

The Biology and Restorative Capacity of Long-Term Denervated Skeletal Muscle

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Abstract

Mammalian skeletal muscle undergoes profound atrophy after denervation. The functional restoration of denervated muscle is a significant clinical problem, and the success of restorative attempts decreases substantially after several months of denervation. Rat extensor digitorum longus muscles are capable of excellent restoration for the first 2-3 months after denervation, but after that time the level of restoration upon reinnervation decreases dramatically. Severe atrophy precedes the loss of restorative capacity. Attempts to understand the basis for the reduced restorative ability have led to an intensive analysis of the biology of long-term denervated muscle. In fast muscles, the satellite cell population undergoes a major increase over the first 2 months after denervation, and thereafter it steadily declines. Atrophying muscle fibers lose nuclei through apoptosis, and some degenerate. New muscle fibers form either alongside atrophying muscle fibers or in place of degenerated ones. The microcirculation undergoes a tenfold diminution over the first year after denervation, and over time denervated muscle is characterized by increasing amounts of interstitial collagen. Various barriers to reinnervation are discussed. Attempts to improve the restoration of long-term denervated muscle have included the stimulation of regeneration and removal of interstitial collagen. Both of these have resulted in significant improvement in the level of functional restoration. Although chronic electrical stimulation maintains an excellent degree of mass and force in a denervated muscle, grafts of such muscles undergo no better restoration than grafts of denervated muscles.

Key words: denervation, regeneration, reinnervation, satellite cells, skeletal muscle, transplantation.

Basic Appl Myol 12 (6): 247-254, 2002

One of the longstanding problems in the field of muscle paralysis is the frequently poor recovery of muscle that has been denervated for prolonged periods. Although there is no exact rule and exceptions have been noted, many clinicians report that if a muscle has been denervated for more than a year restoration is poor, even though regenerating motor nerves may have reached the muscle [1, 3, 48]. This is particularly true after injuries to areas, such as the brachial plexus, where the axons must regenerate for distances of up to half of a meter before they encounter their target denervated muscle. In contrast Irintchev et al. [28] have reported good recovery of mouse soleus muscles that had been denervated for up to 7 months.

A denervated muscle undergoes a rapid decline in mass and an even more rapid decline in force. The rat extensor digitorum longus (EDL) muscle loses almost

two third of its mass and over 90% of its maximum force within a month after denervation (Figure 1). Yet, the success of attempts to restore such denervated muscles does not parallel the course or degree of atrophy. An EDL muscle that has been denervated for one to two months is highly atrophic, but yet its restorative capacity far exceeds that of a similarly atrophic muscle that has been denervated for a longer period (see below).

In an overall approach to improving the restoration of denervated muscle, attention can be focused on both the muscle and the nerve regenerating toward it. For several years our laboratory has investigated properties of the denervated muscle itself, as they may relate to its capacity for functional restoration. To do so, we have used a muscle grafting model.

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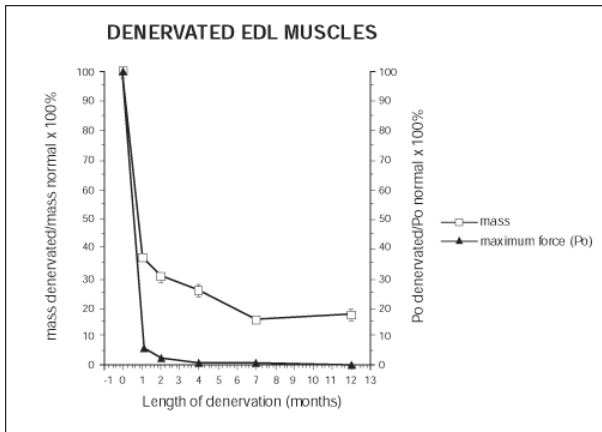


Figure 1. Curves showing the rapid decline in mass and maximum tetanic force in the denervated rat extensor digitorum longus muscle.

Materials, Methods and Experimental Model

The experimental model

The assay model used in many experiments was to remove an EDL muscle from the denervated hindlimb of a rat and graft it in place of the EDL muscle of a normal young host rat of the same inbred strain. After a 60-day period of recovery, functional and morphological properties of the grafts were evaluated [9].

Animals

All experiments were done on a highly inbred (100-120 generations of brother-sister matings) strain (WI/HicksCar) of Wistar rat. Hindlimbs of donor rats were denervated when the animals were 4-months old, and denervated muscles were grafted into 4-month old hosts.

Denervation

In donor rats, the right hindlimbs were denervated by severing the sciatic nerve high in the thigh. Consistent and permanent denervation for periods of up to 25 months without any follow-up operations can be accomplished by tightly tying the sciatic nerve with silk sutures in two places and severing the nerve between the sutures. The free ends of the nerves are then reflected as far from one another as possible and then implanted into nearby muscles. With denervation operations on hundreds of rats, we have seen only one case of partial reinnervation of the lower leg segment.

Muscle grafting

The basic muscle grafting model consists of removing a denervated EDL muscle from a donor limb and grafting it in place of the EDL of a 4-month old host. Both proximal and distal tendons are sutured in place, and the motor nerve of the host, which has been carefully dissected from the host EDL muscle before removal, is implanted into the grafted EDL muscle by inserting it into the motor endplate area. The contralateral EDL muscle

from the same donor is also removed and implanted in the same manner into the corresponding leg of the host. No attempt is made to restore vascular connections. Spontaneous revascularization of EDL grafts occurs over the course of a week [24]. The grafts are allowed to regenerate for 60 days and are then removed for physiological and morphological analysis. Then the recoveries of the grafts of formerly denervated and contralateral control muscles are compared.

Summary of results of grafting previously denervated muscles

The decline in restorative capacity of denervated rat EDL muscles was documented in a series in which the recovery of grafts of EDL muscles that had been denervated for 2, 4, 5 ½, 7, and 12 months prior to grafting was compared with the recovery of non-denervated contralateral control muscles [9]. As measured by maximum tetanic force, the recovery of 2-month-denervated muscle equalled that of grafts of control muscles, but thereafter the level of recovery declined in a linear fashion up to 7-month denervated muscle (Figure 2). There was little change in the level of recovery between 7- and 12-month denervated muscles. These results are very similar to those obtained by Gulati [23].

From these data, three main postdenervation periods were identified (Fig. 2). The first (0-2 months) was characterized by rapid and severe atrophy, but yet the muscle retained the capacity for full regenerative recovery when grafted. The second period (2-7 months) was one in which the already atrophic muscle steadily loses its capacity for regenerative restoration. By seven months (third period), the restorative capacity of the muscle was so low that there was little room for further decline.

These experimental results led to the question, "What is happening to the denervated rat EDL muscle between 2 and 7 months of denervation that so greatly reduces its capacity for regenerative recovery?" A related question is how, despite the occurrence of great atrophy, a muscle denervated for less than two months retains its restorative capacity. These questions led to several years of intensive analysis of the biology of the denervated rat EDL muscle.

Changes in a Denervated Muscle That Could Affect Its Restorative Capacity

There are many possible reasons why a denervated muscle could lose its restorative ability over time. Many of these can be encapsulated within two broad ways of addressing the problem. The first is that something(s) could interfere with the regeneration of motor axons back to the denervated muscle fibers. The interfering factors could act at levels from the neuronal cell bodies to the neuromuscular junction (Fig. 3). The second focuses on the ability of the long-term muscle to respond to a returning motor innervation. This review will concentrate principally on changes occurring within a denervated muscle that could affect its ability to recover from denervation atrophy.

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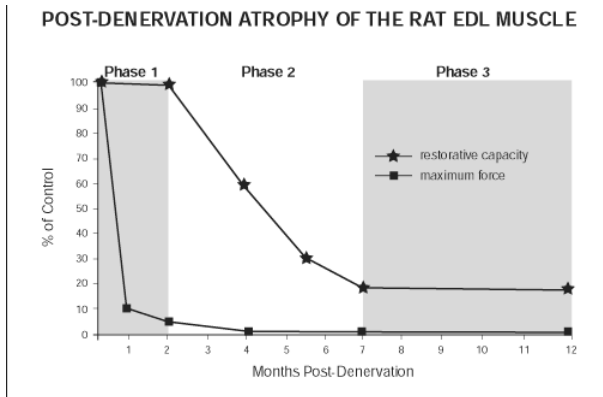


Figure 2. Comparison between the course of denervation atrophy and the level of restoration of grafts of denervated rat extensor digitorum longus (EDL) muscles. The data are based on maximum tetanic force generated by the entire muscle *in vitro*. The line connecting boxes represents a comparison between maximum tetanic force of the muscle denervated for the periods indicated on the X-axis in comparison with that of contralateral control muscles. The line connecting stars represents the return of maximum tetanic force of grafts of muscles that had been denervated for the lengths of time indicated on the x-axis before grafting was done. The return is expressed as percent of return as compared with that of grafts of contralateral control EDL muscles from the same donors into the same hosts. Phase 1 represents the period in which the denervated muscle, despite having undergone severe atrophy, still retains a restorative capacity equal to that of control muscle. Phase 2 is the period when the denervated muscle undergoes a steady decline in its restorative ability. By phase 3 the denervated muscle has already lost most of its ability to recover from atrophy, and little further decline takes place.

Muscle fiber atrophy and nuclear death

Following denervation, individual muscle fibers undergo dramatic atrophy [50], with atrophy of type II muscle fibers more prominent than that of type I during the early months [6, 32]. In young rats these differences are quite pronounced at 2 months, whereas there is much less of a difference in denervated muscles of old rats [10]. By 4 months after denervation the degree of atrophy of types I and II fibers is comparable.

For the first 7 months after denervation rat EDL muscle fibers exhibit a net loss of slightly more than one myonucleus per day [50]. This can be explained by the death of myonuclei in denervated muscle fibers. Individual myonuclei undergo the classic series of morphological changes associated with apoptosis in other cell types [5, 6, 40]. Interestingly, nuclear death does not necessarily lead to death of the entire myofiber, nor do all nuclei die. It is not uncommon to find nuclei with highly condensed chromatin next to myonuclei that are

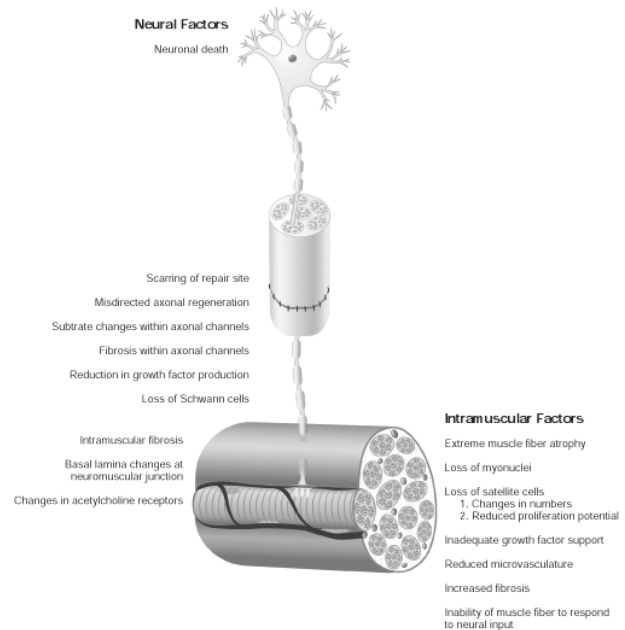


Figure 3. Schematic drawing illustrating where, both within the nerve and within the muscle, blocks to functional reinnervation of a long-term denervated muscle can occur.

structurally very healthy looking. In addition to individual myonuclei, entire muscle fibers also degenerate and die in long-term denervated muscle [2, 6, 44]. In order for a denervated EDL muscle to be restored, it would be necessary to replace the myonuclear population in the atrophic fibers with roughly double the amount of remaining nuclei or form significant numbers of new muscle fibers to replace those that had died.

Satellite cells

Satellite cells are the principal source of new myonuclei in growing muscle fibers, as well as the cellular source of regenerating muscle fibers [22, 26, 46]. The extent to which myogenic stem cells add to or duplicate satellite cell functions is not known. In the normal adult rat, satellite cells constitute approximately 3% of the total nuclei beneath the basal lamina in a fast muscle and 4-6% in slow muscle [20]. Denervation in the adult rat EDL muscle is followed by a rapid rise in satellite cell numbers, with the percentage doubling within the first 1-2 weeks post-denervation ([25, 34], Rengen and Carlson, unpublished data). At longer periods after denervation, both numbers and percentages of satellite cells come to a peak in the rat EDL muscle 2 months after denervation. Four months after denervation the number of satellite cells in the muscle has dropped to control levels, and by 7 months of denervation, the total population of satellite cells has fallen to one third of control levels [50]. After 25 months of denervation satellite cells constitute only 0.5% of all nuclei in the muscle fiber complex, as compared with 1.4% in control muscles from the same 29-month animals [13]. In contrast to

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these findings on the adult EDL muscle, Rodrigues and Schmalbruch [40] found a rather steady decrease in satellite cells in the soleus muscle of rats after denervation during the first 5 weeks after birth and only a small rise in satellite cells in the denervated EDL muscle.

An interesting relationship between steroid hormones and satellite cell frequency after denervation is seen in the testosterone-sensitive levator ani muscle. This muscle undergoes striking atrophy after castration, as well as after denervation. The normal adult levator ani has 1.9% satellite cells in relation to total muscle fiber nuclei [38]. After two months of denervation, the percentage of satellite cells doubles (4.3%), but after the same period of castration it remains the same (2.4%). Following combined denervation and castration, the percentage of satellite cells (2.3%) did not undergo the increase seen after denervation alone, indicating that in this muscle the satellite cell response after denervation requires a hormonal intervention. Administration of testosterone resulted in a significant increase (5.2%) in the satellite cell frequency after two months of castration/denervation [39].

There is no direct information on the maximum proliferative potential of individual satellite cells in long-term denervated muscle. Therefore it is not possible to make quantitative estimates on the capacity of the satellite cell population in such a muscle to restore its functional mass through addition of nuclei to existing muscle fibers or by forming new fibers. Nevertheless, earlier studies with H3-thymidine labeling [34, 36] have shown significant increases in uptake in denervated rodent muscle. More recently, Schmalbruch and Lewis [45] used BRDU labeling to study the reactions of soleus and EDL muscles in young rats to denervation. BRDU labeling was sharply reduced in the denervated soleus as compared with the EDL muscle. The latter results show that caution must be used in extrapolating data collected on different muscles and from animals of different ages.

Satellite cells show abundant evidence of activation after denervation, as reflected in the production of increased amounts of cytoplasm and the expression of myogenic regulatory factors, such as MyoD and myogenin [13, 32]. Even though myogenin levels begin to fall after one month of denervation, they still remain substantially higher than normal [29]. *In toto*, the available evidence suggests that neither depletion nor reduction in individual function of satellite cells is sufficient in itself to account for the dramatic reduction in restorative ability of a long-term denervated rat EDL muscle.

New muscle fiber formation

Contrary to what might be assumed, denervated muscle is a highly active tissue at both the cellular and molecular level. Not only do muscle fibers undergo atrophy and, in some cases, degeneration, but new muscle fibers also form in denervated muscle [6, 40]. Borisov et al. [6] described two modes of new fiber formation in the denervated rat EDL muscle.

The first, beginning between 1 ½ and 3 weeks after denervation and lasting through the second month, consists of new muscle fibers forming between intact, but atrophying muscle fibers and the basal lamina. Morphologically, this resembles the formation of secondary and tertiary myotubes during normal ontogenetic myogenesis. A second mode, which is most prominent between 2 and 4 months after denervation, is the formation of new muscle fibers within empty basal laminae of degenerated muscle fibers. This closely resembles the formation of muscle fibers during regeneration.

It is noteworthy that both of the above modes of neomyogenesis are most prominent during the first four months after denervation, when the denervated muscle still possesses a reasonable degree of restorative capacity. Whether the lack of neomyogenesis during later post-denervation months is due to the inability of the satellite cells of the muscle to produce new myotubes or whether it is a reflection of an inadequate stimulus for new muscle formation remains to be determined.

The Microcirculation

Denervation exerts a profound effect on the microcirculation of a muscle. In normal muscle, capillaries are closely associated with muscle fibers, with a diffusion distance often little greater than that of the diameter of the endothelial cells. Over the course of a year, the capillary/muscle fiber ratio falls to roughly 10% of the normal adult level [7]. In addition to the large drop in capillary density, the capillaries are further removed from the severely atrophic muscle fibers by large deposits of interstitial collagen fibers. This adds to the diffusion distance and could have a highly detrimental effect on the overall nutritional level of the affected muscle fibers. In a transplant model of restoration such a massive reduction in the microcirculation could reduce the number of endothelial cells available for restoring the vascular bed of the grafted muscle. The role of the pericytes remains enigmatic in both the reduction and restoration of the microcirculatory supply. However, concentric layers of basal lamina material around capillaries in long-term denervated muscle [14] provide evidence of repeated cycles of capillary degeneration and regeneration.

Interstitial connective tissue

The deposition of large amounts of interstitial connective tissue in the form of dense mats of collagen fibers is a hallmark of chronically denervated muscle. The increase in deposition of collagen fibers is a reflection of increased proliferation of connective tissue cells after denervation of muscle [45]. This could interfere with restoration of a denervated in a variety of ways. As mentioned above, the collagen fibers very likely interfere with the exchange of nutrients, oxygen and metabolic waste products between the atrophic muscle fibers and the capillary bed. The effect of such fibrosis on the transmission of growth factors or other signaling molecules within muscle tissue has not been carefully exam-

ined. Also it is not certain to what extent such fibrosis depresses the ability of satellite cells to respond to proliferative or migratory signals. Interstitial fibrosis could play a major role in preventing proper reinnervation of a long-term denervated muscle either through acting as a mechanical barrier or by providing a poor substrate for axonal elongation. For a pure denervation-reinnervation model, such fibrosis may be less of an issue, but in a transplant model, in which axons regenerating into the transplant must pass through interstitial tissue, this could have a significant dampening effect.

Intramuscular neural structures

Ideally axons regenerating into a previously denervated muscle will be able to utilize pre-existing neural pathways. For the major intramuscular nerve branches the issues of axonal extension are essentially those that are encountered in the more proximal nerve trunks. These include the production of regeneration-enhancing molecules by Schwann cells [19, 21, 27, 35], alterations in the substrate upon which the axons grow [16, 33, 37], and fibrosis within the channels formerly occupied by motor axons [8, 42, 51]. Schwann cells are strongly affected by nerve damage, both acutely and over long periods [19, 49, 52]. Despite many changes, Schwann cells persist for long periods after denervation. Even 25 months after transaction of the sciatic nerve, the Schwann cells remaining in the distal intramuscular stumps continue to express NCAM and S-100 protein [15].

The normal endpoint of axonal regeneration is the site of the original neuromuscular junction, assuming that the regenerating axons are able to extend down pre-existing basal lamina tubes. The basal lamina at the neuromuscular junction contains a great deal of molecular information, some of which is associated with recognition and stabilization by the regenerating axon terminals [18, 43]. Despite the preference for the original endplate site, axons regenerating into a muscle can readily settle down on non-endplate areas if the zone of motor endplates has been removed [53]. This experiment has not been tried on very long-term denervated muscle, and it is not known if changes in the interstitial connective tissue in this case would reduce the efficiency of formation of neuromuscular junctions *de novo*. Even less is known about the role of acetylcholine receptors or other surface molecules that are expressed on the muscle fibers themselves in attracting or retaining axon terminals, since the basal lamina intervenes between the muscle fiber and the regenerating nerve.

Surprisingly, muscle spindles persist for very long periods after denervation (at least two years in the rat). As long as the spindle capsule remains intact, spindles also survive the degeneration and regeneration that occurs in a muscle transplant, but as indicated by their structure and histochemical pattern, reinnervation is abnormal and incomplete [41].

Denervation in Old Animals

Because partial denervation through motor unit remodeling is a part of the normal aging process [30, 31], old muscle contains populations of muscle fibers that have been denervated for short periods before becoming reinnervated by sprouting of other, preferentially slow, axons or that may be permanently denervated. Recent studies on denervated limb muscles in old rats [10] have shown surprisingly few differences from the reactions of denervated muscles in young rats. The rate of muscle fiber atrophy, especially among type II fibers, is somewhat reduced in old animals, and baseline levels of expression of several molecules, such as peptide elongation factor (eEF1a-1), myogenin, and the γ -subunit of the nicotinic acetylcholine receptor, are somewhat increased over those in young adult rats. Although the levels of these molecules increase after denervation in both young and old rats, the absolute levels in the old animals post-denervation were not higher than those in young animals, suggesting that the increased levels in control old muscle may be due, in part, to the presence of some denervated fibers in these muscles. The frequency of satellite cells in two-year old rats is half of that in young muscle [12], and after two months of denervation the increases in satellite percentage are comparable, showing that aging does not affect the ability of satellite cells to become activated. Therefore this should not be a limitation to the restoration of long-term denervated muscle in old individuals.

Attempts to Restore Long-Term Denervated Muscle

Over recent years, several attempts have been made to restore long-term denervated muscle through regeneration. The first was simply removing a denervated muscle and grafting it into an innervated site. Both Gulati [23] and Carlson et al. [9] have found that in muscle that has been denervated for over 2-3 months there is a steady decline in its restorative capacity (Fig. 2). One potential reason for the results reported is that denervated muscles undergo less muscle fiber breakdown after transplantation than do normal muscles [11, 47]. However, the increased muscle fiber survival under such circumstances is seen in muscle that has been denervated as little as two weeks. Nevertheless, Billington and Carlson [4] tested the hypothesis that restoration of denervated muscles would be increased if greater amounts of regeneration were stimulated. Seven-month denervated rat EDL muscles were soaked in the myotoxic local anesthetic, bupivacaine, before being grafted into normally innervated host legs. Two months after transplantation the grafts of bupivacaine-treated muscles produced 3.3 times the maximum tetanic force of grafts of non-bupivacaine-treated 7-month denervated muscles. However, this degree of restoration was still only 38% that of grafts of normal control muscles.

Testing the hypothesis that the poor recovery of grafts of long-term denervated muscles is due to the prevention of adequate reinnervation because of interstitial fibrosis,

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Carlson (unpublished) treated 7-month denervated rat EDL muscles with collagenase before grafting in order to reduce the integrity of the connective tissue barrier. The collagenase-treated muscles had softened to the point of nearly falling apart at the time of grafting. The grafts of collagenase-treated muscles recovered over twice the maximum tetanic force of non-collagenase-treated denervated muscles, but again the level of recovery was far less than that of non-denervated muscles.

Dow [17] has been able to maintain mass of 4-month-denervated rat EDL muscles at 100% of control levels and maximum force at nearly 80% by chronic electrical stimulation. The muscles were locally stimulated 200 times each 24-hour period with a train of 20 bipolar pulses at 100 Hz with a 9.0V amplitude and 0.4 ms pulse width for the entire four months following denervation. However, when these muscles were grafted into innervated host limbs their recovery was no greater than that of non-stimulated denervated muscles. The reason for the discontinuity between the excellent maintenance of mass and force of the muscles after denervation and the poor response to grafting remains to be determined, but in this case, at least, the poor recovery cannot be attributed to excessive fibrosis.

Conclusions

The restoration of long-term denervated muscle is a complex problem that involves both axonal regeneration into the muscle and the response of the muscle to that reinnervation. This review has concentrated principally on the biology of long-term denervated muscle tissue as it relates to the restorative capacity of a denervated muscle. The experimental data presented above show that there is a significant reduction over time in the ability of a denervated muscle to respond to reinnervation. Therefore there is a major muscular component, as well as a neural component, to the overall problem of restoration. Within a denervated muscle, one potential area of interference with full restoration consists of mechanical or biochemical barriers to regenerating nerve axons. These could consist of changes within the intramuscular nerve trunks and increased interstitial connective tissue that could block the progress of axons regenerating outside pre-existing neural structures. Another intramuscular block to restoration could be an inability to reverse the atrophy of existing muscle fibers or to form new muscle fibers, and for each of these, satellite cell numbers and function could be important determinants.

The existing experimental evidence suggests that while mechanical barriers to reinnervation of denervated muscle fibers could, and probably do in some cases, interfere with restoration, a more fundamental underlying defect may reside in the ability of the denervated muscle to initiate myogenic activity, whether by forming new muscle fibers or by adding nuclei to existing atrophic muscle fibers. Evidence for this consists of the documented reduction in satellite cell numbers in long-term denervated muscles, the reduction in formation of new muscle fibers

seen in a muscle after four months of denervation, and the poor restoration of grafted muscles whose mass and contractile properties had been well maintained after denervation by electrical stimulation. What is needed now is an intensive analysis of individual satellite cells from long-term denervated (over 4 months in the rat) muscles to determine their proliferative potential and their ability to fuse with either other satellite cells or with pre-existing atrophic muscle fibers.

Acknowledgements

Original research reviewed here was supported by NIH grant PO1 AG 10821.

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