INFLUENCE OF IMMUNOSUPRESSION ON NERVE REGENERATION USING ALLOGRAFTS: AN EXPERIMENTAL STUDY ON RATS

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SUMMARY

Purpose. This paper was aimed to study nerve regeneration after allografting using conventional point counting technique.

Introduction. The interest towards nerve allografting has been growing since the recent development of better immunosuppressant drugs.

Methods. Three groups were studied: Group A - Lewis rats receiving nerve grafts from isogenic donors; Group B - Lewis rats receiving nerve grafts from Brown-Norway donor rats and treated with saline solution; Group C - Lewis rats receiving nerve grafts from Brown-Norway donor rats and treated with cyclosporine. Nerve regeneration was evaluated by histologi-cal analysis and by histomorphometric studies after 6 and 12 weeks.

Results. At 6 weeks, nerve fiber density and the percentage of neural tissue in the immunosuppressed allograft group (C) were significantly higher than in group B. Allograft groups (B and C) showed significantly lower nerve fibers density and percentage of neural tissue when compared to the autograft group A at 6 or 12 weeks.

Conclusions. We conclude that the point counting method was simpler to use than the computerized model, and yielded accurate and reproducible results.

Keywords: *Neural Regeneration, Cyclosporine, Microsurgery*

INTRODUCTION

Surgical therapy in patients with peripheral nerve injuries has not presented changes over the last decades, especially due to the use of autologous grafts, to the development of intraoperative magnification, and to the proven deleterious effects of tension at neural repair site.

Despite all the advancements achieved, functional repair results are still imperfect. In addition, the collection of donor nerves produces a new neurological sequel. In extensive defects or in several nerves' defects on a same patient, there may not be enough autologous donor nerve to fill that neural failure.

With the increasing understanding capacity and with the manipulation of the immune system, autologous grafts have been proposed as an alternative method in peripheral nerve reconstructions.

Cyclosporin has been widely employed in organ transplantations in association with other drugs, allowing a significant morbidity reduction when compared to early immunosuppression methods[1]. The use of cyclosporin in the transplantation of non-vital organs such as skin, nerve, muscles and ends has been reported in literature[2-5].

Cyclosporin's mechanism of action in nerve regeneration remains controversial[6-9]. And, thus, cyclosporin has been replaced by the drug FK-506 in several nerve allografting protocols[10]. A study series showed a more favorable regeneration in small peripheral neural allografts with the FK-506 when compared to cyclosporin in experimental models with rats[11,12].

Recently, new strategies in peripheral nerves bioengineering have emerged, combined with cyclosporin, to induce tolerance and to enhance transplanted cells' viability[13,14].

In experimental models, nerve regeneration is being evaluated, especially by means of computer-based methods, although conventional morphometric assessments continue to be used in liver evaluations as well as in other pathologies[2,5,6,15]. The use of conventional morphometric models seems to be logical for the assessment of nervous regeneration, particularly in countries where more sophisticated technologies are not easily available.

The purpose of the present article – an experimental study using nerve allografts on rats – is to assess neural regeneration as a morphometric planimetry technique by scoring in nerve allografts with and without immunosuppression.

MATERIAL AND METHOD

Thirty isogenic Lewis (Lew) and Brown-Norway (BN) rats, weighting 200-300 g were used in the study. These species differ for higher histocompatibility locus level[16].

Three groups were built (Table 1). The group constituted of Lewis rats received nerve grafts from syngenic Lewis donors (Group A)or nerve grafts of allogenic Brown-Norway rats without immunosuppression (Group B). Group C (Lew vs. BN) received postoperative immunosuppression with cyclosporin A solution (CsA) at a dosage of 5 mg/kg/day, administered on a daily basis through subcutaneous injection. In groups A (Lew vs. Lew – autogenous control group) and B (Lew vs. BN – allogenic control group), the animals received daily injections of saline solution. The rats were sacrificed within 6 and 12 weeks postoperatively.

Surgical Procedure

All procedures followed a standard microsurgery technique, under an operating microscope. With the divulsion of gluteus muscles, the sciatic nerve was bilaterally exposed and a 1.5 cm graft was removed on each side of donor animals. Sciatic nerve on the right side of receptor animals was similarly exposed and a 0.5 cm neural failure was produced. Nerve grafts were then transferred and sutured with nylon 10-0 wire with epineural stitches.

Morphometric and Histologic Studies

After sacrifice, a fragment of the sciatic nerve including a portion distally to the graft was removed and fixated by pouring them into a 2% glutharaldehyde solution (wt/vol). The tissue was post-fixated with osmium 4-oxide and soaked into hydroxyethylmetacrylate. Toluidine blue was used to stain 2 μm-thick cross-sections to future study under optical microscope. Slices of the distal segment of the allograft were examined by an independent investigator who assessed the overall nerve architecture, amount and quality of fiber regeneration, and the presence/ absence of Wallerian degeneration.

The quantitative histological test was conducted on cross-sections of receptor nerves 5cm distal to the suture line, using a conventional morphometric technique by planimetry with score counting[17]. Data collection was made by an investigator blinded to the kind of group, superposing a 1 cm^2 reticulum with 100 points and type-II Zeiss lines for histological images seen under microscope. With a magnification of 1000x, 10 histological fields were randomly selected and assessed. All myelinated fibers that were superposed onto reticulum lines were counted. With these primary measurements, the following morphometric rates were calculated: fibers density (number of fibers / μm2 - FD), neural tissue percentage (area occupied by neural tissue/ total area of nerve cross-section – NTP) and mean fiber area (μm2 - MFA) of each neural segment.

Statistical Analysis

An overall analysis of the mean differences among groups was measured by a profile analysis for the three morphometric variables. With this multiple-variated technique, the following basic hypotheses were tested:

H_{01}: the profiles of averages are parallel;
H_{02}: the profiles of averages are matching;
H_{03}: the profiles of averages are constant throughout segments.

Whenever these hypotheses showed statistical significance, an analysis was carried out in order to discriminate differences. The significance level employed was 0.05.

RESULTS

1. After 6 weeks:

Histological signs of nerve regeneration were seen in all groups. However, significant differences were noticed for histological characteristics between allogenous and autogenous. In the autografting group (Figure 1), myelinated fibers were noted with a distribution pattern all over the neural tissue. Oppositely, in the allografting group, where less regenerating fibers were seen, a lower degree of myelination was found (Figure 2) and degenerating fibers were present on the tissue. In groups with immunosuppression (Figure 3), more myelinated fibers were found compared to Group B.

Quantitatively, values for fiber density (FD) rates were found to be significantly higher in group C (grafts with immunosuppression) compared to group B (allografts without immunosuppression), but no significant difference was found when FD on group A was compared. When values for neural tissue percentage (NTP) were assessed, values achieved on group C were significantly higher than on group B. These values were significantly lower than the values seen on group A (Table 2).

| Group	Nerve Graft	Injection	Sacrifice
A (n=10)	Lew X Lew (autogenous control group)	Saline solution	6 weeks (n=5) 12 weeks (n=5)
B (n=10)	Lew X BN (allogenous control group)	Saline solution	6 weeks (n=5) 12 weeks (n=5)
C (n=10)	Lew X BN (treatment group)	Cyclosporin	6 weeks (n=5) 12 weeks (n=5)

Table 1 - Experiment design
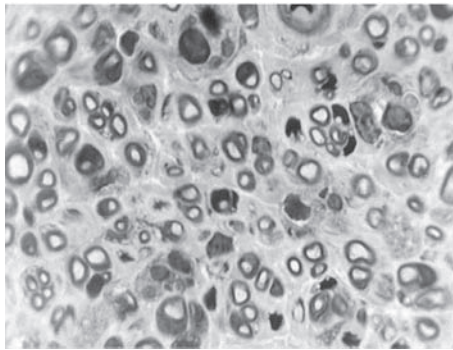
Figure 1 - *Autogenous graft at 6 weeks postoperatively (magnification: 1000 X)*
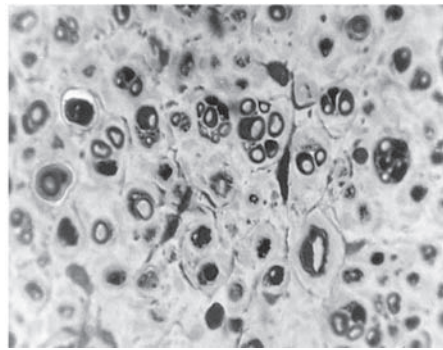
Figure 2 - *Allograft without immunosuppression 6 weeks postoperatively (magnification: 1000 X)*
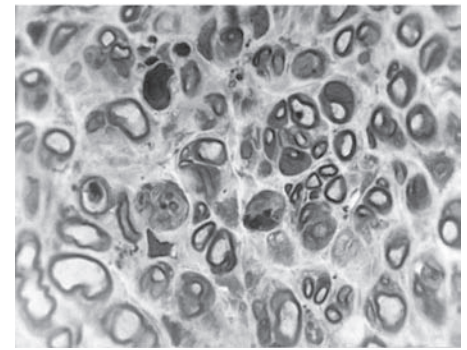
Figure 3 - *Allograft with immunosuppression 6 weeks postoperatively (magnification: 1000 X)*

2. After 12 weeks:

Fiber density (FD) on group A was higher than that observed on the remaining two groups. No difference was found between groups B and C (Table 2). Regarding values for neural tissue percentage (NTP), a superior value was found for group A compared to groups B and C. There was no difference between groups B and C (Table 3).

No differences were found for values for mean fiber area (MFA) among the three groups both in animals sacrificed at 6 or 12 weeks postoperatively (Table 4).

DISCUSSION

The assessment and interpretation of the results achieved with nerve allografts are still controversial due to the uncertain histocompatibility between donor and receptor of different grafting technique and of the complexity of quantitative methods for neural regeneration assessment. Mackinnon et al.[15] proposed an experimental model to study neural regeneration with allografts in rats, applying a computer-based method for assessing results.

In the present study, rat species with established differences on histocompatibility[16], and appropriate cyclosporin dosages[6,18] have been used. Conventional morphometry was employed as a validated alternative to the computer-based method[19].

The use of an electronic apparatus for morphometric analyses has not yet been a target in critical studies for its applications and, particularly, for its limitations[19].

Image modification is a factor that may induce errors when computer-based analysis is used. For the study of tissues such as peripheral nerves, image edition is required for improving

FD		Group A	Group B	Group C
	Average	8.3	3.1	5.8
6 weeks	Standard Deviation	1.9	0.8	2.6
		N=5	N=5	N=5
	Average	15.3	11.4	12.1
12 weeks	Standard Deviation	2.7	2.4	2.1
		N=5	N=5	N=5

Significance level* = 0.05
6 weeks: AXBXC (p=0.0004)* 12 weeks: AXBXC (p=0.0224)*
AXB (p=0.0010)*   AXB (p=0.0097)*
AXC (p=0.0926)   AXC(p=0.0313)*
BXC (p=0.0409)*   BXC (p=0.6070)

Table 2 - *Fibers density- FD (10-3 fibers/µm²)*

NTP		Group A	Group B	Group C
	Average	29.6	9.2	20.8
6 weeks	Standard Deviation	6.11	2.17	7.69
		N=5	N=5	N=5
	Average	51.4	36	38.2
12 weeks	Standard Deviation	6.47	4.06	1.79
		N=5	N=5	N=5

Significance Level* = 0.05
6 weeks: AXBXC (p<0.0001)* 12 weeks: AXBXC (p=0.0001)*
AXB (p<0.0001)*   AXB (p<0.0001)*
AXC (p=0.0133)*   AXC(p=0.0005)*
BXC (p=0.0017)*   BXC (p=0.5105)

Table 3 - *Neural tissue percentage (NTP)*

FMA		Group A	Group B	Group C
	Average	36.45	31.21	37.18
6 weeks	Standard Deviation	5.2	3.82	4.78
		N=5	N=5	N=5
	Average	34.3	32.38	32.2
12 weeks	Standard Deviation	4.95	4.63	5.57
		N=5	N=5	N=5

Significance Level* = 0.05 6 weeks: AXBXC (p=0.8719) 12 weeks: AXBXC (p=0.8719)

Table 4 - *Fibers' Mean Area- FMA (µm²)*

data detection, thus introducing potential biases. Sometimes, it is difficult to assure that the finding on a computer monitor accurately represents its counterpart observed on microscope. Indeed, some of the techniques that are massively used in research which are based on morphologic findings have limited practical application on diagnostic pathologic anatomy[20].

A computer-based morphometry technique does not provide a practical advantage over the conventional counting[19,20], being both methods hard to perform. The reticulum is the most primitive method for image capture, but it can produce accurate and reproducible results[21].

In the present study, the scoring technique[17,22] was regarded as reliable and reproducible. Its experimental validation can be demonstrated by the fact that data obtained in this study are similar to those obtained by Berger et al., who used computer-based morphometric techniques[23].

The use of cyclosporin was associated to a significant increase of fibers density and of the percentage of neural tissue on the distal segment of reconstructed nerves in immunosuppressed animals after 6 weeks postoperatively when compared to the non-immunosuppressed group. At week 12, that difference was no longer significant, a similar finding to that reported by Berger et al[23].

The use of cyclosporin seems to accelerate neural regeneration process only at early stages (6 weeks postoperatively). The rejection process seen on group B (allografts without immuno-suppression) may have been interrupted due to the elimination of Schwann cells from the graft after 6 weeks. Schwann cells might be the element responsible for rejection on peripheral nerves. The rejected graft, however, may serve as a non-cellular neural replacement, considering that there is no additional damage to its connective tissue's structural architecture.

At 12 weeks postoperatively, regenerating fibers on the group without immunosuppression showed a normal growth speed, similarly to the immunosuppressed group. This was probably due to the regeneration promoted by host's Schwann cells that entered into the graft. Schwann cells migration can be demonstrated by the induced rejection response that occurs in nerve segments implanted back into their original donor animals[23].

The faster growth of neural fibers towards their target organs may result in a faster and more effective functional recovery. In clinical practice, functional results observed after peripheral nerve grafting depend on the extension of nerve defect and on its corresponding graft, on the time between injury and repair, and on the quality of functional rehabilitation; and these conditions cannot be simulated on an experimental model. It is clear that the histological results of neural regeneration do not correspond to the degree of functional recovery.

The experimental model presented here is reproducible for the study of nerve allografting, and the morphometric method of score counting may be effectively used for the study of neural regeneration.

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