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Special Issue

Direct muscle neurotization after end-to-end and end-to-side neurorrhaphy

An experimental study in the rat forelimb model

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Abstract

The need for the continuous research of new tools for improving motor function recovery after nerve injury is justified by the still often unsatisfactory clinical outcome in these patients. It has been previously shown that the combined use of two reconstructive techniques, namely end-to-side neurorrhaphy and direct muscle neurotization in the rat hindlimb model, can lead to good results in terms of skeletal muscle reinnervation. Here we show that, in the rat forelimb model, the combined use of direct muscle neurotization with either end-to-end or end-to-side neurorrhaphy to reinnervate the denervated flexor digitorum muscles, leads to muscle atrophy prevention over a long postoperative time lapse (10 months). By contrast, very little motor recovery (in case of end-to-end neurorrhaphy) and almost no motor recovery (in case of end-to-side neurorrhaphy) were observed in the grasping activity controlled by flexor digitorum muscles. It can thus be concluded that, at least in the rat, direct muscle neurotization after both end-to-end and end-to-side neurorrhaphy represents a good strategy for preventing denervation-related muscle atrophy but not for regaining the lost motor function.

Key Words

nerve injury; nerve reconstruction; microsurgery; axonal regeneration; denervation; muscle atrophy; Schwann cells; stereology; grasping test; rats

Research Highlights

(1) Direct muscle neurotization, *i.e.* the possibility to recover motor function by directly suturing motor nerve fascicles to the perimysium of a denervated skeletal muscle, has proven to be effective in experimental animal models and is currently being employed in selected clinical cases.
(2) We show that direct muscle neurotization in the rat forelimb model can be applied also in combination with both end-to-end and end-to-side neurorrhaphy of the proximal nerve stump with the goal of preventing long-term muscle atrophy. By contrast, the possibility of functional recovery is very limited.
(3) Direct muscle neurotization might be useful in the clinical practice for preventing muscle atrophy but not for recovering motor function.

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INTRODUCTION

Motor function recovery still represents a major challenge after posttraumatic peripheral nerve reconstruction[1]. When severe trauma induces complete lost of the nerve continuity, microsurgical repair should be carried out. If no substance loss has occurred, end-to-end neurorrhaphy allows the regenerating axons from the proximal nerve stump to regrowth along the distal stump. However, direct suturing of the two nerve stumps often is not possible due to substance loss and thus various types of nerve prostheses have been devised in order to bridge the nerve gap, including both biological (*e.g*. vein and muscle-vein-combined conduits) and synthetic (*e.g*. polyglycolic acid and chitosan conduits) materials[1]. Unfortunately, in some cases the proximal nerve stump is not available hindering the possibility to reconnect nerve continuity, and thus alternative strategies have been sought for at least partially regaining function of denervated muscles. Among these *ultima ratio* techniques, direct muscle neurotization and end-to-side nerve reconstruction have attracted attention of the researchers. Although direct muscle neurotization was first described by Heineke in 1914[2], the use of this technique was investigated in depth starting from the eighties[3-4] and is today used in selected clinical cases[5-7].

End-to-side neurorrhaphy is an alternative to end-to-end nerve suture when a proximal donor nerve stump is not available[8]. It was first described by Letievant in 1873[9] and then re-discovered in the nineties[10]. Similarly to direct muscle neurotization, end-to-side neurorrhaphy is today used in selected clinical cases[11-14].

In a previous study, the two techniques have been applied simultaneously in the rat hindlimb model showing that reinnervation of the denervated skeletal muscle occurs[15]. However, the experimental model used in that study did not permit to assess motor function recovery and thus no information is available as to if this surgical approach may eventually lead to motor recovery.

The aim of the present study was to repeat the previous experiment in the rat forelimb model (that is particularly suitable for functional testing) comparing the use of direct muscle neurotization in combination with either end-to-end or end-to-side neurorrhaphy.

RESULTS

General status of rats

All animals survived until the end of the experiment and careful assessment of the post-operative consequences of functional nerve loss on animal well-being showed no detectable distress even in the earlier postoperative times. Auto-mutilation and joint retractions were never observed.

Behavioural and muscle mass changes

Behavioural assessment of motor function recovery by means of the grasping test at time of euthanasia (month-10 postoperative) showed that grasping strength, that in normal adult female rats permits to lift about 250 g[6], was partially regained in four out of the five rats after end-to-end neurorrhaphy, though with an average strength of 0.45 ± 0.30 g, while none of the animals of the end-to-side group showed grasping recovery (with the only exception of one rat that managed to lift a minimal weight of 22 g).

By contrast, muscle mass assessment immediately after harvesting showed that muscle trophism was maintained in both experimental groups, though muscle mass was significantly ($P < 0.05$) higher after end-to-end neurorrhaphy (0.371 ± 0.021 g) than after end-to-side neurorrhaphy (0.329 ± 0.016 g).

Histological and stereological changes

Histological analysis showed that axon regeneration occurred in all nerves, both after end-to-end (Figure 1A) and end-to-side (Figure 1B) neurorrhaphy. The presence of small myelinated axons and the typical micro-compartmentalization of regenerated nerve fascicles were detectable in both experimental groups. Flexor digitorum muscle fibers did not show morphological signs of muscle fiber atrophy, namely the reduction of cross-sectional area of muscle fibers accompanied by the increase of the connective tissue (Figure 1C). Yet, the presence of many variously-sized fascicles regenerated axons was detectable in the muscle interstice (Figures 1D–F).

Stereological analysis of regenerated nerve fibers along the graft used for neurotization (Table 1) showed a significantly ($P < 0.05$) higher number of axons in the end-to-end group, accompanied by a significantly ($P < 0.05$) higher axon and nerve fiber diameter.

DISCUSSION

Improvement of peripheral nerve repair and regeneration continues to represent a major challenge both for basic and clinical neuroscientists[16-24]. Previous studies have shown that when a proximal donor nerve stump is not available, both direct muscle neurotization[3-4] and end-to-side neurorrhaphy can be effective in partially regaining the lost function[10, 25-27].

In this experimental study, we wish to verify if direct

muscle neurotization could be effective in preserving muscle atrophy and restoring motor function in combination with either end-to-end or end-to-side neurorrhaphy after very long postoperative time lapse (10 months). For end-to-side neurorrhaphy, an epineurial window was opened in the donor nerve since previous studies indicated that this procedure improves the effectiveness of end-to-side nerve regeneration[26-27].

Although morphological investigation of the regenerated nerve fibers along the distal median nerve stump used for direct muscle neurotization showed that axonal regrowth occurred in both experimental groups (with a higher number and size of regenerated nerve fibers observed after end-to-end neurorrhaphy), behavioural testing showed that motor recovery was very limited. While at least minimal functional regain (around 15% of normal grasping strength) might be expected after direct muscle neurotization combined to end-to-end neurorrhaphy only, our data suggest that combination of direct muscle neurotization with end-to-side suture is not able to lead to any recovery of the motor function even after a long postoperative time lapse. One possible explanation for this failure of functional recovery can be found in the observation that after end-to-side repair predominantly sensory neural regeneration occurs[27].

On the contrary, both repair techniques led to very good results in the prevention of denervation-related muscle atrophy. In fact, while in case of complete muscle denervation muscle weight drops to about 50% of normal weight[28], direct muscle neurotization in combination to both end-to-end and end-to-side neurorrhaphy maintain a normal muscle weight.

In the translational and clinical perspective, our data suggest that direct muscle neurotization in combination with both end-to-end and end-to-side nerve suture should be regarded as an interesting method for preventing denervated-related atrophy in skeletal muscles and thus a useful tool in the hands of the microsurgeon. On the other hand, our results raise doubts as to the possibility that this repair strategy can lead to any recovery of motor function, especially if it is used in combination with end-to-side neurorrhaphy.

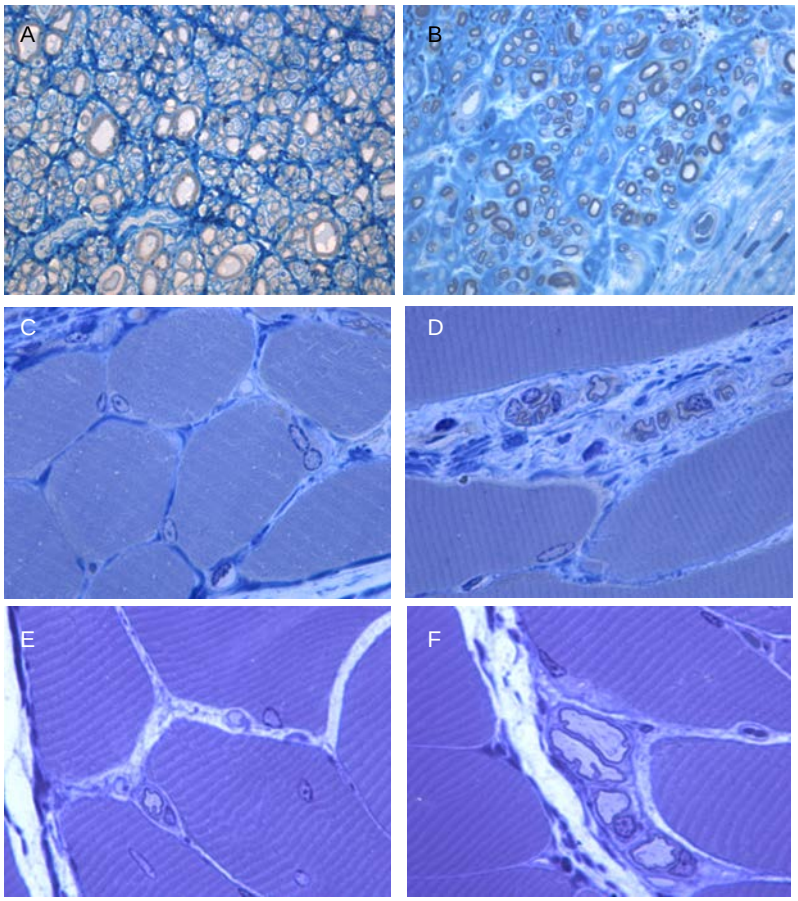

Figure 1 Histological features of the regenerated nerve segments and reinnervated muscles.

In the regenerated nerve segments immediately before entry inside the neurotised muscle, small myelinated axons organized in micro-fascicles can be detected both in end-to-end (A) and end-to-side neurorrhaphy (B) groups. In the reinnervated muscles, no signs of fiber atrophy are present (C) and variously-sized fascicles regenerated axons can be detected (D–F) (light microscopy, ×1 000).

Table 1 Comparison of stereological parameters of regenerated myelinated nerve fibers after direct muscle neurotization in combination with either end-to-end or end-to-side neurorrhaphy

Parameter	End-to-end neurorrhaphy	End-to-side neurorrhaphy
Total number	23 034±4 659	6 692±3 307[a]
Mean fiber density (n/mm^2)	43 350±9 598	42 138±11 002
Mean fiber diameter (μm)	3.25±0.51	2.44±0.22[a]
Mean axon diameter (μm)	2.26±0.52	1.54±0.20[a]
Mean myelin thickness (μm)	0.50±0.06	0.45±0.03
Mean axon-diameter/ fiber-diameter	0.68±0.04	0.61±0.03

[a]P < 0.05, *vs.* end-to-end neurorrhaphy

MATERIALS AND METHODS

Design

The experiments were designed as double-blind study. For both behavioural and morphological analysis, researchers did not know to which group belongs each animal/specimen under analysis.

Time and setting

The experiments reported in this paper were carried out, from January 2008 to October 2008, at the Laboratory of Microsurgery of the School of Surgery of Paris after approval of the local Institution's Animal Care and Ethics Committee.

Materials

The experiments were carried out on 3-month-old Wistar female rats (weight 230–250 g). Animals were bred under identical environmental and feeding conditions. The number of animals used in the experiments was calculated to meet the Ethical Committee requirement for a minimum number of animals used in agreement with the 'Three Rs' (replacement, reduction and refinement of animal studies) concept put forward by Russell and Burch[29] and adopted by the European Community.

Methods

Surgical inteventions

Surgical interventions were carried out under deep anesthesia (ketamine 40 mg/250 g and chlorpromazine 3.75 mg/250 g, i.p.) and in clean conditions. After approaching the median and ulnar nerves of the left upper limb from the pectoral region, complete epineurial transection of the median nerve was performed at a level corresponding to the proximal third of the humerus. The median nerve segment distal to the defect was cut 2 mm distal to its division at the elbow and then inserted and sutured about 1 mm inside the superficial belly of the flexor digitorum muscles, in small clefts created in the perimysium laterally to the point at which the nerve normally enters the muscle (direct muscle neurotization). Animals were then randomly divided into two experimental groups (n = 5 for each group). In group 1 (Figure 2A), the proximal end of the distal median nerve segment was directly sutured by end-to-end neurorrhaphy to the distal end of the proximal median nerve stump by means of two or three 10/0 stitches. In group 2 (Figure 2B), the proximal end of the distal median nerve segment was sutured by end-to-side neurorrhaphy to the neighboring ulnar nerve by means of four 10/0 stitches and after having opened a small window on its epineurium. In order to avoid axonal colonization coming from the proximal stump of the transected median nerve, its distal end was twisted up 180° and sutured to the pectoral muscle.

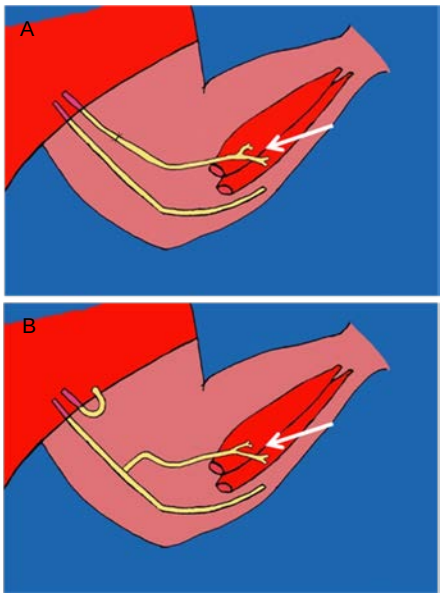

Figure 2 Schematic drawing of the surgical paradigm.

(A) After direct muscle neurotization, the proximal end of the distal median nerve segment was directly sutured by end-to-end neurorrhaphy to the distal end of the proximal median nerve segment (group 1).

(B) After direct muscle neurotization, the proximal end of the distal median nerve segment was sutured by end-to-side neurorrhaphy to the neighboring ulnar nerve (group 2).

The white arrows point to direct muscle neurotization site.

Behavioural assessment

At month-10 after surgery, before euthanasia under deep anesthesia, the recovery of the muscle function was assessed by means of the grasping test[30-31]. In brief, this

behavioural test consists of holding the rat by the tail and lowering the animal towards the device. Then, when the animal grips the grid, it is pulled upward until it loses the grip. The device measures the maximum weight that the animal manages to hold before losing its grip[30].

Muscle mass assessment and histological analysis

The flexor digitorum muscles were then withdrawn together with a 1-cm long segment of the nerve stump used for direct neurotization. Specimens were immediately weighted and then fixed by immediate immersion for 6–8 hours in 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1 M Sorensen phosphate buffer followed by washing in a solution containing 1.5% saccarose in 0.1 M Sorensen phosphate buffer. Then, specimens were post-fixed in 1% osmium tetroxide, dehydrated and embedded in resin[32]. Resin blocks were cut, using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany), and the sections (2.5 μm thick) were collected and stained by Toluidine blue for high resolution light microscopy examination using a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany).

Stereological analysis

Stereology of regenerated nerve fibers was carried out using systematic random sampling and the 2D disector as described in details by Raimondo *et al* [33]. In brief, the first sampling field was randomly selected and then the other sampling fields were identified by systematically jumping to pre-determined distance (calibrated in relation to the nerve size in order to allow for an adequate number of sampled nerve fibers). Afterwards, in each sampling field, a 2D-disector procedure (based on sampling the “tops” of fibers) was adopted in order to avoid the “edge effect”[33]. Mean fiber density was then calculated by dividing the total number of nerve fibers counted in each sampling field by its area, and total fiber number was estimated by multiplying the mean fiber density by the total area of the nerve cross section. Finally, for each selected nerve fiber the fiber and axon diameter, the myelin thickness and the g-ratio (axon-diameter/fiber-diameter) were calculated.

Statistical analysis

One-way analysis of variance was used for the statistical analysis assuming a normal distribution of the values measured. Statistical significance was established as $P < 0.05$. All statistical tests were performed using the software “Statistica per discipline bio-mediche” (McGraw-Hill, Milano, Italy).

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Author contributions: Igor Papalia carried out all microsurgical procedures and behavioural tests. Giulia Ronchi, Luisa Muratori, and Alessandra Mazzucco were responsible for the histological and stereological analyses. Ludovico Magaudda and Stefano Geuna contributed to the study design, the interpretation of the results and preparation of the manuscript.

Conflicts of interest: None declared.

Ethical approval: All animal procedures described in this paper conformed to European Union guidelines for the use of vertebrate animals.

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