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The developing shoulder has a limited capacity to recover after a short duration of neonatal paralysis

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ABSTRACT

Mechanical stimuli are required for the proper development of the musculoskeletal system. Removal of muscle forces during fetal or early post-natal timepoints impairs the formation of bone, tendon, and their attachment (the enthesis). The goal of the current study was to examine the capacity of the shoulder to recover after a short duration of neonatal rotator cuff paralysis, a condition mimicking the clinical condition neonatal brachial plexus palsy. We asked if reapplication of muscle load to a transiently paralyzed muscle would allow for full recovery of tissue properties. CD-1 mice were injected with botulinum toxin A to paralyze the supraspinatus muscle from birth through 2 weeks and subsequently allowed to recover. The biomechanics of the enthesis was determined using tensile testing and the morphology of the shoulder joint was determined using microcomputed tomography and histology. A recovery period of at least 10 weeks was required to achieve control properties, demonstrating a limited capacity of the shoulder to recover after only two weeks of muscle paralysis. Although care must be taken when extrapolating results from an animal model to the human condition, the results of the current study imply that treatment of neonatal brachial plexus palsy should be aggressive, as even short periods of paralysis could lead to long-term deficiencies in enthesis biomechanics and shoulder morphology.

1. Introduction

Mechanical cues generated by muscular contraction are essential to the development and growth of the musculoskeletal system. Numerous studies have shown that the physical loading environment influences the formation of bone architecture and density (Goldstein, 1987; Goldstein et al., 1991; Mullender and Huiskes, 1995; Rodriguez et al., 1992; Wolff, 1892). Furthermore, connective tissues quickly lose mass and strength in the absence of muscle loading (Amiel et al., 1982; Woo et al., 1982). A reduction of muscle load during postnatal development can lead to severe functional defects in bone, tendon, and cartilage. For example, neonatal brachial plexus palsy (NBPP), a common complication of childbirth that causes paralysis in approximately 1 in 250 births, can lead to persistent defects and loss of function in the affected shoulder (Adler and Patterson, 1967; Greenwald et al., 1984). Although paralysis is often temporary, even seemingly short durations of lost muscle force can result in serious long-term complications (Adler and Patterson, 1967; Waters, 1999).

Animal models of NBPP have begun to elucidate the effects of unloading on the developing shoulder joint. Denervation of the upper trunk of the brachial plexus in neonatal mice led to contractures of the elbow as well as internal rotation of the shoulder and shortening of the biceps, brachialis, and rotator cuff muscles (Kim et al., 2009 and 2010; Nikolaou et al., 2011; Weekley et al., 2012). Defects in bone development were seen in the humeral head, consistent with the clinical condition (Kim et al., 2010; Nikolaou et al., 2011; Thomopoulos et al., 2007; Weekley et al., 2012). Formation of the critical connective tissue necessary for force transfer between tendon and bone was also severely impaired; in the absence of muscle load, the fibrocartilage at the tendon enthesis was malformed, mineralization was impaired, and tendon enthesis biomechanics were reduced (Kim et al., 2009 and 2010; Schwartz et al., 2013; Thomopoulos et al., 2007). It remains unclear if the defects that develop after NBPP are permanent, or if the shoulder has a capacity to recover after a short duration of neonatal paralysis.

The purpose of the current study was to determine the effect of reloading after a short duration of paralysis on the post-natal development of muscle, tendon, fibrocartilage, and bone in the shoulder. Our objective was to understand if the reapplication of load to a transiently paralyzed supraspinatus muscle would

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allow for complete recovery of tissue properties. The capacity of the neonatal shoulder to recover after paralysis has important implications for the treatment of NBPP. We hypothesized that long durations of recovery would be necessary to rescue the effects of neonatal muscle unloading.

2. Methods

2.1. Study design

All animal procedures were approved by the Animal Studies Committee at the Washington University in St. Louis. CD-1 mice ($N=170$) were divided into three experimental groups (BtxA, Recovery, and Normal) at four time points (4, 8, 12, and 16 weeks) as shown in Fig. 1. Botulinum toxin A (BtxA) (BOTOX, Allergan Inc) was injected into the left supraspinatus muscles and saline was injected into the right supraspinatus muscles of the recovery and paralyzed groups within 24 h of birth. Continuous BtxA injections were given to the paralyzed group until sacrifice, based on an injection schedule developed previously (Thomopoulos et al., 2007). An initial injection of 0.15 U was given on postnatal day 1 (P1). Subsequent injections of 0.2 U were given biweekly through postnatal day 21 (P21). At P21, injections were lowered to 0.15 U. At P28, the frequency of 0.15 U injections was reduced to once weekly until sacrifice. BtxA is well established as an agent for dramatically reducing force generated by a muscle (Aoki, 2001; Fortuna et al., 2011; Longino et al., 2005; Manske et al., 2011; Stone et al., 2007; Yaraskavitch et al., 2008). The recovery group received only 2 weeks of paralytic and allowed to recover 2, 6, 10, and 14 weeks. A normal group was age matched and received no injections. Animals were separated into 5 groups per time point (paralyzed, paralyzed control, recovery, recovery control, and control). Mice were weighed and monitored throughout the experiment to ensure paralysis.

2.2. Microcomputed tomography (μCT)

Upon sacrifice, humerus–supraspinatus muscle specimens designated for μCT and histology were fixed in 4% paraformaldehyde overnight and subsequently dehydrated to 70% ethanol with a series of graded ethanols. Morphological scans of the bone, muscle, and tendon were performed using a μCT scanner ($N=5$ per group) (Scanco μCT 40; SCANCO Medical AG, Bassersdorf, Switzerland) with an isotropic voxel resolution of $\sim$20 μm. Samples were suspended and scanned in air at 55 kVp, 145 μA, and a 99 ms integration time. The trabecular region of interest proximal to the humeral head growth plate was analyzed using an automated thresholding algorithm. Muscle–tendon volume was calculated for each sample. Bone Volume (BV), Total Volume (TV), Bone Volume to Total Volume ratio (BV/TV), Tissue Mineral Density (TMD), Trabecular Number (Tb.N.), Trabecular Thickness (Tb.Th.), Trabecular Separation (Tb.Sp.), and Muscle Volume (MV) were measured. In addition, samples for biomechanics were scanned to determine the cross sectional area (CSA) of the tendon (measurement made at the point of minimum tendon width).

2.3. Histology

After scanning, samples were dehydrated through 100% ethanol and embedded in either paraffin ($N=3$ per group) or methylmethacrylate ($N=2$ per group). 5 μm thick sections were cut along the coronal plane. Toluidine Blue stain was used to evaluate enthesis morphology and fibrocartilage extent. Tartrate-resistant acid phosphatase (TRAP) staining (Sigma-Aldrich) was performed as previously described (Zhao et al., 1999) and used to identify osteoclasts. A semi-quantitative analysis was performed at the insertion with various grading scales, explained in the legends of Tables 1 and 2, to determine vascularity, muscle atrophy and fatty infiltrate, chondrocyte and osteoclast cellularity, and enthesis maturity and organization. All counts were obtained with a 40 $\times$ objective by a pathologist (NH).

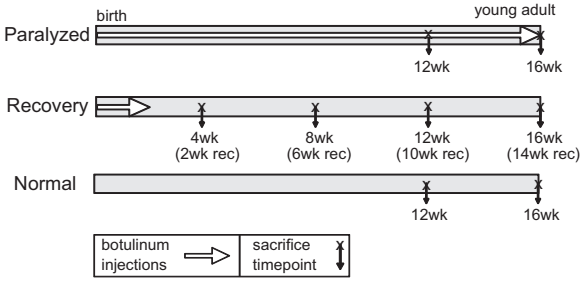

Fig. 1. Study design. Mice were divided into 5 groups: normal, recovery control, recovery, paralyzed control and paralyzed. The paralyzed group received continuous BtxA injections from birth while the recovery group received 2 weeks of BtxA injections followed by varying recovery periods of 2, 6, 10, and 14 weeks. The contralateral shoulder was used as an internal control and received saline injections. The normal group consisted of age matched animals that did not receive injections. A total of 4 time points were examined (4, 8, 12, and 16 weeks).

Table 1
Histology grading of cellular response. Cellularity/PMN/Mono: + < 20 per HPF, ++ 21–50 per HPF, +++ > 50 per HPF; vascularity near tendon enthesis: + > 5 vascular profiles above normal score, ++ > 10 vascular profiles above normal; osteoclasts (TRAP positive) percent of perimeter of epiphyseal surface: + 4–8%, ++ 8–12%, +++ > 12%; PMN: polymorphonuclear cells; Mono: monocytes/macrophages; HPF: high powered field (40 $\times$ objective).

Age (week)	Group	Cellularity	Vascularity	PMN	Mono	Osteoclasts
4	Recovery ctrl	+	+	+	++	+
	Recovery	++	+	+	++	+++
8	Recovery ctrl	+	+	+	+	++
	Recovery	+	+	+	+	++
12	Normal	+	+	+	+	+
	Recovery ctrl	+	+	+	+	++
	Recovery	+	+	+	+	++
	Paralyzed ctrl	+	+	+	+	+
	Paralyzed	++	+	+	++	+
16	Normal	+	+	+	+	+
	Recovery ctrl	+	+	+	+	+
	Recovery	+	+	+	+	+
	Paralyzed ctrl	+	+	+	+	+
	Paralyzed	+++	++	+	++	++

2.4. Biomechanics

After sacrifice, biomechanics samples were frozen until the date of testing ($N=10$–12 per group). Sample randomization was performed to eliminate day-to-day testing variation. Once thawed, dissected, and microCT scanned for CSA, the humeri were potted in epoxy in a plastic vial and the tendon was gripped with a KimWipe and superglue in a saw-tooth grip. Tests were performed in a 37 °C phosphate buffered saline bath. A fine mist of Verhoeff stain was sprayed onto the tendon to aid optical tracking of strain (Illunis VMV-8M Camera; Illunis LLC, Minnetonka, MN). Strain was calculated optically (Qualisys Video Analysis; Qualisys AB, Gothenburg, Sweden) between the tendon grip and the humeral head. Stress was calculated as force divided by initial cross sectional area. Failure mode was determined visually. Force–deformation plots were used to determine maximum load and stiffness. Stress–strain plots were used to determine strength (i.e., maximum stress), yield strain/stress, and modulus. Yield strain and stress were determined as the point where the instantaneous slope dropped to 75% of the maximum. Modulus and stiffness were defined as the linear portions of the stress–strain and load–deformation curves, respectively, prior to yielding. Additional samples were analyzed and added from a previous study at 4 and 8 week timepoints for the normal, paralyzed control, and paralyzed groups (Schwartz et al., 2013).

2.5. Statistics

To compare between groups and over time, a two-factor ANOVA was performed followed by Fisher's least square's differences post-hoc test where appropriate. Significance was set to $p<0.05$; trends are also indicated for $p<0.1$.

3. Results

3.1. Gross observations

Paralysis of the supraspinatus muscle was observed within the first 3 days of BtxA shoulder injections, as determined by a lack of shoulder abduction and external rotation. Continued paralysis was observed in the groups of animals that received additional BtxA injections. Once injections were stopped, shoulder mobilization (i.e., abduction and external rotation) was apparent within 7 days.

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