

Received: 2012.10.19
Accepted: 2013.05.28
Published: 2014.05.08

Pressing the nerve alters muscle fiber types of the peroneus longus in rats: Preliminary evidence for external anal sphincteroplasty

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ACEF 1,2 **Song Shi**
ADG 1 **Hao Liu**
BF 1 **Xiaobin Bai**
AD 3 **Yongxiao Cao**

1 Department of General Surgery, The First Affiliated Hospital, Xian Jiaotong University College of Medicine, Xian, P. R. China
2 Department of General Surgery, Junan People's Hospital, Junan, P. R. China
3 Department of Pharmacology, Xi'an Jiaotong University College of Medicine, Xi'an, P. R. China

Corresponding Author: Hao Liu, e-mail: haoliu@mail.xjtu.edu.cn

Source of support: This study was supported by grants from the Technological Program of Shaanxi Province, China [grant No: 2006K09-G-(1)] and the First Affiliated Hospital, Medical School of Xian Jiaotong University (grant No: 2005YK.7)

Background: Studies have demonstrated that anal reconstruction with a gracilis graft pressing the dominant nerve could be used to treat fecal incontinence. However, the detailed mechanism by this remains unknown. Herein, we evaluated the alteration in muscle fiber types and contractility of the peroneus longus muscle in rats after pressing its dominant nerves.


Material/Methods: The rat soleus and peroneus longus were exposed during surgery. The superficial peroneal nerve was pressed so that the peroneus longus temporarily lost its innervation. The epimysium between the soleus and the peroneus longus was removed. The end point of the soleus was cut off and the epimysium of the contact surfaces of the soleus and the peroneus longus were sutured. Five months later, peroneus longus contractility was recorded by the myograph system, and types of muscle fibers were observed using the myosin ATPase staining method.

Results: The skeletal muscle fiber type underwent adaptive changes due to double innervations with both fast and slow muscle nerves. Compared with other groups, the percentage of type I fibers in the peroneus longus increased significantly in the group of rats with the pressure on the nerve and removal of the sarcolemma. The maximal contraction and relaxation time at the single twitch and complete tetanus of the peroneus longus were also increased.

Conclusions: Our results show that pressing dominant nerves alter the skeletal muscle fiber types of the peroneus longus, which lead to increased maximal contraction and relaxation time, and significantly improve the ability in resistance to fatigue in rats. This study provides a basis for future clinical studies for external anal sphincter reconstruction using gracilis grafts that are doubly innervated by pressing on its dominant nerve.

Keywords: skeletal muscle • double innervation • muscle fibers • fatigue resistance • external anal sphincter

Full-text PDF: <http://www.medscimonit.com/download/index/idArt/884037>

 2723

 2

 1

 21

Background

Abdominoperineal resection with the creation of a permanent stoma is commonly used for low rectal and anal canal cancers located extremely near the anus. These malignancies were associated with lymph node metastasis along the anal levator muscle or in fatty tissue of the ischiorectal fossa [1]. Technically, it was difficult to preserve the anus, and anal function was destroyed after removal of the sphincter muscle [2]. This procedure caused inconvenience and prodigious mental stress, which was unacceptable to patients [3]. On the contrary, constructing a new anal sphincter with normal defecatory function would be more appropriate [4].

Several types of muscles have been transplanted to reconstruct an external anal sphincter to recover or improve anal defecation control [5]. Among these, the gracilis is one of the most extensively used muscles [5]. The gracilis is located in the femoribus internus and includes many congenerous muscles [6] that have strong tensile force and muscular tension, and includes a long blood vessel and neural stalk. The gracilis could be excised easily due to its anatomical position and was extensively used as a graft in reconstructive surgery [7], although an excised gracilis may weaken the function of the thigh [8]. Since 1986, dynamic graciloplasties (DGPs) have been performed to solve end-stage fecal incontinence [9]. Dynamic gracilis transferred anoplasty was the focal point of anal reconstruction after Mile's operation for rectum carcinoma [10,11]. In addition, successful reconstruction of pelvic and perineal defects using bilateral pedicled gracilis, together with inferiorly based rectus abdominal muscle flaps, was reported in a patient with abdominoperineal resection of advanced perineal adenocarcinoma [12].

While the gracilis and external anal sphincter are striated muscles, the gracilis is made of type II fibers that have low ability to persistently contract and low fatigue resistance. Therefore, the outcome of direct transplantation of the muscles to reconstruct the external anal sphincter may not be the best option. The gracilis with crushed nerves and divested epimysium under the temporary condition of denervation are considered as innervation of the outside nerves and, through the neural regeneration, resulted in double innervations. The nerves from the external anal sphincter affected metabolism of the gracilis, resulting in improvement in persistent contraction and fatigue resistance. Furthermore, neural regeneration could decrease the extent of atrophy, thereby reducing the fibrotic changes in the muscle [13]. Through the direct contact of the transplanted muscle with the smooth muscle, their myoelectricity interacts with each other; thus, the gracilis is more suitable for the alternative function of neosphincter. The external sphincteroplasty reconstruction by transferred gracilis with crushed nerve and intact blood vessels has been reported to

cure pediatric fecal incontinence [13]. However, this has not been used in anus reconstruction after Mile's operation for rectum carcinoma.

In this study, we demonstrated that pressing the dominant nerve of the peroneus longus made of type II fibers in Sprague-Dawley rats were doubly innervated with slow nerves from the soleus and its own. Through the changes in peroneus longus contractility and fiber types, we tested whether the gracilis with nerve pressing and remaining blood supply could be transplanted to reconstruct the external anal sphincter after Mile's operation due to rectum carcinoma.

Material and Methods

Animals and reagents

Male Sprague-Dawley (SD) rats weighing 200–250 g were provided by the Animal Center of Xian Jiaotong University College of Medicine (Xian, China). All animal experiments were conducted in accordance with international ethics guidelines. The housing temperature was maintained at 23–25°C, and the humidity was 30–50%. The animal use and care protocols were reviewed and approved by the Animal Use Ethics Committee at Xian Jiaotong University. ATPase was purchased from Sigma (St. Louis, MO). The BioLab-420 type biological function analysis instrument was purchased from Chengdu Taimen Co. (Chengdu, China).

Experimental design and surgical procedure

The rats were randomly divided into 4 groups (12 per group): the sham-operated group, the peroneus longus double innervation group, the peroneus longus temporary denervation group, and the peroneus longus denervation group.

In the peroneus longus double innervation group, the rat superficial peroneal nerve was pressed so that the peroneus longus temporarily lost its innervations. The end point of the soleus was cut off and the epimysium of the contact surfaces of the soleus and the peroneus longus were sutured. The rats were anesthetized with chloral hydrate (10 mg/kg) through intraperitoneal (i.p.) injection. While the rats were placed in the prone position, a longitudinal incision was made in the fossa poplitea skin of the right lower limb. The soleus, peroneus longus, and superficial peroneal nerve were exposed. At 3 mm of the upper access into the peroneus longus, 3 points of the nerve spacing of 2 mm were pressed with mosquito forceps for 5 seconds. There was no reaction when stimulated with acupuncture anesthesia apparatus, thus, the peroneus longus temporarily lost its innervation. The upper part of the pressed nerve was marked with filament thread. The epimysium

between the soleus and peroneus longus was then divested carefully. The end point of soleus was cut off and soleus was drawn close to the peroneus longus; the contact surface of the soleus and peroneus longus was approximately 0.5×1 cm. The anterior and posterior epimysium of the contact surfaces of the 2 muscles were sutured intermittently. The blood supply of the muscles was carefully maintained during the procedure.

In the peroneus longus temporary denervation group, the rat superficial peroneal nerve was pressed the same as that in the double innervation group. However, the end point of the soleus was not cut off, so that the epimysium between soleus and peroneus longus remained intact. The anterior and posterior epimysium of the contact surface of the 2 muscles was not sutured. In the peroneus longus denervation group, the operation was the same as in the double innervation group, but the superficial peroneal nerve innervating the peroneus longus was cut off completely. For the sham-operated group, the procedure was the same, but without interruption of the nerve and blood supply of the muscle.

General observations

Five months after the procedures, the rats were anesthetized and constrained in the prone position. Through the original incision, the proximal end of the pressed nerve was exposed. The general appearance of the nerves of the soleus and peroneus longus and the degree of muscular atrophy of the peroneus longus were observed. The proximal end of the pressed peroneus longus nerve and the nerve of the soleus were stimulated with acupuncture anesthesia apparatus to measure the contraction of the peroneus longus.

Muscle contractility

Muscle contractility was recorded as described in other reports [14,15] with some modifications. Briefly, the right lower limb sciatic nerve-peroneus longus specimen was prepared so that only the sciatic nerve and peroneus longus remained. The end of the peroneus longus was tied with a string 10 cm in length and the other end of the string was attached to the bar of the transducer fixed to the bracket. The sciatic nerve was stimulated with a stimulating electrode. The wave form of the muscle contractility was recorded by the BioLab-420 type myograph system. The maximum stimulus intensity was found under single stimulus with a wave width of 0.2 ms. The single twitch contraction, maximal contraction, and maximal relaxation time of the muscle were recorded. The minimum frequency of stimulation causing muscle tetanus was then calculated via a computer-based system (1000 stimulations/maximal contraction time). The tetanus contraction was induced and maximal contraction time was recorded at 2 and 5 times the maximal stimulus intensity of a single stimulation with the wave of 0.2 ms.

Histological analysis

The peroneus longus was removed quickly, blotted with filter paper (2-mm thick, Sigma, St. Louis, MO), frozen in liquid nitrogen, followed by slicing it into 10- μ m-thick frozen sections. The myosin ATPase staining was performed as described by others [16,17]. Briefly, the frozen sections were incubated with the ATPase incubation buffer at 37°C for 1.5 h, followed by 1% CaCl₂ and 2% CoCl₂ for 6 min. After washing, the sections were immersed in diluted ammonium sulfide for 15 sec and washed for 5 min. After the sections were dehydrated, cleared, and mounted onto the slides, the histology of rat peroneus longus were observed under a microscope at 200× magnification.

Statistical analysis

The experimental data are expressed as mean \pm SD. Two-way analysis of variance (ANOVA) with Dunnett's post-test was used for comparisons among various treatment groups. A P value of less than 0.05 was considered as a statistical significant difference.

Results

Model development and clinical observations

Among rat groups, 2 rats in the double innervation group died of complications unrelated to the operation; 5 rats scarred in the position of suturing so that the peroneus longus had no reaction when stimulated by the acupuncture anaesthesia apparatus; and 2 rats had dehiscence in the suture site. Unlike in the sham-operated group, atrophy of the peroneus longus to various degrees was found in all 3 of the other groups. The muscular atrophy in the double innervation and temporary denervation groups was much less than that in the denervation group. There was no difference between the double innervation and temporary denervation groups.

The nerve was stimulated by an acupuncture anaesthesia apparatus with the same intensity as in the first procedure. The peroneus longus in the double innervation group contracted when pressed and superficial peroneal and soleus nerves were stimulated. In the temporary denervation group, the peroneus longus showed contraction when the superficial peroneal longus nerve, but not soleus nerve, was stimulated. The muscle of the denervation group contracted when the soleus nerve alone was stimulated. The muscle of the sham-operated group could be contracted by stimulating the peroneus longus nerve only. These results indicate that the peroneus longus in the double innervation group was innervated by its own nerve and the nerve from the soleus. The peroneus in the temporary

Table 1. The contractility changes of the double innervating peroneus longus by stimulating sciatic nerve with a single twitch and tetanus stimulus.

Groups	n	Single twitch			Tetanus	
		Contraction force (g)	Maximal contraction time (s)	Maximal relaxation time (s)	Contraction force (g)	Maximal contraction time (s)
Sham control	12	14.34±3.84 ^a	0.11±0.02 ^a	0.20±0.04 ^a	22.80±5.27 ^a	4.46±2.13 ^a
Denervation	9	3.15±0.74	0.17±0.02	0.28±0.06	5.27±2.12	7.38±2.07
Double innervation	8	12.88±3.54 ^{a,b}	0.18±0.04 ^b	0.32±0.08 ^b	18.78±4.38 ^{a,b}	5.42±1.84 ^a
Temporary denervation	10	8.72±2.68 ^a	0.11±0.01	0.21±0.04	10.04±3.24 ^a	4.24±2.07

Data are expressed as mean ±SD of each group, ^a indicates significant difference as compared to the denervation group (P<0.01); ^b indicates significant difference as compared to the temporary denervation group (P<0.01).

denervation group was innervated by its own nerves only, and the peroneus in the denervation group was innervated only by nerves from the soleus. The peroneus not controlled by its own nerve increased the extent of the muscular atrophy.

The contractility of the peroneus longus

The contraction forces of the peroneus longus in the denervation group at the single twitch and complete tetanus was smaller than in the sham group, and the maximal contraction and relaxation time were longer than in the sham group, indicating that the denervation significantly reduced the contraction function of muscle (P<0.01) (Table 1). The contraction forces of the peroneus longus in the double innervation group at the single twitch and complete tetanus were larger than in denervation, and there was no significant difference in contraction force between the double innervation and the sham groups (P>0.05), suggesting that the double innervation improved the contraction function of the denervated peroneus longus. The contraction forces in the temporary denervation group were lower than in the double innervation group (P<0.01).

The maximal contraction and relaxation time in the double innervation group increased significantly compared to that in the temporary denervation and the sham-operated groups (P<0.01), but no statistically significant difference was observed between the denervation and the double innervation groups (P>0.05) at the single twitch. However, the maximal contraction time in the double innervation group decreased significantly compared to that in the denervation group (P<0.01), but there was no statistically significant difference between the double innervation and the temporary denervation groups (P>0.05). These results indicate that the resistance to fatigue in the peroneus longus innervated by its own nerve and nerves from the soleus in the double innervation group was increased without decreasing its strength. The fatigue resistance of the

peroneus longus innervated by nerves from the soleus alone in the denervation group was increased, but its strength was decreased. Both the fatigue resistance and strength of the peroneus longus innervated by its own nerves in the temporary denervation group were decreased.

Histology of the peroneus longus

The percentage of type I fibers of the peroneus longus in the double innervation group (Figure 1A) increased remarkably as compared to that in the sham group (Figure 1B). The ratio of type I/II fibers of the peroneus longus in the double innervation group was greater than in the temporary denervation and control groups (P<0.01), but was not significantly different from in the denervation group (P>0.05) (Table 2). The ratio of type I to type II fibers of the peroneus longus in the denervation group was greater than that in the temporary denervation and control groups (P<0.01). However, the ratio of type I to type II fibers of the peroneus longus in the temporary denervation group had no statistically significant difference compared to the control group (P>0.05). These results indicate that the percentage of type I fibers of the peroneus longus innervated by nerve from soleus in the double innervation and the denervation groups were increased.

Discussion

The organization of mammalian skeletal muscles can be divided into 2 categories: type I fiber that can contract quickly but becomes fatigued easily and type II fiber that contracts slowly but does not easily become fatigued [18,19]. The skeletal muscles are mainly innervated by their own nerves, but can also accept the innervation of other nerves under certain conditions [20,21]. In our study, through the nerve being crushed or mutilated, the peroneus longus made of type II fibers lose

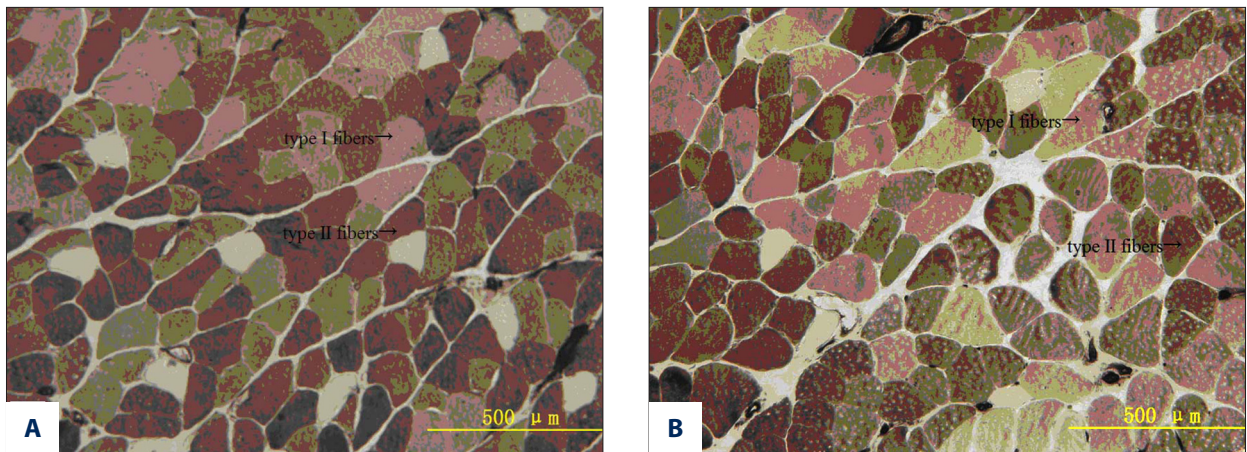


Figure 1. The histology of rat peroneus longus after crushing the nerve. The rat peroneus longus was obtained and sliced into 10- μ m-thick frozen sections. The frozen sections were then incubated with the ATPase buffer at 37°C for 1.5 h, followed by incubating with 1% CaCl_2 and 2% CoCl_2 for 6 min. After washing, the sections were immersed in diluted ammonium sulfide and dehydrated, cleared, and mounted onto the slides. Histology of rat peroneus longus were observed under a microscope at 200 \times magnification. **(A)** The cross section of peroneus longus in the sham group indicated type I fibers were stained 25% of all the fibers, and type II fibers trachychromatic hold 75% of all. **(B)** The cross section of peroneus longus in the peroneus longus after crushing the nerve showed that the stained type I fibers were increased to 45%, while the trachychromatic type II fibers decreased to 55% of all fibers.

Table 2. The changes in the fiber types of the peroneus longus muscle.

Groups	n	The percentage of type I fibers (%)	The percentage of type II fibers (%)
Sham control	12	23 \pm 2	77 \pm 2
Double innervation	8	42 \pm 5 ^a	58 \pm 5 ^a
Temporary denervation	10	25 \pm 3	75 \pm 3
Denervation	9	44 \pm 3 ^a	56 \pm 3 ^a

Data are expressed as mean \pm SD of each group. ^a indicates significant difference as compared to the sham control and the temporary denervation groups ($P < 0.01$).

its innervation temporarily or permanently, thus, it could be innervated by slow nerves made of type I fibers from the soleus. We found that the peroneus longus with nerve crushed could be innervated by slow nerves from the soleus. The peroneus longus could also be innervated by its own nerves through neural regeneration. Through dual innervation by fast and slow muscle nerves, the percentage of type I fibers of the peroneus longus was noticeably increased. Accordingly, the maximal contraction and relaxation time of a single twitch and complete tetanus were increased. Innervation by its own nerve caused a decrease in extent of atrophy, and increase in its contraction force. Although the nerve was completely mutilated, the peroneus longus could also be innervated by nerves from the soleus. The percentage of type I fibers increased and the fatigue resistance improved to almost the same levels of that of crushed nerve. Importantly, because of losing the innervation by its own nerve, the atrophy of the muscle was more apparent and the contraction force was decreased. Only crushed the nerve but the epimysium between soleus and peroneus longus

remained intact, the peroneus longus could be innervated again by its own nerve through neural regeneration. However, the percentage of type I fibers was not increased and fatigue resistance was not improved. Our data indicate that, compared to innervations only of the gracilis nerve, crushing the nerve and double innervations appeared to be better models to optimize the experimental outcomes.

Conclusions

Our results show that the percentage of type I fibers of the peroneus longus doubly innervated was increased from 25% to 42%, and its contraction force was slightly decreased compared to that in the control group. However, there was a tendency toward decreased force of contraction. The main reason for this might be that the contact surface of the peroneus longus and soleus was small (0.5 \times 1 cm). In our study, increases in the contact surface of the peroneus longus and soleus

could influence the blood supply of the muscle. The gracilis has a long blood vessel, thus, if transferred to establish the external anal sphincter, the contact surface can be as large as possible. Although the transformation efficiency of the fiber types was relatively low in our study, the increased contractility of the peroneus longus supports the notion that fiber types were certainly transformed with different functions.

In this *in vivo* rat study, we demonstrated that, through double innervation by fast and slow muscle nerves, the peroneus longus fibers underwent adaptive changes and showed improved

fatigue resistance. After the gracilis with the crushed nerve and the remaining blood supply was transferred to establish the external anal sphincter, the gracilis could also develop adaptive changes and improved fatigue resistance, making it more suitable for the alternative function of neosphincter. Further studies need to be done to further confirm this. More importantly, our results suggest the feasibility of potential clinical applications in transferring the gracilis with crushed nerve and intact blood supply to establish external sphincteroplasty after Mile's operation, which may help to restore and sustain continence, with improvement in the quality of life in patients with rectal carcinoma.

References:

1. Ferenschild FT, Vermaas M, Hofer SO et al: Salvage abdominoperineal resection and perineal wound healing in local recurrent or persistent anal cancer. *World J Surg*, 2005; 29(11): 1452–57
2. Shirouzu K, Ogata Y, Araki Y et al: A new ultimate anus-preserving operation for extremely low rectal cancer and for anal canal cancer. *Tech Coloproctol*, 2003; 7: 203–6
3. Fenech DS, Takahashi T, Liu M et al: Function and quality of life after transanal excision of rectal polyps and cancers. *Dis Colon Rectum*, 2007; 50(5): 598–603
4. Ho K, Seow-Choen J: Dynamic graciloplasty for total anorectal reconstruction after abdominoperineal resection for rectal tumour. *Colorectal Dis*, 2005; 20(5): 38–41
5. Hassan MZ, Rathnayaka MM, Deen KI: Modified dynamic gracilis neosphincter for fecal incontinence: an analysis of functional outcome at a single institution. *World J Surg*, 2010; 34(7): 1641–47
6. Luo S, Raffoul W, Piaget F et al: Anterolateral thigh fasciocutaneous flap in the difficult perineogenital reconstruction. *Plast Reconstr Surg*, 2000; 105: 171–73
7. Baeten CG, Bailey HR, Bakka A et al: Safety and efficacy of dynamic graciloplasty for fecal incontinence. Report of a prospective, multicenter trial. Dynamic Graciloplasty Therapy Study Group. *Dis Colon Rectum*, 2000; 43: 743–51
8. Hashimoto I, Nakanishi H, Nagae H et al: The gluteal-fold flap for vulvar and buttock reconstruction: anatomic study and adjustment of flap volume. *Plast Reconstr Surg*, 2001; 108: 1998–2005
9. Baeten C, Spaans F, Fluks A: An implanted neuromuscular stimulator for fecal continence following previously implanted gracilis muscle: report of a case. *Dis Colon Rectum*, 1988; 31: 134–37
10. Wang W, Li S, Dong HP et al: Differential impairment of spatial and non-spatial cognition in a mouse model of brain aging. *Life Sci*, 2009; 85(3–4): 127–35
11. Ho KS, Seow-Choen F: Dynamic graciloplasty for total anorectal reconstruction after abdominoperineal resection for rectal tumor. *Int J Colorectal Dis*, 2005; 20: 38–41
12. Tsai WP, Shieh SJ, Lin BW: Extensive perineal and pelvic defects reconstructed simultaneously using bilateral pedicled gracilis and rectus abdominis muscle flaps after en-bloc excision of locally invasive perineal mucinous adenocarcinoma 1. *Scand J Plast Reconstr Surg Hand Surg*, 2009; 43(5): 286–90
13. Cui ZM, Chen WX, Chen YL et al: The rebuilding operation of external anal sphincter with gracilis graft in therapy of fecal incontinence. *Chinese J Clin Paediatr Surg*, 2005; 6: 414–16
14. Huang H, Graham D: Matching of sarcoplasmic reticulum and contractile properties in rat fast- and slow-twitch muscle fibers. *Clin Exp Pharmacol Physiol*, 2006; 33: 591–600
15. Bortolotto SK, Stephenson GMM, Stephenson DG: Caffeine thresholds for contraction in electrophoretically typed, mechanically skinned muscle fibres from SHR and WKY rats. *Eur J Physiol*, 2001; 441: 692–700
16. Liang ZJ, Lou WH: Effect of functional neuromuscular stimulation on fiber types of posterior cricoarytenoid muscle after nerve reinnervation in canine. *Chinese J Clin Rehabil*, 2006; 26: 98–100
17. Szentesi P, Zaremba R, van Mechelen W, Stienen GJ: ATP utilization for calcium uptake and force production in different types of human skeletal muscle fibres. *J Physiol*, 2001; 531: 393–403
18. Bottinelli R, Reggiani C: Human skeletal muscle fibres: Molecular and functional diversity. *Prog Biophys Mol Biol*, 2000; 73: 195–262
19. O'Connell B, Nguyen LT, Stephenson GMM: A single-fibre study of the relationship between MHC and TnC isoform composition in rat skeletal muscle. *J Biochem*, 2004; 378: 269–74
20. Bain J R, Veltri KL, Chamberlain D et al: Improved function recovery of denervated skeletal muscle after temporary sensory nerve innervation. *Neuroscience*, 2001, 103: 503–10
21. Baylor SM, Hollingworth S: Sarcoplasmic reticulum calcium release compared in slow-twitch and fast-twitch fibres of mouse muscle. *J Physiol*, 2003; 551: 125–38