Neural morphological effects of long-term implantation of the self-sizing spiral cuff nerve electrode

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Abstract—*The paper reports on the histological effects of chronic implantation of self-sizing spiral cuff nerve electrodes on the cat sciatic nerve. The implantation period is about 4.4 months. Four different experimental conditions are evaluated: control, sham, bare cuff (cuffs without contacts and leads) and full cuff. The total number of axons in the nerves of the control group is compared with the three other groups. The surface occupied by collagen fibres in the nerve section, perineurium thickness, fibre diameter and myelin thickness are also measured. The average number of axons in the control nerves is found to be 16416 (±1509) and does not differ significantly from the three other groups ($p > 0.1$). Collagen measurements show an extrafascicular epineurial fibrosis in the two implanted groups that is found to be significantly different ($p < 0.05$). No differences are encountered in the perineurium thickness analysis. Fibre diameter distributions show a regular bimodal pattern for all groups. Centrality (mean and P_m) and dispersion statistics (P_{25} and P_{75}) extracted from fibre diameter distributions do not reveal significant differences. Myelin thickness distributions are also similar for all groups, as well as centrality and dispersion statistics. The present morphometrical results suggest that the effects produced by a chronic spiral cuff implant on this animal model are negligible.*

Keywords—*Self-sizing spiral cuff electrode, Cat sciatic nerve, Nerve morphometry, Functional electrical stimulation, Nerve electrode*

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1 Introduction

FUNCTIONAL ELECTRICAL stimulation (FES) of intact peripheral nerves has been used to restore some motor functions in paretic/paraplegic patients (CREASEY *et al.*, 1996; UPSHAW and SINKJAER, 1997; CHERVIN and GUILLEMINAULT, 1997; VAN KERREBROECK *et al.*, 1997; SEGAL *et al.*, 1998; BOSNJAK *et al.*, 1999).

This stimulation can be achieved by implantation of a cuff electrode placed around the nerve. Indeed, different nerve electrodes have been designed (SAUTER *et al.*, 1983; AGNEW *et al.*, 1988) with this aim. Some have been used in clinical applications because of their longevity and functionality, but their use has remained restricted, as they have been associated with occasional neural damage (NIELSON *et al.*, 1976; MCNEAL *et al.*, 1977; KIM *et al.*, 1983; YUEN *et al.*, 1984; WEESE-MAYER *et al.*, 1989; BAER *et al.*, 1990).

Lately, new designs, such as the spiral cuff nerve electrode (NAPLES *et al.*, 1988; VERAART *et al.*, 1993), seem to be more adequate, owing to their elastic properties that permit the electrode to fit snugly around the nerve and accommodate the changes produced by the normal inflammatory response that follows surgical implantation.

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Nerve electrodes can cause damage to the nerve by diverse mechanisms, whose influences in the final host response often overlap. A primary, important factor that can change the host response is the surgical implantation procedure. Different types of traumatic interference with the vascular supply can occur acutely during implantation, depending on how much the nerve is separated from the neighbouring structures. Although the internal nerve vascularity is anatomically independent of the surrounding tissues along some nerve distances (SUDERLAND, 1968), the surgical procedure can damage neighbouring arterial beds that feed the external nerve circulatory system (LUNDBORG, 1970; 1975) and possibly also the internal capillary network. Alternatively, this surgical implantation can injure nerve fibres or fascicles directly, resulting in various degrees of functional impairment. In the long term, there can be intra- or extraneural fibrosis that interferes with intraneural blood flow.

Another potential source of damage can be the electrode itself. Of primary relevance is the mechanical restriction produced on the nerve by the electrode. Nerve electrodes exert various degrees of pressure and constriction on the nerve. It is not clear which, of ischaemia or mechanical deformation of the nerve trunk, plays the more important role in the case of nerve injury (FERN and HARRISON, 1994), but undoubtedly such injury depends on the degree of constriction and its duration.

During graded compression of a peripheral nerve, a pressure of 30–50 mm Hg quickly induces inhibition of the axonal transport (DAHLIN *et al.*, 1984) and increases vascular permeability and epineural oedema (RYDEVIK *et al.*, 1981). Moreover,

intraneural microvascular injury and structural damage have been described in large myelinated fibres at the edge of the compressed nerve segment (OCHOA *et al.*, 1971; RYDEVIK and LUNDBORG, 1977). Long-term compression, either at low or high pressure, can result in several intraneural tissue reactions with a variable degree of functional deficit (LEQUESNE and CASEY, 1974; BROWN *et al.*, 1976). Indeed, researchers dealing with the structural effects of chronic entrapment lesions have often used rigid cuffs to simulate compression conditions (WEISL and OSBORNE, 1964; DYCK, 1969; MACKINNON *et al.*, 1984; 1985; DAHLIN and KANJE, 1992; CORNEFJORD *et al.*, 1997; AZIZ *et al.*, 1999). Several degrees of pathological change in the implanted nerves were reported by these authors, ranging from local demyelination, to fibrosis formation in the different layers of the nerve, with loss of axonal continuity.

In contrast, a silicone curling sheath, used as a spiral cuff, which adapts naturally to nerve changes (NAPLES *et al.*, 1988), should restrict these compression effects by expanding or contracting to fit very closely the nerve surface in the immediate and mediate post-operative periods. Spiral cuff electrodes implanted with an initial diameter between 60% and 200% of the nerve diameter have been shown either to expand or to contract to fit neatly around the nerve (MORTIMER, 1990). Thus, the spiral cuff should lessen not only the structural changes due to pure nerve deformation, along with the above described edge effect, but also the nerve ischaemia.

Finally, trauma can also be produced by wires rubbing the nerve surface or tethering the implanted nerve. Indeed, the scarring reaction surrounding the wires would attach them closely to the nerve and neighbouring muscles, producing traction upon the nerve during limb movement and muscle contraction.

In addition, in stimulating cuffs, welded wires and contacts are obviously needed, but they produce a more rigid frame in which the cuff capacity to expand or contract is diminished. Hence, damage can differ, depending on whether the cuff used is devoid of metal contacts or not. In summary, electrode effects form a complex combination of independent factors that deserve to be studied separately.

Electrical stimulation is considered in itself to be a conspicuous element of potential nerve injury. Many investigations have dealt with this issue (AGNEW *et al.*, 1989; AGNEW and MCCREERY, 1990; MCCREERY *et al.*, 1992; 1994), and its effects are well described. The present study was set up to isolate the importance of the respective contributions of surgical exposure, mechanical trauma and contact with electrode material produced when a spiral cuff is implanted around the cat sciatic nerve.

In this work, only passive effects have been considered. Animals were implanted for about four months. After such a period, morphological changes can be considered to be stabilised (MACKINNON *et al.*, 1984; 1985). Four different conditions have thus been distinguished: a control group (CO), a sham group (SH), which included animals having spiral cuffs surgically implanted and immediately removed, a third group, which was implanted with a bare cuff (BC), i.e. a spiral cuff devoid of any lead to single out the effects of the spiral cuff itself, and the last group, which was implanted with a full cuff (FC), including the metal contacts and leads.

2 Methods

2.1 *Subjects*

Adult cats, aged 8 months or more, with weights from 3.0 to 4.5 kg were selected for this study. The experiments were performed on the sciatic nerve. Twelve animals were used, with both sciatic nerves randomly distributed within the four groups, as shown in Table 1.

Table 1 Random distribution of 24 nerves in different experimental groups and implantation duration in days. L and R correspond to left or right implanted sciatic nerve in each of 12 animals

	Experimental groups				
Cat	CO	SH	BC	FC	duration
1	R	L	–	–	133
2	–	–	R	L	134
3	R	–	L	–	132
4	–	R	–	L	134
5	L	R	–	–	134
6	–	–	L	R	136
7	–	R	L	–	133
8	L	–	–	R	133
9	–	–	R	L	131
10	L	R	–	–	131
11	–	–	L	R	137
12	L	R	–	–	137

The sampling procedure was based on the number of detectable axons in the nerve, as it is one of the main goals of this study. However, to the best of our knowledge, no study in the literature specifically mentions the number of axons in the sciatic nerve of the cat. As a consequence, we drew this number indirectly from data in the literature, as follows. The average sciatic nerve diameter is about 2.7 mm (VERAART *et al.*, 1993), fascicles represent about 65% of its volume (SUDERLAND, 1968), and the fibres are distributed from 2 to 16 μm (LLOYD and CHANG, 1948), with an average of 10.15 μm. It was therefore postulated that the number of closely packed fibres would be about 22 500, with a physiological variability of 15% (thus 3375 fibres). In that case, a minimum number of six nerves per group would be required to detect a 25% reduction (5625 fibres) at the 5% significance level, with a power of 0.95.

The advantage of the intragroup randomisation pattern used in the present study is that it cancels out any training effect on the experimenter. If the variability were larger, we could have added as many nerves per group as were necessary to preserve the same statistical power.

The experiments reported here were approved by the Animal Ethics Committee of the School of Medicine of the Université Catholique de Louvain and follow the law of 14 August, 1986, from the 'Ministère de l'Agriculture et des classes moyennes' (Belgium).

2.2 *Electrode manufacture*

Spiral cuff electrodes with an inner diameter from 2.5 to 3.5 mm were fabricated. The spiral cuffs for the BC group were made as follows. First, a 50 μm thick sheet of silicone rubber* was stretched, and a silicone elastomer† was laid upon it. A second, unstretched sheet was then pressed on top. After curing of the silicone elastomer, the silicone rubber sandwich was trimmed, as described in NAPLES *et al.* (1988).

Spiral cuffs for the FC group were made by including four longitudinal tripoles of recessed platinum dot electrodes. The inclusion of contacts and leads was carried out according to VERAART *et al.* (1993), except for the two anodes, which were short-circuited in each tripole. The eight leads (two for each tripole) ran between the sheets and exited the cuff on the same side. Finally, a circular window with a 0.5 mm diameter was trimmed in the inner layer using the sharp section of a hypodermic needle. The cuff was cut at 12 mm width and trimmed between 22 and 35 mm, depending on the final diameter

* Avecor, Surgical Division, Minnesota, USA.

† Nusil Med 4210, Nusil Technology.

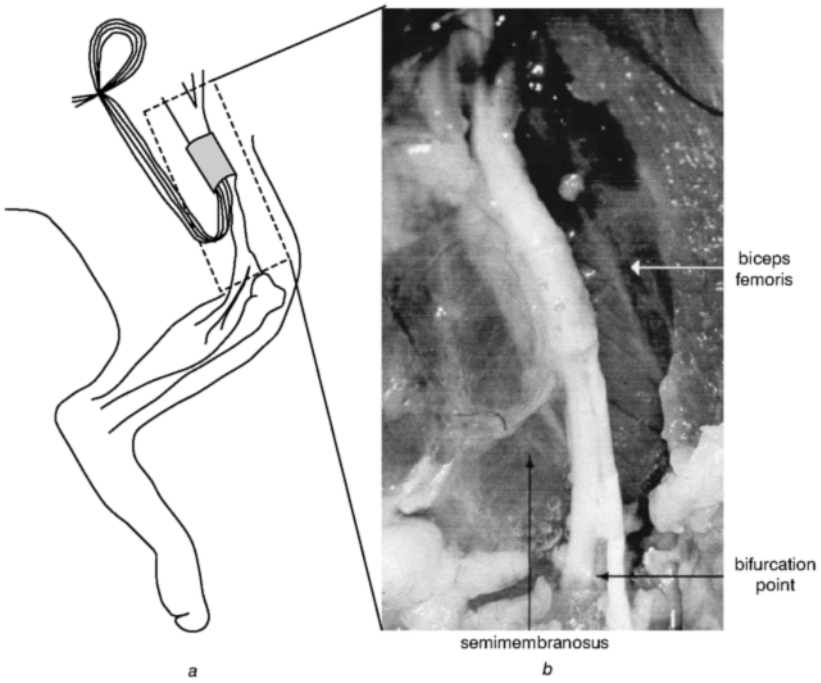

Fig. 1 *View of spiral cuff electrode at explantation time. (a) Schematic diagram indicating the routing of the lead; (b) implantation site showing the cuff electrode, lead cable, nerve and surrounding tissues*

at rest. Electrodes were cleaned using a standard technique (GRILL and MORTIMER, 1994), packed in sterilisation bags and sterilised with vapour at 120 °C for 20 min.

2.3 *Implant procedure*

Animals were anaesthetised with xilazyne hydrochloride (rompun, 0.1 ml kg^{-1}) and ketamine hydrochloride (ketalar, 6–10 mg kg^{-1}). Body temperature was controlled with a heating pad. Bencil-penicilline was administered intramuscularly post-operatively (50 000 I.U./kg). The sciatic nerve was accessed through a long curved incision along the thigh following the caudal border of the biceps femoris. The subcutaneous tissue was dissected, and the muscular plane was exposed. A blunt dissection was made between the biceps femoris and the semi-membranosus muscles to reach the nerve (see Fig. 1). The biceps muscle was gently lifted to reveal the nerve, which lay deep in a groove formed by the semi-membranosus, adductor and biceps femoris muscles. The nerve was then dissected along 40–50 mm in its bed, down to the point where it branched into tibial and peroneal components.

The nerve diameter in the SH, BC and FC groups was determined by measuring the nerve circumference with a 6-0 piece of prolene suture. For this purpose, the nerve was slightly hooked up from its bed and encircled by the suture. The suture was gently tied to the nerve with a double knot and cut as close as possible to the knot. Thus, the suture length corresponded to the nerve circumference. A cuff-to-nerve diameter ratio of 0.9 was set for the implant, and the cuff was installed with its distal end lying about 20 mm above the sural nerve branching point.

For the SH group, the cuff, with its leads, was then immediately removed. For the FC group, the wires left the electrode distally and were sutured to the external fascia of the semi-membranosus muscle bed, forming a loop that reoriented them proximally, as illustrated in Fig. 1. The cable was then led between the biceps and semi-membranosus muscles, routed upward to the back of the thigh up to the subcutaneous tissue, where it was attached and remained for all the implantation period. Special care was taken to avoid any haemorrhage, and the procedure was always performed in sterile conditions.

Animals were maintained under comparable environmental conditions throughout the whole implantation period. In general, no signs of infection, paresis or hyperaesthesia were detected. The 'toe spreading reflex' (STRAUSS and SPRAGUE, 1944) was used as an indirect evaluation of the functional state of the sciatic nerves.

2.4 *Explantation protocol*

Animals were anaesthetised with sodium pentobarbital (30 mg kg^{-1} given intramuscularly), 131–137 days post-implantation (see Table 1). The abdominal aorta was accessed through the abdominal cavity and catheterised between the renal branches and iliac bifurcation. The inferior vena cava was transsected to avoid any excessive pressure. A solution of 10 000 units of heparin‡ and 3.5% Dextran *T*-70** in a PBS phosphate buffer solution was injected for 1 min as a prewash, at a constant flow rate of 250 ml min^{-1}. Thereafter, animals were perfused with 2 l of 4% paraformaldehyde and 3.5% dextran in 0.1 M PBS solution (pH 7.4). The sciatic nerve, accessed along the same path as for the implantation procedure, was dissected starting 20 mm distal from the spinal cord to the popliteal fossa, after the tibial and peroneal bifurcation. Samples were taken from standard sites: a 30 mm long proximal sample was excised starting 10 mm proximal to the cuff, a 30 mm long distal sample was excised starting 10 mm distal to the cuff, and a middle sample was taken from the region underneath the cuff (from about 1 mm proximal to 1 mm distal to the cuff).

2.5 *Histological protocol*

From the most distal end of the proximal, middle and distal samples, 5 mm long fragments were cut out and maintained in Karnovsky fixative (2.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution) for 24 h. The remaining fragments were immersed in Bouin fixative. Karnovsky-fixed fragments were postfixed in 1% osmium tetroxide for 4 h, dehydrated in graded ethanol solutions and

‡ Leo Pharmaceutical Products, Belgium N.V.

** Amersham Pharmacia Biotech. A.B., Uppsala, Sweden.

embedded in epon *Ladd LX*-112††. Semi-thin (1 μm), transverse nerve sections were cut on a Reichert ultracut microtome‡‡ and were stained with toluidine blue (fragments were oriented, as much as possible, perpendicular to the section plane). Bouin Hollande fragments were taken for paraffin embedding; 5–7 μm sections were cut on a 820 Spencer microtome and stained with haemalum-eosin-safran (HES) and Masson's trichrome.

2.6 *Data processing*

Qualitative analysis was performed with the proximal, middle and distal samples. The quantitative analysis was performed with the middle sample only (underneath the spiral cuff).

2.6.1 *Data acquisition:* Photomicrographs of Masson's trichrome sections were taken through a Zeiss microscope at low magnification (× 3.3) for collagen fibre measurements and were digitised by a Nikon 25–1000 software system. The scaling factor was evaluated with a square millimeter calibration grid (Carl Zeiss). For perineurium thickness measurements and axon counting, the Masson's trichrome sections were scanned using a Leitz laborlux D microscope and the images were digitised by a JVC KY-1950*E* video camera on a Vision Etics acquistion card at a magnification of ×1114.

For fibre diameter and myelin thickness measurements, photomicrographs of toluidine blue sections were obtained using a colour system in a Zeiss microscope with a ×40 PLANAPO oil immersion objective. Each photomicrograph represented a $209 \times 140\,\mu m^2$ tissue area. A slight overlap between adjacent microscopic frames was adjusted manually to take care of partial fibres lying on the edge of each photomicrograph. Images were then digitised by a Nikon 25–1000 software system with a 1850×1234 pixel resolution. A microscale* (0.01 mm) was processed in the same way to obtain the scaling factor.

2.6.2 *Definition of nerve compartments:* The total nerve was divided into different compartments defined as follows: the area delimited by drawing straight lines tangential to the external fascicle perimeter (see Fig. 2) was subdivided into a fascicle zone and an interfascicular zone, as illustrated in Fig. 2. The difference between the total nerve and these two zones was defined as the epifascicular zone.

Compartment areas were measured from low-magnification images. Most calculations were performed with the NIH-image free software (available via anonymous FTP from zippy.nimh.nih.gov).

2.6.3 *Collagen fibre measurements:* To measure the build-up of connective tissue changes according to the experimental condition, the areas of the collagen fibres were measured in Masson's trichrome stained sections as follows. Colour images (collagen fibres being stained blue) were converted into grey-scale images. The grey value of pixels corresponding to the collagen fibres was determined for each section in a representative area, outside the fascicle area. Pixels of the corresponding value ranges were thereafter selected and counted. Finally, these data were transformed into area values by scaling.

2.6.4 *Perineurium thickness:* Perineurium thickness was defined as the average of eight measurements performed systematically every 45° on the circumference of each fascicle.

†† Ladd Research Industries, Inc., Burlington, Vermont, USA.
‡‡ Reichert Wien, Austria.
* Wild, Switzerland.

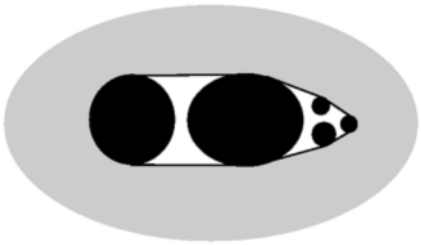

Fig. 2 *The total nerve is split in three different compartments for evaluating the connective tissue reaction: the fascicular zone (■), defined by the area occupied by the fascicles, the interfascicular zone (□), defined as the area between the fascicles, and the epifascicular zone ▭, defined by the surface surrounding the fascicles*

2.6.5 *Fibre counting:* Beginning in the upper left corner of the cross-section, the section was sequentially digitised in a serpentine pattern, creating an ordered list of images, until the entire cross-section had been covered.

To lessen the counting burden, a fibre number estimator was validated on the CO group and applied to all experimental groups, as follows. From a total number of images in one fascicle (n), a random sample of k ($k < n$) images can be obtained by selecting the first k elements from a random list constructed with the first n natural numbers. The estimator N of the total number of fibres in a given fascicle is calculated as the total fascicle area multiplied by the number of profiles per unit area, i.e.

$$N = \frac{N_k}{A_k} \sum_{i=1}^{n} A_i \tag{1}$$

where N_k corresponds to the number of fibres found in k random selected images, and A_k is the area occupied by these fibres in the corresponding images. Profiles touching upper and right borders were not taken into account to avoid overestimation (GUNDERSEN, 1977). An image area is considered as the area within the fascicle zone only, technical flaws or fissures being excluded.

To assess the optimum k value in the six control nerves, first, all their axons were counted exhaustively in every image of a given fascicle. Then, the n images from a random list were grouped in series of k for a given k-size, and the corresponding N_k were computed, as well as the estimators N. Next, a standard deviation was calculated from all estimators N, but with the mean replaced by the known actual axon number.

As for the construction of the study, a physiological variability of 15% was assumed to be normal, and a standard deviation below 7.5% was considered acceptable. The list was reconstructed ten times. For each repetition of the random list, the minimum k-size for which the error of the fibre number estimation remained below 7.5% was evaluated for every fascicle including more than 12 images in the six control nerves (in total 13 fascicles). Fig. 3 illustrates the distribution of the minimum k-sizes according to fascicle dimension. These variables are uncorrelated ($r = 0.18$).

In conclusion, the fibre number in the nerves of the four groups was estimated according to eqn 1, with $k = 13$ (the maximum k-size found for all fascicles; see Fig. 3). This arbitrary selection covered, in some cases, 20% of the fascicle (50 images) and 80% (16 images) in others, but this effect was equally randomly distributed over all groups. Fascicles with less than 13 had all their images counted.

2.6.6 *Fibre diameter and myelin thickness:* Measurements on each nerve were performed with 20 randomly selected images per nerve. An automatic method was used to estimate the fibre diameter and the myelin thickness distributions (ROMERO *et al.*,

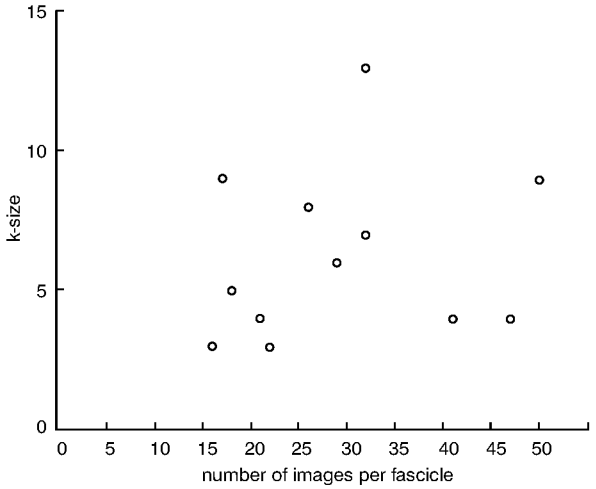

Fig. 3 *Selection of the minimal number of images needed to compute an estimate of the fibre number. As for the construction of the study a biological variability of 15% was assumed to be normal, and the number of images needed to maintain the standard deviation of the estimated total number of axons below 7.5% was determined. The Figure illustrates the relationship between the minimal k-size image group and the number of images per fascicle. Only fascicles with more than 13 images were considered*

2000). Briefly, the image grey level was thresholded from a local analysis of the histogram, to provide a coarse binary pixel classification between myelin (black) and non-myelin (white) pixels. In the resulting binary image, through connectivity tests, pixels became objects that were simplified using connected morphological operators (HEIJMANS, 1999) according to predefined morphological rules, i.e. nerve fibres could be considered as white areas surrounded by black areas with a similar fibre/axon diameter ratio. They also presented analogous area/perimeter ratios for both the fibre and the axon.

To separate connected fibres, the thickness of the myelin ring was evaluated around each candidate area through a Euclidean distance transformation, with a stopping criterion on the pixels in the propagation front. Finally, fibre and axon areas on each detected fibre were computed, and the fibre diameter and myelin thickness were calculated, based on the surface of the equivalent circle. The fibres touching the image edge were not included in the analysis. The results, superimposed on the original grey-level image, were visually controlled.

Raw data were grouped by experimental condition, and frequency histograms were constructed. The positions of the histogram peaks were determined by approximating frequency histograms (bin width $= 0.01\,\mu m$) with the sum of two Gaussian functions.

2.6.7 *Statistical methods:* Statistical analysis was structured as follows:

- Data were reported as *mean* $\pm$ *SD* or quartiles. The statistical significance level was set to 5%. The four experimental groups were compared using a one-way analysis of variance (ANOVA) with *F*-test. A significant ANOVA result led us to perform pairwise comparisons using the Fisher's LSD and Tukey's method with $0.05/6 = 0.008$ as the significance level.
- The relationship between perineurium thickness and fascicle area was assessed using linear regression, and the equality of regression lines along the four experimental groups was tested using analysis of covariance on regression (ANCOVA) with *F*-test.
- For each fibre diameter distribution, statistics such as mean, quartiles and the position of the inflection point between the two main peaks of the histogram (P_m) were calculated to estimate central and dispersion parameters. The four experimental groups were compared for those parameters using ANOVA with *F*-test.

Most analyses were performed with Systat 5.2.† statistical software

3 Results

3.1 *Qualitative morphological observations*

During the implantation period, none of the animals showed any detectable neurological deficit; the motor behaviour and the 'toe spreading reflex' (STRAUSS and SPRAGUE, 1944) were also normal in all 12 animals on both sides.

At explanation time, the spiral cuff nerve electrode implants appeared encapsulated by a greyish white layer of connective tissue. This capsule adhered to the neighbouring muscle fascia and surrounding tissues, i.e. biceps femoris and semi-membranosus fascia, making the dissection difficult. Once the nerve had been dissected, the surrounding connective tissue was cut longitudinally, and the cuff electrode was easily unwrapped from the nerve. Underneath the cuff, the nerve generally appeared reshaped as a cylindrical profile corresponding to the inner surface of the cuff. This change extended about 2 mm proximal and distal to the cuff ends. No macroscopic evidence of tracks or injuries due to the leads was observed in the nerve at the distal level of the cuff electrode.

When sections stained either with haemalum-eosin-safran (HES) or Masson's trichrome were analysed, a layer of dense fibrous tissue was observed in the implanted nerves in contact with the electrode surface. Its thickness ranged from 35 to 300 µm. Usually, fibroblasts and collagen parallel fibres formed in this layer. In three animals, for both implanted nerves (in cats 2, 9 and 11, see Table 1), signs of a chronic inflamatory response were observed close to the inner electrode surface. This reaction consisted of a thin, variable and well delimited layer of mononuclear-cell or macrophage infiltrations and sparse multinucleated giant cells, along with a less organised fibroblastic response. These changes were not observed at the proximal or distal cuff levels.

Typically, the nerves in the CO group were flat structures, with two major fascicles corresponding to the tibial and peroneal branches (letters T and P in Fig. 4) and a cluster of small fascicles. This arrangement was also observed in the SH group. In contrast, in nine of the 12 implanted nerves, the smaller fascicles appeared to have moved from the peripheral position, as observed in the non-implanted nerves (see Figs 4*a* and *c*), to a central position (see Figs 4*b* and *d*), seemingly better to accommodate the cylindrical shape imposed by the cuff. In the other cases, only a thickened connective layer in contact with the cuff was observed.

Some collagen fibres also formed a thin, dense fibrous tissue connected to the external side of the perineurium. Individual nerve fascicles were made of myelinated axons alternating with scattered non-myelinated and smaller axons. Within the fascicles, the endoneurium was preserved, with no sign of injury. These findings were also observed at proximal and distal levels.

3.2 *Comparative areas of nerve compartments*

Tables were constructed from measurements taken from the cuff region, as the major qualitative changes were observed in this zone.

† Systat, Inc.

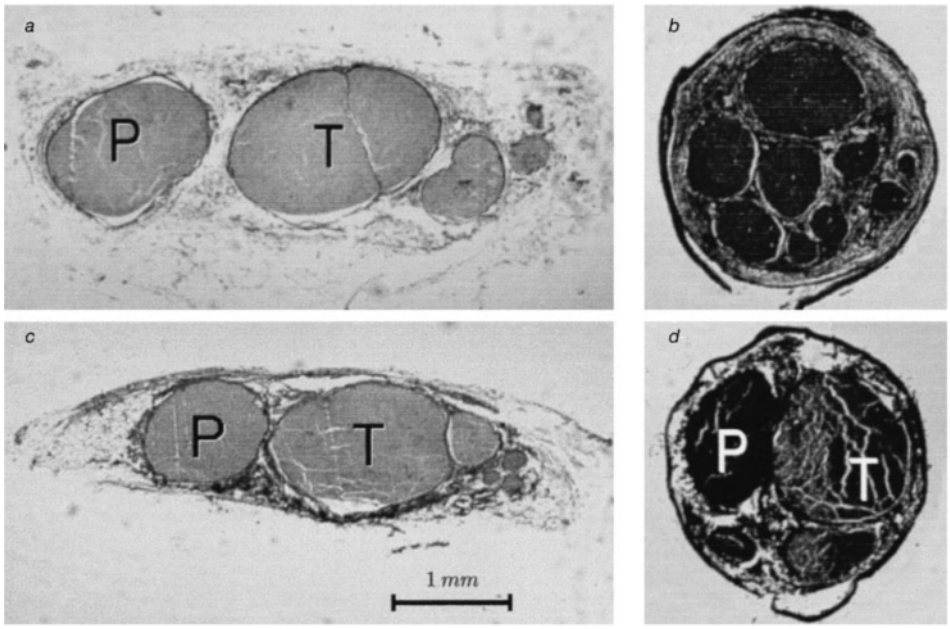

Fig. 4 *Aspects of the sciatic nerve without (a) and (c) or with implant (b) and (d). A pattern can be established regarding the reorganisation of*

Table 2 shows area measurements in different nerve compartments (as defined in Fig. 2). Considering the total nerve and the fascicular + interfascicular nerve area, several trends, but without statistical significance under ANOVA analysis, were observed for both the BC and FC groups. Regarding these areas, a wider range of variation was observed in the sham group. The fascicular + interfascicular nerve area was smaller in both implanted groups, with a smaller variation. The epifascicular zone showed a similar slight increase in the SH, BC and FC groups.

For the fascicle zone area, whereas there were smaller area for implanted groups, the SH group presented larger values. However, variability was too high to reach statistical significance.

3.3 *Collagen reaction*

No difference was detectable in the interfascicular zone (see Table 3). In contrast, in the total nerve, as well as in the epifascicular zone, the intergroup differences were significant ($p < 0.05$), owing to the modifications introduced by the two implanted groups (see Table 3). This result indicates that the collagen build-up reaction occurred at the exterior of the fascicular nerve zone.

3.4 *Perineurium thickness*

Fig. 5 illustrates the linear regressions between the perineurium thickness and fascicle surface, grouped according to experimental condition.

Slopes did not provide evidence for any significant difference (ANCOVA: $F = 2.54$; $p > 0.05$). Analysis of variance (ANOVA) performed on perineurium thickness data did not show significant differences ($F = 2.09$; $p = 0.1$). In contrast, line intercepts were significantly different ($F = 5.31$; $p < 0.001$). Pairwise comparisons between intercepts using Tukey's test ($n_1 = 35$; $n_2 = 29$; $n_3 = 32$; $n_4 = 24$ per condition, respectively) showed significant differences in CO with BC and SH with BC comparisons.

Table 2 Measurements of nerve compartments (mm^2). Intergroup variations were not significant under ANOVA test

Areas of nerve compartments, mm^2				
	experimental groups			
Nerve compartment	CO	SH	BC	FC
Total nerve	5.19 ± 0.65	5.9 ± 1.68	5.22 ± 0.45	5.80 ± 0.57
Epifascicular zone	1.61 ± 0.54	2.19 ± 0.91	2.13 ± 0.68	2.65 ± 0.63
Interfascicular + fascicular zones	3.58 ± 1.10	3.71 ± 1.27	3.09 ± 0.30	3.15 ± 0.38
Fascicle zone	2.50 ± 0.62	2.63 ± 0.61	2.39 ± 0.43	2.32 ± 0.53

*Table 3 Collagen measurements in different nerve compartments. Top and bottom rows correspond to significant differences ($p < 0.05$) using ANOVA * and †correspond to Fisher's LSD and Tukey post-hoc test and follow the next convention: against control group *($p < 0.05$) and against sham group †($p < 0.05$) and ‡($p < 0.01$)*

Collagen area in nerve compartments, mm^2				
	experimental groups			
Nerve compartment	CO	SH	BC	FC
Total nerve	1.5 ± 0.41	1.38 ± 0.41	2.26 ± 0.72*†	2.15 ± 0.57*†
Interfascicular zone	0.62 ± 0.31	0.52 ± 0.26	0.57 ± 0.14	0.47 ± 0.25
Epifascicular zone	0.88 ± 1.18	0.86 ± 0.19	1.69 ± 0.63*‡	1.68 ± 0.37*†

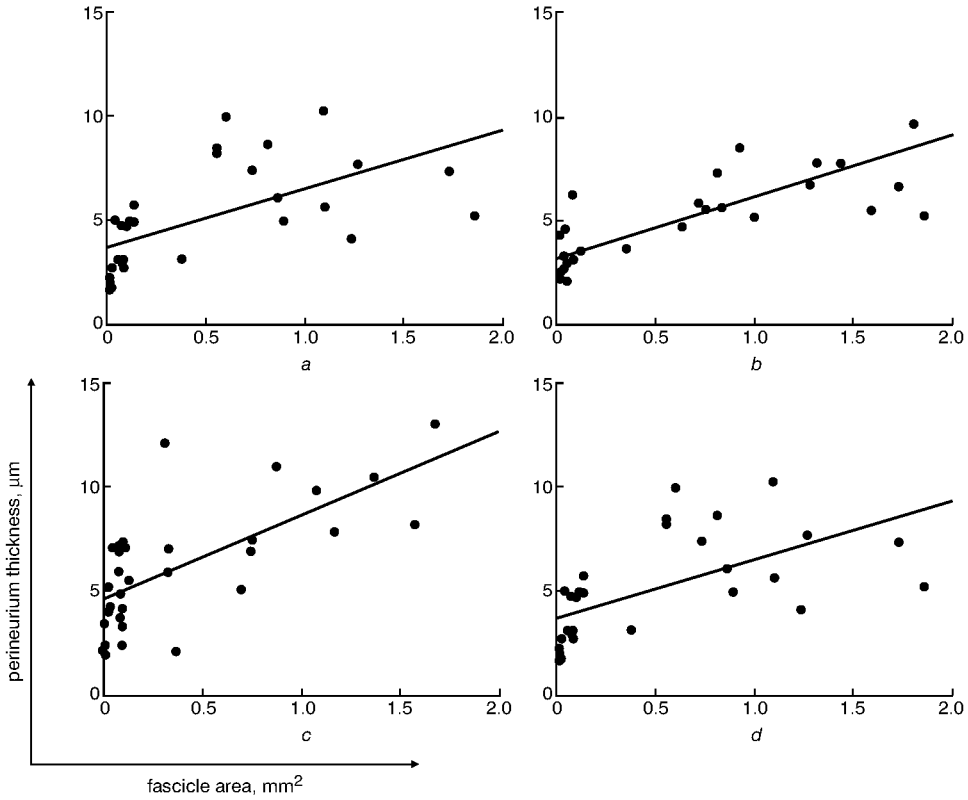

Fig. 5 *Relationship between the fascicle area and the perineurium thickness for the four experimental groups (a) CO group, $r = 0.60$ ($p < 0.05$), $y = 2.79X + 3.64$, $n = 35$; (b) SH group, $r = 0.84$ ($p < 0.05$), $y = 3.00X + 3.23$, $n = 29$; (c) BC group, $r = 0.69$ ($p < 0.05$), $y = 4.09X + 4.55$, $n = 32$; (d) FC group, $r = 0.49$ ($p < 0.05$), $y = 1.56X + 4.33$, $n = 24$*

3.5 *Fibres*

3.5.1 *Number of fibres:* The total estimated numbers of neural fibres in each group are presented in Table 4. For the CO group, the estimation is only 3% different from the actual value (see Section 2). Intergroup comparisons using ANOVA tests showed no statistical difference in the number of fibres between the four groups.

3.5.2 *Fibre diameter and myelin thickness:* Five nerves per group were technically available for fibre diameter and myelin thickness measurements. In fact, epon sections of animals 1 and 6 were discarded for technical reasons (osmium impregnation failure). Usually, about 50% of the total nerve was successfully prepared for the quantitative study. However, on some occasions, up to 80% of the entire nerve section was available for the study. In some images, artefacts such as faint fixation, weak osmium impregnation or unhomogeneous staining could be observed. Thresholds were adjusted to overcome these problems (ROMERO *et al.*, 2000).

Fibre diameter histograms were constructed for each experimental condition (see Fig. 6). A regular bimodal pattern was found in all fibre diameter distributions. All Gaussian approximation functions used to localise the different peaks in all histograms showed correlation coefficients larger than 0.94, when compared with the histogram data. Small fibre population peaks and large fibre peaks were located at 4.28, 4.82, 5.21 and 5.41 μm, and at 10.6, 10.5, 10.9 and 11.5 μm, respectively for the CO, SH, BC and FC groups.

Myelin sheath thickness data were also grouped by experimental condition (see Fig. 7). The first peak appeared at 1.1 μm for the CO group and at 1.3 μm for the other groups. The second peak was at 2.2 μm for the CO, SH and BC groups and at 2.1 μm for the FC group.

Centrality (mean and P_m) and dispersion (P_{25} and P_{75}) statistics were calculated from the raw diameter data and are outlined in Table 5. The ANOVA test performed on these data did not show evidence of any significant difference.

Centrality and dispersion statistics, calculated from the raw myelin thickness data, are depicted in Table 6. ANOVA analysis did not show any significant difference here either.

Table 4 Number of fibres in four experimental groups. ANOVA analysis did not show any significant difference

	Number of fibres	
Experimental group	estimated	actual
CO	16 922 ± 2020	16 416 ± 1509
SH	15 556 ± 1026	–
BC	16 038 ± 2019	–
FC	14 800 ± 1530	–

4 Discussion

Our study shows that, after about four months of spiral cuff implantation on cat sciatic nerves, most morphological parameters remain nearly unaltered, compared with control nerves. Underneath the cuff, the nerve assumes a cylindrical shape, and fascicles tend to gather in the centre of the nerve space. A moderate epineural fibrosis is present, with minimum changes in the perineurium thickness. The numbers of fibres do not show significant variations among the four experimental conditions, neither do the fibre diameters nor the myelin thickness distributions. Qualitatively, proximal or distal levels to the cuff were seen to be not compromised.

In this study we observed that the nerve adapts to the new available space set by the implant in two main ways. First, in nine out of 12 implanted nerves, a fascicle rearrangement was

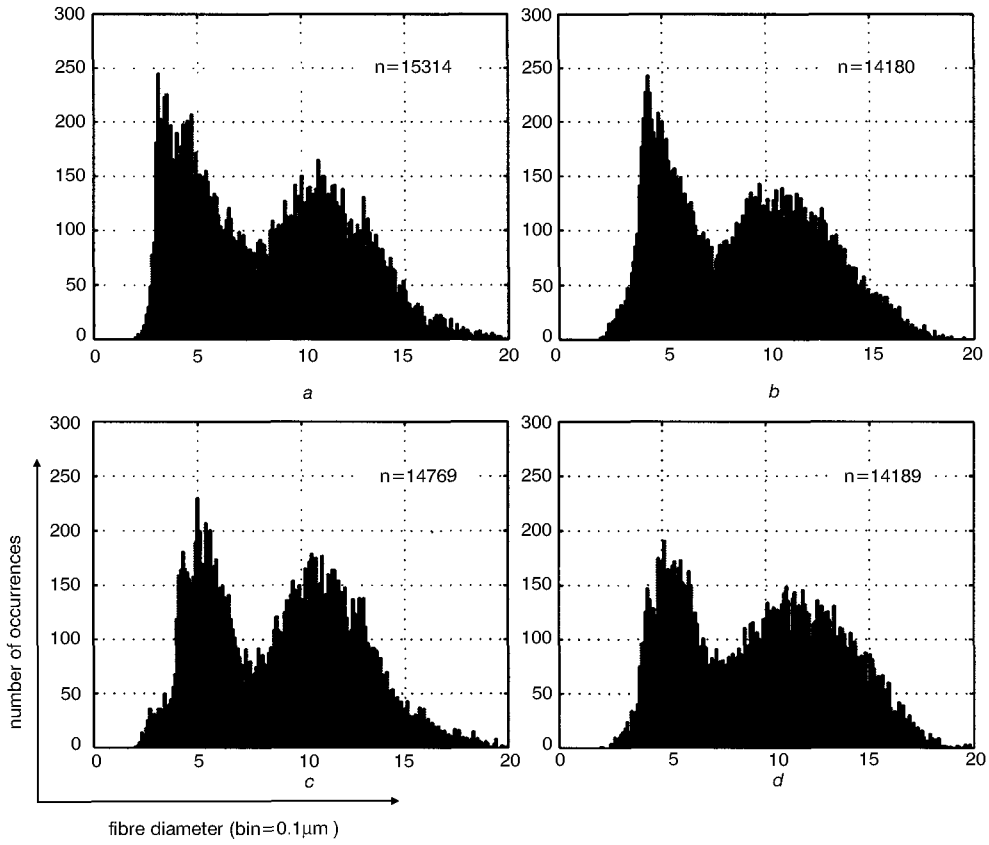

Fig. 6 *Fibre diameter distribution for the four experimental groups. The abcissa corresponds to the fibre diameter in μm (bin width = 0.1 μm) and the ordinate to the fibre frequency ((a) CO: control; (b) SH: sham: (c) BC: bare cuff; (d) FC: full cuff). The total number of fibres involved in the distribution is indicated in the upper right part of each pannel*

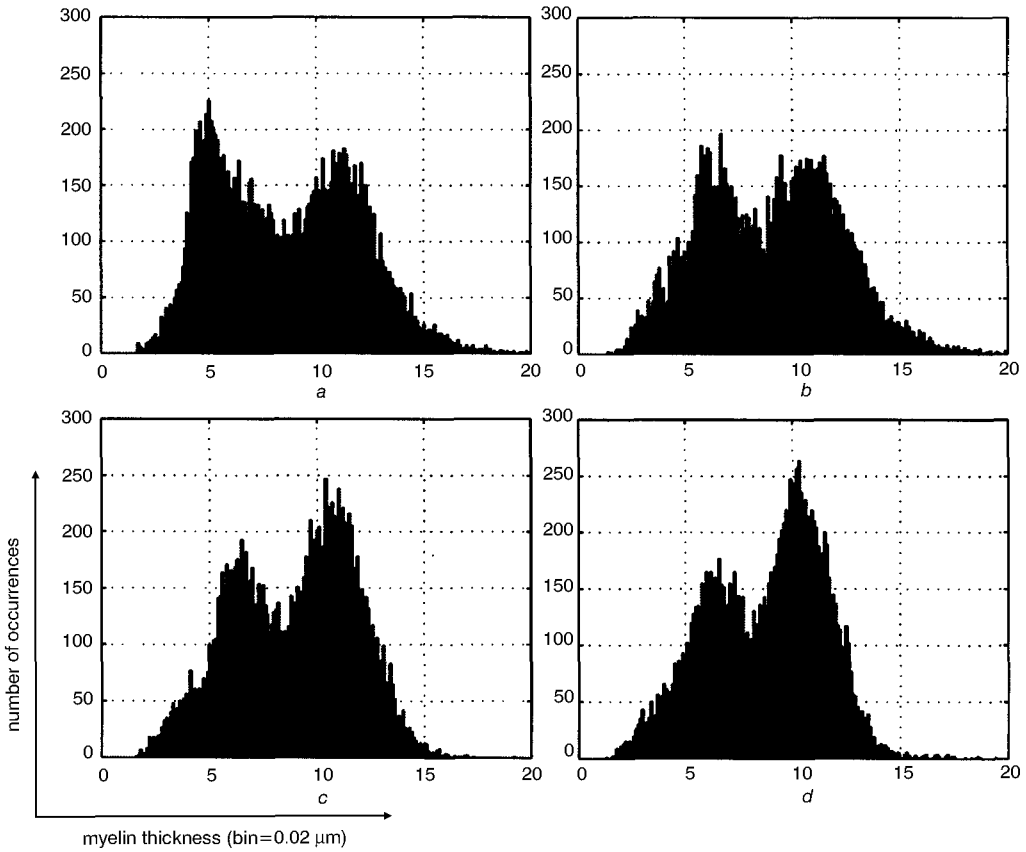

Fig. 7 *Myelin thickness distribution in the four experimental conditions. The abcissa corresponds to the myelin thickness in μm (bin width = 0.02 μm) and the ordinate to the frequency. Other conventions as in Fig. 6. (a) CO group; (b) SH group; (c) BC group; (d) FC group*

Table 5 Central and dispersion statistics for fibre diameter (μm). ANOVA test did not show any significant difference

	CO					SH					BC					FC				
Mean	8.3	8.9	9.6	8.6	8.5	9.4	9.5	9.2	9.5	8.4	9.2	9.7	8.6	9.5	8.6	8.9	9.7	9.0	9.8	9.6
P_m	6.9	8.4	9.5	9.1	7.3	6.9	6.6	7.3	7.9	7.8	7.5	8.3	7.4	7.6	7.3	6.8	8.3	8.5	7.9	8.3
P_{25}	5.7	5.7	6.1	5.4	5.1	6.2	6.3	5.9	6.3	5.1	5.8	6.4	5.8	6.2	5.5	5.9	6.2	5.5	6.4	6.2
P_{75}	10.5	11.6	12.8	11.6	11.4	12.1	12.2	11.9	12.1	11.3	11.7	12.6	11.0	12.0	11.3	11.2	12.7	12.0	12.7	12.6

Table 6 Central and dispersion statistics for myelin thickness (μm). ANOVA test did not show evidence of significant differences

	CO					SH					BC					FC				
Mean	1.7	1.7	1.8	1.7	1.8	1.9	1.7	1.8	1.9	1.7	1.8	1.8	1.7	1.9	1.8	1.8	1.7	1.7	1.9	1.6
P_m	1.6	1.5	1.9	1.6	1.6	1.6	1.5	1.6	1.7	1.4	1.6	1.8	1.4	1.7	1.5	1.6	1.5	1.5	1.7	1.6
P_{25}	1.3	1.3	1.3	1.3	1.1	1.4	1.4	1.4	1.5	1.0	1.3	1.4	1.0	1.5	1.3	1.4	1.2	1.2	1.5	1.1
P_{75}	2.1	2.1	2.2	2.2	2.4	2.3	2.1	2.1	2.2	2.2	2.1	2.2	2.2	2.2	2.2	2.1	2.1	2.2	2.2	2.0

observed. These nerves presented a whole, round aspect where small fascicles had migrated from a lateral position to a central one (see Fig. 4). This fascicle distribution differs from that shown by LARSEN *et al.* (1998), in which rigid nerve cuff electrodes were temporarily implanted around the tibial nerve of rabbits: two weeks post-implant, fascicles tended to occupy the entire available surface delimited by the cuff, which was larger than the nerve. Sixteen months post-implant, this new space occupation seemed also to extend distally. This discrepancy with our observations may result from different pressures exerted on the nerve by rigid as against spiral cuffs, but also from the nerve structure. Indeed, in the tibial nerve, the fascicles are closely packed, with little supporting epineurial tissue, whereas, in the sciatic nerve, fascicles are loosely arranged within an abundant epineurium. A second nerve adaptation observed in the present study was the presence of connective tissue proliferation underneath the implant, mainly present in the space between the fascicles and the inner electrode surface. This kind of reaction has been described in other studies (MACKINNON *et al.*, 1984; NAPLES *et al.*, 1988; LARSEN *et al.*, 1998).

Among the eight long-term implanted animals, three presented a classic foreign body inflamatory reaction close to the electrode. This was probably caused by contamination due either to the surgical procedure, or to the electrode manufacturing process. The fact that all four non-implanted animals did not exhibit such reaction is not in favour of a surgical contamination. Furthermore, all the cuff electrodes were fabricated in laboratory environmental conditions, with the likely subsequent accumulation of allergenic particles in the electrode, in such a way that, even with an exhaustive cleanning process, it was impossible to eliminate them completely. Similar findings have been described in another investigation (NAPLES *et al.*, 1988), in which passive spiral cuffs were implanted in cat sciatic nerves for seven months. Certainly, improving the quality of the fabrication environment should reduce the chronic inflamation element of this immunological response. We are at present manufacturing the cuff electrode under a laminar flow bench. So far, we have not observed a similar reaction in animals implanted with these new electrodes (data not shown).

In the present study, we did not observe any difference in the number of fibres among the four groups. The estimator of the number of fibres (eqn 1) at the mentioned variance (15%) can thus clearly be defined as a uniform random estimator (CRUZ-ORIVE and WEIBEL, 1981). Also, the number of animals was adjusted at the minimum level needed to show fibre losses larger than 25% as a physiological variability of this parameter of about 15% was expected. Although the assumed number of axons and the actual number in the CO group differed, both the variability and the number of axons permitted us to work at the required statistical power level (0.95), with six nerves per experimental condition. The number of axons was fairly constant in the four experimental conditions, i.e. the largest variation was about 12% between the CO group and the FC group. Therefore a principal first conclusion from our study is that the number of fibres was quite similar, with comparable variation ranges for all experimental conditions. In the study mentioned above (LARSEN *et al.*, 1998), no significant changes in the number of axons could be observed, 16 months post-implant, at either of the cuff levels.

Epon sections were used for fibre diameter and myelin thickness measurements. In this study, a random subset from the entire cat sciatic nerve section was first selected, and an equivalent nerve area was then examined. In other investigations (LARSEN, 1998; MAYHEW and SHARMA, 1984*a*, *b*) performed on smaller nerves, the reference system with respect to which the stereological data are expressed is the entirety of the nerve, and a variable subset is chosen for the taking of measurements. The different design chosen in the present study comes from the necessity to obtain reasonably well-stained sections. Epon sections presented more homogeneous cuts under about 2 mm^2. Therefore we decided to cut out an area of about 3–4 mm^2 of the epoxy block, but no *a priori* knowledge of the nerve structure was available when this procedure was performed. We thus consider that our random selection was also unbiased.

Linear regression slopes of the perineurium thickness against the fascicle surface showed no significant differences in any experimental condition. Most individual nerves exhibited a linear relationship between the fascicle area and the perineurium thickness, as was expected (SUDERLAND, 1968), but, at the group level, the correlation between these two variables was weaker. This was probably the result of the biological variability. In contrast, the line intercepts for one implanted group (BC) were found to be significantly different from those of the two non-implanted groups, indicating a perineurium thickening. The other implanted group (FC) also showed a higher value compared with the two non-implanted groups, but without reaching a statistically significant level.

As the fascicle surface remained similar for the four groups, it cannot be considered as a contraction of the perineurium resulting from missing fibres (SUDERLAND, 1968). Thus, this perineurium thickening may well be a primary reaction against the lack of space for the surrounding connective tissue. This finding can be considered a minor pathological change or even may just be incidental, for, as was pointed out before, a high degree of biological variability was present in these measurements. In summary, this finding may well suggest a trend

towards perineurium thickening, but the number of animals is insufficient to sustain that conclusion. It must be stressed that some perineurium thickening has been described in the case of nerve damage due to chronic implantation (MACKINNON *et al.*, 1984; 1985; LARSEN *et al.*, 1998).

Qualitatively, in all groups, fibre diameter histograms presented a biphasic pattern, with peaks situated around the same positions. Our results exhibit different peak locations from those described in other studies, i.e. at 3, 6 and 16 μm (LLOYD and CHANG, 1948) or at 2.5, 4.6 and 11 μm (DYCK *et al.*, 1984), in the same species. These differences can be attributed to the fact that these authors made their measurements from other nerves and that they used different histological protocols. As both peak patterns observed in this study are evenly distributed among experimental conditions, it is considered a minor effect for the conclusions of this investigation.

Regarding the fibre measurements, analysis of variance (ANOVA) on central and dispersion statistics did not show significant differences. Also, fibre diameter histograms indicated no changes among groups. LARSEN *et al.* (1998) found a unimodal pattern for the fibre diameter distribution and reported that, 16 months post-implant, 20% of the largest axons had not regained their original size.

Myelin sheath thickness showed a pattern with two peaks, but with a higher peak for the larger values of myelin thickness in the implanted nerves. This trend is most probably casual, but it may also express a primary defense reaction of some fibres against the external aggression, that is, the physical limitation imposed by the implant. In LARSEN *et al.* (1998), the geometric mean myelin sheath areas as well as the axon area-myelin sheath area, were unchanged 16 months after implantation.

Different degrees of nerve structural changes have been reported after long-term implantation. For instance, MACKINNON *et al.* (1984) implanted rigid cuffs (1.5 mm diameter) in rat sciatic nerves (1 mm diameter) for up to 6 months. After 4 months, stable morphological changes had been established and showed epineurium fibrosis and perineurium thickening, with a decrease in the large myelinated fibres in the periphery of the fascile. Also, LARSEN *et al.* (1998) conducted a study in which rigid cuffs with and without leads were chronically implanted. These investigators reported that, 2 weeks after the implant there were significant losses of myelinated axons underneath, distal to the cuff, with a partial recovery at 16 months. In the present study, 4 months after surgery, considered to be sufficient for most histological changes to stabilise (MACKINNON *et al.*, 1984; 1985), none of these effects was observed, except for the epineurial fibrosis.

When comparing our study with the results obtained by NAPLES *et al.* (1988), with snugly fitted spiral cuffs, we failed to observe similar myelin and axon losses, perineurium thickening, increased intraneural tissue and axonal swelling. Perhaps these discrepancies could be explained by the selection of the implantation site. Indeed, we always performed implantations proximal to the bifurcation point of the sural branch, which is about 4–6 cm from the tibial-peroneal bifurcation. At sites such as the popliteal fossa, the potential trauma to the nerve is larger, as the nerve has to flex, and the surrounding muscles have to move more, relative to the nerve.

The fact that there are more adequate locations for nerve implantation is suggested by other investigators (KIM *et al.*, 1983; GLENN and PHELPS, 1985). KIM *et al.* (1983) evaluated 53 canine phrenic nerves implanted with cuff electrodes. Only two nerves presented focal loss of myelin at the cuff level. Also, AGNEW *et al.* (1989) implanted the circumneural helical electrode array on the sacral nerve roots of dogs for up to two years. Histological evaluation of the nerves revealed very little damage. In contrast, when it was implanted on the cat common peroneal nerve, approximately 40% of the nerves sustained significant injury. Damage consisted of 'crescentic' zones of endoneurial connective tissue, which in some instances apparently had replaced degenerated axons, with remyelinating axons at the margins of these zones. No similar endoneurial damage was observed in the present study.

Besides a nerve electrode implant location distant from joints, another characteristic of an ideal mechanical silent site relates to the surrounding muscle behaviour. In the case of the present investigation, the cuff was implanted close to the caudal face of the biceps femoris belly. The implantation site was a bed formed by the semi-membranosus and biceps femoris muscles in which the nerve course and muscle fibres were nearly parallel. Importantly, these two muscles are both extensors of the thigh, thus producing much less friction during contraction than if they had antagonist function. Furthermore, the biceps femoris is a very large, flat muscle that covers much of the lateral surface of the thigh. Such a muscle would protect the site from any external trauma.

Associated conditions likely to lessen the host reaction are: short interventions, minimum damage to neighbouring muscle masses, limited nerve elevation during cuff wrapping as well as using a specific implant tool. If it is necessary to invade a muscle belly, this should be done by splitting the fibres, and the fascial incision should be made close to the belly region.

In conclusion, our findings indicate that long-term utilisation of the self-sizing spiral cuff electrode does not produce a morphological lesion within the nerve fascicles. Also, our results are in favour of a good nerve tolerance of implanted snugly fitting spiral cuff electrodes at appropriate, well-chosen, nerve locations.

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