Impaired Growth of Denervated Muscle Contributes to Contracture Formation Following Neonatal Brachial Plexus Injury

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Background: The etiology of shoulder and elbow contractures following neonatal brachial plexus injury is incompletely understood. With use of a mouse model, the current study tests the novel hypothesis that reduced growth of denervated muscle contributes to contractures following neonatal brachial plexus injury.

Methods: Unilateral brachial plexus injuries were created in neonatal mice by supraclavicular C5-C6 nerve root excision. Shoulder and elbow range of motion was measured four weeks after injury. Fibrosis, cross-sectional area, and functional length of the biceps, brachialis, and subscapularis muscles were measured over four weeks following injury. Muscle satellite cells were cultured from denervated and control biceps muscles to assess myogenic capability. In a comparison group, shoulder motion and subscapularis length were assessed following surgical excision of external rotator muscles.

Results: Shoulder internal rotation and elbow flexion contractures developed on the involved side within four weeks following brachial plexus injury. Excision of the biceps and brachialis muscles relieved the elbow flexion contractures. The biceps muscles were histologically fibrotic, whereas fatty infiltration predominated in the brachialis and rotator cuff muscles. The biceps and brachialis muscles displayed reduced cross-sectional and longitudinal growth compared with the contralateral muscles. The upper subscapularis muscle similarly displayed reduced longitudinal growth, with the subscapularis shortening correlating with internal rotation contracture. However, excision of the external rotators without brachial plexus injury caused no contractures or subscapularis shortening. Myogenically capable satellite cells were present in denervated biceps muscles despite impaired muscle growth in vivo.

Conclusions: Injury of the upper trunk of the brachial plexus leads to impaired growth of the biceps and brachialis muscles, which are responsible for elbow flexion contractures, and impaired growth of the subscapularis muscle, which correlates with internal rotation contracture of the shoulder. Shoulder muscle imbalance alone causes neither subscapularis shortening nor internal rotation contracture. Impaired muscle growth cannot be explained solely by absence of functioning satellite cells.

Clinical Relevance: The current study suggests a primary role for impaired growth of denervated muscle in contracture pathogenesis following neonatal brachial plexus injury. These findings provide a novel avenue for investigation toward development of rational contracture prevention and treatment strategies.

Neonatal brachial plexus injury occurs in one to three per 1000 live births[1], leaving permanent neurological sequelae in 20% to 30% of affected children[2]. Secondary joint contractures, most notably an internal rotation contracture of the shoulder and flexion contracture of the elbow, are common in these children[3]. These disabling contractures lead to dysplastic changes in adjacent bones, especially at the shoulder[4], and are the most common indication for surgery following neonatal brachial plexus injury.

The etiology of the contractures following neonatal brachial plexus injury is incompletely understood. The internal rotation contracture of the shoulder is believed to result from muscle imbalance between functioning internal rotators and paralyzed external rotators, leading to static internal rotation joint posturing and ultimate joint contracture[5,6]. This theory is supported by a magnetic resonance imaging (MRI) study in patients with neonatal brachial plexus injury, demonstrating a correlation between the degree of shoulder contracture and

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the ratio of cross-sectional area between internal rotator (pectoralis major and subscapularis) and external rotator (infraspinatus and teres minor) muscles[7]. Conversely, other MRI studies have demonstrated that the degree of contracture correlates only with atrophy of the subscapularis muscle, an internal rotator of the shoulder, and not with atrophy of the external rotator muscles[8,9]. In addition, muscle imbalance cannot explain the elbow flexion contracture that develops in the setting of elbow flexor paralysis. In fact, the elbow flexion contracture has been shown to correlate on MRI only with atrophy of the brachialis muscle, an elbow flexor[10]. Further complicating the understanding of contracture pathogenesis is the finding that older children and adults who sustain brachial plexus injuries outside the neonatal period are plagued more by glenohumeral instability than by internal rotation contracture[11,12].

The current study tests the novel hypothesis that contractures following neonatal brachial plexus injury are caused by functional shortening of denervated muscle itself, resulting from impairment of longitudinal muscle growth during the neonatal period of rapid skeletal growth. Previous studies with use of lower limb models of neonatal nerve injury have demonstrated impairment of muscle growth in mass, fiber number, and fiber diameter following denervation[13-15]. However, the effect of neonatal denervation on muscle functional length and joint contracture has not been investigated. Similarly, previous research[16] has demonstrated a loss of muscle stem cells, known as satellite cells[17], following neonatal nerve injury, although the relationship of this satellite cell depletion to muscle growth and joint contracture has not been investigated.

Animal models of neonatal brachial plexus injury have been recently reported in rats[18] and mice[19], producing shoulder deformities similar to those seen in humans. We developed a similar surgical model of neonatal brachial plexus injury in newborn mice to specifically study the relationships between contracture development, impaired muscle growth, and satellite cell depletion. We also developed a surgical model of muscle imbalance at the shoulder to formally test the muscle imbalance theory of the pathogenesis of shoulder internal rotation contracture.

Materials and Methods

Animal Model of Neonatal Brachial Plexus Injury

All procedures were approved by the Institutional Animal Care and Use Committee. Unilateral brachial plexus injuries were created in five-day-old CD-1 mice (Charles River Laboratories International, Wilmington, Massachusetts) by surgical excision of the C5 and C6 nerve roots through a transverse supraclavicular incision after induction of general anesthesia with isoflurane. Motor function was assessed immediately postoperatively to confirm functional deficits consistent with upper trunk paralysis. A simplified motor function scale was used for individual limb movements (with grade 0 indicating absent function; grade 1, partial function; and grade 2, normal symmetric function). Motor function was again assessed immediately before the mouse was killed. Depending on the experiment, animals were killed either at five weekly time points, starting immediately postoperatively, or at a single time point at four weeks postoperatively. Isoflurane overdose was used to kill mice that were five days old, and CO_2 asphyxiation was used for mice that were twelve to thirty-three days old.

Measurement of Joint Contracture

Shoulder external rotation in adduction and elbow extension were measured immediately after the mice were killed in each experiment. Because of the limitations of joint goniometry in immature mice, range of motion measurements were performed digitally with use of AxioVision (release 4.7; Carl Zeiss MicroImaging, Thornwood, New York) on images of the mice positioned under a 10.2-megapixel digital single-lens-reflex camera (Sony, Tokyo, Japan) set on a tripod equipped with a leveled horizontal center column (Manfrotto, Bassano del Grappa, Italy). For external rotation, animals were positioned supine with the forelimbs forward flexed to allow the chest wall to stabilize the scapula during passive external rotation of the glenohumeral joint. With the elbows flexed 90°, the shoulders were passively externally rotated until the torso began to shift. On digital images captured in this position, the angle subtended by the forearm and the animal's midline was used as the measure of maximum glenohumeral external rotation. The forelimbs were then skinned for elbow extension measurement because of the inability to accurately see the humeral segment through the forequarter skin. For each side, the animal was positioned in lateral decubitus and traction was applied to the paw with the glenoid stabilized until the scapula began to rotate. On digital images captured in this position, the angle subtended by the lateral intramuscular septum (representing the axis of the humeral segment) and the digital extensor tendons (representing the axis of the forearm segment) was used as the measure of elbow extension. Elbow extension measurement was repeated immediately following excision of the biceps and brachialis muscles with the joint capsule left intact in order to assess the contribution of these muscles to contracture formation. A similar experiment was not possible at the shoulder because of the confluence of the joint capsule and the intra-articular subscapularis tendon.

A pilot study of eight mice tested the reliability of this method for passive range of motion measurement at four weeks following surgical brachial plexus injury, with both limbs positioned three times each for photographs and each photograph measured twice. Intraclass correlation coefficients were >0.93 for the shoulder and >0.98 for the elbow, suggesting high reliability.

Histological Analysis of Muscle

In twenty mice following range of motion analysis, bilateral biceps, brachialis, and triceps muscles were removed, fixed, and processed for paraffin embedding. Scapulae were also harvested with rotator cuff muscles in situ and decalcified prior to paraffin embedding. Ten μm cut sections were stained with Masson trichrome (American MasterTech Scientific, Lodi, California) and imaged with use of a microscope (Axiovert 100 M; Carl Zeiss MicroImaging) at 2.5× magnification. Quantification of fibrosis in the biceps and triceps muscles was performed with use of ImageJ software (version 1.42q; National Institutes of Health, Bethesda, Maryland) on three regions per muscle, avoiding intramuscular tendons and epimysium. The collagen (blue) and muscle fiber (red) stains were separated by color deconvolution, and the resulting images were converted to binary format for pixel counts to determine the ratio of collagen to muscle area. Use of this ratio controlled for the potential confounding variables of muscle size and stretch.

Measurement of Cross-Sectional Muscle Growth

At weekly time points following C5-C6 nerve root excision, bilateral biceps and brachialis muscles were removed from four to eight mice and processed as described for Masson trichrome staining. The muscles were completely removed from supporting skeletal elements and suspended in formalin for fixation to allow measurement of cross-sectional area without tension applied, minimizing the potential confounding effects on the area of differential response to muscle stretch by means of Poisson's ratio. Following sectioning, staining, and image acquisition, the ten largest sections from each muscle were measured for cross-sectional area with use of AxioVision, and the largest three cross-sectional areas were averaged.

Measurement of Longitudinal Muscle Growth

The method for this measurement was adapted from Felder et al.[20]. For biceps and brachialis muscle length measurements, following elbow range of motion analysis, forequarters were removed bilaterally and pinned to cork cubes at 90° of shoulder internal rotation, 0° of shoulder abduction, 90° of elbow flexion, and neutral forearm rotation. Radiographs were used to confirm a difference of <5° in joint positions between sides. The limbs, positioned on

the cubes, were fixed in 10% neutral buffered formalin for forty-eight hours and washed three times in phosphate-buffered saline solution (twenty-four hours per wash). The biceps and brachialis were then removed from their osseous origins and insertions, suspended in phosphate-buffered saline solution, and digitally imaged with use of AxioVision for length measurement under a calibrated dissecting microscope. Muscles were then digested in 15% H_2SO_4 for thirty minutes, returned to phosphate-buffered saline solution, and dissected into fiber bundles for imaging with use of differential interference contrast microscopy (40× oil immersion) to visualize sarcomeres. For each muscle, six series of ten sarcomeres were measured and averaged. At each time point, muscles were harvested from four to six mice taken from three litters.

For subscapularis muscle length measurements, following range of motion analysis, the forequarters were removed bilaterally and pinned to cork cubes at 0° of shoulder external rotation and 0° of shoulder abduction. Following formalin fixation, the lengths of the upper and lower subscapularis muscle bellies from the medial scapular origin to humeral insertion were digitally measured in situ under a calibrated dissecting microscope. The upper and lower subscapularis muscle bellies were analyzed separately, given their potentially distinct innervations and different orientations on the scapula. The upper and lower subscapularis muscle bellies were then dissected from the scapulae and processed for sarcomere length measurements as above. Subscapularis muscle length was measured at a single time point (four weeks postoperatively) in eight animals from the same litter.

Shoulder Muscle Imbalance Model

In ten five-day-old mice, under general anesthesia with isoflurane, the posterior aspect of the scapula was exposed through a chevron incision in order to minimize the effects of scar contracture. The infraspinatus and teres minor muscles were excised from the musculotendinous junction at the level of the glenoid to the medial border of the scapula to prevent muscle healing. The contralateral side was exposed with the same incision as a sham control. The brachial plexus was not excised in these animals. Four weeks postoperatively, motor function was assessed, the mice were killed, joint motion was analyzed as above, and the subscapularis muscle bellies were processed for muscle and sarcomere length.

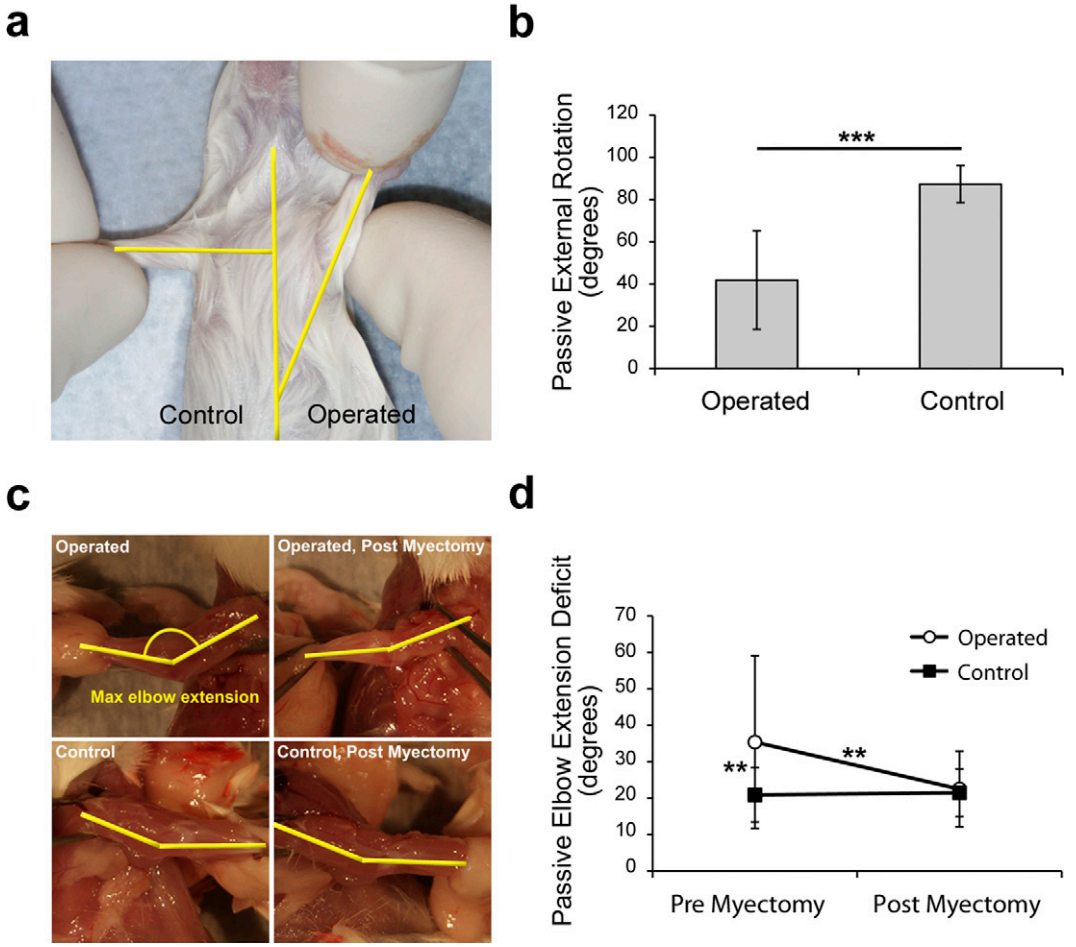

Fig. 1

Clinical contractures following surgical brachial plexus injury. All lines schematically represent the lines used for measurement as described in the Methods section. Four weeks following unilateral surgical brachial plexus injury, the involved limb displayed internal rotation contractures of the shoulder (a), with significantly less passive external rotation on the involved side than the control side (***$p < 0.0001$) ($n = 18$) (b). Given the different orientation of the scapula on the thorax of the mouse compared with the human, the position depicted is analogous to passive external rotation of the shoulder in adduction in humans. Similarly, elbow flexion contractures developed (c), with a significantly greater deficit in passive elbow extension on the involved side than the control side (**$p = 0.008$) ($n = 20$) (d). The deficit in passive elbow extension was completely relieved following excision of the biceps and brachialis (**$p = 0.003$) ($n = 20$) (c and d).

Satellite Cell Culture and Immunohistochemistry

The method was adapted from Rosenblatt et al.[21]. Following elbow motion analysis four weeks after C5-C6 excision, denervated and control biceps muscles of five mice were dissected and digested in phosphate-buffered saline solution containing 0.3% collagenase I (Sigma-Aldrich, St. Louis, Missouri), 5 mM $CaCl_2$, and 5% bovine serum albumin for one hour at 37°C. Digested biceps muscles were then triturated twenty times in phosphate-buffered saline solution containing 5% bovine serum albumin in two cycles and then triturated twenty times in one final cycle in Dulbecco modified Eagle medium (DMEM) (GIBCO, Grand Island, New York). The released single myofibers were cultured in eight-chamber slides coated with Matrigel (BD Biosciences, San Jose, California) in growth medium (10% horse serum, 0.5% chick embryo extract, 100 U/mL penicillin, and 100 μM/mL of streptomycin in DMEM), and incubated at 37°C. Cultures were fixed after seven days in 4% paraformaldehyde in phosphate-buffered saline solution for fifteen minutes. Cells were permeabilized in 0.1% Triton X-100 for ten minutes, blocked in 10% normal donkey serum for three hours, incubated in primary antibody diluted in 1.5% normal donkey serum for twelve hours at 4°C, incubated in secondary antibody diluted in 1.5% normal donkey serum for four hours, and mounted in VECTASHIELD medium (Vector Laboratories, Burlingame, California). Primary antibodies used were mouse anti-Pax-7 (1:50; sc-81648), rabbit anti-MyoD (1:50; sc-760), and goat anti-myogenin (1:50; sc-31945) (all from Santa Cruz Biotechnology, Santa Cruz, California). Secondary antibodies used were donkey anti-mouse IgG-AMCA (1:200; 715-155-150), donkey anti-rabbit IgG-DyLight 649 (1:800; 711-495-152), and donkey anti-goat IgG-DyLight 549 (1:800; 705-505-147) (all from Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). Multichannel images were made with use of a microscope (Axiovert 100 M; Carl Zeiss MicroImaging) at 20× magnification.

Statistical Analysis

Power calculations were performed for each dependent variable in order to ensure at least 80% power for each statistical test. Continuous variables confirmed to be normally distributed with the Lilliefors test were compared with use of the Student t test and with use of paired t tests when the contralateral limb was used as a control. Correlation coefficients were calculated to test correlation of continuous variables.

Source of Funding

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Results

Joint Contractures

To establish the animal model, unilateral C5-C6 nerve root excision was performed in twenty CD-1 mice at five days of age. Postoperative examination confirmed paralysis to be limited to the upper trunk of the brachial plexus (absent shoulder abduction and external rotation, absent elbow flexion, and preserved elbow extension and grasp) in all mice. Twelve mice (60%) recovered partial elbow flexion by four weeks postoperatively, but no mouse recovered shoulder abduction or external rotation. Four weeks postoperatively, the involved limb demonstrated significantly less passive external

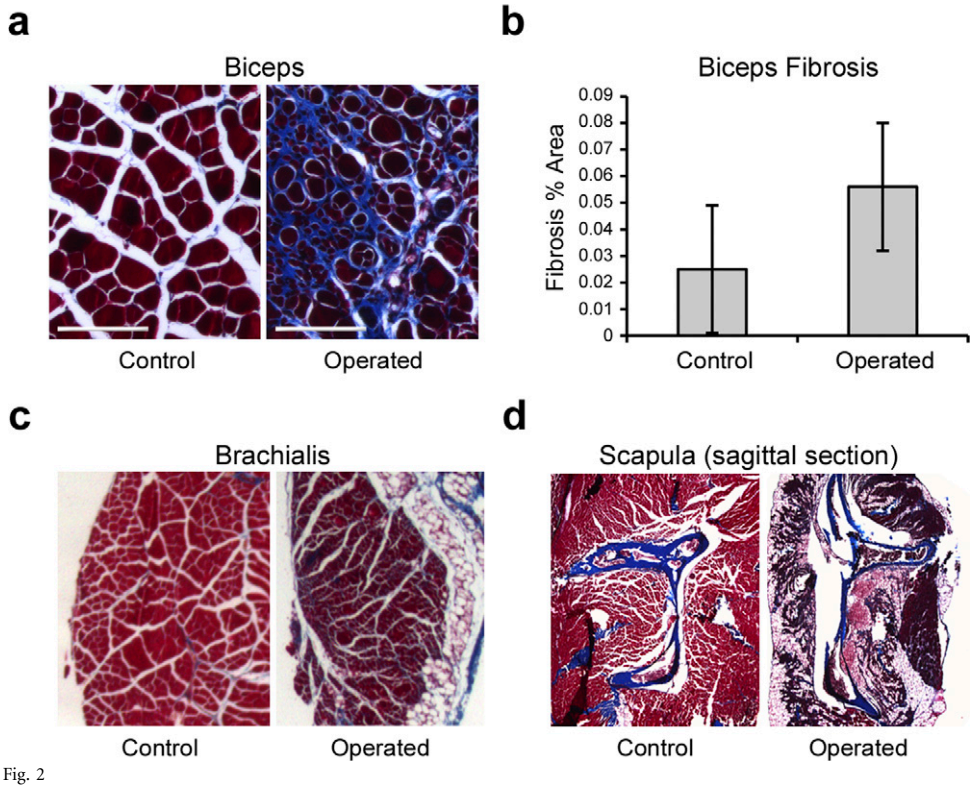

Fig. 2

Histological specimens of muscle stained with Masson trichrome to assess fibrosis following denervation. Red staining represents muscle fibers; blue represents collagen. The biceps from the involved side displayed dense fibrosis histologically (a), with a significantly greater collagen content by area on the involved side than the control side (b). The predominant histopathological change in the brachialis (c) and muscles of the rotator cuff (d) was fatty infiltration. The bar in (a) denotes 100 μm.

rotation ($p < 0.001$, two-tailed paired t test) in eighteen mice (Fig. 1, *a* and *b*) and less passive elbow extension ($p < 0.01$, two-tailed paired t test) in twenty mice (Fig. 1, *c* and *d*) than the contralateral, control limb. The elbow flexion contractures were completely relieved with removal of the biceps and brachialis muscles, leaving the joint capsule intact ($p < 0.01$, two-tailed paired t test) in twenty mice (Fig. 1, *d*), suggesting that these muscles may be primarily responsible for the flexion contracture at the elbow.

Muscle Histological Analysis

In the initial twenty mice, four weeks following C5-C6 nerve root excision, the denervated biceps muscle histologically demonstrated dense fibrosis, with significantly greater collagen deposition than that in the control biceps muscle ($p < 0.001$, paired t test; Fig. 2, *a* and *b*). However, the brachialis and rotator cuff muscles demonstrated no fibrosis histologically, with fatty atrophy the predominant histopathological finding (Fig. 2, *c* and *d*).

Muscle Growth

Cross-sectional growth of bilateral biceps and brachialis muscles was measured at weekly intervals following unilateral C5-C6 nerve root excision in an additional fifty-seven mice (four to eight mice per muscle per time point). The biceps and brachialis muscles on the involved limb failed to grow in cross-sectional area over four weeks, despite growth of the contralateral, control muscles by 492% and 302%, respectively ($p < 0.01$, two-tailed paired t tests) (Fig. 3, *a* and *b*).

Longitudinal growth of the biceps and brachialis muscles was assessed in an additional fifty-five mice (four to eight mice per muscle per time point). No difference was found in the growth of the humerus between involved and control limbs over four weeks ($p = 0.64$, paired t test; power of >90% to detect a 0.2 mm or 5% difference). However, the longitudinal growth of the biceps muscle belly was reduced on the involved side ($p < 0.005$, two-tailed paired t test; four to five mice per time point; Fig. 3, *c*), with no difference detected in the length of sarcomeres ($p = 0.17$, paired t test; 90% power to detect a

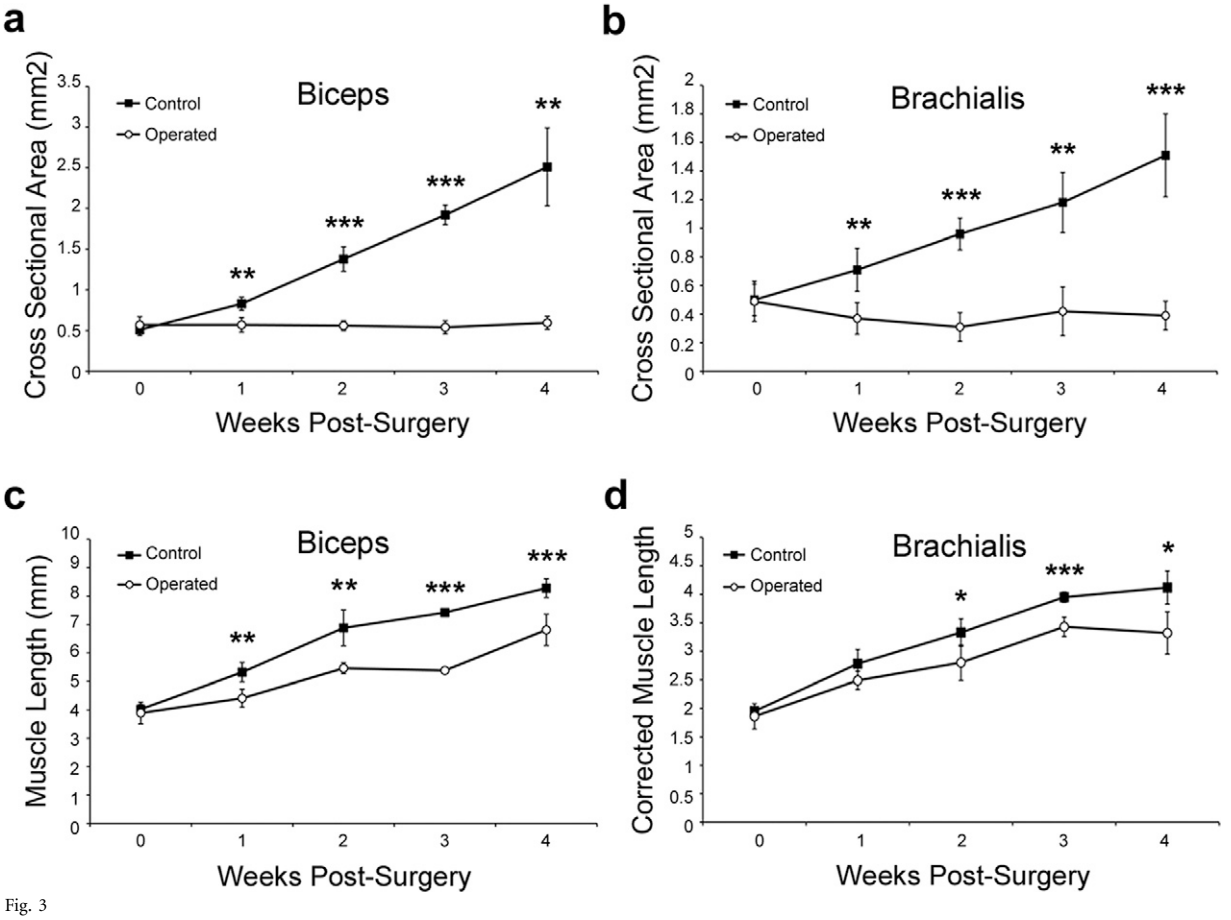

Fig. 3

Elbow flexor muscle growth following surgical brachial plexus injury. Following unilateral surgical brachial plexus injury, the involved limb displayed significantly impaired biceps and brachialis muscle cross-sectional growth (a and b) (four to eight mice per time point) compared with the contralateral, control side. Longitudinal growth of the biceps muscle was impaired in overall muscle belly length (c), and brachialis muscle longitudinal growth was impaired when corrected for sarcomere length (d) (four to six per time point). All data presented as the means and the standard deviations. *$p < 0.05$, **$p < 0.005$, and ***$p < 0.0005$.

0.2-μm difference). In contrast, the longitudinal growth of the brachialis muscle belly was also reduced on the involved side ($p < 0.02$, two-tailed paired t test) (Fig. 3, *d*), but with longer sarcomeres on the involved side ($p < 0.001$, two-tailed paired t test), suggesting that the brachialis muscle was functionally shorter as it was fixed in a more stretched state at the same joint position as the control side.

Longitudinal growth of the subscapularis muscle was assessed in eight mice at four weeks following brachial plexus injury. The upper subscapularis muscle was shorter on the involved side than the control side ($p < 0.001$, two-tailed paired t test), with longer sarcomeres on the involved than on the control side ($p < 0.002$, two-tailed paired t test). Moreover, the degree of upper subscapularis muscle shortening, corrected for sarcomere length and expressed as a percentage of the corrected length of the contralateral upper subscapularis muscle, was significantly correlated with the degree of internal rotation contracture ($p < 0.05$; correlation coefficient, $R^2 = 0.54$) (Fig. 4). A similar correlation was not seen with the lower subscapularis muscle belly.

Shoulder Muscle Imbalance

Surgical excision of the shoulder external rotators (infraspinatus and teres minor) in ten mice produced permanent impairment of active shoulder external rotation, with only one mouse recovering weak external rotation by four weeks. However, internal rotation contractures of the shoulder did not develop in any mice, with passive external rotation on the involved side not significantly different from the control side ($p = 0.380$; power of >90% to detect a 10° difference between sides). Similarly, there was no significant difference between the two sides in upper subscapularis muscle belly length ($p = 0.305$; power of >90% to detect 1-mm difference) or sarcomere length ($p = 0.518$; power of >90% to detect a 0.5-μm difference). Adding these data to the plot of passive external rotation over subscapularis corrected length following denervation further strengthened the correlation between subscapularis length and passive external rotation ($R^2 = 0.82$) (Fig. 4).

Satellite Cell Culture

When stained immunohistochemically for markers of satellite cells in various stages of differentiation (Fig. 5, *a*), cultures of single myofibers from bilateral biceps muscle harvested four weeks following C5-C6 nerve root excision revealed satellite cells in all stages of activation, proliferation, differentiation, and myotube formation in vitro (Fig. 5, *b*), even in cultures of muscles from the denervated limbs of mice with severe elbow

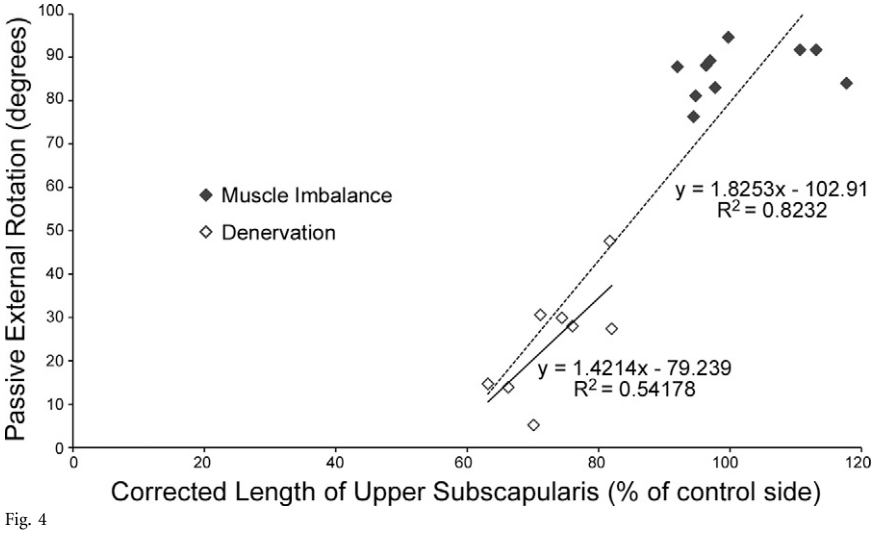

Fig. 4

The relationship between passive external shoulder rotation and length of the subscapularis muscle four weeks following C5-C6 neurectomy versus external rotator excision. The length of the upper subscapularis muscle corrected for sarcomere length and presented as a percentage of the corrected length of the control side was plotted against the passive external rotation at the shoulder on the involved side. The open diamonds represent subscapularis muscles ($n = 8$) evaluated four weeks after C5-C6 neurectomy, where a significant correlation ($p < 0.05$, $R^2 = 0.54$) was found between joint passive motion and amount of subscapularis muscle shortening (solid regression line). The closed diamonds represent subscapularis muscles evaluated four weeks following surgical excision of the external rotator muscles with the brachial plexus left intact, where passive external rotation and subscapularis length remained normal. When plotted together, these data points fall on a similar regression line (dashed line), strengthening the correlation between subscapularis length and passive external rotation ($p < 0.001$, $R^2 = 0.82$).

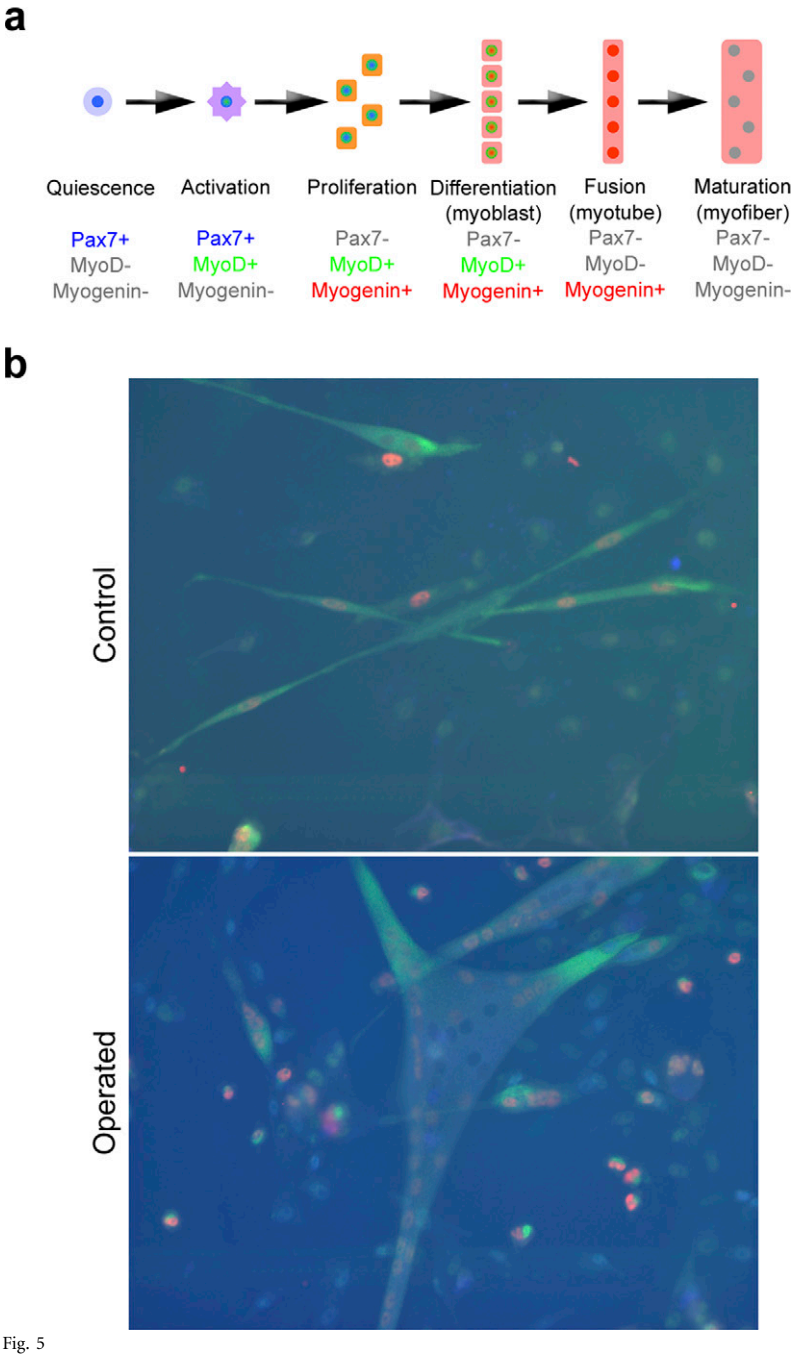

Fig. 5

The presence of functionally myogenic satellite cells in the biceps following neonatal denervation. *a:* Schematic representation of the life cycle of a satellite cell with the transcription factor expression pattern that marks each specific cell state. (Adapted, with permission, from: Zammit PS, Partridge TA, Yablonka-Reuveni Z. The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem. 2006;54:1177-91.) *b:* Representative immunohistochemistry results of seven-day in vitro culture of satellite cells from single myofibers derived from the control and the involved side of the biceps of a mouse at four weeks after neonatal brachial plexus injury. Satellite cells are identifiable at each stage of differentiation and formed myotubes with mature, unstained myonuclei.

flexion contractures. These data, while not quantitative, refute the possibility that the absence of myogenically capable satellite cells is responsible for the relative muscle shortening and contracture formation.

Discussion

The current investigation suggests that impaired growth of neonatally denervated muscle may be at least in part responsible for loss of passive joint range of motion following

neonatal brachial plexus injury. These findings challenge the notion that the involvement of denervated muscle in contractures following neonatal brachial plexus injury is secondary only to the lack of passive stretch of those muscles, resulting from muscle activity imbalance across a given joint[8,22].

Impaired postnatal development of skeletal muscle following neonatal denervation has been previously demonstrated. In a lower limb denervation model in rats, Betz et al.[13] found no increase in the number of muscle fibers over four weeks following neonatal total denervation and found diminished muscle fiber production following partial denervation. A similar impairment of muscle growth following neonatal nerve injury in the lower limb in rats has been demonstrated by others in terms of muscle mass, cross-sectional area, fiber number, and fiber diameter[14,15,23,24]. Our findings of a lack of growth in cross-sectional area of the biceps and brachialis muscles confirm those in previous studies involving experiments in the lower limb, and they do so in a clinically relevant model of brachial plexus injury in the upper limb.

More importantly, to our knowledge, the current study is the first to investigate the effects of neonatal denervation on longitudinal muscle growth and to correlate longitudinal growth impairment with loss of passive range of motion of a joint. Controlling for limb length, joint position, and sarcomere length, we found the biceps, brachialis, and subscapularis muscles were shorter on the involved side than the control side. It is unlikely that the shortening of the elbow flexors is due to lack of passive stretch, given the preserved active elbow extension in our model. It is also similarly unlikely that the shortening of the subscapularis muscle following neonatal brachial plexus injury is due solely to external rotator weakness since neonatal excision of the external rotators without C5-C6 denervation failed to produce internal rotation contractures or subscapularis shortening. While it is possible that excision of the shoulder external rotators may lead to contractures at later time points not studied, muscle imbalance alone cannot explain the contractures that reliably occur four weeks following denervation in our model of neonatal brachial plexus injury and in those used by others[18,19].

The impaired longitudinal growth of denervated muscles corresponded to the clinical contractures. At the elbow, normal passive elbow extension was restored following removal of the denervated elbow flexors, suggesting a primary role for these shortened muscles in the etiology of the elbow flexion contracture. While a similar experiment could not be performed at the shoulder because of the confluence of the joint capsule and the upper subscapularis tendon, the significant correlation between the corrected length of the upper subscapularis and passive external rotation of the shoulder supports a relationship between muscle shortening and loss of passive joint motion. It is more likely that this correlation points to the subscapularis shortening as a cause of, rather than result of, the loss of passive shoulder external rotation, given the lack of subscapularis shortening following external rotator muscle excision.

Several important questions are raised by this study. The various muscles studied appeared to be shorter by different architectural mechanisms: the biceps muscle bellies were shorter but with sarcomeres of normal length, whereas the brachialis and subscapularis muscles were shorter with longer sarcomeres. In addition, the biceps muscle displayed fibrotic histopathology compared with fatty infiltration of the brachialis and subscapularis muscles. These differential responses of skeletal muscles to neonatal denervation could be due to different fiber-type composition[24] or different levels of denervation[13,15]. Neurofilament and rhodamine-conjugated alpha bungarotoxin staining were used in pilot experiments to confirm isolated denervation in the upper trunk distribution in our model. However, the degree of reinnervation, which may occur by recruitment and remodeling of redundant innervation pathways normally occurring in the newborn period[25], was not quantified. This partial reinnervation may be more likely in the brachialis and subscapularis muscles, which have more variable innervation contributions than the biceps muscle in humans, potentially explaining differences in muscle histopathology and architecture. Additional experiments are under way to quantify denervation and reinnervation at the shoulder and elbow following partial and global plexus injury and repair, in an effort to directly correlate quantitative denervation of specific muscles with shortening and contractures. Nonetheless, fibrosis was not a consistent feature of denervated muscles and cannot alone account for the contractures.

Our study also aimed to investigate the possibility that loss of satellite cell populations was responsible for the impaired muscle growth and resulting contractures. However, we were able to culture myogenically capable satellite cells from denervated biceps muscles four weeks following denervation, even from mice with severe elbow flexion contractures. Therefore, the impairment of in vivo muscle growth occurs in the presence of myogenically capable satellite cells in vitro, suggesting an antimyogenic milieu in the neonatally denervated muscle. Characterization of that milieu may allow development of rational prevention and treatment strategies for contractures following neonatal brachial plexus injury.

Other animal models of neonatal brachial plexus injury, with use of upper trunk neurotomy in rats[18] and mice[19], and with use of supraspinatus botulinum toxin injection in mice[26], have recently been reported. The first of these studies demonstrated internal rotation contractures and glenohumeral deformity, but did not evaluate elbow flexion contractures or muscle histological changes[18]. The mouse neurotomy study[19] evaluated only passive shoulder abduction and elbow flexion, but not passive external rotation and elbow extension, the two most common disabling contractures in humans. Furthermore, despite a detailed analysis of glenohumeral osseous anatomy, the only muscle analyzed was the supraspinatus. The study describing the botulinum toxin injection model[26] only quantified supraspinatus muscle atrophy. Our study specifically evaluated the contractures most commonly encountered in humans and the muscles most likely to be responsible for those

contractures on the basis of empirical clinical treatments[27-29] and previous MRI studies in humans[8-10].

We realize that our study has several limitations. First, our study did not address glenohumeral dysplasia, which is common in humans following neonatal brachial plexus injury and has been demonstrated in other animal models of neonatal brachial plexus injury[18,19]. While the current experiments were not designed to evaluate glenohumeral dysplasia, we were able to axially visualize the glenohumeral joint on radiographs made to confirm joint position for the subscapularis muscle length measurement experiments. No glenohumeral dysplasia was seen in the mice that underwent external rotator muscle excision, suggesting that muscle imbalance alone is not sufficient to produce glenohumeral dysplasia. Additional experiments are under way to quantitatively assess glenohumeral dysplasia in various experimental conditions and to correlate dysplasia with muscle growth. Second, not all muscles of the upper limb were evaluated; rather, specific muscles were chosen for analysis on the basis of previously described clinical studies. Third, we cannot yet mechanistically explain the variability in histopathological findings and growth impairment between different muscles, although experiments are ongoing as described above. Finally, extrapolation of anatomical findings in the forelimb of the quadrupedal mouse to the upper limb of the bipedal human must be done with caution. Nonetheless, the use of a murine model allows investigation into potentially conserved molecular and physiologic mechanisms of denervation-induced myocontractures.

Since the original description of birth-related brachial plexus injury over a century ago, numerous authors have described conflicting surgical and nonsurgical treatments for the shoulder internal rotation contracture, variably highlighting the role of the subscapularis muscle[27-29], the pectoralis major muscle[3], or the joint capsule[30-32]. Even worse, there is a conspicuous absence of clinical reports demonstrating effective treatment of the elbow flexion contracture. These controversies can be attributed at least in part to the historically empirical derivation of treatment strategies from clinical observations rather than from a clear a priori understanding of the physiologic mechanisms of contracture pathogenesis.

The current study, while not providing the final answer, is the first, as far as we know, to demonstrate a potential primary role for impaired growth of neonatally denervated muscle in contracture formation following neonatal brachial plexus injury. The many questions raised by the findings of this study should stimulate ongoing research into neuromuscular, cellular, and molecular mechanisms governing the relationship among neonatal denervation, muscle growth, and contracture formation. Regardless of these uncertainties, however, we can no longer ignore the effects of neonatal denervation on postnatal growth and development of skeletal muscle in this unique pediatric neuromuscular condition. ■

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References

1. Foad SL, Mehlman CT, Ying J. The epidemiology of neonatal brachial plexus palsy in the United States. J Bone Joint Surg Am. 2008;90:1258-64.

2. Pondaag W, Malessy MJ, van Dijk JG, Thomeer RT. Natural history of obstetric brachial plexus palsy: a systematic review. Dev Med Child Neurol. 2004;46:138-44.

3. Waters PM, Peljovich AE. Shoulder reconstruction in patients with chronic brachial plexus birth palsy. A case control study. Clin Orthop Relat Res. 1999;364:144-52.

4. Waters PM, Smith GR, Jaramillo D. Glenohumeral deformity secondary to brachial plexus birth palsy. J Bone Joint Surg Am. 1998;80:668-77.

5. Waters PM. Update on management of pediatric brachial plexus palsy. J Pediatr Orthop. 2005;25:116-26.

6. Pearl ML. Shoulder problems in children with brachial plexus birth palsy: evaluation and management. J Am Acad Orthop Surg. 2009;17:242-54.

7. Waters PM, Monica JT, Earp BE, Zurakowski D, Bae DS. Correlation of radiographic muscle cross-sectional area with glenohumeral deformity in children with brachial plexus birth palsy. J Bone Joint Surg Am. 2009;91:2367-75.

8. Hogendoorn S, van Overvest KL, Watt I, Duijsens AH, Nelissen RG. Structural changes in muscle and glenohumeral joint deformity in neonatal brachial plexus palsy. J Bone Joint Surg Am. 2010;92:935-42.

9. Pöyhiä TH, Nietosvaara YA, Remes VM, Kirjavainen MO, Peltonen JI, Lamminen AE. MRI of rotator cuff muscle atrophy in relation to glenohumeral joint incongruence in brachial plexus birth injury. Pediatr Radiol. 2005;35:402-9.

10. Pöyhiä TH, Koivikko MP, Peltonen JI, Kirjavainen MO, Lamminen AE, Nietosvaara AY. Muscle changes in brachial plexus birth injury with elbow flexion contracture: an MRI study. Pediatr Radiol. 2007;37:173-9.

11. Blaauw G, Muhlig RS, Vredeveld JW. Management of brachial plexus injuries. Adv Tech Stand Neurosurg. 2008;33:201-31.

12. Hentz VR, Doi K. Traumatic brachial plexus injury. In: Green DP, Hotchkiss RN, Pederson WC, Wolfe SW, editors. Green's operative hand surgery. 5th ed. Philadelphia: Elsevier; 2005. p 1319-71.

13. Betz WJ, Caldwell JH, Ribchester RR. The effects of partial denervation at birth on the development of muscle fibres and motor units in rat lumbrical muscle. J Physiol. 1980;303:265-79.

14. Fisher TJ, Vrbová G, Wijetunge A. Partial denervation of the rat soleus muscle at two different developmental stages. Neuroscience. 1989;28:755-63.

15. Schmalbruch H. Growth and denervation response of skeletal muscle fibers of newborn rats. Muscle Nerve. 1990;13:421-32.

16. Rodrigues Ade C, Geuna S, Rodrigues SP, Silva MD, Aragon FF. Satellite cells and myonuclei in neonatally denervated rat muscle. Ital J Anat Embryol. 2002;107:51-6.

17. Zammit PS, Partridge TA, Yablonka-Reuveni Z. The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem. 2006;54:1177-91.

18. Li Z, Ma J, Apel P, Carlson CS, Smith TL, Koman LA. Brachial plexus birth palsy-associated shoulder deformity: a rat model study. J Hand Surg Am. 2008;33:308-12.

19. Kim HM, Galatz LM, Das R, Patel N, Thomopoulos S. Musculoskeletal deformities secondary to neurotomy of the superior trunk of the brachial plexus in neonatal mice. J Orthop Res. 2010;28:1391-8.

20. Felder A, Ward SR, Lieber RL. Sarcomere length measurement permits high resolution normalization of muscle fiber length in architectural studies. J Exp Biol. 2005;208:3275-9.

21. Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA. Culturing satellite cells from living single muscle fiber explants. In Vitro Cell Dev Biol Anim. 1995;31:773-9.

22. Einarsson F, Hultgren T, Ljung BO, Runesson E, Fridén J. Subscapularis muscle mechanics in children with obstetric brachial plexus palsy. J Hand Surg Eur Vol. 2008;33:507-12.

23. Lowrie MB, Krishnan S, Vrbová G. Recovery of slow and fast muscles following nerve injury during early post-natal development in the rat. J Physiol. 1982;331:51-66.

24. Mousavi K, Miranda W, Parry DJ. Neurotrophic factors enhance the survival of muscle fibers in EDL, but not SOL, after neonatal nerve injury. Am J Physiol Cell Physiol. 2002;283:C950-9.

25. Wyatt RM, Balice-Gordon RJ. Activity-dependent elimination of neuromuscular synapses. J Neurocytol. 2003;32:777-94.

26. Kim HM, Galatz LM, Patel N, Das R, Thomopoulos S. Recovery potential after postnatal shoulder paralysis. An animal model of neonatal brachial plexus palsy. J Bone Joint Surg Am. 2009;91:879-91.

27. Newman CJ, Morrison L, Lynch B, Hynes D. Outcome of subscapularis muscle release for shoulder contracture secondary to brachial plexus palsy at birth. J Pediatr Orthop. 2006;26:647-51.

28. van der Sluijs JA, van Ouwerkerk WJ, de Gast A, Nollet F, Winters H, Wuisman PI. Treatment of internal rotation contracture of the shoulder in obstetric brachial plexus lesions by subscapular tendon lengthening and open reduction: early results and complications. J Pediatr Orthop B. 2004;13:218-24.

29. Pedowitz DI, Gibson B, Williams GR, Kozin SH. Arthroscopic treatment of posterior glenohumeral joint subluxation resulting from brachial plexus birth palsy. J Shoulder Elbow Surg. 2007;16:6-13.

30. Pearl ML. Arthroscopic release of shoulder contracture secondary to birth palsy: an early report on findings and surgical technique. Arthroscopy. 2003;19:577-82.

31. Pearl ML, Edgerton BW, Kazimiroff PA, Burchette RJ, Wong K. Arthroscopic release and latissimus dorsi transfer for shoulder internal rotation contractures and glenohumeral deformity secondary to brachial plexus birth palsy. J Bone Joint Surg Am. 2006;88:564-74.

32. Waters PM, Bae DS. The early effects of tendon transfers and open capsulorrhaphy on glenohumeral deformity in brachial plexus birth palsy. J Bone Joint Surg Am. 2008;90:2171-9.