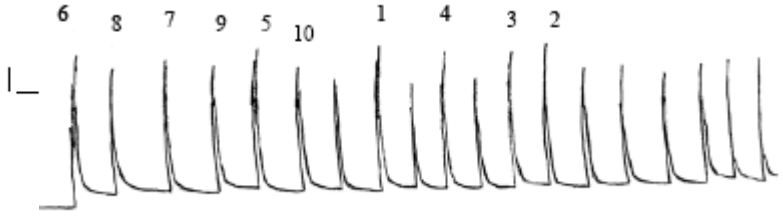

Figure 2 . Typical WBT record from a nondystrophic mouse showing the top 10 FPTs during a particular recording session. Calibrations: Vertical - 65 gms; horizontal – 2 seconds.

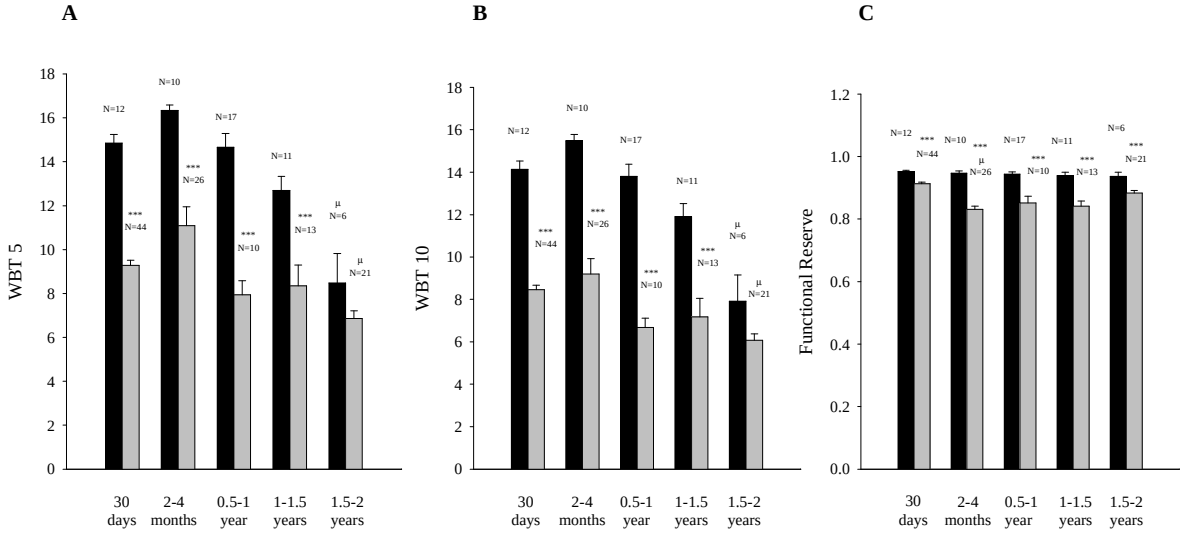

Figure 3. Baseline values for (A) WBT5, (B) WBT10, and (C) FR for a large sample of nondystrophic (black histobars) and mdx (gray histobars) mice aged between 30 days and 2 years (from Siegel et al., 2009).

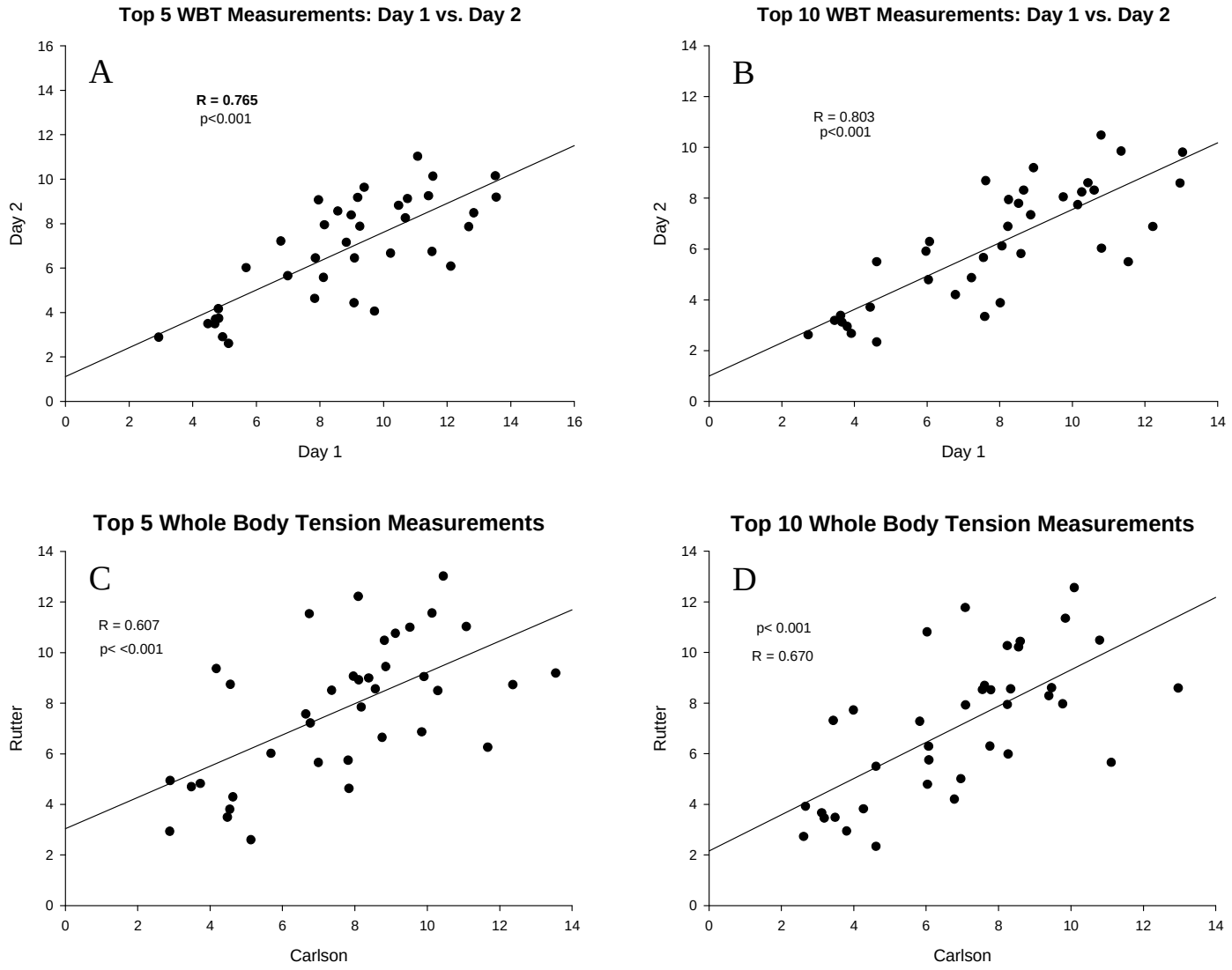

Figure 4 . Tests for reliability of WBT5 and WBT10 measures on a sample of 40 mdx and nondystrophic mice of various ages. The mice were tested on 2 successive occasions (Day 1 and Day 2) at an interval of 2 days by 2 different examiners (Carlson, Rutter). One of the mice did not reach criterion (15 FPTs in 5 minutes) for the first trial and was therefore excluded from the study leaving an N of 39 mice. The two examiners performed the WBT procedure on separate groups of 20 mice (10 mdx, 10 nondystrophic) on each of the two trial days. The mice examined by Carlson on Day 1 were examined by Rutter on Day 2 and those examined by Rutter on Day 1 were examined by Carlson on Day 2. (A) and (B) represent the inter-trial reliability for the WBT5 and WBT10 measures obtained on the sample of 39 mice. The Pearson correlation coefficients (R) for each test are indicated along with the level of significance for the correlations ($p < 0.001$). Since the results for day 2 were less than those for day 1 (A and B), it is recommended that a longer period between measurements (1 month or more) be used for the WBT test in cases where multiple tests are performed on individual mice. Since the correlation coefficients between trials (A and B) were larger than those between examiners (C and D), it is recommended that a single examiner perform all of the

measurements for a given treatment trial in order to reduce variability (from Carlson et al., 2010).