

Effects of Tenotomy on Regenerating Anterior Tibial Muscle in Rats

Evelio Luque, Ignacio Jimena, Fuensanta Noguera, Luis Jiménez-Reina and José Peña

Department of Morphological Sciences, Section of Histology, University of Córdoba, Spain

Abstract

The effect of tenotomy on the regeneration of anterior tibial muscle was studied in Wistar rats. Regeneration was induced by intramuscular injection of a local anesthetic, and tenotomy was performed at varying intervals post-injection. Muscles were examined using light and electron microscopy 30 days after sectioning the tendon. Results showed that tenotomy failed to prevent new muscle fibers formation, although it did hinder the recovery of normal muscle architecture, prompting atrophy and degeneration of regenerating fibers as a result of fibrosis, which led to defective reinnervation and vascularization. These alterations affected the deep or red region of the muscle; regeneration in the superficial region was not significantly modified.

Key words: muscle atrophy, muscle regeneration, rat anterior tibial muscle, tenotomy.

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Regeneration is an essential process in the healing of injured muscle; the progress and efficiency of regeneration depend on adequate revascularization and reinnervation [10]. Moreover, muscle tension must be maintained through the integrity of tendinous connections, enabling satisfactory restoration of the internal architecture of the regenerating muscle [5].

Since the tendon is a common injury site in both traumatology and sports medicine [11], it might be useful to ascertain the consequences of tension deficiency on muscle regeneration. Muscle regeneration following partial rupture of muscle tissue near the myotendinous junction has been studied using a strain model in mouse gastrocnemius muscle; in this model, muscle injury was simultaneous with severing from the tendon, leading to incomplete healing due to the development of fibrosis at the injury site [18, 19]. In free-graft and autograft models, despite the severing of tendinous junctions, complete denervation and devascularisation of skeletal muscle is also reported [6], which masks the possible effects of tension deficiency on muscle regeneration.

Experimental tendon transection is often used to study tension deficiency and its histological effects are widely documented in the literature. Although tenotomy is a routine procedure in traumatological and orthopedic surgery [12], little information is available regarding the effects of tension deficiency on regenerating muscle.

This study was performed using rat anterior tibial muscle, which has only one, distal, tendon; its size and accessibility also make it ideal for studying muscle regeneration. This muscle consists of two clearly distinct regions:

a deep "red" region (with a high proportion of type 1 and 2a fibers) and a superficial "white" region (predominantly type 2b fibers) [2, 23]. Given this characteristic distribution of fiber types, it becomes easier to determine whether, within the same muscle, tenotomy affects regeneration differently depending on the predominant fiber type. Red muscles are known to be more susceptible to the effects of tenotomy [9, 24] and display poorer regenerative properties than white muscles [4]. In order to ascertain whether any phase of the regenerative process was more susceptible to tenotomy-induced damage, muscles were examined at various stages of regeneration.

Material and Methods

Animals

A total of 42 male Wistar rats weighing roughly 300 g were used. All experimental procedures were performed under ether anesthesia and approved by the University of Córdoba Ethical Committee on Animal Experimentation.

Experimental procedure

Induction of regeneration

Anterior tibial muscles were injected with 50 µl mepivacain (Scandinibsa, Inibsa, Barcelona): the needle was inserted close to the tendon and pushed gently in a proximal direction; as the needle was withdrawn, anesthetic was gradually released over the whole muscle.

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Tenotomy

After anesthesia, the tendon was exposed and a 3 mm section extracted.

Distribution into experimental and control groups

Normal control group (NC)

Four rats not subjected to any form of handling.

Tenotomized control group (TC)

Four rats killed 30 days after tenotomy of the anterior tibial muscle.

Regenerating control group (RC)

Four rats killed 30 days after induction of regeneration in anterior tibial muscles.

Tenotomized regenerating group (TR)

30 rats in which regeneration had been induced were subjected to tenotomy at varying stages of the regenerative process (1, 3, 6, 10 and 20 days post-injection). At 30 days post-tenotomy, all rats were decapitated. Five subgroups were thus formed, each containing six rats: TR₁, TR₃, TR₆, TR₁₀ and TR₂₀.

Light and electron microscopy

Anterior tibial muscles from both legs were dissected and the muscle belly immediately frozen in isopentane precooled in liquid nitrogen. Serial sections 8-10 μ m thick were cut on a cryostat and stored at -20°C until examination. Sections were stained with hematoxylin-eosin, modified Gomori trichrome, NADH-tetrazolium reductase, acid phosphatase and acridine orange. Desmin immunostaining was performed using a monoclonal antidesmin antibody (1:50, Desmin, DE-R-11, Dako, Denmark).

Small muscle samples for electron microscopy were fixed by immersion in 2.5% buffered glutaraldehyde, postfixed in osmium tetroxide, embedded in Araldite and cut on an ultramicrotome. Semithin sections were stained with toluidine blue and ultrathin sections were contrasted with uranyl acetate/lead citrate and examined through a Philips CM10 transmission electron microscope.

Morphometric analysis

Morphometric analysis was performed using a Leitz Dialux 20 microscope with built-in camera, connected to a PC running an image-analysis program (IMAGO, Grupo SIVA, University of Córdoba, Spain).

H&E-stained sections were examined at x250, since it was impossible to demonstrate histochemical fiber types in the experimental group, particularly in the deep region. Two regions were distinguishable in the anterior tibial muscle cross-section: a deep region (DR) and a superficial region (SR). In four fields randomly selected from each of these, the mean fiber cross-sectional area and the percentage area occupied by connective tissue were measured. The total area covered in each muscle region was around 1mm².

Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM). The statistical significance of inter-group differences was determined by ANOVA, and was accepted at $p < 0.05$. The Student-Newman-Keuls test was used after ANOVA. When the normalized test failed, the Kruskal-Wallis one-way analysis of variance, followed by the Mann-Whitney Rank Sum Test, were performed.

Results

Microscopy

Normal control group (NC)

Histochemical examination of anterior tibial muscle revealed an unequal distribution of fiber types: a superficial region composed largely of type 2b fibers and a deep region in which type 2a and type 1 fibers predominated (fig. 1).

Tenotomized control group (TC)

Muscles in the TC group displayed certain changes with respect to normal controls, these changes being more marked in the deep region: small, rounded fibers were observed alongside other apparently normal fibers. An increase in endomysial connective tissue was apparent in the deep region. NADH-tr staining clearly distinguished fiber types in both regions, and highlighted the existence of core lesions, which were also desmin-positive (fig. 2). These lesions were more abundant in the superficial region.

Regenerating control group (RC)

Fiber morphology, size and fascicular arrangement appeared normal. The only features of interest were centralized nuclei indicative of regeneration and a slightly increased endomysial space. NADH-tr staining revealed the recovery of fiber types and their characteristic distribution in superficial and deep regions. Reactions to anti-desmin and acridine orange staining were negative.

Tenotomized regenerating group (TR)

Changes in the response of regenerating tibial anterior muscle following tenotomy were similar, regardless of the stage of regeneration at which tension deficiency was induced. However, differences were recorded between the deep and superficial regions of these muscles.

Fibers in the superficial region displayed apparently-normal morphology, polygonal outline and internalized nuclei (fig. 3). NADH-tr staining enabled clear differentiation of fiber types; type 2b fibers predominated. However, fiber type grouping was observed (fig. 4) and some fibers displayed changes in myofibrillar pattern, assuming a moth-eaten aspect. Fibers in this muscle region were ultrastructurally normal, except for the presence of a central nuclei and some focal loss of myofibrillar alignment (fig. 5).

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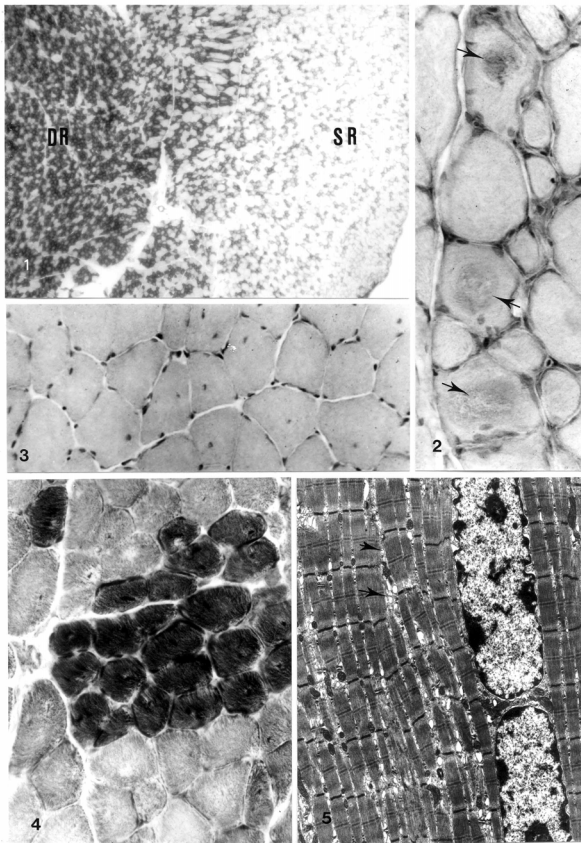


Figure 1. A low magnification view of the normal anterior tibial muscle to demonstrate the superficial (SR) and deep regions (DR). NADH-tr. 10x.

Figure 2. Desmin immunoreactivity is increased in core lesions (arrows). 40x.

Figure 3. Polygonal fibers with central nuclei are present in superficial region. H&E. 25x.

Figure 4. Fiber type grouping in superficial region. NADH-tr 25x.

Figure 5. This electron micrograph shows the nuclei in regenerating fiber within the centre of the fiber. Arrows point to no linear arrangement of Z-lines. 4 900x.

In the deep region, small fiber with internalized nuclei were observed. At all stages of regeneration, some areas contained clusters of highly-atrophied, angular fibers, surrounded by a considerable increase in connective tissue, largely affecting the perimysium (fig. 6), in which adipose cell clusters were also occasionally observed. In this region fiber types could not be differentiated (fig. 7); atrophic fibers were positive to acid phosphatase. All fibers were negative for acridine orange and desmin. Ring fibers were visible in areas where the increase in connective tissue was most marked, and were more apparent in groups TR₁₀ and TR₂₀. Light and electron microscopic features of these ring fibers have been reported previously [22].

Ultrastructurally, atrophic regenerating fibers displayed internal, irregularly-shaped myonuclei, mitochondria and thin myofibrils (fig. 8). Anomalous triad profiles were apparent in some atrophic fibers. A total of 9.8% of tenotomized regenerating fibers showed ultrastructural signs of degeneration, in the form of pre-necrotic changes such as dilation of the sarcoplasmic reticulum or hypercontraction of myofibrillar material (fig. 9). Degenerating fibers were generally found in association with capillaries displaying enlarged, electron-lucid endothelial cells indicative of cell damage (fig. 10); in other cases, degenerating regenerative fibers were surrounded by abundant collagen, with no capillaries apparent in the vicinity. There was no visible evidence of invasion of degenerating fibers by phagocytic cells.

Morphometry

Control groups

Table 1 shows the results of morphometric analysis in control groups. TC group muscles displayed atrophy affecting both regions, and proliferation of deep-region connective tissue with respect to normal controls.

Mean fiber area in the RC group was similar to that of NC group; no significant difference was observed between deep regions, but mean area in the superficial region was 17% smaller in the RC group, which also displayed an increase in connective tissue in both regions.

Table 1. Morphometry of control groups

	DR		SR	
	Csa (μm^2)	Ict (%)	Csa (μm^2)	Ict (%)
NC	1887.4 $\pm$ 23.3	8.54 $\pm$ 0.79	3184.20 $\pm$ 50.6	8.10 $\pm$ 0.32
TC	1171.0 $\pm$ 25.9*	14.08 $\pm$ 2.07*	2204.3 $\pm$ 50.6*	9.48 $\pm$ 0.48
RC	1747.7 $\pm$ 29.0¥	15.54 $\pm$ 0.81*	2652.7 $\pm$ 60.6*¥	13.90 $\pm$ 1.04*¥

Csa: cross sectional area; Ict: intramuscular connective tissue; SR: superficial region; DR: deep region; NC: normal control group; TC: tenotomy control group; RC: regenerating control group.

*: significant differences for $p < 0.05$ with NC

¥: significant differences for $p < 0.05$ with TC

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Tenotomized regenerating group (TR)

A comparison of the results for groups RC and TR is shown in Table 2. Results clearly show greater involvement of the deep region in TR muscles, evident in a significant drop ($p < 0.05$) in fiber area and an equally significant increase ($p < 0.05$) in the proportion of connective tissue. In the superficial region, results were similar to those obtained for group RC, no significant differences being recorded.

Discussion

Our results clearly showed that tenotomy affected muscle regeneration, although the effect was not uniform in the two regions of the anterior tibial muscle. In the superficial region, regeneration appeared to be completed normally, whilst in the deep region there was evidence of atrophy and degeneration of regenerating fibers, as well as endomysial and perimysial fibrosis. This response was apparent regardless of the timing of tension deficiency, suggesting that no single stage of regeneration is especially susceptible to tenotomy.

The differing response of the two muscle regions may be due to the varying proportions of fiber types of which the superficial and deep regions are composed [2, 23]. White muscles, such as the extensor digitorum longus, are known to regenerate better than red muscles such as the soleus [4]. Red muscles are also reported to undergo greater atrophy [1] and fibrosis [14, 15] following both immobilisation and tenotomy. This varying response to regeneration and tenotomy would account for differences in behavior between the two regions of the anterior tibial muscle. Recently, it has been demonstrated that tenotomy alter the expression of mature myosin heavy chains in regenerating slow skeletal muscle, but no in a fast regenerating muscle [21].

The possibility that the small size of regenerating fibers in the deep region might be due to immaturity or delayed growth was ruled out by negative staining to desmin and acridine orange, as well as by ultrastructural findings, indicating that these fibers had atrophied.

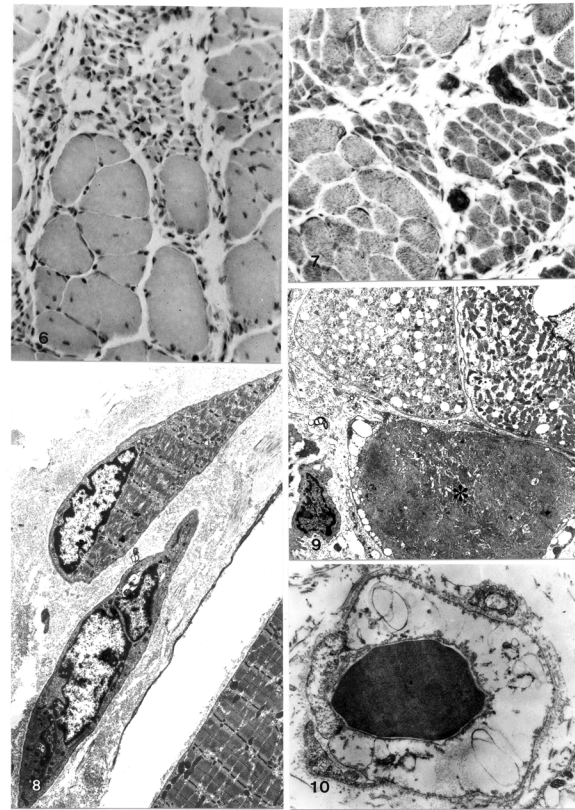


Figure 6. Perymysial fibrosis and atrophy of several muscle fascicles in deep region. H&E 25x.

Figure 7. Deep region showing lack of fiber type differentiation. NADH-tr. 25x.

Figure 8. Two small fibers are angular in outline. Strands of collagen surround them. 2750x.

Figure 9. A group of small fibers that appears to be degenerating. Two fibers show dilated sarcoplasmic reticulum. Another fiber (asterisk) contains clumped myofibrils and collections of subsarcomellar vesicles. 1900x.

Figure 10. This electron micrograph shows an endothelial cell with pale and enlarged cytoplasm. 8900x.

Table 2. Morphometry of experimental group.

	DR		SR	
	Csa (μm^2)	Ict (%)	Csa (μm^2)	Ict (%)
RC	1747.7 $\pm$ 29.0	15.54 $\pm$ 0.81	2652.7 $\pm$ 60.6	13.90 $\pm$ 1.04
TR ₁	864.5 $\pm$ 20.4*	33.11 $\pm$ 4.31*	2340.0 $\pm$ 39.5	16.41 $\pm$ 1.38
TR ₃	809.0 $\pm$ 13.4*	26.63 $\pm$ 3.28*	2351.7 $\pm$ 40.0	11.80 $\pm$ 0.76
TR ₆	908.7 $\pm$ 18.5*	30.09 $\pm$ 4.3*	2361.5 $\pm$ 30.3	11.30 $\pm$ 0.81
TR ₁₀	939.4 $\pm$ 18.8*	28.0 $\pm$ 4.13*	2414.6 $\pm$ 45.4	12.57 $\pm$ 0.67
TR ₂₀	743.9 $\pm$ 13.7*	30.95 $\pm$ 2.67*	2412.8 $\pm$ 46.8	11.82 $\pm$ 0.61

Csa: cross sectional area; Ict: intramuscular connective tissue; SR: superficial region; DR: deep region; RC: regenerating control muscle; TR₁: tenotomized regenerating muscle 1 day after injury; TR₃: tenotomized regenerating muscle 3 days after injury; TR₆: tenotomized regenerating muscle 6 days after injury; TR₁₀: tenotomized regenerating muscle 10 days after injury; TR₂₀: tenotomized regenerating muscle 20 days after injury.

*: significant differences for $p < 0.05$ with RC.

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Given that the degree of atrophy was similar in all groups, regardless of the timing of tenotomy, it must be concluded that early stages of fiber regeneration were not affected by tension deficiency, and that tenotomy therefore induced atrophy of regenerating fibers only at more advanced stages of maturation.

The results obtained here point to a twofold origin of the atrophy affecting the deep region. Whilst some fibers displayed rounded profiles indicative of a myopathic atrophy similar to that observed in tenotomized control muscles, completely-atrophied bundles were also visible, with fibers displaying the angular outline characteristic of neurogenic atrophy [7]. As occurs with immobilization, where fibrosis impedes the regenerative response [13], tenotomy-induced fibrosis was probably responsible for the neurogenic atrophy observed in the present study, by preventing effective re-innervation of regenerating muscle fibers. In this connection it has been reported that, in chronically-denervated muscles, axons are unable to cross the barriers created by collagen fibers [20].

Watkins and Cullen [25] suggest that the accumulation of connective tissue may impede the growth of regenerative fibers in Duchenne muscular dystrophy, by reducing oxygenation and nutrient exchange with the vascular system. The degeneration of regenerating fibers observed in the present study may also have been prompted by fibrosis. Observation of degenerating capillaries and of fibers unassociated with capillaries would seem to suggest that degeneration could result from impaired vascular supply. Fibrosis reduces the supply of oxygen and nutrients to the muscle fiber, not only by increasing the diffusion distance but also by enhancing capillary degeneration [1, 8]. This would account for the decreased capillary density reported after tenotomy and immobilization [14, 15].

It is likely that fiber regeneration was completed in the superficial region since the absence of fibrosis ensured normal vascularization and reinnervation. However, the presence of core lesions in these fibers confirmed that they had regenerated despite tension deficiency. Core fibers are reported in adult tenotomized muscles [3, 16], and it has been shown that they can be prevented by simultaneous denervation [17]. This may account for the absence of core lesions in the deep region of the muscle, due to impaired reinnervation of regenerating fibers.

To conclude, the results obtained here show that regeneration of anterior tibial muscle was not significantly impaired by tension deficiency in the superficial or white region of the muscle, while in the deep or red region it was seriously affected. This unequal response does not appear to be governed by the timing of tension deficiency. Fibrosis arising in the deep region of the muscle was in all likelihood responsible for impaired reinnervation and revascularization, which gave rise to neurogenic atrophy and degeneration of regenerating muscle fibers. Since sectioning of the tendon is a widespread practice in traumatology and orthopedic surgery

[12], the results reported here may be of clinical value; if tenotomy is performed on a previously-injured muscle, ensuing irreversible atrophy may seriously affect muscle recovery, basically in red muscles.

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Address correspondence to:

José Peña, Department of Morphological Sciences, Section of Histology, Faculty of Medicine, Av. Menéndez Pidal s/n, E-14071, Córdoba, Spain, fax +34 57 218246, tel. +34 57 218264, Email cm1peamj@uco.es.

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