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# UNIVERSITÀ DEGLI STUDI DI TORINO

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#### CAN REGENERATED NERVE FIBERS RETURN TO NORMAL SIZE? A LONG-TERM POST-TRAUMATIC STUDY OF THE RAT MEDIAN NERVE CRUSH INJURY MODEL

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Whether post-traumatic regeneration can eventually result in rat peripheral nerve fibers regaining their pretrauma size is still an open ques-tion. While it has been shown that, after a sufficient duration in posttraumatic time, the number of regenerated rat peripheral nerve fibers can return to pretrauma numbers and the animal can regain normal prelesion function, no information regarding long-term changes in the size parameters of the regenerated nerve fibers is available. To fill this gap, we have investigated the post-traumatic changes in myelinated axon and nerve fiber diameter, myelin thickness, and g-ratio (the ratio of the inner axonal diameter to the fiber diameter) at three different time points following nerve injury: week-6, week-8, and week-24. A standardized nerve crush injury of the rat median nerve obtained using a nonserrated clamp was used for this study. The results showed that, consistent with previous studies, fiber number returned to normal values at week-24, but both axon and fiber diameter and myelin thickness were still significantly lower at week-24 than prelesion, and the g-ratio, which remained unchanged during the regeneration process, was significantly reduced at week-24 in comparison to the prelesion value. On the basis of these results, the hypothesis that regenerated rat peripheral nerve fibers are able to return spontaneously to their normal pretrauma state, provided there is a sufficiently long recovery time postaxonotmesis, is not supported

Although peripheral nerves retain a high regeneration potential throughout adulthood,<sup>1,2</sup> it is not known whether regenerated nerve fibers can return to normal levels after a sufficient period of post-trauma recovery time. Mackin-non et al.<sup>3</sup> showed that rat nerve fiber numbers return to normal given a long enough period of time following neurorrhaphy, but as far as we are aware, the possibility that the size parameters of regenerated nerve fibers can return to normal levels has never been investigated. To fill this gap, we have carried out a long-term stereological study on a standardized rat median nerve crush (axonotm-esis) model. Since in a previous study<sup>4</sup> we showed that, in these experimental conditions, the morphological size parameters were still significantly different from controls at week-6 postoperatively, we extended the observation up to 8-week and 24-week time-points. We test the hy-pothesis that the main size parameters of rat peripheral nerve fibers (axon and nerve fiber diameter, myelin thick-ness and g-ratio) are able to return to prelesion values provided that sufficient recovery time postaxonotmesis is allowed.

#### MATERIALS AND METHODS

#### Animals and Surgery

Ten 8-week-old female Wistar rats (Charles River Lab-oratories, Milano, Italy), each weighing 250 g, were used for this study. The animals were housed in large cages in a temperature and humidity controlled room with 12-h light/12-h dark cycles. They were fed with standard food and water ad libitum. Adequate measures were taken to minimize pain and discomfort, taking into account human endpoints for animal suffering and distress. All procedures performed were in accordance with the Local Ethical Committee and the European Communities Council Direc-tive of 24 November 1986 (86/609/EEC).

All surgical procedures were carried out under deep anaesthesia using Tiletamine and Zolazepam (Zoletil) i.m. (3 mg/kg). Each animal was subjected to a median nerve crush injury of the left forelimb by approaching it from the axillary region toward the elbow. The crush lesion was applied at the middle of the arm, using a nonserrated clamp, by compressing the nerve for 30 seconds with a final pressure of 17.02 MPa.<sup>4</sup> Animal wellbeing was assessed by careful surveillance of passive and active movement, auto-mutilation, skin ulcers, and joint contrac-ture, especially during the early postoperative period. Animals were sacrificed by lethal i.m. injection of tilet-amine and zoletil at week-8 (n 5 5) and week-24 (n 5 5) after crush injury. Data were compared to values obtained from a previous study,<sup>4</sup> where 8-weeks-old female rats were subjected to the same nervecrush injury under the same experimental conditions, and six crushed left median nerves and six uninjured control left median nerves were harvested at week-6 postoperatively.

### Resin Embedding and Design-Based Quantitative Morphology

At the time of euthanasia, a 10-mm-long median nerve segment was harvested immediately distal to the injury site in the axonotmesis groups, and at the corre-sponding level in uninjured controls. A 4/0 stitch was used to mark the proximal stump of the nerve segment. Nerve samples were fixed by immediate immersion for 6–8 h in 2.5% purified glutaraldehyde/0.5% saccarose in 0.1M Sorensen phosphate buffer. Specimens were then washed in a solution containing 1.5% saccarose in 0.1M Sorensen phosphate buffer, postfixed in 1% osmium te-troxide,<sup>5</sup> dehydrated and embedded in resin. From each resin block, 2.5 Im thick series of semithin transverse sections were cut, starting from the distal stump of each median nerve specimen, using an Ultracut UCT ultrami-crotome (Leica Microsystems, Wetzlar, Germany). Sec-tions were stained using Toluidine blue for high-resolu-tion light microscopy examination and design-based ster-eology.

Design-based stereological analysis was carried out using one randomly selected toluidine blue-stained semi-thin section. A DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager sys-tem (Leica Microsystems, Wetzlar, Germany) was used for stereology. On the randomly selected section, the total cross-sectional area of the nerve was measured, and 12–16 sampling fields were selected using a systematic random sampling protocol.<sup>6–8</sup> In each sampling field, a two-dimensional disector procedure, which is based on sampling the "tops" of fibers, was adopted to avoid the "edge effect." The total number of myelinated fibers (N), the mean diameter of each fiber (D) and axon (d), as well as mean myelin thickness [(D 2 d)/2] and g-ratio (d/D), were estimated.

#### **Statistical Analysis**

Statistical analysis was performed by one-way analy-sis of variance and tested using the software "Statistica per discipline bio-mediche" (McGraw-Hill, Milano, Ita-Iia). Values are expressed as mean 6 standard deviation (SD). The level of significance was set at P 0.05 (\*), P 0.01 (\*\*), and P 0.001 (\*\*\*).

#### RESULTS

For stereological analysis, the results obtained from median nerves harvested at 8 and 24 weeks postlesion were compared to data obtained in a previous study,<sup>4</sup> which included nerves harvested 6 weeks postoperatively, and uninjured control median nerves, all harvested from 8-week-old female rats.

Figure 1 shows high-resolution light photomicro-graphs of a normal rat median nerve and nerves harvested at 6 weeks, 8 weeks, and 24 weeks postinjury. After 6 weeks from the injury, the regenerated myelinated axons were smaller with a thinner myelin sheath, compared to normal nerves (Fig. 1A), and microfasciculation typical of regenerated nerve fibers was detected; few degenera-tion signs could still be observed among the regenerated fibers (Fig. 1B). After 8 weeks, the regenerated myelin-ated fibers were still smaller compared to control nerves, and almost no more degenerating fibers were detectable (Fig. 1C). The time course of nerve fiber maturation was clearly detectable, progressing to a qualitative morpholog-ical appearance that at week-24 (Fig. 1D) was similar to controls.

Time course morphological changes were confirmed by the results of the stereological assessment of myelin-ated nerve fibers (Fig. 2). The total number of myelinated fibers was significantly (P 0.05) higher at 6 and 8 weeks postinjury, whereas at week-24, it did not signifi-cantly (P 0.05) differ from controls. Regarding axon and fiber diameter, both parameters decreased signifi-cantly (P 0.001) 6 and 8 weeks postlesion. Twenty-four weeks after the injury, the data showed that the fiber size had increased compared to the previous time points analyzed, but it was still significantly (P 0.01) smaller than in the control nerves. The myelin thickness results demonstrated that this parameter follows the same trend as axon and fiber diameter. Interestingly, while the mean g-ratio was not significantly (P 0.05) different from controls at both week-6 and week-8, statistically signifi-cant changes (P 0.05) were detectable at week-24, when the g-ratio was lower than prelesion values.



Figure 1. Photomicrograph of semithin section of normal (A), 6 weeks (B), 8 weeks (C), and 24 weeks (D) postinjury median nerves. Mag-nification bars 5 20 lm.

#### DISCUSSION

Because functional recovery is achieved very quickly after a rat axonotmesis lesion, experimental studies on nerve regeneration after a crush lesion are always based on short-term post-traumatic endpoints: usually 6–8 weeks, and never longer than 12 weeks, as far as we could determine from the relevant literature. However, in spite of the very fast functional recovery, morphological differences persisted even at week-12 post-trauma,<sup>8</sup> leav-ing open the question of whether post-traumatic regenera-tion may eventually lead rat peripheral nerve fibers back to normal.

To answer this question, we prolonged the regenera-tion time in the rat median nerve crush model up to 24 weeks. This is a model in which full functional recovery can already be observed much earlier, namely at week-4 post-trauma,<sup>4</sup> and it allows the following optimal regener-ation conditions: (1) pure axonotmesis lesion with no need for surgical neurorrhaphy; (2) no mismatch between regenerating axons and the respective original distal bands of Bu<sup>¬</sup>ngner; and (3) complete functional recovery reached very quickly (already at week-4 post-trauma)

Contrary to our expectations, even extending the obser-vation period for so long post-trauma under optimal regen-eration conditions, we did not detect complete recovery or the return to normal preinjury size of the regenerated axons. In fact, we showed that only total fiber number returned to normal. These findings on fiber number are in agreement with the observations of Mackinnon et al.<sup>3</sup> in the neurotmesis model, while all the other morphological predictors of regeneration that we investigated (axon diam-eter, fiber diameter, myelin thickness, and g-ratio) had still not returned to normal values at the end of our study. Data about changes in the quotient axon diameter/fiber diameter (g-ratio), a measure of the myelination process,<sup>9</sup> deserve particular mention. The observation that the g-ratio remains unchanged in early regeneration stages supports the view that axons and myelin sheaths grow synchronously. On the other hand, at the latest post-traumatic stages, when regen-eration is stabilized, the g-ratio is significantly reduced.

This observation indicates that myelin sheath enlargement has overcome axon enlargement and supports the view that the limited post-traumatic recovery of peripheral nerves must mainly be attributable to the neuronal component (axons) rather than the glial one.

Our results are important from both the biological and the clinical perspectives. From a basic science point of view, they provide a better understanding of the nerve regeneration potential by adding an original piece of infor-mation, namely that adult peripheral nerve fibers do not retain the capacity for complete regeneration, i.e., a return to prelesion size, even if optimal regeneration conditions and a long-term observation window are guaranteed. It could be argued that, if we had further extended post-traumatic follow-up, thus leaving still more time for the regeneration process, nerve fibers could have returned to normal. However, in view of the long-term stabilization of functional recovery detectable 24 weeks postoperatively in the adopted experimental model, we can reasonably rule out the possibility that the partial recovery of nerve fiber size reflects too little time still being allowed for regeneration to occur.



**Figure 2**. Results of the stereological assessment of peripheral nerve fibres in control rat median nerves (CTRL) and in regenerated rat median nerves, at week 6, 8, and 24 postaxonotmesis. All data reported are presented as means 6 standard deviation. \* P 0.05, \*\*P 0.01, and \*\*\* P 0.001

In the translational clinical perspective, our results tell us that, without treatment, complete post-traumatic recov-ery of nerve morphology in a patient should not reason-ably be expected, even if surgery is perfectly adequate. This evidence is in line with the observation that although axon regeneration in peripheral nerves is very

active,<sup>1,2</sup> electrophysiological parameters do not return to normal levels after nerve lesion and repair,<sup>10,11</sup> and that

clinical recovery in patients is usually partial and often frankly unsatisfactory.<sup>12</sup> It can thus be concluded that the persistence of abnormal electrophysiological and/or clini-cal outcomes after nerve reconstruction does not mean that surgery was inadequate. Our results further support the need to seek effective treatment for improving post-traumatic peripheral nerve fiber regeneration.<sup>13,14</sup>

Our results could influence the selection of regenera-tion predictors in animal nerve repair studies, supporting the view that fiber size data should always be considered to reduce the risk of a discrepancy between the preclini-

cal stage and the following translation into human sub-jects.<sup>15,16</sup> Finally, it is interesting to note that the total number of myelinated nerve fibers at week 6 and week 8 postinjury is higher than the number of fibers in the con-trol group. This observation, which could be interpreted as the result of multiple axonal sprouts during the early regeneration phases followed by a late pruning of the branches that did not reach their proper target, supports the need for sufficiently long post-traumatic observation time-points for monitoring the progression of the morpho-logical predictors of recovery.

In conclusion, the hypothesis that a regenerated nerve is able to return spontaneously to a normal (prelesion)

condition, provided that a long enough recovery period is allowed, can be supported on the basis of fiber number and functional recovery data, but not on the basis of axon diameter, fiber diameter, myelin thickness, and g-ra-tio data. Therefore, in the absence of effective treatment, complete post-traumatic recovery, as far as rat peripheral nerve fiber morphology is concerned, is not an achievable goal.

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