NEURODYNAMIC MOBILIZATION REDUCES INTRANEURAL FIBROSIS AFTER SCIATIC CRUSH LESION IN RATS

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ABSTRACT | Peripheral nerve lesions may be associated with abnormal scarring that lead to regenerative failure and dysfunction. Neurodynamic mobilization (NM) imposes controlled mechanical loads on the peripheral nerve and may influence inflammation and collagen deposition after a lesion. However, there is lack of experimental data to support these claims. **Objective:** To evaluate the impact of NM in the intraneural number of mast cells, collagen deposition and number of blood vessels after an ischiatic crush lesion in rats. **Methods:** This is a laboratory animal study, where 20 rats *(Rattus norvegicus)* were randomly divided into two groups, NM (n=10) and control (n=10), submitted to a right ischiatic nerve lesion. A tensioning NM began 10 days after lesion, and was maintained once a day, six times a week, for three weeks. After this period, the animals were euthanized and the nerves assessed for the number of mast cells, collagen area and number of blood vessels. **Results:** NM led to a lower number of degranulated mast cells *(Kruskal-Wallis* = 0.29 $p<0.05$), higher organization of collagen deposition *(Kruskal-Wallis* = 0.01, $p<0.05$). There was no influence of NM on the number of intraneural blood vessels *(Kruskal-Wallis* = 0.46 $p<0.05$). **Conclusion:** NM started 10 days after a ischiatic nerve crush lesion modulates the inflammatory process and prevents random deposition of collagen at the lesion site, but has no influence on blood vessels formation.

Key-words: peripheral nerves; nerve regeneration; mast cells; stress,mechanical; collagen.

INTRODUCTION

Peripheral nerve lesions are among the most debilitating neurological problems, and may result in anatomic, sensory and motor changes[1]. Abnormal scar formation at the lesion site is one of the factors associated with failure of the regenerative processes, and may delay or prevent function restoration[2].

After a traumatic episode, disease or lesion, inflammatory reaction develops to restore the structure and function. Mastocytes, blood flow and angiogenesis play a fundamental role in the inflammatory cascade. On one hand, mastocytes produce and release a variety of chemical mediators related to vascular proliferation and vasodilatation[3]. They also produce proteolytic enzymes responsible for the degradation of the basal membrane and extracellular matrix, which contribute to fibroblast activation and collagen deposition[4]. On the other hand, blood flow and angiogenesis help with tissue nutrition and create an adequate microenvironment for the repair process[5].

Intraneural inflammation can result in excessive scarring, which may reduce nerve cross-sectional area, increase intrafascicular pressure, and inhibit regenerative capacity[6]. Scar formation and edema resulting from impaired microcirculation will alter mechanical properties of the nerve (neurodynamics), leading to abnormal mechanical behavior (pathomechanics)[7,8] which in turn, can prevent peripheral nerve regeneration.

Whereas pathomechanical behavior can be an impeditive factor in the recovery of a peripheral nerve lesion, optimal mechanical loads would have the oposite effect. Mechanical stimuli change nerve cells behavior[9]. Mechanical distention of the cell membranes modulates the activity of cation transport from the ionic channels sensitive to tension[10]. In vitro studies have demonstrated that mechanical loads are capable of modulating the inflammatory process[11].

Therefore, passive maneuvers that promote tensioning or sliding of the peripheral nerve, known as neurodynamic mobilization (NM), might be used to restore peripheral nerve mechanical properties after a lesion[12]. NM may influence the circulation of fluids and fibrosis of an injured nerve, as well as the inflammatory response. However, there are few experimental studies that corroborate these hypotheses[13].

Previous studies have investigated the effect of mechanical loads on inflammation and behavior of mastocytes[14], but no study has specifically evaluated the role of NM in inflammation and the formation of endoneural fibrosis, a key element to peripheral nerve regeneration. Hence, the main aim of this study was to evaluate the impact of NM on inflammation, collagen deposition and intraneural blood circulation after an ischiatic crush lesion. Specifically, we also aimed to investigate the effect of the most tensioning NM maneuvers, as they may promote nerve regeneration, but also harm due to excessive intraneural pressure.

METHOD

Declaration by the Ethics Committee

Experimental conditions were in compliance with the Brazilian College of Animal Experimentation and the Federal Council of Veterinary Medicine guidelines. The study was approved by the BAHIANA School of Medicine and Public Health (EBMSP) Ethics Committee on the use of Laboratory Animals under Protocol # 002/2008. The results follow the ARRIVE guidelines for reports on researches involving laboratory animals.

Experimental Design and Surgical Procedure

We used in this study 20 male rats (Rattus norvegicus) with body weight between 240g and 340g. Animals were randomly divided into Neurodynamic Mobilization (NM, n=10) and Control (GC, n=10) groups. Randomization and blinding of researchers involved with the intervention and data analysis were done by numbering the rats. An axonotmesis lesion was performed by crushing the right ischiatic nerve. The surgical procedure was performed

after anesthesia with an intraperitoneal injection of ketamine (60 mg / kg) and xylazine (7 mg / kg). Efficacy of the anesthesia was evaluated by the absence of reflexes from the tail and ipsilateral paw, as well as flexor reflexes in responses to noxious stimuli (pinching).

After trichotomy and asepsis, a longitudinal incision was performed in the lateral surface of the right thigh, below the trochanter of the femur, to expose the ischiatic nerve. The nerve was crushed once for 30 seconds, with a smooth needle holder forceps (without teeth), in the first lock, at the ischiatic nerve notch. The same investigator performed the technique in all the animals. After injury, the muscles were sutured with catgut 4.0 and the skin with nylon 4.0. In the post-operative period, all animals were placed in a cot warmed to 37°C to prevent hypothermia. The animals were housed one per box, under a 12-hour light/dark cycle, in a room with controlled temperature (22°C) and 45% humidity. They received food and water ad libitum.

Neurodynamic Mobilization

Neurodynamic mobilization began 10 days after the lesion and was performed on an acrylic platform, according to a previously established protocol[15]. During the procedure, the neck, back, tail and left posterior limb were fixed with belts, while the right posterior limb remained free of movements in the control group, or was treated in accordance with the NM protocol. A tensioning NM was performed, in three series of one minute each block, with plantar and dorsiflexion movements at 1 Hz (one complete cycle/second).

This procedure was repeated six times a week, for three weeks. The movements were - executed to the final range of the paw movement. Animals received a light sedation with a mixture of isoflurance and oxygen (1 L / min), during procedures to prevent stress and movements that could interfere with the results.

Experimental Outcomes

The animals were submitted to euthanasia by deep anesthesia and transcardiac perfusion, 31

days after the lesion, with a fixative solution (4% paraformaldehyde - PA, and 2% glutaraldehyde - GA, in a cacodylate buffer 0.1 M and pH 7.4, 70 mL / animal). The ischiatic nerves ipsilateral to the lesion were harvested and dissected for evaluation of the crushed region. A symmetrical section of the contralateral intact nerve was evaluated as the parameter of normality. The tibial nerve was chosen to express the morphology of the ischiatic nerve, due to its proportional size. The nerves were evaluated with regards to the absolute number of intact and degranulated mastocyte, areas of collagen and blood vessels. For this purpose, these nerves were fixed with 10% formalin incorporated into paraffin wax and stained with toluidine blue for mastocyte count, or *picrosirius red* to the analysis of collagen content.

Once the slides were obtained, a light microscope (Olympus BX 51) was used to systematically capture 10 images of each nerve, at 40x magnification. Mastocytes count was performed automatically using an image processing software program (Image J, NIH, USA). The area of collagen for each image was estimated with an image edition software program and was calculated automatically (Motic Image Plus 3.0).

Immunohistochemistry

To investigate the area of blood vessels, the slides were deparaffinized with xylol in three consecutive baths of 10 minutes each. Immediately afterwards, they were dehydrated twice with absolute alcohol, for five minutes each time, washed with tap and distilled water. To expose the site, antigenic retrieval was used at a temperature of 97°C for 30 minutes. After this, we used hydrogen peroxide to block endogenous peroxidase for 20 to 30 minutes. Next, the unspecific bonds were eliminated with serum free protein block (DAKO-X0909). The slides were incubated with primary rabbit antibody PECAM-1(CD31 1:1000) and placed in a damp chamber at 4°C during the night. The slides were washed with PBS, five times, every five minutes, and incubated with secondary antibody (DAKO labeled polymer-HRP anti-rabbit K4011). They remained at room temperature for two hours, and afterwards were washed with PBS, five times, every five minutes, counterstained with hematoxylin, and finally mounted

with Canada balsam.

Statistical Analysis

The intervention (NM, control/normal) was the independent variable. The dependent variables were the number of mastocytes, area of collagen and area of blood vessels. As the data did not present normal distribution, median and interquartile intervals were used to describe the results. The number of mastocytes, area of collagen and area of blood vessels were evaluated between groups by the Kruskal-Wallis test, followed by the Dunn multiple comparisons test. The study power was considered to be 90% and the alpha value set as 5%. Analyses were performed using the GraphPad Prism 6.0 statistical software.

RESULTS

Animals were randomly divided into the two study groups and had a mean weight of 277g in the control group and 275 g in the experimental group. The contralateral paw of each group was used as a parameter of normality (group without injury and without NM). No signs of suffering were detected throughout the entire study. We initially had 10 rats on each group. One animal of the control group died after surgery, and its initial data were excluded from the analysis. During sample processing, the tissue from other two animals of the control group were damaged. The final sample had 10 animals in the NM Group and seven animals in the control group. In the group of normal nerves (contralateral) 14 nerves were analyzed.

Histologic Evaluation

Mastocytes

Mastocytes were classified in their intact and degranulated forms. Normal nerves (without lesion or mobilization) presented a lower quantity of mast cells, limited to areas neighboring blood vessels (Figure 1A). In the control nerves, that suffered lesion but were not mobilized, we observed a larger number of degranulated mastocytes smaller in size,

dispersed, and not limited to the surroundings of the blood vessels (Figure 1B). Crushed and mobilized nerves presented a smaller number of degranulated mastocytes than the control group. They also showed a higher number of intact with grains of preserved vasoactive amines (Figure 1C).
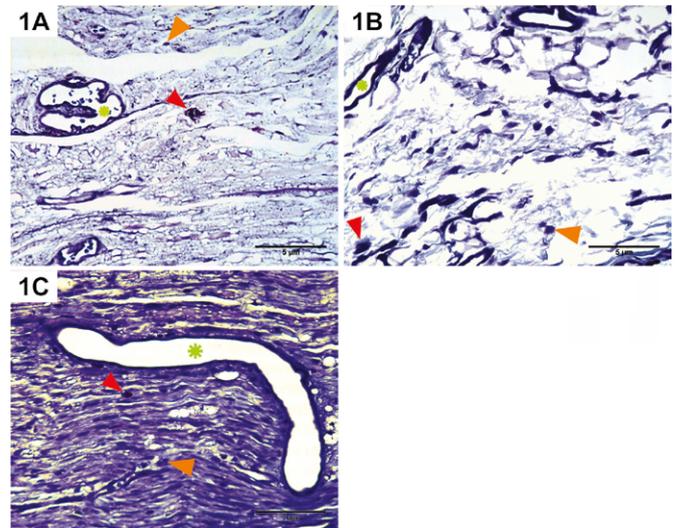

Figure 1. Mastocytes arrow head present in the normal ischiatic nerves (A) (without lesion or neurodynamic mobilization), (B) controls (crushed, but without receiving neurodynamic mobilization) and (C) treated with neurodynamic mobilization (crushed and treated). Mastocytes were present in the intact and degranulated forms, with oval shape and blue-violet color.

Collagen

Figures 2A, 2B, and 2C represent the quality and formation of epineural and endoneural collagen at the lesion site. In the normal nerves, the collagen fibers were aligned, in an organized form, with little staining, representing a low density of collagen (Figure 2A). Conversely, the control group had a disorganized tissue, with no alignment configuration, and disordered deposition. In this group, it was difficult to differentiate nerve connective tissue layers (Figure 2B). In the NM group, collagen was deposited in the epineural and endoneural areas. Collagen fibers showed a robust and organized configuration, deposited longitudinally to the nerve structure, and resembling the normal group (Figure 2C).

Blood Vessels

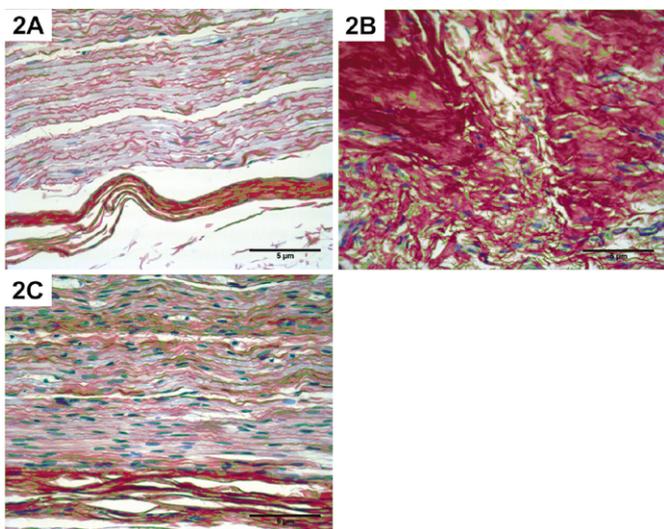Crushed and untreated nerves showed a high variability of blood vessels area, also distant from

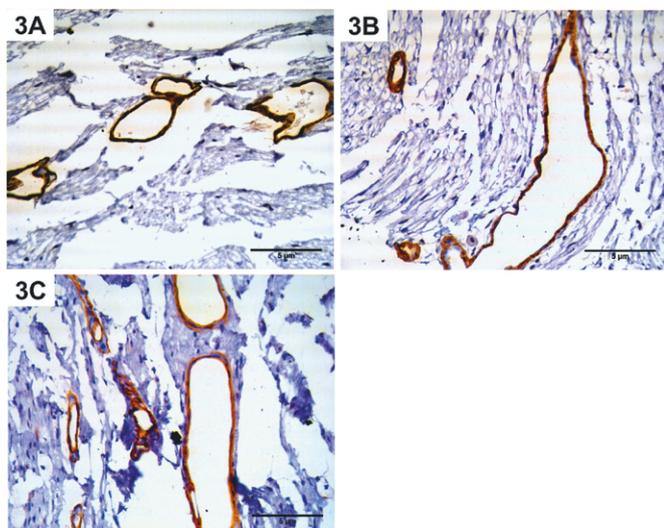**Figure 2.** The images represent (A) normal ischiatic nerves (without lesion or neurodynamic mobilization), (B) controls (crushed, but without receiving neurodynamic mobilization) and (C) treated with neurodynamic mobilization (crushed and treated). Note that both normal (A) and treated with neurodynamic mobilization (C) nerves present longitudinal collagen deposition. However, crushed nerves not submitted to neurodynamic mobilization (B) had deposit of collagen in a disorganized manner through the nerve structure.

each other. Conversely, in normal nerves and in crushed and treated with NM there was less area variability. Blood vessels on the NM group were more grouped (Figure 3).

Morphometric Analysis

The nerves that suffered lesion (Control and treated with NM) presented a larger number of degranulated mastocytes than the normal group (Kruskal-Wallis + Dunn, $p < 0.05$). The number of degranulated mastocytes was larger in the two lesioned groups regardless of NM (Figure 4A). Number of intact mastocytes was not different among the three groups (Figure 4B).

Regarding collagen area, the NM group was similar to the normal group. Crushed nerves not treated with NM presented a larger collagen area (Figure 5). There was no difference in the cross-sectional area of blood vessels among the evaluated groups (Figure 6).
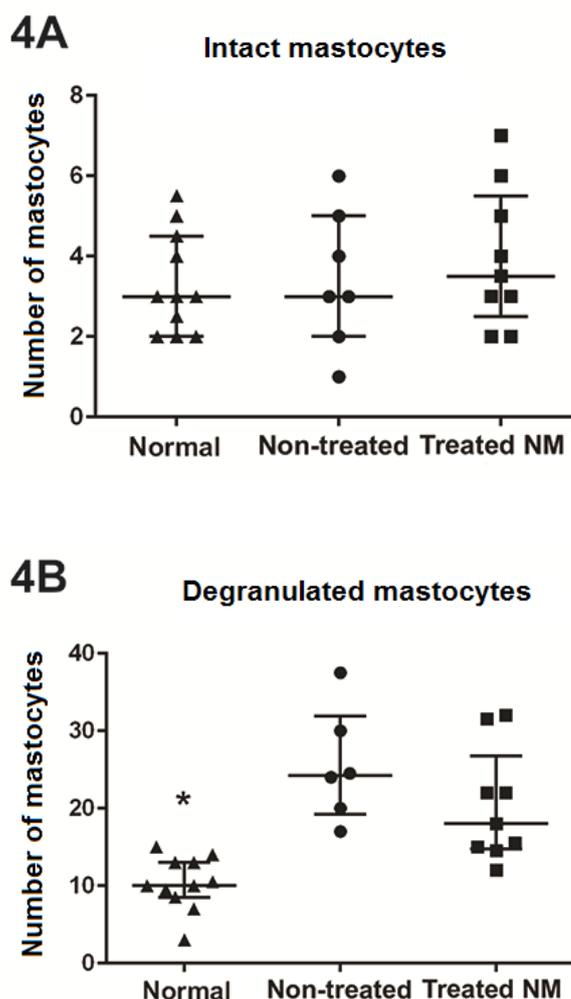

Figure 4. The degranulated mastocyte count was higher in the crushed when compared to normal nerves. There were no differences between nerves treated with neurodynamic mobilization and controls. Intact mastocytes count was not different among the three studied groups. Data is shown as means and quartiles. *Kruskal-Wallis, p<0.05.

Figure 3. The images represent the normal ischiatic nerves (A) (without lesion or neurodynamic mobilization), (B) controls (crushed, but without receiving neurodynamic mobilization) and (C) treated with neurodynamic mobilization (crushed and treated). Caliber of blood vessels was larger in crushed nerves (B and C), despite neurodynamic mobilization.
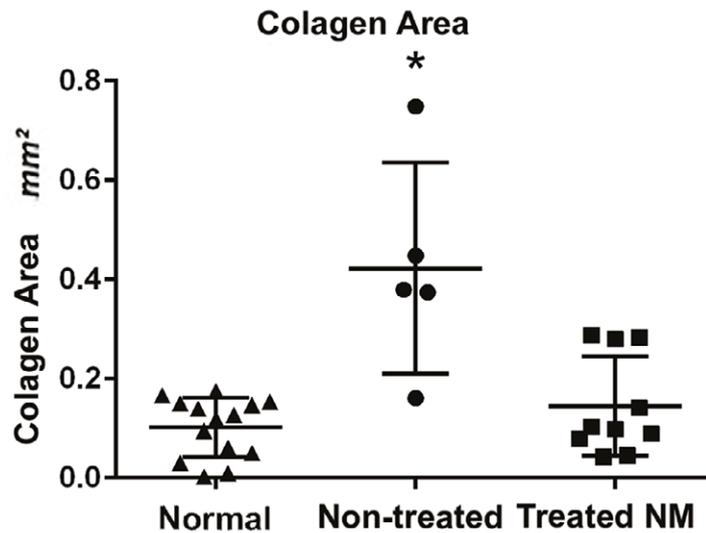
Colagen Area

Figure 5. The nerves treated with neurodynamic mobilization had a smaller area of collagen when compared to the untreated group. The former had a similar area to that of the normal group. Data expressed in terms of means and quartiles. *Kruskal-Wallis = 0.01, $p < 0.05$
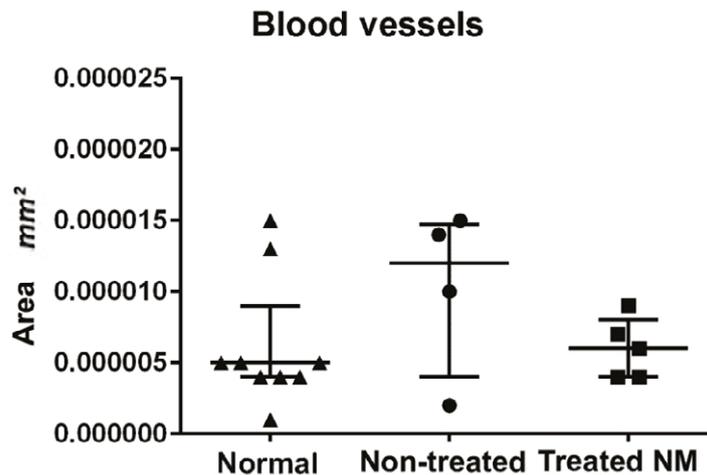

Blood vessels

Figure 6. The area of endoneural blood vessels was similar among the three studied groups . Data is presented as means and quartiles. (Kruskal-Wallis=0).

DISCUSSION

The aim of this study was to evaluate the influence of tensioning NM on variables related to inflammation and nerve repair after an ischiatic crush injury. Specifically, the influence of NM on the quantity of mastocytes and area of collagen and blood vessels was studied. The main results suggest that tensioning NM initiated 10 days after lesion could modulate the inflammatory process and decrease intraneural fibrosis, organizing collagen deposition.

In our study, NM appears to have prevented a disperse distribution of the amount of degranulated mastocytes through the intraneural space. We did not find that this treatment influenced the increase or decrease in the number of these cells. Mastocytes take a fundamental part in tissue repair and peripheral nerve regeneration, as they are capable of releasing vasoactive substances when in the degranulated form[16]. This could lead to an

increase in blood flow and presence of collagen synthesizing cells[4]. However, if these phenomena are excessive, or collagen fibers deposited in a random manner at the site of the lesion, local fibrosis could occur[17]. Our results are opposite to those found in other studies that caused mechanical tension in cells cultures[14,18]. This may have occurred due to the type of intervention, intensity and time of use of NM maneuvers.

Previous studies[19,16] have described that the application of mechanical loads on an injured peripheral nerve would lead to an increase in the number and function of mastocytes, with beneficial consequences for the healing process. These findings suggest that the imposition of mechanical loads to an injured tissue may control the inflammatory cascade. However, an excessive increase in the number of mastocytes could trigger disorders and increase cell signaling. In a paracrine manner, this could increase the synthesis and deposition of collagen, which is seen in some diseases, as neurofibromatosis[20]. NM could therefore play a role in the control of inflammation after a peripheral nerve injury. However, due to the diversity of this type of lesion, other models must be studied to elucidate whether these results are consistent.

Fibrosis formation involves the disorganized deposition of collagen, which may harm or inhibit regeneration. In our results, NM oriented collagen deposition in the epineural and endoneural layers, conducting the area of collagen to normal values. In addition, not mobilized crushed nerves presented a disorderly collagen deposition in the intraneural compartment. This suggests that NM prevents the formation of endoneural and epineural fibrosis. Recent studies have supported these results by demonstrating that the gradual elongation of the nerve is beneficial to peripheral nerve regeneration[21,22]. As our results suggest that the lack of mobilization after a peripheral nerve lesion increases the amount of collagen and may prevent regeneration, graded nerve mobilization may be an important procedure to prevent those maladaptive changes[23].

On the other hand, the imposition of excessive loads may harm regeneration. The increase in tension may generate an increase in collagen deposition, and consequently an abnormal healing, preventing

peripheral nerve regeneration[24]. The lack of balance between the forces of internal and external tension appears to be a fundamental factor in the formation of an abnormal scar[25]. Therefore, NM may play a role that could simultaneously be important or harmful to peripheral nerve regeneration. This will probably depend on the nature of the lesion and mobilization. Future studies must use different types of peripheral nerve lesions and mobilizations to evaluate this question.

In spite of neural mobilization influencing the dispersion of fluids through the peripheral nerve in cadavers, in our study we did not observe the structural influence of NM on the caliber of blood vessels. As our study involved fixated and no longer living blood vessels, the differences in pressure and flow were not studied.

CONCLUSION

Our results suggest that NM started 10 days after an ischiatic nerve crush lesion decreased intraneural fibrosis, without interfering in nerve inflammation or vascularization.

REFERENCES

1. Burnett MG, Zager EL. Pathophysiology of peripheral nerve injury: a brief review. Neurosurg Focus. 2004; 16(5):E1.

2. Abe Y, Doi K, Kawai S. An experimental model of peripheral nerve adhesion in rabbits. Br J Plast Surg. 2005;58(4):533-40. doi: 10.1016/j.bjps.2004.05.012

3. Nienartowicz A, Sobaniec-Łotowska ME, Jarocka-Cyrta E, Lemancewicz D. Mast cells in neoangiogenesis. Med Sci Monit. 2006;12(3):RA53-56.

4. Monk KR, Wu J, Williams JP, Finney BA, Fitzgerald ME, Filippi M et al. Mast cells can contribute to axon-glial dissociation and fibrosis in peripheral nerve. Neuron Glia Biol. 2007;3(3):233-44. doi: 10.1017/S1740925X08000021

5. Hobson MI, Green CJ, Terenghi G. VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy. Journal of anatomy. 2000;197(Pt 4):591-605. doi: 10.1046/j.1469-7580.2000.19740591.x

6. Brown CL, Gilbert KK, Brismee J-M, Sizer PS, Roger James C, Smith MP. The effects of neurodynamic mobilization on fluid dispersion within the tibial nerve at the ankle: an unembalmed cadaveric study. J Man Manip Ther. 2011;19(1):26-34. doi: 10.1179/2042618610Y.0000000003

7. Shacklock M. Clinical Neurodynamics. 2005.

8. Topp KS, Boyd BS. Peripheral nerve: from the microscopic functional unit of the axon to the biomechanically loaded macroscopic structure. J Hand Ther. 2012;25(2):142–51. doi: 10.1016/j.jht.2011.09.002

9. Ingber DE. Cellular mechanotransduction: putting all the pieces together again. FASEB J. 2006;20(7):811-27. doi: 10.1096/fj.05-5424rev

10. Chiquet M, Gelman L, Lutz R, Maier S. From mechanotransduction to extracellular matrix gene expression in fibroblasts. Biochim Biophys Acta. 2009;1793(5):911-20. doi: 10.1016/j.bbamcr.2009.01.012

11. Wong VW, Paterno J, Sorkin M, Glotzbach JP, Levi K, Januszyk M et al. Mechanical force prolongs acute inflammation via T-cell-dependent pathways during scar formation. FASEB J. 2011;25(12):4498-510. doi: 10.1096/fj.10-178087

12. Coppieters MW, Butler DS. Do "sliders" slide and "tensioners" tension? An analysis of neurodynamic techniques and considerations regarding their application. Man Ther. 2008;13(3):213-21. doi: 10.1016/j.math.2006.12.008

13. Schmidhammer R, Zandieh S, Hopf R, Mizner I, Pelinka LE, Kroepfl A et al. Alleviated Tension at the Repair Site Enhances Functional Regeneration: The Effect of Full Range of Motion Mobilization on the Regeneration of Peripheral Nerves??? Histologic, Electrophysiologic, and Functional Results in a Rat Model. J Trauma. 2004;56(3):571-84.

14. Fowlkes V, Wilson CG, Carver W, Goldsmith EC. Mechanical loading promotes mast cell degranulation via RGD-integrin dependent pathways. J Biomech. 2013;46(4):788-795. doi: 10.1016/j.jbiomech.2012.11.014

15. Boyd BS, Puttlitz C, Gan J, Topp KS. Strain and excursion in the rat sciatic nerve during a modified straight leg raise are altered after traumatic nerve injury. J Orthop Res. 2005;23(4):764-770. doi: 10.1016/j.orthres.2004.11.008

16. Gottwald T, Coerper S, Schäffer M, Köveker G, Stead RH. The mast cell-nerve axis in wound healing: a hypothesis. Wound Repair Regen. 1998;6(1):8-20. doi: 10.1046/j.1524-475X.1998.60104.x

17. Barros CS, Franco SJ, Müller U. Extracellular matrix: functions in the nervous system. Cold Spring Harb Perspect Biol. 2011;3(1):a005108. doi: 10.1101/cshperspect.a005108

18. Komiyama H, Miyake K, Asai K, Mizuno K, Shimada T. Cyclical mechanical stretch enhances degranulation and IL-4 secretion in RBL-2H3 mast cells. Cell Biochem Funct. 2014;32(1):70-6. doi: 10.1002/cbf.2973

19. Younan GJ, Heit YI, Dastouri P, Kekhia H, Xing W, Gurish MF et al. Mast cells are required in the proliferation and remodeling phases of microdeformational wound therapy. Plast Reconstr Surg. 2011;128(6):649e-58e. doi: 10.1097/PRS.0b013e318230c55d

20. Tucker T, Riccardi VM, Sutcliffe M, Vielkind J, Wechsler J, Wolkenstein P et al. Different patterns of mast cells distinguish diffuse from encapsulated neurofibromas in patients with

neurofibromatosis 1. J Histochem Cytochem. 2011;59(6):584-590. doi: 10.1369/0022155411407340

21. Nishiura Y, Yamada Y, Hara Y, Ichimura H, Yoshii Y, Ochiai N. Repair of peripheral nerve defect with direct gradual lengthening of the proximal nerve stump in rats. J Orthop Res. 2006;24(12):2246-53. doi: 10.1002/jor.20280

22. Udina E, Cobianchi S, Allodi I, Navarro X. Effects of activity-dependent strategies on regeneration and plasticity after peripheral nerve injuries. Ann Anat. 2011;193(4):347-53. doi: 10.1016/j.aanat.2011.02.012

23. Atkins S, Smith KG, Loescher AR, Boissonade FM, O'Kane S, Ferguson MWJ et al. Scarring impedes regeneration at sites of peripheral nerve repair. Neuroreport. 2006;17(12):1245-9. doi: 10.1097/01.wnr.0000230519.39456.ea

24. Ip WY, Shibata T, Tang FH, Mak AF, Chow SP. Adhesion formation after nerve repair: an experimental study of early protected mobilization in the rabbit. J Hand Surg Br. 2000;25(6):582-4. doi: 10.1054/jhsb.2000.0480

25. Ogawa R. Mechanobiology of scarring. Wound Repair Regen. 2011;19 Suppl 1:s2-9. doi: 10.1111/j.1524-475X.2011.00707.x