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Effects of Denervation on Fast-Twitch Extensor Digitorium Longus and Slow-Twitch Soleus Muscles of the Rat

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EFFECTS OF DENERVATION ON FAST-TWITCH EXTENSOR DIGITORIUM LONGUS AND SLOW-TWITCH SOLEUS MUSCLES OF THE RAT

by

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The undersigned certify that they have read, and recommend to the faculty of graduate studies and research for acceptance, a thesis entitled Effects of Denervation on Fast-Twitch Extensor Digitorium Longus and Slow-Twitch Soleus Muscles of the Rat, submitted by Kadhem Alkhenaizi in partial fulfillment of the requirement for the degree of Master's of Science in Anatomy.

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ABSTRACT

This study is set out to examine the response of two different muscles to loss of innervations. Serial frozen sections (10 μm thick) of fast-twitch extensor digitorium longus muscles (EDL) and slow-twitch soleus muscles (SOL) of the denervated and contralateral control hindlimbs at different time points (one, two and three weeks) were compared using different enzyme histochemical and immunohistochemical techniques. It was hypothesized that the fiber types in the two muscles would respond similarly to the loss of neuronal influence. Succinate dehydrogenase (SDH) was used to determine the oxidative capacity, while phosphorylase (PHRL) was used to determine the glycolytic capacity. The expression of myosin heavy chain (MHC) isoforms were determined by immunohistochmistry. Based on the enzyme histochemcial and immunohistochemical reactions, type 1 and 2A fibers in the denervated SOL showed severe progressive atrophy with time relative to the contralateral control SOL. Different fibers in the denervated EDL showed different extents of atrophy with time, type 2B fibers atrophied the most, then type 2X fibers and type 2A atrophied to a lesser extent relative to the contralateral control EDL. In addition, type 2B, 2X, and 2A fibers showed decreased glycolytic activities with time. In contrast, denervated type 1 fibers of the EDL maintained their size during the three week of denervation and expressed glycolytic activities. Thus, the results of the study showed that the fast-twitch EDL and slow-twitch SOL muscles responded differently to the loss innervation.

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Introduction

The overall properties of the skeletal muscle depend on the different skeletal muscle fiber types within the muscle. There are different ways to categorize skeletal muscle fibers, for example, by the myosin ATPase reactions (Brooke and Kaiser, 1970), by their metabolic and contractile properties (Peter et al., 1972), or by the expression of different myosin heavy chain (MHC) isoforms (Pierobon-Bormioli et al., 1981). Muscle fibers differentiate during development and aging. They are able to adapt to changed conditions. The adaptation of muscle fibers to different physiological and pathological conditions, such as denervation, occurs in the MHC isoforms by transformation, the metabolic activities, and the size of different muscle fiber types. In addition, hybrid fibers that show coexpression of MHC isoforms exist after denervation (Michel et al., 1996; Huey and Bodine, 1998).

Skeletal muscle fibers can be classified as type 1, 2A, and 2B based on the enzyme histochemcial assay of myosin adenosine triphosphatase (mATPase) (Brooke and Kaiser, 1970). The enzyme is located in the globular head of the myosin heavy chain. It hydrolyzes the adenosine triphosphate (ATP) to adenosine diphosphate (ADP) plus phosphate, and the energy released is used to release the myosin head from the myosin binding site on the actin filaments during the cross-bridge cycle of sarcomere shortening or muscle contraction (Huxley, 1964).

The enzyme histochemical method used to demonstrate mATPase activity is based on the differential liability of the activity of mATPase to different preincubation pHs (Brooke and Kaiser, 1970). Muscle fibers containing the mATPase that is acid-stable

and alkali-labile stain darkly at pH 4.3 or lower, whereas all other fibers not containing this mATPase are unstained (Fig. 1A). The stained fibers are called type 1 fibers. Muscle fibers containing the mATPase that is alkali-stable stain darkly at pH between 9.5-10.2, whereas fibers not containing this mATPase are unstained (Guth and Samaha, 1969). These stained fibers are referred to as type 2 fibers. Type 2 fibers can be further subdivided to types 2A and 2B on the basis of the differential liability of the mATPase at pH 4.5-4.6, where type 2A fibers are lightly stained and type 2B fibers are intermediately stained. At this preincubation pH type 1 fibers retain their dark staining (Fig. 1B). Table 1 summarizes the reaction of myofibril ATPase at different preincubation pHs.

Table 1. Comparative myosin ATPase staining of fiber types after different preincubation pH.

	Myofibril ATPase staining		
Fiber type	pH 4.3	pH 4.55	pH 9.5-10.2
1	Dark	Dark	Light
2A	Light	Light	Dark
2B	Light	Intermediate	Dark

Peter et al. (1972) added the metabolic profiles (oxidative and glycolytic enzyme activities) to the myosin ATPase reaction as a contractile speed marker to classify skeletal muscle fibers. This led to formulation of three categories: slow-twitch-oxidative (SO), fast-twitch-glycolytic (FG), and fast-twitch-oxidative-glycolytic (FOG),. This classification scheme was formulated on the basis of: 1) the contraction

speed of the fiber relative to the others within the same animal, 2) glycolytic capacity, and 3) oxidative capacity.

Slow-twich-oxidative (SO) fibers are characterized by low glycolytic activities, such as phosphorylase (PHRL) (fiber 1 in Fig. 1C), and low glycogen content (as determined using Periodic Acid Schiff reaction (PAS) (fiber 1 in Fig. 1D). Their oxidative capacity is manifested by cytochrome a, succinate dehydrogenase (SDH), and nicotinamide adenine dinucleotide tetrazolium reductase (NADHtr) and is intermediate between the FG and FOG fibers (Peter et. al., 1970); (see fiber 1 in Fig. 1E & 1F). The SO fibers have high capillary density (Mai et al., 1970) and myoglobin concentration (Reis and Wooten, 1970). These fibers are physiologically characterized by long contraction times, slow-twitch speed, and great fatigue resistance (Burke et al., 1973). Based on these metabolic and contraction criteria, the SO fibers rely primarily on aerobic metabolism.

Fast-twitch-glycolytic (FG) fibers have high glycogenolytic and glycolytic enzyme activites, such as PHRL (see fiber 3 in Fig. 1C) and lactate dehydrogenase (LDH), respectively. They also have high glycogen content (see fiber 3 in Fig. 1D) (Fairchild and Fournier, 2004). The oxidative capacity of FG fibers is low (see fiber 3 in Fig. 1E & 1F). These fibers have low capillary to fibers ratio compared to SO and FOG fibers (Mai et al., 1970). The FG fibers also have low myoglobin concentration (Reis and Wooten, 1970). Physiologically FG fibers are characterized by fast-twitch speed, short contraction time and ready fatigability fibers (Burke et al., 1973). All these

characteristics indicate that FG fibers rely mainly on anaerobic metabolism for the production of ATP.

Fast-twich-oxidative-glycolytic fibers (FOG) have high glycolytic and oxidative capacities, and high glycogen concentration (Peter et al., 1970) (fiber 2 in Fig. 1C-1F). FOG fibers have high capillary density (Mai et al., 1970) and myoglobin concentration (Reis and Wooten, 1970). Physiologically, these fibers are characterized by intermediate twitch speed, long contraction times, and fatigue resistance (Burke et al., 1973). The characteristics of FOG fibers indicate the possession of anaerobic and aerobic metabolism for energy generation.

Close (1972) assumed that there was a relationship between the myofibril ATPase classification scheme of Brooke and Kaiser (1970) and the metabolism-contraction classification scheme of Peter et al. (1972). The correlation, while not exact, was that type 1, 2A, and 2B fibers are roughly related to SO, FOG, and FG fibers, respectively. Type 1 and 2A fibers were reasonably comparable with SO and FOG fibers. Type 2B fibers did not match FG fibers because a large population of 2B fibers showed high oxidative capacity (Nemeth and Pette, 1980 and 1981). Fibers with high oxidative capacities (fiber 4 in Fig. 1) were later classified as type 2X fibers (Schiaffino et al.,1989; Gorza, 1990), or type 2D fibers (Termin et al., 1989; Hamalainen and Pette, 1993) using immunohistochemical procedures with specific antibodies against myosin heavy chain isoforms (Pierobon-Bormioli et al., 1981).

Myosin heavy chain (MHC) isoform (thick filaments) is the most closely correlatable structural protein to the speed of contraction of muscle fibers. It is composed of two major components, an alpha-helical coil rod-shaped part and a globular-head part containing the myosin ATPase activity and binding site for actin (Berne et al., 2004). Different MHC isoforms exist (Pierobon-Bormioli et al., 1981). Therefore, skeletal muscle fibers can be classified based on the type of MHC isoform into type 1, 2A, 2X (2D), and 2B fibers by using monoclonal anti-MHC isoforms. Each MHC isoform is encoded by a distinct gene (Mahdavi et al., 1987, and DeNardi et al., 1993).

Type 2X fibers represent a major population in rat's hindlimb muscles (Punkt, 2002). They have oxidative capacity intermediate between type 2A and 2B fibers and can only be identified by immunohistochemical analysis with anti-MHC antibodies (Gorza et. al. 1990). Type 2X MHC isoform were first identified using serial sections, each of which was stained with one specific MHC monoclonal antibody and then performing a comparison of fiber staining in these serial sections. This allowed the identification of any fiber that was unstained in all serial sections (DeNardia et al., 1993; Gorza, 1990; Schiaffino et al., 1989). Lucas et al. (2000) used monoclonal antibodies against MHC 2X that the previously classified as 2B fibers in human muscles were identified as being in fact type 2X fibers based on their MHC content. Therefore, classification of skeletal muscle fibers based on the MHC isoform content as type 1, 2A, 2X, and 2B is now considered as the most complete MHC classification scheme (Pette and Staron, 2001).

However, two or more MHC isoforms may coexist in the same fiber. For example, MHC 2X is coexpressed with MHC 2A (Gorza, 1990; DeNardi et al., 1993). Collectively, the pure and such hybrid fibers form a continuum from slow to fast. Hybrid fibers can fill the gap between the pure fibers.

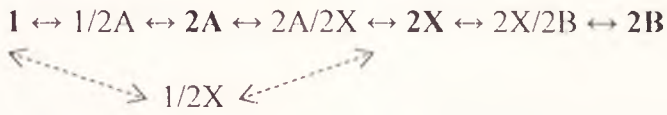


Figure 1: Diagrammatic relationship showing the pure (bold) and hybrid fibers.

Multiple MHCs coexpression in the hybrid fiber is not restricted to the nearest neighbor scheme; for example, type MHC 2X is coexpressed with type 1 MHC after high frequency stimulation of the SOL without passing through MHC 2A (Schiaffino et al., 1989). Table 2 summarizes the characteristics of skeletal muscle fiber types.

The MHC composition of skeletal muscle fibers changes throughout development. The same is true for the metabolic activities. These changes are different from one muscle to another. For example, slow-twitch soleus (SOL) muscles and fast-twitch extensor digitorium longus (EDL) muscles of the rat hindlimb, which are often used for investigation for physiological or pathological effects, show different pathways of fiber type transformation and metabolic activities with development (Ariano, 1973; Armstrong and Phelps, 1984).

Table 2: Summary of the characteristics of skeletal muscle fiber types.

Characteristics	Fibers type			
	1	2A	2X	2B
Contraction speed*	Slow-twitch	Moderately Fast-twitch	Fast-twitch	Very fast-twitch
Fatigue resistance**	High	Fairly high	Moderate	Low
Oxidative capacity*	High	High	Moderate	Low
Glyoclytic capacity*	Low	Moderate	Moderate	High
Glycogen concentration*	Low	Moderate	Moderate	High
Myoglobin concentration†	High	High	Moderate	Low
Capillary density††	High	High	Moderate	Low
Myosin heavy chain ‡	MHC 1	MHC 2A	MHC 2X	MHC 2B
Metabolic activity*	Aerobic	Aerobic/ anerobic	Aerobic/ anerobic	Anaerobic

* Peter et al. (1970). ** Burke et al. (1973). †Reis and Wooten (1970). †† Mai et al. (1970). ‡ DeNardi et al. (1993)

Before birth (21st embryonic day), the SOL and the EDL muscles of the rat are undifferentiated immunohistochemically for fiber types and enzyme histochemical for metabolic profiles (Punkt et al., 2004). However, the embryonic MHC isoform is initially expressed before birth and gradually declines and is replaced by the neonatal MHC isoform (Bulter-Brown and Whalen, 1984). These developmental MHC isoforms are not detected in the EDL and the SOL from 3 weeks to 3 months after birth, respectively (Whalen et al., 1981).

One day after birth, slow and fast fibers were typed using antibodies against slow and fast MHC. At postnatal day 8, the different myosin ATPase activities of slow and fast fibers after alkaline pH 10.4 preincubation were detected histochemically. At postnatal day 21, skeletal muscle fibers could be classified into type 1, 2A, and 2B with myosin ATPase reaction at a preincubation pH of 4.6. At this age also, oxidative and glycolytic metabolic profiles were distinguishable for the first time in the SOL and the EDL after delivery and muscle fibers were able to be classified into SO, FOG, and FG fibers (Whalen et al., 1981, and Punkt et al., 2004).

The muscle fibers of the SOL and EDL of the rat were transformed with aging. In the SOL, there was an increase in type 1 fibers and decrease in type 2A fibers, whereas, in the EDL, there was decrease in type 1 and 2A and increase in type 2B fibers with aging (Ansved and Edstrom, 1990). This transformation from fast to slow fibers in the SOL and from slow fibers to fast fibers in the EDL indicated the readiness of the muscle for the higher demands for mobility of the hindlimb and changes in posture.

It has also been observed that there was a topographic variation in fiber types along the longitudinal and the transverse axis form one muscle to another. For example, in the SOL of the rat, the population of the SO fibers increases from the origin of the muscle (60%) to the insertion of the muscle (90%). In the EDL, the FG fibers increased from the origin (50%) to the insertion of the muscle (65%) at the expense of the FOG fibers. There was also continuous increase in the oxidative capacity along the transverse axis form the superficial to the deep region of the SOL muscle. In

contrast, there was increase in the glycolytic capacity from the superficial to the deep region of the EDL muscle (Punkt, 2002).

Skeletal muscle fiber transformation and differentiation was under the control of many factors, including genetic makeup, innervation, hormones, mechanical loading and unloading, exercise, and aging (Pette and Staron, 2001). Among all these factors, innervation appeared to play a major role in muscle fibers transformation as indicated by the study of Buller et al. (1960). Their study of cat hind limb flexor digitorium longus (FDL) and soleus (SOL) of 6 day old kittens to 6 month old adult cats involved cross-reinnervating a denervated fast-twitch FDL muscle with a nerve that previously supplied the slow-twitch SOL muscle, while the latter was reinnervated by FDL muscle nerve. The result of their study showed the transformation of the FDL from a fast-twitch muscle to a slow twitch muscle, and the transformation of the SOL muscle form a slow-twitch muscle to a fast twitch muscle.

Various experimental models can be employed for the decrease or inactivation of the neuromuscular activities. The administration of tetradotoxin (TTX) drug in the nerve caused the blockage of the sodium channels and thus the inhibition of the action potential (Michel et al., 1996). Spinal cord transection (cordotomy) was used to disturb the upper motor neuron while the lower motor neuron was intact (Otis et al., 2004). In denervation experiments, the lower motor neuron was disturbed. Even though TTX administration, cordoctomy, and denervation decrease the supply of nerve impulse to the muscle, each experimental model induces numerous changes in the muscle that are quite distinct from each other.

The effects of denervation on different muscle types are not the same. For example, the rate of denervation atrophy of the slow-twitch muscle, such as the SOL, was faster than atrophy of the fast-twitch muscle, such as gastrocnemus, tibialis anterior, and EDL (Michel et al., 1996; Huey and Bodine, 1998; Jakubiec-Puka et al., 1999). The reduction in the total MHC content was greater in the denervated SOL muscle than in the denervated EDL muscle (Jakubiec-Puka et al., 1999).

There is an agreement among investigators that slow-twitch muscle convert to a faster muscle and a fast-twitch muscle convert to a slower muscle following denervation (Ansved and Larsson, 1990; d'Albis et al., 1994; Huey and Bodine, 1998; Jakubiec-Puka, 1999; Pette and Staron, 2001; Huey et al., 2003). Thus, denervation caused decrease in slow MHC and increase in fast MHC in slow-twitch muscle, and decrease in fast MHC and increase of slow MHC in fast-twitch muscle.

The results of denervating a slow-twitch soleus muscle were a significant decrease in the muscle mass, decrease in MHC 1 and increase in the MHC 2A and 2X expression of total MHC content (d'Albis et al., 1994; Huey and Bodine, 1998; Jakubie-Puka et al., 1999). There was a significant decrease in MHC 1 at the mRNA level that is faster than the decrease in the expression of MHC 1 protein (Huey et al., 2003). It was also observed that there was an increase in the hybrid fibers (fibers simultaneously expressing more than one MHC isoforms) after denervation (Michel et al., 1996; Huey and Bodine, 1998). In addition, there was an increase in the expression and/or coexpression of developmental MHC (embryonic and neonatal

MHCs) after two weeks of denervation (Albis et al., 1994). Other investigators (Michel et al., 1996), however, reported that there was no change in the developmental MHC isoform in 2 weeks denervated SOL muscle compared to the control muscle.

The effect of denervation on fast-twitch muscle was different from one fast-twitch to another fast-twitch muscle. For example, in two week denervated EDL, MHC 1, 2A, and 2X increased while 2B decreased and over-coexepression of embryonic MHC was seen, mainly with MHC 2A (Michel et al., 1996). However, Jacubiec-Puka et al. (1999) found that there was a decrease in MHC 1, 2X, and 2B, and an increase in MHC 2A two weeks after denervation. Bobinac et al. (2000) reported that denervation of EDL muscle caused progressive atrophy of all fast fiber types (i.e., type 2A, 2X, and 2B fibers), while type 1 fibers initially showed reduction in size but regained the size of the control fibers by 30 days of denervation. In addition, it was reported that the phosphorylase enzyme activites degraded after EDL denervation (Turner and Manchester, 1972; Beynon et al., 1986; Layland et al., 1990). In this study, the metabolic activities and the MHC isoforms are evaluated in the fast-twitch EDL and slow-twitch SOL muscles after the removal of the neuronal influence and compared to control muscles at different time points.

Skeletal muscle is very plastic and adaptable to different physiological and pathological conditions. For example, when a slow-twitch soleus muscle was stimulated by a high frequency pattern, type 1 fibers, which represent a major component of the soleus muscle, transformed to type 2A fibers that have the potential

for faster contraction speed. In contrast, when a fast-twitch muscle, such as EDL that consist mainly of type 2X and 2B fibers, was stimulated by a low frequency pattern, type 2X and 2B fibers begin to transform to slower type 2A and 1 fibers that have the potential for slower contraction speed (Pette and Staron, 2001). Therefore, slow- and fast-twitch muscles are capable of adapting by switching from one fiber type to another when exposed to different conditions.

The purpose of this study was to determine if the two different types of muscles, SOL and EDL, responded similarly to denervation from the perspective of:

(1) The fiber type composition of the fast-twitch EDL and the slow-twitch SOL muscles after unilateral denervation.

(2) The fiber cross sectional area of different fiber types in fast-twitch EDL and the slow-twitch SOL muscles after unilateral denervation.

(3) The metabolic activities (oxidative and glycolytic activities) of different fiber types in the fast-twitch EDL and slow-twitch SOL muscles after unilateral denervation.

Hypothesis

It was hypothesized that the different fiber types of the fast-twitch EDL and the slow-twitch SOL muscles would respond similarly to the loss of innervations in the following manner:

1. The fiber types in the slow-twitch SOL and fast-twitch EDL muscles would show similar changes in fiber sizes following denervation.
2. The changes in the fiber type proportions would be similar in the slow-twitch SOL and fast-twitch EDL following denervation.

Materials and Methods

Animal care and experimental groups

Twenty young female Wister rats (90-100 gm body weight) were used in this study. The experimental protocol was approved by Barry University Institutional Animal Care and Use Committee (IACUC). The animals were obtained from Charles River Inc. (Wilmington, MA) and kept in an environment maintained at 22°C on 12 hours light and 12 hours dark cycle provided with regular rat chows and water *ad libitium*. The bedding was changed every two days. The rats were allowed to acclimatize for 5 days before experimentation.

The rats were randomly separated into two groups: completely unoperated naïve control (n=8) and a group of (n=12) in which the left hindlimb muscles were denervated. Two naïve unoperated control rats were sacrificed at each of four time points: 0 day, 7 days, 14 days, and 21 days starting from the day of denervation. The denervated group was subdivided into three: 7 days, 14 days, and 21 days starting from the day of denervation. Four denervated rats were sacrificed at each of these time points.

Week 0: Denervation of 12 rats and sacrifice of 2 unoperated control rats.

Week 1, 2, and 3: Sacrifice of 4 denervated and 2 unoperated control rats at each time point.

Denervation Surgery

All surgeries were carried out using aseptic techniques. Immediately prior to the experiments, each rat was weighed and then anesthetized with an intraperitoneal injection of Ketamine (9 mg per 100 g body weight) using a 26 gauge needle attached to 1.0 ml syringe. The anesthetic agent was diluted with sterilized saline. After anesthesia, the posterior side of the left thigh was shaved with an electric hair clipper (Oster Golden A5, Milwaukee, Wisconsin) that met Occupational Safety and Health Administration (OSHA) specification. A 2 cm longitudinal incision was made in the midthigh , the fascia and the thigh muscles were separated with fine dissecting scissors. The sciatic nerve was located deep to the thigh muscles before the bifurcation of the tibial and peroneal branches. An approximately 3 mm long segment of the sciatic nerve was removed. Care was taken not to damage the surrounding blood vessels. The proximal end of the cut nerve was reflected and sutured into the nearby thigh muscle to insure that it did not reinnervate the hindlimb muscles between the time of operation and sacrifice some days later. The skin incision was closed and sutured using a 3/8 circle reverse cutting needle (16.2mm) and 6-0 nylon black nonfilament suture (45 cm) (Cincinnati Surgical Co., Cincinnati, OH) and an antibacterial ointment (containing polymyxin B sulfate, bacitracin zinc, neomycin sulfate and pramoxine HCl (Walgreen Co., Deerfield, IL) was applied to the incision site to prevent infection. The rats were returned to their cages with heating pads (Sunbeam Products, Inc., FL) underneath that were adjusted to the medium heat level. Ten drops of concentrated Tylenol (McNeil Consumer and Specialty Pharmaceuticals, Fort Washington, PA) were added to the water bottles for

suppression of any postoperative fever and pain. The rats had recovered from anesthetic and were able to move freely within 2 hours post operation.

Muscle Procurement

On the day of the denervation, two control rats were sacrificed (0 day). At each subsequent time point (7 days, 14 days, and 21 days) after denervation, four denervated and two unoperated control rats were sacrificed using a combination of an over-dose of Ketamine (18 mg per 100 gm body weight) and cervical dislocation. The body weight of each rat was determined before sacrifice and recorded.

Following sacrifice, the skin of the posterior compartment of the left leg was peeled off from the ankle to the thigh, and the soleus (SOL) muscle was carefully dissected free of the surrounding fascia and muscles and removed. The rat was then turned over and the skin of the anterior compartment was peeled off from the ankle to the thigh. The retinaculum that holds the distal tendons of tibialis anterior (TA) and the extensor digitorium longus (EDL) was cut. The muscles were separated from the surrounding fascia and each other and the EDL was removed.

The SOL and the EDL muscles were placed in weighing boats and separately weighed (Mettler AE 100, Mettler Instrument Corp., Highstown, New Jersey). All body weights and muscles weights were recorded. The muscles were laid on 2 x 2 cm corks with tissue freezing medium (TBS, Triangle Biomedical Sciences, Durham, NC). The muscle name and rat number was inscribed on the cork. The distal ends of the muscles were inked for identification.

Freezing and Storage

The cork and muscle were frozen in melting isopentane (2-methylbutane) (Sigma-Aldrich, Inc., St. Louis, MO) previously frozen by immersion in liquid nitrogen. Alternatively, the isopentane was cooled to -80°C by placing small pieces of the dry ice into a glass beaker containing isopentane and storing the beaker in the dry ice. The time required for freezing of muscles by either method was between 5-10 seconds. After freezing, the specimens were transferred to a -75°C freezer (Queue cryostar -75C, Queue Systems, Parkersburg, WV) on dry ice and stored there until used for the study.

Cryosectioning

It was reported that the frozen muscles can be stored at -70°C for an indefinite amount of time, retrived, resectioned, and can retain satisfactory staining results with non-enzyme, enzyme, and immunohistochemistry decades after its initial freezing process (Mitchell and Waclawik, 2007). The muscles were transferred to the Leica CM 1850 cryostat (Leica Microsystems, Wetzlar, Germany) on dry ice and allowed to warm to the cryostat temperature (-20°C) for a minimum of 15 minutes before sectioning. A 5 mm segment of the muscle belly was cross cut with a cold single edge blade. This steak extended 5 mm from the middle of the belly to the distal end of the muscle. It was embedded vertically onto a cooled cork with the middle of the muscle's belly facing up to permit cross-sections to be cut. The cork was labeled with the rat number and muscle name. Chilled small forceps, tissue freezing medium and rapid freezing spray (Decon laboratories, Inc., GA) were used for the orientation and the preliminary attachment of the specimen on the cork. The muscle was held lightly to avoid any

damage or compression to the tissue. The cork, with the embedded tissue, was attached to the cryostat chuck using tissue freezing medium. Serial frozen sections (10 μm thick) were cut and collected on cover glasses (22 mm x 22 mm, Daigger microscope cover glass, Vernon Hille, IL) for enzyme histochemisty and on labeled charged glass slides (Laboratory Storage System, Inc., St. Peters, MO) for general morphology and immunohistochemistry. The mounted sections on the cover glass and the glass slides were air dried at room temperature for 30 minutes before staining. All the staining techniques were performed within three to four hours after sectioning. Two extra slides with mounted sections were stored at -20°C freezer for repeat or future analysis.

Staining: General Morphology

Hematoxylin and eosin (see Appendix 1A for method) and one-step trichrome (see Appendix 1B) stains were routinely used to determine the general morphology and the quality of the tissue section.

Staining: Enzyme Histochemistry

The myosin adenosine triphosphatase (mATPase, E.C. 3.6.4.1) activity was demonstrated after acidic preincubation (pH 4.22 and pH 4.55) to determine type 1, 2A, and 2B (including 2X) fibers (see Appendix 2A). To evaluate the metabolic potential, phophorylase (PHRL, E.C. 2.4.1.1, see Appendix 2B) and succinate dehydrogenase (SDH, E.C. 1.3.5.1, see Appendix 2C) were used to determine the glycolytic and the oxidative activities, respectively. Nicotinamide adenine dinucleotide tetrazolium reductase (NADHtr, E.C. 1.6.99.3), was used as an oxidative

capacity marker in some muscles (see appendix 2D). Since the result of NADHtr method was similar to SDH method (Fig 1E and 1F), the SDH was used as the sole oxidative capacity marker on all control and experimental muscles.

Staining: Immunohistochemistry

Monoclonal antibodies (MABs) raised against four myosin heavy chain (MHC) isoforms: 1, 2A, 2X, and 2B MHCs were used. A4.840 MAB is anti-type 1 MHC (Hughes et al., 1993), and 2F7, 6H1, and 10F5 MABs are anti-type 2A, 2X, and 2B MHCs, respectively (Lucas et al., 2002). These MABs were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. The secondary antibodies (Dako, Carpinteria, CA) used were labeled with horse-radish peroxidase (HRP) conjugated polymer (see appendix 3A). Table 2 summarizes the specificities of monoclonal antibodies to the different MHC isoforms.

Table 3: Comparison of immunohistochemical reactivity of different monoclonal antibodies with various types of MHC isoforms.

Monoclonal Antibodies	MHC isoforms			
	1	2A	2X	2B
A4.840	+	-	-	-
2F7	-	+	-	-
6H1	-	-	+	-
10F5	-	-	-	+

Photomicroscopy

Stained sections were routinely examined under a light microscope (Olympus CH30, Olympus Optical LTD. Co., Japan). Photographs were taken using a digital camera system (DP70, Olympus America, Inc., Center Valley, PA) attached to a light

microscope (Olympus BX 60, Olympus Optical LTD. Co., Japan) and connected to a PC computer (Dell Computer Corporation, Austin, TX). The images were saved on a flash memory card (Kingston Corp., Fontain Vally, CA) and then transferred to a computer for fiber selection, counting, and statistical analysis.

Fibers selection and counting

Sixty muscle fibers were randomly selected from the SOL and the EDL and classified as type 1, 2A, 2X, and 2B fibers based of the myosin heavy chain antibody staining characteristics.

For the random fibers selection, a grid was created on the immunohistochemistry negative control using Adobe Photoshop CS ver. 8.0 (Adobe Systems Incorporated, US) and 6 squares were randomly selected using random number generating program written for this purpose using Microsoft Excel, 2004 (Microsoft Corporation, Redmond, WA). The selected areas (squares) were compared with serial frozen sections stained with anti-MHC isoforms antibodies, SDH, and PHRL. A maximum of 10 fibers were counted from each square and total of 60 counted. The proportion of type 1, 2A, 2X, and 2B fibers was determined within this population based on the MHC isoforms staining characteristics. The metabolic activities were also determined in serial-cross sections.

Fiber cross sectional area measurement

Sizes of individual muscle fibers were obtained by tracing their outlines on images of muscle cross-sections on a Mac G5 (Apple corporation, Cupertino, CA) using Image J software (Wayne Rasband, National Institute of Health, MD).

Statistical Analysis

Three-way analysis of variance (ANOVA) was used to determine effect of denervation on different muscle fiber types at different time points. If a significant duration effect was found, Post-Hoc Holm test was carried out to compare contralateral control with experimental data. A significance level of $p < 0.05$ was used to indicate statistical significance.

Results

Body and muscle weights

Several hindlimb denervation studies (Jakubiec-Puka et al., 1999; Albis et al., 1994; and Ansved and Larsson, 1990) have shown that in experimental models such as the one used in this study, the properties of the contralateral control muscle in the surgical animals are the same as those of the muscle in unoperated animals. Therefore, in this study, the contralateral muscles of the experimental animals were used as an internal control. Table 4 shows that there was no significant difference between the raw EDL and SOL weights of the naïve rats and the contralateral internal control of the operated rats.

Table 4: Muscle weight of the SOL and EDL muscles of the contralateral control and the naive rats.

Time points (wk)	Muscle weight (g)			
	CONTRALATERAL		NAIVE RAT	
	SOL	EDL	SOL	EDL
0	-	-	51.8 ± 3.9	55.5 ± 3.3
1	77.6 ± 3.9	73.5 ± 2.6	75.7 ± 5.5	71.2 ± 7.2
2	85.2 ± 14.6	81.1 ± 6.9	86.5 ± 7.1	79.4 ± 1.2
3	98.3 ± 11.0	90.3 ± 10.7	106.6 ± 9.6	93.4 ± 5.2

The muscle weight is expressed as mg. Values are means ± SD. Total of 4 muscles were weighed at each time points.

Contralateral control and denervated EDL

Denervation for three weeks caused progressive decrease in the EDL and SOL raw weight compared to their contralateral control muscles.

In the control EDL, the raw muscle weight at one- and three-week time points were 73.5 ± 2.6 mg and 90.3 ± 10.7 mg, respectively, with 23% increase in weight. When expressed as a ratio of the muscle weight (mg) to the body weight (g), the ratio essentially unchanged (Table 5).

It should be noted that the body weight as well as all the contralateral muscles weight progressively increased with time. Therefore, when the contralateral muscles weight were expressed relative to the body weight they remained unchanged at all time points. In contrast, the ratio of the denervated muscles to the body weight declined because it was affected largely by the progressive decrease in the denervated muscle weight and the increase in the body weight (Tables 5 and 6).

In the denervated EDL, the raw muscle weight declined gradually from 64.1 ± 14.5 mg at one-week to 41.9 ± 4.7 mg at three-week time. When expressed with respect to body weight, muscle weight decreased by 45% after three weeks of denervation (Table 5).

Compared to the contralateral control EDL, the denervated EDL weight decreased by approximately 13%, 44%, and 54% after one, two, and three weeks of denervation, respectively, regardless of method used for comparison (Table 5).

Table 5. Body and muscle weight of the contralateral control and denervated EDL.

Time after operation (wk)	Body weight (g)	Muscle weight (EDL)			
		CONT		DEN	
		mg	mg/g	mg	mg/g
1	166 ± 13	73.5 ± 2.6	0.44 ± 0.02	64.1 ± 14.5	0.38 ± 0.06*
2	178 ± 15	81.1 ± 6.9	0.44 ± 0.03	45.5 ± 5.0	0.26 ± 0.05*†
3	198 ± 16	90.3 ± 10.7	0.45 ± 0.02	41.9 ± 4.7	0.21 ± 0.02*†

The muscle weight is expressed as mg and as mg/g body weight. n=4. Values are means ± SD. CONT, contralateral control muscle. DEN, denervated muscle. *Significantly different from contralateral control muscle. †Significantly different from 1 week denervated muscle at $p < 0.05$

Contralateral control and denervated SOL

The weight of the control SOL muscle was 77.6 ± 3.9 mg and 98.3 ± 11.0 mg at one- and three-week time, respectively, and the percentage of increase was 26%. When expressed with respect to body weight, the muscle weight was increased by 5.4% by three-week time (Table 6).

Denervated SOL showed gradual decrease in muscle weight from 42.1 ± 4.7 mg at one-week to 24.1 ± 5.5 mg at three-week time points, with 43% decline in muscle weight. When expressed in relative to body weight, the muscle weight decreased by 52% after three weeks of denervation (Table 6).

When the denervated SOL is compared to the contralateral control SOL, the denervated SOL weight decreased by 46%, 71%, and 76% to the contralateral control SOL after one, two, and three weeks of denervation, respectively, regardless of the method used for comparison (Table 6).

Table 6. Body and muscle weights of the contralateral control and denervated SOL.

Time after operation (wk)	Body weight (g)	Muscle weight (SOL)			
		CONT		DEN	
		Mg	mg/g	mg	mg/g
1	166 ± 13	77.6 ± 3.9	0.47 ± 0.04	42.1 ± 4.7	0.26 ± 0.04*
2	178 ± 15	85.2 ± 14.6	0.48 ± 0.09	25.0 ± 5.1	0.14 ± 0.03*†
3	198 ± 16	98.3 ± 11.0	0.50 ± 0.04	24.1 ± 5.5	0.12 ± 0.03*†

The muscle weight is expressed as mg and as mg/g body weight. N=4. Values are means ± SD. CONT, contralateral control muscle. DEN, denervated muscle. *Significantly different from contralateral control muscle. †Significantly different from 1 week denervated muscle at $p < 0.05$

The data show that the progress of weight loss was greater in the denervated SOL muscle than in the EDL muscle. One week after denervation, the SOL muscle lost 46% of its muscle mass, while the EDL muscle had lost only 13%. After three weeks of denervation, the SOL and the EDL muscles had lost 76% and 54% of muscle mass, respectively.

Fiber type composition

Fiber typing methodology

The classification of individual fiber types within the EDL muscle into type 1, 2A, 2X, and 2B muscle fibers and within the SOL muscle into type 1 and 2A fibers was based on the direct correlation of their myosin ATPase pH 4.55 enzyme histochemical with MHC isoforms immunohistochemical characteristics.

Type 1 fibers in both contralateral control EDL and SOL muscles, were classified based on their dark staining characteristics with the myosin ATPase reaction (Fig. 2

and 3). They were also characterized by the presence of MHC-1. Some of type 1 fibers coexpressed MHC-2A and MHC-2X. Fiber 1 in figure 4 shows darkly stained type 1 fibers with myosin ATPase and the coexpression of MHC-2A and MHC-2X. After denervation, type 1 fibers continued to show their dark staining with myosin ATPase pH 4.55 at all time points (Fig. 8 and 9).

Type 2A fibers in the contralateral control SOL muscle were classified based on their light staining characteristics with mATPase pH 4.55 and MHC-2A expression. Type 2A and 2B fibers in the contralateral control EDL muscle were classified based on their expression of MHC-2A and MHC-2B, respectively. Some fibers, expressed only MHC-2X, and were classified as type 2X fibers. When stained with myosin ATPase, type 2A fibers were stained lightly while type 2X and 2B fibers stained with intermediate intensity. In the contralateral control EDL, MHC-2X was also coexpressed in some of type 2A and rarely in type 2B fibers. In figure 2, fiber 2, 3, and 4 are type 2A, 2X, and 2B fibers, respectively, based on the expression of MHC isoforms.

The subclassification of type 2 fibers was possible until the second week of denervation (Figures 5 and 6). By the third week of denervation, some fibers expressed two or more MHC isoforms. For example, figure 7 is a three week denervated EDL, and fibers 2 and 4 coexpress both MHC-2A and MHC-2B that made it difficult to subclassify type 2 fibers. Therefore, all fibers other than type 1 fibers were classified as type 2 fibers at the 3-week denervation time point.

Contralateral control and denervated EDL

In the control EDL, the proportion of type 2B fibers was the highest, while the proportion of type 1 fibers was lowest at all time points. The proportion of type 2X was higher than type 2A fibers during the experimental periods (Table 7).

Table 7. Fiber type composition in contralateral control and denervated EDL.

Time after operation (wk)	EDL%			
	1	2A	2X	2B
1				
CONT	3.3 ± 1.3	22.1 ± 5.8	35.4 ± 4.4	39.2 ± 6.2
DEN	5.8 ± 2.2*	26.7 ± 3.9	29.2 ± 4.4	38.3 ± 8.3
2				
CONT	3.9 ± 2.6	20.0 ± 3.3	35.0 ± 6.7	41.1 ± 4.8
DEN	5.3 ± 0.6*	24.4 ± 5.1	33.4 ± 2.9	36.9 ± 3.1
3				
CONT	3.8 ± 2.1	20.5 ± 2.5	32.1 ± 4.6	43.8 ± 7.5
DEN	7.5 ± 0.9*		92.5 ± 0.8‡	

Values are means ± SD. n=4. CONT, contralateral control muscle. DEN, denervated muscle. ‡The proportion of individual type 2 fibers is not reported because fiber types could not be typed by either myosin ATPase or MHC isoforms. *Significantly different from contralateral control muscle at $p < 0.05$

In comparing denervated EDL muscles to their contralateral control, Type 1 fibers were higher than their proportion in the contralateral control EDL at every point (Table 7, $p < 0.05$). In contrast, the proportions of type 2A, 2X, and 2B fibers in the denervated EDL did not show statistical difference when compared to their respective contralateral control fibers (Table 7).

Contralateral control and denervated SOL

The proportion of type 1 fibers in the control SOL muscle was higher than type 2A fibers during the experimental period. The proportion of type 1 fibers increased while the proportion of type 2A fibers decreased with time. From week one to week three time points, type 1 fibers increased by 19%, while type 2A fibers decreased by 51% (Table 8).

Table 8. Fiber type composition in contralateral control and denervated SOL.

Time after operation (wk)	SOL%	
	1	2A
1		
CONT	75.4 ± 1.6	24.6 ± 1.6
DEN	73.2 ± 14	26.7 ± 14
2		
CONT	90.0 ± 7.8	10.0 ± 7.8
DEN	81.7 ± 12.4	18.4 ± 12.4
3		
CONT	87.9 ± 4.2	12.1 ± 4.2
DEN	70.8 ± 5.2*	29.2 ± 5.2*

Values are mean ±SD. n=4. CONT, contralateral control muscle. DEN, denervated muscle.
*Significantly different from contralateral control muscle

After denervation, the proportion of type 1 fibers remained higher than the proportion of type 2A fibers. However, in contrast to the contralateral control SOL, the proportion of type 1 and 2A fibers did not show significant statistical difference between different time points (Table 8).

In comparing the denervated SOL with the contralateral control SOL, the proportion of type 1 fibers decreased and type 2A fibers increased. After 3 weeks of denervation, the proportion of type 1 fibers decreased by 19% and the proportion of type 2A fibers was more than double that in the contralateral control SOL (Table 8).

Fiber cross sectional area

Contralateral control and denervated EDL

The cross sectional area (CSA) measurement of different fiber types in the control EDL showed that type 2B fibers were the largest. Type 1 fibers had the smallest CSA with type 2A and 2X fibers being of an intermediate size (Table 9).

All type 2 control fiber types increased in size during experimental period. The CSA of type 2B fibers increased the most by 44%, type 2A and 2X fibers increased by 42% and 38%, respectively. The CSA of type 1 control fibers was not changed during the experimental period (Table 9).

In comparison to the control EDL muscles, at week one, the CSA of denervated type 2B fibers was smaller by 20%. At week two, the CSA of type 2A, 2X, and 2B fibers was lower by 25%, 44%, and 58%, respectively, when compared to the contralateral control muscle fibers. At week three, the CSA of all type 2 fibers was lower than their contralateral muscles fiber types by 74% (Table 9). Figures 4A, 5A, 6A, and 7A, respectively, show mATPase stained images of 21-day contralateral control EDL, one-week, two-week, and three-week denervated EDL; they show the gradual decrease in CSA of type 2A, 2X, and 2B fibres with time compared to the control

Table 9. Cross sectional area of contralateral control and denervated EDL.

Time after operation (wk)	Cross sectional area (μm^2)			
	1	2A	2X	2B
1				
CONT	694.8 ± 113.8	611.1 ± 72.5	929.0 ± 58.8	1498.2 ± 89.9
DEN	734.1 ± 69.9	733.8 ± 62.9	875.7 ± 90.9	1198.5 ± 315.7*
2				
CONT	757.7 ± 65.4	797.0 ± 43.6	1115.4 ± 16.1	1886.3 ± 122.8
DEN	736.0 ± 85.9	595.7 ± 51.2*†	627.8 ± 81.0*†	789.8 ± 178.9*†
3				
CONT	789.3 ± 121.5	865.1 ± 151.5	1280.8 ± 241.9	2149.7 ± 423.2
DEN	675.5 ± 108.6		415.2 ± 51.3‡	

Values are mean ±SD. N=4. CONT, contralateral control muscle. DEN, denervated muscle. ‡The CSA of individual type 2 fibers populations is not reported because fiber types could not be distinguished by either myosin ATPase or MHC isoforms. *Significantly different from contralateral control muscle. †Significantly different from 1 week denervated muscle at $p < 0.05$

EDL muscle. However, type 1 fibers remain almost unchanged at all time points. The images in figure 8 show mATPase stained sections of contralateral control and denervated EDL muscles at all time points, they show the unchanged size of darkly stained type 1 fibers of the contralateral and denervated EDL at all time points.

Type 1 fibers in the contralateral control and denervated EDL

The type 1 fibers represent a very small population in the EDL muscle, and the results of the measurement of CSA of the few randomly selected type 1 fibers seemed to show that they atrophied the least relative to their control with time compared to other fiber types (Table 9). In order to validate this finding, twenty type 1 fibers were

randomly selected from each contralateral control and denervated EDL at the three time points and their CSA measured (Table 10).

The result confirms that there was no statistically significant difference in the CSA of type 1 fibers between the contralateral control and the denervated EDL muscles at all time points nor was there a significant change in mean CSA of the fibers with time (Table 10).

Table 10. Type 1 fiber CSA in the contralateral control and denervated EDL muscles.

Time after operation (wk)	Cross sectional area (μm^2)	
	CONT	DEN
1	701.5 ± 60.2	734.8 ± 71.5
2	785.0 ± 82.5	718.1 ± 97.2
3	824.1 ± 122.3	725.8 ± 81.2

Values are means ±SD. Twenty fibers from each of four muscles were measured. CONT, contralateral control muscle. DEN, denervated muscle.

Contralateral control and denervated SOL

Compared to their respective contralateral control muscles, the CSA of type 1 fibers in the denervated SOL decreased by 52%, 78%, and 88% after one, two, and three weeks of denervation, respectively. In type 2A fibers the decrease in the CSA at corresponding times was 48%, 83%, and 88%, respectively (Table 11).

Table 11. Cross sectional area of the contralateral control and denervated SOL.

Time after operation (wk)	Cross sectional area (μm^2)	
	1	2A
1		
CONT	2262.2 ± 349.7	1610.7 ± 339.7
DEN	1094.5 ± 329.3*	840.5 ± 254.6*
2		
CONT	2477.7 ± 269.6	1748.8 ± 163.6
DEN	549.2 ± 8.6*†	293.3 ± 73.4*†
3		
CONT	2692.6 ± 216.1	1729.0 ± 220.1
DEN	324.7 ± 85.3*†	213.3 ± 35.6*†

Values are mean ±SD. n=4. CONT, contralateral control muscle. DEN, denervated muscle. *Significantly different from contralateral control muscle. †Significantly different from 1 week denervated muscle at $p < 0.05$

Oxidative capacities

Contralateral control and denervated EDL

In the control EDL, the distribution of the succinate dehdrogenase (SDH) activities in different fiber types from the highest to the lowest was as follow: 2A≥1>2X>2B. Fibers 1, 2, 3, and 4 in figure 2E show the SDH activities in type 1, 2A, 2X, and 2B fibers.

This distinction of the SDH activity between the different fiber types seemed to partially disappear after denervation. However, type 1 (fiber 1 in figures 5F, 6F, and 7F) and type 2A (fiber 2 in figures 5F, 6F, and 7F) fibers of the denervated EDL

continued to show higher SDH activities than type 2X and 2B fibers at all time points when examined under the light microscope.

Contralateral control and denervated SOL

In the control SOL, the SDH activity was higher in type 2A than in type 1 fibers (Fig 3E). In one week denervated SOL, type 2A fibers continued to show higher SDH activity than type 1 fibers (Fig 4A). At two and three week time, this distinction of SDH activity between type 1 and 2A was significantly decreased (Fig 4C and 4E).

Glycolytic capacities

Contralateral control and denervated EDL

In control EDL, phosphorylase (PHRL) activities in the muscle fibres from the highest to the lowest were as follow: 2B>2X>2A>1. Fibers 1, 2, 3, and 4 in figure 2F show the PHRL activities in type 1, 2A, 2X, and 2B fibers, respectively.

After denervation, the EDL muscle showed a gradual decrease in PHRL activity in many type 2X (fiber 4 in figures 5G an 6G) and 2B fibers (fiber 6 in figure 6G) with time. Three weeks after denervation, most of type 2 fibers showed a decrease in PHRL activity (figure 7G). In contrast, type 1 fibers (fiber 1 in figures 5G, 6G, and 7G) showed relatively high PHRL activity at all times.

Contralateral control and denervated SOL

In the control SOL, PHRL activities were low in type 1 fibers, while 2A fibers

showed slightly higher activity (figure 3E). At one week denervated SOL, type 2A fibers continued to show higher PHRL activity than type 1 fibers (figure 4B). At two weeks, this distinction of PHRL activity between type 1 and 2A was significantly decreased (figure 4D) and disappeared by three weeks (figure 4F).

Discussion

Twenty adult rats were used in this study; the left leg was denervated by removing a segment of the sciatic nerve in the middle of the thigh. The right leg served as an internal control. One, two, and three weeks after denervation, the extensor digitorium longus (EDL) and the soleus (SOL) of the contralateral control and denervated legs were analyzed using enzyme histochemical and immunohistochemical procedures to study the effect of denervation on the two muscles.

Several hindlimb denervation studies (Jakubiec-Puka et al., 1999; Albis et al., 1994; and Ansved and Larsson, 1990) have previously shown that the properties of the contralateral control muscle in experimental animals were the same as those of muscles of the naive animals. Therefore, in this study, the unoperated muscles from the contralateral limb of the experimental animals were used as control and are referred to as "control" muscles in the remainder of this discussion.

Body and muscle weights

The change in muscle weight after denervation have been evaluated in the literature by two methods, the denervated muscle weight compared to its control muscle (Ansved and Larsson 1990, Huey and Bodine, 1998, Jakubiec-Puka et al., 1999, and Degens et al., 2008) and the denervated muscle weight compared to the body weight (Michel et al., 1994, and Comery et al., 2000). Both parameters of the muscle weight expression were used in this study (Tables 5 and 6).

The ratio of the control muscle weight to the body weight was almost identical at all time points, thus indicating the normal growth of the animal. This shows that both muscles are growing at a rate very similar to the whole body. This is in agreement with the studies of Jakubiec-Puka et al. (1999), d'Albis et al. (1994), and Ansved and Larsson (1990).

Denervation caused an unequal reduction in the muscle weights of the fast-twitch EDL and the slow-twitch SOL muscles relative to their control muscles (Appendix 4A). One week after denervation, the proportion of weight loss of the denervated SOL was about three times greater than that of the weight loss of the denervated EDL (46% vs. 13%). The reduction in muscle weight of both denervated muscles was the greatest between the first and the second week of denervation. This suggests that the extent of atrophy caused by the loss of innervation is not the same in different muscles. This result is consistent with the previous EDL and SOL denervation studies (Jakubiec-Puka et al., 1999; and Degens et al., 2008). This study examine the different respond of different muscles to denervation by evaluating the composition, the size, and the metabolic activities of different fiber types in the fast-twitch EDL and the slow-twitch SOL muscles.

Fiber type composition

In the control SOL, there was an increase in type 1 fibers and a decrease in type 2A fibers with time. In the control EDL, there was an increase in type 2B fibers and almost no change in type 1 fibers at all time points. These results are in agreement with Ansved and Edstrom (1990) in which the fast-twitch EDL muscle becomes

faster by the increase in type 2B fibers and the slow-twich SOL muscle become slower by the increase in type 1 fibers and the decrease in type 2A fibers with time.

The proportion of type 1 fibers in the denervated fast-twitch EDL muscle was increased by almost 100% when compared to the control muscle by the third week of denervation. Type 2B fibers decreased by 10% compared to the control muscle by the second week of denervation (Table 7). This suggests that the fast-twitch EDL muscle become slower following denervation by the increase of the proportion of the type 1 fibers the decrease in the proportion of type 2B fibers.

The denervated SOL muscle showed a decrease in the proportion of type 1 fibers and an increase in the proportion of type 2A fibers relative to the control muscle at all time points, which suggested that the slow-twitch SOL muscle becomes faster following denervation by the increase of the proportion of type 2A fibers and the decrease of the proportion of the type 1 fibers.

These results are consistent with the previous skeletal muscle denervation findings that demonstrated fast-twitch EDL muscle becomes slower by the increase of type 1 fibers and decrease of type 2B fibers and the slow-twicth SOL muscle becomes faster by the increase of type 2A fibers and decrease of type 1 fibers (Ansved and Larsson, 1990; Albis et al., 1994; Huey and Bodine, 1998; Jackubiec-Puka, 1999; Pette and Staron, 2001; and Huey et al., 2003)

Fiber size characteristics

The cross sectional area (CSA) of all fiber types of the control EDL and SOL muscles increased with time indicating the normal growth of the muscle. However, the percentage of increase in the fiber size of different types in a given muscle was not the same.

In the control EDL, the percentage increase with time was the highest for type 2B fibers (44%) and lowest in type 1 fibers (18%). In the control SOL the percentage increase in the CSA was greater for of type 1 fibers (19%) was greater than for type 2A fibers (7%).

This indicate that the fast-twitch EDL muscle does not only depend on the high percentage of type 2B fibers but also on the rate of type 2B muscle fiber's growth that is higher than any other fiber type in the EDL muscle to give the EDL muscle its fast-twitch characteristic. This assumption is the same for the SOL muscle that depends not only on the high proportion of type 1 fibers but also the rate of type 1 fibers growth that is higher than type 2A fibers to give the SOL muscle its slow-twitch characteristic.

After denervation, type 2B fibers in EDL atrophied the most when compared to their control muscle (Table 9). Denervated type 2A and 2X fibers were smaller than their control muscle after two weeks as well. Type 1 fibers in the denervated EDL maintained their size at all time points (Table 10; Appendix 4C). In the denervated SOL, type 1 and 2A fibers were both severely atrophied by denervation (Table 11).

Taken together, the severe reduction in the SOL muscle weight and of the CSA of its fibers following denervation compared to the EDL muscle, suggest that the denervation-induced muscle weight loss and the fiber atrophy is not only dependent the loss of innervation but also the type of muscle fiber. The slow-twitch SOL muscle is severely affected while the fast-twich EDL muscle was less affected by the loss of innervation. It indicates that the slow-twitch SOL is more susceptible to nerve loss than the fast-twitch EDL muscle and is in agreement with previous studies by Jakubiec-Puka et al. (1999) and Wroblewski et al. (1989).

Bobinac et al. (2000) reported that the denervation-induced atrophic response of different fibers in the EDL was more in all type 2 fibers than in type 1 fibers, for example type 2B fibers atrophied the most at all time points, then type 2X fibers, and type 2A fibers the least. They also reported that type 1 fibers decreased in size for12 days after denervation but than returned to the size of type 1 fibers in the control muscle by 30 days.

This study extended these findings and showed that the response of the same fiber types to denervation is muscle specific. Type 1 fibers in the control SOL and EDL muscle had the same metabolic characteristics, high oxidative and low glycolyitc capacities, they showed the same dark staining intensity to myosin ATPase stain pH 4.55, and expressed the same MHC isoform, but the SOL muscle type 1 fibers atrophied severely after denervation, while in the EDL muscle, type 1 fibers maintained their size after the same period of denervation. Therefore, type 1 fibers in

the slow-twitch SOL muscle are more sensitive to denervation than in the fast-twitch EDL muscle. This is more remarkable given the fact that type 1 fibers in control SOL muscles were 2-3 times larger than those in control EDL muscles (Appendix 4B).

The results of this study also demonstrated that type 2B fibers in the EDL, the predominant fiber type, atrophied more than any other fiber type after denervation. By contrast, type 1 fibers in the SOL, the predominant fiber type, atrophied more than type 2A fibers after denervation when compared to their respective control muscles. This suggests that the fibers of the dominant fiber type of the muscle are affected the most by changed condition, an observation that was also made by Punkt (2002). Moreover, the muscle fibers that determine the general properties of the muscle are affected the most by the removal of the neuronal influence. In addition, this research shows that the rate of atrophy was more in type 2X than type 2A and more in type 2A than in type 1 fibers in the denervated EDL.

The above suggest that the muscle fibers that the EDL muscle depends most on atrophy most severely: The very fast type 2B fibers atrophied the most, then the fast type 2X fibers; type 2A fibers atrophied to a lesser extent than type 2X and 2B fibers, while type 1 fibers which have minor function in the EDL activities are not affected by denervation. Therefore, the extent of atrophy of different fiber types in the denervated EDL is directly related to the twitch speed of the muscle fibers, the faster the muscle fiber, the more atrophy of the muscle fiber.

Metabolic activities characteristics

Phosphorylase (PHRL) and succinate dehydrogenase (SDH) enzymatic activities have been used as reliable markers to determine the glycolytic and oxidative capacities of skeletal muscle fibers (Mitchell and Waclawik, 2007). The determination of the glycolytic and oxidative capacities has been used to classify skeletal muscle fibers into slow-oxidative (SO), fast-oxidative-glycolytic 1 (FOG1), fast-oxidative-glycolytic 2 (FOG2), and fast-glycolytic (FG) that correspond to type 1, 2X, 2A, and 2B fibers (Punkt, 2002).

The results of this study showed that the metabolic characteristics of different fiber types in the contralateral control EDL and SOL muscles correspond to the metabolic classification scheme. Figures 1 and 2 shows the normal metabolic activites of different fiber types in the control EDL and SOL muscles.

Several studies, whether in vivo (Beynon et al., 1986) or on homogenates of skeletal muscle tissue (Layland et al., 1990; and Turner and Manchster, 1972), showed degradation of PHRL and SDH activites after denervation. In this study, after denervation, the PHRL activities in the EDL decreased gradually in some type 2X and 2B fibers. In contrast, type 1 fibers and some of type 2A fibers showed high PHRL activities at one- and two-week of denervation (Figures 5H and 6H). By the third week of denervation, some of the type 1 fibers expressed low PHRL and SDH activities, while type 2A, 2X, and 2B fibers showed very low PHRL and SDH activities (figure 7H).

The results of the study of PHRL activity here are in agreement with the previous studies that showed a decline of the PHRL activity after denervation in general. However, this study shows that the decline is selective because some fibers appear to have increased PHRL activity. Type 2X and 2B fibers that represent major population of fiber types in the EDL, showed decrease in PHRL activity while type 1 and some of type 2A fibers, which represent smaller population in the EDL muscle, showed higher PHRL activity after denervation.

Taken together the increase of the proportion of type 1 fibers, maintaining their size up to the third week of denervation, and the expression of PHRL activity after denervation and the maintenance of expression SDH activity up to the third week of denervation suggests that type 1 fibers may play an important role in adaptation of the EDL muscle to denervation.

On the other hand, the SOL muscle was severely atrophied after denervation and metabolic activities of type 1 and 2A fibers were indistinguishable by microscopic examination.

Conclusion

In order to evaluate the effect of denervation on different type of skeletal muscle, fast-twitch EDL and slow-twitch SOL muscles were denervated by cutting a segment of the sciatic nerve in the middle of the thigh of the rat. The denervated and the contralateral control muscles were removed and studied using different enzyme histochemical and immunohistochemical staining techniques.

The results of studying the composition, the size, and the metabolic properties of different fiber types in the fast-twitch EDL and slow-twitch SOL muscles showed differential effects of denervation on the fast-twitch EDL and the slow-twitch SOL muscles. Denervation caused severe reduction in the muscle weight and the cross sectional area of type 1 and 2A fibers of the SOL muscle relative to contralateral control muscle.

The effect of denervation on the fast-twitch EDL muscle was to a lesser extent than the SOL muscle. All type 2 fibers atrophied and showed decreased in the glycolytic activities as represented by PHRL activity compared to the contralateral control EDL. Type 1 fibers maintained their size but also showed relatively increase in the expression to the glycolytic activities until the end of the three-week experimental period. Therefore, slow-twitch type 1 fibers in the fast-twitch EDL muscle, which represent the lowest percentage, seem to play an important role in adaptation to denervation.

The results of this study may be useful in evaluating the response of different type of skeletal muscle to peripheral nerve injuries and perhaps determine the proper course of treatment to reduce or prevent muscle atrophy associated with loss of innervations in different type of muscles.

Figures

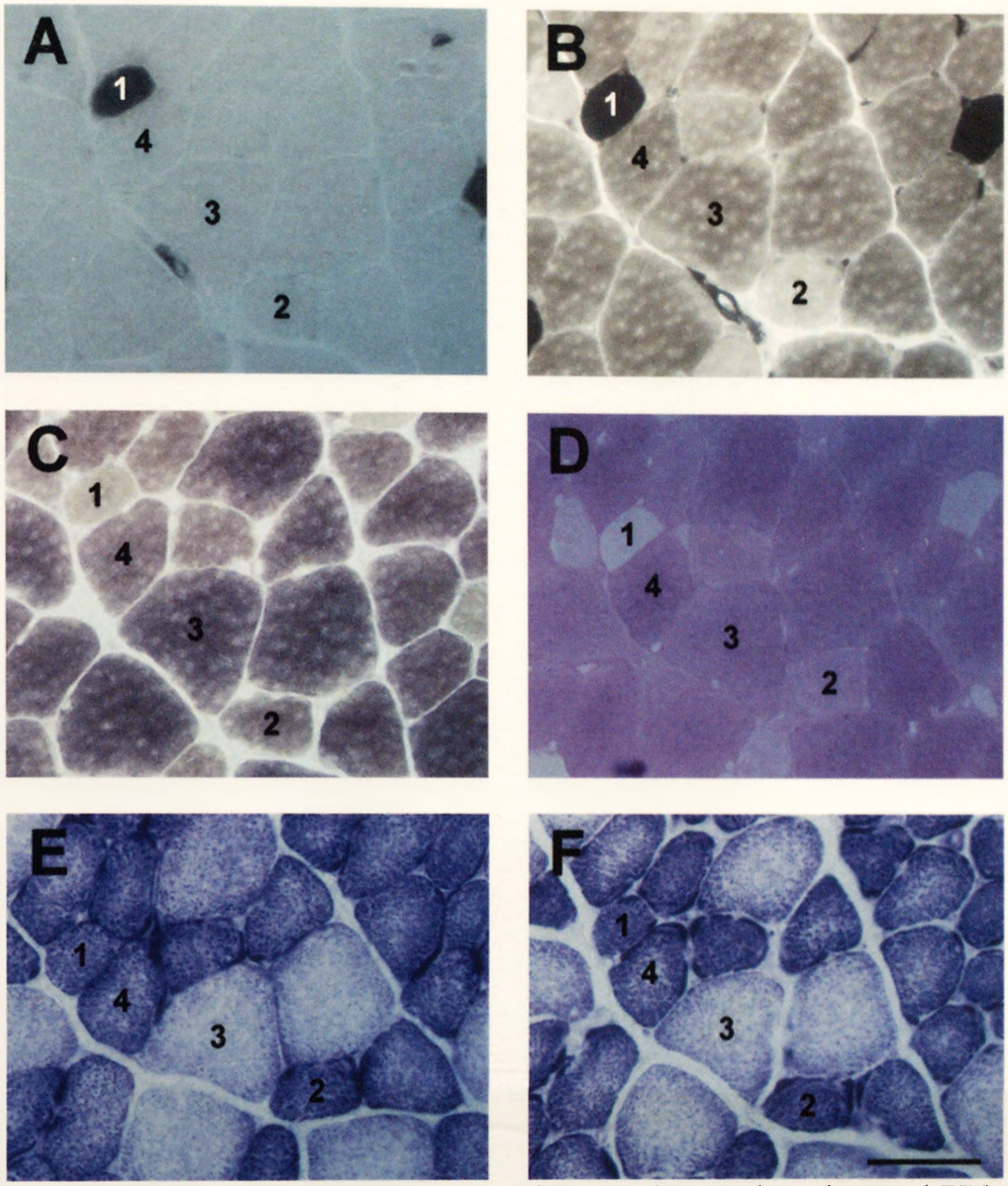


Figure1. Micrographs of serial sections of two week contralateral control EDL. (A) myosin ATPase with preincubation at pH 4.22, (B) myosin ATPase with princubation with pH 4.55, (C) phosphorylase (PHRL), (D) periodic acid Schiff (PAS) stain, (E) succinate dehdrogenase (SDH), and (F) NADHtr. Labelled fibers based on ATPase pH 4.55, (1) type 1, (2) type 2A, (3 and 4) type 2B.
Scale bar = 50μm

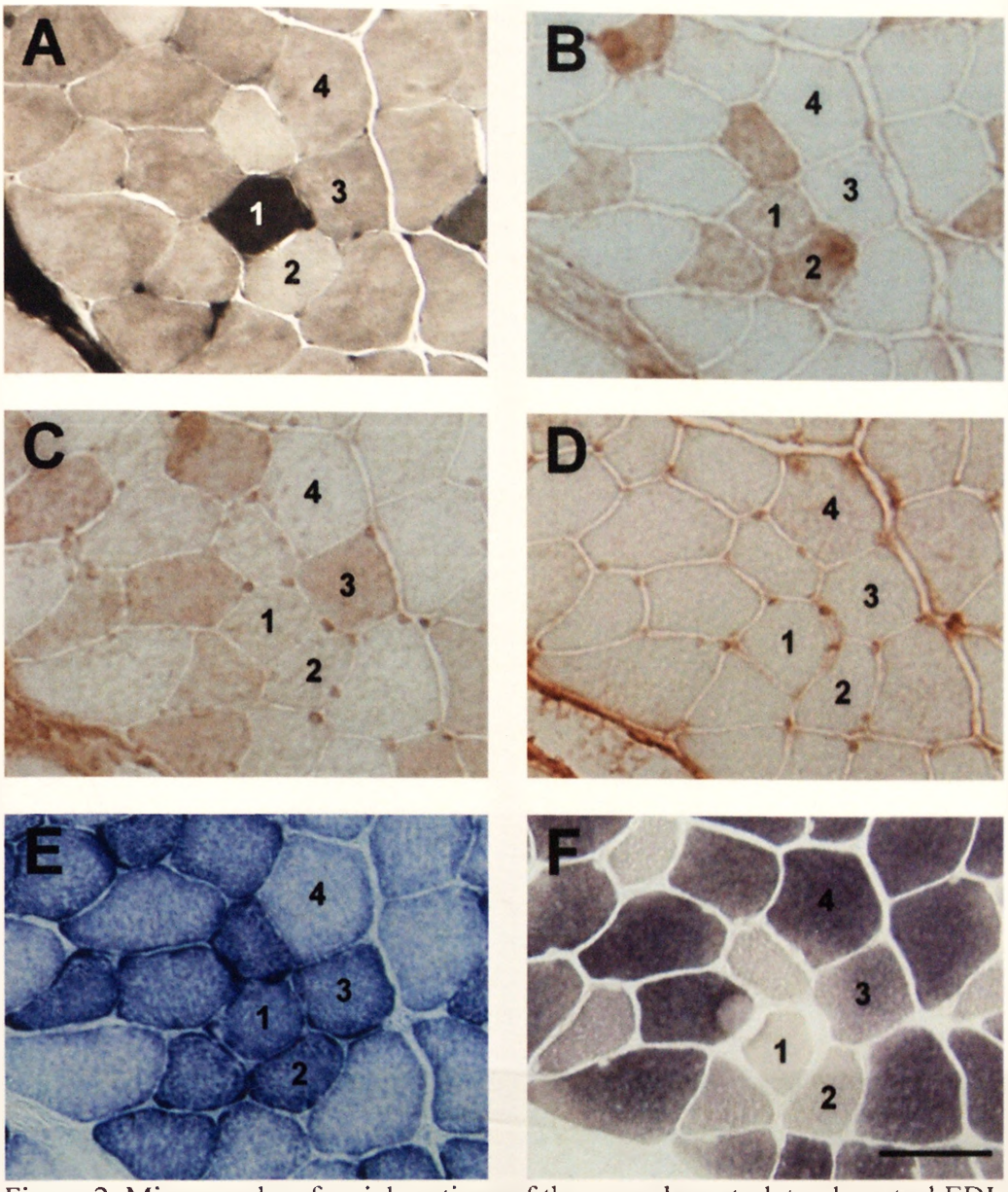


Figure 2. Micrographs of serial sections of three-week contralateral control EDL. (A) myosin ATPase with preincubation at pH 4.55, MAbs against MHC-2A(B), MHC-2X (C), MHC-2B (D), SDH (E), and PHRL (F). Labeled fibers, (1) type 1 fiber and (2) type 2A, (3) type 2X, and (4) type 2B. Scale bar = 50μm.

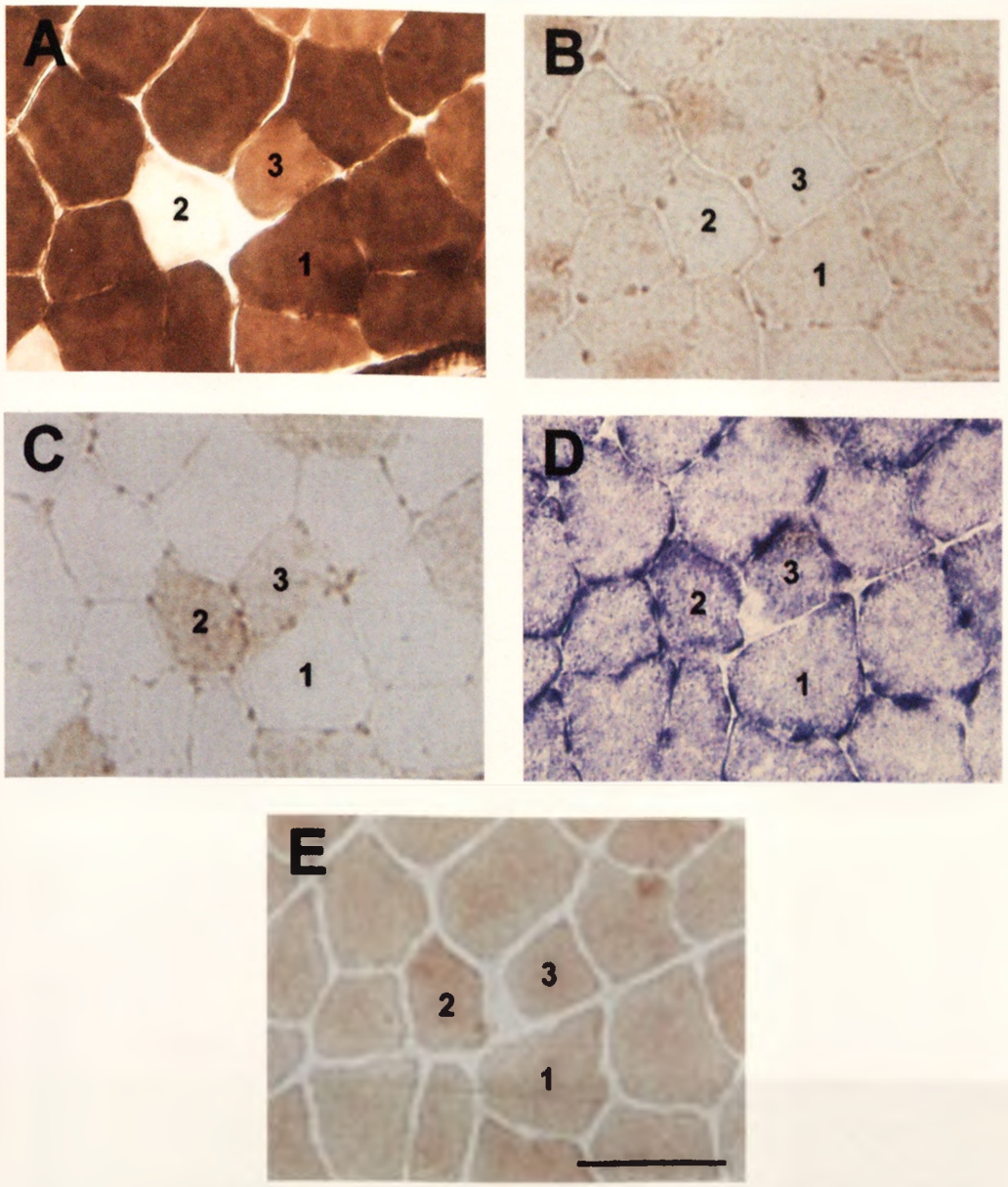


Figure 3. Micrographs of serial sections of two week contralateral control SOL. (A) myosin ATPase with preincubation at pH 4.55, MAbs against MHC-1(B), MHC-2A (C), SDH (D), and PHRL (E). Labeled fibers, (1) type 1 and (2 and 3) type 2A. Scale bar = 50μm.

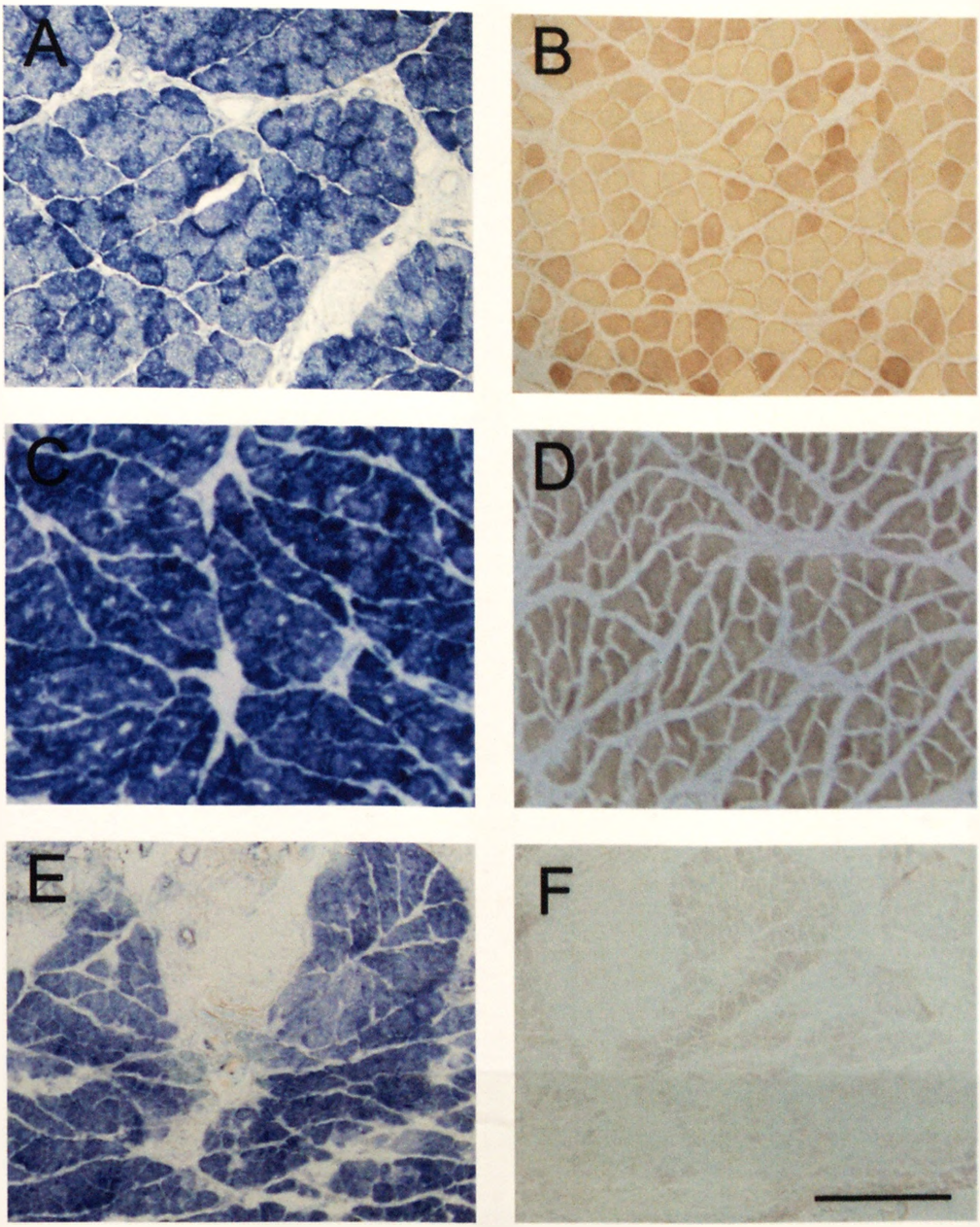


Figure 4. Micrographs of serial sections of one, two, and three week denervated SOL. Images A, C, and E are SDH and B, D, and F are PHRL at one, two, and three weeks of denervation, respectively. Scale bar = 100μm.

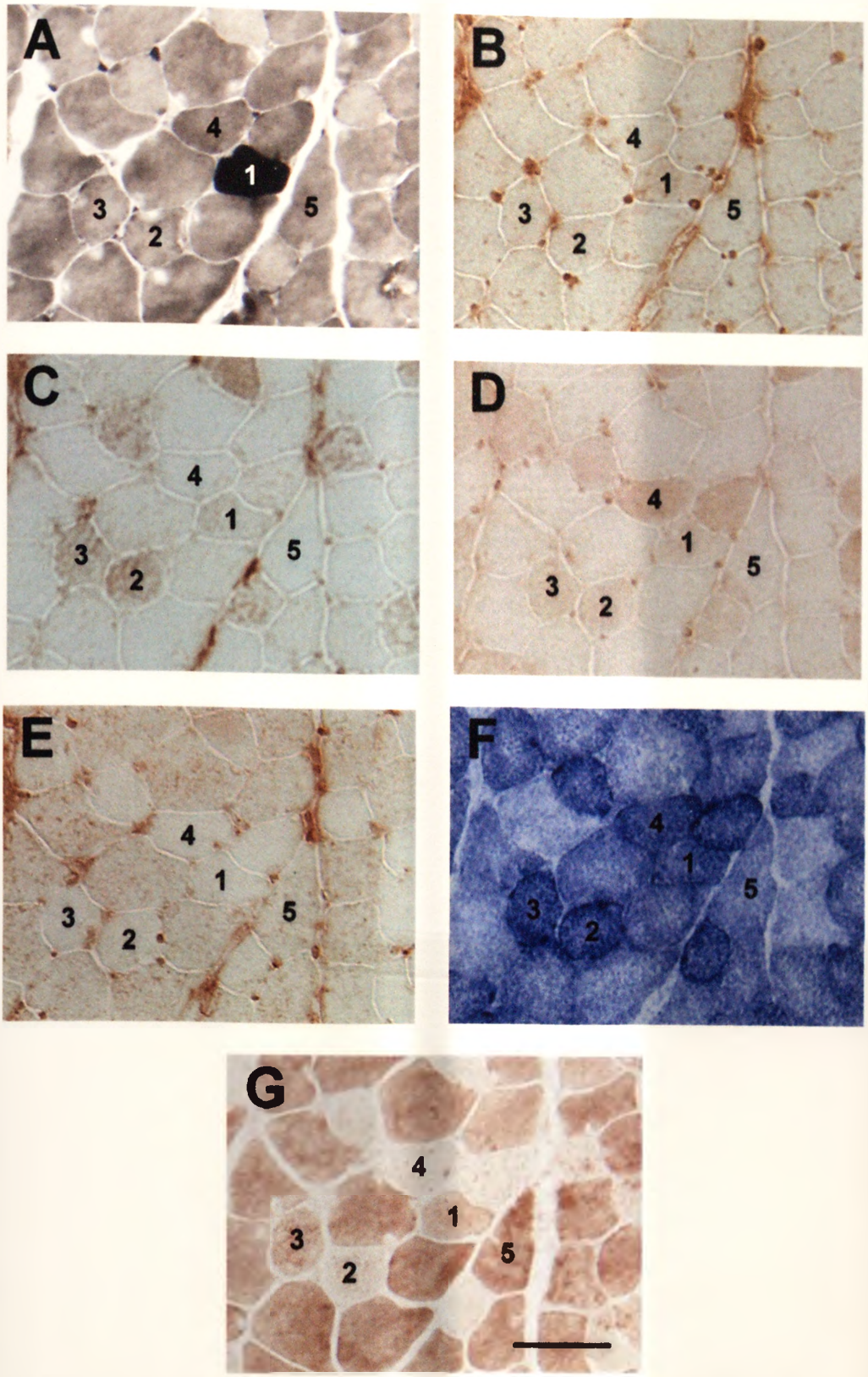


Figure 5. Micrographs of serial sections of one week denervated EDL. (A) myosin ATPase with preincubation at pH 4.55, MAbs against MHC-1(B), MHC-2A (C), MHC-2X (D), MHC-2B (E), SDH (F), and PHRL (G). Labeled fibers, (1) type 1, (2 and 3) type 2A, (4) type 2X, and (5) type 2B. Scale bar = 50μm.

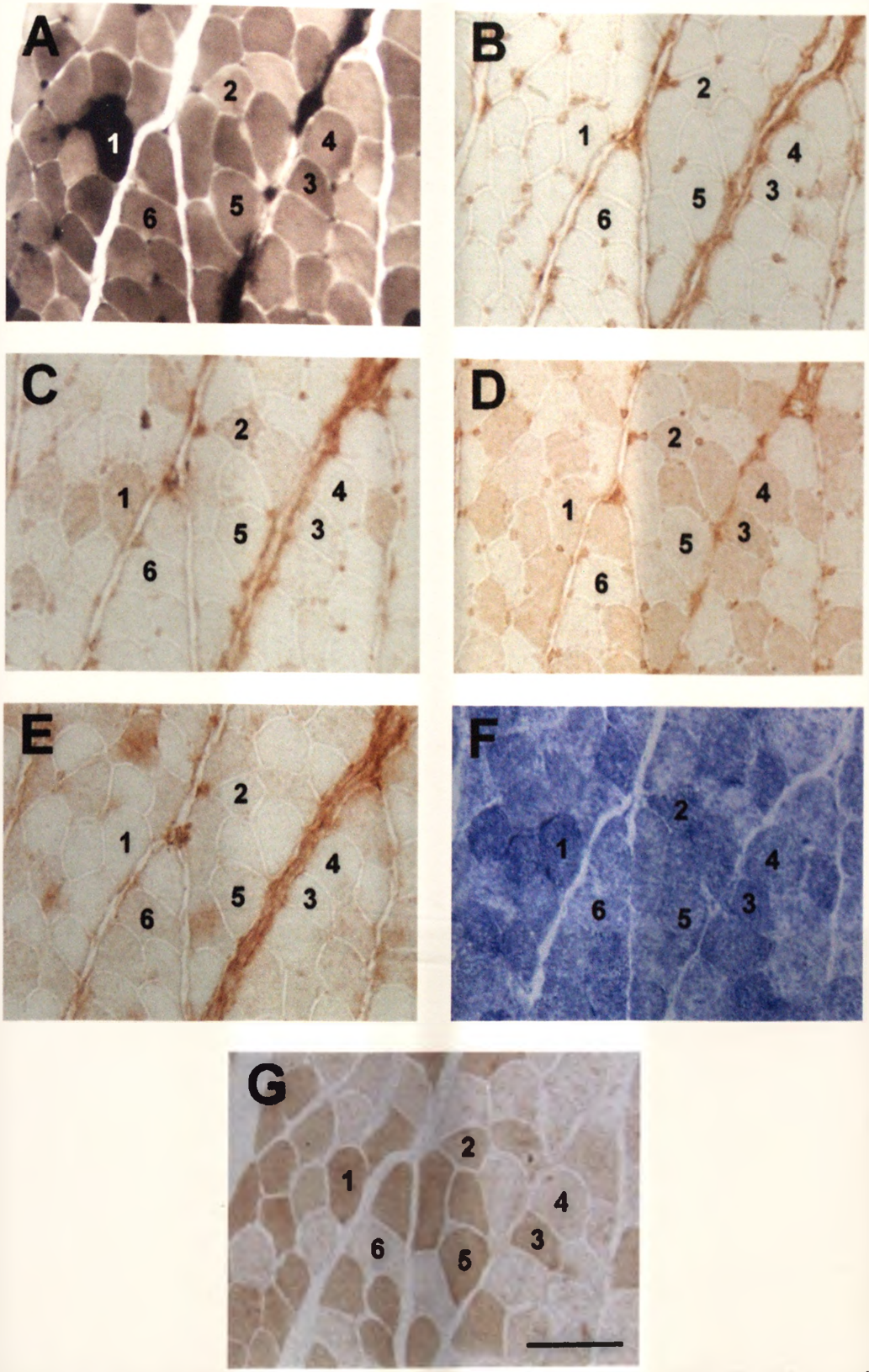


Figure 6. Micrographs of serial sections of two week denervated EDL. (A) myosin ATPase with preincubation at pH 4.55, MAbs against MHC-1(B), MHC-2A (C), MHC-2X (D), MHC-2B (E), SDH (F), and PHRL (G). Labeled fibers, (1) type 1, (2) type 2A, (3 and 4) type 2X, and (5 and 6) type 2B. Scale bar = 50μm.

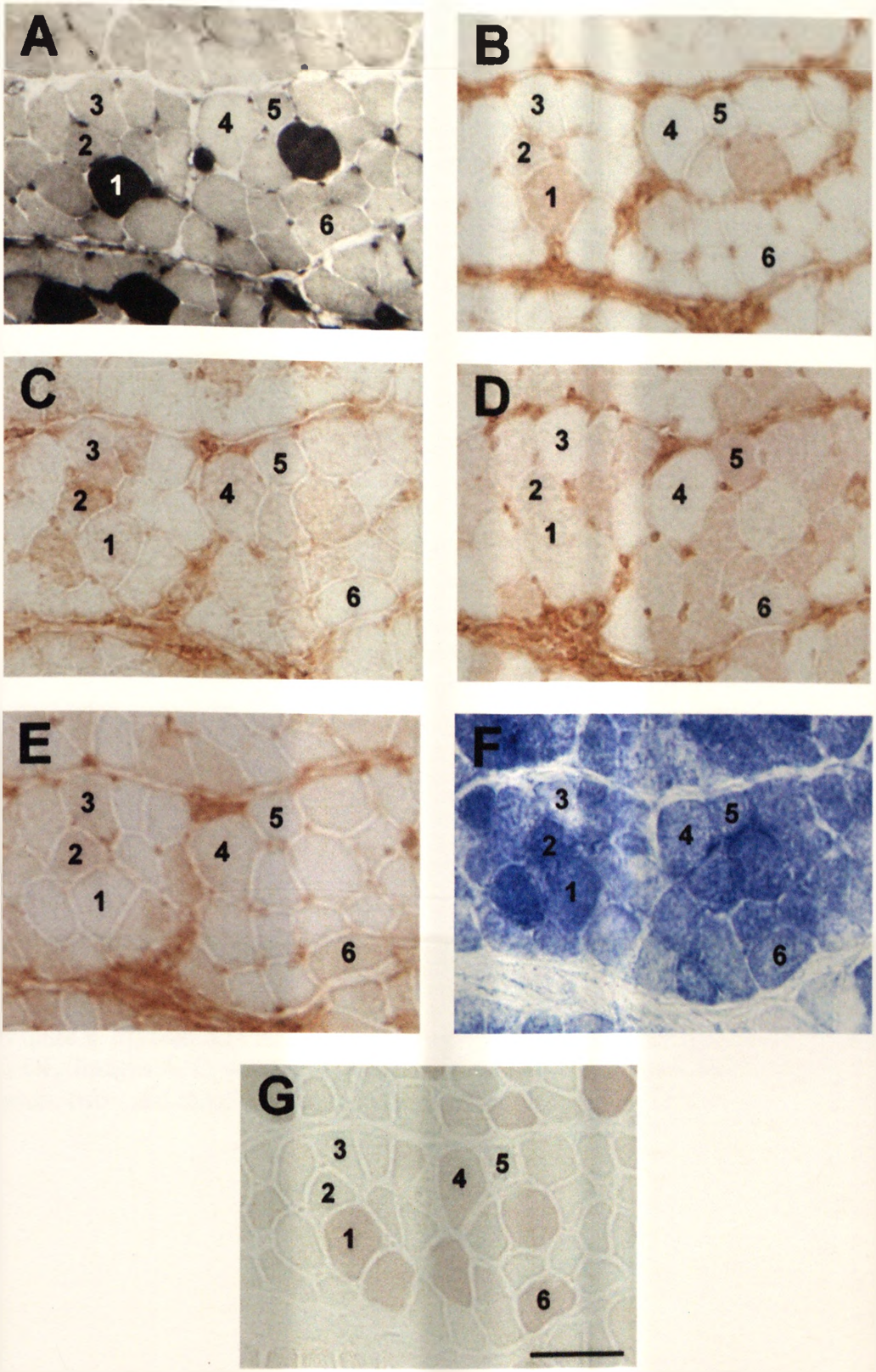


Figure 7. Micrographs of serial sections of three-week denervated EDL. (A) myosin ATPase with preincubation at pH 4.55, MAbs against MHC-1(B), MHC-2A (C), MHC-2X (D), MHC-2B (E), SDH (G), and PHRL (H). Labeled fibers, (1) type 1, (2, 3, and 4) type 2, (5) type 2X, and (6) type 2B. Scale bar = 50μm.

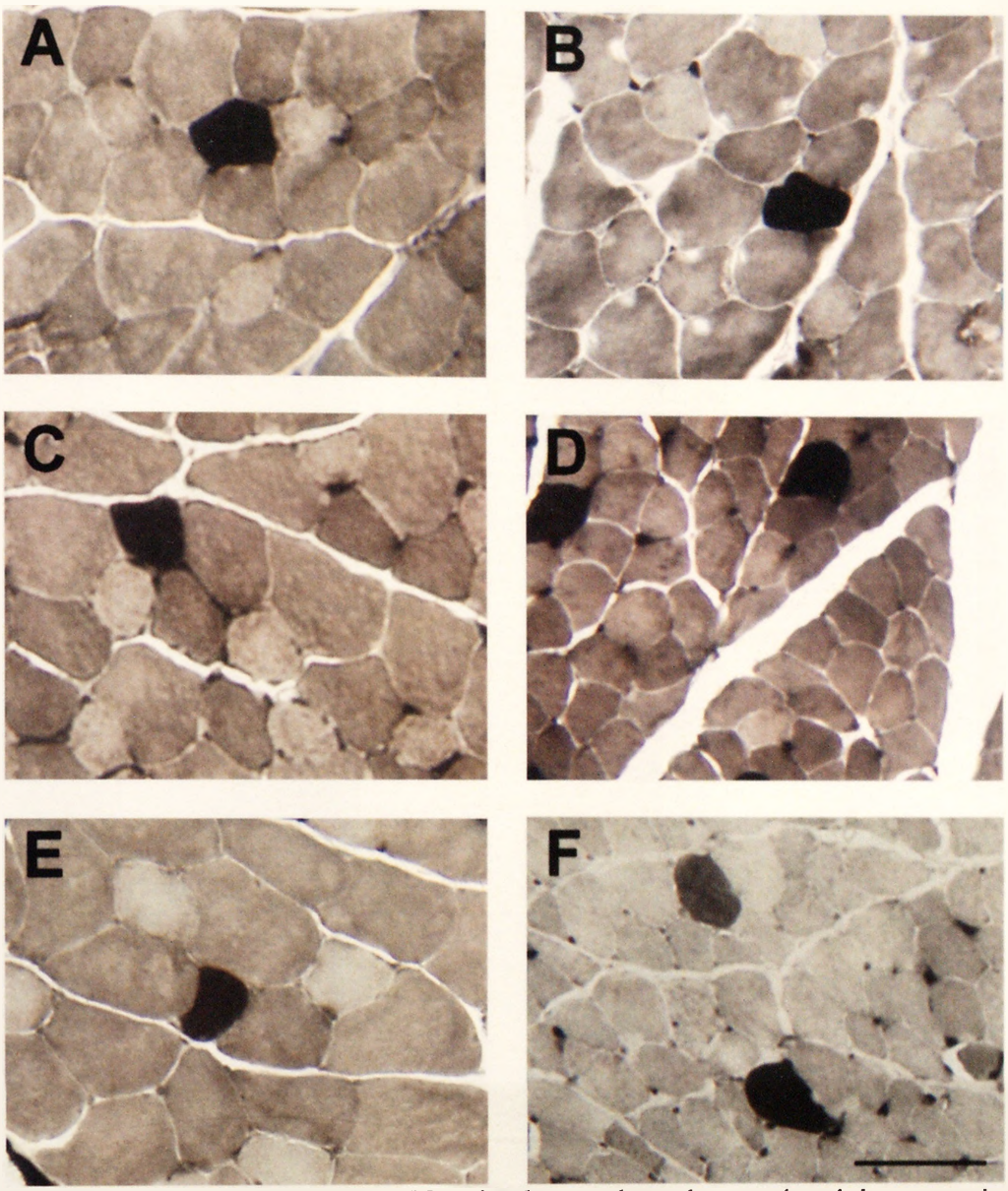


Figure 8. Myosin ATPase pH 4.55 stained contralateral control and denervated EDL. Images A, C, and E are control EDL and B, D, and F are denervated EDL at one-, two-, and three-week time, respectively. Scale bar = 50μm.

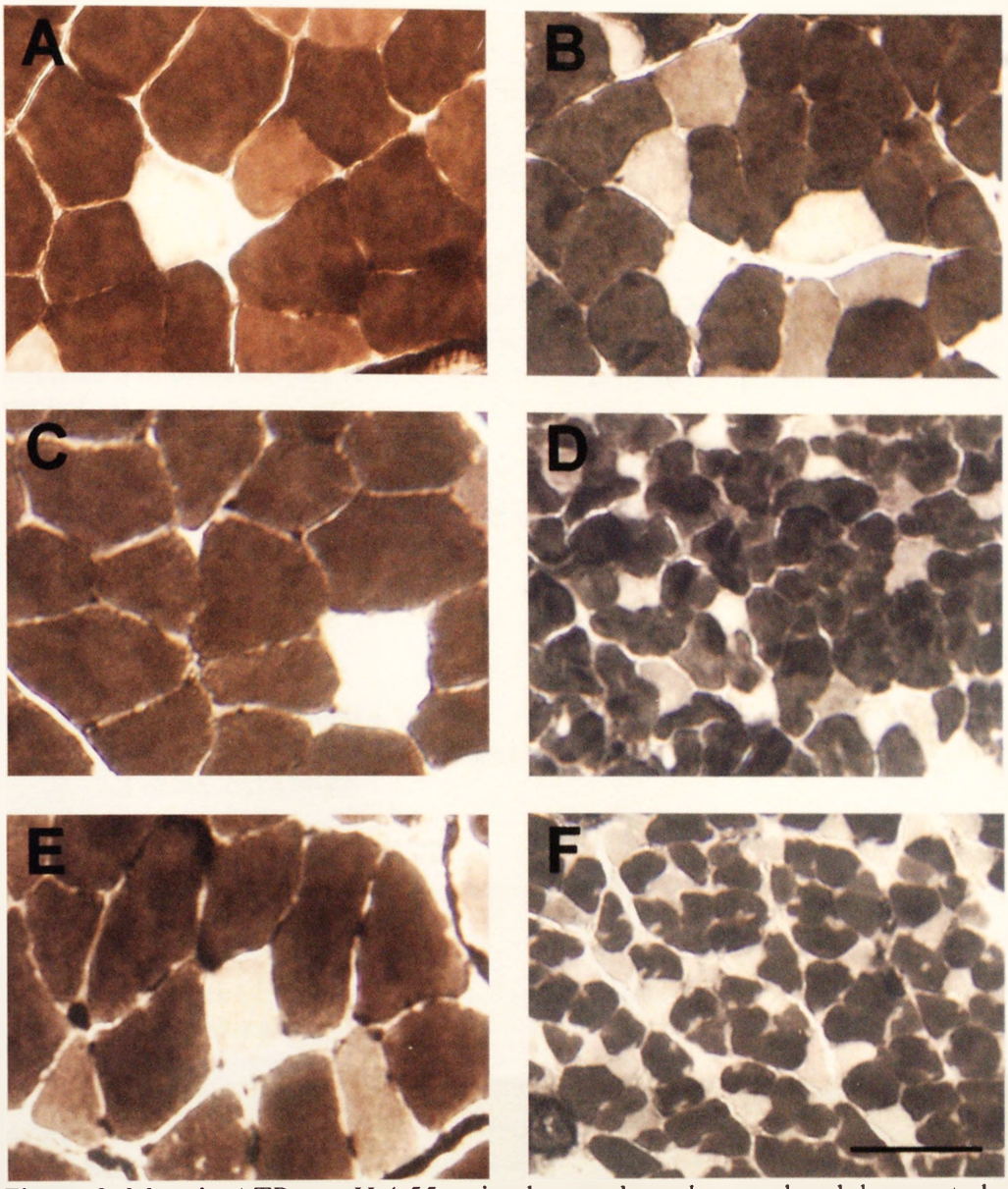


Figure 9. Myosin ATPase pH 4.55 stained contralateral control and denervated SOL. Images A,C, and E are control SOL and B, D, and F are denervated SOL at one-, two-, and three-week time, respectively. Scale bar = 50μm.

Appendix 1: General Morphology

A. Hematoxylin and Eosin

Purpose

To give general morphology of the tissue section.

Principle

Hematoxylin and Eosin stain is a routine histological stain that consists of two staining solutions. Hematoxylin is a basic dye that stains the nuclei and basophilic granules blue-purple. Eosin is an acidic dye that stains the cytoplasm and extracellular components different shades of pinkish color. Hematoxylin is used regressively and decolorized with 1% acid alcohol.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on charged slides.

Quality control

Reagents

1- Harris Modified Hematoxylin solution
   Commercially prepared by Fisher Scientific.
   Lot No. 012439-12

2- Eosin Yellowish solution, 1% w/v
   Commercially prepared by Fisher Scientific.
   Lot No. 012980-24

3- 1% Acid Alcohol

Distilled water	99 ml
Hydrochloric Acid	1 ml

Procedure

1- Fix frozen section in 95% alcohol for 1 min.
2- Rinse in running tap water briefly.
3- Stain with Harris Modified Hematoxylin solution for 2 min.
4- Rinse in tap water briefly.
5- Decolorize in 1% Acid Alcohol quickly (one dip).
6- Rinse in running tap water for 5 min.
7- Counterstain with Eosin Yellowish solution for 15 sec.
8- Dehydrate, clear and mount with permanent mounting media.

Results

Nuclei and basophilic components : Blue-Purple
Cytoplasm and other cellular components: Different shades of pinkish color

B. One-StepTrichrome stain

Purpose

To distinguish connective tissue from muscular tissue.

Principle

One-Step Trichrome stain is a combination of three stains. Weigert Iron Hematoxylin is used to stain nuclei blue-black, light green stains connective tissue green and, chromotrope 2R stains cytoplasm, muscle, and RBC red. Light green and chromotrope 2R and combined in one staining solution.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on charged slide.

Quality control

Reagents

1. Bouin's Fluid

Picric Acid, saturated aqueous solution	75 ml
Formaldehyde	25 ml
Acetic Acid	5 ml

2. Weigert Iron Hematoxylin

Solution A: Hematoxlin	1 g
95% Ethyl alcohol	100 ml
Solution B: Distilled water	95 ml
Hydrochloric Acid	1 ml
29% Ferric Chloride	4 ml
Working Weigert hematoxylin:	
Solution A	1 ml
Solution B	1 ml

3. One-Step Trichrome stain

Commercially prepared from American Master Tech Scientific, Inc.
Item # STOSTG

4. 0.5% Acetic Acid

Acetic Acid	0.5 ml
Distilled water	99.5 ml

Procedure

1. Fix in Bouin's fluid over night at room temperature.
2. Rinse in running tap water for 5-10 min.
3. Stain with Weigert Iron hematoxylin for 10 min.
4. Rinse in tap water for 5 min.
5. Stain with One-Step Trichrome stain for 20 min.
6. Rinse in 0.5% Acetic Acid.
7. Clear, dehydrate and mount with permanent mounting media.

Results

Collagen	: Green
Muscle, cytoplasm, and RBC	: Red
Nuclei	: Blue to Black

Appendix 2: Enzyme histochemistry techniques

A. ATPase (Brooke and Kaiser, 1970)

Purpose

To distinguish between type 1, type 2A, 2B, and 2C fibers, with sodium barbital acetate buffer acidic preincubation (pH 4.5 and pH 4.2).

Principle

Myosin adenosine triphosphatase (ATPase) is a phosphatase enzyme, belongs to hydrolase group that catalyzes the hydrolysis of adenosine triphosphate (ATP) by breaking the bond between an alcohol and a phosphate group giving adenosine diphosphate (ADP) and phosphoric acid. Phosphoric acid is simultaneously captured by calcium chloride forming calcium phosphate that deposits as a colorless insoluble compound at the site of enzyme activity. Calcium phosphate is soluble at low pH; therefore, this reaction must be performed in an alkaline solution (pH 9.4). Calcium phosphate reacts with cobalt chloride to form cobalt phosphate, which is still invisible with the light microscopy, so addition of ammonium sulfide develops a black-brown insoluble cobalt sulfide.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on 22 x 22 mm coverslip.

Negative control

Omit the substrate (ATP) from the incubating medium for the negative control.

Reagents

1- 0.1 M Sodium Barbital

Sodium Barbitone	2.06 g
Distilled water upto	100 ml

2- 0.18 M Calcium Chloride

Calcium Chloride ($CaCl_2.2H_2O$)	2.65 g
Distilled water upto	100 ml

3- 1% Calcium Chloride

Calcium Chloride ($CaCl_2.2H_2O$)	1 g
Distilled water upto	100 ml

4- 2% Cobalt Chloride

Cobalt Chloride (hexahydrate)	2 g
Distilled water upto	100 ml

5- Barbital Acetate Solution

Sodium Barbitone	1.47 g
Sodium Acetate ($NaC_2H_3O_2.3H_2O$)	0.97 g
Distilled water upto	50ml

6- 1 N Sodium Hydroxide

Sodium Hydroxide (Anhydrous Pellets)	4 g

Distilled water upto	100 ml

7- 0.1 N Sodium Hydroxide

1 N Sodium Hydroxide	1 ml
Distilled water	9 ml

8- 1 N Hydrochloric Acid

Hydrochloric Acid	10.4 ml
Distilled water upto	125 ml

9- 0.1 N Hydrochloric Acid

1 N Hydrochloric Acid 10 ml
Distilled water upto 100 ml

10- Preincubating Solution for ATPase pH 4.5 and pH 4.2 (Prepare fresh)

Barbital Acetate Solution	2.5 ml
0.1 N Hydrochloric Acid	5 ml
Distilled water	2 ml

Adjust to pH 4.5 and pH 4.2 with 1 N HCL or 1 N NaOH

11- Incubating solution (Prepare fresh)

ATP powder (disodium salt)	20 mg
0.1 M Sodium Barbital	2 ml
Distilled water	7 ml
0.18 Calcium Chloride	1 ml

Adjust to pH 9.5 with 1 N HCL or 1 N NaOH

12- 2% Ammonium Sulfide (Prepare fresh in the fume hood)

Ammonium Sulfide	0.2 ml
Distilled water	9.8 ml

Procedure

1- Preincubate freshly cut section in the freshly prepared preincubating solutions (pH 4.5 and pH 4.2) for 5 min.
2- Rinse briefly in distilled water.
3- Incubate in freshly prepared incubating solution at 37°C for 30 min.
4- Wash with three changes of 1% Calcium Chloride for 10 min.
5- Immerse in 2% Cobalt Chloride for 10 min.
6- Wash with three changes of 0.05 M Sodium Barbital Solution (5ml of 0.1 M Sodium Barbital + 95 ml of Distilled water). The initial wash should turn a faint blue in color.
7- Rinse in five changes of distilled water.
8- Immerse in freshly prepared 2% Ammonium Sulfide solution for 30 seconds (in fume hood).
9- Rinse well in running tape water.
10- Dehydrate, clear and mount with Canada balsam.

Results

Type 1 fibers : Dark
Type 2A fibers: Light
Type 2B fibers: Intermediate
Type 2C fibers: Between light and intermediate

B. Phosphorylase

Purpose

To determine the glycolytic capacity of skeletal muscle fibers.

Principle

Phosphorylase is a transferase enzyme that transfers phosphate groups from one compound to another. The presence of high concentration of glucose-1-phosphate (substrate) in the incubating medium forces the phosporylase to synthesis unbranched polysaccharide chains, and the low concentration on glycogen is used as a primer and adenosine-5'-monophosphate acts as an activator in the incubating medium. Alcohol is added to the incubating medium to prevent the action of the branching enzyme. The unbranched polysaccharide chains react with the iodine solution giving various shades of brown color that is proportional to the length of the polysaccharide chains and phosphorylase activity.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on 22 x 22 mm coverslip.

Negative control

Omit the substrate (glucose-1-phosphate) form the incubating medium for the negative control.

Reagents

1- 0.1 M Sodium Acetate Buffer, pH 5.6

Sodium acetate trihydrate (mw 181) (1.36g/100 ml)	90.5 ml
	9.5 ml
Stock A: Sodium Acetate trihydrate (mw 181)	1.36 g
Distilled water up to	100 ml
Stock B: Acetic Acid	0.575 ml
Distilled water up to	100 ml

Working 0.1 M Sodium Acetate Buffer, pH 5.6

Stock A	90.5 ml
Stock B	9.5 ml
Adjust to pH 5.6 with 0.1 N HCl or 0.1 N NaOH	

2- 0.02% Glycogen in 0.1 M Sodium Acetate Buffer

Glycogen	0.02 g
0.1 M Sodium Acetate Buffer	100 ml
Store at -20°C in 5 ml aliquots	

3- 1% Glucose-1-Phosphate

Glucose-1-Phosphate	1 g
Distilled water	100 ml
Store at -20°C in 2.5 ml aliquots	

4- 0.2% Adenosine Monophosphate (AMP)

AMP	0.2 g

Distilled water	100 ml
Store at -20°C in 2.5 ml aliquots	

5- Incubating medium: Prepare fresh
Thaw and mix 2,3,and 4 in a glass beaker, add 1.5 ml of 100% ethanol, and adjust to pH 5.6.

6- Lugol's Iodine solution: store in an amber bottle.

Potassium iodide	1 g
Iodide crystals	0.5 g
Distilled water upto	25 ml

7- Working Iodine solution

Lugol's Iodine solution	6-8 drops
Distillted water	10 ml

Procedure

1- Incubate freshly cut section in freshly prepared incubating solution at 37°C for 45 min.
2- Wash in 3 changes of distilled water.
3- Stain in working Iodine solution until section turns dark in color.
4- Dehydrate in 0.1% alcohol, clear in 0.1 xylene, and mount in 0.1% histoclad mounting medium.

Results

Sites of phosphorylase activity: Brown

C. SDH (Nachlas et. al., 1957)

Purpose

To determine the oxidative capacity of skeletal muscle fibers.

Principle

Succinate dehydrogenase (SDH) is a dehydrogenase enzyme, belongs to oxidoreductase group, which catalyzes the oxidation (removal of hydrogen atoms) of an organic substrate (sodium succinate) in the incubating medium. The hydrogen atoms are simultaneously captured by an artificial hydrogen accepter (nitro blue tetrazolium "NBT") in the incubating medium forming water-insoluble, blue formazan complex at the site of enzyme activity.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on 22 x 22 mm coverslip.

Negative control

Omit the substrate (sodium succinate) from the incubating medium for the negative control.

Reagents

1- 0.2 M Phosphate Buffer, pH 7.6

Stock A: 0.2 M Sodium Phosphate monobasic (mw 137.99)

Sodium Phosphate monobasic (mw 137.99)	2.76 g
Distilled water up to	100 ml

Stock B: 0.2 M Sodium Phosphate dibasic heptahydrated (mw 268.07)

Sodium Phosphate dibasic heptahydrated (mw 268.07)	5.361 g
Distilled water up to	100 ml

Working 0.2 M Phosphate Buffer, pH 7.6

Stock A	13 ml
Stock B	87 ml
Adjust to pH 7.6 with 0.1 N HCl or 0.1 N NaOH	

2- 0.2 M Sodium Succinate. Prepare fresh

Sodium Succinate (mw 270.1)	0.135 g
Distilled water	2.5 ml

3- 0.2% Nitor Blue Tetrazlium (NBT). Store in refrigerator

Nitro Blue Tetrazolium	0.1g
Distilled water	50 ml

4- Incubating medium

0.2 M Phosphate Buffer	2.5 ml
0.2 M Sodium Succinate (freshly prepared)	2.5 ml
0.2% Nitro Blue Tetrazolium (NBT)	2.5 ml
Distilled water	2.5 ml

5- Physiological saline

Sodium Chloride	0.9 g
Distilled water up to	100 ml

6- 10% Fomalin-saline solution. Prepare fresh

Formaldehyde	1 ml
Physiological saline	9 ml

Procedure

1- Incubate freshly cut section in freshly prepared incubating solution at 37°C for 30 min.
2- Rinse in Physiological saline.
3- Fix in 10% Formalin-saline solution for 10 min.
4- Rinse in 15% alcohol for 5 min.
5- Mount with aqueous mounting medium and seal the coverslip with nail polish.

Results:

Sites of SDH activity: Blue

D. NADHtr (Carson, 1997)

Purpose

To determine the oxidative capacity of skeletal muscle fibers.

Principle

Nicotinamide adenine dinucleotide tetrazolium reductase or diaphorase is a oxidoreductase enzyme that catalyzes the oxidation (removal of hydrogen atoms) form the reduced coenzyme (NADH) in the incubating medium. Hydrogen atoms are simultaneously captured by an artificial hydrogen acceptor (nitro blue tetrazolium "NBT") in the incubating medium forming a water-insoluble, blue formazan complex at the site of enzyme activity.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on 22 x 22 mm coverslip.

Negative control

Omit the coenzyme (NADH) from the incubating medium for the negative control.

Reagents:

1- Phosphate Buffer, pH 7.4

Stock A: Sodium Phosphate monobasic (mw 137.99)	0.69 g
Distilled water up to	50 ml
Stock B: Sodium Phosphate dibasic (mw 141.96)	1.41 g
Distilled water up to	100 ml

Working Phosphate Buffer, pH 7.4

Stock A	19.5 ml
Stock B	80.5 ml
Adjust to pH 7.4 with 0.1 N HCl or 0.1 N NaOH	

2- Physiological saline

Sodium Chloride	0.9 g
Distilled water up to	100 ml

3- 0.2% Nitor Blue Tetrazlium (NBT). Store in refrigerator

Nitro Blue Tetrazolium	0.1g
Distilled water	50 ml

4- Incubating medium: Prepare fresh

NADH, 10 mg/vial (Sigma No. 340-110)	1 vial
Distilled water	4 ml
Physiological saline	2 ml
Phosphate Buffer, pH 7.4	2 ml
0.2% NBT solution	10 ml

Procedure

1- Incubate freshly cut section in freshly prepared incubating solution at 37°C for 30 min.

2- Rinse well in distilled water.
3- Mount with aqueous mounting medium and seal the coverslip with nail polish.

Results

Sites of NADHtr activity: Blue

Appendix 3: Immunohistochemistry

A. ABC detection system

Purpose

To identify antigens in tissue section.

Principle

Immunohistochemistry is used to identify a specific antigen in tissue section by applying the antigen's specific primary antibody followed by the application of the biotinylated secondary antibody, next the addition of the Avidin-Biotin complex (ABC), and then the application of Diaminobenzidine (DAB) chromogen for the final color development. Endogenous peroxidase activities is quenched by incubating the section with 3% hydrogen peroxide. Normal horse serum is used to minimize the non-specific antibody-antigen reaction. Phosphate buffer normal saline (PBS) is the wash buffer solution between steps.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on charged slides.

Negative control

Omit the primary antibody step for negative control

Reagents

1- 0.01M Phosphate Buffered normal saline (PBS) with Tween 20

Sodium Chloride	3.95 g
Sodium Phosphate monohydrate (monobasic)	0.325 g
Sodium Phosphate heptahydrate (dibasic)	2.013g
Distilled water up to	500 ml
Adjust to pH 7.4 with 1 N HCl and 1 N NaOH	
Prepare PBS with 0.1% Tween 20 for washing steps	

2- 3% Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide (30%)	5.0 ml
PBS	45.0 ml

3- Blcoking serum

PBS	10 ml
Normal horse serum	150 µl

4- Primary antibodies dilutions

Nitric oxide synthase (NOS) isoforms:

nNOS (NOS-1)	1:100
iNOS (NOS-2)	1:200
eNOS (NOS-3)	1:100

Myosin isoforms:

A4.840	1:200
N2.261	1:200
A4.1519	1:200
N1.551	1:200
A4.74	1:200

F1.652	1:200
The diluent is PBS with out Tween 20	

5- Biotinylated secondary antibody

PBS	10 ml
Normal horse serum	150 µl
Biotinylated antibody stock	50 µl

6- Vectastain *Elite* ABC reagent

PBS	5 ml
Reagent A	2 drops
Reagent B	2 drops
Allow the reagent to stand for about 30 mins	

7- Diaminobenzidine (DAB) substrate

Distilled water	5 ml
Buffer stock solution	2 drops
DAB stock solution	4 drops
Hydrogen peroxide	2 drops
Mix well after adding each reagent.	
Add 2 drops of Nickel solution if grey-black stain is desired.	

Procedure

1- Place freshly cut section in cold acetone for 10 min.
2- Wash in PBS briefly.
3- Place the slides in 3% hydrogen peroxide for 5 min.
4- Wash in PBS for 5 min.
5- Incubate in diluted normal blocking serum for 20 min.
6- Blot excess serum from the sections. Do not wash in buffer.
7- Incubate in the appropriate primary antibody overnight at 4°C.
8- Wash in PBS for 5 min.
9- Incubate in the Biotinylated secondary antibody for 30 min.
10- Wash in PBS for 5 min.
11- Incubate in VECTASTAIN *Elite* ABC reagent for 30 min.
12- Wash in PBS for 5 min.
13- Incubate in DAB substrate for 2-10 min or until desired stain intensity develops.
14- Rinse in tap water briefly*.
15- Dehydrate, clear and mount in permanent mount.

Results

Areas of antigen-antibody reaction: Brown

Comments:

*If nuclear counterstain is desired, stain in Mayer hematoxylin for 5 min then wash in running tap water for 10 min.

B. Labeled polymer detection system

Purpose

To identify antigens in tissue section.

Principle

Immunohistochemistry is used to identify a specific antigen in tissue section by applying the antigen's specific primary antibody followed by the application of the peroxidase labeled polymer, and then the application of Diaminobenzidine (DAB) chromogen for the final color development. Endogenous peroxidase activities is quenched by incubating the section with 3% hydrogen peroxide. Normal horse serum is used to minimize the non-specific antibody-antigen reaction. Phosphate buffer normal saline (PBS) is the wash buffer solution between steps.

Specimen

Frozen section of unfixed skeletal muscle tissue cut at 10 µm and mounted on charged slides.

Negative control

Omit the primary antibody step for the negative control.

Reagents

1- 0.01M phosphate buffered normal saline (PBS) with Tween 20

Sodium chloride	3.95 g
Sodium phosphate monohydrate (monobasic)	0.325 g
Sodium phosphate heptahydrate (dibasic)	2.013g
Distilled water up to	500 ml
Adjust to pH 7.4 with 1 N HCl and 1 N NaOH	
Prepare PBS with 0.1% Tween 20 for washing steps	

2- 3% hydrogen peroxide (H_2O_2)

Hydrogen peroxide	5.0 ml
PBS	45.0 ml

3- Blcoking serum

PBS	10 ml
Normal horse serum	150 µl

4- Primary antibodies dilutions

Nitric oxide synthase (NOS) isoforms:

nNOS (NOS-1)	4:100
iNOS (NOS-2)	4:100
eNOS (NOS-3)	4:100

Myosin isoforms:

A4.840 (type 1 fibers)	4:100
N2.261 (type 1 and 2A fibers)	4:100
2F7 (type 2A fibers)	4:100
10F5 (type 2B fibers)	4:100
6H1 (type 2X fibers)	4:100
F1.652 (embryonic fibers)	4:100

5- Labeled Polymer

Commercially prepared peroxidase labeled polymer from Dako Cytomation Code K4000

6- Diaminobenzidine (DAB) substrate

Distilled water	5 ml
Buffer stock solution	2 drops
DAB stock solution	4 drops
Hydrogen peroxide	2 drops

Mix well after adding each reagent.

Add 2 drops of Nickel solution if grey-black stain is desired.

Procedure

7- Place freshly cut section in acetone for 10 min.
8- Wash in PBS briefly.
9- Place the slides in 3% hydrogen peroxide for 5 min.
10- Wash in PBS for 5 min.
11- Incubate in diluted normal blocking serum for 20 min.
12- Blot excess serum from the sections. Do not wash in buffer.
13- Incubate in the appropriate primary antibody overnight at 4°C.
14- Wash in PBS for 5 min.
15- Incubate in Labeled polymer reagent for 30 min.
16- Wash in PBS for 5 min.
17- Incubate in DAB substrate for 2-10 min or until desired stain intensity develops.
18- Rinse in tap water briefly*.
19- Dehydrate, clear and mount in permanent mount.

Results

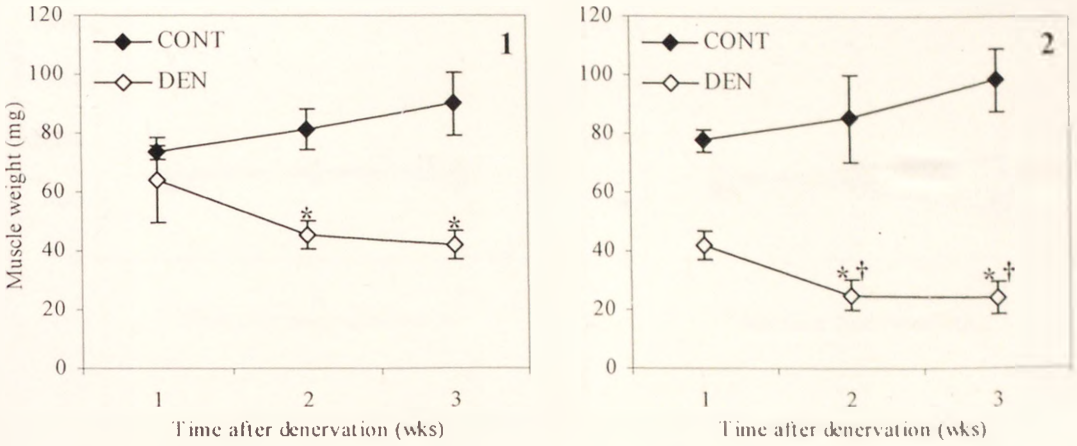
Areas of antigen-antibody reaction: Brown

Comments

*If nuclear counterstain is desired, stain in Mayer hematoxylin for 5 min then wash in running tap water for 10 min.

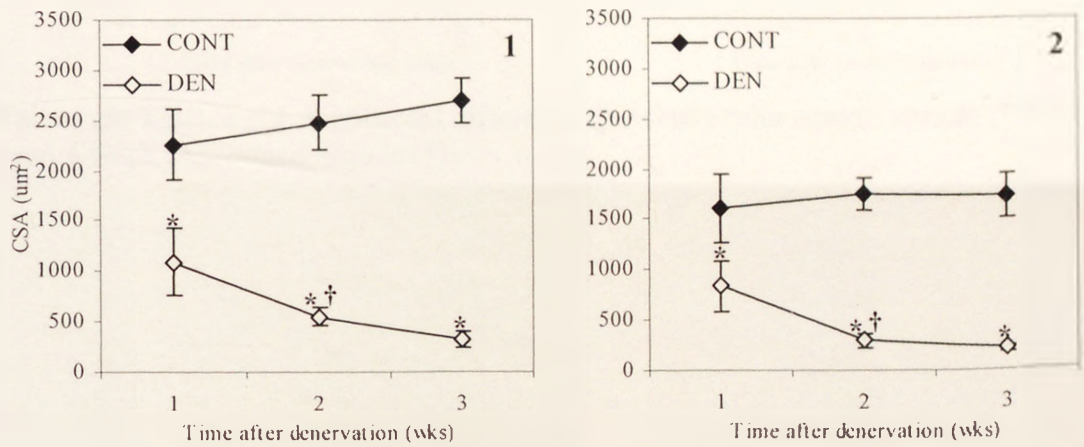
Appendix 4: Additional data of muscle weight and cross sectional area of different fiber types.

A. Changes in muscle weight of control (CONT) and denervated (DEN) extensor digitorum longus (EDL) (1) and soleus (SOL) (2) muscles at one, two, and three weeks after denervation.



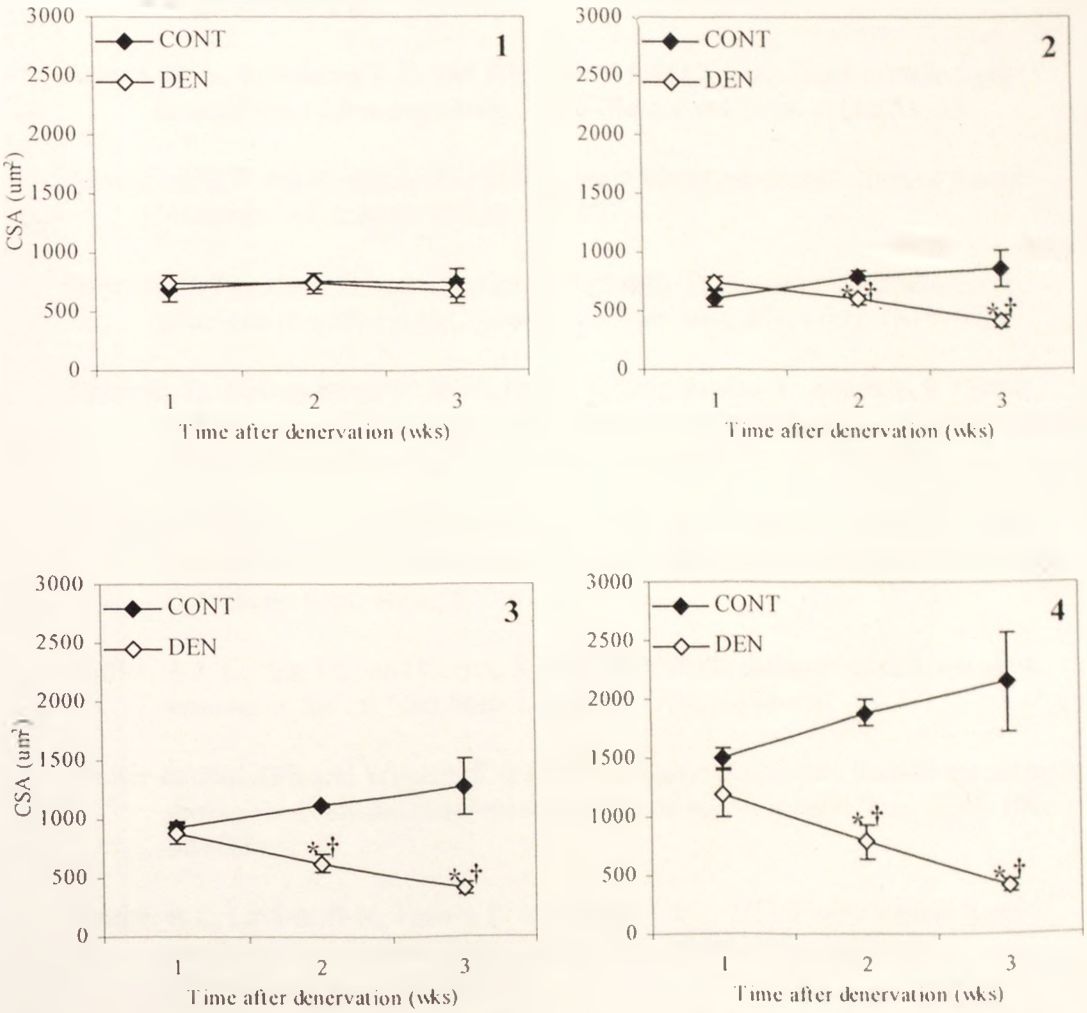
Values are ± SD. n = 4. Significant difference ($p < 0.05$) from control muscle (*) and from 1 week denervated muscle (†).

B. Changes in cross sectional area (CSA) of control (CONT) and denervated (DEN) type 1 (1) and type 2A (2) fibers of soleus (SOL) muscles at one, two, and three weeks after denervation.



Values are ± SD. n = 4. Significant difference ($p < 0.05$) from control muscle (*) and from 1 week denervated muscle (†).

C. Changes in cross sectional area (CSA) of control and denervated type 1 (1), type 2A (2), type 2X (3), and type 2B (4) fibers of extensor digitorum longus (EDL) muscles at one, two, and three weeks after denervation.



Values are ± SD. n = 4. Significant difference ($p < 0.05$) from control muscle (*) and from 1 week denervated muscle (†).

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