NEUREGULINS 1-ALPHA AND 1-BETA ON THE REGENERATION THE PERIPHERAL NERVES

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ABSTRACT

Objective: To evaluate the effect of the neuregulins 1-alpha and 1-beta on the regeneration the sciatic nerves of male adult C57BL/6J mice, using the tubulization technique. Methods: Eighteen animals were used, divided into three groups. A polyethylene prosthesis was implanted in a 4.0 mm defect of the left sciatic nerve, as follows: group 1 containing only purified collagen (Vitrogen®); group 2, collagen with neuregulin 1-alpha; group 3, collagen with neuregulin 1-beta. The control group consisted of six segments of right sciatic nerves. After four weeks, the animals were sacrificed. A segment from the midpoint of the nerve regenerated inside the prostheses was extracted; histological sections were standardized, and slides were made up for histomorphometric analysis. Results: the results were statistically compared using the Tukey multiple comparisons test and The Student's t test. The animals treated with neuregulins had greater numbers of myelinized axons, with a statistically significant difference in relation to the collagen-only group. There was no statistical difference between the neuregulin 1-alpha and 1-beta groups. Conclusion: The addition of neuregulins provided a significant increase in the number of myelinized fibers.

Keywords: Neuregulins. Peripheral nerves. Sciatic nerve.

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INTRODUCTION

Traumatic lesions of the nervous system constitute a serious public health problem, as they generally affect individuals at the productive age, demand high hospitalization and rehabilitation costs as well as long periods of leave from work and determine a large number of sequelae. In Brazil, there are no reliable statistics on the number of patients victims of posttraumatic neurological lesions. It is estimated that there are 500,000 new cases per year in the United States, with a cost of hospital treatment ranging from 15,000 to 60,000 dollars per patient.[1]

Since the first report of treatment of peripheral nervous system lesions, the obtainment of satisfactory results after nerve repair has constituted the greatest challenge to reconstructive surgery and to microsurgery. The introduction of microsurgical techniques in the treatment of nerve lesions, the development of specific material (forceps, scissors, tweezers, etc.), the advance of microscopy technology and specialized training, do not yet afford complete neurological regeneration, causing sensibility and motricity deficits.[2,3] The approximation of injured stumps followed by epineural repair under magnification, the absence of excessive tension, use of appropriate threads and instruments, constitutes the gold standard of treatment in the acute phase. After this period, the coaptation of the injured extremities is impossible without causing excessive tension.[4]

In these situations, the autograft, using the sural nerve, the medial cutaneous nerve of the forearm, the saphenous nerve, and others, is widely employed. This technique provides results that fall short of normal standards, and always injures intact structures, giving rise to sequelae, such as paresthesiae, anesthesiae, hyperesthesiae, scars, neuromae and chronic pain.[5]

A promising alternative to the autograft is the tubulization technique, which consists of the introduction of the nerve extremities in cylindrical prostheses, aiming at axon guidance, protection of the growth cone of the adjacent fibrosis, and longitudinal orientation of the neovascularization. It also enables the containment of endogenous and exogenous neurotrophic factors, producing low morbidity and the reduction of surgical time.[4] These days several types of prosthesis have been described and used in experimental research, including polyethylene, collagen, silicon, polyesters, veins, arteries, human amnion, and others.[6,7]

All the authors declare that there is no potential conflict of interest referring to this article.

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The exogenous substance containment capacity promoted by this technique allows the study of countless neuronal regeneration promoting factors, such as aFGF, cardiotrophin-1, oncostatin-M, GM1, CNTF, GDNF, NT-3, NGF, and neuregulins.[8,9] Neuregulins constitute a family of growth and differentiation factors responsible for playing numerous roles in the nervous system.[10] In the Schwann cells, neuregulins promote proliferation, differentiation, survival, maturation and myelination.[11,12]

The physiology of the peripheral nervous system depends directly on the integrity of the myelin sheath, where the Schwann cells play an essential role, from remyelination after injury to functional balance. The activity of neuregulins in the Schwann cells is said to result from their ability to activate the erbB receptors, which apparently determine the actions mentioned.[13]

In spite of the vast potential in the regeneration process of the peripheral nervous system, research on neuregulins in vivo is practically nonexistent.[11,14] The goal is to evaluate by morphometry the axonal myelination of sciatic nerves of C57BL/6J mice, employing neuregulins 1-alpha and 1-beta, using the tubulization technique.

MATERIALS AND METHODS

Animals and experiences

The experimental model adopted employed 18 mice of the C57BL/6J lineage from the Biotherium of the Department of Cellular Biology and Development of the Institute of Biomedical Sciences of Universidade de São Paulo. The treatment dealt out to the animals and the study protocol were analyzed by the Institutional Review Board of the University, and are in accordance with the guidelines of the American Veterinary Medicine Association (AVMA). Lineage C57BL/6J was used due to the ease of manipulation, the availability and the high isogenicity, which conferred uniformity to the study groups.

The study included male mice with mean age of eight weeks, and healthy under macroscopic examination. The presence of infections, cutaneous lesions, musculoskeletal anomalies or other diseases, such as autophagia and consumption of the operated limb or sudden death constituted exclusion criteria.

To form the study groups, the mice were chosen and distributed independently and at random, in order to guarantee unbiased results. The left sciatic nerve was sectioned in all the animals to create a segmental defect 4mm in length, which was treated pursuant to the following description. Two animals were selected, also at random, from each one of the three groups and, in these, a segment of the sciatic nerve from the contralateral (unoperated) thigh was isolated, extracting the total number of myelinized fibers. These animals formed the control group.

Polyethylene tubes containing only purified collagen (Vitrogen®, Collagen Corporation, Palo Alto, CA, USA) were used in the first study group, designated collagen group, to treat the neurological lesion. In the second study group, neuregulin 1-alpha was added to the collagen (250 ng), in the dilution of 100 μg/ml. In the third study group, neuregulin 1-beta was employed in addition to the collagen. The neuregulins were acquired from R&D Systems Inc. (Minneapolis, MN, USA). Each one of the groups was formed by six animals.

The animals received general anesthesia with Avertin® (500 mg of tribromoethanol and 250 mg of 2-methyl-2-butanol, dissolved in 19.5 ml of distilled water), in the dose of 0.02 ml/g of body weight, by intraperitoneal route, in the lower abdomen. Next, the animals were trichotomized and the surgical site was washed with a disinfectant solution of 2% chlorhexidine gluconate, with the animals in prone position on the dissection plate.

A longitudinal incision was made on the posterior side of the left thigh and, by divulsion of the biceps femoris, the sciatic nerve was exposed. It was sectioned crosswise in the middle third, and 1.0mm of each one of the extremities was invaginated in a polyethylene tube (Sigma Chemical Company, St Louis, MO, USA) and joined by a stitch (10.0 mononylon thread, 75μm needle, Ethicon®) and fibrin sealant (Tissucol®, Baxter, Immuno AG, Vienna, Austria). The tubes were 6.0mm long, with an internal diameter of 0.75mm and an external diameter of 1.22mm. Accordingly the distance between the stumps after the procedure was 4.0mm. This procedure was performed with 20x magnification, using a microscope (Zeiss OPM 240F). (Figure 1) The tubes were filled using a Hamilton microsyringe (10μl). The needle was fully introduced in the prosthesis, until it reached the opposite extremity. The material was injected while the needle was slowly withdrawn, taking care not to form air bubbles inside the tube. Next, the tubulization of the opposite extremity was concluded with the application of fibrin sealant, in order to contain any material overflow. After this, the muscle layers were coapted and the skin was sutured with separate stitches (5.0 mononylon thread, Ethicon®). This stage was concluded with another wash using 2% chlorhexidine gluconate.

Postoperative period

The animals were kept in cages with minimum area of 200 cm^2 and adequate quantity of shavings, with no more than three in each unit, in the biotherium of the university, with cycles of 12 hours of light and darkness and a temperature around 20 °C to 25 °C. They received an adequate quantity of food and water *ad libitum*.

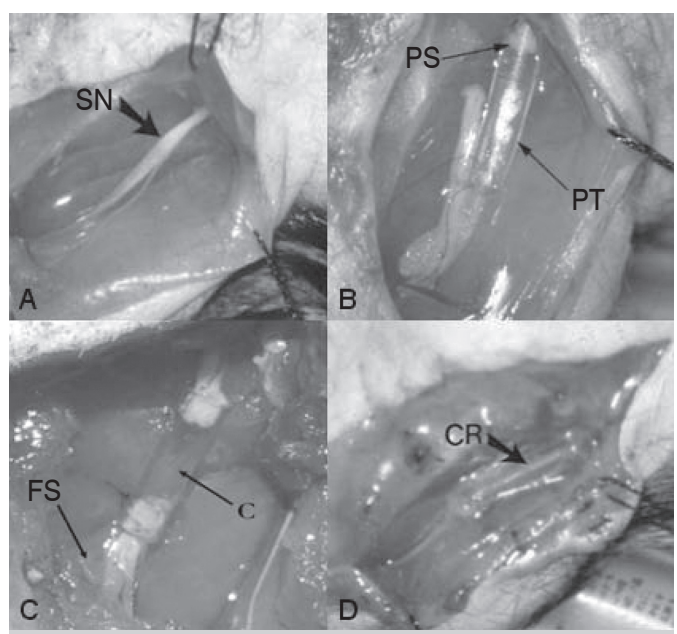

Figure 1 – **A)** *Exposure of the left sciatic nerve (SN).* **B)** *Proximal stump (PS) invaginated in the polyethylene tube (PT).* **C)** *Tube that contains collagen (C) and presence of fibrin sealant (FS) at the extremities.* **D)** *Exposure of the polyethylene prosthesis that contains regenerated nerve (CR).*

Staining and collection of the material

Four weeks after the surgical procedure, the animals were once again submitted to general anesthesia with Avertin®, starting the collection process. A longitudinal incision was made in the middle and upper third of the abdomen, with dissection by planes, and injection of 0.2ml of heparin solution (5000UI/ml) in the spleen. This was followed by the thoracotomy, where a catheter was introduced into the left ventricle, injecting 10ml of heparinized (5000UI/ml) saline solution, followed by 15ml of anti-fade solution comprised of paraformaldehyde (1%) and glutaraldehyde (2%), in a 0.1 M sodium phosphate buffer solution, pH 7.3.

The participants then carried out the exploration of the tubular prostheses, which contained the regeneration cable (regenerated nerve), and were dissected, removed and kept in the abovementioned anti-fade solution, at the temperature of 4°C, for 24 hours. The material was post-fixed in osmium tetroxide (2% in 0.1 M sodium phosphate buffer, pH 7.3) for two hours at 4 °C. Afterwards dehydration was executed through an increasing series of ethyl alcohol, whitened in propylene oxide and embedded in epoxy resin. Transversal 1-μm cuts thick were made at the middle point of the tubular regenerated nerve, stained with 0.5% toluidine blue.

The control group, formed by the contralateral sciatic nerve, was constituted in this phase.

After fixation on slides, the images containing the myelinized fibers were collected by digital camera, interconnected to a PC by stage controller. A digitizer table (Summargraphics) connected to the PC was used for obtainment of the images by the observer. At the end of the surgical stage, the animals were euthanized through an overdose of Avertin® (0.2ml/g), according to criteria established by the American Veterinary Medical Association - AVMA.

Histological study

The middle point of the tube was standardized to carry out the count of the number of myelinized axons present in the regenerated nerve, on each slide. (Figure 2) The count was executed by means of the marking of each axonal unit with the cursor, thus avoiding the counting of the same fiber more than once. With this method in each field, the participants arrived at the total number of fibers in the segment. The Sigma Scan Pro Image Analysis V.5.0.0 software (SPSS Inc. 1987-1999) was used for the count.

Statistical analysis

A confidence interval of 5% ($\alpha = 0.05$) was obtained. The normality of distributions was evaluated with the Kolmogorov-Smirnov test for continuous variables. Parametric distribution was accepted in the cases in which the Kolmogorov-Smirnov test did not present a significant result and in those where Pearson's coefficient of variation was below 30%. All the distributions were accepted as normal (Gauss). Parametric tests were also used.

The variance analysis (three unrelated samples with parametric distribution) was used for the comparisons among the mean axon counts in the transversal cuts of the regenerated sciatic nerves of the mice. The differences among mean values were distinguished by the paired comparison of the samples, with Tukey multiple comparisons test. Student's t-test (unrelated pairs of samples with parametric distribution) was used to infer the differences of the mean numbers of axons formed inside each kind of tube, in relation to the number of normal axons (control).

The GraphPad Prism version 2.01 software (GraphPad® Software, Inc., 1996) was used for the statistical analysis.

RESULTS

No systemic immunological reactions or exuberant local inflammations were observed. Neither there was excessive fibrosis or release of the nerve extremities in the paws of the mice that underwent surgery. The regenerated nerve was found in all cases.

Table 1 shows the total number of regenerated axonal fibers in the control group, which was higher than in the mice that received

Table 1 – *Count of axons in the transversal cuts of the sciatic nerve.*

Sciatic nerve	Number of axons			
	Control	Collagen	Collagen + Neuregulin 1-alpha	Collagen + Neuregulin 1-beta
Mean	4354.7	2685.5	3400.5	3486.2
Standard deviation	47.2	161.7	357.6	371.3
Standard error of the mean	19.3	66.0	146.0	151.6
Maximum	4417	2847	3812	3802
Minimum	4291	2461	2914	2837
n	6	6	6	6
Kolgomorov-Smirnov	0.17	0.23	0.19	0.23
Pearson's coefficient of variation	1.1	6.0	10.5	10.6

Student's t-test: control x collagen $t = 24.28$; $p \cong 0.00$
Student's t-test: control x collagen + neuregulin 1-alpha $t = 6.48$; $p \cong 0.00$
Student's t-test: control x collagen + neuregulin 1-beta $t = 5.68$; $p = 0.0002$

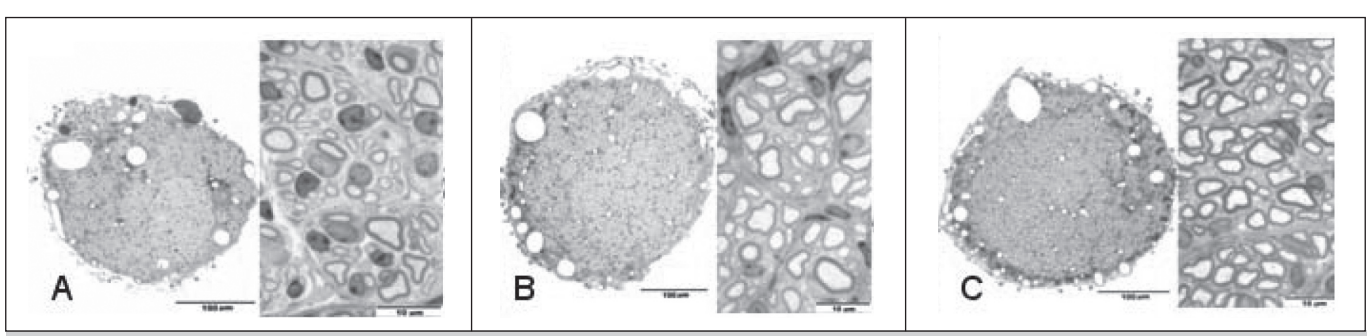

Figure 2 – **A)** *Microphotography of histological cut of the sciatic nerve of a mouse from the collagen group.* **B)** *Collagen plus neuregulin 1-alpha group.* **C)** *Collagen plus neuregulin 1-beta group. Staining with toluidine blue. 100 X magnification.*

collagen plus neuregulin 1-alpha and collagen plus neuregulin 1-beta, after four weeks of evolution. It was also verified that the groups with increase of neuregulins presented a larger quantity of myelinized fibers than the group that received only collagen. On the other hand, the groups with neuregulins 1-alpha and 1-beta had similar total numbers of myelinized fibers. The results show that there was a statistically significant difference between the collagen group and the neuregulin groups. No significant difference was verified between the neuregulin 1-alpha and 1-beta groups. Statistically significant differences were found between the control group and the other groups.

DISCUSSION

Lesions that affect the nervous system are the main challenge of reconstructive surgery and microsurgery, whether resulting from trauma or from neoplasia.[2] The high cost of treatment, the long duration of rehabilitation, the prolonged time of absence from work, the high incidence among individuals at a productive age and the current impossibility of reaching normal sensory and motor patterns after treatment place an undesirable emphasis on posttraumatic neurological lesions.

The complex physiology of the peripheral nervous system during rehabilitation after traumatic events is a topic that has been intensely investigated and insufficiently understood. Many factors are implicated in this panorama, from Wallerian regeneration of the distal segment to abnormalities of the cerebral cortex,[15] including modifications of the body of the neurons, migration of cells, chromatolysis, metabolic interactions, proliferation of Schwann cells and activation of neurotrophic factors.[16] Important items in post-treatment prognosis are: extension of damage in the adjacent tissue, degree of epineural rupture, extension of associated lesions (especially vascular lesions) and age and general state of the patient.[3,17] Many studies were conducted aiming to obtain further knowledge of these events and their interrelations.

The introduction of microsurgical techniques and their development in recent years has contributed to the obtainment of better results.[2] The ideal treatment should be epineural neurorraphy from one extremity to the other during the acute phase, without excessive tension. Nevertheless, even if a procedure of this nature is guided by meticulous technical standards, it would be unable to promote total functional recovery, as the surgeon does not act directly on the physiology of the damaged nerve.[3] This situation has produced many lines of study, such as the addition of fibrin sealant to neurorraphy, association with the neurotrophic factors NGF, GDNF and aFGF, electroacupuncture, low-intensity ultrasound and phototherapy.[16,18]

Evolution to the chronic phase or the impossibility of compatibilizing nerve stumps favors the poor prognosis. Excessive tension entails unsatisfactory results, as the increase of intraluminal pressure inhibits neovascularization and hinders the axoplasmatic flow, the action of endogenous neurotrophic factors, cell migration and the advance of the growth cone. The gold standard of treatment in these cases is autograft interposition to eliminate excessive tension. The main donor nerves are the sural nerve, the saphenous nerve, the medial cutaneous nerve of arm and forearm, the lateral femoral cutaneous nerve and the posterior femoral cutaneous nerve, great auricular nerve the and part of the distal posterior interosseous nerve.[7]

In spite of the widespread use of autograft, this technique produces results that are below par, as the growth cone should cross two suture lines in a hypovascularized segment, and there is a limit for the quantity of donor tissue. This method is associated with undesirable situations, such as increase of surgical time, need for adjacent anesthetic procedures, hemorrhages and lesions in places that were previously normal, thus causing paresthesia, hyperesthesia, anesthesia, scars, neuromae and chronic pain.[4,19,20]

The tubulization technique is a promising alternative to autograft. It consists of the invagination of the extremities of damaged nerves in cylindrical conduits, aiming to provide a favorable microenvironment for the guidance of the segment being regenerated, to support the migration of Schwann cells, macrophages and fibroblasts, to permit the absence of excessive tension on the stumps, to avoid associated dissection and to reduce surgical trauma.[7,20,21] Moreover, the presence of angiogenesis inside the tubes, originating from the proximal and distal segments, acts as a biological support for cell migration and for the migration of intrinsic neurotrophic factors.[22]

The containment capacity provided by the tubes makes it possible to conduct a wide range of studies in pursuit of further knowledge regarding the molecular, cellular and psychochemical interactions directly or indirectly related to the regenerative process. Therefore, several substances that favor neural regeneration can be tested, such as collagen, laminin, IGF-1, aFGF, BDNF, NT-3, CNTF, LIF, GGF, GDNF, NGF and neuregulin 1-beta. The segmental interposition of autologous grafts, muscles and autografts enriched with Schwann cells was also analyzed.[8,9,16,22,23]

Studies of the composition of conduits have not yet conceived the "ideal tube", which would be characterized by easy production, with adequate internal diameter and wall thickness, easy implantation and sterilization, presenting good flexibility, biodegradability and low toxicity. Among the most frequently studied materials are tubes of polyethylene, silicone and bioabsorbable collagen.[20,24] Some structures were also tested, such as arteries, veins, human amnion and other biodegradable materials.[25,26] Both the experimental series[21,22,27,28] and the clinical series[4,5] did not demonstrate statistically significant differences between tubulization and conventional autografts.

The presence of the distal stump is a decisive factor for the development of the proximal stump, possibly on account of neurotrophism.[29,30]

Schwann cells play a fundamental role in the regeneration of the peripheral nervous system. Some examples of their roles: migration to the damaged site on both stumps; production of neurotrophic factors;[31] regulation of axonal growth that precedes remyelinization; supply of spatial orientation;[32] organization of the compartments around the axons, by the recruitment of adhesion molecules and enabling the accumulation of protein and the formation of ion channel;[33] driving of axons between and among contiguous synapses and provision of conditions for maturation of the neuromuscular synapses.[34] Among the substances capable of causing stimulation of Schwann cells, those that merit special emphasis are the neuregulins. These proteins, which have not yet been studied much, play a crucial role in the stimulation of mitoses, of initial growth of the neural crest, survival, proliferation, development, differentiation, migration, maturation and myelination of Schwann cells.[10,11,35,36]

Schwann cells are activated through tyrosine kinase receptors from the erbB family, especially erbB2 and erbB3.[13,37]

In the experimental model adopted, the polyethylene prostheses[24] were used due to availability, safety and ease of handling, from their sterilization through to the surgical procedure. The other surgical intervention was performed four weeks later,[23] and the regenerated nerve can be observed in all the cases, surrounded by liquid inside the tube, as observed by Lundborg et al.[29] and Fields et al.[6]

The regeneration evaluation method through the count of the number of myelinized axons is safe, reliable and direct, characterizing the trustworthiness of the direct and objective analyses of the images studied.[18,19,23,27,29,38]

The total number of myelinized fibers in the control group of our study was similar to the findings of Henry et al.[19]. Cai et al.[9] published a study in which they performed tubulization in a 1.4cm defect in the sciatic nerve of rats, using prostheses that contained microfilaments, Matrigel® and neuregulin 1-beta. They demonstrated superior results in the groups with microfilaments (with or without neuregulins) and, among them, better results using associations between neuregulins and microfilaments.

While Holmes et al.[39] suggested that the alpha subtype is more powerful than beta, Jones et al.[40] found that the beta subtype was more powerful than alpha when investigating the specificity and affinity of neuregulins by the erbB receptors. All these studies were carried out in vitro. In this study, no statistically significant differences were verified between the 1-alpha and 1-beta subtypes. The hypothesis derived from these results is that neuregulins allegedly stimulate the migration and the mitosis of Schwann cells, thus allowing greater speed and quality of neural regeneration.

Neuregulins need to be studied in future projects, aiming to corroborate the hypothesis proposed by means of associations with other neurotrophic factors, using the tubulization method or the addition of sealant fibrin, in termino-terminal, termino-lateral or autograft-associated neurorraphy procedures.[38]

CONCLUSION

Neuregulins 1-alpha and 1-beta promote improvement in the nerve regeneration process, expressed by the number of regenerated nerve fibers.

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