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EFFECT OF LASER THERAPY ON EXPERIMENTAL REPAIR OF PERIPHERAL NERVE INJURY AFTER TUBULIZATION TECHNIQUE FILLED WITH ADIPOSE TISSUE

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Abstract: Peripheral nerve injuries are common and can cause great sensory and functional loss in the patient. In order to minimize this loss and the final functional prognosis of this patient with quality of life, many surgical and post-surgical techniques and treatments are studied. Therapy with stem cells derived from adipose tissue is one of them, since they are pluripotent mesenchymal cells with the capacity to differentiate into several lineages. However, this method still has limited success in vitro, impacting the clinical area as it is an invasive technique, with risks of mutagenesis, in addition to high costs. Another technique that was used was end-to-end neurorrhaphy (NTT), used in the repair of peripheral nerve injuries to unite the injured proximal and distal stumps using a suture, but if the distance between the stumps is approximately 5 cm, the use of an autologous nerve graft is recommended, a nerve repair strategy considered the gold standard in humans, however, it will result in sensory loss for the patient. As a complementary therapeutic resource, laser therapy has gained prominence as it is a non-invasive treatment and has positive results in regeneration and functional recovery. This research aimed to evaluate the effect of laser therapy on the repair of peripheral nerve injury after the tubulation technique filled with adipose tissue. We used 60 male Wistar rats, 80 days old, provided by the Vivarium of the ``*Universidade do Sagrado Coração*``, randomly divided into six experimental groups with 10 animals each: Control Group (CG), Denervated Group (GD), Tubulization Group (GT), Fat Tubing Group (GTG), Tubing and Laser Group (GTL) and Fat Tubing and Laser Group (GTGL). The GTL and GTGL groups underwent laser treatment three times a week lasting 90 seconds for 90 days. In the morphometric evaluations of the Soleus, Extensor Digitis Longus and Tibialis Cranial muscles and in the functional evaluation, the GTGL group obtained the best result. Therefore, with the results obtained, it was possible to conclude that the association of fat in the tubing, as well as the use of laser therapy, provided positive results in the process of peripheral nerve repair and functional improvement.
Keywords: Peripheral Nerve Injuries; Muscle Regeneration; Tubing; Adipose Tissue; Lasertherapy.

INTRODUCTION

Peripheral nerve injuries, whether traumatic, inflammatory, neoplastic, toxic, metabolic or genetic, are common and frequent in clinical and physiotherapeutic environments, and can trigger great sensory and functional loss in the patient, compromising their routine and professional activities (DALY; YAO; ZEUGOLIS, 2012).

The incidence of peripheral nerve injuries is higher in individuals aged 25 to 40 years, thus causing important economic and social consequences due to early functional disability (WHITLOCK, 2009 cited by MELO, 2011).

End-to-end neurorrhaphy (NTT) is a technique used in the repair of peripheral nerve injuries to unite the injured proximal and distal stumps through a suture, as long as the sectioned nerve has not suffered tissue loss, and its stumps remain visible and integrity (CHEN, 2015; CEDERNA; KUZON; ROVAK,2001).

The autologous graft technique has negative consequences for the patient, such as loss of sensory function in the donor area, formation of neuromas and risk of infections (LICHTENFELS; SEBBEN; SILVA, 2011).

As an effective therapeutic resource in the complementary treatment of injuries, laser therapy has gained prominence in physiotherapeutic intervention protocols mainly because it is a non-invasive treatment and has positive results in regeneration and functional recovery. Among these benefits can be mentioned the anti-inflammatory and anti-edematous effect, the potential for wound healing, pain relief, increased mitochondrial respiration, increased ATP synthesis, increased fibroblast proliferation, stimulates the proliferation of Schwann cells that They secrete neurotrophic factors for nerve regeneration, among other factors (HUANG, 2011; MARTINS, 2015; WANG, 2014, 2015).

In view of the data presented, the purpose of this study is to evaluate the effect of laser therapy on the repair of peripheral nerve injury and functional recovery after the tubulization technique filled with adipose tissue.

METHODOLOGY

All procedures were in accordance with the Ethical Principles in Animal Experimentation adopted by the Brazilian College of Animal Experimentation (COBEA), and were analyzed by the Ethics Committee on the Use of Animals at ``*Universidade do Sagrado Coração*`` – USC, registered by protocols, number: 8853300315 and number: 4474300315 approved at a meeting on 04/14/2015.

The animals were provided by the Vivarium of ``*Universidade Sagrado Coração*``. 60 male Wistar rats (*Rattusnorvergicus*), aged 80 days, were used. The 60 animals were randomly distributed into six groups:

Control Group (CG): In this group, the animals did not undergo any type of intervention.

Denervated Group (DG): To ensure denervation, the common peroneal nerve was sectioned and its stumps were inverted 180 degrees, the proximal stump was passed through an incision in the gluteal muscles and sutured in its external portion in order to prevent motor reinnervation. The distal stump was sutured to the subcutaneous tissue.

Tubulization Group (GT): In this group, the animals were subjected to the tubulization technique without filling.

Fat Tubulization Group (GTG): In this group, the animals underwent the tubulization technique with adipose tissue filling.

Tubulation and Laser Group (GTL): In this group, the animals underwent the tubulation technique without filling and underwent a laser therapy protocol.

Fat Tubulization and Laser Group (GTGL): In this group, the animals underwent the tubulization technique with adipose tissue filling and underwent the laser therapy protocol.

SURGERY

In the groups that applied tubulization techniques, a unilateral paramedian incision of approximately 3 centimeters was made in the neck, where 2 cm of the External Jugular vein was removed. In this same location, adipose tissue was collected to fill the vein in the GTG and GTGL groups. In a second surgical procedure, the sciatic nerve on the right side was exposed and, with the help of a surgical magnifying glass, a segment of approximately 0.5 cm in length was sectioned and removed from said nerve. After section, the right sciatic nerve received the external jugular vein graft with or without filling, reestablishing communication between the proximal and distal stump. The lesion space between the stumps was 1.5 cm.

LASER APPLICATION

The animals in the GTGL and GTL groups received treatment with the AsGa (Gallium Arsenide) Laser, with a continuous pulse, with a wavelength of 830 nm, 6J/cm2, for 24 seconds/site applied, at four points on the operated site. The laser was kept in contact with the animal's skin, making the total application time 96 seconds.

The laser was applied immediately after surgery and three times a week, until the period corresponding to euthanasia.

FUNCTIONAL ASSESSMENT OF GAIT

Before electrophysiological analysis and euthanasia 90 days after repair surgery, the animals underwent functional gait assessment. The distance between the impressions of the hind limbs was evaluated according to the equation described by Bain; Hunter; Mackinnon (1989), based on studies by De Medinaceli; Freed; Wyatt (1982). For this evaluation, the animals walked through a device called CatWalk®, in which the footprints on the floor were automatically identified and used for functional analysis.

STRENGTH TEST

For muscle strength analysis, after 90 days, the animals were anesthetized with Ketamine Hydrochloride (100 mg/kg) and Xylasine Hydrochloride (10 mg/kg), applied intramuscularly, and immobilized on cork plates in the prone position. Asepsis and a wide incision were made in the right hind limb allowing access to the distal tendon of the Tibialis Cranial muscle where it was sectioned and connected to a force transducer FT03 Grass Technologies Company, Astro-Med, Inc. (West Warwick, RI) using a monofilament thread 4-0 nylon. The ideal muscle length was maintained at a pre-tension load of 0.18 N (newton) and which was reset between tests.

Electrical stimulation was generated directly on the exposed cranial tibialis muscle. The test consists of three sequential applications of one second duration, with muscle tension readjusted between measurements and tetanic contraction was performed with 100 mA and a frequency of 100 Hz.

MORPHOMETRIC ANALYSIS

The digitized images of the muscles were analyzed using measurements of fiber area and minimum fiber diameter, for which 220 muscle fibers from each animal were used.

Measurements were made semi-automatically using Sigma Pro Image Analysis software, version 5, from Jandel Scientific Corporation.

RESULTS

In animals undergoing denervation surgery, paralysis of the pelvic limb called "*Waiterstiphand*" (waiter's hand) was observed, similar characteristics found in high injuries of the brachial plexus (C5; C6). The characteristic was more pronounced in the GD group.

Evaluating the nerve fibers, more irregularities were observed in fibers from the GT group, followed by the GTL and GTG groups and later by the GTGL group. Regardless of the irregularity, at the graft level, myelinated and unmyelinated nerve fibers of heterogeneous diameters were identified. The GD group did not present nerve fibers, thus guaranteeing the efficiency of the denervation surgery.

In the muscular layers, a slight atrophy of the fibers of the experimental groups was observed when compared to the control group. In GTGL the spacing between muscle fibers and fascicles was smaller. In the GT, GTL and GTG groups the spacing was greater, caused by the increased presence of connective tissue in the perimysium and endomysium region.

All animals showed mass gain (g) during treatment. Table 1 shows the initial and final masses and percentage gain.

GROUPS	Initial mass (g)	Final mass (g)	gain percentage (%)
GC	274,5	482,1	75,6
GD	278,3	487,6	75,2
GT	272,4	479,9	76,2
GTG	269,9	485,1	79,7
GTL	270,2	479,4	77,4
GTGL	275,7	484,7	75,8

Table 1: Average, initial and final masses, and weight gains in percentage.

(GRAPH)

In the functional analysis using the catwalk device, it was observed that no group obtained a result similar to the CG (-9.82 ± 6.95). GD obtained the worst result (-93.05 ± 23.61), confirming the effectiveness of denervation and the acquired functional impairment. Evaluating the surgical groups, the GTGL group presented the best result (-38.58 ± 20.19) followed by the GTG group (-50.47 ± 18.73), GTL (-63.84 ± 16.88), and GT (-78.31 ± 19.54).

Groups	Gear values	
	Average	D.P
GD	-93,05[a]	23,61
GC	-9,82[b]	6,95
GT	-78,31[c]	19,54
GTG	-50,47[d]	18,73
GTL	-63,84[e]	16,88
GTGL	-38,58[f]	20,19

Table 2: Average results of the functional analysis of the groups studied.

(GRAPH)

To analyze the results of the strength test, Analysis of Variance and Tukey's Test were used to compare the 6 groups, with a significance level of 5% ($p<0.05$). Groups with the same letter have no statistical difference.

Groups	Power test (N)	
	Average	D.P
GD	0,14[a]	0,02
GT	0,71[b]	0,03
GTG	0,98[c]	0,02
GTL	0,83[d]	0,02
GTGL	1,17[e]	0,03
GC	1,32[f]	0,02

Table 3: Average strength test results (Newton).

(GRAPH)

In the strength test, no surgical group obtained a result similar to the CG (1.32±0.02) and the GD group obtained the worst result (0.14±0.02).

In the analysis of the surgical groups, the GTGL group presented the best result (1.17±0.03) followed by GTG (0.98±0.02), GTL (0.83±0.02) and GT (0. 71±0.03).

To analyze the morphometric data, Analysis of Variance and Tukey's Test were used to compare the 6 groups, with a significance level of 5% ($p<0.05$). Groups with the same letter do not have a statistically significant difference between them.

Group	m. EDL		m. TC		m. SO	
	Average	DP	Average	DP	Average	DP
GD	419[a]	22	584[a]	28	160[a]	31
GT	1322[b]	122	1284[b]	135	992[b]	179
GTG	1881[c]	143	1931[c]	147	1307[c]	188
GTL	1614[d]	139	1726[d]	133	1101[bc]	204
GTGL	2211[e]	152	2831[e]	142	1829[d]	192
GC	2791[f]	33	3981[f]	39	2418[e]	41

Table 4: Average fiber areas (μm^2) in cross-section of the EDL, TC and SO muscles.

(GRAPH)

When evaluating the EDL, TC and SO muscles, we were able to observe that none of the surgical groups presented values similar to the CG (2791±33 / 3981±39 / 2418±41). GD presented the worst result in all muscles (419±22 / 584±28 / 160±31).

Among the surgical groups, GTGL presented the best results (2211±152 / 2831±142 / 1829±192). In the m. EDL and m.TC the GTG group appears next (1881±143 / 1931±147), followed by the GTL group (1614±139 / 1726±133) and the GT group (1322±122 / 1284±135) respectively.

In the m. SO GTGL also presented the best result (1829±192), however, the GTG and GTL groups obtained similar results (1307±188 / 1101±204), a result that was repeated between the GTL and GT groups (1101±204 / 992 ±179).

DISCUSSION

With the intention of obtaining more reliable results, some variables during the project design deserved special attention, as they are essential for the standardization of procedures. The processing of all samples followed specific protocols according to the purpose of the material. To process the muscles for morphometric purposes, the protocol of the Histology Laboratory of ``*Universidade do Sagrado Coração*`` was followed. Furthermore, the previous experience obtained from previously developed work, as well as the infrastructure of the vivarium and the laboratories where the techniques used were carried out contributed to obtaining reliable results.

For this experiment, rats (*Rattus norgegicus*) of the Wistar lineage were used, normally used by several researchers in this same line of investigation, involving injury, repair and regeneration of peripheral nerves and striated skeletal muscles (ITOH et al., 1996; MACKINNON et al. 1985; SCHMALBRUCH & LEWIS, 2000). Furthermore, they are easy to manage and have low maintenance costs (FERREIRA et al., 2005).

Hems & Glasby (1992) chose to use rabbits as an experimental model, and the justification given was that the regenerative capacity was lower compared to rats, making this model closer to humans. However, more recent studies show that the degeneration and regeneration process in rats resembles that of humans (FERREIRA & FERREIRA, 2003).

The animals used in this research were young, this standardization is important, as in adult animals the speed of regeneration is slower (LUNDBORG, 1987; FAWCETT, 1992; VAUGHAN, 1992; MIDRONI et al, 1995; VERDÚ et al., 1995; BATTISTON et al., 2000; TOS et al., 2007; RODRIGUES & SILVA, 2001;

The animals, although of the same age, showed small, non-significant variations in weight before the start of the experiment. On the day of surgery, the animals were drawn and divided between control and experimental groups, allowing a random distribution of the sample between the groups (VIEIRA & HOSSNE, 2002).

The sacrifices took place 90 days after surgery. This time was standardized, as some authors believe that the greatest functional and histomorphometric responses occur up to the 12th week post-surgery, in longer periods no significant changes are found that justify a period longer than this (FRAHER et al., 1990; WANG et al., 1993 and 1995, FOIDART-DESSALLE et al., 1997; al., 2003; WALKER et al. With a period of 90 days, equivalent to 12 weeks, we believe that the functional and histomorphometric responses are already established, therefore, we can obtain real values of the neuromuscular response through the new innervation.

The use of the vein as a graft is due to the fact that, according to the literature consulted, they present superior results to non-biological materials (BATTISTON et al., 2005; LEE et al., 2006; Geuna et al., 2007). To remove the animal's external jugular vein, a 0.06mm stainless orthodontic wire was used, bent so that its tip was "blunt". Some researchers (ROQUE, 2007) and (ROQUE, 2008) used another technique to remove the vein, using dental instruments. In our work we used orthodontic wire, which allowed better handling of the graft. The external jugular vein was used due to its easy access and manipulation, its caliber and low morbidity (ULKUR et al., 2003; KELLEHER et al., 2001).

The sciatic nerve was also chosen because it is easily accessible, is the largest nerve in the body, is richly vascularized, and also has intraneural vessels similar to human peripheral nerves (PAASSEN et al., 2005).

Regarding the type of injury performed, neurotmesis with tissue loss was chosen, as it is the most serious injury and most difficult to recover from. In this type of injury, it is necessary to use a tube or tubulization technique to guide the axonal sprouts from the proximal stump to the distal stump (Hall, 1997). In an attempt to standardize the lesion using other studies consulted, we found a very large variation between 5mm and 15mm. As in most studies the lesions were between 10mm and 15mm, we opted for a 15mm lesion between the proximal and distal stump (VITERBO et al. 1993; KARACAOGLU et al., 2001; COSTA et al., 2006; FERNANDES et al., 2007).

To allow better adaptation of the grafted vein with the nerve stumps, 10-0 nylon monofilament suture thread was used. Other researchers used 11-0 or even 12-0 monofilament thread such as Schultes et al (2001). We believe that 10-0 thread performs the same function as other threads, in addition to being more economically viable than 11-0 and 12-0 threads.

When observing macroscopic aspects, such as the animals' gait, there is difficulty in locomotion. In the first post-surgical week, there is greater difficulty in positioning the sole of the paw as the animal only drags the operated limb. After the third week, he stops dragging and begins to rest the sole of his paw on the walking surface again. This phenomenon caused by denervation of the animal was studied by Ulkur (2003), who also observed an improvement after the third week, due to a new communication between the nerve and the muscle being reestablished.

When we compare the proportion of myelinated nerve fiber development, taking into consideration, the axon area and making a relationship with the area and/or thickness of the myelin sheath, we observed an irregularity in the development of fibers from the GEEVcg150L and GEEVcg150 groups.

Post-treatment with Gallium Arsenide Laser was due to the fact that its anti-edematous, healing and analgesic properties are easily proven in the literature (POSTEN et. al., 2005).

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