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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

Dystrophin serves to buttress the muscle cell membrane. In the absence of dystrophin, the membrane is prone to tearing during minimal exercise. Dystrophic muscles are particularly prone to injury subsequent to eccentric (lengthening) contractions.[1] As an example, mdx mice have near-normal absolute force measurements but demonstrate greater-than-normal force decrement after eccentric muscle contractions.[2] We have previously shown that GRMD dogs also have a greater-than-normal force decrement with eccentric contractions.[3]

2. SCOPE AND APPLICABILITY

As discussed in the GRMD tibiotarsal joint force protocol, phenotypic variation in affected dogs confounds statistical analysis, necessitating larger group sizes to show significance. With localized treatments, the effect of phenotypic variation is less of a concern because the untreated opposite limb can serve as the control. With systemic treatments, baseline measurements should be established prior to treatment so that each dog serves as its own control. Evaluation of the force decrement incurred due to eccentric muscle contraction offers another option by which each dog can be used as its own control.

3. CAUTIONS

Dogs must be anesthetized. A nerve stimulator, specialized, custom-made sterotactic frame, and servomotor are required. See METHODS and EVALUATION AND INTERPRETATION OF RESULTS (below) for guidance on interpretation.

4. MATERIALS

Separate personnel are utilized for anesthetic management and force measurements. Dogs are anesthetized and positioned in dorsal recumbency with one of the pelvic limbs placed in a custom-made stereotactic frame such that the hip (coxofemoral), knee (stifle), and hock (tibiotarsal) joints are all at 90^{o} angles (see GRMD tibiotarsal joint force protocol and **Figure 1**). In an initial study, eccentric contractions were induced in flexor muscles of the cranial tibial compartment by stimulating the sciatic trunk in the mid-thigh area.[3] This caused contraction of both the tibiotarsal joint flexors and extensors. Because the extensors are more powerful, eccentric (lengthening) contractions were induced in the flexors.

More recently, we have developed a technique whereby the common peroneal nerve is stimulated while simultaneously extending the tibiotarsal joint with a servomotor (Aurora Scientific, Ontario, Canada) coupled to a lever arm. The servomotor extends (lengthens) muscles of the cranial tibial compartment. Movement of the lever arm is controlled by use of a computer and customized LabView software (Aurora Scientific). Before beginning the experimental contractions, isometric forces from single twitches are measured. The common peroneal nerve is stimulated with optimally-positioned percutaneous electrodes (Model S48

Solid State Square Wave Stimulator, Grass Instruments, Quincy, MA, USA). Voltage is adjusted until twitch force (Pt) reaches a maximum. To maximally activate the muscles of interest, stimulation voltage is 50% greater than necessary to achieve Pt. Stretches are performed 10 times, once every 5 sec. Following a 4 minute rest, the series of 10 stretches is repeated. Thus, the muscles of the cranial tibial compartment are repeatedly stretched to induce mechanical damage.

5. METHODS

5.1. Anesthetic protocol (Note, in a preliminary study, mean alveolar concentration [MAC] values for isoflurane did not significantly affect force measurement values [Schueler RO, Koch J, Kornegay JN, unpublished data]).

20-30 minutes prior to anesthesia induction:

- Pre-anesthetic agents:
  - Atropine sulfate (0.04 mg/kg, IM)
  - Acepromazine maleate (0.02 mg/kg, IM) for dogs weighing greater than 5 kg
  - Butorphanol tartrate (0.4 mg/kg, IM)

Anesthetic induction:

- Anesthetic agents:
  - Propofol (up to 3 mg/kg, IV – slowly!) for dogs weighing greater than 5 kg
  - Isoflurane or sevoflurane (to effect, inhaled) (avoid masking down)

Anesthetic monitoring:

During anesthesia, ECG, heart and respiratory rate, blood pressure, end tidal (Et)CO_2, and saturation of hemoglobin by peripheral oxygen (SpO_2) are monitored continuously with a pulse oximeter (Cardell Multiparameter Monitor 9405, Minrad International, Inc, Orchard Park, NY). These values, as well as capillary refill time and anesthetic setting, are recorded every 15 minutes.

Anesthetic recovery:

Monitor affected and carrier dogs closely during anesthetic recovery until fully awake and in sternal recumbency.

- Naloxone (up to 0.4 mg/kg, SQ) for rapid recovery; given in ½ dose increments (1st dose given while the dog is still intubated and breathing O_2; 2nd dose, if necessary, after extubated and/or if respiration drops below 7 breaths per minute).

5.2. Position the dog in dorsal recumbency.

5.3. Measure the tibiotarsal joint angle (see separate GRMD protocol).

5.4. Calibrate the force transducer before each measurement.

5.5. Place one of the pelvic limbs in a stereotactic frame such that the hip (coxofemoral), knee (stifle), and hock (tibiotarsal) joints are all at 90^{o} angles (see GRMD tibiotarsal joint torque force protocol).

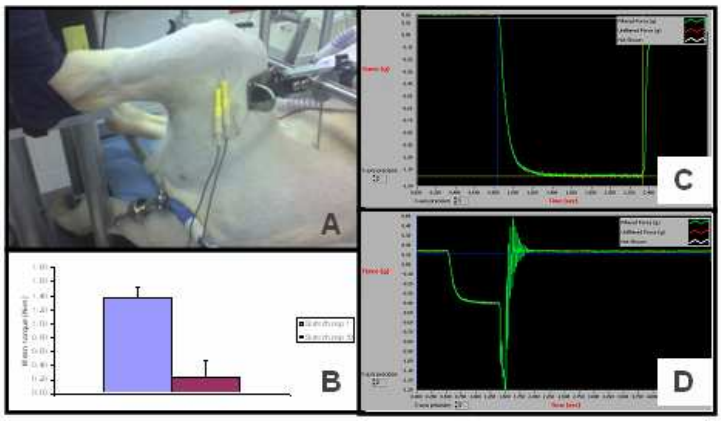

Figure 1. A. Left pelvic limb of a 6-month-old GRMD dog immobilized in a stereotactic frame, with needles positioned to stimulate the peroneal nerve. B. Histogram showing mean torque (N-m) generated by tetanic tibiotarsal joint flexion during a series of 10 tetanic contractions (50 pps, pulse width 0.001 sec, duration 1.7 sec, each being separated by 10 secs). At the conclusion of each tetanic contraction, these maximally-stimulated cranial tibial compartment muscles were forcibly stretched by the servomotor as the tibiotarsal joint was extended a further 30 degrees at a rate of 290 degrees/sec (~ 1 muscle length/sec) (see D). The mean value of the initial contractions (blue bar) was ~ 1.4 N-m, while the tenth contraction (red bar) was ~ 0.2 N-m, representing an 85% decrement. C. The characteristic tetanic mechanical potential produced by tibiotarsal joint flexion is illustrated. D. A single tetanic mechanical potential analogous to that seen in C is followed by a sharp further deflection reflecting the eccentric contraction induced by the servomotor, with an immediate return to baseline. The baseline deflection following the potential is due to vibration artefact. Note, data shown here were collected using a modification of the method described. The tetanic run was 700 msec, with the first 500 isometric followed by 200 msec of stretch.

5.6. Palpate the common peroneal nerve just distal to the head of the fibula as it courses across the bone. Paired stimulating and reference 27-gauge monopolar needle electrodes are placed subcutaneously on either side of the nerve **(Figure 1A)**. The electrodes are optimally positioned and the voltage is adjusted until twitch force (Pt) reaches a maximum. To maximally activate the muscles of interest, stimulation voltage is 50% greater than necessary to achieve Pt. (generally, supramaximal pulses of 150 V are applied). An electromyography (EMG) unit (TE42, Teca Corp, Pleasantville, NY, USA) can be used to record electrical activity in tibiotarsal joint flexor muscles (cranial tibialis and long digital extensor) to ensure proper needle placement. Eccentric contractions are induced using square wave pulses of 100 µsec duration in a tetanic run for 1 sec at a frequency of 50 Hz. The contraction is held isometric at the optimal fiber length (L_o) for the first 900 msec. In the final 100 msec, the muscles of the cranial tibial compartment are stretched by the servomotor at a rate of 0.7 muscle lengths/sec, such that the muscles are stretched to 107% of L_o. Stretches are performed 10 times, once every 5 sec. Following a 4 minute rest, the series of 10 stretches is repeated. Thus, the muscles of the cranial tibial compartment are repeatedly stretched to induce mechanical damage. Force is recorded by the computer software. Contraction-induced injury is quantified by the force deficit (P_d), which is defined as the percent difference between maximum isometric force (P_o) before and after exercise (in this case, eccentric stretches). Force deficit is expressed as a percent change of the initial value of P_o, using the following equation:

$$\text{Force deficit } (P_d) = [P_o - P_{o\ \text{after stretch}}/P_o] \text{ X } 100$$

6. EVALUATION AND INTERPRETATION OF RESULTS

Based on results from mdx mice, we expected that GRMD dogs would have a greater decrement in force following eccentric contractions of muscles of the cranial tibial compartment. In an initial study, GRMD dogs (n = 10) decremented significantly more (-63% ± 4; $p < 0.0001$) than either carrier (n = 7; - 13 ± 1) or normal (n = 5; - 8 ± 3) dogs after a series of 30 eccentric contractions administered in three groups of 10 (see technique above) (**Figure 2**). However, in subsequent studies, we have found that the decrement in normal dogs can be ~ 25%, approaching that of GRMD dogs (Kornegay JN, unpublished). It may be useful to compare data from the first 10 eccentric contractions alone, since the differential is greater at that point. Data also still must be correlated with histologic markers such as Evans blue dye exclusion before definitive conclusions can be drawn.

Potential Advantages/Disadvantages of the Methodology

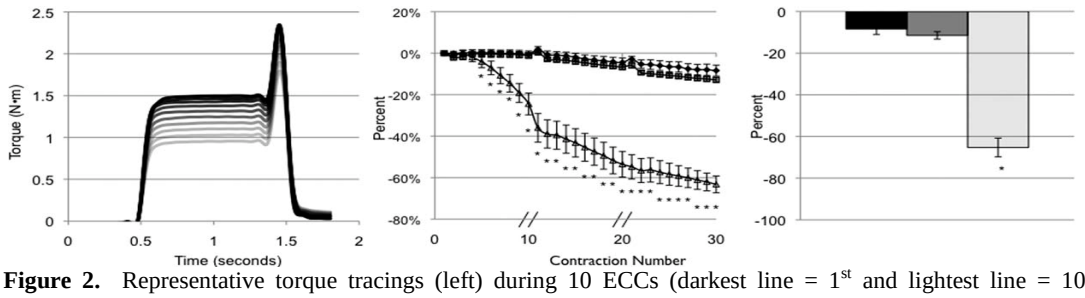

Figure 2. Representative torque tracings (left) during 10 ECCs (darkest line = 1st and lightest line = 10th contractions). Contraction torque rapidly decrements (middle) in GRMD dogs (triangles, bottom trace) rapidly compared to normal (diamonds, top trace) and carrier (squares, middle trace) dogs. Broken lines through the x-axis indicate a 4-min rest period. After 30 contractions (right), GRMD dogs have lost 63% of their original torque, whereas carriers lost13% and normals lose 8%. Black = normal; gray = carrier; light = GRMD. *Significantly different (one-way ANCOVA) compared to the other genotypes ($P < 0.05$). From reference 4.

Advantages

As with the tibiotarsal joint force method, this technique allows serial, non-invasive evaluation of contractile force. The effects of phenotypic variation are offset because the force decrement is individualized for each dog.

Disadvantages

Instrumentation is more expensive and must be customized. Muscles of the cranial tibial compartment may undergo functional hypertrophy subsequent to initial injury. Thus, it is not clear that functional test results will accurately predict overall response to treatment.

4. REFERENCES

1. Edwards RHT, Jones DA, Newham DJ, Chapman SJ: Role of mechanical damage in the pathogenesis of proximal myopathy in man. Lancet 1:548-552, 1984.

2. Moens P, Baatsen PHWW, Marechal G: Increased susceptibility of EDL muscles from mdx mice to damage induced by contractions with stretch. J MusRes Cell Motil 14:446-451, 1993.

3. Childers MK, Okamura CS, Bogan DJ, Bogan JR, Petroski GF, McDonald K, Kornegay JN: Eccentric contraction injury in dystrophic canine muscle. Arch Phys Med Rehabil 83:1572-1578, 2002.

4. Tegler CJ, RW Grange, DJ Bogan, CD Markert, D Case, **JN Kornegay**, MK Childers: Eccentric contractions induce rapid isometric torque drop in dystrophic-deficient dogs. Muscle Nerve 42:130-132, 2010.