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Regenerative Capacity and Histomorphometric Changes in Rat Sciatic Nerve Following Experimental Neurotmesis

Capacidad Regenerativa y Cambios Histomorfométricos en el Nervio Ciático de Ratas Luego de una Neurotmesis Experimental

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SUMMARY: Through a wide range of cellular and molecular events, the peripheral nervous system is endowed with great regenerative capacity, responding immediately to injuries that occur along the length of the nerve. The aim of this study was to histomorphometrically assess the degree of maturity of the nervous tissue and possible microscopic changes in newly formed nerve segments 60 days after experimental neurotmesis of the sciatic nerve in rats. Control Group (CG) and an Injury Group (IG) were used. IG underwent neurotmesis of the sciatic nerve of the right foot, with immediate surgical repair using the tubulization technique. 60 days following experimental surgery, animals from both groups had their sciatic nerves collected for histomorphometric analysis. Statistical analysis was performed, using the Student t-test for independent samples, expressed as mean ± standard deviation, with 5% significance. In the event of injury, peripheral nerve tissue is mobilized in an intrinsic self-healing process. 60 days following of nerve regeneration in neurotmesis injury, the peripheral nerve presents a segment joining the newly formed neural stump. The new stump has a number of regenerated axons compatible with an intact nerve, but which still show great immaturity in the axonal structural layers of the nerve.

KEY WORDS: Nerve Tissue; Nerve Regeneration; Peripheral Nerve Injuries; Vasa nervorum; Histology.

INTRODUCTION

Due to its length, path and external location to the bone framework that protects the central nervous system, the peripheral nerve is extremely vulnerable to injury (Yegiyants *et al*., 2010) resulting from direct trauma, stretching, laceration with sharp objects, as well as bone fractures and iatrogenesis (Eser *et al*., 2009).

Thus, the intensity of nerve injury depends directly upon the degree of impairment in the functional unit of the nerve, the nerve itself, and the framework of connective fiber, which includes the: epineurium, perineurium and endoneurium (Lee & Wolfe, 2000; Yegiyants *et al*.).

Several studies have attempted to investigate possible non-invasive methods that can contribute to the regenerative process (Chang *et al*., 2005; Teodori *et al*., 2011; Medalha *et al*., 2012). However, the peripheral nervous system is intrinsically endowed with regenerative capacity, so that the occurrence of peripheral nerve injury in cells belonging to the injured nerve tissue – such as Schwann cells – are essential for rapid identification and response to injury. This is achieved via the release of cytokines to signal the recruitment of macrophages to the site of injury (Shamash *et al*., 2002).

This coordinated series of cellular and molecular events is part of the repair mechanism of the peripheral nerve, triggered by the Wallerian degeneration process, which presents a true cleaning of the affected segment following injury. This creates space for the formation of new tissue that is intact and functional (Be'eri *et al*., 1998; Shamash *et al*.).

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Moreover, the Schwann cells, located in the full portion of the stump proximal to injury, alter their phenotype and acquire proliferative andmigratory capacity. They move distally and cross the perineurium of nerves during regeneration (Cheng & Zochodne, 2002) to form longitudinal bands – Büngner bands – which serve as a framework for the growth of each nerve fiber, and lead to their regeneration (Son & Thompson, 1995; Cheng & Zochodne).

When evaluating the regeneration process, it is important to take into account the autonomy of regenerative nerve tissue itself, so that we can identify and intervene at the time that the physiological regeneration process is not able to repair the injured tissue. In order to establish a morphological pattern that can explain how the nervous tissue can be repaired independently under viable conditions for regeneration, studies that evaluate histomorphometric parameters of the newly formed nerve tissue are important.

The aim of this study was to evaluate the body's intrinsic ability to repair nerve tissue by measuring variables patterns of histomorphometry in peripheral nerve tissue. The sciatic nerve underwent neurotmesis and the regenerative process was free of external interference.

MATERIAL AND METHOD

The sample consisted of 9 male albino Wistar rats, weighing 254-290 g obtained from the animal laboratory of the Department of Nutrition at the Federal University of Pernambuco – UFPE. The animals were housed in plastic, propylene cages (49 x 34 x 16 cm) and kept in laboratory for experimentation by the Department of Anatomy at UFPE. The temperature was kept at 23±2 °C with a 12:12 h reversed light/dark cycle. The animals had free access to food and filtered water.

This study was approved by the Ethics Committee on Animal Experimentation at the Federal University of Pernambuco (Letter No. 359/11– Case No. 23076.008415/ 2012-95).

Experimental Groups. The animals were randomly divided into 2 groups: Control Group (CG, n= 4), animals with sciatic nerve intact; and Injury Group (IG, n= 5), animals whose sciatic nerves underwent neurotmesis. After inducing peripheral nerve injury to facilitate neurotmesis, the animals underwent the tubing technique to facilitate nerve regeneration.

Surgical Procedure. When they reached about 60 days, the animals were pre-anesthetized with atropine (0.044 mg/kg) 10 minutes prior to being anesthetized with a solution of 2% xylazine (Rompum® – Bayer) and ketamine (Ketalar®) 1:1 intramuscularly in 0.2 ml of solution per 100 g of body weight. Afterwards, an incision was made in the posterior paw in order to pull back the gluteus medium and maximus, and hamstring muscles for visualization of the sciatic nerve.

Nerve transection was performed using surgical scissors, with no loss of nerve tissue at the time of the experimental incision. Immediately after sectioning, the neural stumps experienced little spontaneous retraction. Thus, the stumps could be introduced, without traction, into a polyethylene tube (9 mm x 0.8 mm) filled with a solution of matrigel (BD MatrigelTM Bioscience). The neural stumps were separated 5 mm apart and formed a closed compartment. The epineurium was sutured 2 mm from the end of the tube (Braga-Silva *et al*., 2006).

Processing and Data Analysis. The animals were anesthetized 60 days after nerve injury, using the same procedures described above in the initial surgical procedure. This was followed by the collection of the newly formed nerve fragment present inside the polyethylene tube.

The fragment of the sciatic nerve was pre-fixed in situ by administering 1 ml of Karnowisky solution (2.5% glutaldehyde, 4% paraformaldehyde and 0.1 M sodium cacodylate buffer , pH = 7.4) 1 minute prior to nerve removal. A 5 mm segment of nerve was collected and maintained for 24 hours in Karnowisky solution and post-fixed with 1% osmium tetroxide in a 0.1 M (pH 7.4) sodium cacodylate buffer for two hours. It was also immersed in 5% uranyl acetate for 24 hours, dehydrated in solutions of increasing acetone (50%, 70%, 90% and 100%) and embedded in epoxy resin.

The transverse cuts of the nerve were stained with toluidine blue solution (1%) and analyzed using an optical microscope (Olympus – BX50, 1000x magnification), and connected to a video camera (Samsung – SHC – 410 NAD) and a computer with TV Tuner Application software to capture images.

The following programs were used: Mesurim Pro 0.8 to count the number of fibers and blood vessels (vasa nervorum), and ImageJ for measurement of the area of the transverse section of the sciatic nerve, diameter of myelinated fibers and axonal diameter. Then, the variables of fiberdensity, thickness of the myelin sheath, and g-ratio were calculated.

Statistical Analysis. Data were analyzed using the Prism 5.0 program and the Student t-test for independent samples. Results were expressed as Mean ± standard deviation, with 95 % as the level of reliability.

RESULTS

At 60 days post injury, the regenerated nerve fragments present in the Injury Group showed smaller values of axonal diameter, diameter of myelinated fibers and thickness of the myelin sheath than the Control Group ($p<0.05$). The newly formed tissue also showed intense vascularization (Table I).

Despite the immaturity of the regenerated myelinated fibers, the g-ratio showed that nerve fibers in the newly formed segment presented with normal mylelinization, proportional to the size of the regenerated myelinated axon formation, thus, giving rise to the capacity to conduct nerve impulses.

With respect to the absolute number of myelinated fibers, we found that the newly formed segment, at 60 days of nerve regeneration, showed a similar amount of myelinated fibers as in intact nerve. However, with regard to the g-ratio we found that the density of myelinated fibers in damaged nerve was significantly greater; demonstrating clearly that the cross-sectional area of this group presented numerically lesser than the Control Group (Table I).

Table I. Histomorphometric analysis of the parameters evaluated in the groups.

Groups	Myelinated Fibers (µn)	*Vasa nervorum*	Density of the Fibers (µn/µm_)	Ø axonal (µm)	Ø of the Myelinated Fibers	Myelin Thickness (µm)	G-ratio
CG	8749 ± 754	52 ± 7	31 ± 2	2.49 ± 0.09	3.62 ± 0.26	0.56 ± 0.09	0.69 ± 0.03
IG	10442 ± 1327	238 ± 72*	83 ± 15*	1.53 ± 0.20*	2.02 ± 0.19*	0.25 ± 0.03*	0.75 ± 0.04*

CG= Control Group; IG= Injury Group; Ø= diameter. *Differs from CG. $p<0.05$

DISCUSSION

Neurotmesis is the most severe traumatic injury that can occur in the peripheral nervous system. It can cause disruption to the nerve, prevent that spontaneous regeneration occurs, and to restore function in the denervated limb. Thus, surgical repair is essential to enable aviable regeneration in this type of injury (Lee & Wolfe; Siqueira, 2007, Campbell, 2008).

In the present study, using the tubilization technique, the neural stumps in regeneration had their growth guided and protected from infiltration by scar tissue, which could have invaded the space of the nerve growth factor (Pfister *et al*., 2007). This reduced the influence of external factors, allowing only cells and tissue elements normally present in the nerve trunk to influence the regeneration process (Belkas *et al*., 2004).

The results of this study showed that the variables of thickness of the myelin sheath, axonal diameter and myelin fiber diameter, were statistically lower than those found in a healthy nerve. This demonstrated that the 60-day period of regeneration is insufficient to reach the stage of axonal maturation, which is described as the last stage of the regenerative process, and which is vital to restoring the size of the axon and returning to nerve impulse conduction (Verdú *et al*., 2000).

This process is supported by progressive metabolic changes with increased synthesis of lipids and protein constituents of myelin, in order to increase the thickness of the myelin sheath and the size of regenerated axons (Sunderland, 1991).

The high metabolic activity still present in these regenerating fibers is marked by intense vascularization, where the amount of vasa nervorum in the injured nerve excessively exceeds the values shown in healthy nerve. This reflects the importance of angiogenesis for nervous tissue regeneration via the enabling of the delivery of oxygen and nutrients needed for the maturation of regenerated tissue (Carmeliet & Storkebaum).

However, as first described by Schmidt & Bear in 1937, the evaluation of myelinization of regenerating axons must not be limited to assessing myelin thickness. It is also important to consider the proportion of the myelin formed in relation to the size of the respective axon. Thus, the g-ratio variable (axonal diameter/overall fiber diameter) relates to the way in which the nerve impulse is transmitted in a saltatory fashion, presenting normally myelinated axons with g-ratio values ranging between 0.65 and 0.80. The higher ratio values represent a thinner myelin, while lower values represent a thicker myelin (Ansselin *et al*., 1997; Stopiglia *et al*., 1998).

G-ratio in the Control Group showed a thicker myelin, but no significant differences when compared to the Injury Group. This indicates that at 60 days post-neurotmesis, despite the presence of axonal immaturity, fibers can be normally myelinated, facilitating nerve impulse conduction (Stopiglia *et al*., 1998).

Inside the guide tube, nerve regeneration is guided by Büngner Bands. Attempting to connect to the target organ, axons will mature and return to a diameter close to normal (Fawcett & Keynes, 1990). Numerous axonal segments begin to sprout from the terminals of the nodes of Ranvier, with myelinated and unmyelinated fibers intact in the segment proximal to the injury (Fawcett & Keynes; Belkas *et al*.).

This process is called poly-innervation, which has its peak between 21 and 25 days after injury (Sobral *et al*., 2008), and is responsible for the final number of axons that will integrate with the regenerated nerve. Axons that can establish a connection to the target organ will mature and return to a diameter close to normal (Fawcett & Keynes). Conversely, those that do not effectively reach the target organ are removed during the process of synaptic elimination (Favero *et al*., 2007; Fawcett & Keynes).

After the absolute numbers of myelinated fibers that make up the nerve were measured, no significant difference in the number of myelinated fibers in intact and injured nerves was found (Sobral *et al*., 2008).

Despite presenting a compatible number of myelinated fibers with intact nerve, the fiber density per cross-sectional area not only revealed the immaturity of the axons, as previously noted, but also the connective tissue layers of the nerve. This was noted mainly in the peri and epineurium, where strong and intact nerve appears as dense layers, which give the nerve support, cushioning and resistance to tension (Bové, 2008; Mizisin & Weerasuriya, 2011; Yegiyants *et al*.). These factors contributed to integrating the most robust cross – sectional area identified in the Control Group.

In the event of injury, peripheral nerve tissue is mobilized in an intrinsic self-healing process. After 60 days of nerve regeneration in neurotmesis injury, the peripheral nerve presents a segment joining the newly formed neural stump. The newly formed nerve segment has a number of regenerated axons compatible with an intact nerve, but which still show great immaturity in the axonal structural layers of the nerve.

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RESUMEN: Mediante diversos procesos celulares y moleculares, el sistema nervioso periférico tiene una gran capacidad regenerativa, respondiendo inmediatamente a las lesiones ocurridas a lo largo de su extensión. El objetivo de este estudio fue evaluar histomorfométricamente el grado de madurez del tejido nervioso y los posibles cambios microscópicos en los segmentos nerviosos recién formados 60 días después de la neurotmesis experimental en el nervio ciático de ratas. Se utilizaron 9 ratas (Wistar) separadas en grupo control (GC, n= 4) y Grupo lesión (GL, n= 5). A los 60 días de vida, el grupo GL fue sometido a neurotmesis del nervio ciático de la miembro posterior derecho, con inmediata corección quirúrgica con la técnica de tubolización. Completados 60 días luego de la cirugía experimental, los animales de ambos grupos fueron anestesiados y sus nervios ciáticos seccionados para el análisis histomorfométrico. Se realizó un análisis estadístico utilizando la prueba t de Student para muestras independientes, expresado como media ± desviación estándar, con un 5% de significancia. A los 60 días de la lesión por neurotmesis, el nervio ciático del GL presentó alteraciones histomorfométricas significativas para las variables: número de *vasa nevorum*, densidad de fibras mielínicas, diámetro axonal y de fibras mielínicas, espesor de la vaina de mielina y razón G, con similitud solamente para los números absolutos de fibras mielínicas regeneradas. El nervio periférico durante su proceso regenerativo, pasa por grandes alteraciones estructurales, siguiendo una secuencia coordinada de acciones, que dependiendo de las condiciones del microambiente donde ocurre esta regeneración, podrá ser clave para el nivel de regenerecion nerviosa periférica.

PALABRAS CLAVE: Tejido Nervioso; Regeneración Nerviosa; Traumatismos de los nervios periféricos; Vasa nervorum; Histología.

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