Force Deficit in Neurovascular Muscle Transfer is Partially Explained by a Denervated Muscle Fiber Population

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Abstract

Neurovascular muscle transfer restores functional loss from injury or disease but a significant muscle force deficit results. This work determined whether atrophy and denervated muscle fibers account for this force deficit. The proximal and distal extensor digitorum longus (EDL) muscle tendons were transected and immediately repaired in a rat neurovascular muscle transfer model. The peroneal nerve was either simply exposed for the SHAM group or divided and repaired. A TOTAL repair group had all the proximal nerve included in an end to end coaptation while a REDUCED group had very few fascicles from the proximal end included in the repair to the entire distal nerve. Whole EDL muscle force was quantified. Muscle cross sections were labeled for neural cell adhesion molecule, a marker for fiber denervation. EDL muscle "corrected" specific force was calculated by normalizing force to the innervated muscle fiber cross sectional area (CSA). The EDL muscle absolute force was significantly decreased and the percentage of CSA occupied by denervated fibers was significantly increased as the number of axons available for muscle reinnervation was reduced. The TOTAL group had a 10.3% deficit in "corrected" specific force (254 ± 49 kN*m⁻²) and the REDUCED group had a 34.6% deficit (185 ± 64 kN*m⁻²) relative to the SHAM group mean (283 ± 47 kN*m⁻²). In both the TOTAL and the REDUCED groups, muscle fiber denervation accounted for approximately 3% of the specific force deficit. Muscle atrophy and fiber denervation do not fully explain the force deficit in neurovascular muscle transfer.

Abbreviations: CSA: muscle cross sectional area, "corrected" CSA: innervated physiological muscle cross sectional area, innervated-CSA (%): percentage of fiber area determined to be innervated relative to the total CSA, EDL: extensor digitorum longus muscle; L_f: muscle fiber length, L_0: optimal muscle length, SHAM: control surgical exposure of peroneal nerve muscle transfer group, TOTAL: total peroneal nerve repair muscle transfer group, REDUCED: Reduced peroneal nerve repair muscle transfer group, NCAM: neural cell adhesion molecule, P_0: maximal tetanic muscle force, specific P_0: P_0 normalized to muscle CSA, "corrected" specific P_0: P_0 normalized to innervated muscle fiber CSA.

Key words: muscles, neurovascular muscle transfer, NCAM, reinnervated muscle.

Basic Appl Myol 14(3): 179-189, 2004
some of the force (P_0) deficit but a specific force (specific P_0) deficit remains after P_0 is normalized to total muscle fiber cross section area (CSA) [20, 30, 35-37, 45].

The surgical procedures of a neurovascular muscle transfer have adverse affects on the muscle. Tenotomy can result in increased intramuscular connective tissue, muscle fiber necrosis, fiber denervation, and fibers with fewer sarcomeres [1, 3-5, 20, 29]. Nerve lesion also results in denervated muscle fibers, muscle atrophy, and upregulation of neural cell adhesion molecule (NCAM) [12, 17, 21]. During neurovascular muscle transfer the nerve is repaired so that regenerating motor axons can reach the denervated muscle and re-establish operational neuromuscular junctions for muscle transfers to resume controlled function. If insufficient numbers of motor axons reach the muscle fibers or if synaptogenesis is impaired, a population of muscle fibers may remain denervated after nerve reinnervation is stabilized [9, 16]. Therefore, several months after neurovascular muscle transfer, there may be a population of normal appearing but none the less denervated muscle fibers that do not contribute to force production, but do contribute to the muscle cross sectional area (CSA) [29]. As a result, when whole muscle P_0 is normalized to the whole muscle CSA, a deficit in specific P_0 is observed as the CSA is not fully comprised of contractile elements.

The technique of polyclonal NCAM antibody immunohistochemistry can distinguish between normal and denervated muscle fibers as only denervated fibers express NCAM in the extrajunctional sarcolemma [13, 31, 54]. Increased levels of the sarcolemmal protein NCAM are observed in chronically denervated skeletal muscles and in old muscles [2, 8, 12, 54]. Increases in extracellular matrix NCAM can be detected for up to approximately 10 months after muscle denervation [12]. Though NCAM is present on the unmyelinated terminals and synaptic portions of adult neuromuscular junctions it is undetectable at portions of normal innervated muscle fibers distant from synapses [12, 13]. Thus immunohistochemistry is used to differentiate and quantify both denervated and innervated muscle fiber areas. By excluding the denervated fiber area from CSA measurement, the calculation of a "corrected" specific P_0 is possible [31, 54].

The purpose of this study was to determine whether denervated muscle fibers account for the specific P_0 deficit seen following neurovascular muscle transfer. In a rat orthotopic neurovascular muscle transfer model, the proximal and distal extensor digitorum longus (EDL) muscle tendons were cut and repaired. The peroneal nerve providing motor innervation to the EDL muscle was treated with one of the following surgeries: 1) the peroneal nerve was isolated from the surrounding tissues but not divided (SHAM), 2) the peroneal nerve was completely divided and repaired (TOTAL), 3) the peroneal nerve was completely divided and only a small portion of the proximal nerve was repaired to the distal nerve (REDUCED). Our null hypothesis was that following EDL muscle transfer the specific P_0 deficit seen for the TOTAL and REDUCED nerve repair groups compared to the SHAM group is fully explained by excluding denervated fiber CSA in the "corrected" specific P_0 dependent values.

Materials and methods

Thirty-two, male, specific pathogen-free, 8 to 9 month old, Fisher 344 rats (Charles River Laboratory; Portage, MI) were entered into the study. Procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan and were in strict accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals [26]. Rats were individually housed in a barrier-protected facility administered by the Unit for Laboratory Animal Medicine at the University of Michigan that maintained ongoing surveillance. Rats were free to move about their cages where rodent chow and water were available ad libitum. During surgical procedures and force evaluations, rats were anaesthetized with an initial intraperitoneal injection of pentobarbital sodium 65 mg/Kg body mass. Supplementary injections were administered as necessary to maintain a deep plane of anesthesia. Additionally, atropine sulfate (0.02 mg/Kg body mass) and saline (20 cc/Kg body mass) were given during muscle force evaluation to prevent mucus secretion and dehydration in the airways. All surgical procedures were performed by a reconstructive microsurgeon.

Rats were randomly assigned to one of three, orthotopic neurovascular muscle transfer groups based on the type of surgical nerve repair. The EDL muscle was surgically isolated with care taken to avoid injury to the vascular pedicle. The proximal and distal EDL tendons were cut and immediately repaired orthotopically with 6-0 suture (Ethibond-Extra, Ethicon, Somerville, NJ) in all (SHAM, TOTAL, and REDUCED) groups. A longitudinal incision at the level of the knee was made to expose the peroneal branch of the sciatic nerve. The peroneal nerve was exposed usingatraumatic microsurgical technique and manipulated as follows. In two groups the peroneal nerve supplying the EDL muscle was divided 1cm proximal to the muscle and repaired with 10-0 nylon epineural sutures (Micrins, Lake Forest, IL) coapted by neurorrhaphy to the distal peroneal nerve stump. The two groups were distinguished by grading the amount of proximal stump neural tissue included in the repair (Figure 1). Either the entire proximal nerve stump was included in the nerve repair (TOTAL) or approximately 20% of the proximal nerve stump was included in the repair (REDUCED). The excluded proximal portion of the REDUCED peroneal nerve stump was reflected proximally and stitched to the biceps femoris musculature. In the
Figure 1. Schematic of the EDL neurovascular muscle transfer surgery. The model includes all surgical steps in a true neurovascular muscle transfer except the vascular supply is not severed and repaired. The proximal and distal EDL muscle tendons are cut and repaired. Nerve repair surgery creates three groups by varying the number of innervating motor axons as follows: 1) exposing the peroneal nerve (SHAM), 2) dividing and repairing the total peroneal nerve (TOTAL), or 3) drastically reducing the number of axons included in the proximal branch of the peroneal nerve repair (REDUCED).
sufficient to detect rat NCAM. Sections were again rinsed three times with pH 7.4 PBS which was followed by incubation with CTM3-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (12.5 µg/mL) (Jackson ImmunoResearch, West Grove, PA) as a secondary antibody. Fluorescein labeled α-bungarotoxin (Sigma Chemicals, St Louis, MO) which binds to acetylcholine receptors in the postsynaptic membrane was mixed with the secondary antibody (10 to 20 µg/mL) to identify motor endplates. Sections were again rinsed with pH 7.4 PBS, dried overnight, and covered with DPX (Fluka Chemi, Neu-Ulm, Switzerland) and a cover-slip.

Individual EDL muscle sections were viewed through a Leica Laborlux S fluorescent microscope (Leica, Wetzlar, Germany) with filters selective for either CY™3 (BP 515-560) or fluorescein (FITC) (BP 450-490). Muscle sections were digitally captured in sequential 24-bit RGB TIFF format images (BioQuant-R&M Biometrics, Inc, Nashville, TN) with an Optronics DEI-759, 3-chip color camera (Optronics Engineering; Goleta, CA) mounted to the microscope (Fig 2). Both the CTM3 and FITC labeled views were individually viewed sequentially with Computer digitizing software (BioQuant – True Color Windows Software, Bioquant-R&M Biometrics, Inc. Nashville, TN). The entire muscle cross section was imaged and viewed frame by frame in montage fashion. Muscle fiber CSA was measured for each muscle fiber in the entire muscle cross section. A cell was classified as NCAM-positive if the muscle fiber surface (or muscle fiber surface and intracellular accumulation) showed CY™3 fluorescence but did not show FITC binding to acetylcholine receptors; the rest of the cells were classified as NCAM-negative. The FITC background fluorescence facilitated identification of fibers which were NCAM-negative and not visible under the BP 515-560 (CTM3 label) filter. All cells were classified as either NCAM-positive or NCAM-negative. A muscle fiber was defined as denervated if it labeled positively for NCAM. Areas positive for α-bungarotoxin (FITC label) were very rarely also positive for NCAM (CTM3 label) but these rare fibers were classified as innervated, (NCAM-negative). Denervated (NCAM-positive) and innervated (NCAM-negative) fiber counts were totaled during planimetry as all fibers were measured and classified in each typical cross-section. Denervated-CSA was the total CSA for all NCAM-positive fibers in a cross section as measured with planimetry.

The mean CSA was determined by dividing the muscle wet mass (mg) by the product of Lf (mm) and 1.06 mg.mm⁻³ which is the density of mammalian skeletal muscle (Equation 1) [44, 51, 53]. Specific P₀ was calculated by dividing P₀ by the CSA (Equation 2). The relative total area of NCAM-negative muscle fibers in each cross section represented the innervated-CSA (%), (Equation 3). A “corrected” specific P₀ or P₀ per innervated-CSA (kN/m²) was calculated in Equation 4

\[ \text{CSA} = \frac{\text{mmass} \times \cos \Theta}{\rho \times L_o \times 0.35} \]

Equation (1)

where: CSA = total muscle fiber cross sectional area

\[
\text{mmass} = \text{wet muscle mass} \\
\theta = \text{angle of pennation for rat EDL muscle (less than 3°)} \\
\rho = \text{density of mammalian skeletal muscle (1.06 g/cm³)} \\
L_o = \text{optimal muscle length during force production} \\
0.35 = \text{measured fiber length to muscle length ratio for rat EDL muscle} \\
\]

\[ \text{specific} P_o = \frac{P_o}{\text{CSA}} \]

Equation (2)

where: specific P₀ = maximal specific force (kN/m²)

\[ P_o = \text{maximal tetanic force} \\
\text{CSA} = \text{total muscle fiber cross sectional area} \]

\[ \text{Innervated – CSA (%)} = \frac{(\text{Innervated – CSA})}{(\text{Innervated – CSA}) + (\text{Denervated – CSA})} \times 100 \]

Equation (3)

where: Innervated-CSA (%) = percentage of CSA considered to be innervated

\[ \text{Innervated-CSA} = \text{digitized CSA which is negative for NCAM protein} \\
\text{Denervated-CSA} = \text{digitized CSA which is positive for NCAM protein} \]

\[ "\text{corrected}" \text{specific} P_o = \frac{P_o}{\text{CSA}} \times \frac{1}{(\text{innervated – CSA}(%))} \]

Equation (4)

where: "corrected" specific P₀ normalizes each P₀ to only the percentage of CSA which is innervated

\[ P_o = \text{maximal tetanic force} \\
\text{innervated-CSA} (%) = \text{percentage of CSA considered to be innervated} \]

based on the percentage (innervated-CSA (%)) derived from the NCAM digitized data in Equation 3.

Statistical tests were performed using SAS for Windows (SAS Institute Inc., Cary, NC). The mean and standard deviation were determined for each variable by neurovascular muscle transfer group. A general linear model (GLM) analysis determined the significance of the differences between the means for the measured dependent descriptive, force, and histological variables in the SHAM, TOTAL, and REDUCED groups. If a significant main effect difference were found, a paired t-test with a Bonferroni correction for multiple comparisons was used to determine which group comparisons were significant. An α-level equal to 0.05 was selected a priori as being appropriately stringent.
Force deficit in neurovascular muscle transfer

Results

Three rats of the thirty-two originally entered in the study died during the neurovascular muscle transfer operation leaving twenty-nine rats that successfully completed the study. Muscle mass was missing for one SHAM group rat. Muscle force was missing for another SHAM group muscle. NCAM data were missing for two REDUCED and one SHAM group muscles. All available data were included in the analysis.

Descriptive morphology data (mean ± SD) are summarized in Table 1 by neurovascular muscle transfer group. There was no significant main effect for body mass, $L_0$, or $L_f$. Body mass and muscle mass from rats in the SHAM group were similar to previously reported values [9, 55, 57, 58]. EDL muscle mass, CSA, and innervated CSA for rats in the REDUCED group were significantly smaller than for rats in the SHAM and TOTAL groups (Table 1).

In situ force measurements (mean ± SD) and statistical results are summarized in Table 2. The neurovascular muscle transfer group main effect was significant for $P_0$, specific $P_0$, and "corrected" specific $P_0$. The absolute $P_0$ was significantly decreased when compared with the SHAM group $P_0$ (3766 ± 363 mN) > TOTAL group $P_0$ (2608 ± 448 mN) > REDUCED group $P_0$ (1602 ± 776 mN) as the number of axons included in the nerve repair was reduced. The REDUCED group $P_0$ was also significantly decreased from the TOTAL group $P_0$. Thus following muscle transfer, the EDL muscle $P_0$ decreased as the number of axons available for muscle reinnervation was reduced.

It is de rigour to normalize $P_0$ values to muscle CSA (including muscle mass) when muscle atrophy or growth is a consideration (Equations 1 and 2) [30, 40]. The trend seen with increased absolute $P_0$ force deficits across transfer groups was also noted with increased specific $P_0$ deficits as the number of axons available for reinnervation was reduced. The specific $P_0$ for the REDUCED group (173 ± 54 kN*m$^{-2}$) was significantly decreased compared to both the SHAM (278 ± 43 kN*m$^{-2}$) group and the TOTAL group (242 ± 47 kN*m$^{-2}$). However, the mean specific $P_0$ of EDL muscles from rats in the TOTAL group was not significantly different when compared with that for the SHAM group.

Photomicrographs in Fig. 2 are representative portions of EDL muscle cross sections from rats in the SHAM, TOTAL, and REDUCED groups. Column A photomicrographs are the fluorescent images captured using the BP 515-560 (CY™3 label) filter while the right hand column is the same cross section when viewed with the BP 450-490 filter (fluorescein; FITC). The CY™3 is a conjugated Ig-g secondary label binding to the anti-NCAM primary anti body. Large arrows indicate fibers labeled positively for NCAM protein (denervated fibers). FITC is bound to $\alpha$-Bungarotoxin that labels intact motor endplates if present. Above right hand views indicate that fibers positive for NCAM were not also positive for $\alpha$-Bungarotoxin Small arrows point to myoneural junctions. This indicates that myoneural junctions, when present, were not false indicators of denervation. Scale bars are 100 um.

In Fig. 2. Photomicrographs of EDL muscle cross sections. Cross sections "A1" and "B1" belong to the SHAM group, "A2 and "B2" belong to the TOTAL group, and "A3" and "B3" belong to the REDUCED group. The left hand column is the cross section captured using the BP 515-560 (CY™3 label) filter while the right hand column is the same cross section when viewed with the BP 450-490 filter (fluorescein; FITC). The CY™3 is a conjugated Ig-g secondary label binding to the anti-NCAM primary anti body. Large arrows indicate fibers labeled positively for NCAM protein (denervated fibers). FITC is bound to $\alpha$-Bungarotoxin that labels intact motor endplates if present. Above right hand views indicate that fibers positive for NCAM were not also positive for $\alpha$-Bungarotoxin Small arrows point to myoneural junctions. This indicates that myoneural junctions, when present, were not false indicators of denervation. Scale bars are 100 um.
Force deficit in neurovascular muscle transfer

Table 1. Descriptive data and statistical test summary for Fischer 344 Rat EDL neurovascular muscle transfer experimental groups.

<table>
<thead>
<tr>
<th>Neurovascular Muscle Transfer Group</th>
<th>Body mass† (g)</th>
<th>Muscle mass (mg)</th>
<th>L₀ (mm)</th>
<th>Lₚ (mm)</th>
<th>EDL CSA (mm²)</th>
<th>EDL Innervated-CSA ‡** (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM (n = 9)</td>
<td>423 ± 19</td>
<td>173 ± 21</td>
<td>35 ± 1</td>
<td>12 ± 0.3</td>
<td>14 ± 2</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>TOTAL (n = 7)</td>
<td>424 ± 26</td>
<td>140 ± 20</td>
<td>35 ± 2</td>
<td>12 ± 0.2</td>
<td>11 ± 2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>REDUCED (n=13)</td>
<td>413 ± 30</td>
<td>114 ± 38*</td>
<td>34 ± 1</td>
<td>12 ± 0.5</td>
<td>9 ± 3*</td>
<td>8 ± 3*</td>
</tr>
</tbody>
</table>

Notes: g = grams, mg = milligrams, mm = millimeters, L₀ = optimal muscle length, Lₚ = muscle fiber length, EDL = extensor digitorum longus muscle, EDL CSA = total muscle cross sectional area. Data are means ± SD. Comparison groups are sham neurovascular muscle transfer (SHAM), neurovascular muscle transfer with total nerve repair (TOTAL), and neurovascular muscle transfer with a nerve repair in which a reduced number of axons is included in the nerve anastomosis (REDUCED). * indicates REDUCED group is different from the SHAM and the TOTAL groups (p < 0.05). †SHAM group has n=10 for body mass variable and n = 8 for EDL denervated-CSA, innervated-CSA, ‡ REDUCED group has n = 11 for EDL denervated-CSA, innervated-CSA variable.

REDUCED groups were noted to have large groups of denervated fibers (Fig 2). The mean denervated-CSA was 1.1 ± 0.4 % for the SHAM group, 4.7 ± 3.7% for the TOTAL group, and 8.4 ± 3.2% for the REDUCED group. Denervated-CSA for both TOTAL and REDUCED groups was significantly increased when compared to denervated-CSA for the SHAM group; the REDUCED group denervated-CSA was also significantly increased relative to the TOTAL group. Our neurovascular muscle transfer model successfully leads to a gradation in the severity of muscle denervation seen across groups once the transfers were stabilized. The increases in denervated muscle fiber number, P₀ deficit, and specific P₀ deficit in the TOTAL and REDUCED are clinically relevant. The REDUCED group resembles clinical situations where reduced numbers of axons are available for muscle reinnervation which may be seen in nerve avulsion injuries, nerve and muscle tissue injuries, or when a small donor muscle or nerve is transferred to reconstruct a large defect.

To test the hypothesis that the specific P₀ deficit seen for the TOTAL and REDUCED groups is fully explained by the presence of denervated fibers occupying the muscle CSA but not contributing to force production, the non contractile effect of denervated muscle fibers is removed from the specific P₀ by calculating a "corrected" specific P₀ (Equation 4). The results show that accounting for increases in denervated fiber CSA in the TOTAL and REDUCED groups does not completely equalize the "corrected" specific P₀ to that of the SHAM group. Though not significantly lower, the TOTAL group still had a 10.3% deficit in "corrected" specific P₀ (254 ± 49 kN*m⁻²) and the REDUCED group had a significant 34.6% deficit in "corrected" specific P₀ (185 ± 64 kN*m⁻²) relative to the SHAM group mean (283 ± 47 kN*m⁻²). We reject the null hypothesis as a significant unexplained deficit remains for the REDUCED group even after correcting for denervated, non-contracting muscle fiber area.

Discussion

A deficit in "corrected" specific P₀ remains to be explained even after correcting for muscle fiber atrophy and for denervation. It is important to point out that atrophy and denervated fibers do account for differences (force deficits) of 20.5% and 22.9% in force between the P₀ value and the "corrected" specific P₀ for the TOTAL and REDUCED groups respectively relative to SHAM muscles. In both the TOTAL and the REDUCED groups, muscle fiber denervation only

Table 2. In Situ EDL muscle contractile force properties and statistical test summary by neurovascular muscle transfer group.

<table>
<thead>
<tr>
<th>Neurovascular Muscle Transfer Group</th>
<th>P₀ (mN)</th>
<th>specific P₀ (kN/m²)</th>
<th>&quot;corrected&quot; specific P₀ (kN/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM (n = 9)</td>
<td>3766 ± 363</td>
<td>278 ± 43 (n = 8)</td>
<td>283 ± 47 (n = 7)</td>
</tr>
<tr>
<td>TOTAL (n = 7)</td>
<td>2608 ± 448*</td>
<td>242 ± 47(n = 7)</td>
<td>254 ± 49 (n = 7)</td>
</tr>
<tr>
<td>REDUCED (n=13)</td>
<td>1602 ± 776*†</td>
<td>173 ± 54*† (n = 13)</td>
<td>185 ± 64* (n = 11)</td>
</tr>
</tbody>
</table>

Notes: mN = millinewtons, kN = kilinewtons, m = meter, P₀ = maximal tetanic muscle force, specific P₀ = maximal specific force = P₀ normalized to muscle CSA, "corrected" specific P₀ = P₀ normalized to innervated muscle fiber cross sectional area. Values are means ± SD. *Indicates significantly different from SHAM group. † Indicates significantly different from the TOTAL group. p < 0.05.
accounted for approximately 3% of the specific $P_0$ deficit even though the percentage of CSA occupied by denervated fibers was significantly different across neurovascular muscle transfer groups, i.e., 1.1%, 4.7%, and 8.4% for the SHAM, TOTAL, and REDUCED groups respectively. It is possible that establishing a robust myoneural junction is of greater importance in this model than whether a fiber is reinnervated or not.

The unique finding is that the absolute force deficit observed after neurovascular muscle transfer is only partially attributable to muscle fibers which remain denervated following the transfer's stabilization. Muscle atrophy is confirmed as a contributor but there are still unexplained deficits of 10.3% and 34.6% in the TOTAL and REDUCED groups. Muscle fiber denervation explains approximately 20% of the absolute $P_0$ deficit regardless of surgical group while the unexplained force deficit is a much greater percentage in the REDUCED group where the nerve repair contains very few motor axons.

Previous studies demonstrate that deficits in both $P_0$ and specific $P_0$ remain after muscle transfer [18-20, 30]. Numerous investigations have evaluated the contribution of tenotomy and repair, muscle denervation and reinnervation, and vascular repair on the resulting force deficit. Tenotomy alone has a detrimental effect on muscle force production due to a number of factors including muscle belly and sarcomere length shortening and necrotic muscle segments near the myotendinous junctions [1, 5, 28]. Denervated muscle fibers are observed as a result of tenotomy alone [28]. A muscle length approximating the original physiological length must be reestablished at the time of tendon repair to optimize functional recovery and to reduce muscle fiber denervation [28]. Tenotomy and repair may constitute up to a 40 percent force deficit in stabilized larger muscle grafts such as the medial gastrocnemius muscle but have no long term effect on the smaller extensor digitorum longus (EDL) muscle [1, 20, 30, 42]. Approximately 5 percent of EDL muscle fibers are lost as a result of the tenotomy and repair process but the fibers are thought to be replaced with regenerated muscle fibers after muscle graft stabilization [20, 42, 48]. Thus in the rat EDL, one would expect a force deficit but little to no persistent muscle fiber denervation attributable to muscle tenotomy if the tenotomy is repaired and the muscle is allowed to adapt to the new muscle length.

In this study the proximal and distal EDL tendons were transected and repaired for each neurovascular muscle transfer. Post repair tensile loading can affect tendon cellular response with loading contributing to an increased collagen matrix and low cell to matrix ratio [27]. One would expect the effect of tenotomy to be equivalent across the groups however SHAM group muscles were never denervated and thus continued to contract during a latent period when TOTAL and REDUCED muscles were denervated and not actively contracting. As the breaking strength of tendons increases with both motion and tension, it is possible the SHAM group muscles were advantaged during tendon healing [34]. Also while tendons heal adhesions between the tendon and sheath impair the gliding mechanism of tendons and result in poor range of motion [47]. Adhesion formation was noted in this study, but no attempt was made to verify muscle-tendon compartment organization or the extent of scar formation at recovery. No obvious changes in the rat locomotion were observed prior to the final evaluation. Still one expects limitations in tendon excursion to

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**Table 3. Summary for rat EDL neurovascular muscle transfers immunologically labeled for NCAM protein.**

<table>
<thead>
<tr>
<th>Neurovascular Muscle Transfer Group</th>
<th>Innervated-CSA (%)</th>
<th>Denervated-CSA (%)</th>
<th>Innervated individual fiber area (um$^2$)</th>
<th>Denervated individual fiber area (um$^2$)</th>
<th>Total fiber count</th>
<th>Innervated fiber count</th>
<th>Denervated fiber count</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM (n = 9)</td>
<td>98.9 ± 0.4</td>
<td>1.1 ± 0.4</td>
<td>2590 ± 617</td>
<td>2539 ± 452</td>
<td>2281 ± 559</td>
<td>2256 ± 555</td>
<td>24 ± 9</td>
</tr>
<tr>
<td>TOTAL (n = 7)</td>
<td>95.3 ± 3.7*</td>
<td>4.7 ± 3.7*</td>
<td>2099 ± 584</td>
<td>1768 ± 449*</td>
<td>1776 ± 372</td>
<td>1681 ± 362</td>
<td>94 ± 74*</td>
</tr>
<tr>
<td>REDUCED (n = 11)</td>
<td>91.6 ± 3.2*†</td>
<td>8.4 ± 3.2*†</td>
<td>1686 ± 511*</td>
<td>1380 ± 376*</td>
<td>2270 ± 883</td>
<td>2049 ± 788</td>
<td>220 ± 104*†</td>
</tr>
</tbody>
</table>

Notes. CSA = cross sectional area, innervated-CSA (%) = percentage of fiber area determined to be innervated relative to the total CSA or (sum of innervated fiber area) / ((sum of denervated fiber area) + (sum of innervated fiber area)) when digitized, um = micrometers, total fiber count = average number of fibers which were measured for cross sectional area, innervated fiber count = average number of NCAM-negative fibers which were measured for cross sectional area, denervated fiber count = average number of NCAM-positive fibers which were measured for cross sectional area. Values are means ± SD. *Indicates group is significantly different from SHAM group. †Indicates group is significantly different from TOTAL group, p < 0.05.
change muscle function but this was not tested.

Peripheral nerve injury eliminates all nervous system influences resulting in rapid and progressive decline in the maximal muscle force and integrated muscle function [21, 33]. Following nerve repair, muscle fiber reinnervation occurs but the pattern of innervation is changed as seen by a reduction in the average number of motor units, motor units occupying larger territories and displaying larger innervation ratios, and a non-random arrangement of motor unit fibers [2, 15, 16, 32, 39, 49, 56]. The physiologic motor unit types also transform with reinnervation resulting in a larger percentage of fatigue intermediate or unclassifiable motor units indicating that the reinnervating motoneuron does not entirely regulate the motor unit properties [2, 15, 16, 32, 39, 49, 56]. These cumulative changes in the muscle following peripheral nerve division and repair result in significant force deficits and specific P0 deficits as we have seen in this study [15, 16, 23, 41]. Animal models specifically studying peripheral motor nerve transection with an immediate repair also show deficits in EDL muscle P0 and specific P0 similar to those seen in this study [9, 57, 58].

Others report rat medial gastrocnemius neurovascular muscle transfer data showing significant decreases in P0 (between 15% and 43%) and specific P0 decreases (between 17% and 27%) compared to control muscle [37, 38, 45, 46]. Even when synergistic muscles are removed, surprisingly, muscle transfer hypertrophy does not occur and P0 and specific P0 deficits persist for the medial and lateral gastrocnemius muscles but not for the smallest and most overloaded plantaris muscle [45]. Our P0 and specific P0 decreases for a similar transfer condition (TOTAL group when compared with the SHAM group) are in line with the findings of these studies.

During clinical neurovascularized muscle transfer, the vascular supply is divided and repaired ideally with only a brief period of muscle ischemia followed by reperfusion. No vascular repair was included in this study due to reports that vascular repair with ischemia up to three hours does not contribute to the functional deficits that occur in neurovascular muscle transfers [20, 43]. Though we acknowledge that ischemia and reperfusion can cause significant muscle damage if prolonged specifically if intraoperative ischemia of greater than four hours occurs, vessel spasm or obstruction may occur and contractile force recovery may be adversely affected [35, 36]. Following recovery from muscle transfer, total muscle blood flow does not differ between control muscle and muscle which underwent a vascular division and repair [7]. Thus vascular repair, ischemia time, and blood flow following muscle transfer are ruled out as factors affecting the recovery of maximal P0 capability following muscle transfer.

Once a muscle transfer stabilizes a population of denervated muscle fibers may persist due to incomplete synaptogenesis, incomplete reinnervation, or temporary reinnervation with subsequent axonal pruning [10, 20, 30, 35-37, 45]. Differentiation between denervated and innervated muscle fibers is possible using NCAM immunohistochemistry as only denervated fibers express NCAM in the extrajunctional sarcolemma [52]. Other studies have used NCAM immunohistochemistry to identify denervated muscle fibers and to exclude these fibers from the CSA measurements of muscles [31, 54]. After correcting muscle CSA for the presence of denervated fibers, a "corrected" specific P0 deficit of 25% is still seen two weeks following the partial denervation [31]. Thus other mechanisms must play a role in the size of the "corrected" specific P0 deficit. Rat EDL muscle fiber degeneration is usually minimal during denervation [22]. Degenerated muscle fibers can be identified as vacuolar fibers distinct from normal fibers [22]. Vacular fibers in this study were noted to label positively for NCAM. Regenerating muscle fibers (satellite cells) also label NCAM positive [29]. Thus degenerated and regenerating fibers are included in the denervated fiber mechanism. Other mechanisms which may contribute to the "corrected" specific P0 deficit are alterations in the excitation contraction coupling, deficits in force production by individual fibers that remain innervated, alterations in muscle architecture due to the tenotomy and repair, and alterations in muscle force transmission due to the formation of scar tissue.

During the first weeks of life, rat skeletal muscle myoneuronal junctions are innervated by more than one nerve axon at the end plate [6]. During early maturation there is withdrawal from the polyneural innervation state leaving one axon per myoneuronal junction per muscle fiber in normal rodents by postnatal day 11 [50]. Tenotomy during polyneural withdrawal delays polyneuronal synapse elimination [14]. When adult rats undergo total muscle denervation with the possibility for subsequent reinnervation there is a return to the polyneuronal innervated state that regresses with time but persists for up to 21 weeks following a nerve repair [25]. There are indirect indications that muscle fibers with polyneural innervation show abnormal contractile muscle action potentials [50]. In transplanted muscles force capacity stabilizes by 16 weeks (also the recovery period for this study) but polyneural innervation might continue [25, 46]. Thus the existence of muscle fibers with polyneural innervation which are considered innervated but which show limitations in contractile function may add to the specific P0 deficit seen in this study.

Based on the NCAM protein labeling, muscle fibers were designated as either innervated or denervated. The NCAM labeling did not differentiate between innervated and reinnervated muscle fibers. Denervated
fibers were assumed capable of producing zero force while innervated fibers were assumed to produce a uniform specific $P_{0}$. The innervated fibers in the SHAM group were assumed to maintain their original motor unit myoneural network while innervated fibers in the TOTAL and the REDUCED groups are "reinnervated" muscle fibers. It is possible that reinnervated fibers have deficits in force production due to changes in the fiber contractile proteins, the cytoskeleton of the fiber, or the organization of the myoneural junction. The acetylcholine receptor number per myoneural junction is known to be decreased while the myoneural junction also shows abnormalities even at 30 weeks following muscle transfer [24]. Though the TOTAL and REDUCED group fibers are reinnervated, aspects of the neuromuscular synaptic transmission may not resemble mature myoneural junctions with regard to size, presynaptic and postsynaptic membrane alignment and organization, and the ability to potentiate muscle fiber contraction. These factors could account for the unexplained "corrected" specific $P_{0}$ deficit seen in this study.

In summary following vascular muscle transfer the muscle produces more force and has fewer denervated muscle fibers if more of the native nerve motor axons are included in the nerve repair. Neurovascular muscle transfers even when completed under optimal conditions are included in the nerve repair. Neurovascular muscle transfer [24]. Though the TOTAL and the REDUCED groups are "reinnervated" muscle fibers. It is possible that reinnervated fibers have deficits in force production due to changes in the fiber contractile proteins, the cytoskeleton of the fiber, or the organization of the myoneural junction. The acetylcholine receptor number per myoneural junction is known to be decreased while the myoneural junction also shows abnormalities even at 30 weeks following muscle transfer [24]. Though the TOTAL and REDUCED group fibers are reinnervated, aspects of the neuromuscular synaptic transmission may not resemble mature myoneural junctions with regard to size, presynaptic and postsynaptic membrane alignment and organization, and the ability to potentiate muscle fiber contraction. These factors could account for the unexplained "corrected" specific $P_{0}$ deficit seen in this study.

In summary following vascular muscle transfer the muscle produces more force and has fewer denervated muscle fibers if more of the native nerve motor axons are included in the nerve repair. Neurovascular muscle transfers even when completed under optimal conditions where motor axons are regenerating in an endoneurial environment allowing for rapid reinnervation have force deficits which are only partially explained by muscle atrophy and fiber denervation.

Acknowledgments

This work was supported by the National Institute of Neurological Disorders and Stroke Grant NS 34380. Dr. Cederna received support as the John E. Hoopes Academic Scholar of the American Association of Plastic Surgeons.

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