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ORIGINAL ARTICLE

HAND SURGERY AND RECONSTRUCTIVE MICROSURGERY

EFFECT OF HYPERBARIC OXYGEN THERAPY ON NERVE REGENERATION IN RATS

EFEITO DA TERAPIA HIPERBÁRICA DE OXIGÊNIO NA REGENERAÇÃO NERVOSA EM RATOS

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ABSTRACT

Objective: To evaluate histological changes in peripheral nerves of rats after sciatic nerve neurorrhaphy, according to the time of exposure to hyperbaric oxygen chamber treatment. Methods: Twenty-five Wistar rats were divided into 5 groups according to the amount of exposure to hyperbaric oxygen chamber treatment. Group 1 was the control and there was no use of hyperbaric oxygen chamber; group 2 received one week of therapy; group 3, two weeks; group 4, three weeks; and group 5, four weeks. After the fourth postoperative week, the animals were submitted to euthanasia and a sciatic nerve sample sent for histological analysis. Axons proximal and distal to the neurorrhaphy were counted with axonal regeneration index measurement. Results: We observed that the number of axons distal to neurorrhaphy increases with the amount of hyperbaric oxygen chamber exposure, the results were more expressive from the third week of treatment. However, the statistical analysis found no significant difference between the groups. Conclusion: The descriptive analysis suggests benefit of using hyperbaric oxygen chamber directly proportional to the time of therapy. The study, however, did not present statistically relevant results, probably due to the reduced sample size. Subsequent studies with more significant sampling would be of great value. ***Level of Evidence II, Prospective Comparative Study.***

Keywords: Peripheral Nerves. Hyperbaric Oxygenation. Nerve Regeneration. Rats.

RESUMO

Objetivo: Avaliar as alterações histológicas nos nervos periféricos após neurorrafia do nervo ciático de ratos, de acordo com o tempo de exposição ao tratamento com câmara hiperbárica de oxigênio. Métodos: Vinte e cinco ratos da raça Wistar foram divididos em cinco grupos conforme o tempo de exposição ao tratamento com câmara hiperbárica de oxigênio. O grupo 1 não recebeu o tratamento; o grupo 2 recebeu uma semana de terapia; o grupo 3, duas semanas; o grupo 4, três semanas; e o grupo 5, quatro semanas. Após quatro semanas de pós-operatório, os animais foram submetidos à eutanásia e uma amostra do nervo ciático foi enviada para análise histológica. Foram feitas contagens do número de axônios proximalmente e distalmente à neurorrafia, com medição do índice de regeneração axonal. Resultados: Observamos que o aumento do número de axônios distais à neurorrafia foi diretamente proporcional ao tempo de exposição à câmara hiperbárica de oxigênio, sendo mais expressivo a partir da terceira semana de tratamento. Entretanto, a análise estatística não encontrou diferença significativa entre os grupos. Conclusão: A análise descritiva sugere benefício do uso da câmara hiperbárica de oxigênio. Porém, devido à amostra reduzida, o estudo não apresentou resultados estatisticamente relevantes, sendo necessária a realização de estudos subsequentes com amostragem mais significativa. ***Nível de Evidência II, Estudo Prospectivo Comparativo.***

Descritores: *Nervos Periféricos. Oxigenação Hiperbárica. Regeneração Nervosa. Ratos.*

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INTRODUCTION

Peripheral nerves are important for the maintenance of sensory and motor function in living beings; an injury to it results in partial or complete elimination of these functions. Despite several technological advances, including the use of a surgical microscope and epineural and perineural suture without tension of nerve endings, complete sensory and functional recovery of the nerve cannot yet be guaranteed.[1-5]

Innovative methods have been tested in an attempt to obtain better results in the treatment of nerve lesions. Among them, the treatment with hyperbaric oxygen chamber (HOC), which aims to increase oxygenation of peripheral tissues, deserves particular attention. The main objective being the elimination of hypoxia, avoiding the accumulation of substances with toxic effect that promote the process of ischemia and inflammation.

All authors declare no potential conflict of interest related to this article.

The study was conducted at Universidade de São Paulo, Medical School, Hospital das Clínicas, Institute of Orthopedics and Traumatology, Hand Surgery and Reconstructive Microsurgery Group.

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The proper functioning of aerobic mechanisms must be ensured while maintaining the vitality of the region.[6,7]

Treatments with HOC has been reported to reduce ischemic lesions in various tissues.[8-11] In the peripheral nervous system, HOC treatment rescued nerve fibers in test subjects with experimentally impaired microcirculation.[12,13] In peripheral nerve injury, it has been shown that oxygen at high pressures has favorable effects on the recovery of mechanically damaged nerves, either by nerve sectioning, crushing, or both, in animal models and in humans.[14-22]

On the other hand, there were unsatisfactory functional results in nerve recovery after adjuvant treatment with HOC in rats with transfixing lesion or with crushed peripheral nerves.[22,23] Thus, we observed that there is still no consensus on the benefits obtained with the use of HOC for nerve lesions.

In this study, we evaluated the histological alterations presented in the regenerating nerve according to the duration of HOC treatment. Something not yet established in the studies found.

MATERIALS AND METHODS

This is an experimental study in rats, approved by the Ethics Committee of the Department of Orthopedics and Traumatology of HC/FMUSP, under protocol no. 15026. In total, 25 rats were used and the following inclusion criteria were adopted: Wistar rats; young adult males (120 to 140 days of life); weight between 250 and 330 grams; normal general condition (coat and clinical status) and motricity. Regarding exclusion criteria: females, death after injury; autophagy, and impossibility of reversing infection. We defined as criteria of interruption (suspension) and closure: weight loss greater than 10% during the postoperative period in a significant number of animals (greater than 10%), death of animals during the postoperative period in more than 10% of the total, and adverse reactions to the hyperbaric chamber (including changes in animal behavior and convulsion).

Anesthesia protocol

The animals received preanesthetic tramadol hydrochloride medication at a 5 mg/kg dose associated with meloxicam 2 mg/kg subcutaneously and, after 15 minutes, were anesthetized with xylazine 10 mg/kg + ketamine 100 mg/kg intraperitoneally. The deep anesthetic state was confirmed by the lack of corneal reflexes and lack of reaction to compression of the tail and hind legs. An anesthetic reinforcement was performed with one third of the initial dose in the specimen that still presented reflexes during the procedure.

Surgical procedure

Following the anesthesia, the rat was positioned in ventral decubitus on a wooden board, immobilizing the thoracic and pelvic limbs. Subsequently, trichotomy of the right hind paw was performed, followed by degermation with alcoholic chlorhexidine solution. Through an incision in the posterior facet of the right paw, the musculature was removed until the sciatic nerve was exposed from its middle portion to the distal portion of its branches: fibular, sural, and tibial nerve. The nerve was cross-sectioned with microscissors, five millimeters proximal to the trifurcation of the sciatic nerve. Nerve repair was performed immediately after the injury, always by the same surgeon. The end-to-end external epineural neurorrhaphy technique was used with two stitches using mononylon thread size 10.0. The skin was subsequently sutured with mononylon thread size 5.0, in simple stitches.

Postoperative

After cleaning the surgical scar, a layer of healing ointment was applied, composed of fibrinolysin, deoxyribonuclease, and chloramphenicol (Fibrase ®). After surgery, meloxicam 2 mg/kg was administered for seven days and tramadol hydrochloride 5 mg/kg, for five days; both with a daily subcutaneous dosage. Feed and water were offered as soon as the animal was fully awake and was freely available throughout the treatment period. The rats were submitted to the hypothermia prevention protocol through the use of controlled temperature chambers of 25 to 28°C for 30 minutes in the immediate postoperative period.

Hyperbaric chamber

HOC treatment was initiated after 24 hours of surgery. The test subjects were divided into groups of five. The therapy adopted was the permanence in HOC for 30 minutes daily, at 2.5 ATA, according to the duration protocol per group of animals. The groups were separated as follows:

1) Control (no therapy in HOC)
2) Treatment with HOC for one week
3) Treatment with HOC for two weeks
4) Treatment with HOC for three weeks
5) Treatment with HOC for four weeks

At the end of the fourth week, the rats were sacrificed following the ethical principles in animal experimentation established by the Brazilian College of Animal Experimentation, Federal Law No. 6,638, of 1979. After euthanasia, a piece of the operated sciatic nerve was collected and sent, in a 10% formaldehyde solution, to the Pathology department, where they were examined by an evaluator who was not aware of the procedure to which the animal was submitted or of the previous division of the groups.

After receiving the pieces in 10% formaldehyde solution they were embedded in blocks of paraffin, followed by routine tissue processing. Slices sized five micrometers were performed with the microtome device and were stained with hematoxylin and eosin, toluidine blue, Masson and actin (immunohistochemistry) for microscopic evaluation. Specimens were examined under a light microscope.

After the process of fixation and staining of the pieces, the number of axons proximal and distal to the neurorrhaphy was checked. With these data we estimated the axonal regeneration index (RI), which consists of the ratio between the number of axons distal to the neurorrhaphy and the ones proximal to it.

Statistical procedures

First, the distribution of the data was tested with Shapiro-Wilk test, after which the means of the axonal regeneration index (RI) of the groups in their respective hyperbaric chambers were analyzed by the ANOVA test, with its variances homogeneity tested by the Levene test and Tukey's *post-hoc* test. The data were analyzed with the software SPSS version 24.0, considering as significant a $p \leq 0.05$.

RESULTS

We observed a higher number of axons distal to neurorrhaphy and a higher axonal regeneration rate in all groups submitted to HOC when compared to the control. The increase occurred according to the therapy exposure time, so that group 5 presented the best results.

Table 1 shows the results of the axon count proximal and distal to the neurorrhaphy, as well as the RI of each specimen in groups 1 to 5.

After the ANOVA test we obtained the following result: $F(4, 20) = 2.797$, $p = 0.054$, $\eta^2 p = 0.35$ leading to the conclusion that no significant differences were observed between the groups. (in which F is the function of the result obtained by ANOVA test).

Table 2 shows the RI mean and the standard deviations for each group.
Although the results were not statistically significant, probably due to the small sampling, we noticed that between the second and third week of therapy the increase was more expressive, as shown in Figures 1 and 2.

Table 1. Axons count proximal and distal to the neurorrhaphy of the sciatic nerve of the test subjects after 4 weeks of follow-up in the 5 different groups, n = 25.

	Group 1			Group 2			Group 3		
Nº of specimen	Nº of Axons			Nº of Axons			Nº of Axons		
	P	D	RI	P	D	RI	P	D	RI
1	345	115	0.333	412	189	0.459	368	110	0.299
2	344	224	0.651	321	144	0.449	400	299	0.748
3	396	203	0.513	374	214	0.572	362	205	0.566
4	325	189	0.582	398	328	0.824	398	421	1.058
5	412	330	0.801	347	247	0.712	347	125	0.360
Mean	364	212	0.576	370	224	0.603	375	232	0.606
SD	37	78	0.173	37	69	0.163	23	130	0.308

	Group 4			Group 5		
Nº of specimen	Nº of Axons			Nº of Axons		
	P	D	RI	P	D	RI
1	287	247	0.861	325	298	0.917
2	408	399	0.978	353	288	0.816
3	362	278	0.768	348	315	0.905
4	484	360	0.744	410	297	0.724
5	352	272	0.773	344	330	0.959
Mean	379	311	0.825	356	306	0.864
SD	73	65	0.096	32	17	0.094

P: proximal; D: distal; RI: axonal regeneration index; SD: standard deviation.

Table 2. Mean comparison of the axonal regeneration index (RI) in the sciatic nerve between groups with different durations of HOC treatment and the control group, n = 25.

Groups	Mean RI	SD
Group 1	0.576	0.173
Group 2	0.603	0.163
Group 3	0.606	0.308
Group 4	0.825	0.096
Group 5	0.864	0.094

SD: standard deviation.

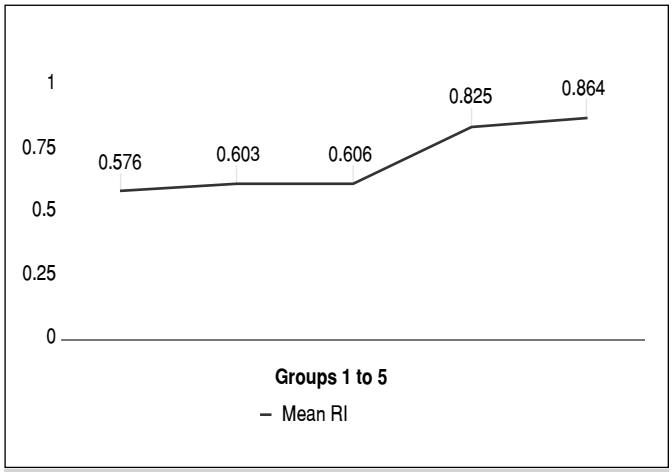

Figure 1. Mean axonal regeneration index per study group.

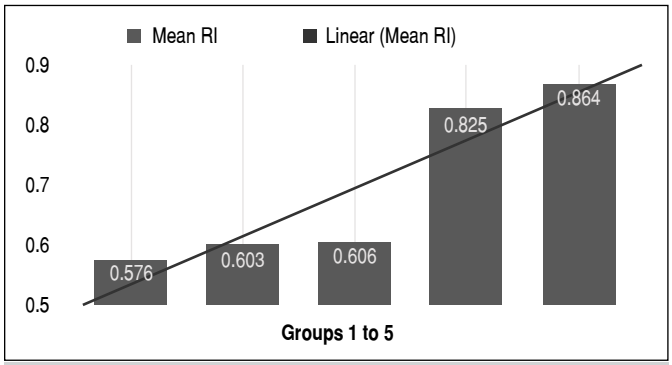

Figure 2. Trend of the mean axonal regeneration index form the data of the 5 groups studied.

DISCUSSION

Several studies have sought a correlation between HOC therapy and axonal regeneration in traumatized nerves. This study sought to understand if there is real evidence of increased and/or improved regeneration in traumatized nerves.[14,16,18,23-26]
During the study, each group was submitted to a different period of treatment, except for the control group, which was only medicated for pain according to the protocol adopted. It was possible to identify that the specimens submitted to HOC had an increase in RI compared to the control group and greater expression in groups 4 with 43% and 5 with 50% increase when compared to the control group. In relation to groups 2 and 3, the increase was not significant (4% in group 2 and 5% in group 3).
Despite these differences, there was no statistically relevant evidence between the control groups and those submitted to HOC. Therefore, there was no evidence of a dose-dependent effect of HOC treatment on the early regeneration of axons using the adopted protocol.
Eguiluz-Ordoñez et al.[7] also evaluated sciatic nerves of rats, in which the animals were sacrificed after seven postoperative weeks (control group and HOC group) or after 14 weeks (another control group and another HOC). In their study, HOC therapy was performed twice a day for ten days, with 100% oxygen, using 2 ATA. As for the animals evaluated at seven weeks, there was an increase in the number of axons in rats treated with HOC compared to the control group. Differently from our study, Eguiluz-Ordoñez et al. observed statistically significant difference, but only in the absolute number of axons, the RI was not considered. After 14 postoperative weeks, the groups showed only a slight difference in the number of axons.
Bradshaw et al.[24] conducted a study in which rabbits were exposed to different oxygen concentrations and to different atmospheric pressures in oxygen therapy. In this study, he noticed that the animals treated with compressed oxygen at 202, 242, and 303 kPa and 100% oxygen presented nerve recovery, with organized fibers similar to uninjured nerves. Animals submitted to 100% oxygen therapy at ambient pressure, compressed air and ambient air did not present a morphology similar to uninjured nerves.
Ince et al.[25] conducted a study to determine the effect of different durations of hyperbaric oxygen treatment application on nerve regeneration in rats. They found that rats submitted to HOC in the first hour after neurorrhaphy (group 2) had better functional gait results when compared to the other study groups (group without HOC therapy – group 1; HOC starting in the first week after neurorrhaphy – group 3; HOC started in the second week after neurorrhaphy – group 4), when evaluated in the eighth postoperative week. In the gait evaluation in the sixteenth postoperative week, group 2 continued with the best gait score

and group 3 presented better results when compared to groups 1 and 4. In conclusion, the benefit of early initiation of HOC after neurorrhaphy has been suggested.

The real cause of the difference in the number of distal axons in groups 4 and 5 is unknown. They may be related, however, to the known physiological effects of HOC, such as increased partial oxygen pressure not only in the blood, but in interstitial fluids and tissues; which causes increased oxygen tension, even in sites with low perfusion, such as the region distal to nerve damage.[27,28] Moreover, HOC is known to improve local perfusion of ischemic tissues, as it decreases the formation of local edema. Although we know the result, the mechanism itself for preventing edema is still poorly understood, but may be associated with the fact that HOC causes temporary vasoconstriction and reduced post-ischemic vascular permeability.[29-34]

There are no conclusive studies on the efficacy of HOC use for the treatment of peripheral neural regeneration. However, some studies, ours included, have provided intriguing results that allow for further study.

Our study suggests that with three weeks or more of HOC, the axonal regeneration index increases in the sciatic nerve of rats, but no statistically relevant HOC effect was demonstrated, probably due to our reduced sample, something that may be proven by conducting a study with a larger sample.

CONCLUSION

Our study did not present statistically relevant results on the use of HOC in sciatic nerve regeneration in rats. Although groups 4 and 5 showed an important improvement in the axonal regeneration index, the limited number of samples (5 rats per group) may have precluded a statistically significant result. Therefore, a greater number of experimental studies with more significant samples would be of great value, mainly aiming to understand HOC's mechanism of action in regions related to nerve damage with a higher incidence of ischemia.

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