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Morphofunctional analysis of sciatic nerve and motor performance of rats after cryotherapy with liquid nitrogen

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ABSTRACT

Objective. This work evaluated sciatic nerve regeneration after cryotherapy.

Study design. Rats underwent surgical access of the sciatic nerve and subsequent cryotherapy, crush lesion or no manipulation. Walking-track, electroneuromyographic and histomorphometric analyses were performed at 15, 30 and 70 postoperative days.

***Results*.** At 15 days, the crush and cryotherapy groups showed significant morphofunctional impairment. At 30 days, functional loss was significant in the walking track group, but at 70 days, there were no significant differences between the groups. Amplitude was near zero for the crush group at 15 and 30 days, and zero for the cryotherapy group. Measurement of latency was not possible in this latter group. Crush and cryotherapy groups showed greater amounts of myelinated fibers (by 30^{th} day), with axonal diameter and width of the myelin sheath being less than in controls.

Conclusion. Sciatic nerve lesion by application of liquid nitrogen is classified as axonotmesis, which is reversible*.*

INTRODUCTION

The jaws can be the site of some benign lesions such as ameloblastoma, odontogenic keratocystic tumor, odontogenic myxoma and central giant cell lesion, whose locally aggressive behavior leads to potential recurrences. These recurrences are related to tissue remnants of the lesion in the bone margins.[1] Therefore, the conventional treatment in these cases is surgery with safety margins or resection, which can result in mutilation and difficult esthetic and functional rehabilitation.[2]

The therapeutic approach can be conservative, through surgical enucleation or curettage, if these procedures are complemented by applying to the residual bone cavity an agent capable of eliminating the cellular remnants. Cryotherapy is an efficient method of tissue destruction by freezing, which induces bone necrosis, where the inorganic scaffold remains intact, allowing osteoconduction.[3] For this reason, it has been used as complementary therapy for locally aggressive bone lesions.[4] It produces safety margins without esthetic and functional sequelae, with the major advantage of a conservative treatment, preserving important anatomical structures such as the inferior dental nerve and infraorbital nerve.[1] However, in cases of extensive lesions, low temperature can also exert some effects on nerve branches causing injury to peripheral nervous tissue.[5]

Some clinical studies suggest the reversibility of neurosensory changes associated with cryotherapy applied near or directly to peripheral nervous tissue.[3,6] Nevertheless, the issue is controversial and most studies supporting this idea are clinical, with no experimental *in vivo* studies reported. Research involving humans generally shows high variability, whereas the rat sciatic nerve lesion is the best

experimental model to test the morphophysiologic effects of different protocols such as crushing and cryotherapy.[7] The present work investigated rat sciatic nerve regeneration after cryotherapy with liquid nitrogen by means of walking-track, electroneurophysiologic and histomorphometric analyses.

MATERIAL AND METHODS

The present study was approved by the Ethics Committee for Animal Use of the Pontifical Catholic University of Rio Grande do Sul. The sample comprised 54 adult male Wistar rats weighing an average of 272 g. The animals were randomly allocated into 9 groups of 6 rats each, and classified according to the type of lesion produced in the peripheral nerve and the time of analysis. Groups 1 (15 days), 2 (30 days) and 3 (70 days) comprised rats without sciatic nerve lesion (controls); groups 4 (15 days), 5 (30 days) and 6 (70 days) comprised rats subjected to a sciatic nerve crush lesion; and groups 7 (15 days), 8 (30 days) and 9 (70 days) comprised rats subjected to cryotherapy lesion of the sciatic nerve.

Sciatic nerve approach

The animals were anesthetized intraperitoneally with a solution of 5% ketamine (90 mg / kg, Vetbrands, Jacareí, SP, Brazil) and 2% xylazine (15 mg/kg, Vetbrands, Jacareí, SP, Brazil). The right sciatic nerve was surgically accessed and exposed at its emergence from the distal bifurcation. A styrofoam device was positioned between the sciatic nerve and dissected muscles, in order to isolate the area receiving cryotherapy. The lesions (crush and cryotherapy) were performed in the sciatic nerve at 1 cm proximal to its bifurcation into the tibial and common peroneal

nerves. The control groups were subjected to the surgical access and placement of the styrofoam device, without producing any sciatic lesion, either crush or cryotherapy. A Cry-Ac cryogenic system (Brymill Cryogenics Systems, Ellington, CT, USA) for liquid nitrogen spray was used in the cryotherapy groups. Two applications of nitrogen, lasting 10 seconds each, with a 2.5-minute interval between them, were performed. In the crush groups, the sciatic lesion was performed with a calibrated curved Halstead mosquito forceps, also in 2 applications of 10 seconds with a 2.5-minute interval in between. The wound was closed in layers with interrupted 4-0 mononylon sutures.

Walking-track analysis

At 15, 30 and 70 days after the surgery, the animals underwent a postoperative walking-track analysis, based on the protocol described by De Medinaceli et al.[8] The rats were trained to walk over a white sheet of paper covering the bottom of a wooden alley (43 x 9 x 7 cm) ending in a dark goal box. Afterward, the animals had their ventral hindpaw painted with dark dye, and then they were placed on the track to walk. The rats paw prints were used to determine the following measurements: (1) distance from the second to the fourth toe (intermediate toe spread, ITS); (2) distance from the first to the fifth toe (toe spread, TS); and (3) distance from the heel to the third toe (print length, PL). These measurements were then inserted in the sciatic functional index (SFI) formula according to Bain et al.[9]

Electroneurophysiologic analysis

After the walking track, animals underwent electroneurophysiologic analysis, in which the motor neuroconduction of the sciatic nerve was determined. With the

animals hand held, an electric stimulus of 0.2 milliseconds and 30 mA was applied in the proximal region of the sciatic nerve. In this way, a bipolar stimulator (Medelec Synergy, San Francisco, CA, USA) was positioned parallel to the long axis of the sciatic, and electrodes were placed on the dorsal and ventral surfaces of the hind leg. Another surface electrode was placed on the tail, serving as a ground electrode. The variables amplitude and latency of action potential of the right paw were measured.

Euthanasia of animals and histologic processing

After the walking-track and electroneurophysiologic analyses at the different times of observation, the animals were anesthetized and humanely killed. A 1-cm segment of the right sciatic nerve was excised at a point distally located in relation to the lesion site. The specimen was fixed by 24-h immersion in a modified Karnovsky solution (Sigma Chemicals, St Louis, MO, USA). Next, it was bathed in 0.05 M sodium cacodylate, post-fixed in 1% osmium tetroxide (Sigma Chemicals, St Louis, MO, USA) for 2 hours, dehydrated in an increasing graded series of acetone (Electron Microscopy Sciences, Hatfield, PA, USA), and embedded in epoxy resin (Araldite, Durcupan, Fluka, Buchs, Switzerland), which was then polymerized at 60°C. Semi-thin cross-sections (1 μm) were obtained, using an ultramicrotome (MT 6000-XL, RMC, AZ, USA) and stained with 1% toluidine blue (Merck, Darmstadt, Germany) in 1% sodium tetraborate (Ecibra, Curitiba, PR, Brazil).

Histomorphometric analysis

The histologic images were digitized using a videocamera (Axiocam-Zeiss, Zeiss, VA, USA) coupled to a light microscope (Imager A1-Zeiss, NY, USA) and to a computer (Compaq, Pentium 4, NY, USA) with x 5 and x 100 objectives. Histomorphometric analysis was performed by a calibrated blind examiner using Image Pro Plus 4.1 software (Media Cybernetics, MD, USA). Myelinated fibers were counted in each image and their density (no. of fibers/area) was calculated. Myelin sheath thickness, axonal area and axonal diameter were calculated by means of a specific software tool.

Statistical analysis

The results obtained were compared between the groups (control, crush and cryotherapy) at the different times evaluated. Data were analyzed by descriptive statistics, the Kruskal-Wallis test (complemented by its multiple comparison test) and *one-way* ANOVA (complemented by Tukey's test), setting the level of significance at 5%. The statistics were processed by the SPSS 10.0 software (Statistical Package for the Social Sciences, Chicago, IL, USA).

RESULTS

Walking-track analysis

The walking track results are presented in Table I. At 15 days, the three groups differed, with the cryotherapy group showing the lowest SFI values. At 30 days, the cryotherapy and crush groups showed SFI values significantly lower compared to

the control group. At 70 days, there was no significant difference between the groups (Kruskal-Wallis, multiple comparison test, α=0.05).

Electroneurophysiologic analysis

The results for electroneurophysiologic analysis are presented in Tables II and III.

Action potential amplitude of sciatic nerve

There was a significant difference in this variable at 15 and 30 days, at which time the crush and cryotherapy groups did not differ from each other, but both had lower values compared to the control group. At 70 days, there was no significant difference between the groups (Kruskal-Wallis, multiple comparison test, α=0.05).

Action potential latency of sciatic nerve

There was a significant difference in the action potential latency of the sciatic nerve between the three groups at 70 days, with the cryotherapy group showing the highest values (Kruskal-Wallis, multiple comparison test, α=0.05).

Histomorphometric analysis

The results for histomorphometric analysis are presented in Figures 1 and 2.

Myelinated fibers

For the 30-day period, the cryotherapy group showed number of myelinated fibers/nerve significantly higher than those of the control and crush groups, but there was no significant difference at the other evaluation times (one-way ANOVA, Tukey's test, α=0.05). For the 30- and 70-day period, myelinated fiber density (no. of fibers/mm^2) was significantly higher for the cryotherapy group compared to the control and crush groups, but these latter two did not differ significantly from each

other. There was no significant differences for this variable at 15 days (one-way ANOVA, Tukey's test, α=0.05).

Myelin sheath thickness and axonal diameter

At 15, 30 and 70 days, the cryotherapy and crush groups did not differ from each other, but had significantly lower values of myelin thickness compared to the control group (one-way ANOVA, Tukey's test, α=0.05).

At 15 and 70 days, the control group had an axonal diameter that was significantly greater compared to the cryotherapy and crush groups, whereas the latter two did not differ significantly from each other. At 30 days, the cryotherapy group had significantly lower values than those of the control, but did not differ from the crush group, which in turn did not differ significantly from the control (one-way ANOVA, Tukey's test, α=0.05).

DISCUSSION

The results of walking-track analysis suggest a significant functional deficit in the crush and cryotherapy groups in the 15-day period, with an improvement at 30 days and recovery at 70 days. The observed functional loss reflects a decrease or absence of nerve endings that innervate the muscles involved.[10] This finding corroborates other studies,[11-13] in which the functional recovery of crush injuries only started on the thirtieth day after the injury. However, other authors reported complete recovery during the third and fourth weeks.[14-16] Comparing the walking-track results between the crush and cryotherapy groups, a significant lower SFI was observed in the latter at 15 days. At 30 and 70 days, these two groups had

close values without significant difference. These data demonstrate that freeze injury, from a functional point of view, is more serious in the first 15 days, and that injuries from crushing and freezing have similar recovery within 30 days.

One animal in the cryotherapy group was excluded from the walking-track analysis because it had suffered autotomy of two digits of the paw in the operated limb. This finding has been associated in the literature with neuropathic pain caused by injuries of great magnitude in peripheral nerves[17,18] or with cryoneurolysis.[19,20] The injury resulting from freezing disrupts axonal integrity, which blocks the transmission of pain.[6,21] Some authors are opposed to the use of this technique, arguing that cryotherapy of the peripheral nerve tissue can lead to subtantial neuralgia.[22,23] Thus, we cannot rule out the possibility that cryotherapy causes, in some patients, neurosensory changes such as hyperesthesia, neuralgia and allodynia during the postoperative period, which were not evaluated in the present study. The tests applied here are morphofunctional ones and evaluate motor performance; they are not suitable for neurosensory evaluation. Nevertheless, they are considered the gold standard for animal models and efficiently evaluate the damage to the peripheral nerve.[24] On the other hand, sensory tests in animal models are challenging with regard to quantification and are still not reliable, whereas clinical studies applying questionnaires to patients are limited due to their subjectivity.[25]

Higher amplitude represents a greater number of functional axons, which in turn represents the number of motor nerve fibers regenerated.[26] We found amplitude values close to zero in the crush group at 15 and 30 days, and zero for the cryotherapy group. At 70 days, these values were not significantly different

between the groups. Kerns et al.[15] also assessed freeze and crush lesions, and obtained amplitude values similar to those of our research in the first 15 days. Nevertheless, the authors found values close to the control group as early as 30 days. This difference may result from lower intensity lesions induced by those authors, who used jeweler's forceps for the crush injury and the indirect method (probe) for freezing, where the temperature of liquid nitrogen, when reaching the site of application, is not as cold as in the spray method.[27]

The measurement of latency was not possible in the cryotherapy group at 15 and 30 days, suggesting an absent pulse in nerve fibers and, therefore, an important functional deficit in the affected nerve. The measurement was possible at 70 days, when the values in this group were significantly higher than in the control and crush groups. This finding corroborates the study by Wolthers et al.[28] in which there was an increase in latency after crush injuries of up to 150 postoperative days.

The crush and cryotherapy groups showed more myelinated fibers than the control group at 30 and 70 days. This is due to the fact that each injured axon may give rise to two or three axonal extensions during the regenerative process, which progress towards the muscle to restore the connection and function.[29,30] After 70 days, the number of myelinated fibers in the crush and cryotherapy groups was lower than at 30 days. This finding is consistent with the report of Gorio et al.[31] Accordingly, in crush injury of the sciatic nerve of rats, the number of fibers remains high until the twenty-sixth postoperative day. At this time, the process of synaptic elimination starts, causing a reduction in fibers in the segment distal to the lesion.

The maturation of the regenerated axons can be determined under a microscope by means of axonal diameter and thickness of the myelin sheath.[32] The decrease in the caliber of the axon in the injured nerve is accompanied by thinning of the myelin sheath.[31,33,34] This effect was also observed in the present study, where the thickness of the myelin sheath in the crush and cryotherapy groups was significantly less than in the control group at all times evaluated. The axonal diameter behaved similarly. Axonal diameter and thickness of the myelin sheath increased in the crush and cryotherapy groups with time, but they did not reach the values shown by the control group, which means incomplete maturation of the nerve during the regeneration process in these two groups. Verdú et al.[35] demonstrated that, even for longer assessment periods, the axonal diameter and thickness of the myelin sheath did not reach normal values after crush injury. According to these authors, discrete dimensional changes in these features do not indicate functional alterations.

According to the statistical analysis, the crush and cryotherapy groups showed similar results. Considering that the crush injury is classically an axonotmesis, one can infer that this is also the type of lesion occurring in the cryotherapy group. Axonotmesis, in turn, refers to the break in the continuity of the axon with the maintenance of an intact epineural sheath,[36,37] a characteristic whose detection requires analysis by scanning electron microscopy.[38] The results indicate that the peripheral nerve injury induced by liquid nitrogen spray directly on the neural structure is not confined to the myelin sheath, which disagrees with Schmidt and Pogrel,[5] who classified this lesion as neuropraxia. A possible explanation for this discrepancy is the fact that these authors conducted a clinical

study performed by means of tests and questionnaires about neurosensorial disorders, whose subjectivity can be considered an important bias. These authors, besides classifying the injury resulting from cryotherapy as neuropraxia, also stated that its average time of regeneration in patients was 90 days. However, the results of our study indicate that the freeze lesion is characterized as axonotmesis, in which a 90-day period is insufficient for complete neurosensory recovery.[39]

In the cases of cryotherapy reported in the literature, either the open (spray) or the closed (probe) system of liquid nitrogen was used.[4,6,15,27] Nevertheless, the spray system is the most applied technique in cryosurgery of jaw lesions, where its use is supported by some clinical studies.[1-3,5] The present study pioneers the use of this technique (spray) in animal models attempting to experimentally reproduce the peripheral nerve injury resulting from the use of liquid nitrogen as a complementary therapy to surgical treatment of bone lesions. The choice for the open system was based on the higher frequency of this modality in such cases.

It is also important to state some considerations about the sciatic nerve compared to the inferior dental nerve, which is probably the most affected nervous structure in these jaw lesions needing cryotherapy. The sciatic nerve is classified as a mixed nerve, whereas the inferior dental nerve is a sensory one. This is the main difference between them. Nevertheless, sensory and motor fibers are histologically and morphologically similar. That is, the sciatic nerve of rat and human inferior dental nerve are constitutionally similar[40] and therefore prone to the same structural effects with cryotherapy. The morphological injury in the sciatic nerve evaluated here is representative of injury to the inferior dental nerve.

Actually, the functional result from this injury would be different depending on the physiology of each one. An injury to a sensory nerve will result in sensory disturbances (paresthesia, hyperesthesia), whereas an injury to a motor nerve will result in motor disturbances (loss of muscle and glandular function).[5] Our aim in this study using the sciatic nerve was to evaluate the magnitude of the structural damage to the nerve caused by cryotherapy, which in this gold standard model, needs to be evaluated by the tests performed here, including motor tests, as the sciatic is a mixed nerve.

The present study suggests that the use of cryotherapy with liquid nitrogen as a complementary therapy for locally aggressive lesions of the maxillomandibular complex requires caution. The damage to the nervous tissue caused by cryotherapy seems to be more severe than that indicated by clinical studies. Therefore, in the postoperative care of the patient, the time for neurosensory recovery might be longer than that indicated in the literature. Moreover, according to a risk/benefit analysis, cryotherapy combined with surgical enucleation of large bone lesions appears to be well indicated. The procedure allows for less mutilating surgery, where the damage to the nerve structure is reversible.

CONCLUSIONS

The results of this study let us draw the following conclusions: (a) the damage caused by the direct application of cryotherapy using liquid nitrogen spray on the rat sciatic nerve is characterized as an axonotmesis injury; (b) cryotherapy with

liquid nitrogen spray applied directly on the peripheral nerve causes reversible neural changes; (c) the freeze lesion displays the development of a regenerative process similar to that observed with crush injury.

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FIGURE LEGENDS

Fig. 1. Microscopic analysis of regenerating rat sciatic nerve fibers. Graphs show the total number of myelinated fibers/nerve (A), myelinated fiber density (B)**,** myelin sheath thickness (C) and axonal diameter (D). Ct=control group; Ch=crush group; Cr=cryotherapy group; 15d=15 days; 30d=30 days; 70d=70 days.

Fig. 2. Image of semithin cross-sections (1 µm) obtained from regenerating sciatic nerves in: control group at 15 (A), 30 (B) and 70 (C) days; in crush group at 15 (D), 30 (E) and 70 (F) days; and in cryotherapy group at 15 (G), 30 (H) and 70 (I) days (toluidine blue stain, x 100, bar =10 µm).

(The figures are not available in this version).

Table I. Sciatic functional index (SFI) for control, crush and cryotherapy groups at 15, 30 and 70 days

Group	15 days			30 days			70 days		
	M	SD	Med	M	SD	Med	M	SD	Med
Control	-12.07	7.82	-10.64[A]	-8.39	8.77	-11.35[A]	-7.11	19.90	-7.02
Crush	-45.85	15.17	-44.53[B]	-22.00	10.66	-18.90[B]	-14.75	11.04	-12.24
Cryotherapy	-66.42	15.49	-67.61[C]	-22.91	6.35	-25.20[B]	-11.21	3.30	-13.26
p value	0.002			0.038			0.593		

Kruskal-Wallis, multiple comparison test (α=0.05)
M= mean; SD= standard deviation; Med= median
Medians followed by different letters represent groups that differed significantly

Table II. Sciatic action potential amplitude for control, crush and cryotherapy groups at 15, 30 and 70 days

Group	Action potential amplitude								
	15 days			30 days			70 days		
	M	SD	Med	M	SD	Med	M	SD	Med
Control	6.02	2.01	5.75[A]	8.12	2.69	8.25[A]	4.58	1.10	4.55
Crush	0.20	0.28	0.10[B]	0.25	0.36	0.05[B]	3.83	0.19	3.80
Cryotherapy	0.00	0.00	0.00[B]	0.00	0.00	0.00[B]	3.50	1.27	3.20
p value	0.001			0.002			0.209		

Kruskal-Wallis, multiple comparison test (α=0.05)
M= mean; SD= standard deviation; Med= median
Medians followed by different letters represent groups that differed significantly

Table III. Sciatic action potential latency for control, crush and cryotherapy groups at 15, 30 and 70 days

Group	Action potential latency								
	15 days			30 days			70 days		
	M	SD	Med	M	SD	Med	M	SD	Med
Control	2.16	0.48	2.33	1.98	0.21	1.95	1.83	0.51	1.83 [A]
Crush	1.55	1.86	1.20	0.69	1.07	0.00	2.82	0.31	2.78 [B]
Cryotherapy	--*	--*	--*	--*	--*	--*	3.54	0.46	3.58 [C]
p value	0.394			0.132			0.001		

Kruskal-Wallis, multiple comparison test (α=0.05)
M= mean; SD= standard deviation; Med= median
Medians followed by different letters represent groups that differed significantly
* Measurement was not possible