Published in final edited form as:

J Biomech. 2014 July 18; 47(10): 2314–2320. doi:10.1016/j.jbiomech.2014.04.036.

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 micro computed 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.

Keywords

neonatal brachial plexus palsy; enthesis; rotator cuff; supraspinatus tendon; botulinum toxin A

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Conflict of Interest Statement

The authors of this manuscript have no conflicts of interest related to the content of the study.

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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., 2010; Kim et al., 2009). 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., 2010; Kim et al., 2009; 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 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.

Methods

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 Figure 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 hours 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 the proper degree of paralysis.

Micro computed tomography (μ CT)

Upon sacrifice, humerus-supraspinatus muscle specimens designated for μ CT 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 μ CT scanner (N=5 per group) (Scanco μ CT 40; SCANCO Medical AG, Bassersdorf, Switzerland) with an isotropic voxel resolution of $\sim 20 \mu\text{m}$. Samples were suspended and scanned in air at 55 kVp, 145 μA , and a 99ms 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. Additionally, samples for biomechanics were scanned to determine the cross sectional area (CSA) of the tendon (measurement made at the point of minimum tendon width).

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 1–2, to determine vascularity, muscle atrophy and fatty infiltrate, chondrocyte and osteoclast cellularity, and enthesis maturity and organization. All counts were made with a 40x objective by a pathologist (NH).

Biomechanics

After sacrifice, biomechanics samples were frozen until the date of testing (N=10–12 per group). Sample randomization was preformed 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 (Illuniv VMV-8M Camera; Illuniv 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).

Statistics

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

Results

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. No significant differences were observed in mouse weight when comparing groups at a particular timepoint.

MicroCT Morphology

Continuous paralysis of the shoulder led to severe defects in humeral head bone outcomes and supraspinatus muscle size (paralyzed group in Figures 2 and S1 and Table S1). At 12 and 16 weeks, there were significant changes in nearly all parameters measured compared to the contralateral paralyzed controls and the recovery groups. Animals transiently paralyzed for the first two postnatal weeks showed persistent defects in trabecular architecture through 8 weeks, and recovery of some properties by 12 weeks (recovery group in Figures 2 and S1). However, some defects persisted in the recovery group at 16 weeks compared to normal and recovery controls (e.g., trabecular number, humeral head bone volume; Figures 2 and S1 and Table S1). Muscle volume recovery showed similar trends, although the differences were often not statistically significant (Figure 2). Bone and muscle morphologic measures for the normal group were similar to those of saline controls.

Biomechanics

All samples failed at or near the tendon entheses. Biomechanical properties increased with time in all groups, consistent with rapid growth and maturation of the tendon enthesis through the first 16 postnatal weeks (Figure 3 and Table S2). Continuous paralysis of the shoulder led to significant decreases in structural (maximum load, stiffness) and material

(strength, modulus) properties at all timepoints compared to controls (Figure 3; note that normal, paralyzed control, and paralyzed group data for 4 and 8 weeks are taken from a previous publication (Schwartz et al., 2013)). Animals transiently paralyzed for the first two postnatal weeks showed persistent defects in mechanical properties at 4 weeks compared to normal, and these differences were generally resolved by 8 weeks (Figure 3).

Histology

Continuous paralysis of the shoulder led to increased osteoclast numbers, increased cellularity, an atrophied supraspinatus muscle with fatty infiltrate, and an immature and disorganized enthesis compared to control groups (Tables 1–2, Figures 4–5). Animals transiently paralyzed for the first two postnatal weeks showed recovery of some of these defects through 16 weeks of postnatal development. Enthesis immaturity and disorganization and a poor mineral transition was seen in the recovery groups compared to controls, even at 16 weeks (Table 2, Figure 4). Osteoclast numbers were higher in the recovery groups through 12 weeks compared to normal, but these differences were resolved by 16 weeks (Table 1, Figure 5). No clear differences were evident in chondrocyte morphology or vascularity between groups. Inflammatory cell numbers were low in all groups.

Discussion

The rotator cuff was paralyzed for the first two weeks of post-natal development using BtxA and subsequently allowed to recover for up to 4 months. The paralysis model mimicked the postnatal paralysis seen in human infants suffering from NBPP and provided insight into the development and recovery potential of the shoulder subjected to this pathology. The results confirmed previous studies showing the deleterious effects of neonatal paralysis (Das et al., 2011; Kim et al., 2010; Kim et al., 2009; Nikolaou et al., 2011; Schwartz et al., 2013; Thomopoulos et al., 2007; Weekley et al., 2012) and demonstrated that these effects can be reversed given enough recovery time. Surprisingly, some defects remained after long-term recovery, despite a relatively short period of paralysis.

During the first two weeks of post-natal development, the critical structural components of the enthesis (e.g., a fibrocartilage interface with a gradient in mineral) are initiated (Schwartz et al., 2012), setting the stage for effective transfer of load between the muscle and bone. In the current study, we paralyzed the shoulder during these critical first two weeks, expecting that persistent defects may result from the loss of early loading. Importantly, previous studies have shown that there are no defects in bone architecture or enthesis morphology after 2 weeks of postnatal paralysis, and that at least three weeks of paralysis are necessary for differences to develop (Das et al., 2011; Kim et al., 2009; Thomopoulos et al., 2007). Furthermore, a previous study demonstrated that soft tissue contractures were completely resolved after 2 weeks of paralysis followed by 14 weeks of recovery, whereas 4 weeks of paralysis followed by 12 weeks of recovery resulted in only partial resolution of the soft tissue contractures (Kim et al., 2009). In the current study, we found that tendon enthesis mechanical properties were normal by the longest recovery period. In contrast, 2 weeks of paralysis was sufficient to cause persistent defects in bone

architecture and enthesis morphology. It is likely that genetic factors were perturbed during the early period of unloading, leading to tissue-level changes that did not recover at later timepoints.

Paralysis led to defects in muscle volume, tendon biomechanics, fibrocartilage morphology, and bone architecture and morphology. Supraspinatus muscle volume was decreased due to unloading, consistent with previous work (Das et al., 2011; Schwartz et al., 2013; Thomopoulos et al., 2007). This was accompanied by fatty changes and atrophy of the muscle. The biomechanics of the tendon insertion were also significantly affected by unloading. Similar to a previous report at earlier timepoints, paralysis led to significantly lower material and structural properties of the tendon enthesis (Schwartz et al., 2013). Based on microCT analysis, humeral head bone architecture was dramatically affected by muscle unloading, with fewer trabeculae spaced further apart and lower bone volume. This was likely due to increased osteoclast numbers, as measured on histologic sections in the current study and previously (Thomopoulos et al., 2007). The morphology of the enthesis after 12 and 16 weeks of paralysis showed the highest levels of immaturity, disorganization, and fibrocartilage mineral transition compared to all other groups. The tissue changes seen in the recovery group at similar time points were less severe than the fully paralyzed group, indicating a temporal opportunity for reversibility of defects.

Local paralysis of the supraspinatus muscle was confirmed through daily observations of the muscular range of motion. Contralateral saline controls maintained full mobility throughout the study. Decreases in supraspinatus muscle volume provided further evidence for a decrease in force across the BtxA shoulder. BtxA pharmacokinetics are age and species dependent. Consequently, younger mice recovered faster from BtxA injections compared to older mice, and the duration of peak effect BtxA was much shorter in the neonatal mouse (on the order of days) than in adults (on the order of weeks) (Carruthers et al., 2004; Matarasso and Matarasso, 2003; Wong, 1998, 2003; Wong et al., 2002). The cause of a faster recovery in younger mice is not known; however it is speculated that neonatal mice have a faster ability to generate new and functional nerve terminals (Bambrick and Gordon, 1989). Regardless, the shoulders of mice in the recovery group were fully mobile within a few days of cessation of BtxA injections. Furthermore, neurotomy of the upper trunk of the brachial plexus in neonatal mice resulted in outcomes similar to those induced by BtxA chemical denervation, supporting its use in the current study for muscle unloading (Kim et al., 2010).

There were a number of limitations to our study. Animals with paralyzed shoulders demonstrated unstable gait and likely had decreased access to food, although animal weights were not significantly different among groups. This limitation was partly alleviated by the paired contralateral control for all paralyzed groups, which allowed for within-animal paired statistical comparisons. Any trauma caused by needle insertion and fluid injection was also controlled for with the contralateral shoulder, which received saline injections. A further limitation is the lack of joint-level function analysis during paralysis and during recovery. Although technically very difficult, future studies should evaluate the *in vivo* kinematics of the shoulder, as has been done previously for the rat shoulder (Sarver et al., 2009). Finally, as with any animal model, there are significant physiologic and anatomic differences

between mice and humans. Mice have shorter life spans, faster growth and maturation rates, continued growth throughout their lifetime, and quadruped posture and gait. However, rodents are appropriate animals to use for the study of the shoulder due to similarities in bony structure and musculature (Soslowsky et al., 1996).

In conclusion, this study demonstrated that shoulder defects get progressively worse with longer durations of paralysis and that these defects can be mostly rescued given long durations of recovery. Importantly, some morphological defects remained, even after 16 weeks of recovery. These results suggest that that early return of muscle load is critical to prevent permanent functional impairment in infants with NBPP. However, further study is necessary to verify these murine animal model results in larger animal models and in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Doreen Sheen for assistance in data analysis. The study was funded by the National Institutes of Health (R01 AR055580).

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Figure 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 two, six, ten, and fourteen weeks. The contralateral shoulder acted as an internal control and received saline injections whenever an injection was given. 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).

Figure 2.

Bone and muscle morphology was examined using microCT. Continuous paralysis (red bars) resulted in bone and muscle defects. Transient paralysis (pink bars) resulted in bone defects at early timepoints that resolved for some parameters by 12 weeks. Solid lines over bars represent $p < 0.05$ and dashed lines over bars represent $p < 0.1$.

Figure 3.

Tendon enthesis structural (maximum load, stiffness) and material (strength, modulus) properties were examined using tensile tests to failure. Continuous paralysis (red bars) resulted in mechanically deficient tendon entheses at all timepoints. Transient paralysis (pink bars) resulted in lower mechanical properties at 4 weeks compared to normal (green bars); these differences resolved with increased recovery time. Solid lines over bars represent $p < 0.05$ and dashed lines over bars represent $p < 0.1$.

Figure 4.

Representative toluidine blue-stained sections of the humeral head-supraspinatus tendon enthesis are shown for all groups. Increased purple staining intensity indicates higher levels of proteoglycans (normally present at the enthesis but not in the tendon and bone). A demarcation line (the tidemark) is often evident at the tendon-to-bone interface. Continuous paralysis led to an immature and disorganized enthesis compared to control groups. Animals transiently paralyzed showed recovery of these defects by 16 weeks. t: tendon, b: bone.

Figure 5.

Representative tartrate-resistant acid phosphatase stained sections of the humeral head-supraspinatus tendon enthesis are shown. Continuous paralysis of the shoulder led to increased osteoclast number. Animals transiently paralyzed demonstrated high osteoclast numbers at 4 weeks, with a progressive decline through 12 weeks. t: tendon, b: bone.

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 (40x objective).

Age	Group	Cellularity	Vascularity	PMN	Mono	Osteoclasts
4 Week	Recovery Ctrl	+	+	+	++	+
	Recovery	++	+	+	++	+++
8 Week	Recovery Ctrl	+	+	+	+	++
	Recovery	+	+	+	+	++
12 Week	Normal	+	+	+	+	+
	Recovery Ctrl	+	+	+	+	++
	Recovery	+	+	+	+	++
	Paralyzed Ctrl	+	+	+	+	+
16 Week	Paralyzed	++	+	+	++	+
	Normal	+	+	+	+	+
	Recovery Ctrl	+	+	+	+	+
	Recovery	+	+	+	+	+
	Paralyzed Ctrl	+	+	+	+	+
16 Week	Paralyzed	+++	++	+	++	++

Histology grading of enthesis, muscle, and mineral. Enthesis immaturity was evaluated on degree of metachromasia: + high (normal level) of purple, ++ intermediate level of purple, +++ little to no purple staining; Enthesis disorganization was evaluated based on alignment and dispersion of cells in fibrocartilage and mineralized cartilage: + normal level of organization, ++ intermediate level of dispersion, +++ high levels of dispersion; Hypertrophic chondrocytes: + < 10, ++ 10–15, +++ > 15; Muscle atrophy: + little to no atrophy, ++ intermediate level of atrophy, +++ high levels of atrophy; Mineral transition was evaluated based on presence or absence of dividing line (“tidemark”) between fibrocartilage and mineralized cartilage: + tidemark present (normal), ++ tidemark not present (abnormal).

Table 2

Age	Group	Enthesis Immaturity	Enthesis Disorganization	Hypertrophic Chondrocytes	Muscle Atrophy	Mineral Transition
4 Week	Recovery Ctrl	+	+	+	+	+
	Recovery	+	++	++	+	++
8 Week	Recovery Ctrl	+	+	+	+	+
	Recovery	++	+++	++	+	++
12 Week	Normal	+	++	+	+	+
	Recovery Ctrl	+	++	+	+	+
	Recovery	+	+	++	+	+
	Paralyzed Ctrl	+	++	+	+	+
	Paralyzed	++	++	+	+++	++
16 Week	Normal	+	+	+	+	+
	Recovery Ctrl	+	+	+	+	+
	Recovery	++	++	++	+	++
	Paralyzed Ctrl	+	++	+	+	+
	Paralyzed	+++	++	++	+++	++