Neurovascular Transfer Does Not Cause Skeletal Muscle Fiber Degeneration

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Background: The mechanisms responsible for the incomplete recovery of muscle function after microneurovascular transfer have not been fully determined. Because fiber degeneration and regeneration can impact muscle mechanical function, the authors tested the null hypothesis that ischemia-induced fiber degeneration is not responsible for the force deficits observed after neurovascular muscle transfer.

Methods: Rats were assigned to one of three groups: orthotopic, nonvascularized grafting of the extensor digitorum longus muscle (STD group); orthotopic, neurovascular transfer of the extensor digitorum longus muscle with no intraoperative ischemia (NV-0 group); or orthotopic, neurovascular transfer of the extensor digitorum longus muscle with 3 hours of intraoperative ischemia (NV-3 group). At 1 and 2 weeks, extensor digitorum longus muscle cross-sections were labeled for developmental myosin heavy chain isoforms, markers of fiber regeneration.

Results: In extensor digitorum longus muscles from animals in the STD group, many small cells strongly labeled for developmental myosin heavy chain were observed and identified as myoblasts, indicating recent muscle fiber necrosis with subsequent regeneration. Extensor digitorum longus muscles from rats in the NV-0 and NV-3 groups contained no cells labeled for developmental myosin heavy chain.

Conclusions: After neurovascular muscle transfer (with ischemia times up to 3 hours), ischemia-induced muscle fiber degeneration and regeneration does not occur. Muscle fiber degeneration is not responsible for the force deficits observed after microneurovascular skeletal muscle transfer. (*Plast. Reconstr. Surg.* 117: 1455, 2006.)

Microneurovascular transfer of skeletal muscle has been used clinically for the reconstruction of finger flexion, finger extension, facial animation, and other functional deficits.[1–12] A significant factor limiting the application of this technique to relatively few clinical circumstances is the observation that, in both clinical and experimental studies, a force deficit is almost always observed in muscles that have been subjected to neurovascular transfer.[3,13–24] The mechanisms responsible for muscle mechanical dysfunction after neurovascular transfer have not been fully determined.[25,26]

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Received for publication September 20, 2004; revised December 8, 2004.

DOI: 10.1097/01.prs.0000206356.77759.1a

In vivo, death of individual muscle fibers is followed by a well-described process of muscle fiber degeneration and regeneration.[27–29] Degeneration and regeneration of skeletal muscle fibers has been proposed as a mechanism contributing to the mechanical deficit in neurovascular muscle transfers for two reasons. First, ischemia, tenotomy, and denervation have all been observed to result in at least some degree of muscle fiber degeneration,[30] and all three of the factors attend microneurovascular muscle transfer operations. Second, available experimental evidence suggests that, under most circumstances, muscles with many regenerated fibers manifest a deficit in mechanical function.[26,31] Note that, although available evidence suggests that intraoperative ischemia times less than 3 hours in duration do not correlate with the recovery of muscle function after microneurovascular transfer,[13] this evidence does not preclude the possibility that ischemia-induced degeneration and regeneration of individual muscle fibers results in a

population of muscle fibers with suboptimal mechanical function. As a result, a longstanding hypothesis in this area of research has been that muscle fiber degeneration and regeneration may account, at least partially, for the mechanical dysfunction in muscle after neurovascular transfer.

Using histologic criteria, Guelinckx et al. estimated that less than 5 percent of muscle fibers had undergone degeneration and regeneration when rabbit rectus femoris muscles were observed 8 days after undergoing microneurovascular transfer.[18] In this study, most degenerating fibers were identified near the site of tendon repair rather than in the center of the muscle. Prendergast et al. made similar, qualitative observations in muscle biopsy specimens taken 1 week after neurovascular transfer of the canine tibialis anterior transfer muscle.[32] This suggested that, under clinically relevant circumstances, fiber necrosis, degeneration, and regeneration do not occur to a significant extent after microneurovascular transfer and are therefore unlikely to be responsible for observed force deficits. However, histologic criteria may not be sufficient to identify all fibers undergoing a cycle of degeneration and regeneration. We therefore sought to observe muscles shortly after neurovascular transfer for more direct evidence of muscle fiber degeneration. We made the assumption that in the setting of neurovascular transfer, myocytes lethally injured by the procedure would undergo necrosis followed by degeneration and regeneration. Therefore, we used immunohistochemical visualization of the developmental isoforms of the myosin heavy chain, a marker of muscle fiber regeneration, to indicate that some myocytes had undergone degeneration at the time of muscle transfer. By observing muscles in the first 2 weeks after neurovascular transfer, we tested the following hypothesis: In neurovascular muscle transfers (with ischemia times up to 3 hours), ischemia-induced degeneration followed by regeneration of muscle fibers does not occur.

MATERIALS AND METHODS

Animal Care

Experiments were performed on 10-month-old Lewis rats (Charles River Laboratories, Wilmington, Mass.). Rats were housed individually in a specific-pathogen–free animal facility at the University of Michigan. The animals were provided food and water ad libitum and were subjected to a 12-hour light/dark cycle. All animal care and operative procedures were performed in accordance with the United States Public Health Service *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23) and were approved by the University Committee on the Use and Care of Animals. Rats were anesthetized for all surgical procedures with an intraperitoneal injection of sodium pentobarbital (60 mg/kg); supplementary doses were administered as needed to maintain a deep state of anesthesia. Surgical procedures were conducted under aseptic conditions.

Experimental Design

Rats were assigned randomly to one of three groups: NV-0, NV-3, or STD. Rats in the NV-0 group underwent a vascularized orthotopic transfer of the extensor digitorum longus muscle, leaving the vascular pedicle undisturbed (no ischemic interval). Rats in the NV-3 group underwent orthotopic vascularized transfer of the extensor digitorum longus muscle with a 3-hour intraoperative ischemic interval (vascular pedicle clamped for 3 hours). Rats in the STD group underwent standard, nonvascularized orthotopic grafting of the extensor digitorum longus muscle.[28,31,33] Note that we deliberately chose a rat model because, in the rat, muscle fiber degeneration does not occur to a significant extent in response to denervation alone.[34] Therefore, comparison of the NV-0 and NV-3 groups allowed isolation of the effect of intraoperative ischemia. Because virtually all muscle fibers in nonvascularized extensor digitorum longus grafts undergo ischemic necrosis followed by regeneration, grafted muscles from animals in the STD group served as the positive control. The contralateral intact extensor digitorum longus in each rat served as the negative control.

Neurovascular Transfer and Standard Grafting Procedures

For all groups, a longitudinal incision was made to allow isolation of the extensor digitorum longus muscle in one randomly chosen limb. The tendons of origin and insertion were severed and repaired with 7-0 nylon. In all three experimental groups, the extensor digitorum longus was denervated by sectioning the peroneal nerve. An epineural repair with 10-0 nylon suture was performed for each nerve. For rats in the NV-0 and NV-3 groups, the vascular pedicle was not severed. In the NV-3 group, a 3-hour period of intraoperative ischemia was induced by means of a microvascular clamp occluding the pedicle for 3 hours

before being removed, allowing muscle reperfusion. In animals in the STD group, the vascular pedicle was severed to induce global, permanent muscle ischemia. At the end of the procedure, the leg incision was closed in layers with 4-0 chromic suture and animals were returned to standard cages and allowed to recover.

MHC Immunohistochemistry

At 1 and 2 weeks after the initial procedure, animals from each group were killed and the extensor digitorum longus was harvested from both legs. The muscles were frozen in isopentane cooled with liquid nitrogen (–160°C) and stored at –90°C before being sectioned at 10 to 12 μm with a cryostat. Sections of each muscle were mounted on glass slides and stained with hematoxylin and eosin for morphological examination. To detect developmental myosin heavy chain isoforms, additional sections of each muscle were labeled with the monoclonal antibody NCL-MHCd (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), which binds to embryonic and fetal myosin heavy chain isotypes. The specificity of this antibody, which has been previously described,[35,36] was confirmed with the monoclonal antibody F1.652 (Developmental Hybridoma Bank, University of Iowa, Iowa City, Iowa).[37,38] F1.652 reacts with embryonic and fetal (neonatal) myosin heavy chain and is derived from hybridomas prepared from mice immunized with myosin heavy chain from human leg muscles. To label sections for developmental myosin heavy chain isoforms, the 10- to 12-μm sections were air-dried, blocked with 2% horse serum in phosphate-buffered saline for 20 minutes, and incubated with the developmental myosin heavy chain antibody for 1 hour at room temperature. This was followed by incubation with biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, Calif.) and then with biotin-avidin-peroxidase complex (diluted in 1% horse serum as per the Vectastain ABC kit; Vector Laboratories) at room temperature for 30 minutes each. The sections were then washed for 30 minutes using three changes of phosphate-buffered saline. Phosphate-buffered saline washes were also used between each of the individual steps. The sections were developed by 5 minutes of incubation in 0.5 mg/ml diaminobenzidine tetrahydrochloride, 0.01% H_2O_2 in phosphate-buffered saline. Sections were then mounted in Permount (Fisher Scientific, Springfield, Ill.).

Extensor Digitorum Longus Muscle Section Evaluation

Muscle cross-sections from each extensor digitorum longus muscle were examined with a standard light microscope under magnifications ranging from 80× to 250×. All sections were examined by a single observer, blinded to the group assignment of the animal from which the muscle was harvested.

Cells that had the morphological characteristics of myoblasts (small cells arranged in palisades or columns) *and* that had cytoplasm that was strongly labeled for the developmental isoform of the myosin heavy chain were identified as myoblasts involved in the regeneration of muscle fibers that had undergone prior necrosis and degeneration. If sections from a muscle were noted to contain even one developmental myosin heavy chain–positive myoblast, that muscle was designated as containing regenerating fibers (regeneration-positive). If no developmental myosin heavy chain–positive cells were observed in a given section, the muscle was designated as not containing regenerating fibers (regeneration-negative). Note that the methods used to label myosin heavy chain isoforms and to identify regenerating muscle fibers have been used in previous studies reported by our group.[39]

Statistical Analysis

Chi-square analysis was used to determine whether group assignment and the regenerating fiber status assigned to each muscle were independent variables (chi-square test of independence; Sigma Scan, Jandel Scientific, San Raphael, Calif.). For this analysis, alpha was set at 0.05 and beta was set at 0.80.

RESULTS

Twenty-two animals were entered into the study. One animal in the NV-3 group died during the initial operation and was replaced, resulting in seven animals in each group. In each group, three animals were killed 1 week after the initial surgical procedure, and four animals were killed 2 weeks after the initial surgical procedure.

Figures 1 and 2 present representative extensor digitorum longus muscle sections from animals in all groups. Table 1 presents the results of assignment of individual muscles to regeneration-positive or regeneration-negative status. Chi-square analysis indicated that group assignment and regeneration status were strongly related ($p < 0.001$, power = 0.94),

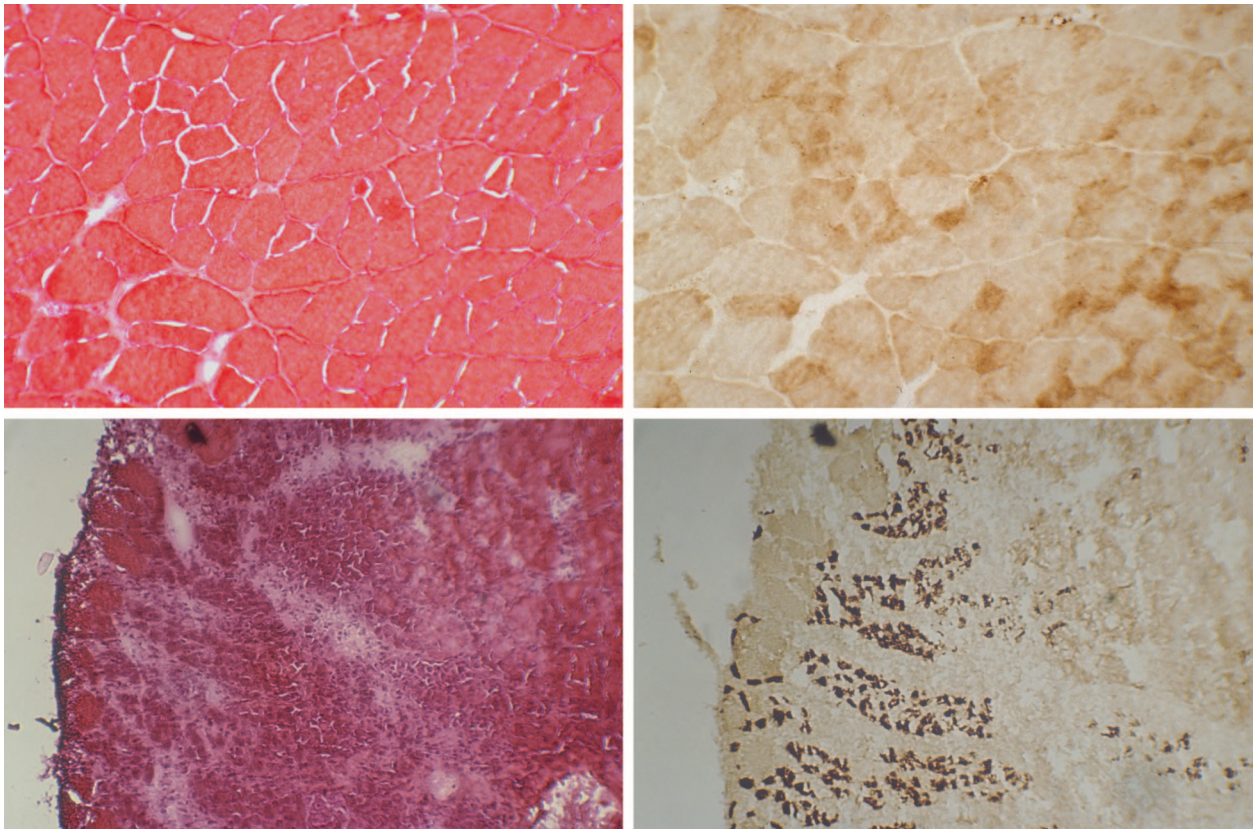

Fig. 1. Representative sections from extensor digitorum longus muscles in the contralateral control limbs (*above*) or from the experimental limbs of animals in the STD (*below*). Sections in the right hand column are stained with hematoxylin and eosin; sections in the left hand column are labeled for developmental myosin heavy chain. Note that the magnifications have been varied for each frame to best illustrate the relevant morphology. (*Above*, *left*) Normal extensor digitorum longus muscle (original magnification, ×156). Normal fascicular organization observed. (*Above*, *right*) Normal extensor digitorum longus muscle (original magnification, ×156). Note absence of fibers strongly staining for developmental myosin heavy chain (negative control). (*Below*, *left*) extensor digitorum longus muscle from rat in the STD group harvested 1 week after grafting (original magnification, ×125). Note peripheral, surviving myocytes, central necrotic core, and intermediate zone with small, mononuclear cells. (*Below*, *right*) extensor digitorum longus muscle from a rat in the STD group (1 week; original magnification, ×125). Note small, mononuclear cells in intermediate zone labeled strongly for developmental myosin heavy chain. These regenerating myocytes serve as a positive control.

In the negative controls (contralateral leg extensor digitorum longus muscle), normal fiber architecture was observed and no fibers were identified as being labeled for developmental myosin heavy chain (Fig. 1, *above*). As expected, hematoxylin and eosin–stained sections of extensor digitorum longus muscles from rats in the STD group revealed a central, necrotic core with a peripheral rim of surviving fibers in a pattern characteristic of regenerating, standard extensor digitorum longus muscle grafts (Fig. 1, *below*, *left*). In addition, extensor digitorum longus muscles from rats in the STD group had numerous cells labeled positively for developmental myosin heavy chain (Fig. 1, *below*, *right*) at both 1 and 2 weeks of recovery, indicating that regenerating fibers are successfully detected using our antibody (positive control).

No cells were identified that were strongly labeled for developmental myosin heavy chain in extensor digitorum longus muscle sections from animals in either the NV-0 (Fig. 2, *above*) or the NV-3 group, at either time point examined (Fig. 2, *below*). In sections from animals in both the NV-0 and NV-3 groups, some cells that had morphological characteristics of adult myocytes (large diameter, multinucleated cells, polygonal shape) were observed to be very weakly labeled for developmental myosin heavy chain. Because the morphological identity of these cells as adult myocytes was unambiguous, the weak developmental myosin heavy chain labeling was not considered to be

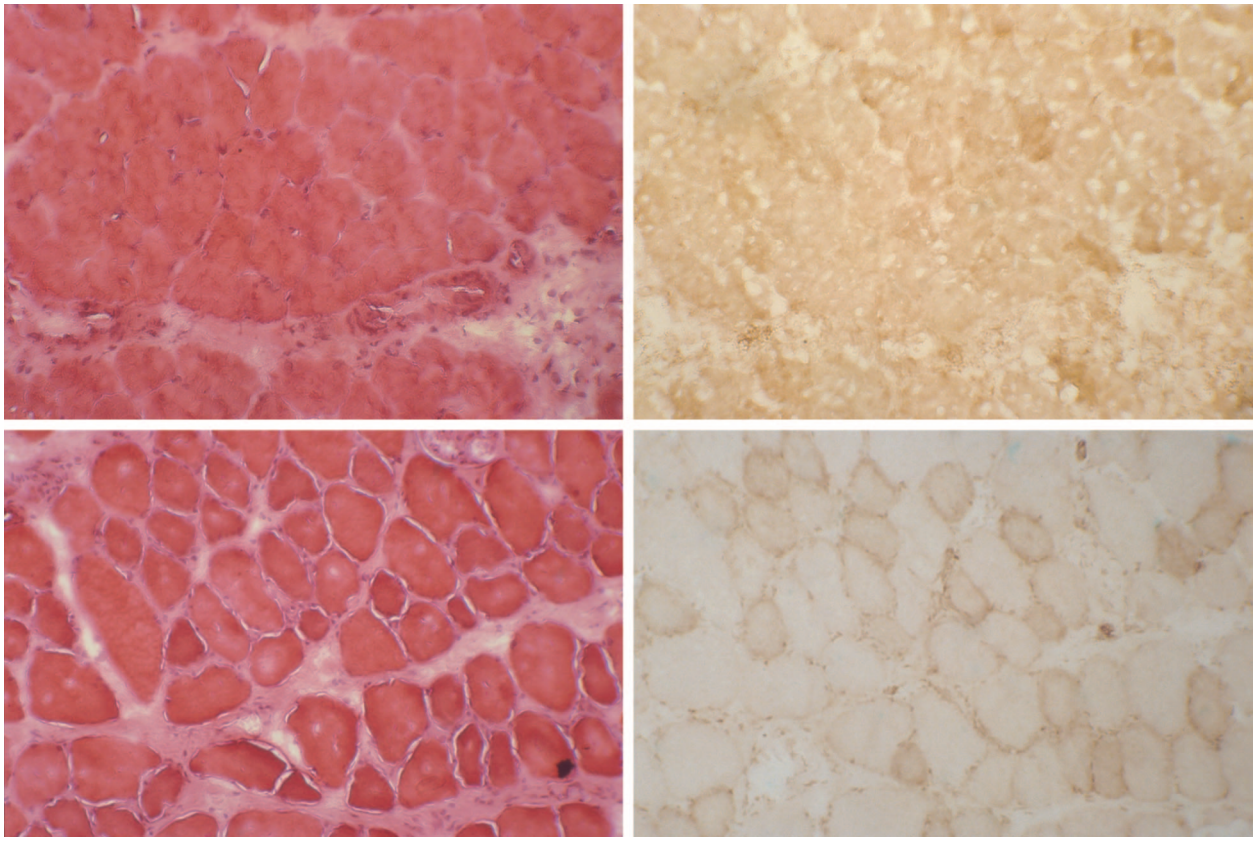

Fig. 2. Representative sections from extensor digitorum longus muscles from the experimental limbs of animals in the NV-0 (*above*), and NV-3 (*below*) groups. Sections in the right hand column are stained with hematoxylin and eosin; sections in the left hand column are labeled for developmental myosin heavy chain. Note that the magnifications have been varied for each frame to best illustrate the relevant morphology. (*Above*, *left*) extensor digitorum longus muscle from a rat in the NV-0 group (1 week; original magnification, ×125). Normal fiber architecture. (*Above*, *right*) extensor digitorum longus muscle from a rat in the NV-0 group (1 week; original magnification, ×125). No fibers strongly labeled for developmental myosin heavy chain. (*Below*, *left*) extensor digitorum longus muscle from a rat in the NV-3 group (2 weeks; original magnification, ×250). Thickening of interfascicular septae; normal fiber architecture. (*Below*, *right*) extensor digitorum longus muscle from a rat in the NV-3 group (2 weeks; original magnification, ×250). No fibers strongly labeled for developmental myosin heavy chain.

evidence of muscle cell regeneration. The reason for the weak developmental myosin heavy chain labeling observed was not determined and will require further study.

DISCUSSION

Our observations support our hypothesis that neurovascular muscle transfer procedures with up to 3 hours of ischemia time do not result in necrosis, degeneration, and regeneration of muscle fibers. This finding leads us to conclude that necrosis, degeneration, and regeneration of muscle fibers at the time of neurovascular transfer is very unlikely to be a major mechanism contributing to the mechanical deficits observed in neurovascular muscle transfers. This conclusion is in agreement with histologic evidence from previous studies.[18,32] Note again that this conclusion is reliant on the assumption that degeneration of muscle fibers will result in fiber regeneration after neurovascular transfer.

The observation times of 1 and 2 weeks posttransfer were chosen deliberately based on abundant data indicating that, in vivo, necrotic muscle fibers will induce proliferation and fusion of satellite cells (so-called adult myoblasts) within this time frame.[29] Developmental myosin heavy chain–positive cells in extensor digitorum longus muscles from animals in the STD group had the typical appearance of myoblasts and were found in a pattern consistent with previous morphological descriptions of regenerating rat extensor digitorum longus muscles.[29] As in previous work from our

Table 1. Frequency of Regeneration-Positive and Regeneration-Negative Extensor Digitorum Longus Muscles from Animals in Each Experimental Group

Regeneration Status	Group			
	Control	STD	NV-0	NV-3
Positive	0	7	0	0
Negative	7	0	7	7

Data values are the number of extensor digitorum longus muscles that contained cells that were positively labeled for developmental myosin heavy chain (regeneration-positive) or that contained no cells strongly labeled for developmental myosin heavy chain (regeneration-negative). The chi-square test of independence indicates that regeneration status and group assignment are not independent ($\chi^2 = 14.0$, $p < 0.001$, power = 0.94).

laboratory, this positive control confirmed that the antibody chosen allowed identification of regenerating muscle fibers.[39] The absence of positive cells in the contralateral extensor digitorum longus muscles in all groups (i.e., the negative controls) strengthens this conclusion. Based on these controls, we believe it is unlikely that our observations were confounded by any factors related to the immunohistochemical methodology.

The absence of regenerating fibers in the extensor digitorum longus muscles from animals in both the NV-0 and NV-3 groups in this study supports data from previous studies indicating that, for ischemia times up to 4 hours, ischemia is not a significant determinant of the contractile deficit usually observed after microneurovascular muscle transfer.[13] Data from clinical observations also indicate that there is no relationship between intraoperative ischemia time ranging from 1 to 4 hours and the quality or rapidity of onset of the recovery of function in neurovascular transfers used for facial reanimation.[40] Therefore, available data do not support the hypothesis that intraoperative ischemia, by means of muscle fiber degeneration or other mechanisms, is a major factor contributing to the force deficit in muscle transfers. Of course, this statement is only applicable for ischemia times within the clinically "acceptable" range of less than 4 hours. It is also important to note that our study does not exclude the possibility that nonlethal injury to myocytes may impair their mechanical function. To study this possibility, we are currently measuring force production in individual, permeabilized fibers from muscles after neurovascular transfer.

If injury sufficient to cause necrosis of myocytes is not the cause of the mechanical deficits observed in neurovascular muscle transfers, other mechanisms must be responsible. Because both capillary geometry and blood flow are largely unchanged after neurovascular muscle transfer, it is unlikely that changes in muscle perfusion account for the mechanical deficits observed in muscle transfers.[41,42] In contrast, available data suggest that, under many circumstances, denervation/reinnervation and tenotomy and repair can result in deficits in skeletal muscle contractile function.[16,18,26,43–45] These factors must remain the prime independent variables in future studies of mechanical function in neurovascular muscle transfer.

CONCLUSIONS

We did not observe regenerating muscle fibers after neurovascular transfer of the rat extensor digitorum longus muscle with up to 3 hours of intraoperative ischemia. The data indicate that mechanisms other than injury sufficient to cause degeneration of myocytes must be largely responsible for the functional deficits observed after neurovascular muscle transfer.

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ACKNOWLEDGMENTS

This study was supported by National Institute of Neurologic Disease and Stroke grant NS34380. The F1.652 hybridoma was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, Iowa City, Iowa.

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