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Abstract

Quantitative estimation of myelinated nerve fiber number, together with fiber size parameters, is one of the most important tools for nerve regeneration research. In this study we used a design-based stereological method to evaluate the regenerative process in two experimental paradigms: crush injury and autograft repair. Samples were embedded in resin and morphometric counting and measurements were performed using both light and electron microscopy.

Results show a significant difference in myelinated fiber number estimation between light and electron microscopy, especially after autograft repair; light microscopy significantly underestimates the number of fibers due to the large number of very small axons that can be detected only in electron microscopy. The analysis of the size parameters also shows a higher number of small fibers in electron microscopy analysis, especially in regenerated nerves.

This comparative study shows that the integration of data obtained in light microscopy with those obtained in electron microscopy is necessary in revealing very small myelinated fibers that cannot be detected otherwise. Moreover, the difference in the estimation of total number of myelinated fibers between light and electron microscopy must be considered in data analysis to ensure accurate interpretation of the results.

Key words: Electron microscopy; Light microscopy, Stereology, Nerve regeneration

Introduction

The evaluation of regeneration of the peripheral nervous system in animal models often includes the estimation of myelinated nerve fiber number and fiber size parameters (axon diameter, fiber diameter, myelin thickness and g-ratio), which are important morphological indications of the functional success of the regenerative process (*Geuna, et al., 2001*).

Most of the morphometric and stereological analysis in the peripheral regenerative field are performed using light microscopy (*Acar, et al., 2008; Ayranci, et al., 2013; Jeronimo, et al., 2008; Raimondo, et al., 2009; Ronchi, et al., 2013*), because it is less expensive and less time consuming compared to electron microscopy.

On the other hand, electron microscopy is usually employed to study the ultrastructural changes occurring after nerve degeneration and regeneration from a qualitative point of view (*Papalia, et al., 2013; Piskin, et al., 2009; Ronchi, et al., 2009; Varejao, et al., 2004*) and only few studies have used this method to perform quantitative analysis (*Biscoe and Lewkowicz, 1982; Einheber, et al., 2012; Soltanpour, et al., 2012; Taveggia, et al., 2008*).

However the question is: can all myelinated nerve fibers be recognized at light microscopy? A few studies comparing light and electron microscopy in the quantitative investigation of the peripheral nerves have been performed in the 1970s. In particular, Bronson and co-workers (*Bronson, et al., 1978*) analyzed 500 axon circumferences of the third cranial nerve of a rat showing that the number of small axons was significantly underestimated by the light microscope compared with the electron microscope. The same results were obtained by Eldred and Moran (*Eldred and Moran, 1974*), who investigated one of the two major femoral nerve trunks of the cockroach *Blaberus discoidalis* showing an underestimation of the total number of axons in light microscopy, due to a large number of very small axons (60% of the total axons measured with electron microscopy were 0.2 μm or less in diameter).

As far as our knowledge is concerned, no study so far has compared light and electron microscope nerve fiber estimations in regenerated nerves, a condition where fiber number and size varies significantly in comparison to controls (*Geuna, et al., 2009; Raimondo, et al., 2009*).

We therefore asked the question whether discrepancies between light and electron microscopy might be even more relevant in regenerated nerve fibers quantitative evaluation. To give an answer, we estimated the total number of myelinated nerve fibers and their size following two types of nerve injury and regeneration paradigms: crush injury and autograft repair. In order to obtain unbiased estimation, a design-based stereological approach was adopted (*Kaplan, et al., 2010*).

Materials and Methods

Surgical procedure

Fifteen female Wistar rats (Charles River Laboratories, Milano, Italy), each weighing approximately 200 g, were divided into three experimental groups: crush injury (n=5), autograft repair (n=5), and control group (n=5). Animals were housed in plastic cages with free access to food and water. Their room was maintained at constant temperature and humidity under 12 hours light/ 12 hours dark cycle. Adequate measures were taken to minimize pain and discomfort; all procedures performed were in accordance with the Local Ethical Committee and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Rats were put under deep anesthesia using Tiletamine and Zolazepam (Zoletil) i.m. (3 mg/kg). After shaving the surgery area, an incision was made in the skin to expose the left median nerve. In the crush group, the crush lesion was applied at the middle of the arm, using a non-serrated clamp, by compressing the nerve for 30 seconds (*Ronchi, et al., 2009*). In the autograft group, 1 cm segment of the nerve was resected, rotated 180° and then sutured at the proximal and distal nerve stumps. Finally, the skin was sutured and the animals were allowed to recover.

Animals were sacrificed by lethal i.m injection of tiletamine and zoletil at week-12 after injury. The left median nerves from 5 female healthy animals were collected and used as controls. A 6/0 stitch was used to mark the proximal stump of the nerve segment.

Resin embedding

Nerve specimens were fixed by immediate immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 to 6 hours in 4° C. Samples were then postfixed in 2% osmium tetroxide for 2 hours and carefully dehydrated in passages in ethanol from 30% to 100% . After two passages of 7 min each in propylene oxide and overnight in a 1:1 mixture of propylene oxide and Glauerts' mixture of resins, specimens were embedded in Glauerts' mixture of resins, which was

made of equal parts of Araldite M and the Araldite Harter, HY 964. In the resin mixture, 0.5% of the plasticizer dibutylphthalate was added. For the final step, 2% of accelerator 964 was added to the resin in order to promote the polymerization of the embedding mixture.

Light microscopy

Cutting and staining

From each nerve, 2.5μm 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 with 1% Toluidine blue for high resolution light microscopy examination and design-based stereology.

Design-based quantitative morphology

A DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany) was used for stereology.

On one randomly selected toluidine blue stained semi-thin section, the total cross-sectional area of the whole nerve was measured at the light microscopic level and 12–16 sampling fields were selected using a systematic random sampling protocol (*Geuna, 2000; Geuna, et al., 2000; Larsen, 1998*). 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/mm^2). Total fiber number (N) was finally estimated by multiplying the mean fiber density by the total cross-sectional area of the nerve. Moreover, both fiber and axon area were measured and the 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 diameter ratio, the g-ratio (D/d).

Electron microscopy

Cutting and staining

Ultra-thin sections (70nm thick) were cut from the same samples used to obtain the semi-thin sections using the same ultramicrotome and stained with saturated aqueous solution of uranyl acetate and lead citrate.

Design-based quantitative morphology

Ultra-thin sections were analyzed using a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a Mega-View-III digital camera and a Soft-Imaging-System (SIS, Münster, Germany) for the computerized acquisition of the images.

On one randomly selected ultra-thin section, 10-15 fields were selected using a systematic random sampling protocol, with a magnification of 2500X. The number and the size of myelinated fibers were quantified using the same protocol used for the quantification in light microscopy.

Axon recognition at the electron microscopic level

To verify axon recognition, pairs of semi-thin and ultra-thin sections spaced close enough to allow exact ultrastructural identification of all structure profiles detectable (or not) in light microscopy were cut. Briefly, prior to ultra-thin cutting, one final semi-thin "reference" section was cut and then immediately followed by one ultra-thin section. In this way, all identifiable structures in the semi-thin sections could be observed and matched at electron microscopic levels.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and tested using the software "SPSS".

Results

We first analyzed semi-thin Toluidine blue-stained sections from control nerves (fig. 1A), crushed nerves (fig. 1B) and autograft repaired nerves (fig. 1C) three months after the injury in light microscopy using design-based quantitative morphology. Figure 1D shows the quantification of the total number of myelinated fibers: as expected, no significant differences were seen between control and crush groups, whereas significant differences ($p \leq 0{,}001$) were seen both between control and autograft groups and between crush and autograft groups. We then used the same design-based quantitative morphology protocol to quantify the total number of myelinated fibers on ultra-thin sections using electron microscopy (fig. 2). Intriguingly, in addition to the differences already seen in light microscopy analysis, results showed a significant difference ($p \leq 0{,}05$) also between control and crush groups (fig. 2D), not detected in light microscopy.

Figure 3 shows the comparison between the analysis obtained in light and electron microscopy. Data show that there is a significant difference in the estimated number of myelinated fibers between light and electron microscopy in the autograft group. This difference is solely due to the different resolutions in light and electron microscopy since the same nerves are counted with both methods. Light microscopy, with its lower resolution, underestimates the number of fibers in the autograft repaired group because it contains a higher number of very small fibers which are only detectable with electron microscopy. Also in the control and crush group the total number of myelinated fibers are underestimated using light microscopy, but in these groups the differences with electron microscopy is not significant ($p > 0{,}05$). The difference in resolution is illustrated in Figure 3A-A', 3B-B', 3C-C' where two sections, one for light and one for electron microscopy, are cut adjacent to each other making it possible to observe that some small myelinated fibers are not detectable in light microscopy but only in electron microscopy, especially in the autograft group. We then calculated the average percentage increase in number of myelinated fibers when the nerves are counted with electron microscopy instead of light microscopy (fig. 4). In the control group,

myelinated fiber number quantified in electron microscopy was increased by 13,1 percent (SD ± 6,8) when compared with light microscopy analysis; in the crush group the difference between light and electron microscopy was of 22,7 percent (SD ± 4,2) and, finally, in the autograft group the difference was even more 33,0 percent (SD ± 10,4).

After fiber quantification, we evaluated the size parameters (axon diameter, fiber diameter and myelin thickness) in both light and electron microscopy. Results are summarized in Figure 5: as expected, after regeneration (both in the crush and in the autograft group), axon and fiber diameters were smaller compared to control, both with light and electron microscopy analysis. Regarding the myelin thickness, light microscopy analysis showed a significant difference only between control group and the other two groups (where both crush and autograft groups showed thinner myelin thickness). Interestingly, when the analysis was performed using the electron microscopy, a significant differences was detectable also between crush and autograft groups (difference not observed in light microscopy).

Figure 6 represents the frequency distribution histograms of fiber diameter as measured in the control (fig. 6A), crush (fig. 6B) and autograft (fig. 6C) groups. For each experimental group, the frequency distribution histograms obtained in both light (black histograms) and electron (red histograms) microscopy are represented. In the control groups, the bimodal distribution can be seen both in light and electron microscopy analysis, with some differences: in light microscopy the two main peaks are at 5-6µm and 10-11µm, whereas in electron microscopy the peaks are shifted to the left (towards smaller diameter), at 2-3µm and 5-6µm. As expected, in the crush group, histograms are shifted to smaller nerve diameters. Inside this experimental group, red histograms (electron microscopy) are more shifted to the left. Finally, in the autograft group, histograms are even more shifted to the left, especially those obtained with electron microscopy analysis, showing that more smaller fibers can be detected and measured with electron microscopy compared to light microscopy. When we focused on fibers with a diameter smaller than 2 µm (fig. 7), we observed that in the control group only 0,2 percent of the total number of fibers showed a diameter smaller

than 2 µm if analyzed in light microscopy. The percentage increased up to 3,4% when the measurement is done with electron microscopy. In the crush group the percentages of fibers with a diameter smaller than 2 µm are 4% (in light microscopy) and 14 % (in electron microscopy). Finally, in the autograft, the two percentages are 20,4 % and 38,8 % in light and electron microscopy, respectively.

We finally analyzed the g-ratio (axon diameter/fiber diameter, a measure of relative myelin thickness) in each group. As expected, plotting g-ratios against axonal diameters (fig. 8) showed an increase in the slope of the linear regression line in the regenerated nerves compared to the control (the slope is more prominent in the autograft group). Moreover, inside each group, the scatterplot obtained with light and electron microscopy analysis were different, due to the increasing number of smaller axons evaluated in electron microscopy.

Discussion

After injury to the peripheral nerves, the regenerative and repair processes occur almost immediately and spontaneously. One of the most important neuropathological predictors of nerve regeneration is the total number of myelinated axons (*Kaplan, et al., 2010; Raimondo, et al., 2009; Vleggeert-Lankamp, 2007*). Moreover, fiber size parameters (especially, fiber and axon diameter and myelin thickness) are key tools for nerve regeneration assessment since they have proven to be correlated to conduction velocity (*Ikeda and Oka, 2012; Raimondo, et al., 2009*).

After the introduction of the stereological principles in biomedical research (*Geuna, 2005; Sterio, et al., 1994*), several advancements have been made regarding the procedures for the unbiased estimation of quantitative parameters in histological sections. Unbiased stereology has also been applied successfully to the quantification of peripheral nerve fibers (*Geuna, et al., 2001; Kaplan, et al., 2010*). Whereas stereology allows to estimate unbiasedly the number and size of nerve fibers, a major problem is their identification with light microscopy. In other words: are we able to recognize, for counting and measuring , all myelinated nerve fibers even when they are analyzed in high resolution pictures taken from semi-thin sections (*Kaplan, et al., 2010*)? And can recognition be even more problematic for regenerated nerve fibers?

In order to give an answer to these questions, in this study we compared myelinated axon number and size estimation obtained at light microscopic level with those obtained at the electron microscopic level (where all nerve fibers can be unequivocally recognized). The comparison was carried out not only in normal nerves but also in regenerated nerves in two injury models (crush injury and autograft repair). The two injury models differ significantly in relation to the regenerative process of nerve fibers. In fact, crush injury involves loss of the continuity of the nerve fibers only while connective tissue of the nerve is preserved (*Bridge, et al., 1994; Sarikcioglu, et al., 2007; Varejao, et al., 2004*); regeneration is thus particularly fast and effective. On the other hand, after autograft nerve repair, the “gold standard” technique for repairing nerve defects

(*Battiston, et al., 2009*), the whole nerve continuity is lost and thus regeneration is slower and mismatched when compared to crush injury (*Geuna, et al., 2009*).

Our results revealed that light microscopy estimations significantly underestimate the total number of myelinated fibers compared to the evaluation made with electron microscopy. The discrepancy is particularly relevant in the autograft group since axon regeneration after this severe injury takes longer compared to crush injury and therefore contains a higher number of very small fibers which are only detectable with electron microscopy.

Noteworthy, on the set of data obtained with electron microscopy, it is possible to reveal statistically significant differences between normal and regenerated nerves that are not detectable in light microscopy (for example significant difference in the total number of myelinated fibers between control and crush groups).

When the size parameters (axon diameter, fiber diameter, myelin thickness) are measured, it can be observed that the higher number of fibers counted in electron microscopy are due to the high number of very small axons whose diameter lies beyond the limit of resolution of the light microscope. Indeed, by comparing axon diameter distributions determined with the two methods, it was shown that small fibers are significantly underrepresented by the light microscopic technique. Since the percentage of very small axons increases after regeneration (crush and autograft groups), discrepancy between light and electron microscopy are more relevant in these conditions.

Whereas this study is the first light-electron microscope comparison carried out in regenerated nerve fibers, a couple of previous studies compared light and electron microscopy in the investigation of normal peripheral nerves (*Bronson, et al., 1978; Eldred and Moran, 1974*) and both showed, in agreement with our results, that the number of small axons was significantly underestimated by the light microscope compared with the electron microscope. In particular, Bronson and co-workers (1978) analyzed 500 axon circumferences of the third cranial nerve of a rat showing that the number of small axons was significantly underestimated by the light microscope.

The same result was obtained by Eldred and Moran (1974), who described the morphology of one of the two major femoral nerve trunks of the cockroach *Blaberus discoidalis*, showing a 65% underestimation of the total number of axons in light microscopy due to a large number of very small axons. However, those studies did not employ stereology for quantification and thus results could be biased (*Kaplan, et al., 2010*). Results of the present stereological study are thus an important confirmation of the previous findings as regards un-lesioned peripheral nerves. Interestingly, in the present study the underestimation of axon number in control nerves was only 13%. Although this difference can be related to the different animal species (rat vs cockroach), the results of our study also suggest that, as expected, the use of non-stereological counting methods leads to biased results, in terms of an overestimation of the light/electron microscope discrepancies in axon counts.

Taken together, our results raise a further question: can we still rely on nerve fiber quantitative assessment obtained at light microscopic level? The answer, in our opinion, is yes, provided that researchers are aware that failure in identifying some of the fibers smaller than 2um might underestimate fiber number and also influence size parameters (namely limited small fiber recognition might lead to an overestimation of the mean size). This information should also be taken into consideration when interpreting and discussing results of a histomorphometric study of myelinated nerve fibers carried out with light microcopy analysis only.

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Figure legends

Figure 1: Toluidine blue-stained light micrographs of transverse semi-thin sections of control (A), crush (B) and autograft (C) nerves three months after nerve injury. Bar = 10 µm. D shows the total number of myelinated fibers estimated applying a design-based stereological method in light microscopy. Values are expressed as mean ± standard deviation. ***= $p<0.001$.

Figure 2: Transmission electron micrographs of transverse ultra-thin sections of control (A), crush (B) and autograft (C) nerves three months after nerve injury. Bar = 2 µm. D shows the total number of myelinated fibers estimated applying a design-based stereological method in electron microscopy. Values are expressed as mean ± standard deviation. *= $p<0.05$; ***= $p<0.001$

Figure 3: The histogram shows a graphical representation of the comparison between the total number of myelinated fibers obtained in light (black histograms) and electron (red histograms) microscopy. Values are expressed as mean ± standard deviation. **= $p<0.01$. A and A', B and B', C and C' illustrate pictures of the control nerve (A-A'), crushed nerve (B-B') and autograft repaired nerve (C-C') used for the stereological analysis obtained with the light microscope and the electron microscope, respectively. The pictures show that some myelinated fibers (pointed out with red arrows) can be seen in the electron microscope but not in the light microscope. Bar A,B,C = 10 µm; bar A',B',C' = 2 µm.

Figure 4: Histograms show the average percentage increase in number of myelinated fibers in each group when counted with electron microscopy compared to light microscopy. Values are expressed as mean ± standard deviation. *= $p<0.05$; **= $p<0.01$.

Figure 5: Histograms represent the size parameters (axon diameter, fiber diameter and myelin thickness) in the three experimental groups (ctrl, crush and autograft) obtained in light (A) and electron (B) microscopy. Values are expressed as mean ± standard deviation. **= $p<0.01$; $=p<0,001 compared to the other two experimental groups.

Figure 6: Diameter-frequency histograms of myelinated fibers in the control (A), crush (B) and autograft (C) experimental groups evaluated in light (black bars) and electron (red bars) microscopy. Note the marked shift to the left in the spectrum of fiber diameters in the regenerated nerves, especially when analyzed with electron microscopy.

Figure 7: Histograms show the percentage of fibers with a diameter ≤ 2 μm. Black bars represent data obtained in light microscopy; red bars represent data obtained in electron microscopy.

Figure 8: Scatter plot of individual g-ratios as a function of the respective axon size in control (A), crush (B) and autograft (C) group. The lines represent linear fits to pooled data from all rats for each experimental group.