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Stretch-induced nerve injury: a proposed technique for the study of nerve regeneration and evaluation of the influence of gabapentin on this model

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Abstract

The rat models currently employed for studies of nerve regeneration present distinct disadvantages. We propose a new technique of stretch-induced nerve injury, used here to evaluate the influence of gabapentin (GBP) on nerve regeneration. Male Wistar rats (300 g; n = 36) underwent surgery and exposure of the median nerve in the right forelimbs, either with or without nerve injury. The technique was performed using distal and proximal clamps separated by a distance of 2 cm and a sliding distance of 3 mm. The nerve was compressed and stretched for 5 s until the bands of Fontana disappeared. The animals were evaluated in relation to functional, biochemical and histological parameters. Stretching of the median nerve led to complete loss of motor function up to 12 days after the lesion ($P<0.001$), compared to non-injured nerves, as assessed in the grasping test. Grasping force in the nerve-injured animals did not return to control values up to 30 days after surgery ($P<0.05$). Nerve injury also caused an increase in the time of sensory recovery, as well as in the electrical and mechanical stimulation tests. Treatment of the animals with GBP promoted an improvement in the morphometric analysis of median nerve cross-sections compared with the operated vehicle group, as observed in the area of myelinated fibers or connective tissue ($P<0.001$), in the density of myelinated fibers/mm^2 ($P<0.05$) and in the degeneration fragments ($P<0.01$). Stretch-induced nerve injury seems to be a simple and relevant model for evaluating nerve regeneration.

Key words: Nerve regeneration; Median nerve; Motor activity; Rat model

Introduction

Peripheral nerve injury has a prevalence of 1-3% among adult patients with polytrauma, with a higher proportion of affected males than females, and has consequences that often lead to a functional loss of the affected limb (1,2).

Rat models of nerve injury are the most prevalent type used in studies designed to evaluate nerve regeneration (3), and they serve as a basis for studies aimed at seeking new forms of treatment as well as those intended to improve understanding of the neurophysiological mechanisms by which drugs act in the treatment of such conditions. The use of forelimbs in these experiments is clinically interesting since it approximates what is usually observed in patients with brachial plexus nerve injury (4,5). However, the major kinds of experimental lesion to this nerve are axonotmesis by crushing (6) or neurotmesis followed by microsurgical nerve reconstruction (7). These techniques present a number of disadvantages, such as a fast regeneration process and the need for microsurgical techniques, respectively. Thus, the development of techniques to overcome these difficulties is justified, given the importance of their application in studies of nerve regeneration.

Another important area of investigation is the contribution to functional nerve recovery of substances with clinical application in these conditions. Patients with

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peripheral nerve injury, in addition to having paralysis of the affected limb, frequently present with neuropathic pain, which is a cause of great suffering, generally leading the clinicians to employ one or more classes of analgesics for their treatment (8,9). Among the substances commonly used in such cases, gabapentinoids such as gabapentin (GBP) and pregabalin are the first-line treatments of choice (10). These agents are associated with very satisfactory pain relief, but very little is known about their effects on nerve regeneration, which could be another aspect of their action.

In view of the issues discussed above, the present research proposes a new rat model of median nerve injury that induces a later onset of functional recovery compared with the results previously obtained with crush injury alone. The possible influence of gabapentin was investigated in this model.

Material and Methods

Animals

A total of 36 male Wistar rats weighing about 300 g were divided into three experimental groups of 12 animals each: 1) sham-operated, animals that underwent surgery and exposure of the median nerve, but were not subjected to nerve injury, and were treated with GBP; 2) operated-vehicle, animals that underwent surgery and exposure of the median nerve with subsequent nerve injury by crushing followed by stretching (stretch-induced nerve injury as described below), and were treated with the GBP vehicle; 3) operated-GBP, animals that underwent surgery and exposure of the median nerve with subsequent nerve injury, and were treated with GBP.

Treatment with vehicle (sterile isotonic saline used to dissolve GBP) or GBP (300 mg/kg; Sigma, Brazil) administered orally was performed daily from the day following surgery up to 30 days after the procedure.

Stretch-induced nerve injury

The animals were anesthetized with a mixture of 100 mg/kg ketamine + 10 mg/kg xylazine, by intramuscular injection, and remained under spontaneous breathing during the operative period. After verifying the general anesthesia, the rats were placed in the supine position under a plank of Formica (30×35 cm), with the forelimbs immobilized. Trichotomy and antisepsis with 2.0% chlorhexidine were done on the inner surface of both forelimbs. The nerve lesion was performed by crushing followed by stretching of the median nerve of the left forelimb of the animals as follows. The distance between the proximal and distal clamps was 2 cm. A sliding action of 3 mm was used, and the nerve was kept compressed and stretched for 5 s (Figure 1). These measurements were chosen empirically, with the goal of achieving an injury by axonotmesis, which was more pronounced than that caused only by nerve crush. As anatomical orientation for the crushing procedure, the reference for the proximal segment (infraclavicular region) was the origin of the median nerve along with the ulnar nerve, just below the common trunk. In the distal segment (the region of the cubital fossa), the reference was the point immediately before the emergence of the branch to flexor digitorum sublimis (between the two heads of the branch) and the deep flexor muscles. In addition, resection of the ulnar nerve of both the right and left forelimbs was performed. This procedure was aimed at preventing any interference in the measurement of the grasping force of the left paw during the evaluation of the functional activity in the grasping test (see below).

After recovering from anesthesia, the animals received postoperative analgesia (100 mg/mL dipyrone for 4 days; Eurofarma, Brazil) in water, which was offered freely. Additionally, the animals were weighed and monitored daily for discomfort and possible infection. All protocols were approved by the Ethics Committee for Animal Use from Universidade do Sul de Santa Catarina (#11.005.4.01. IV).

Nerve regeneration assessment

Functional analysis of the injured nerve was performed by using the grasping test and the response of the animals to mechanical or electrical stimulation. The grasping test is a simple procedure, originally described for the quantitative assessment of peripheral nerve regeneration in rats (4). In this test, the rats were lifted by the tail allowing them to grab a grid of wires with dimensions of 1.5 mm diameter and 8×14 cm overall, which was fixed on an electronic balance. The animals were assessed daily up to 30 days after surgery and results were recorded for the day after surgery in which full flexion of the toes of the operated (left) paw of the animal was observed. This outcome was then registered as the day of nerve recovery after injury. Furthermore, at the exact moment that the animals grabbed the railing, a drift perpendicular to the plane of the wire grid was undertaken and the traction was steadily increased until the moment that the animal dropped the grid. A negative weight was then registered on the balance at the point where the animals released the grid, and was recorded as the grasping force (in g). As described earlier, the right forelimb did not influence this measurement because it was denervated.

The animals' responses to mechanical stimulation were assessed using the von Frey filaments method. In this test, the animals were assessed daily up to 30 days after surgery and the result was recorded when the animal withdrew the injured paw upon the application of a nylon monofilament corresponding to a pressure of 10 g. This pressure was chosen because it was the force that promoted a greater frequency of response in the sham-operated group from the day immediately after nerve injury. The animals were placed in individual boxes with a

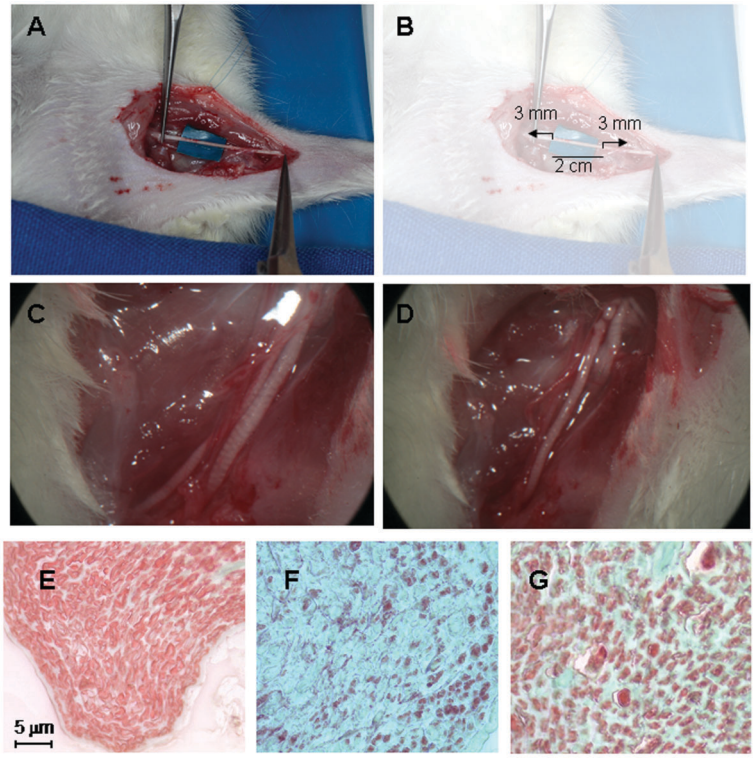

Figure 1. Photograph illustrating the procedure of crushing followed by stretching of the median nerve in rats, and light micrographs showing changes in the nerve structure of animals receiving different treatments. *A*, Isolation of the median nerve. *B*, Characteristics of the procedure for crushing followed by stretching of the median nerve: distance between the clamps 2 cm proximal and distal, sliding of 3 mm and the nerve was kept compressed and stretched for 5 s, until the bands of Fontana disappeared. Bands of Fontana in the non-injured (*C*) or injured (*D*) specimens. *E-G*, Light micrographs of cross-sections of the distal portion of the median nerve on the 30th day after stretch-induced injury in animals from sham-operated, operated-vehicle or operated-GBP groups, respectively. In *E*, note the apparent bimodal spectrum of fibers, myelinated small and large size (red), and little connective tissue (green) in the space between endoneurial myelinated fibers. In *F*, the predominance of small diameter fibers as well as an increase in endoneurial connective tissue space is suggestive of endoneurial fibrosis. In *G*, myelinated fibers similar to those from the sham-operated group. The images were obtained with 1000× magnification.

wire-mesh base and the filaments were applied through the mesh in the plantar center of the operated paws of the animals. This was repeated on a daily basis until a withdrawal response and shake of the paw could be seen. This was recorded as the day after surgery that the animals responded to this kind of stimulus.

To evaluate the response of the animals to electrical stimulation, a neurostimulator was used to deliver stimuli with intensities of 5 or 39 V on the plantar surface of the operated paw. The animals were assessed daily up to 30 days after surgery, with the onset of a response being recorded as the day that they had a withdrawal response of the forelimb to one of the two voltages evaluated. This was recorded as the day after surgery that the animals responded to an electrical stimulus.

Histology

On day 30 after median nerve injury (crushing and stretching) and after functional assessments, the rats were deeply anesthetized with isoflurane and killed by decapitation. The median nerve was removed by incision at the site of its insertions (axillary and cubital regions). The distal portion of the left median nerve was excised and immediately immersed in a buffered fixative solution of zinc-formalin (1.6% zinc chloride, 4% formaldehyde, 20% calcium acetate) for 24 h. After fixation, the samples were placed in 5% potassium dichromate solution for 5 days. They were then put in running tap water overnight to wash out all traces of dichromate before dehydrating in graded concentrations of ethanol. All samples were embedded in paraffin wax; 5-µm thick sections were obtained and mounted, and the slides were stained with Cason's trichrome (11,12).

Morphological analysis

Once stained, the sections were observed and photographed under light microscopy. Four parameters were quantified: 1) degeneration debris (%): to measure this, we took a photo at 100× magnification and calculated the total cross-sectional area of the nerve and then we identified areas of debris, connective tissue, and myelinated fibers; 2) area of connective tissue (%); 3) area of myelinated fibers (%), and 4) density of myelinated fibers/mm^2 as measured in photographs at 1000× magnification. The histological examination was restricted to the endoneurium and the myelin sheath area. Fields with folds or poorly preserved tissue components in histological sections were excluded. Morphological analysis was performed blinded with respect to group assignment. Digital images were acquired using a light microscope (Olympus, BX-41; Olympus America, USA), a digital camera (3.3 Mpixel QCOLOR3C, QImaging, Canada) and image acquisition software (Qcapture Pro 5.1, QImaging). The images were digitized (initially 1000× and further amplified 200× for analysis). The images were captured by the Image Pro Plus Software

6.0 (Media Cybernetics, USA). In each case, photomicrographs of 1600 × 1200 pixels were obtained from non-coincident and consecutive fields.

Measurement of neurotrophic factor levels in the median nerve

On day 30 after median nerve injury, proximal portions of the left median nerve were obtained as described above and weighed. They were homogenized in a glass homogenizer (Dounce Tissue Grinders, Omni International, USA) in a phosphate-buffered saline (PBS) solution containing Tween 20 (0.05%), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 2 ng/mL aprotinin, and 0.1 mM benzethonium chloride. The homogenates were transferred to 1.5-mL Eppendorf tubes, centrifuged at 3000 *g* for 10 min at 4°C, and the supernatant obtained was stored at –70°C until further analyses. The total protein content in the nerve samples (in mg) was measured in the supernatant using the Bradford method, this serving as the reference for the quantification of the neurotrophic factors. Sample aliquots of 100 µL were used to measure the brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels using rat neurotrophin enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (USA) according to the manufacturer's instructions. The absorbance for all of the neurotrophins studied was measured using a microplate reader at 450 and 550 nm.

Data analysis

The results are reported as means ± SE and were analyzed in Graphpad Instat using ANOVA followed by Tukey or Newman Keuls tests for continuous values, and the Kruskal-Wallis test for ordinal values (e.g., days after surgery for nerve recovery). Differences were considered statistically significant for $P \leq 0.05$.

Results

The procedure for the induction of median nerve injury in rats, by crushing followed by stretching, led to a loss of function of the median nerve. It can be seen in Figure 2 that compared with the sham-operated group, animals of both groups with nerve injury had decreased grasping force when evaluated in the grasping test. This effect lasted up to day 12 after nerve injury ($P<0.001$), while the average power grab of these groups was equal to zero. Although the grabbing force gradually increased after day 12 in the groups with nerve injury, it did not reach values similar to those observed in the sham-operated group until 30 days after injury ($P<0.05$). Daily treatment of the animals with GBP (300 mg/kg, orally, for 30 days after surgery) did not alter the function of the injured nerve. The values observed in these animals in the gasping test over the 30 days of examination did not differ from those observed in the groups with experimental injury that were treated daily with vehicle.

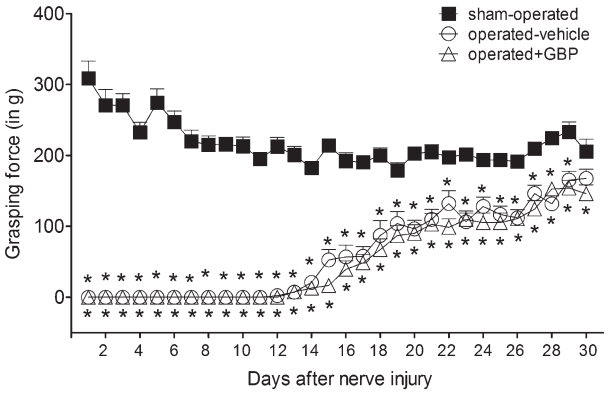

Figure 2. Analysis of the motor nerve function using the grasping test after stretched-induced nerve injury in rats subjected to different treatments. Functional recovery was measured up to 30 days after nerve injury and recorded as the grasping force (g). The loss of functional activity of the nerve caused by the injury was assessed by comparison of the animals from the operated-gabapentin (GBP) group in relation to the sham-operated group. The influence of GBP (300 mg/kg, orally, for 30 days) was assessed in relation to operated-vehicle animals. Data are reported as means ± SE for n = 6-12 animals per group. *$P<0.05$, compared to sham-operated group (two-way ANOVA followed by the Bonferroni test).

As can be seen in Table 1, stretch-induced nerve injury also caused an increase in the time of sensory recovery compared with the sham-operated group. In both the electrical and the mechanical stimulation tests, treatment of animals with GBP did not inhibit this effect. In addition, GBP did not lead to changes in the levels of neurotrophins detected in the median nerve of rats in relation to the other groups.

However, with respect to histological findings, treating the animals with GBP (same conditions) led to improvement in some parameters compared with operated animals that were treated with vehicle, as can be seen in photographs obtained in the morphometric analysis of median nerve cross-sections (Figure 3). The quantitative data from this analysis (Figure 4) show that the area of myelinated fibers in the operated-vehicle group (12.2 ± 1.2% of the cross-sectional area) was significantly less than that seen in the sham-operated group (57.1 ± 3.1%, $P<0.001$), and there was a partial reversal of this effect in the GBP-operated group (38.6 ± 3.0%, $P<0.001$). Analysis of the percentage of the area of connective tissue observed in sections from the median nerve (Figure 4B) demonstrated a statistically significant difference between the sham-operated and operated-vehicle groups (9.3 ± 1.2 and 76.5 ± 4.2%, respectively, $P<0.001$), with a significant increase in the latter group. Again, this effect on nerve injury observed in the sham-operated group was reduced by treating the animals with

Table 1. Analysis of the nerve recovery and neurotrophin levels from the median nerve of rats submitted to different treatments.

Group	Mechanical stimulus (day of recovery)	Electrical stimulus (day of recovery)		Neurotrophins (pg/mg protein)	
		5 V	39 V	NGF	BDNF
Sham-operated	3.5 ± 0.5	4.3 ± 0.5	2.4 ± 0.3	0.0163 ± 0.0035	0.6651 ± 0.1087
Operated-vehicle	13.1 ± 0.2*	17.7 ± 0.8*	14.0 ± 1.3*	0.0143 ± 0.0048	1.2050 ± 0.3484
Operated-GBP	14.6 ± 0.5*	16.4 ± 0.7*	14.1 ± 0.7*	0.0197 ± 0.0038	0.7897 ± 0.2218

Data are reported as means ± SE for n = 6-12 animals per group. GBP: gabapentin; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor. * $P<0.05$, compared to sham-operated group (ANOVA followed by the Kruskal-Wallis test).

GBP (63.6 ± 5.2%, $P<0.05$). This beneficial role for GBP on nerve regeneration was also observed in relation to the density of myelinated fibers/mm^2. As can be seen in Figure 4C, the operated-vehicle group had an average density of 18,789 ± 3568 fibers/mm^2, not statistically significantly different from that of the sham group (22,982 ± 885.6 fibers/mm^2). On the other hand, a significant increase in the density of myelinated fibers was observed in the operated-GBP group in relation to the other groups, with an average value of 29,600 ± 2822 fibers/mm^2 ($P<0.05$). Finally, Figure 4D shows that while virtually no fragments of degeneration were seen in the sham-operated group (0.25 ± 0.2%), an increase of this index in the operated-vehicle group (5.3 ± 1.0%, $P<0.01$) was observed. Again, a partial reversion of this effect was promoted by treatment with GBP (2.5 ± 0.8%, $P<0.05$).

Discussion

The present study proposes a new rat model of stretch-induced median nerve injury that could be a useful avenue for evaluating approaches to the investigation of nerve regeneration. This outcome was assessed by functional and histological measurements, with differences between control (sham-operated) and experimental (operated-vehicle) groups being observed in both cases.

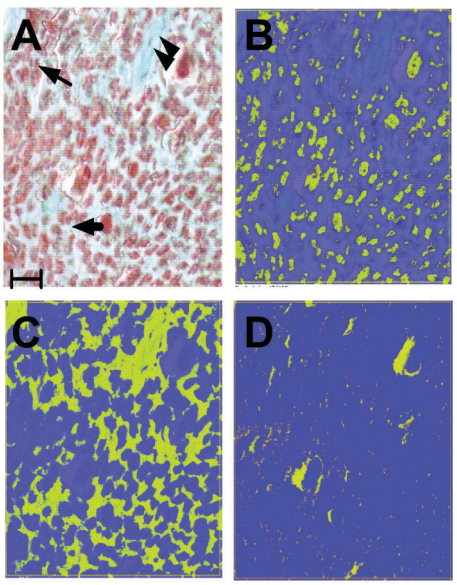

Figure 3. Morphometric analysis of cross-sections of the rat median nerve on the 30th day after stretch-induced nerve injury. Quantification of the area of myelinated fibers [thick arrow (*A*) and green (*B*)], connective tissue area [thin arrow (*A*) and green (*C*)] and quantification of fragments of degeneration [double arrowheads (*A*) and green (*D*)]. The sections of the nerves were stained with Mason trichrome staining, which allowed a clear distinction between myelinated tissue (red) and tissue (green). Magnification bar: 5 μm.

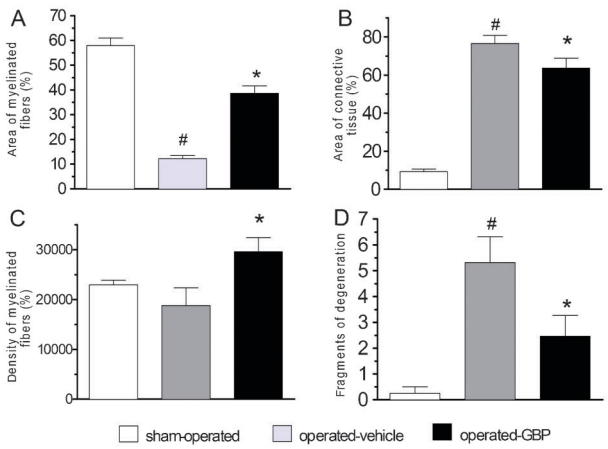

Figure 4. Quantitative analysis of histological cross-sections obtained from the median nerve of rats on the 30th day after stretch-induced nerve injury. The parameters measured were the area of myelinated fibers (*A*) and connective tissue (*B*), the density of myelinated fibers/mm^2 (*C*) and the fragments of degeneration (*D*). Data are reported as means ± SE for n = 6-12 animals per group. Measurements were made at 1000× magnification GBP: gabapentin. $^{\#}P<0.05$, compared to sham-operated; *$P<0.05$, compared to operated-vehicle (ANOVA followed by the Tukey test).

In the first case, recovery of motor function was mainly assessed using the grasping test, which presents some advantages for clinical practice since the large majority of the surgical interventions for repairing a damaged human nerve are performed at the upper limb level. The welfare of the animals is also better preserved following lesion of the median nerve compared with sciatic nerve lesions (3,4). When proposing the grasping test as a method to evaluate motor function, the authors cited above compared it with the crush-injury technique. Here, stretch-induced nerve injury promoted a longer recovery time as measured by the recovery of motor function, with recuperation first seen 8 days after lesion in the other authors' study, compared with an average of 12 days in this work. Thus, it seems that stretch-induced nerve injury promotes more pronounced morphological and/or biochemical alterations than have been observed previously by others (4), suggesting that a lesion of greater severity was obtained by axonotmesis, which must be responsible for the delayed recovery of the nerve.

The above-cited feature can be useful in studies related to nerve regeneration, since by prolonging the onset of nerve recovery it can better differentiate the effects provided by different treatments. In the present study, this difference was reflected in the histological samples obtained from the groups with nerve injury but which had received different treatments: with vehicle or with GBP. This evaluation permitted the visualization of a significant effect of GBP, augmenting the area or the density of myelinated fibers, as well as reducing the area of degenerated tissue. This is an important observation since, in a previous study involving sciatic nerve injury using only the crush procedure in rats, the authors were unable to demonstrate any significant effect for pregabalin on nerve regeneration, as evaluated in behavioral and morphometric tests 21 days after the injury (13). Consequently, modification of the technique presented here could be responsible for this different outcome, which has allowed visualization of important effects of GBP as noted above. It is also an interesting model since, unlike the injury that occurs with crushing, the stretch injury is more likely to happen in reality (14).

On the other hand, in the present study, prevention of changes in the median nerve of rats induced by the stretch lesions treated with GBP were not related to improvement in the indices of functional recovery in the different tests. Measurements of neurotrophic factors BDNF or NGF 30 days after the nerve lesion, which were evaluated as possible components of GBP mechanisms of action, were also not altered in relation to the control group. Although the role of the neurotrophins in the survival and growth of nerve *in vivo* is already well established (15), a review of similar studies (16) can help us to explain these contradictory results. In accordance with those authors, differences in experimental results on nerve regeneration with axotomized motor neurons or distal nerve stumps can be attributed to the temporal pattern of expression of the neurotrophic factors and their receptors. Taking that into account, in the present study, the first set of results might indicate a need to assess the effect of higher doses of GBP, which could reflect its action on nerve function. In the second case, the findings suggest the need to evaluate the levels of these neurotrophic factors at different times after the induction of nerve injury, including times closer to the time of injury.

Besides the highlighted differences, the results presented here seem to be very promising because, although the role for GBP or pregabalin in pain control appears to be correlated to its blocking of calcium channels (17), the action of these substances on nerve regeneration has been investigated to a lesser degree. In accordance with our findings, recent preliminary data indicate a greater regeneration of the sciatic nerve in rats treated with GBP (100 or 300 mg/kg, *ip*, for 21 days) and nitroglycerin (1 mg/kg) compared with control animals, as assessed using parameters such as the proportion of neuronal cells, cell shape or differences in the sizes of these cells, and their connections (18).

This study is proposed as a pilot study and some aspects remain to be explored in future work. These include the investigation of the best procedure for stretching the nerve, compared with the 3-mm gliding length and 2-cm medial nerve segment used here, to determine whether nerve recovery would be worse or indeed whether this animal model would still be effective if the nerve was stretched by more than 3 mm. The use of a larger number of animals would contribute to investigating the dose-response effect of GBP on the parameters of motor function, which could also be assessed by electrophysiological studies. Furthermore, the determination of the levels of neurotrophins in the injured nerve measured at different times from that used here would also contribute to discovering whether changes in their levels could be a possible mechanism of action for this drug. A comparison between treated animals and a sham-vehicle group would also allow better elucidation of the effect of GBP on the non-injured nerve. Finally, a comparison of the GBP treatment with treatment with other drugs well known for their action on nerve regeneration would strengthen the method's validation.

In conclusion, this pilot study presents a new technique of stretch-induced nerve injury with important advantages for studies evaluating rat nerve regeneration. Further research would help to improve the technique proposed here in order to make it more consistent as a tool in the field of developing strategies for the clinical enhancement of nerve regeneration.

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