Video Article

An *in vivo* Rodent Model of Contraction-induced Injury and Non-invasive Monitoring of Recovery

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Abstract

Muscle strains are one of the most common complaints treated by physicians. A muscle injury is typically diagnosed from the patient history and physical exam alone, however the clinical presentation can vary greatly depending on the extent of injury, the patient's pain tolerance, etc. In patients with muscle injury or muscle disease, assessment of muscle damage is typically limited to clinical signs, such as tenderness, strength, range of motion, and more recently, imaging studies. Biological markers, such as serum creatine kinase levels, are typically elevated with muscle injury, but their levels do not always correlate with the loss of force production. This is even true of histological findings from animals, which provide a "direct measure" of damage, but do not account for all the loss of function. Some have argued that the most comprehensive measure of the overall health of the muscle in contractile force. Because muscle injury is a random event that occurs under a variety of biomechanical conditions, it is difficult to study. Here, we describe an *in vivo* animal model to measure torque and to produce a reliable muscle injury. We also describe our model for measurement of force from an isolated muscle *in situ*. Furthermore, we describe our small animal MRI procedure.

Protocol

1. *in vivo* injury Model and measurement of isometric torque.

1. These procedures can be used for rats or mice[7,17,18]. To begin, place the animal supine under inhalation anesthesia (~ 4-5% isoflurane for induction in an induction chamber,then ~ 2% isoflurane via a nosecone for maintenance) using a precision vaporizer (cat # 91103, Vet Equip, Inc, Pleasanton, CA). Apply sterile ophthalmic cream (Paralube Vet Ointment, PharmaDerm, Floham Park, NJ) to each eye to protect the corneas from drying. During the procedure, the animal is kept warm by use of a heat lamp placed outside the cage and kept at least 6 inches from the animal at all times.
2. Prep the skin by removing hair and by cleaning with alternating scrubs of betadine and 70% alcohol to prevent seeding skin bacteria into the soft tissue or bone. Confirm proper anesthesia by lack of a deep tendon reflex (no foot withdrawal in response to pinching the foot). A needle is manually placed through the proximal tibia in order to stabilize the limb onto the rig (25G or 27G for mouse). The needle should not enter the anterior compartment of the leg.
3. Lock the needle into a fixed position, such that the animal is supine and the toes are facing straight up. A custom-made device is used to secure the needle and thereby stabilize the leg.
4. Place the foot of the limb onto a custom-machined footplate (Figure 1). The axis of the footplate is attached to a stepper motor (model T8904, NMB Technologies, Chatsworth, CA) and a torque sensor (model QWFK-8M, Sensotec, Columbus, OH). The foot should initially be aligned so that it is orthogonal to the tibia, as in Figure 1.
5. Use transcutaneous electrodes (723742, Harvard Apparatus, Cambridge, MA) or subcutaneous electrodes (J05 Needle Electrode Needles, 36BTP, Jari Electrode Supply, Gilroy, CA) to stimulate the fibular nerve near the neck of the fibula, where the nerve lies in a superficial position. Visually confirm isolated dorsiflexion by performing a series of twitches (0.1 ms pulse for the mouse and 1 ms pulse for the rat) before the foot is secured. Once the foot is secured to the footplate with adhesive tape, an increase in twitch amplitude in response to an increase in voltage confirms that opposing muscles (plantarflexors) are not being simultaneously stimulated.
6. Before injury, and at selected time points after an injury, the maximal force producing capacity of the dorsiflexors is recorded as the "maximal isometric torque" (torque without a change in muscle length). Torque measurements are performed on the same rig that is used to induce injury. Before recording maximal isometric torque, the pulse amplitude is adjusted to optimize twitch tension and the optimal position of the ankle is determined by giving twitches at different lengths of the dorsiflexors. After obtaining a torque-angle curve to determine the optimal length of the dorsiflexors (resting length, aka Lo), a torque frequency plot is obtained by progressively increasing the frequency of pulses during a 200 ms pulse train. A maximal fused tetanic contraction is obtained usually at 90-100 Hz. Three separate twitches and tetanic contractions are recorded and saved for further analysis.
7. Use commercial software to (Labview version 8.5, National Instruments, Austin, TX) to synchronize contractile activation, onset of ankle rotation, and torque data collection. Stimulation of the dorsiflexor muscles occurs while the computer-controlled motor simultaneously moves the footplate into plantar flexion, thus leading to a lengthening contraction (also called "eccentric" contraction, which causes injury of the muscle). The specific protocol depends on the desired magnitude of injury desired by the investigator. The magnitude of injury, or tissue damage, can be regulated by manipulation of variables such as angular velocity, timing of muscle activation, range of motion, and the number of lengthening contractions.
8. To induce injury, superimpose a lengthening contraction onto a maximal isometric contraction, varying the range of motion, velocity of lengthening, and timing of stimulation as needed. For example, a maximal isometric contraction is obtained in the dorsiflexors and after 200 ms they are lengthened at a selected velocity to approximate normal movement (900°/sec). We have previously shown that activation before the movement and the degree of lengthening are important factors in obtaining an injury[14]. The majority of torque produced by the dorsiflexors is from the TA[11] and we have shown previously that this model results in injury to this muscle[5,13-15]. The TA remains stimulated throughout lengthening.

9. After injury, the animal is removed from the apparatus. The tibial pin is removed, the leg is cleaned again, and the animal is returned to the cage (placed on a temperature-controlled heating block at 37°C) and monitored until recovery. This includes waiting until the animal is awake and mobile. The animals suffer no observable pain during the procedure and there are no visible changes in gait (e.g. lameness) after injury induced by lengthening contractions. However, appropriate anti-pain treatment is administered subsequently (buprenorphine 0.05 - 0.1 mg/kg every 12 hours for 48 hr after surgery).

2. *In situ* measurement of whole muscle tension.

1. The animal is prepared and the tibia is stabilized as described above in section 1.1 through 1.3. All instrumentation is turned on at least 30 min prior to testing for proper calibration and to minimize thermal drift of the force transducer.
2. Incise the skin anterior to the ankle and sever the tendon of the tibialis anterior muscle (TA). Carefully tie 4.0 Ethicon silk non-absorbable suture to the tendon and attach the vicryl suture to the load cell via the provided S-hook (weight = 0.1g), model FT03, Grass Instruments, Warwick, RI). Alternatively, a custom clamp (weight = 0.5g) can be used to attach the tendon to the vicryl suture (Figure 2).
3. The load cell is mounted to a micromanipulator (Kite Manipulator, World Precision Instruments Inc, Sarasota, FL) so that the TA could be adjusted to resting length and aligned properly (a straight line of pull between the origin and insertion). The TA is protected from cooling by a heat lamp and from dehydration by mineral oil. The signals from the load cell (calibrated before each test) are fed via a DC amplifier (model P122, Grass Instruments, Warwick, RI) to an A/D board to be collected and stored by acquisition software (PolyVIEW version 2.1, Grass Instruments, Warwick, RI).
4. Attach the TA to the load cell and apply single twitches (rectangular pulse of 1 ms) at different muscle lengths in order to determine L_0. Muscle resting length, measured using calipers, is defined as the distance between the tibial tuberosity and the myotendinous junction. At this length, gradually increase the pulse amplitude and then the pulse frequency to establish a force-frequency relationship. A maximally fused tetanic contraction is obtained at approximately 90-100Hz (300 ms train duration comprised of 0.1 ms or 1 ms pulses). Use 150% of the maximum stimulation intensity to activate the TA in order to induce maximal contractile activation (P_0). Maximal tetanic contractions can be performed repeatedly and expressed as percentage of P_0, providing an index of fatigue at any desired point in time.

3. *in vivo* MR imaging and/or spectroscopy of rodent skeletal muscles.

All MRI and MRS is performed on a Bruker Biospin (Billerica, MA) 7.0 Tesla MR system equipped with a 12 cm gradient insert (660 mT/m maximum gradient, 4570 T/m/s maximum slew rate) running Paravision 5.0 software.

1. The animal is anesthetized with vaporized isoflurane as described above in 1.1. An MR-compatible small-animal monitoring and gating system (SA Instruments, Inc.) is used to monitor respiration rate and body temperature. Mouse body temperature is maintained at 36-37°C using a warm water circulator. A custom-made holder is used to position the mouse in the supine position with both legs parallel to the bore of the magnet from knee to foot. A four-channel receive-only surface coil is placed within a 72 mm linear 1H resonator. The resonator coil is tuned and matched to the sample.
2. MR Imaging: After localizers, the following MR scans are performed: T1-weighted rapid acquisition with relaxation enhancement (RARE) with the following parameters: TE = 9.52 ms, TR = 1800, echo train length = 4, in-plane resolution 100x100 µm, and slice thickness = 750 µm. Dual-echo PD/T2 RARE: TE=19.0/57.1 ms, TR=5000 ms, echo train length = 4, in-plane resolution 100x100 µm, and slice thickness = 750 µm. Spin echo (SE) diffusion tensor image data using 12 non-colinear directions: b-value = 350 s/mm^{-2}, TE = 26 ms, TR = 4500 ms, in-plane resolution 150x150 µm, and slice thickness = 750 µm. Multi-slice multi-echo (MSME) T2 parametric mapping data using 16 TEs = 11.4 ms to 182.5 ms with ΔTE = 11.4 ms, TR = 10000 ms, in-plane resolution 150x150 µm, and slice thickness = 750 µm.
3. Image processing: Diffusion tensor reconstruction and tractography is performed using TrackVis (Martinos Center for Biomedical Imaging; Massachusetts General Hospital; Boston, MA) to create mean diffusivity (MD), fractional anisotropy (FA) images as well as tractography maps. T2 mapping is performed using custom software written in MATLAB (The Mathworks; Natick, MA) using non-linear least squares to fit the measured data at each pixel to the canonical T2 signal equation. Regions of interest measurements are performed to assess parameter values within the TA.
4. 1H spectroscopy: Automated shimming is performed on a 1 x 1 x 4 mm^3 voxel in the TA. A point-resolved spectroscopy (PRESS) pulse sequence (TR/TE=2000/18 ms) is used to acquire spectra from the same voxel with 1024 averages. Data acquisition is 34 minutes on each leg. Spectral data are processed using the LCModel package[16]. 31P spectroscopy: A dual-tuned (1H, 31P) surface coil is used to perform non-localized (using a single-pulse experiment) or localized spectroscopy using the image selected *in vivo* spectroscopy (ISIS) pulse sequence.

4. Harvesting and storing muscles.

TAs are harvested after at the end of experiments, weighed, snap frozen in liquid nitrogen, and then stored at -80°C. This can be performed at any point in time after the *in vivo* experiments. Muscles are harvested immediately after the *in situ* experiments, as this is a terminal procedure. For detailed morphological studies, the animal is fixed with 4% paraformaldehyde via perfusion through the left ventricle.

5. Representative results.

Figure 3 shows representative data from a rat in the *in vivo* apparatus. *The in vivo* apparatus is used to obtain maximal torque generated by the dorsiflexor muscles; it is also used to induce injury to these same muscles. Due to the length-tension relationship of muscle, maximal isometric torque typically occurs when the ankle joint is positioned at approximately 20° of plantarflexion (with the foot positioned orthogonal to the tibia considered 0°). After maximal isometric torque is obtained, the foot can then be placed into any position to begin the injury protocol. Figure 3 represents an injury protocol of 30 repetitions with an arc of motion from 0° - 70°. Note the steady decline in torque generated from the isometric phase (filled arrow) and lengthening phase (open arrow) during the contraction-induced injury protocol. Torque is recorded in units of Nmm, but the absolute value depends on the size of the animal and its condition (e.g., injured muscle, fatigued muscle, or muscle lacking a certain protein due to homologous recombination).

Figure 4 shows representative data from a rat in the *in situ* apparatus. Our *in situ* apparatus does not involve lengthening contractions; rather, it allows us to isolate, properly align, and measure maximal tension produced by an individual muscle at a known length. Figure 4 shows the gradual loss of force that occurs during a fatigue test in a tibialis anterior muscle of a rat. In this particular example, titanic contractions were performed once every second for 5 minutes. Tension (force) is typically recorded in Newtons (or grams), but like torque, the absolute value

depends on the size and condition of the animal. Because muscle weight is obtained immediately after this procedure, the force can be normalized (called "specific force") to muscle cross sectional area.

Figure 5 shows representative data from *in vivo* imaging of a mouse, such as T1-weighted and T2 parametric mapping (A), 3D tractography from diffusion tensor imaging (B), ^{1}H spectroscopy (C), and ^{31}P spectroscopy. Details are provided in the figure legend.

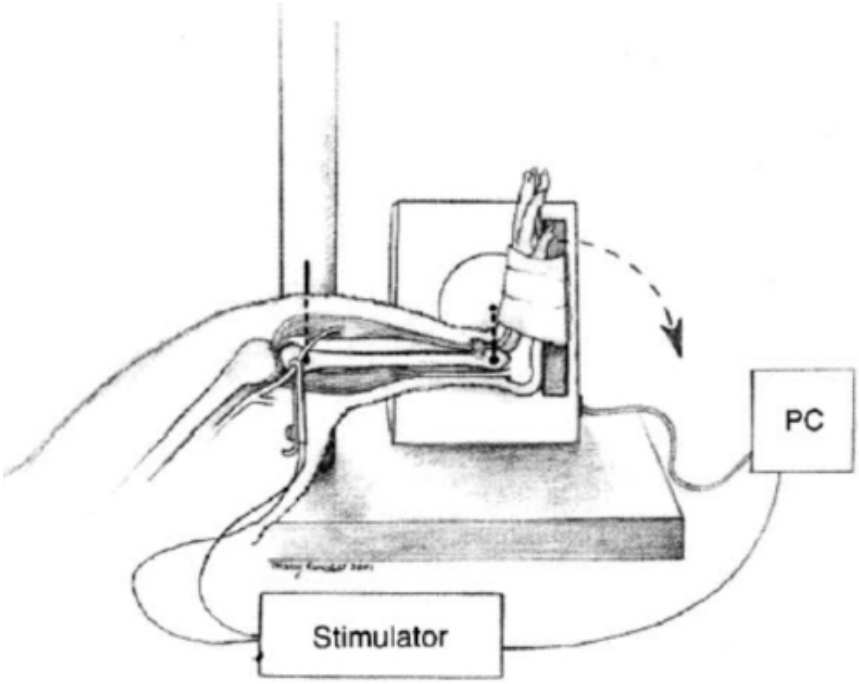

Figure 1: *in vivo* apparatus.* To produce the injury, the tibia is stabilized and the foot attached to a motor-driven plate. The ankle dorsiflexors are stimulated via the fibular nerve while the footplate forces the foot into plantar flexion (dotted arrow).
* *Lovering & De Deyne, J Biomechanics 2005, used with permission.*

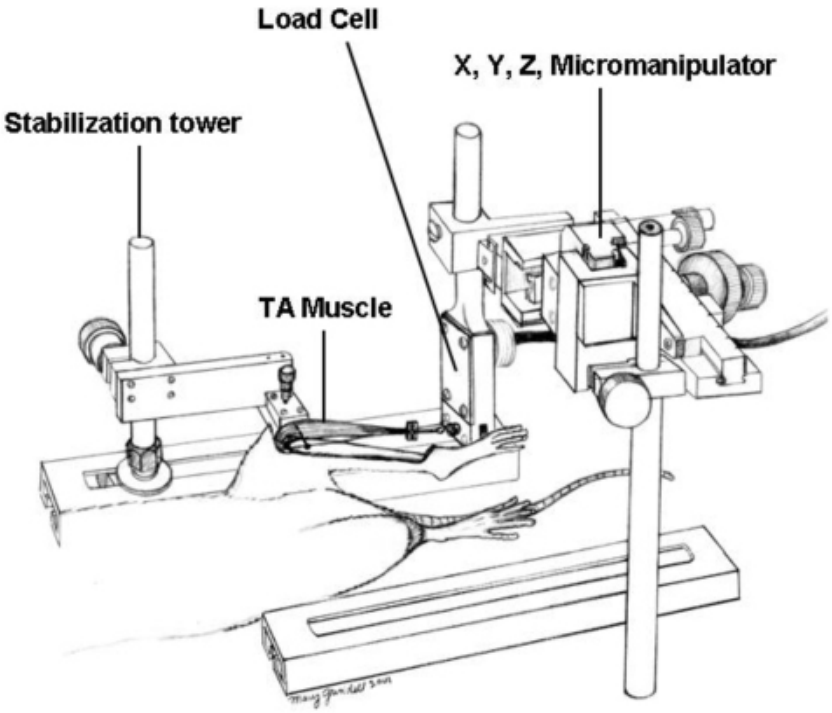

Figure 2: *In situ* apparatus The load cell is mounted to a micromanipulator so that the TA could be adjusted to resting length and aligned properly in the X, Y, and Z directions. The distal tendon of the TA is attached to the load cell and single twitches are induced at different muscle lengths in order to determine L_0. A maximal tetanic contraction is obtained to determine maximal contractile activation (P_0). Maximal tetanic tension can be performed repeatedly and expressed as percentage of P_0, providing an index of fatigue at a desired point in time.

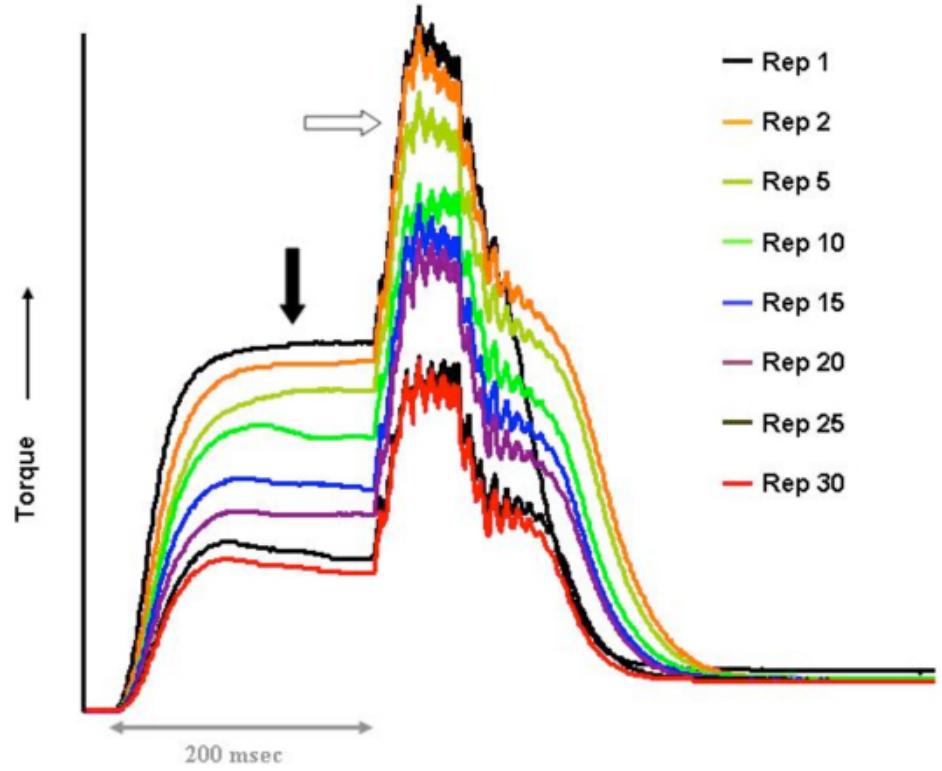

Figure 3: Torque data from *in vivo* apparatus Representative trace recordings of torque from lengthening contractions in the rat. In this particular example, muscles were stimulated for 200 milliseconds to induce a peak isometric contraction (filled arrow) before lengthening (open arrow) by the footplate through a 70° arc of motion at an angular velocity of 900°/s.

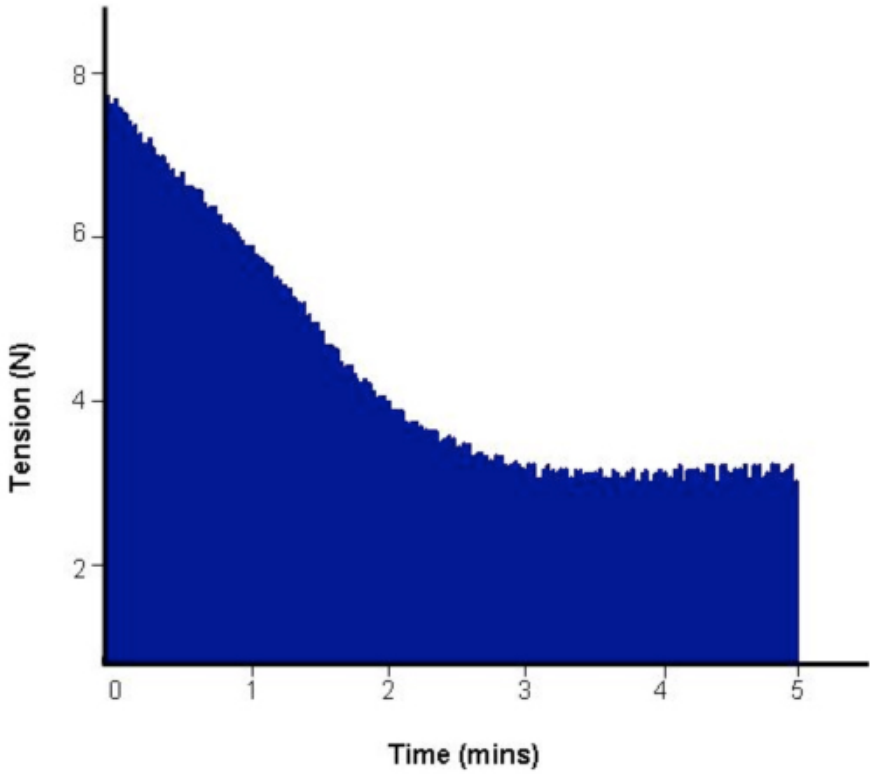

Figure 4: Tension data from in situ apparatus Representative data showing the decline in maximal isometric tetanic tension during repeated stimulation of the tibialis anterior muscle (TA) in a rat. In this example, the TA was isolated, adjusted to optimal length (L_0), and then stimulated with a 200 ms tetanic contraction once every second for 5 minutes.

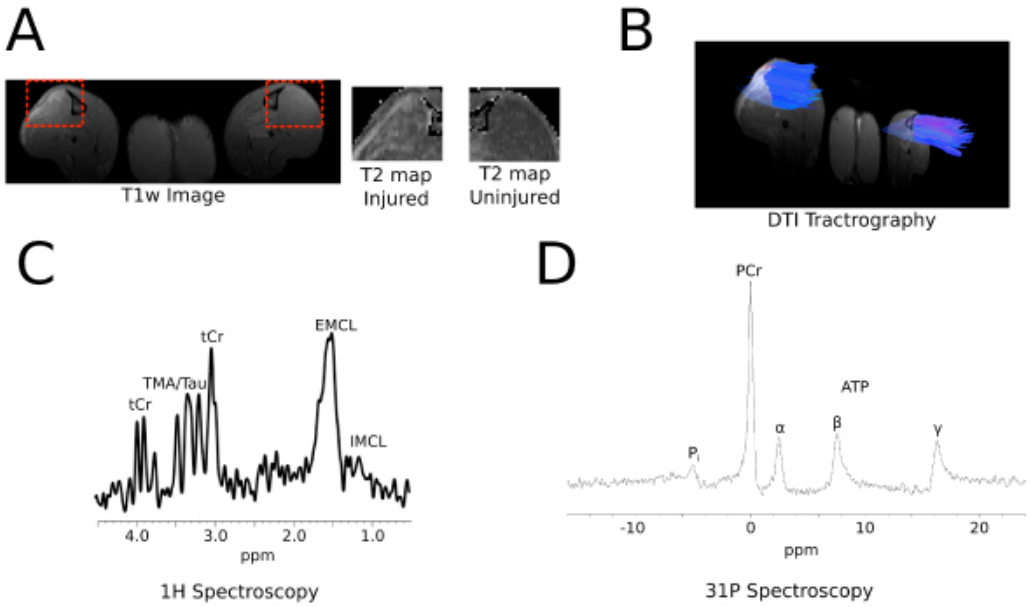

Figure 5: *in vivo* imaging A: The images show transverse (axial) sections of T1-weighted and T2 parametric mapping from the tibialis anterior muscle (TA). The dotted red box surrounds the TA to show increased increased T2 in the injured (left side) versus uninjured (right side). **B:** Representative 3D tractography from diffusion tensor imaging (DTI). **C:** The ^{1}H spectrum of a mouse TA shows several detectable lipid resonances; differentiation between intramyocellular (IMCL) and extramyocellular lipid (EMCL) peaks is obtained using this method. **D:** The ^{31}P MR spectrum of the rat TA shows phosphocreatine (PCr), inorganic phosphate (Pi), and the three resonances (α, β, γ) of adenosine 5'-triphosphate (ATP).

Discussion

"Muscle damage" has been defined and measured in many ways. Structural damage is evident in histological findings[6,9], but one problem with many of the biological markers used to assess muscle injury, including those used in animal studies, is that they usually do not correlate with the loss of force. Muscle damage is often defined within the context of the assay used to examine it and no one finding can account for the changes in contractility after injury. Since full contractile function can persist despite the presence of injury markers, loss of force may be the most valid measure of injury[3], and probably the most relevant.

It is difficult to study muscle injuries in humans, as the incidence is a random event that is difficult to predict and the clinical presentation varies greatly. Therefore much of the data regarding muscle injuries have been ascertained from studies on animals, which provides control over many variables and the ability to study mechanisms of injury and recovery. The *in vivo* injury apparatus we have described provides a method for assessing contractile function without dissecting the muscle, and thus without the need to euthanize the animal under study. Our custom-designed injury model (patent pending) is based on the same principles used by other to establish contraction-induced injury in animals [5,12,15,24]. Despite the availability of models in the marketplace, there is little instruction beyond use of the hardware. Our model has specifications in terms of available range of motion and angular velocity that are advantageous[17], but our main goal is to share the methods; we have tried to describe procedures from start to finish for producing an injury. Benefits of the *in vivo* model are that the muscle, anatomy and biomechanics are not altered and that the procedure is not terminal. We use the same location in the tibia for all torque measurements, following sanitary procedures and using a sterile needle for each measurement. The leg can be stabilized without the use of a transosseus pin, but we have found the pin to be superior in terms of reliability and eliminating extraneous movement during the lengthening contractions.

The apparatus used for *in vivo* torque measurements has several additional advantages. It does not involve any dissection, so there is no need to euthanize the animal under study. The result is that one can measure contractility in the same animal over time, and/or with *in vivo* imaging such as MRI. Other advantages are that normal anatomy is not altered, the nerve is not bypassed for stimulation (such as for in vitro preparations), and the muscle remains in its normal environment, so the effects of inflammation, hormones, or other factors can be studied. Because it requires the use of fewer animals, whose muscles are subjected to fewer manipulations (e.g., dissection prior to assay of function), we prefer to use the torque measurements whenever possible. The moment arm of the mouse TA is known[4] and the muscle can be weighed when the animal is sacrificed. There are some limitations however, compared to isolating the muscle. For example, it is difficult to know the exact length changes that occur during lengthening contractions, and the muscle mass cannot be measured until it is harvested (although it can be estimated based on volume measured via MRI)[8].

To determine the "specific force" (force per unit of cross sectional area) of an individual muscle, that muscle needs to be isolated and positioned properly; this also avoids force transmission from nearby muscles[10]. The *in situ* apparatus was designed for this purpose. It provides an alternative for measuring contractility of only one muscle with a known length and mass. However this method too has limitations. Although the *in situ* apparatus provides more experimental control when measuring the force of an individual muscle, the trade-off is that the experiment becomes less physiological. *In situ* force measurements require a surgical release of the TA muscle, which can alter the anatomy and affect force transmission. The experiment is also terminal, so the muscle cannot be monitored over time.

Diffusion tensor imaging (DTI) is potentially an even more sensitive and earlier marker for muscle damage than standard T2-weighted MRI. The variables obtained with DTI, at least in other tissues such as the brain (1), show a strong and rapid response to damage, whereas the T2 signal can take a prolonged period to change. DTI is based on measurement of the apparent diffusion of water in tissues. The DTI technique has been compared to actual longitudinal sections of the rat TA and it has been shown that DTI directions actually represent local muscle fiber directions in the rat TA muscle[19].

MRS provides information on the chemical composition of muscle non-invasively[12]. Depending on the observed nucleus, MRS allows observation of high-energy phosphates (^{31}P MRS) or lipids (^{1}H MRS). ^{31}P MRS is an ideal tool for the investigation of muscle metabolism because it is non-invasive and can be easily applied to *in vivo* studies of skeletal muscle. Alternative approaches to the biochemical assay of *in situ* muscle metabolites, such as needle biopsy, can give significant overestimates of Pi and apparent reductions of PCr[1]. An animal model provides the obvious benefit of using a controlled injury and comparing *in vivo* MRS changes to findings in the biochemistry, morphology, and function of the tissue. Changes in high-energy phosphate metabolism are encountered in diseases leading to muscular degeneration[2,20]. Intracellular pH, as well as the MR signal intensity ratios Pi/PCr (inorganic phosphate [Pi] to phosphocreatine [PCr]), and PDE/PCr (phosphodiester [PDE] to PCr), may provide valuable information regarding the stage and severity of muscle degeneration.

Disclosures

No conflicts of interest declared.

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