

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 ($254 \pm 49 \text{ kN} \cdot \text{m}^{-2}$) and the REDUCED group had a 34.6% deficit ($185 \pm 64 \text{ kN} \cdot \text{m}^{-2}$) relative to the SHAM group mean ($283 \pm 47 \text{ kN} \cdot \text{m}^{-2}$). 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.

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Neurovascular muscle transfers are surgically used to reconstruct functional and structural defects most often with autogenic tissue. Neurovascular muscle transfers resurface, cover, and protect body areas as well as provide functional, purposeful and spontaneous movement to compensate for paralyzed, injured, or missing motors. To achieve the desired functional

outcome, neurovascular muscle transfer may incorporate division and repair of any of the following structures: muscle fibers, tendons, nerves, fascia, veins and arteries. Once a neurovascular muscle transfer is stabilized, the recovered muscle contractile function is incomplete [10, 20, 30, 35-37, 45]. Muscle atrophy associated with neurovascular muscle transfer explains

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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 muscle 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 using atraumatic 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

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control (SHAM) group the peroneal nerve was surgically exposed but not transected. The EDL muscle vascular supply was not disturbed in any surgery. All wounds were closed in layers with absorbable chromic gut suture.

A four month recovery period was allowed following muscle transfer and then maximum force capacity was evaluated using established methods [9, 54, 57]. Each rat was anesthetized as described above. The sciatic nerve was exposed through a skin and muscle splitting incision in the hip proximal to the previous site of peroneal nerve division and repair. The peroneal branch was isolated and preserved while the tibial and sural nerve branches of the sciatic nerve were cut. A second incision was made over the anterior compartment of the lower leg from the knee to the toes exposing the EDL muscle and its distal tendons. The distal tendons were dissected free, folded proximally, and distal to the musculotendinous junction forming a distal tendon loop. The EDL muscle blood and nerve supplies and the tendon of origin were not disturbed. Distal tendons of the tibialis anterior and extensor hallucis longus muscles were cut. The rat was placed prone on a water bath heated platform maintained at $37 \pm 1^\circ \text{C}$. The platform contained braces to stabilize the rat knee and to maintain the ankle at a fixed 90° . A skin pouch was formed around the EDL muscle that was filled with warmed mineral oil to maintain muscle temperature at $37 \pm 1^\circ \text{C}$. A bipolar, silver chloride coated electrode (Harvard Apparatus, South Natick, MA) was placed

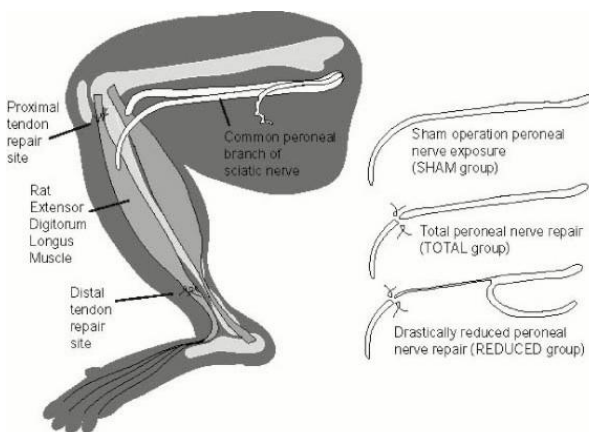


Figure 1. Schematic of the EDL neurovascular muscle transfer surgery. The model includes all surgical steps in a true neurovascular muscle transfer except the vascular supply is not severed and repaired. The proximal and distal EDL muscle tendons are cut and repaired. Nerve repair surgery creates three 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).

across the peroneal nerve proximal to the nerve repair site. The EDL muscle tendon loop was connected to a strain gauge (BG-1000: Kulite Semiconductor Products, Leonia, NJ, USA). A signal conditioning amplifier (Vishay Measurements Group, Raleigh, NC) was in series with the strain gauge to allow precise measurements within an expected force range. Tensions between 50 and 5000 mN could be reliably recorded.

Maximal isometric tetanic P_0 (mN) was quantified for the EDL muscle using established methods [9, 11, 54, 57]. Synchronization of stimulation trigger and force recording were controlled by a microcomputer (Dell Computer Corp., Austin, TX) equipped with a digital-to-analog converter (Data Translation, Marlboro, MA) and appropriate, custom software (ASYST Software Technologies, Inc., Rochester, NY). Force voltage was also monitored and displayed on a storage oscilloscope (Gould Inc., Romulus, MI). Square wave electrical current pulses, 0.2 ms in duration were delivered from a Grass S88 stimulator (Grass Instrument CO, Quincey, MA) and amplified by a Crown current amplifier (Crown International, Elkhart, IN). The stimulation voltage was gradually increased until the twitch force reached a plateau. All subsequent stimuli were at this supra-maximal voltage. Muscle length was adjusted until the optimal length for the development of maximum twitch force was identified. This adjusted muscle length was defined as the optimum muscle length (L_0) for force production. With the muscle held at L_0 and using maximal twitch voltage, the stimulation train duration was increased to 300 ms for measurements of maximal force. A force frequency curve was constructed by delivering a train of stimuli at each frequency between 50 to 200 Hz for a period of 300 ms. A two-minute recovery was imposed between each contraction measurement. The stimulation frequency was incremented until the force reached a plateau or declined. The maximal force recorded was defined as P_0 (mN). At the completion of force testing, the muscle was further exposed above the knee, L_0 was directly, the EDL muscle was harvested, tendons were trimmed, and the muscle was weighed and frozen in isopentane cooled to -160° with liquid nitrogen. An average ratio of fiber length to optimal muscle length (L_f/L_0) of 0.35 was used from established data [57].

Denervated and innervated muscle fibers were identified in EDL muscle cross sections (6 to 10 μm) using immunohistological labeling [31, 52, 54]. Sections were fixed with methanol for 2-5 minutes at -10°C , air dried, incubated for one hour in 5% donkey serum in phosphate buffered saline (PBS) pH 7.4 (Sigma, St Louis, MO), rinsed three times with PBS for a total rinse time of 30 minutes, and incubated overnight in affinity purified, polyclonal, rabbit anti-neural cell adhesion molecule, (AB5032, Chemicon, Temecula) at 4°C . A concentration of 5 $\mu\text{g/mL}$ primary antibody in a 10% solution of bovine serum albumin – PBS was

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sufficient to detect rat NCAM. Sections were again rinsed three times with pH 7.4 PBS which was followed by incubation with CYTM3-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 Leitz Laborlux S fluorescent microscope (Leica, Wetzlar, Germany) with filters selective for either CYTM3 (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 CYTM3 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 CYTM3 fluorescein 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 (CYTM3 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 αBungarotoxin (FITC label) were very rarely also positive for NCAM (CYTM3 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 L_f (mm) and 1.06 mg.mm⁻³ which is the density of mammalian skeletal muscle (Equation 1) [44, 51, 53]. Specific P_0 was calculated by dividing P_0 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 P_0 or P_0 per innervated-CSA (kN/m²) was calculated in Equation 4

$$CSA = \frac{m_{mass} * \cos \Theta}{\rho * L_o * 0.35}$$

Equation (1)

where: CSA = total muscle fiber cross sectional area

m_{mass} = wet muscle mass

Θ = angle of pennation for rat EDL muscle (less than 3°)

ρ = density of mammalian skeletal muscle (1.06 g/cm³)

L_o = optimal muscle length during force production

0.35 = measured fiber length to muscle length ratio for rat EDL muscle

$$specific P_0 = \frac{P_o}{CSA}$$

Equation (2)

where: specific P_0 = maximal specific force (kN/m²)

P_o = maximal tetanic force

CSA = total muscle fiber cross sectional area

$$Innervated - CSA (\%) = \frac{(Innervated - CSA)}{(Innervated - CSA) + (Denervated - CSA)} * 100$$

Equation (3)

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

Innervated-CSA = digitized CSA which is negative for NCAM protein
Denervated-CSA = digitized CSA which is positive for NCAM protein

$$"corrected" specific P_0 = \frac{P_o}{CSA} * \frac{1}{(innervated - CSA(\%))}$$

Equation (4)

where: "corrected" specific P_0 normalizes each P_0 to only the percentage of CSA which is innervated

P_o = maximal tetanic force

innervated-CSA (%) = 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.

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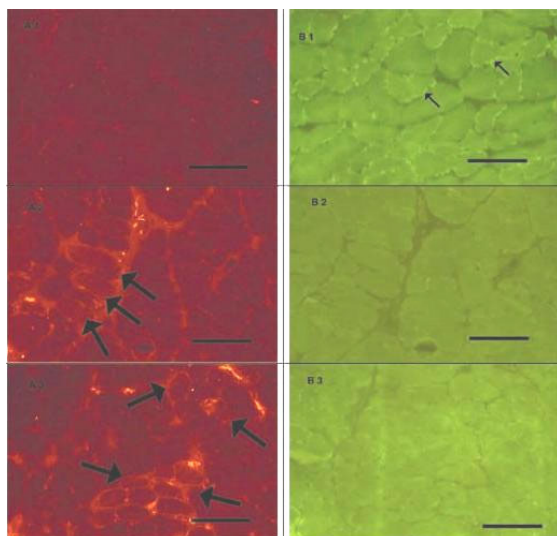


Fig. 2. Photomicrographs of EDL muscle cross sections. Cross sections "A1" and "B1" belong to the SHAM group, "A2" and "B2" belong to the TOTAL group, and "A3" and "B3" belong to the REDUCED group. The left hand column is the cross section captured using the BP 515-560 (CYTM3 label) filter while the right hand column is the same cross section when viewed with the BP 450-490 filter (fluorescein; FITC). The CYTM3 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 α -Bungarotoxin that labels intact motor endplates if present. Above right hand views indicate that fibers positive for NCAM were not also positive for α -Bungarotoxin. Small arrows point to myoneural junctions. This indicates that myoneural junctions, when present, were not false indicators of denervation. Scale bars are 100 μ m.

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 $\pm$ 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 $\pm$ 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 rigor* 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 $\cdot$ m⁻²) was significantly decreased compared to both the SHAM (278 ± 43 kN $\cdot$ m⁻²) group and the TOTAL group (242 ± 47 kN $\cdot$ 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 filter which allows visualization of the CYTM3 label, the positive indicator of NCAM protein. Column B contains the same cross section when viewed with the BP 450-490 filter showing the fluorescein conjugate on the α -Bungarotoxin. Large arrows indicate fibers labeled positively for NCAM protein (NCAM-positive cells). The small arrows point to α -Bungarotoxin bound to intact motor endplates. That muscle fibers positive for NCAM were not also positive for α -Bungarotoxin is seen by comparing column A photomicrographs with those in column B. The lack of double labeled fibers indicates that myoneural junctions, when present, were not false indicators of denervation.

The NCAM immunohistological data are summarized in Table 3. The mean total number of fibers measured in each muscle cross section was very similar for each neurovascular muscle transfer group (SHAM = 2281 ± 559 cells; TOTAL = 1776 ± 372 cells; REDUCED = 2270 ± 883 cells ($p > 0.2$)). Neurovascular muscle transfer group assignment had a significant effect on the percentage of EDL muscle CSA that was denervated. Muscles from rats in the SHAM group were noted to contain relatively few denervated (NCAM-positive) fibers and when present these were isolated and surrounded by innervated (NCAM-negative) fibers. Reinnervated EDL muscles from the TOTAL and

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

Neurovascular Muscle Transfer Group	Dependent variables					
	Body mass [†] (g)	Muscle mass (mg)	L ₀ (mm)	L _f (mm)	EDL CSA (mm ²)	EDL Innervated-CSA ^{†**} (mm ²)
SHAM (n = 9)	423 ± 19	173 ± 21	35 ± 1	12 ± 0.3	14 ± 2	13 ± 2
TOTAL (n = 7)	424 ± 26	140 ± 20	35 ± 2	12 ± 0.2	11 ± 2	11 ± 2
REDUCED (n=13)	413 ± 30	114 ± 38*	34 ± 1	12 ± 0.5	9 ± 3*	8 ± 3*

Notes: g = grams, mg = milligrams, mm = millimeters, L₀ = optimal muscle length, L_f = 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, $4.7 \pm 3.7\%$ for the TOTAL group; and $8.4 \pm 3.2\%$ 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 force 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.

Neurovascular Muscle Transfer Group	Dependent variables		
	P ₀ (mN)	specific P ₀ (kN/m ²)	"corrected" specific P ₀ (kN/m ²)
SHAM	3766 ± 363 (n = 9)	278 ± 43 (n = 8)	283 ± 47 (n = 7)
TOTAL	2608 ± 448* (n = 7)	242 ± 47(n = 7)	254 ± 49 (n = 7)
REDUCED	1602 ± 776* [†] (n = 13)	173 ± 54* [†] (n = 13)	185 ± 64* (n = 11)

Notes: mN = millinewtons, kN = kilonewtons, 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. $p < 0.05$.

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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

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

Neuro-vascular Muscle Transfer Group	Dependent Variables						
	Innervated-CSA (%)	Denervated-CSA (%)	Innervated individual fiber area (μm^2)	Denervated individual fiber area (μm^2)	Total fiber count	Innervated fiber count	Denervated fiber count
SHAM (n = 9)	98.9 $\pm$ 0.4	1.1 $\pm$ 0.4	2590 $\pm$ 617	2539 $\pm$ 452	2281 $\pm$ 559	2256 $\pm$ 555	24 $\pm$ 9
TOTAL (n = 7)	95.3 $\pm$ 3.7*	4.7 $\pm$ 3.7*	2099 $\pm$ 584	1768 $\pm$ 449*	1776 $\pm$ 372	1681 $\pm$ 362	94 $\pm$ 74*
REDUCED (n = 11)	91.6 $\pm$ 3.2*†	8.4 $\pm$ 3.2*†	1686 $\pm$ 511*	1380 $\pm$ 376*	2270 $\pm$ 883	2049 $\pm$ 788	220 $\pm$ 104*†

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, μm = 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 $\pm$ SD. *Indicates group is significantly different from SHAM group. †Indicates group is significantly different from TOTAL group, $p < 0.05$.

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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 P_0 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 P_0 and specific P_0 similar to those seen in this study [9, 57, 58].

Others report rat medial gastrocnemius neurovascular muscle transfer data showing significant decreases in P_0 (between 15% and 43%) and specific P_0 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 P_0 and specific P_0 deficits persist for the medial and lateral gastrocnemius muscles but not for the smallest and most overloaded plantaris muscle [45]. Our P_0 and specific P_0 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 effected [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 P_0 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 P_0 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 P_0 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]. Vacuolar 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 P_0 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 myoneural 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 myoneural junction per muscle fiber in normal rodents by postnatal day 11 [50]. Tenotomy during polyneural withdrawal delays polyneural 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 P_0 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 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.

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