Original Research
Anatomy

Guided neural regeneration with autologous fat grafting and oxygen hyperbaric therapy

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Abstract: The loss of continuity of the nerve structure interrupts the transmission of nerve impulses and leads to the disorganization of functional activities. Many methods, as the use of neurogenic factors, aid in the process of neural regeneration by accelerating or improving peripheral nerves neoformation. The adipose tissue is abundant in the human body, and it has presented promising results in the regeneration of peripheral nerves. We carried out a randomized controlled study in 9 months, using 45 male Wistar rats, 80 days old, and the sciatic nerve was chosen for analysis. The control animals were divided into three groups – Initial group (IG), Final group (FG), and denervated group (DG) – with seven animals each. The experimental groups, with twelve animals each, were polyethylene tube filled with fat (EGF) and polyethylene tube without filling (EGwf). All groups, except IG, were submitted to 10 sessions of hyperbaric oxygen treatment of 1h 45 min in alternating days. Functional evaluation by walking-track was assessed using the Catwalk XT® software and tissues were harvested and stained with 1% toluidine blue for histological analysis. Quantitative data were first analyzed with the Kolmogorov Smirnov normality test. Comparison between the four groups was analyzed by ANOVA followed by Tukey Test. We concluded that hyperbaric oxygen therapy had positive results on morphometric and functional parameters. However, no significant differences were found regarding the use of autologous fat graft.

Keywords: Nerve Regeneration; Neurons; Nervous System; Neuronal Plasticity; Peripheral Nerves.

Introduction

Peripheral nerve injuries (PNI) are among the most disabling conditions in the working age population, especially those with sequelae. Traumas that cause a nerve laceration or stretching, or nerve root avulsion, produce important impairs in skin or muscle receptors, including the dorsal root ganglion in the medulla, changing the cortical portion of the central nervous system (CNS). Impairments can be transient or permanent (irreversible damage).

Seddon[1] classified peripheral nerve injuries into three types – Neuropraxia – mild injury, which recovers in weeks to months; Axonotmesis – injuries commonly caused by crushing or stretching and follows a complete peripheral degeneration; Neurotmesis – the nerve has all essential structures severed.

Some methods are available for the recovery of injured nerves. Primary or end-to-end neurorrhaphy stands out as the resource that presents the best histological and functional results for neural repair.[2] However, in some cases the suture of nerves is impracticable due to loss of structure, with the formation of a gap, making it necessary to use other methods for the rehabilitation of the injured region.[3,4]

The tuberization technique using a nonabsorbable or absorbable tube has been showing satisfactory results, but its clinical use has been limited since it needs to surgically approach another region of the organism. Therefore, researchers have been investigating new approaches, as different types of tube, biomaterials, and the ideal nerve conduit for peripheral nerve healing. A study carried out by Toledo[3] suggested using the conduit filled with autologous fat removed in the same surgical field. Some technical inconveniences occurred such as clogging by excess fat, displacement of the tube, and loosening the suture. Rejections with the type of material used to fill the tube are also possible. The present work proposed to develop a porous polyethylene tube with the placement of autologous fat as a neurogenic factor. Also, the use of a hyperbaric chamber was suggested with the expectation of helping the regeneration process.

Based on this information, a randomized controlled study was conducted to evaluate the morphometric and functional effects of hyperbaric oxygen therapy (HOT) in animals submitted to neural repair through porous polyethylene tubes with and without autologous fat filling.

Methodology

Trial designs

This is a randomized controlled study, divided into two experimental groups and three controls, submitted to morphometric analysis and functional analysis through the software Catwalk XT®. There were no deviations from the recommended protocol, as can be seen in the flowchart below (Figure 1).

The polyethylene tube used in the present study was made by Engimplan Medical devices (Rio Claro, SP, Brazil) exclusively for this work. The tube was 12 mm in length, 0.25 mm in thickness, and 2.5 mm in diameter, and the pores were made by laser in an equidistant manner (Figure 2).

Animals

Male rats were selected due to the absence of hormonal instability found in female animals. Young animals, within 80 days of life, were used because of greater regenerative capacity.

The first surgical procedure occurred when the animals were 90 days old. After 24 hours of the first surgery, the animals started the 10 sessions of hyperbaric therapy in alternating days for 150 days. The hyperbaric chamber was made by Seaway

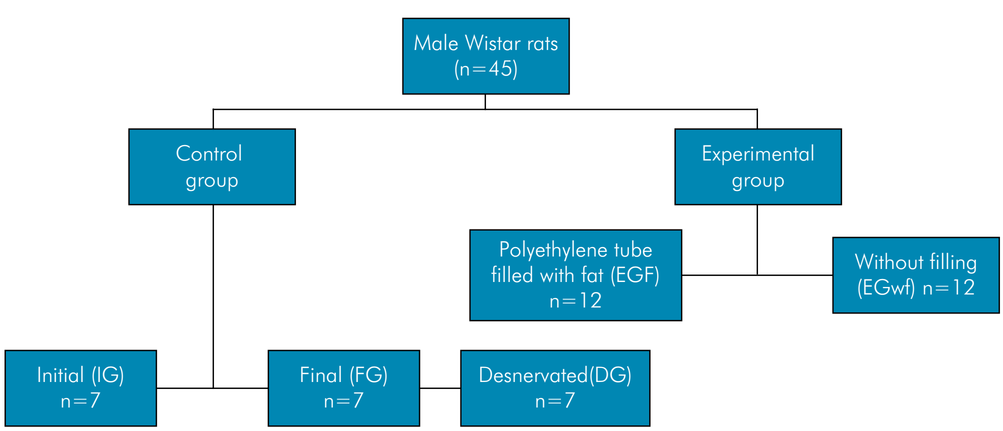

Figure 1. Study flow chart.

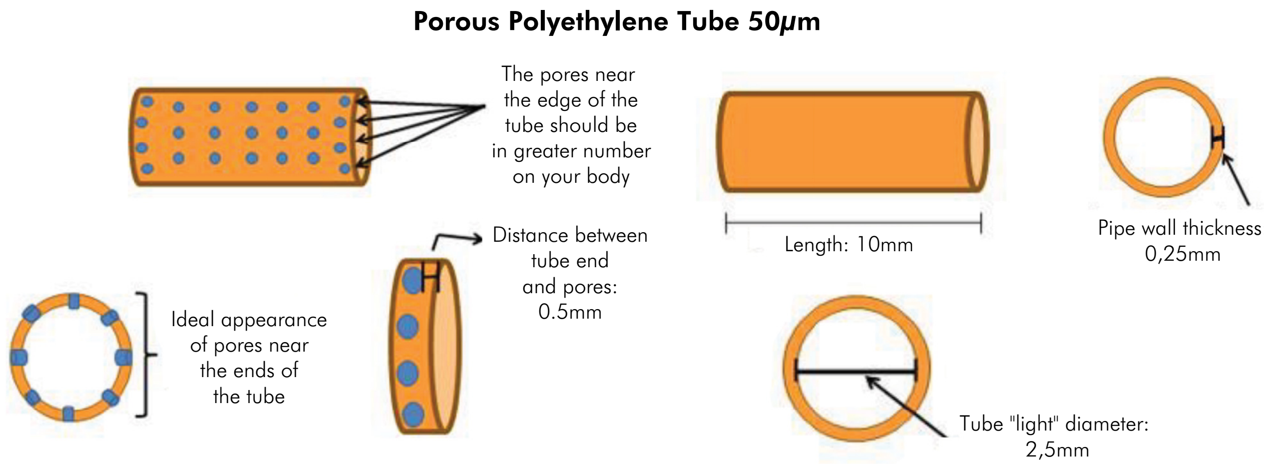

Figure 2. Schematic demonstration of the porous polyethylene tube.

Diver Ind Met e Mont Ltda (Santa Rosa, Brazil). The chamber is located at Beneficência Portuguesa Hospital, Bauru, Brazil.

Before the beginning of the study, the researchers (including the principal investigator) involved were duly calibrated. Suffering of the animals was minimized, and the protocol of the Animal Research Committee (ARC) of the University of São Paulo was followed (Approval ECAER- Proc. N°021/2013).

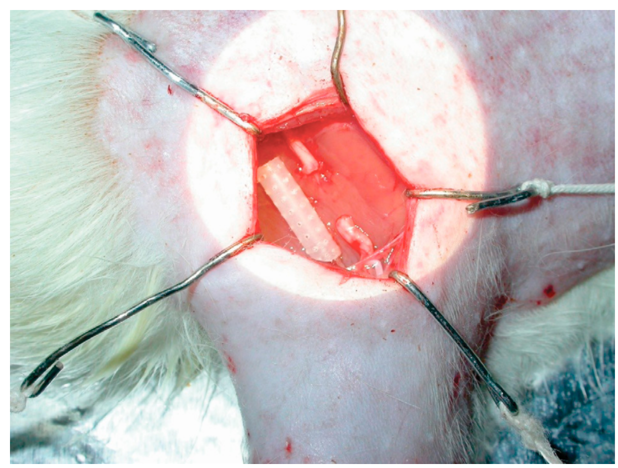

Figure 3. Relationship of the porous polyethylene tube to the defect, demonstrating that the tube must be larger than the defect itself to avoid trapping.

Interventions

A total of 45 male Wistar rats weighing about 300g were randomly selected. The animals were housed in appropriate boxes, divided into three control groups and two experimental groups. The control groups were the initial (IG), final (FG), and denervated (DG) groups with seven animals each, and the two experimental groups with 12 animals were the polyethylene tube filled with fat (EGF) and the polyethylene tube without filling (EGwf) groups. All groups underwent hyperbaric therapy, except for the initial control group. The same anesthetic protocol and preoperative preparation were performed by a single person in all groups. The fat was inserted *in nature* with a thin layer being placed on the inner walls of the tube, to serve as a neurogenic promoting factor, in such a way that it did not obliterate the tube lumen (Figures 3 and 4).

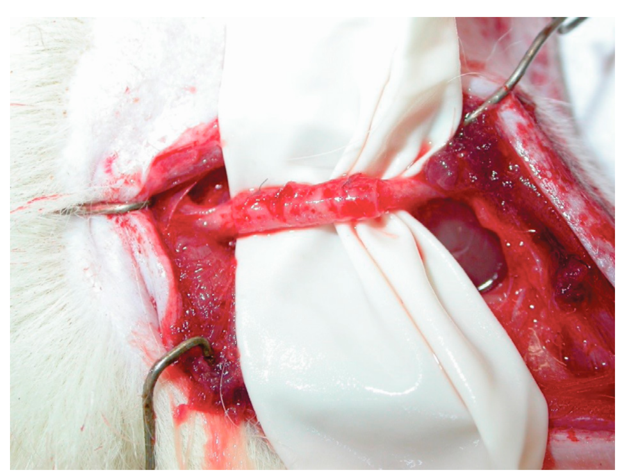

Figure 4. Demonstration of the presence of fibrous scar tissue.

Animals received water and feed *ad libitum*, without restrictions of movement, respecting the

12-hour dark/light cycles, and maintained a constant average temperature of 24°C.

The animals were weighed and submitted to general anesthesia by intramuscular injection of Tiletamine Hydrochloride associated with Zolazepam Hydrochloride (50 mg/kg) in the lateral dorsal region of the left thigh. The aseptic technique was adopted in all surgical procedures. Animals were euthanized after 150 days of the first procedure, with 230 days of life, with an overdose of the anesthetic Zoletil 50® (Virbac from Bauru, Brazil) containing the ratio of 1:1 of Tiletamine hydrochloride and Zolazepam hydrochloride (0.15 mL/kg, IM).

A 10 mm section of the right sciatic nerve of the IG were collected for posterior morphometry. In the DG, the proximal end was sutured in the adjacent musculature, while the distal end was fixed in the subcutaneous tissue. After 150 days, a new surgery was performed with a similar incision, but no evidence of regeneration was found. In the FG, the nerves were collected with 230 days of life, and the samples were used for morphometry. A final standard of comparison was established from this group.

The experimental EGwf, operated at 80 days of age, had the right sciatic nerve exposed using a stereomicroscope (DF Vasconcelos, Rio de Janeiro, Brazil), removing about 10 mm of the nerve. The defect was connected with a porous polyethylene tube without filling, providing a continuity solution between the distal and proximal stump. After 150 days of the first procedure the animals were reoperated, the tube was exposed and sectioned and the regeneration tube removed for histological evaluation. The EGF was distinguished from EGwf only by the placement of fat inside the tube.

The hyperbaric chambers were adapted to the procedure with an entrance for the oxygen cannula placement and an exit. To hinder the dispersion of gases, a diaphragm was inserted, simulating a conventional oxygen mask. The experimental groups were submitted to ten consecutive sessions, each lasting 1 h and 15 min.

Outcomes

The samples were collected one week before euthanasia and the animals were submitted to functional tests through the catwalk test. Four animals from each experimental group, with and without fat filling, and three from each control group (FG and DG) were randomly selected for examination totaling 12 animals evaluated.

The intensity of the footprints and the quality of the steps were verified and compared with the FG. Subsequently, the material was collected and the histological and morphometric analysis was performed.

Sample size

The sample size was based on articles with similar study design, and based on the coefficient of variation (CV). The assessment was considered correct since there were significant differences between evaluated groups.

Randomization

Block randomization was performed by random sequence, Groups had a predetermined size including two experimental groups with twelve animals each and three control blocks with seven animals each. With this strategy, the experimental groups and the control groups were balanced regarding the number of animals.

Allocation

The animals were previously weighed and separated into boxes, being randomly divided into two experimental groups. The control groups were separated in a box with four and another with three animals, in a total of seven. All boxes were labeled, marked with the date of birth, surgical procedures (if performed), in addition to the expected date for euthanasia.

Implementation

The random allocation, recording, and intervention assignment were attributed by the principal investigator.

Blinding

The study was not blinded.

Statistical methods

The Kolmogorov-Smirnov Normality Test was used for the study of nerve morphometry, and for comparison among the four groups the ANOVA followed by Tukey's Test was applied. The significance level of 5% ($p < 0.05$) was adopted.

Results

Outcomes and estimation

Macroscopic evaluation

Both in the experimental (EGf and EGwf) and control groups there was paralysis of the limbs that underwent interventions, similar to "Waiter's tip hand" deformaty. Some cases of autophagy were observed. Interestingly, one week after the hyperbaric chamber therapy there was a marked growth of hair in the trichotomy region of the dorsolateral side of the right thigh. There were no intercurrences during the surgical approach.

Collection of Samples

In the collection procedures, the presence of cicatricial fibrous tissue was noticed during the exposure of the polyethylene tube. Macroscopically, there was a difference in the nerve thickness in the experimental groups when compared to the control groups. Likewise, this difference was apparent when analyzing the musculature of the thigh region showing signs of atrophy in the experimental group. When the samples were removed, a capillary invasion in the pores of the tubes as well as of connective tissue is evident by a slight hemorrhage when performing the cut in the tube to collect the regeneration cable (Figure 5). The tubes were removed with no signs of damage.

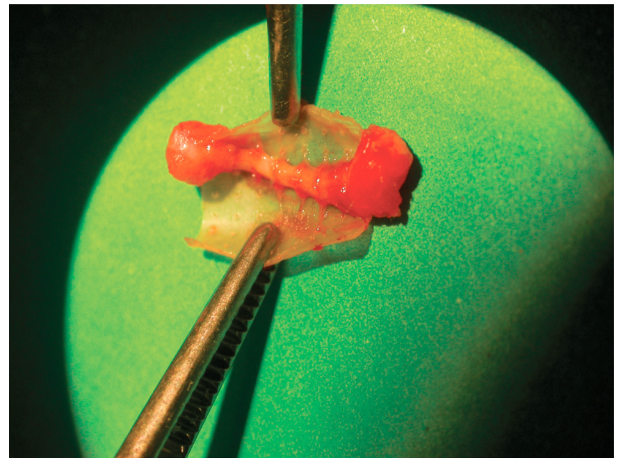

Figure 5. An hourglass-shaped regeneration cable, abundantly surrounded by blood capillaries, was removed from the FG.

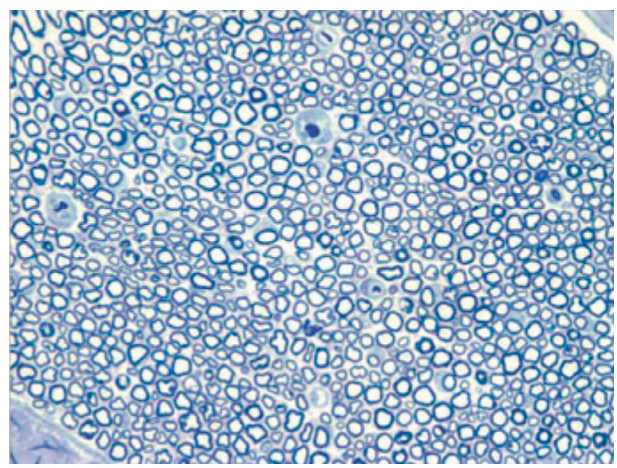

Figure 6. Histological section of the sciatic nerve of the GF, in 400X magnification, where myelin fibers are stained by toluidine blue.

Histological evaluations

Concerning the morphological aspects, heterogeneity in the diameter of the fibers was observed. The staining with toluidine blue was homogeneous in each cut (Figure 6).

Morphometric evaluation

Measurements were performed by a properly trained operator, including Area of the fiber (AF), Axon Area (AA), Fiber Diameter (FD), Axon Diameter (AD), Fiber Area (FA), and the Sheath Area (SA).

Regarding the diameter of the sciatic nerve fibers, no statistically significant differences were observed between the experimental groups. Table shows the diameter of the sciatic nerve axons of the experimental groups was significantly different in comparison with the final control group. However, there was no difference between the experimental groups and the initial control group.

The areas of the nerve fibers of the sciatic nerve showed no statistically significant difference between the experimental groups, but it was different from the final control group (Table). Also, no significant differences were observed between IG when compared to the experimental groups.

Regarding the evaluation of the axon areas in the sciatic nerve none of the experimental groups were statistically significant when compared to the IG. However, there was significance concerning GCF. In addition, differences between experimental groups and FG were presented, but not between the experimental groups and IG (Table). Finally, the thickness of the myelin sheath as a result of the fiber and axon diameter subtraction showed results similar to those evaluated.

Functional evaluations

For gait evaluation, the animals walked along a glass walkway (100 cm long x 15 cm wide x 0.6 cm thick), located in a dark room. A fluorescent lamp showed only the pressure of the animals' feet in contact with the glass floor. The floor of this corridor was monitored by a camera equipped with an angular lens capable of detecting the average intensity in pixels. The intensity signal varied according to the pressure applied by the animal's paw; the higher the pressure, the higher the intensity in pixels. These signals were processed by the CatWalk program (Noldus Inc., Wageningen, The Netherlands) (Figure 7).

Initially, the animals of the FG were verified to establish the parameters for the evaluation of the experimental groups. For the experiment, the following variables were measured: maximum contact, print length, print width, area printout, maximum intensity, minimum intensity, and average intensity, among others.

Table. Morphometric data referring to the ischial nerve.

Groups	AF	AA	FD	AD	FA	SA
	Measure	Measure	Measure	Measure	Measure	Measure
IG (n=7)	22.67^{b}	8.54^{b}	4.98^{b}	2.94^{bcd}	14.12^{b}	2.04^{b}
DG (n=7)	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
FG (n=7)	49.50^{c}	17.78^{c}	7.37^{c}	3.87^{bc}	31.72^{c}	3.49^{b}
EGwf (n=12)	20.92^{b}	9.37^{ab}	6.52^{b}	3.07^{bd}	11.54^{b}	3.45^{b}
EGF (n=12)	31.82^{b}	8.38^{b}	6.42^{b}	3.11^{bd}	23.44^{b}	3.30^{b}

Where AF: Area of the fiber; AA: Axon area; DF: Diameter of the fiber; DA: Diameter of the axon; SA: Sheath area; ET: Sheath thickness.

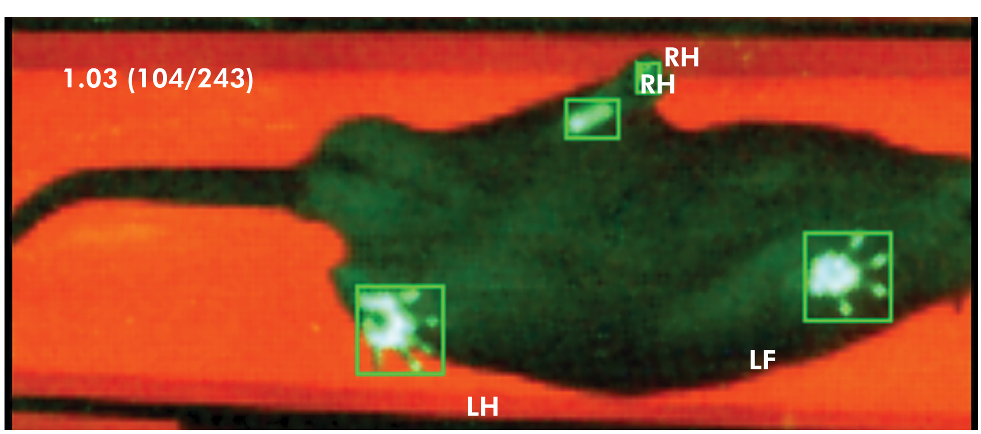

Figure 7. Animal of the experimental group with porous polyethylene tube associated with autologous fat (GEEPcg). Differently from what occurred in the control group, we observed the narrowing of the footprint with intensity maintenance.

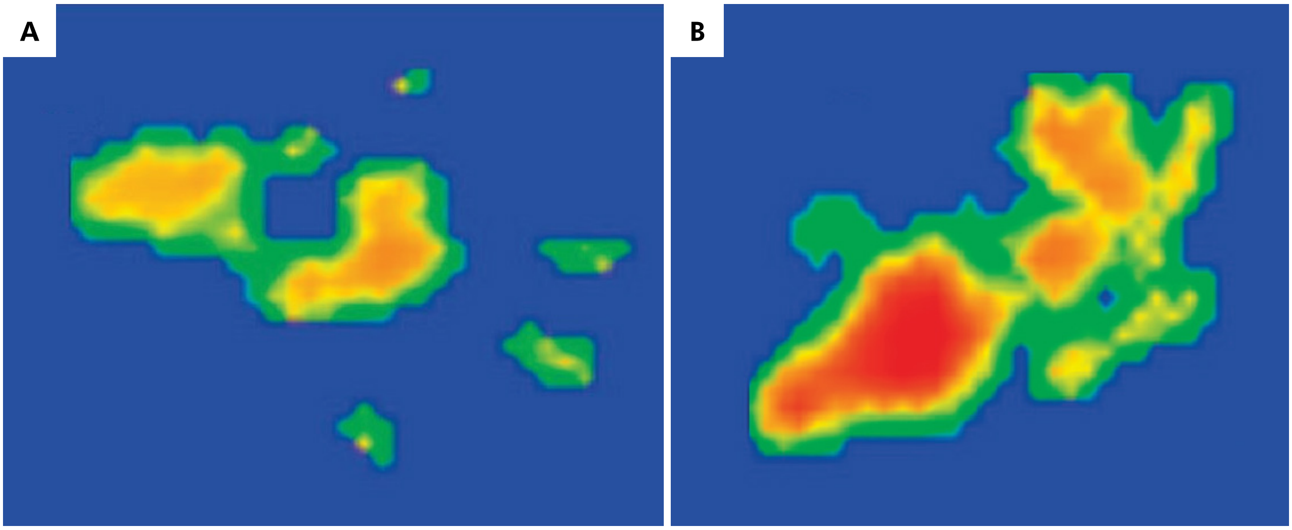

Figure 8. A) Image of the footprint of the right paw of the final control group. The finger impressions with a homogeneous distribution of the footprint force are shown. B) Image of the footprint of the right paw of the experimental group porous polyethylene without fat graft (EGPwg), showing the intensity of the footprint but the distribution of fingerprints is not clear.

When compared to FG, the experimental groups showed statistically significant differences. The differences found between the experimental groups with filling and without filling of autologous fat were not statistically significant, especially in the minimum intensity results (Figure 8).

Harms

There was no harm or unwanted effects in the groups.

Discussion

The peripheral nervous system (PNS) establishes a communication pathway between the central nervous system and the organs.[2,4] The Wistar mouse was used because it has a similarity with the structure and anatomical arrangement of the peripheral nerves when compared to humans.[4]

The sacrifices were performed at 180 postoperative days since functional and histomorphometric responses occur within such time. After this, no significant changes are found.[5]

Schwann cells, one of the main constituents of the PNS, originate in the neural crest and have a flattened morphology and an elongated nucleus. Their primary function is myelinization of the axons and the cells are also responsible for the classification of nerve fibers in types I, II, III, and IV.[6] The importance of these cells led us to measure the thickness of the myelin sheath, obtaining results on the index of myelination which, comparing the experimental groups and the initial control group, did not present significant differences.

Three layers of connective tissue surround nerves and the outermost is called epineurium, which has vessels, fibroblasts, and type I collagen fibers.[7,8] The connective tissue is used to fix autologous and alloplastic grafts, such as polyethylene tubes[3]. Corroborating with the literature, we selected this region for the fixation of the porous polyethylene tube graft, to alow axonal budding, as well as the formation of neuromas.

Peripheral nerve lesions are among the most disabling lesions that affect individuals of productive age.[9] In this paper, to simulate more severe trauma, neurotmesis was generated.

Scientists are seeking more satisfactory results in the clinical evaluation of ambulation, such as ischiatic nerve affections.[10] For a more effective regeneration, techniques are increasingly more sophisticated. For the present study, the Catwalk was used, a highly accurate and reliable tool to evaluate the effects in the animals used.

It is known that the most indicated technique for nerve recovery in cases of neurotmesis is the end-to-end neurorrhaphy.[10] However, when simple connection is not possible due to the loss of structure and presence of a gap, the technique of autologous grafting is used.[11] This technique is capable of guiding axonal growth and joining the ends of the distal and proximal stumps.[12] The application of this therapy implies some disadvantages such as the need to approach another surgical site, definitive (anesthesia) or partial (paresthesia) loss of sensitivity of the donor area, the possibility of amputation neuroma formation in the region that received the graft, among others. To avoid these inconveniences, researchers decided to use other devices such as autologous vein grafts[13] and absorbable synthetic tubes such as polyethylene, chitosan, collagen tubes, and PGLA (1998). A porous polyethylene tube was used in the present work.

The use of synthetic tubes form a primary framework for guiding the migration of fibroblasts, Schwann cells, and eventually axonal process.[9,14,15] Thus, the presence of axons outside the boundaries of the porous polyethylene tubes was not verified.

Adopose tissue is richly found at the surgical site and is an accessible source of adult stem cells that have generated considerable interest as a candidate for autologous cell transplantation and is currently being used in clinical trials for a wide range of indications.[13] In the present study, the use of a porous polyethylene tube with fat filling was applied, integrated with the use of the hyperbaric chamber. The tubes had pores to facilitate even more the invasion of blood capillaries, which was confirmed by our study, since at the time of removal there was blood leakage through the pores. Nevertheless, there were no significant differences between the groups with and without autologous fat filling, which indicates there was no benefits of stem cells present in the fat tissue in the regeneration and emphasizes the necessity of further alternatives.

Neovascularization in the nerve grafts is fundamental in the regenerative process,[13] being potentially useful for the repair of peripheral nerves in humans.[16] Accordingly, pores were made in the polyethylene associated to a hyperbaric chamber protocol.

The treatment in a hyperbaric chamber increases the oxygenation of the tissues, reducing the effects of the lesion[17] and improving microvascular perfusion. The use of Oxygen Hyperbaric Therapy (OHT) inhibits neutrophil adhesion and post-ischemic vasoconstriction.[18] Histological satisfactory results were obtained in our study since the experimental groups did not present a significant difference with the initial control group. From the functional point of view, verified through the Catwalk test, among the experimental groups there was no significant difference.

Skeletal nerve crush injuries were performed and rats were submitted to hyperbaric oxygen therapy in the postoperative period. The results were quantified through functional evaluation by the "walking-track analysis" method. The functional recovery indexes did not differ statistically from those observed in the control group. Therefore, it was verified that hyperbaric oxygen therapy in the proposed scheme did not influence the functional recovery after neural damage by crushing.[19] This work disagrees with the previous study, since it demonstrated positive results of the use of OHT, although this study was based on a different type of trauma, the neurotmesis, a more severe injury than the previous study.

The histological analysis of the middle third of the grafts and the distal stump of all groups showed heterogeneous myelinic nerve fibers, the perineurium neoformation, and the intraneural organization of fascicles with varying sizes and numbers, in agreement with previous authors.[4]

In this experiment, regenerating axons were observed in all animals of all experimental groups. Indeed, the axons grew from the proximal stump of the sciatic nerve, crossed the intersegmental space, and reached the distal stump of this nerve since in all the analyzed variables the parameters were higher in the middle third of the graft than in the region of the distal stump.

No significant difference between the groups with and without fat filling was found, a fact not supported by a previous study,[3,8,9] which a better result for the group with porous polyethylene graft without fat filling. Two possible hypotheses are suggested to explain this contradiction. The first is the functional evaluation, which despite having qualities it is inaccurate an inacurate; the second aspect is the hyperbaric chamber that contributed to the results of the experimental groups making the functional evaluation statistically not relevant.

In the morphometry analysis, no significant difference between the experimental groups and between these and the initial control group was found, differently than with the final control group, agreeing with most of the other experiments that evaluated the initial and final control group.[3,8,13,20,21,22]

Conclusion

Morphometric results showed that the experimental groups treated with a tube with and without fat filling had morphometric and functional results but without statistically significant differences. However, when they were compared with the final control group, differences were significant. In light of the evidence found and the supporting literature, it can be concluded that the hyperbaric chamber had a positive effect verified by the similar results of the two experimental groups, both regarding morphometry and function.

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