

## EFFECTS OF AGGREGAN ON SCHWANN CELL MIGRATION *in vitro* AND NERVE REGENERATION *in vivo*

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### ABSTRACT

Although the role of many small proteoglycans in regeneration of the nervous system has been established, little is known about the involvement of large proteoglycans. In this study, we evaluated the effects of aggrecan, a high molecular mass proteoglycan, on Schwann cells *in vitro* and investigated its effects on axonal regeneration after sciatic nerve tubulization. The number of regenerated axons and their morphometrical parameters were determined *in vivo*. Aggrecan increased the number and viability of Schwann cells *in vitro*. Similarly, the number of regenerated fibers increased significantly when aggrecan was applied *in vivo*, but there were no alterations in the morphometrical parameters. These results indicate that aggrecan contributes to the regeneration of peripheral axons and has a positive effect on the Schwann cells.

**Key words:** Cell culture, extracellular matrix, glycosaminoglycans, proteoglycans, tubulization

### INTRODUCTION

Peripheral regeneration is a complex phenomenon that involves different cell types in extensive remodeling. Schwann cells are key players in this process and produce several of the molecules responsible for oriented axonal regrowth [4,5]. Various molecules contribute to regeneration, including collagen, laminin, fibronectin, nidogen and tenascin, which have been identified *in situ* or applied in various ways to peripheral lesions [1,16,18,19, 23,24].

Proteoglycans, which are proteins with one or more covalently attached glycosaminoglycan chains, exert positive and negative effects on axonal regeneration, depending on their structure, concentration and location in the nervous system [6,7,9,13,20]. Most of the knowledge about proteoglycans is restricted to those with a low molecular mass. Little is known about the properties of high molecular mass proteoglycans, such as aggrecan, in the regeneration of peripheral nerves.

Cell cultures and models *in vivo* can be helpful for understanding the role of aggrecan in nerve regeneration. Assays *in vitro* have the advantage of using isolated Schwann cell and may provide a more accurate understanding of cell multiplication, viability and migration. Experiments *in vivo* can be used to assess the effects of aggrecan on axonal sprouting and on the interaction of Schwann cells with newly formed axons. Nerve tubulization was used for studies *in vivo* and has the advantage that the tube itself acts as a micro chamber that isolates the lesion from the surroundings [11,14]. Additionally, the tube provides mechanical orientation for the regenerating fibers from the proximal stump to reach the distal end of the nerve and can be filled with different substances [15].

In this study, we examined the effects of aggrecan on nerve regeneration by using primary cultures of Schwann cells obtained from explants of adult rat sciatic nerves and by surgically sectioning the sciatic nerves of mice and bridging the stumps with polyethylene tubes prefilled with purified aggrecan.

### MATERIAL AND METHODS

#### *Preparation of aggrecan*

Chicken xyphoid cartilage was minced and homogenized in PBS (0.15 M NaCl in 50 mM phosphate buffer, pH 7.4, containing 50 mM EDTA and 1 mM phenylmethylsulfonyl fluoride - PMSF). The homogenate was then centrifuged at 2,000

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This work is part of a Masters Dissertation by A.P.

x g in a Beckman JA-20 rotor and the pellet was extracted with 15 volumes of 4 M guanidinium chloride (GuHCl) in 0.05 M acetate buffer, pH 5.8, containing 50 mM EDTA and 1 mM PMSF. The extraction was done at 4°C for 20 h, with constant stirring. The extract was subsequently centrifuged at 25,000 x g in a Beckman JA-20 rotor, and the large proteoglycans present in the supernatant were separated using a CsCl gradient density, starting with a density of 1.35 mg/ml, in the presence of 4 M GuHCl, as described by Heinegård and Sommarin [12]. Ultracentrifugation was done in a Beckman 80 Ti rotor, at 82,000 x g for 61 h, at 15°C. The gradient was divided into four equal fractions, D1, D2, D3 and D4 (in ascending order). Only fraction D1 (rich in aggrecan) was used. Agarose-polyacrylamide gel electrophoresis was used to visualize the extracted aggrecan. The protein concentration was determined by the Bradford method [2], and sulfated glycosaminoglycans were determined as described by Farndale *et al.* [10].

#### *In vitro study*

##### *Sciatic nerve explants*

Sciatic nerves from adult (4 months old, n=10) male Wistar rats were used and were predegenerated in order to increase the number of Schwann cells migrating from the explant. For this, the sciatic nerves were exposed and transected at mid-thigh level one week before the culturing procedures (modified from [21]). After predegeneration, the nerves were removed, cut into fragments about 1 cm long, and washed in Ham F-10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 20% fetal calf serum (FCS, Nutricell - Nutrientes Celulares Ltda., Campinas, SP, Brazil) and 100 µg of gentamicin/ml (Schering-Pough S.A., Rio de Janeiro, RJ, Brazil). The fragments were then sectioned into smaller pieces 2 mm long and cultured in culture plates with 24 wells (Corning/Costar Corporation, Cambridge, MA, USA) at 37°C with 5% CO<sub>2</sub> for 20 days. Four experimental conditions were used: 1) Ham F-10 medium supplemented with 100 µg of gentamicin/ml and 20% FCS, 2) Ham F-10 medium supplemented with 100 µg of gentamicin/ml and 20% FCS plus 510 µg of aggrecan/ml, 3) Ham F-10 medium supplemented with 100 µg of gentamicin/ml and 40% FCS, and 4) Ham F-10 medium supplemented with 100 µg of gentamicin/ml and 40% FCS plus 510 µg of aggrecan/ml. New medium (all groups) plus 510 µg of aggrecan/ml (groups 2 and 4) were added every second day. All of the experiments were done in triplicate.

##### *Cell counting*

During the period of culturing, the total number of cells migrating from explants was evaluated on days 7, 9 and 11 for all experimental conditions. Cell counting was done using an Olympus IX-50 inverted microscope with a phase contrast system. The number of non-adherent cells in the different samples was also evaluated, when detachment was observed. At this point, the culture medium in the wells was collected and the non-adherent cells were counted with a hemocytometer.

##### *Immunocytochemistry*

After 20 days in culture, the cells were fixed in 10% formalin for 1 h (Reagen Quimibras Ind. Química, Rio de Janeiro, RJ, Brazil), and washed in 0.1 M phosphate-buffered saline (PBS), pH 7.2, at 37°C. To block nonspecific staining, the specimens

were incubated for 1 h with 1% bovine serum albumin (BSA; by Sigma) in PBS. The preparations were then incubated overnight with a monoclonal anti-S-100 antibody (dilution 1:300) in a moist chamber at 4°C. The samples were rinsed in PBS at 37°C, and incubated with an anti-rabbit CY-3 labeled secondary antibody for 1 h. The cells were then observed with an inverted microscope (Olympus IX-50) equipped for fluorescence analysis. Control experiments were done by omitting the primary antibody.

#### *In vivo study*

##### *Animals*

Twenty 8-10-week-old male C57BL/6J mice were divided into two groups. In the first group (n=10), purified aggrecan gel was applied after sciatic nerve tubulization while in the second group (n=10), the tubes were left empty and served as the control. All procedures were done in accordance with the guidelines of the institutional ethics committee (CEEA-IB/Unicamp).

##### *Surgical procedures*

Under deep anesthesia (pentobarbital, 50 mg/kg, i.p.), the left sciatic nerve was exposed and transected at the mid-thigh level. The proximal stump was introduced into a 6-mm long polyethylene tube and sutured with an epineural stitch (9-0 nylon suture) to the end of the tube. In the first group, the tube was filled with 2 µl of purified avian aggrecan gel (8.5 µg), and in group 2, the tube was left empty. The distal stump was then sutured to the distal end of the tube, leaving a 4 mm gap. The musculature and the skin were closed with 6-0 silk sutures and the mice were maintained for five weeks with food and water *ad libitum*.

##### *Specimen preparation and morphometrical analysis*

Five weeks after tubulization, the mice were perfused transcardially with Karnovsky solution (2% glutaraldehyde, 1% paraformaldehyde) (n=4 for each group) or with 10% formalin (n=3 for each group). The regenerated and the contralateral nerves were removed and, for Karnovsky fixed mice, the specimens were post-fixed with 2% osmium tetroxide and processed for Araldite embedding. Transverse semi-thin sections (0.5 µm thick) obtained at the midpoint of the polyethylene tube were stained with toluidine blue and the total number of regenerated axons was counted. For each specimen four fields were photographed with a light microscope (100X), corresponding to an area at least 30% of the nerve cross-section. Sampling bias was avoided by spreading the micrographs systematically over the entire cross-section, according to the scheme proposed by Mayhew and Sharma [17]. Using Image Tool software (Version 2.00, The University of Texas Health Center in Santo Antonio, USA), the diameter of the myelinated fibers (FD) and of the regenerated axons (AD), and the myelin thickness (MT), were calculated for the regenerated fibers and for contralateral nerve, as appropriate. The "g" ratio was also calculated using the formula "g" = AD/FD. The axon diameter (D) was calculated from the perimeter (P) by applying the formula  $D = P/\pi$ . The data are shown as the mean ± SD. One-way ANOVA and the Newman-Keuls test ( $p < 0.05$ ) were used for statistical analysis. Ultra-thin sections were also cut for ultrastructural analysis. The formalin-fixed specimens were frozen in tissue-tek and used for immunocytochemical labeling.

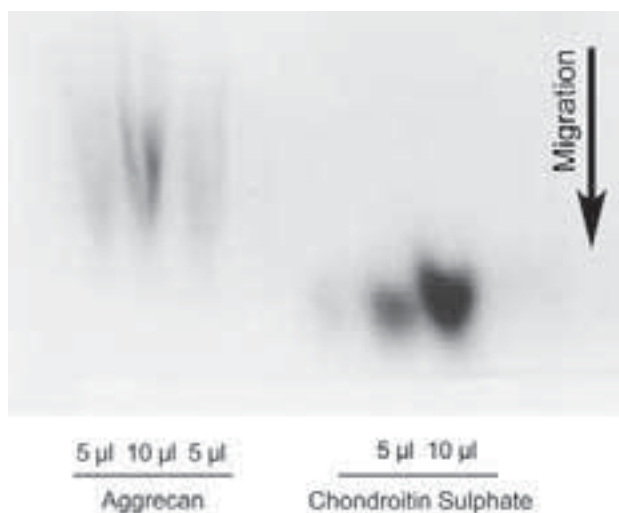
### Immunocytochemistry

Longitudinal nerve sections were obtained in a cryostat and the endogenous peroxidase was inactivated with 3% H<sub>2</sub>O<sub>2</sub> in distilled water for 5 min at room temperature. The slides were transferred to a humidified chamber and incubated with S-100 antibody (Dako, Carpinteria, CA) overnight at 10°C. After several rinses with PBS, the sections were incubated for 1 h with Envision peroxidase labeled polymer (Dako, K1491). Peroxidase was detected using a solution of 3,3',5,5'-diaminobenzidine solution, and the slides were then washed in distilled water, counterstained with hematoxylin and mounted in Entelan (Merck). The sections were examined with an Olympus BX60 microscope.

## RESULTS

### Characterization of the extracted aggrecan

Figure 1 shows an agarose-polyacrylamide gel counterstained with toluidine blue. Aggrecan stained as a polydisperse band, because of the marked variation in the length of the glycosaminoglycan chains. The protein concentration of the D1 aggrecan-enriched fraction was 0.116 mg/ml and the concentration of glycosaminoglycans was 8.5 mg/ml.



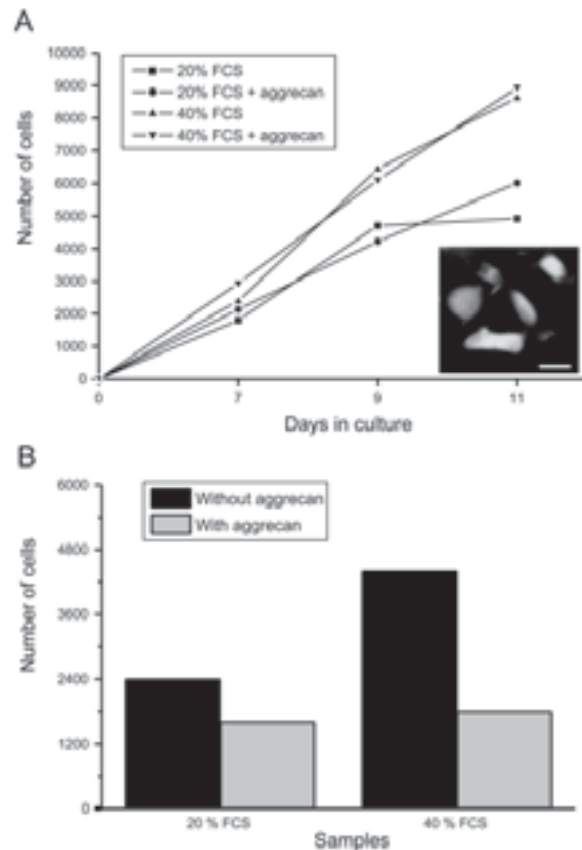
**Figure 1.** Agarose-polyacrylamide gel stained with toluidine blue. Aggrecan stained as a polydisperse band. Chondroitin sulfate was used as a molecular mass marker (15-25 kDa).

### In vitro study

#### Immunocytochemistry and number of migrant and non-adherent cells

The anti-S-100 immunocytochemical analysis of migrating cells confirmed the presence of Schwann cells in conditions used (Fig. 2). The groups supplemented with 40% FCS showed a greater number of migrating cells. The addition of aggrecan did not increase the cell number (Fig. 2A), but greatly reduced cell detachment (Fig. 2B). When the cells

were supplemented with 20% FCS, the number of cells increased slightly after the addition of aggrecan (Fig. 2A). In this case, the cell viability also improved with aggrecan (Fig. 2B).



**Figure 2.** A. Migration of cells from sciatic nerve explants under different experimental conditions. Note the slight increase in cell number when aggrecan was added together with the 20% fetal calf serum (FCS). The inset shows cultured Schwann cells immunolabeled with S-100 antibody in the presence of aggrecan. Bar = 50 µm. B. Total number of non-adherent cells found in the culture medium after 14 days in culture. Note that aggrecan enhanced the cell viability, especially when associated with 40% FCS.

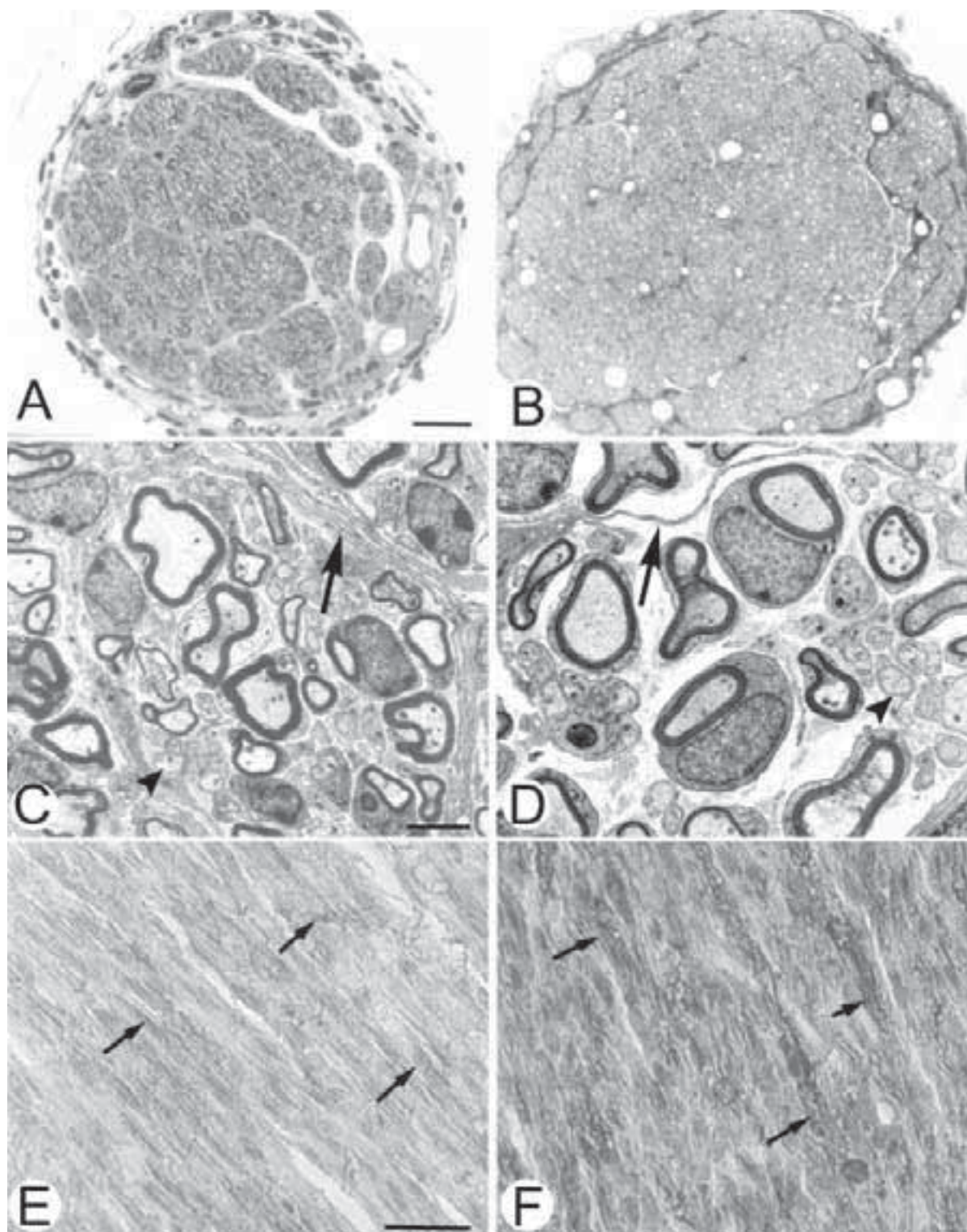
### In vivo study

#### Morphology and immunocytochemistry

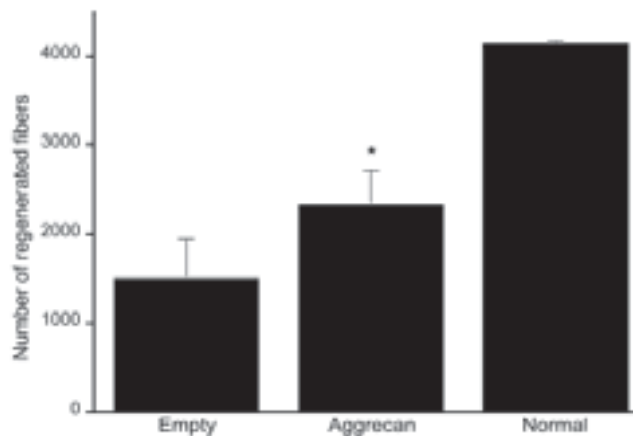
Transverse sections of the regenerated nerve at the midpoint of the tube showed a number of myelinated fibers organized into small bundles surrounded by perineural-like cells and fibroblasts (Fig. 3A,B). At the ultrastructural level, myelinated and non-myelinated axons were also seen (Fig. 3C,D).

The S-100 staining showed the presence of Schwann cells organized longitudinally along the axons. This labeling was stronger in the specimens treated with aggrecan and was concentrated on the Schwann cells projections (Fig. 3E,F).





**Figure 3.** **A and B.** Transverse section of a regenerated nerve at the tube midpoint, five weeks after tubulization. **A.** Empty Tube. **B.** Tube filled with aggrecan gel. Bar = 50  $\mu$ m. **C.** Electron micrograph of a regenerated nerve in an empty tube. Note that the mini-fascicles are surrounded by thick perineural-like cell processes (**arrow**). The arrowhead indicates non-myelinated axons. Bar = 5  $\mu$ m. **D.** Electron micrograph of a regenerated nerve from the aggrecan-treated group. Note the relative looseness of the endoneurial sheath (**arrow**), and the thin perineural-like cell processes. The arrowhead indicates non-myelinated axons. **E and F.** S-100 immunohistochemistry. Note the stronger labeling of the Schwann cell processes when the tube was filled with aggrecan (see in **F**, **arrows**) as compared with the empty tube (**E**). Bar = 15  $\mu$ m.



**Figure 4.** Number of regenerated fibers five weeks after tubulization. Note the greater number of myelinated axons when aggrecan was added (\*  $p < 0.05$  compared to empty tubes).

#### Number of regenerated fibers and morphometric data

A significantly greater number of myelinated fibers were seen in regenerated nerves treated with aggrecan when compared with the empty tube (Fig. 4). In contrast, there were no significant differences in the axonal and fiber diameters or in the myelin thickness (Table 1). In addition, the “g” ratio indicated that the myelin thickness was proportional to the axonal diameter in all groups (Table 1).

## DISCUSSION

Proteoglycans are expressed in the nervous system and may exert positive and negative influences on axonal outgrowth *in vivo* and *in vitro* [6,7]. These properties are attributable mainly to the glycosaminoglycan chains, and several studies have focused on the ability of different glycosaminoglycans to regulate neurite growth [9,13,20]. However, most of the literature so far has dealt either with the glycosaminoglycans alone or with low molecular mass proteoglycans, such as decorin and versican [3]. In our study, we used aggrecan extracted from avian xyphoid process and purified by ultracentrifugation.

Since this proteoglycan was used in its native form, the protein domains and the oligosaccharides remained intact, so that the molecule retained its normal characteristics. The conserved properties were useful for investigating the effects on regeneration in the peripheral nervous system *in vitro* and *in vivo*.

The addition of aggrecan together with 20% FCS stimulated Schwann cells migration and increased cell viability *in vitro*. Schwann cell migration was not seen with 40% FCS, although the viability was greatly augmented. These effects probably reflect the matrix-forming properties of aggrecan and its ability to create a neurotrophic-rich environment, especially at a higher concentration of FCS. Interestingly, aggrecan contains a domain with homology to trophic factors and lectins that may increase cell adhesion [12,22]. In addition, the sulphated glycosaminoglycan chains of aggrecan effectively concentrate negative charges that promote a high degree of hydration, which is important for the storage of neurotrophic factors at the lesion site [12]. As a result, aggrecan can increase the viability of non-neuronal cells during nerve regeneration.

The results obtained *in vivo*, after sciatic nerve tubulization, agreed with the observations *in vitro*. The group in which the tube was filled with aggrecan gel showed the best regeneration in terms of the number of axons and the immunocytochemical staining for the Schwann cells. Interestingly, under similar experimental conditions, the effects of aggrecan were much better than for hyaluronic acid, which also occurs in peripheral nerves [8]. We hypothesize that aggrecan can stimulate axonal sprouting and Schwann cell survival, and possibly accelerates the initial steps of regeneration.

The results of this study reinforce the hypothesis that aggrecan can contribute to the regeneration of peripheral axons, and that this molecule enhances Schwann cell multiplication and migration.

**Table 1.** Morphometrical data for regenerated axons five weeks after sciatic nerve tubulization.

Group	n	Axon diameter ( $\mu\text{m}$ )	Fiber diameter ( $\mu\text{m}$ )	Myelin thickness ( $\mu\text{m}$ )	“g” ratio
Aggrecan	1006	$3.01 \pm 0.10$	$3.86 \pm 1.22$	$0.36 \pm 0.10$	$0.76 \pm 0.06$
Empty	744	$3.40 \pm 1.37$	$4.53 \pm 1.56$	$0.44 \pm 0.11$	$0.74 \pm 0.08$
Normal	613	$4.13 \pm 1.82$	$6.00 \pm 2.38$	$0.77 \pm 0.28$	$0.68 \pm 0.08$

There were no significant differences between empty and aggrecan-filled tubes. The results are the mean  $\pm$  SD of the number of fibers measured (n).

## ACKNOWLEDGMENTS

A. P. was supported by a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This work was supported by FAEP/UNICAMP.

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Received: June 6, 2004

Accepted: July 26, 2004