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Abstract

The availability of effective experimental models for investigating nerve regeneration and designing new strategies for promoting this unique repair process is important. The aim of this study was to standard-ize a rat median nerve crush injury model using a non-serrated clamp exerting a compression force of 17.02 MPa for a duration of 30 s. Results showed that functional recovery, evaluated by grasping test, was already detectable at day-12 and progressively increased until day-28 after which animal performance plateaued until the end of testing (day-42), reaching a range of 75–80% of pre-operative values. Morphological analysis on the median nerve segments, distal to the crush lesion, which were withdrawn at the end of the experiment showed that regenerated nerve fibers are significantly more numerous and densely packed; they are also smaller and have a thinner myelin sheath compared to controls. Together, these results provide a baseline characterization of the crush median nerve injury experimental model for its employment in the investigation of nerve regeneration research, especially when a reproducible regeneration process is required, such as for the study of biological mechanisms of peripheral nerve fiber regeneration or development of new therapeutic agents for promoting posttraumatic nerve repair.

1. Introduction

Peripheral nerve regeneration is a challenging scientific field (Lundborg, 2005; Battiston et al., 2005; Battiston et al., 2005; Brunelli, 2005; Chalfoun et al., 2006; Geuna et al., 2006; Pfister et al., 2007) with relevant clinical implications since nerve injuries are much more frequent than spinal cord injuries (Evans, 2001; Ciardelli and Chiono, 2006). The availability of reliable experimen-tal models is very important for peripheral nerve research, as well as in any other neuroscience field. Until recently most peripheral nerve regeneration studies had been mainly carried out using an experimental model which employs the rat sciatic nerve, proba-bly because it is the largest peripheral nerve (Varejão et al., 2004; Nichols et al., 2005; Luis et al., 2007; Baptista et al., 2007, 2008; Kalbermatten et al., 2008). Although the sciatic nerve model has formed the foundation of most behavioral tests for motor function assessment (Varejão et al., 2004; Nichols et al., 2005; Baptista et al., 2008; Bozkurt et al., 2008a,b), recent years have shown an increasing interest towards the employment of major forelimb nerves for experimental microsurgical repair studies (Papalia et al., 2003, 2006; Bontioti et al., 2005; Galtrey and Fawcett, 2007; Geuna et al., 2007; Santos et al., 2007; Sinis et al., 2007, 2008; Wang et al., 2008). In particular, the median nerve attracted the attention of peripheral nerve researchers because of the availability of a behavioural test which appears to be simpler and more reliable than the behavioural tests commonly used for assessing sciatic nerve regeneration, specifically the sciatic functional index (SFI) (Papalia et al., 2003; Lutz et al., 2000; Sinis et al., 2006; Lee et al., 2007; Tos et al., 2007).

So far, the median nerve has mostly been used to investigate complex microsurgical nerve reconstruction strategies, such as end-to-side neurorrhaphy (Lutz et al., 2000; Papalia et al., 2007) and tubulization (Sinis et al., 2006; Tos et al., 2007). Another widely employed experimental approach in peripheral nerve regeneration research is the induction of a crush injury, which interrupts nerve fibers without severing the connective tissue of the nerve trunk (Bridge et al., 1994; Varejão et al., 2004;

Sarikcioglu et al., 2007). In this way, the injured axons are provided with an optimal regeneration pathway, represented by the nerve segment distal to the injury (which undergoes Wallerian degeneration), without the need for the microsurgical repair by epineurial suture. This experimental approach is therefore less technically challenging, a great advantage for all peripheral nerve researchers not trained in microsurgery.

While the rat sciatic nerve crush injury model has been widely adopted in many laboratories, to the best of our knowledge only three studies have employed the crush injury in the rat median nerve model (Bertelli and Mira, 1995; Bontioti et al., 2003; Galtrey and Fawcett, 2007). Since none of these studies used a standardized method for inducing the crush lesion, this study was aimed at investigating the rat median nerve crush injury model using a standardized and reproducible method, in terms of force and pressure exerted as well as duration of the compression. This method has already been successfully used in the rat sciatic nerve model (Varejão et al., 2004; Luis et al., 2007; Luís et al., 2008; Amado et al., 2008).

2. Materials and methods

2.1. Animals and surgery

We used twenty 2-month-old female Wistar rats (Charles River Laboratories, Milano, Italy), weighing approximately 250 g. Animals were housed in large cages in a temperature and humidity con-trolled room with 12-h light/12-h dark cycles. The animals were fed with standard chow 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 Directive of 24 November 1986 (86/609/EEC).

All surgical procedures ($n = 14$) were carried out under deep anaesthesia using Tiletamine + Zolazepam (Zoletil) i.m. (3 mg/kg), after trichotomy. In all animals, the surgery was performed in two steps. Firstly, the median nerve of the right forelimb was approached from the axillary region to the elbow. Secondly, the nerve was transected at the middle third of the brachium and its proximal stump was sutured to the pectoralis major muscle to avoid spontaneous reinnervation. This second surgical step was done in order to prevent interferences during the grasping test (Papalia et al., 2003, 2006). After one-week, animals were again anesthetized to exert the crush lesion on the contra-lateral forelimb. The median nerve of the left forelimb was approached from the axillary region to the elbow and carefully exposed from its origin at brachial plexus until the elbow. The crush lesion was applied using the non-serrated clamp showed in Fig. 1A manufactured by the Institute of Industrial Electronic and Material Sciences, University of Technology, Vienna, Austria (Beer et al., 2001). The clamp is equipped with three dif-ferent springs and two washers which can be used in different combinations in order to exert different forces to the nerve accord-ing to a table provided by the manufacturer. For this study the spring no. 43 with both washers was used so that the force on the nerve was 61.3 N giving a final pressure of 17.02 MPa. This force was applied for a period of 30 s to the middle brachial third of the median nerve (Fig. 1B). The duration of compression was selected on the basis of our previous experience using this device (Varejão et al., 2004; Luis et al., 2007; Luís et al., 2008; Amado et al., 2008). Immediately after the acute compression injury, the crushed areas of all median nerves appeared flattened, however the nerve con-tinuity was intact. In the remaining six animals, 1-cm segment of the median nerve was withdrawn at the middle of the arm to be used as control. Animal well-being assessment was carried out by careful animal surveillance of passive and active movement, auto-mutilation, skin ulcers, and joint contracture, especially during the early post-operative period.

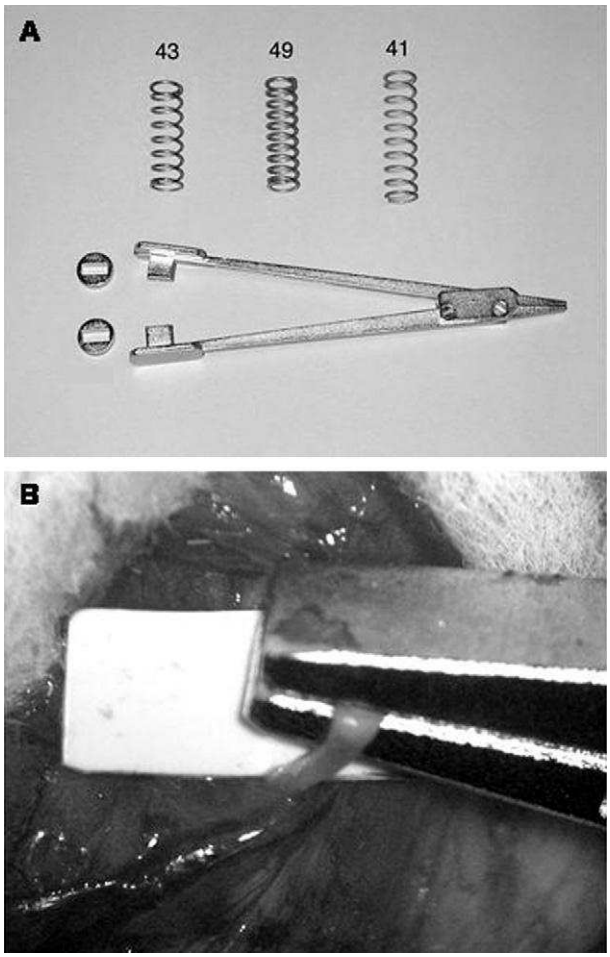

Fig. 1. The device used to produce the crush injury. The final force exerted by the non-serrated clamp can be regulated by changing the number of washers and the three different springs according to a table provided by the manufacturer. For this study the spring no. 43 with both washers has been used exerting a force to the nerve of 61.3 N and a final pressure of 17.02 MPa.

2.2. Grasping test assessment of postoperative functional recovery

In six animals, starting from day-2 postoperative, grasping test sessions were carried out every 5 days until day-42. Grasping test was carried out following the same procedure previously described (Papalia et al., 2003) using the BS-GRIP Grip Meter (2Bio-logical Instruments, Varese, Italy). In brief, the test was carried out by holding the rat by its tail and lowering it towards the device and then, when the animal grips the grid, pulling it upward until it looses its grip (Fig. 2). The balance records the maximum weight that the animal managed to hold before loosing its grip. Each animal was tested three times and the average value was recorded.

2.3. Resin embedding and electron microscopy

Animals were separated into three groups. In one group (n = 4) median nerves were withdrawn at day-5. In another group (n = 4) nerves were withdrawn at day-15 postoperative. Finally, in a third group (n = 6) nerves were withdrawn at day-42 postoperative. Animals were euthanasized and a 10-mm-long segment of the median nerve distal to the injury site was removed. A 4/0 stitch was used to mark the proximal stump of the nerve segment. The 42-day end-point was chosen since functional recovery mea-sured by the grasping test was stabilized by this post-operative

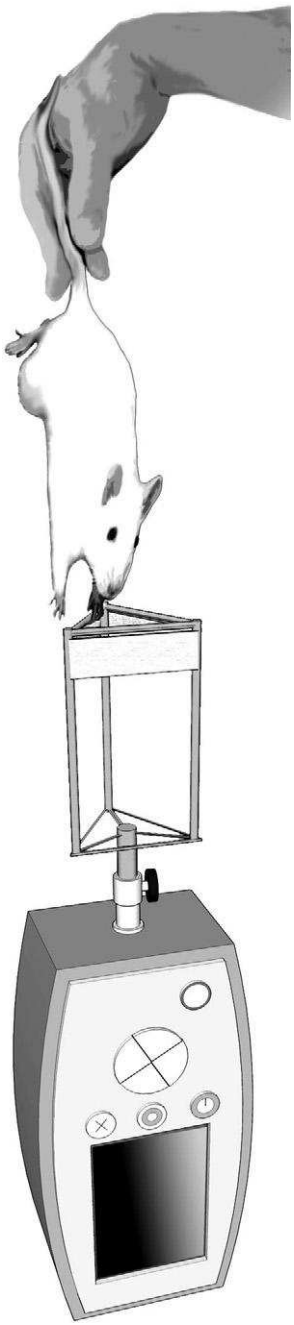

Fig. 2. Schematic drawing illustrating the grasping test. The rat is approached to the device holding it by its tail and, when the animal grips the grid, it is pulled by until it looses the grip. The electronic balance records the maximum weight that the animal manages to hold up before loosing the grip.

time. A 10-mm segment of uninjured left median nerve from a corresponding level was also withdrawn from the 6 control ani-mals.

Nerve samples were then fixed and prepared for design-based stereological analysis of myelinated nerve fibers and for electron microscopy. Nerve samples were fixed by immediate immersion in 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1 M Sorensen phosphate buffer for 6–8 h. Specimen were then washed in a solu-tion containing 1.5% saccarose in 0.1 M Sorensen phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated and embedded in resin. From each nerve, 2- m thick series of semi-thin transverse sections were cut starting from the distal stump of each median nerve specimen, using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained using Toluidine blue for

high resolution light microscopy examination and design-based stereology. For transmission electron microscopy, ultra-thin sec-tions were cut using the same ultramicrotome and stained with saturated aqueous solution of uranyl acetate and lead citrate. Ultra-thin sections were analyzed using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan).

2.4. Design-based quantitative morphology of nerve fiber regeneration

In median nerves from the six 42-day post-crush rats and from the six control rats design-based stereological analysis was carried out using one randomly selected toluidine blue stained semithin section. A DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany) was used for stereology. The final magnifica-tion was 6600× enabling accurate identification and morphometry analysis of myelinated nerve fibers. On the randomly selected sec-tion the total cross-sectional area of the nerve was measured and then 12–16 sampling fields were selected using a systematic ran-dom sampling protocol (Larsen, 1998; Geuna et al., 2000; Piskin et al., 2008). In brief, once the first sampling field was randomly chosen, the other sampling fields were identified by systematically jumping a given distance from the field prior. The inter-field dis-tance is not the same for all nerve profiles, but had to be calibrated based on the nerve size, in order to allow for an adequate num-ber of fields (12–20) and consequently of nerve fibers (300–500) (Geuna et al., 2001; Geuna, 2005). In each sampling field, a two-dimensional disector procedure which is based on sampling the “tops” of fibers was adopted in order to avoid the “edge effect” (Geuna et al., 2000). Mean fiber density was then calculated by dividing the total number of nerve fibers within the sampling field by its area (N/mm2). Total fibers number (N) was finally estimated by multiplying the mean fiber density by the total cross-sectional area of the whole nerve cross section.

Two-dimensional disector probes were also used to select an unbiased representative sample of myelinated nerve fibers. In each fiber, both fiber and axon area were measured and the circle-fitting diameter of fiber (D) and axon (d) were calculated. These data were used to calculate myelin thickness [(D – d)/2], myelin thickness/axon diameter ratio [(D – d)/2d], and axon/fiber diam-eter ratio, the g-ratio (D/d). The precision of the estimates was evaluated by calculating the coefficient of error (CE) as previously described (Schmitz, 1998; Geuna et al., 2000). The sampling scheme was designed in order to keep the CE below 0.10, which assures enough accuracy for neuromorphological studies (Pakkenberg and Gundersen, 1997).

2.5. Statistics

The number of animals used in the experiments (20) was cal-culated taking into account the expected biological variability of 10% (Pakkenberg and Gundersen, 1997) and to meet the Ethi-cal Committee requirements for a minimum number of animals used in agreement with the ‘Three Rs’ (replacement, reduc-tion and refinement of animal studies) concept put forward by Russell and Burch (1992) and adopted by the European Commu-nity.

Statistical analysis was performed using the one-way repeated measures analysis of variance (RM-ANOVA) test on the values from the different time-point assessments. Statistical significance was established as $p < 0.05$. All statistical tests were performed using the software “Statistica per discipline bio-mediche” (McGraw-Hill, Milano, Italia).

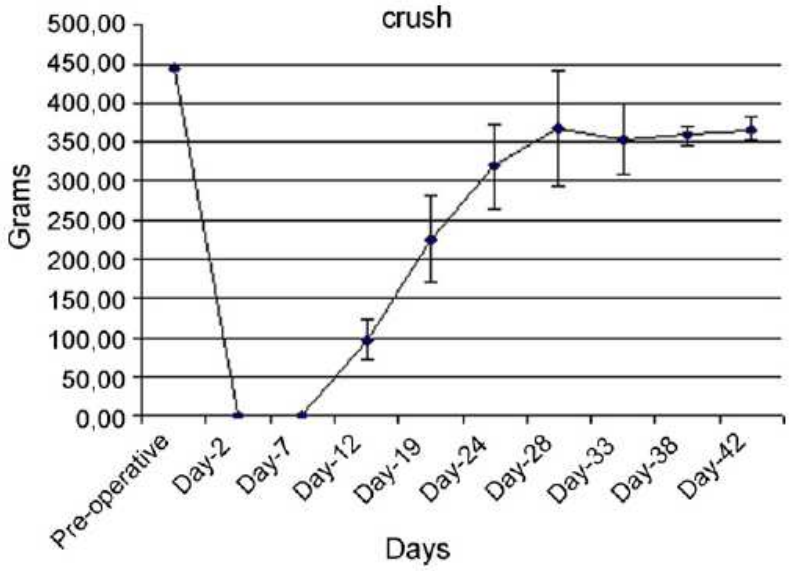

Fig. 3. Line graph reporting the posttraumatic time course of functional recovery as assessed by the grasping test. Values are mean ± standard deviation.

3. Results

All animals survived until the end of the experiments and post-operative animal surveillance showed that animal well-being was maintained over the whole postoperative period. Auto-mutilation, ulcers and joint contractures were not observed in our experiment. In addition, distress caused by grasping test administration was minimal since the test is very quick and does not cause any painful sensation.

3.1. Functional assessment of postoperative functional recovery

Functional results of the grasping test throughout the 42-day postoperative period are reported in Fig. 3. At day-2 and day-7 post-crush, animal performance in the behavioral test dropped to zero confirming a complete nerve fiber transection from the crush lesion. The function of finger flexor muscles innervated by the median nerve already begun to recover at day-12 and pro-gressively increased arriving at 75% of the pre-operative value by day-28 after which animal performance stabilized, ranging between 75% and 80% of pre-operative values, until the end of the experiment (day-42). The RM-ANOVA test disclosed overall statistically significant differences between time-point assess-ments.

3.2. Light and transmission electron microscope analysis

Under light microscopy observation the comparison between normal rat median nerve (Fig. 4A) and regenerated median nerve withdrawn at day-5 (Fig. 4B), and day-42 (Fig. 4C) after crush injury showed that Wallerian degeneration had occurred. At the end-point of this experiment, the presence of smaller myelinated axons with a thinner myelin sheath, compared to normal nerves, and micro-fasciculation typical of regenerated nerve fibers was detected.

Under transmission electron microscopy observation (Fig. 5) the occurrence of Wallerian degeneration at day-5 postoperative (Fig. 5A and B) was confirmed by the presence of clear signs of myelin degeneration and the presence of phagocytes containing myelin debris.

At day-15 (Fig. 5C and D) the presence of small nerve fibers in early stages of myelinization was detectable, suggesting that regeneration had already occurred distal to the crush injury site. At day-42 postoperative (Fig. 5E–H) few degeneration features could still be observed among the many regenerated fibers, both myeli-nated (Fig. 5E and F) and un-myelinated (Fig. 5G and H). The presence of fibers that were still in the very early phase of myelin-ization (Fig. 5G) suggested that at week-6 post-crush injury the process of maturation of regenerated nerve fibers, though very fast in these experimental conditions, is still incomplete.

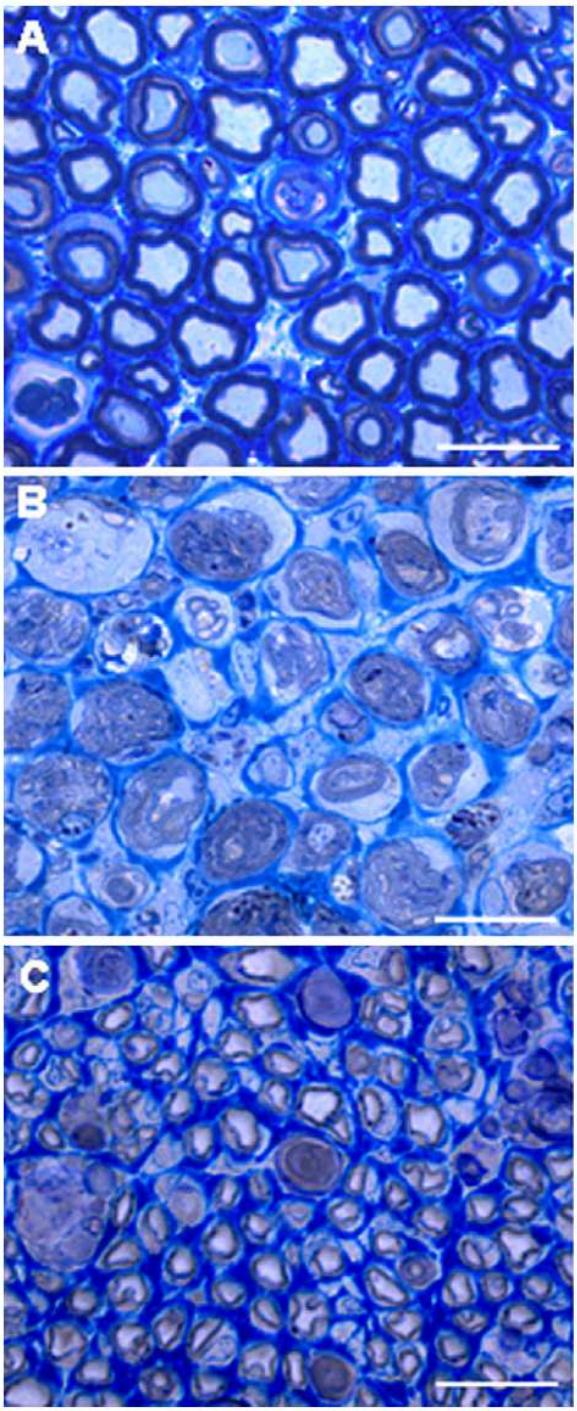

Fig. 4. Photomicrograph of semithin sections cut transversely to the main axis of normal (A), day-5 (B), and day-42 post-operative (C) median nerves. At day-5, many axons with myelin degeneration can already be detected (Wallerian degeneration). At day-42, the presence of many small myelinated nerve fibers organized in minifas-cicles can be detected although some Wallerian degeneration figures are still present. Scale bars = 10 m.

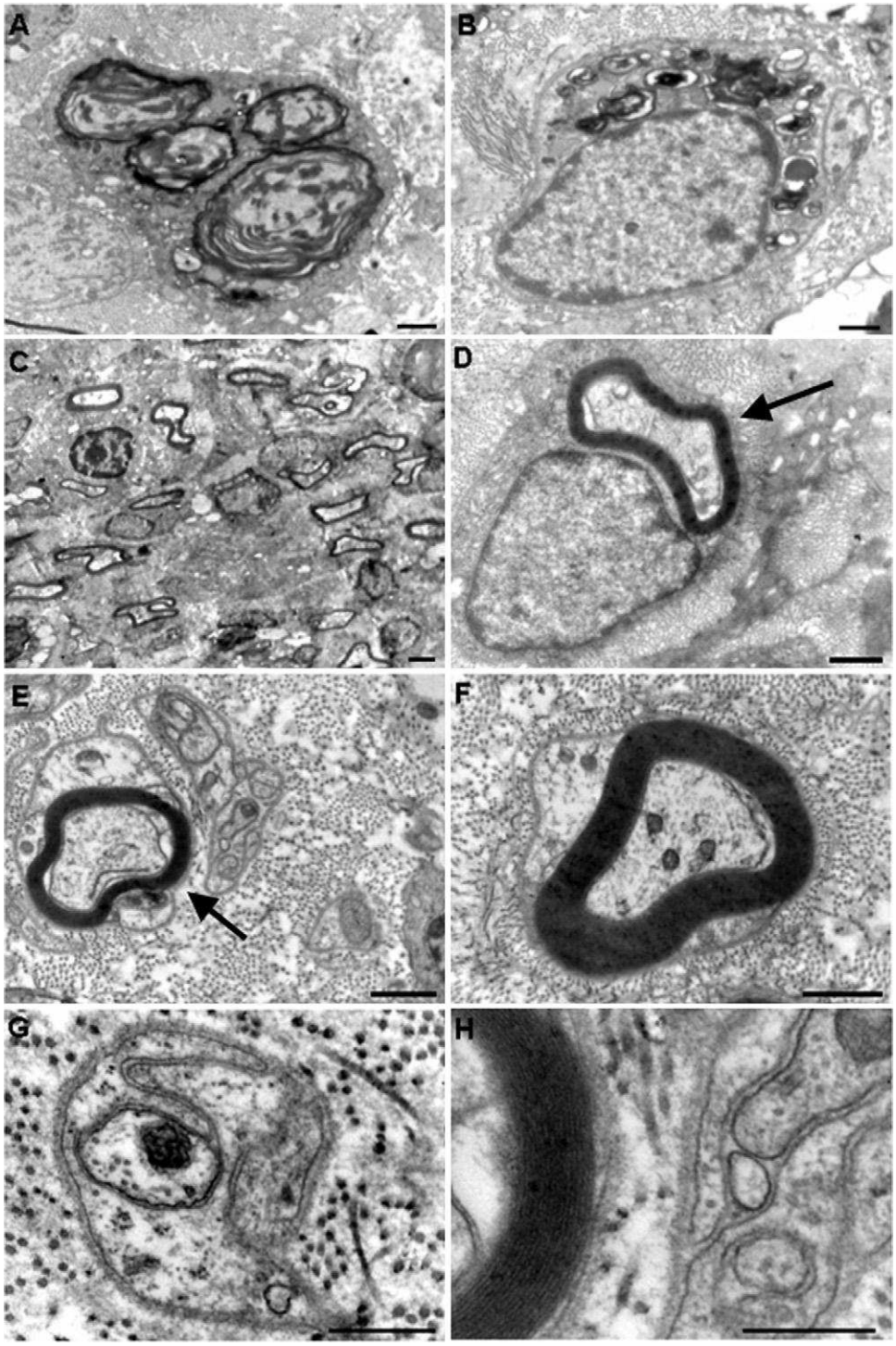

Fig. 5. (A and B) Electron microscope images at day-5 post-operative. Wallerian degeneration is shown by nerve fibers with clear signs of myelin degeneration and phagocytosis. (C and D). Electron microscope images at day-15 post-operative. Small fibers in early stages of myelinization with few myelin layers can be observed (arrows). (E–H) Electron microscope images at day-42 post-operative. Pictures E and F show regenerated myelinated axons. Regenerated un-myelinated fibers are shown in pictures G and H. Scale bars: A, B, D, E, and F = 1 m; C = 2 m; G and H = 0.5 m.

3.3. Design-based quantitative morphology of myelinated nerve fibers

Results of the design-based stereological analysis of normal and regenerated sciatic nerves (Table 1) at day-42 post-crush showed that, unlike functional recovery measured by the grasping test, regenerated

nerves have significantly ($p < 0.05$) higher mean den-sity and total number of myelinated axons as well as significantly ($p < 0.05$) lower mean fiber and axon diameter and myelin thickness. Only g-ratio had already regained a mean value not significantly ($p > 0.05$) different from normal.

4. Discussion

The axonotmetic lesion (crush) of the rat sciatic nerve is a com-monly used experimental model in peripheral nerve research and various methods have been reported in the literature to adminis-ter the crush injury, including using various surgical instruments (Chen et al., 1992; Kingery et al., 1994) and compression devices (Navarro and Kennedy, 1989; Rydevik and Lundborg, 1977; Oliveira et al., 2001; Sarikcioglu et al., 2007) with different crush dura-tions. Unfortunately, there is no standardized method of inducing this nerve injury (Varejão et al., 2004). Recently, a non-serrated clamp which allows the exertion of a final standardized pressure to the nerve has been devised and tested in vivo (Beer et al., 2001; Varejão et al., 2004; Luis et al., 2007). This device allows the stan-dardization of a nerve crush injury method in terms of pressure and duration of the compression. In the present study the rat median nerve axonotmesis model was used, instead of the com-monly used sciatic nerve, in line with a recent trend towards the employment of forelimb as an alternative to hindlimb nerve mod-els of nerve regeneration research. Peripheral nerve regeneration research is usually based on the employment of the sciatic nerve model which still represents a valid experimental approach due to the several behavioral functional tests available (Varejão et al., 2004; Nichols et al., 2005) and specifically the computerized gait analysis (Deumens et al., 2007; Luis et al., 2007; Bozkurt et al., 2008b). Nevertheless the use of the rat median nerve has been recently sustained mainly because animal welfare is more pre-served (Papalia et al., 2003, 2006). Moreover, experimental results are more likely to be translated to the clinical practice (the main goal of most studies on experimental surgery) since the major-ity of surgical interventions for repairing a damaged human nerve are performed at the upper limb level. Additionally, the grasping function requires fine, skilled finger movement and the behavior is quite similar between rodents and humans (Whishaw et al., 1992). The main limitation of this model is the small size of the median nerve which requires advanced microsurgical skills for performing epineurial suturing without causing any epineurial damage. There-fore, it should be emphasized that our present knowledge not allow us to conclude that one of these two models is superior to the other and that researchers must choose the experimental model based on their specific requirements and expertise, knowing each model's limitations and using the results within those limitations, rather than hewing to a more rigid point of view about which model is best.

Table 1
Comparison of stereological parameters of myelinated nerve fibers in normal and regenerated median nerves (42-day post-injury). Values are means ± S.D.

	Normal median nerve	Regenerated median nerve
Total number of myelinated fibers	4535 ± 72	5.184 ± 412[a]
Density of fibers (#/mm^{2})	14923 ± 959	18287 ± 5125[a]
Fibers diameter (m)	6.89 ± 0.41	4.31 ± 0.45[a]
Axons diameter (m)	5,06 ± 0.23	3.20 ± 0.43[a]
Myelin thickness (m)	0.95 ± 0.10	0.55 ± 0.04[a]
g-ratio	0.76 ± 0.03	0.73 ± 0.03

[a] $p < 0.05$.

From a practical viewpoint, the way to hold animals while per-forming the grasping test is particularly important for carrying out the grasping test. In fact, differences in how hard and quick the investigator pulls the rat by its tail after it achieves the grasp can influence the measurements obtained and thus cannot be stan-dardized (Papalia et al., 2003). Besides the general indication to perform the pulling act gently and uniformly, the main requirement for obtaining reliable data is that the test should always be per-formed by the same person who should try to reproduce the same strength and quickness for each test. In addition, if the experimen-tal design allows, blindness of the investigator who lifts animals should be sought. Yet, the induction of a contra-lateral nerve lesion necessary to carry out functional testing deserves mention since centrally mediated responses might modulate contra-lateral func-tional outcomes in behavioral testing and compensatory effects from the initial injury might confound functional results. To cope with the potential influence of the contra-lateral nerve transection on functional assessment, the non-operated paw can covered by an adhesive textile tape during the grasping test, instead of being den-ervated, thus making the additional operation unnecessary (Sinis et al., 2008). This alternative strategy is valid although it has the dis-advantage that paw wrapping could cause repeated distress to the animal. In addition, the animal might focus its attention more on attempting to remove the wrap rather than on grasping the device (Papalia et al., 2006).

Despite the sciatic nerve crush lesion model is widely employed for nerve regeneration research (Kingery et al., 1994; Navarro and Kennedy, 1989; Rydevik and Lundborg, 1977; Oliveira et al., 2001; Varejão et al., 2004; Nichols et al., 2005; Sarikcioglu et al., 2007), only three studies have employed the crush injury in the rat median nerve model, although without using a standardized pres-sure exerted (Bertelli and Mira, 1995; Bontioti et al., 2003; Galtrey and Fawcett, 2007) In our study, after a final standardized pres-sure of 17.02 MPa was exerted on the nerve, a complete functional deficit was found in all animals, subsequently the grasping test score increased up to 75% of normal values at day-28 and then remained stable until the end of the experiments. In contrast to our experiment, others reported a full recovery during the third week post-crush (Bontioti et al., 2003; Galtrey and Fawcett, 2007). In these experiments, fine forceps and microneedle holder were used to induce the axonotmetic lesion in the median nerve. The difference in the rate of functional recovery may thus relate to the different patho-physiological response of peripheral nerves to the magnitude of different pressure and duration of the compression (Lundborg and Dahlin, 1992) and thus the lesions produced in the above-mentioned studies might have been incomplete. In this view, it is interesting to note that, using footprint analysis it was found that injury to the median or ulnar nerve alone had no effect on the toe spread while the combined crush of both median and ulnar nerves produced a decrease in the toe spread (Bontioti et al., 2003; Galtrey and Fawcett, 2007). Yet, it is interesting to note that, despite the preservation of continuity of the epineurium, full restitutio ad integrum (both functional and morphological) is never achieved after complete axonotmetic lesion, in line with previous observa-tions in the rat sciatic nerve model (Varejão et al., 2004; Luis et al., 2007).

The main advantages of the crush lesion in comparison to nerve transection followed by microsurgical reconstruction are that: (1) it is much simpler to be carried out because it does not require the microsurgical skills that are required for epineurial suturing: (2) the inter-individual variability in the regeneration response is much lower; (3) good functional recovery can be expected providing a good model for establishing the ontogeny of motor recovery.

Finally, as for other experimental models, the use of both func-tional and morphological methods of analysis is recommended for a more global assessment of nerve regeneration and functional recov-ery in peripheral nerve regeneration studies.

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