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An *in vivo* rodent model of contraction-induced injury in the quadriceps muscle

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Abstract

Most animal studies of muscle contractile function utilize the anterior or posterior crural muscle (dorsiflexors and plantarflexors, respectively). An advantage to using these muscles is that the common fibular and tibial nerves are readily accessible, while the small size of the crural muscles is a disadvantage. Working with small muscles not only makes some *in vivo* imaging and the muscle testing techniques more challenging, but also provides limited amounts of tissue to study. The purpose of this study was to describe a new animal muscle injury model in the quadriceps that results in a significant and reproducible loss of force. The thigh of Sprague Dawley rats (N = 5) and C57BL/10 mice (N = 5) was immobilized and the ankle was attached to a custom-made lever arm. The femoral nerve was stimulated using subcutaneous electrodes and injury was induced using 50 lengthening ("eccentric") contractions through a 70° arc of knee motion. This protocol produces a significant and reproducible injury, with comparable susceptibility to injury in the rats and mice. This novel model shows that the quadriceps muscle provides a means to study whole muscle contractility, injury, and recovery *in vivo*. In addition to the usual benefits of an *in vivo* model, the larger size of the quadriceps facilitates *in vivo* imaging and provides a significant increase in the amount of tissue available for histology and biochemistry studies. A controlled muscle injury in the quadriceps also allows one to study a muscle, with mixed fiber types, which is extremely relevant to gait in humans and quadruped models.

Keywords

Skeletal Muscle; Injury; Quadriceps

Introduction

Muscle strains are one of the most common complaints treated by physicians and account for the majority of all sports-related injuries.^{1, 2} It is difficult to study muscle injuries in humans, as the incidence is a random event that is difficult to predict, and the clinical

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presentation varies greatly. Much of the data regarding muscle injuries has therefore been ascertained from studies on animals, which provide control over many variables and the ability to study mechanisms of injury and recovery. An *in vivo* injury provides a method for assessing contractile function without dissecting the muscle, and thus without the need to euthanize the animal under study. In the laboratory setting, investigators have used toxins,^{3, 4} lacerations,^{5, 6} freeze damage, and contusions^{7, 8} to study muscle damage, but by far the majority of muscle injuries in humans are attributable to excessive strain of an activated muscle (e.g., forceful lengthening, or “eccentric”, contractions). Submaximal lengthening contractions are used in everyday activities, but it is well known that high force lengthening contractions are associated with muscle damage and pain.⁹

One problem with many of the biological markers used to assess muscle injury, including those used in most animal studies, is that they usually do not correlate with loss of force. Since full contractile function can persist despite the presence of injury markers, loss of force may be the most valid measure of injury,¹⁰ and probably the most relevant. Most animal studies of *in vivo* or *in situ* muscle contraction utilize the dorsiflexor or plantarflexor muscles (the crural muscles), which offer the advantage of accessibility to the nerves innervating them, and the ability to compare results to previous literature.¹¹⁻¹⁵ A disadvantage of using the crural muscles is their small size (especially in mice), which not only makes the contractility assay and injury more challenging, but also provides limited amounts of harvested tissue for examination. Variation from muscle to muscle with regards to architecture, fiber type, regeneration properties, etc., also underlies the importance of studying different muscle groups. Despite a common nerve supply and insertion, the quadriceps muscle is often described as four muscles (rectus femoris, vastus lateralis, vastus intermedius and vastus medialis) due to the different points of origin. The quadriceps femoris was chosen as an alternative to crural muscles due to its large mass and multiple fiber types. For example, approximately half the fibers in the vastus intermedius are slow (Type I), but the other “heads” of the quadriceps contain primarily fast (Type II) fibers.¹⁶ An injury model using the quadriceps allows the study of a larger muscle that has a significant role in gait, even in the quadruped.

The purpose of this study was to describe a novel muscle injury model in the quadriceps that results in a significant and reproducible loss of force. Force production and force loss are compared in both mouse and rat quadriceps. To the best of our knowledge, this is the first study to utilize a controlled lengthening contraction-induced injury model in the quadriceps femoris.

Materials and Methods

Injury

All protocols were approved by the University of Maryland Institutional Animal Care & Use Committee (IACUC). We have previously described a model to injure the tibialis anterior muscle (TA) of rats and mice, which results in a significant and reproducible injury.¹⁷⁻²⁰ Here we tested a modified version of this apparatus on the quadriceps muscles. We used five age-matched male mice (Jackson Laboratory, Bar Harbor, ME C57BL/10ScSn, N = 5, 28.5 ± 1.4g) and five age-matched male rats (Charles River Laboratories, Wilmington, MA, Sprague-Dawley, N = 5, 242.3 ± 5.5 g). Animals were anesthetized (~ 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) for all experiments. Sterile ophthalmic cream (Paralube Vet Ointment, PharmaDerm, Floham Park, NJ) was applied to each eye to protect the corneas from drying. During the procedure, the animal was kept warm by use of a heat lamp.

With the animal supine, the thigh was stabilized with a fine (27 gauge) needle through the femoral condyles and the ankle was secured onto a lever arm. The quadriceps lever arms (Figure 1B and 1C) for the mice and rats were custom-made and machined from aluminum. The mouse lever arm has a total length of 32.16 mm and weighs 2.3 grams. The protruding rod adjusts from 16 - 24 mm at increments of 0.84 mm and is adjusted until it lies anterior to the ankle joint. The rat lever arm has a total length of 49.71 mm and weighs 3.7 grams. The rat ankle rod adjusts from 28 - 40 mm, also at increments of 0.84 mm. The mouse or rat lever arm connects to a stepper motor and rotates through a pre-determined motion (up to 360°, Figure 1A). For experiments, the axis of the knee was aligned with the axis of the stepper motor (model T8904, NMB Technologies, Chatsworth, CA) and a torque sensor (QWFK-8M, Sensotec) to measure torque in newton millimeters (Nmm). The femoral nerve was stimulated via subcutaneous needle electrodes (J05 Needle Electrode Needles, 36BTP, Jari Electrode Supply, Gilroy, CA). Proper electrode position was determined by a series of isometric twitches and by observing isolated knee extension in the anesthetized animal. We have compared the subcutaneous electrodes used here to subminiature electrodes (cat# 501650, Harvard Apparatus, Holliston, MA) clamped directly to the femoral nerve after surgical dissection and found no changes in maximal torque (data not shown). Impulses generated by an S48 square pulse stimulator (Grass Instruments, West Warwick, RI) were 1 ms in duration and passed through a PSIU6 stimulator isolation unit (Grass Instruments, West Warwick, RI). A custom program based on commercial software (Labview version 8.5, National Instruments, Austin, TX) was used to synchronize contractile activation and the onset of forced knee flexion. Injury resulted from 50 forced lengthening (plantarflexion) contractions through a 40° - 110° arc of motion (with full knee extension considered 0°) spaced 1 minute apart. This range of motion is identical to one used in human studies.²¹ Sham procedures (contractions without lengthening, or passive lengthening without contractions, both with knee immobilized) were also performed.

Outcome measures

In preliminary studies, we measured isometric torque every 10° through a range from 0° - 110°. Since 40° was the position that resulted in maximal torque, a maximal isometric contraction (200 ms duration) of the quadriceps was used to measure maximal torque before each lengthening contraction at this position. For each animal, maximal isometric torque was also measured 5 minutes post injury to minimize any possible contributions toward force loss from fatigue.

MRI

Magnetic resonance imaging (MRI) was performed as described.^{18, 19} High-resolution (195 μm in-plane at 1.25-mm slice thickness) dual-echo proton density and T2- weighted rapid acquisition relaxation-enhanced (RARE) MR images (TE1/TE2/TR/ETL/NEX ¼ 17.4 ms/ 52.1 ms/5000 ms/4/8) were on a 7 Tesla Bruker Biospec 7T/30 MR system (Biospec 7T/30; Bruker Biospin, Billerica, Massachusetts) with a four-channel phased array surface coil to assess muscle damage on the day of injury. Standard T2-weighted images were acquired on both mice (N=3) and rats (N=3).

Histology

Animals were euthanized 1 to 2 hours after undergoing the injury protocol and the quadriceps muscles were dissected, weighed, and snap-frozen in liquid nitrogen cooled isopentane. In a subset of animals (2 mice and 2 rats), tissue was harvested at day 4 and day 8 after injury to assess tissue changes over time. Frozen muscle tissue was sectioned at 8 μm in thickness and stained with hematoxylin and eosin using standard techniques. Sections were evaluated using a Nikon Eclipse 50i microscope (Nikon, Melville, NY) and digital

images were acquired using Image-Pro Express 6.3 software (Media Cybernetics Inc., Bethesda, MD).

Results

We modified a previously described tibialis anterior muscle (TA) injury apparatus to induce injury to the quadriceps muscles. The new custom designed lever arm is described in the methods and in Figure 1. Table 1 lists the body weights and quadriceps muscle masses of animals used in this study. The mass of the TA from the same animals is also provided for reference.

The uninjured rat quadriceps generated a mean peak torque of 58.3 ± 8 Nmm (Figure 2B). This is approximately an 8-fold increase compared to the mean peak torque generated by the mouse quadriceps (7.2 ± 2 Nmm, Figure 2A). Both animals showed a significant loss in force after injury; the mouse quadriceps had an $82 \pm 1\%$ loss in force, compared to a $73 \pm 4.4\%$ loss in the rat quadriceps (Figure 2C). Figure 2D shows the percent of isometric torque lost after each lengthening contraction for both mouse and rat.

Approximately 1-2 hours after injury, *in vivo* MRI was performed as described in the Methods. In the injured quadriceps, there was an increase in the signal intensity on T2-weighted *in vivo* MRI, which was presumably due to hemorrhage and edema¹⁹ and was greatest distally near the muscle-tendon junction (MTJ) (Figure 3B and 3D). Interestingly, this increased T2 signal was confined primarily to the vastus medialis and vastus lateralis. The more proximal aspects of the quadriceps did not show any detectable changes.

Histological examination with H&E was performed to determine if morphological changes accompanied the observed loss of force (Figure 4). Uninjured muscle contained peripherally nucleated muscle fibers and limited variation in fiber size, consistent with healthy muscle. Approximately 1-2 hours after injury (Figure 4, Day 0), foci of mild lymphocytic inflammation were found surrounding small perimysial blood vessels, without evidence of myonecrosis, fibrosis, or other inflammation. These foci of inflammation were readily identifiable in the distal aspect of the rat quadriceps, but less so in the mouse quadriceps. In the more proximal areas of the muscle that did not show inflammation, the histology of the muscle appeared normal in both mouse and rat. In contrast to the mild inflammation observed immediately after injury, inflammation was more easily detected several days after injury, and began to resolve within one week for both mouse and rat.

Discussion

To date, most animal studies using contraction-induced injury do so using muscle groups that move the ankle. Some studies have described injury in the quadriceps; however, they use methods such as downhill running and treadmill exercise.²²⁻²⁴ Such methods may cause muscle damage, but also increase the possibility of unwanted variables including partial fiber recruitment or variable injury. We describe an animal injury model where variables including muscles recruited, amount of contraction, arc of motion (degree of muscle lengthening), and angular velocity (speed of muscle lengthening) are all controlled. Using this model, we have shown not only that isolated knee extension is possible with properly placed electrodes, but also that one can produce a reliable and significant injury to the quadriceps muscles (Figure 2C). Loss of force may be the most valid measure of injury,¹⁰ and since many biological markers can exist without a change in contractile function, we operationally define injury as a significant loss of force that does not recover after a short period of rest. Although the focus of this work was simply to describe the model and focus

on contractile data, we also include some additional findings (Figures 3 and 4) to describe some changes in histology and imaging.

Isometric contractions were performed at an optimal length of 40^0 . This point in the range of motion was determined after generating torque-length curves for the quadriceps of both mice and rats. This is presumably due to the optimum length (L_0) of the muscle, but other factors such as the moment arm of the quadriceps tendon at this angle need to be considered. This model demonstrated similar relative losses in force for both mice and rats with continued repetitions (Figure 2D). We used a large number of maximal repetitions because we did not know how the quadriceps would respond in this model. As seen in Figure 2D, both mouse and rat quadriceps were significantly injured early on, with very little increase in loss of force after ~ 25 repetitions. Toward the end of the injury protocol, there was a greater loss in force in the mouse compared to the rat, although the exact mechanisms for this difference are unknown.

We examined MRI and H&E staining to characterize the muscle damage. MRI provides an *in vivo*, non-invasive measurement of muscle disruption.^{19, 25, 26} The visible damage was, surprisingly, minimal throughout most of the quadriceps muscles. The images we examined showed an increased T2 signal in the distal aspect of the muscle, near the MTJ. The MTJ has been cited by some as the primary area of damage after a contraction-induced muscle injury,^{27, 28} but this is not without debate.¹⁹ Whether or not the muscle belly versus the MTJ is injured could also depend on the muscle, its architecture, and how it is being used. This could be due to the myofibers' proximity to the moving joint, where the strain is increased. For example, it has been suggested that the displacement in the proximal and distal halves of the quadriceps aponeuroses is different during knee flexion-extension tasks.²⁹

Histological evaluation of injured and uninjured muscles found only mild focal lymphocytic inflammation around perimysial blood vessels 1-2 hours after injury (Figure 4, Day 0). This was also confined to the distal third of the muscles and more prevalent in rat muscles than mouse muscles. This inflammation was consistent with the MRI findings, but the limited amount of inflammation and visible myofiber damage was likely limited due to the short interval between the injury and the sacrifice of the animal. Because the inflammatory response peaks 24-48 hours after injury,²⁸ we examined the histology at later time points and our findings confirm this delayed response.

This study had a relatively small sample size, and future experiments are needed to study recovery over time. Further investigation and increased sample sizes are also needed to confirm location of damage in the mouse and rat quadriceps, especially with different injury protocols. Changes in the biomechanical parameters, such as angular velocity, arc of motion, the number of repetitions, and timing of muscle activation can all affect the loss of force and amount of histological damage.³⁰ Such issues, and the variety of models used to induce injury, are an ongoing problem in trying to interpret muscle injury literature. However, the focus of this work was to describe the quadriceps contraction-induced injury model. We have provided a proof-of-principal that the quadriceps muscle can be used to study quadriceps contractility and muscle injury *in vivo*, which we hope will provide the basis for further studies into the mouse and rat quadriceps muscles contractile function. It is clear that, at least with the biomechanical parameters used in this study, 20 to 30 maximal lengthening contractions is sufficient to induce significant and consistent injury to the quadriceps muscles.

We have also provided data to show that mouse and rat skeletal muscle do not respond identically to the same contraction-induced injury. To fully describe the inflammation response, the ideal follow-up would involve daily assessment of MRI and histology. Our

model would also be useful to measure contractile force in the quadriceps muscles pre- and post-running, which is often used as a modality to induce injury.³¹

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Figure 1. Injury model and lever arm specifications

The cartoon describes the injury model for the quadriceps. **A.** Animals are positioned supine on a raised bed (not shown) with a fine needle through the femoral condyles. The needle is secured into the thigh stabilizer, which can be adjusted in multiple planes and then stabilized. The needle/thigh stabilizer complex is aligned with the axis of the torque cell (dotted red line). The lever arm is adjusted so that the protruding arm is positioned over the anterior aspect of the ankle joint. Electrodes (not shown) are used to stimulate the quadriceps muscle. The isometric torque (toward extension) is recorded. For lengthening contractions, a stepper motor superimposes flexion (red arrow) onto a maximally contracting quadriceps muscle. **B.** Mouse lever arm has a total length of 32.16 mm and weighs 2.3 grams. Protruding ankle rod adjusts from 16 - 24 mm at increments of 0.84 mm. **C.** Rat lever arm has a total length of 49.71 mm and weighs 3.7 grams. The rat ankle rod adjusts from 28 - 40 mm also at increments of 0.84 mm. Both lever arms connect to the stepper motor and rotate through a pre-determined motion (up to 360°).

Figure 2. Torque data from *in vivo* apparatus

Representative trace recordings of torque during lengthening contractions in the mouse (**A**) and rat (**B**). Specifically, muscles were stimulated maximally for 200 milliseconds to induce a maximal isometric contraction before lengthening by the lever arm through a 70° arc of motion and at an angular velocity of 900°/s. The representative trace recordings show peak isometric (1) and eccentric (2) torque in newton millimeters (Nmm) from a single repetition. **C.** The histogram shows mean torque production before and after injury from both the mouse and rat quadriceps muscles. The rat showed an approximately 8-fold torque production over that of the mouse. Both animals showed a significant (* = $p < 0.05$) loss of force (82 ± 1% in the mouse and 73 ± 4.4% in the rat) after 50 repetitions. **D.** The histogram shows the mean percent loss of torque with each repetition in the mouse and rat over 50 repetitions.

Figure 3. *In vivo* imaging

We used magnetic resonance imaging (MRI) to examine the quadriceps in the mouse (**A-B**) and rat (**C-D**) quadriceps muscles. Representative T-2 weighted images of the healthy (**A**, **C**) versus injured (**B**, **D**) quadriceps are shown. The primary location of increase signal intensity was in the distal quadriceps, close to the muscle-tendon junction (MTJ). Asterisk indicates the vastus intermedius for purposes of orientation. Interestingly, the majority of damage was localized to the vastus medialis and lateralis, while the vastus intermedius was generally spared (belly of the rectus femoris is not seen in these distal cross-sections). The MRI was repeated for 3 mice and 3 rats with similar findings (mild T2 changes confined to the distal aspect of the quadriceps).

Figure 4. Histology

Standard H&E staining of cross-sections of un-injured and injured quadriceps in the mouse and rat. The injured tissue was assessed immediately after injury (Day 0), 4 days after injury (Day 4) and 8 days after injury (Day 8). Uninjured muscle contained peripheral nuclei and limited variation in fiber size, consistent with healthy muscle. Injured muscle at Day 0 showed foci of mild lymphocytic inflammation (arrows) surrounding small perimysial blood vessels, but no evidence of myonecrosis, fibrosis, or other inflammation. Such findings were magnified at a later time point (Day 4); however inflammation was unremarkable by Day 8. Bar = 50 microns

Table 1**Tissue weights**

Table 1 shows average tissue weight for all animals. Note the average rat muscle mass and body weight is about 10 fold greater than for mice.

	Mouse	Rat	Fold Increase
Tibialis Anterior	50 ± 7 mg	510 ± 60 mg	10.2
Non-Injured Quadriceps	263 ± 25 mg	2.4 ± 0.35 g	9.1
Injured Quadriceps	269 ± 27 mg	2.67 ± .19 g	9.9
Body Weight	28.5 ± 1.4 g	242.3 ± 5.5 g	8.5