ABSTRACT
Objective: To study the effects of low intensity ultrasound irradiation applied on the spinal cord on the regeneration of the rat’s sciatic nerve after a controlled crush injury, evaluating the functional results of the sciatic functional index as measured on the video recorded images of the foot sole. Methods: Eighteen rats were submitted to a controlled crush injury of the right sciatic nerve and divided into two groups according to the treatment: Group 1 (n=9), simulated irradiation; Group 2 (n=9), effective irradiation. Low-intensity ultrasound irradiation was started on the 7th postoperative day and applied daily for 6 weeks. Images of the animals’ foot sole were video recorded on a see-through treadmill type walking belt machine at weekly intervals until the 6th week of irradiation and the corresponding sciatic functional index (SFI) was measured with specific software. Results: The SFI during the first and last week of treatment was -59.12 and -12.55 in Group 1, -53.31 and -1.32 in Group 2, indicating a 79% and 97% improvement, respectively, but differences between groups were only significant (p<0.05) during the third week. Conclusion: The authors conclude that low intensity therapeutic ultrasound enhances nerve regeneration, with significance during the 3rd week of treatment. Level of Evidence: Level II, prospective comparative study.

Keywords: Rats. Sciatic nerve. Spinal cord. Crush syndrome. Ultrasonic therapy.

INTRODUCTION
Although spontaneous recovery occurs in most cases, post-traumatic regeneration of the peripheral nerves is a very slow and frequently incomplete process, suffering the influence of numerous factors, such as patient age, type of trauma, lesion location, denervation time, type and diameter of the nerve fibers involved, method employed for nerve repair, intercurrence of chemical agents, temperature and other individual variables. Ultrasound is a physical resource widely used in medicine, both in diagnostic equipment, and in therapy apparatuses. It is defined as acoustic mechanical vibrations of high frequencies, which produce thermal and non-thermal physiological effects, and, since its introduction as a therapeutic resource more than six decades ago, has rapidly become of commonplace use within physiotherapy. Its beneficial effects have been demonstrated in the treatment of a wide variety of conditions, with diverse objectives such as promoting the healing of skin ulcers, stimulating neovascularization in ischemic tissues, promoting the integration of full thickness skin grafts, accelerating the consolidation of fractures and pseudarthroses and the healing of tendons. In fact, therapeutic ultrasound induces physiological tissue changes, such as fibroblast activation, collagen synthesis and the decrease of inflammatory cells, by acceleration of the cellular metabolism. When applied adequately, it also favors pain reduction. Spontaneous or stimulated posttraumatic regeneration of the peripheral nerves has been the motive of experimental investigations by our group, mostly using the rat model of sciatic nerve crush injury, including investigations into the use of therapeutic ultrasound, which confirmed the results of other authors, according to which ultrasonic irradiation effectively stimulates or accelerates conduction speed and the actual regeneration of the peripheral nerves. Ultrasonic or laser irradiation are preferably applied on the injured segment, but irradiation at another site of the nerve chain, such as the spinal cord, was also tested with equally positive results. From the anatomophysiological point of view, irradiation at the spinal cord level is logical, as it is targeted at the cell bodies of the motor and sensory neurons, already located. However, the data available in literature do not yet allow us to accept such a fact as definitive.

All the authors declare that there is no potential conflict of interest referring to this article.

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Degenerative and regenerative processes of the peripheral nerves can be evaluated by histological, histomorphometric and electrophysiological studies, while functional studies are rarely used for obvious reasons. However, a functional analysis method based on animal footprint analysis was introduced by De Medinaceli et al. 24,25 enabling the measurement of the so-called Sciatic Functional Index (SFI) by means of a mathematical formula, in which the parameters measured in the footprints are introduced. The SFI is, in actual fact, a negative indicator of the degree of dysfunction of the sciatic nerve of rats, ranging from zero (0), which indicates normal function, to -100, which corresponds to complete dysfunction. The original formula of De Medinaceli and collaborators24 was later modified by Carlton and Goldberg26, who also developed the functional indices of the tibial (TFI) and peroneal (PFI) nerves, and by Bain et al.27 who introduced new correction factors in the formulas of the three indices established previously. Later on it was demonstrated that the Sciatic Functional Index is directly correlated with histomorphometric parameters of the sciatic nerve of rats during the regeneration process after crush injury, and can also be employed as an isolated evaluation method with a high degree of reliability.15

It was, therefore, the objective of this investigation to test the hypothesis that ultrasonic irradiation on the spinal cord stimulates or accelerates regeneration of the sciatic nerve of rats after controlled crush injury, assessing the results by measuring the sciatic functional index (SFI) in footprints of the rear paws of the animals obtained by video recording.

MATERIAL AND METHOD

The survey project was approved by the Committee of Ethics in Animal Experiments of the institution of origin of the investigators involved (Protocol 190/2008). Twenty male Wistar rats with mean weight of 250 grams (variation: 200 - 300 grams) were used in the study. During the experimental period, the animals were kept in collective cages, with five animals each, and free access to food and water. They were kept in the new environment for 48 hours to acclimatize before the surgical procedure. The animals were divided into two groups, according to the type of procedure performed. Group 1: crush injury, followed by simulated treatment, with the ultrasound equipment turned off (n=10); Group 2: crush injury, followed by effective treatment with the ultrasound (n=10).

Surgical procedure:

The animals were anesthetized with a mixture of 10% ketamine (0.1ml/100g body weight) and 2% xylazine (0.07ml/100g body weight), administered intraperitoneally. They were positioned lying on the left side and fastened to the operating table, with the right pelvic limb facing upwards, and the lateral side of the thigh was prepared routinely for the operation (trichotomy and antisepsis with a solution of 20% iodized alcohol). The right sciatic nerve was approached through a rectilinear longitudinal incision on the lateral surface of the thigh and exposed all along its length, from the emergence underneath the gluteus maximus muscle up to its trifurcation at knee level. To produce the crush injury, we employed a portable deadweight device,28-30 assembled with a load of 5,000 g. (Figure 1) With the nerve exposed, the animal was positioned in the device and the load was applied for 10 minutes, covering a segment measuring 5mm in length located 5mm below the nerve emergence point.15,28 After production of the injury, the animal was removed from the crush device, the nerve was positioned in its anatomical bed and the surgical wound was sutured. The stitches were removed on the 7th day, when ultrasonic irradiation was also started.

Figure 1. Crush device using “deadweight”.

Postoperative procedure:

Ultrasound irradiation was performed with portable ultrasound equipment of clinical use, which features a specific transducer for small areas (1.35cm in diameter, continuous mode, frequency of 1 MHz, intensity of 0.16 W/cm² SATA). To prevent interference by the hairs, the trichotomy was periodically repeated at the irradiation application site, with the use of coupling gel to maximize the contact of the transducer with the skin from the region and to facilitate sliding. During the application, the rats were contained manually, taking care to keep the transducer permanently positioned at 90° in relation to the surface of the region and for its irradiation surface to remain always parallel to the external massage; in Group 2, the irradiation was effective, with the equipment connected according to the abovementioned specifications. The coupling gel and the dynamic irradiation technique were used for both groups, with the transducer moved continuously in circles with a diameter of around 1 cm over the region.
Video recording and analysis of footprints

The footprints of the animals from both groups were filmed on a motorized transparent treadmill, designed at our laboratory and built by a specialized local company. (Figure 2) The transparent treadmill, made of flexible polycarbonate, runs between two horizontally positioned, parallel cylinders and on a transparent acrylic plate, designated work area. One of the cylinders is craven by a dual voltage electric motor controlled by a potentiometer, allowing the treadmill speed to be adjusted from 0 to 14 m/min, so that the animal under analysis walks permanently on the work area, in an attempt to run on the treadmill in movement; the speed employed in this investigation was 3 m/min. A mirror is fastened underneath the work area, at an angle of 45° between this mirror and a webcam type video camera connected to a computer and fixed at an adjustable distance so as to focus the mirror horizontally. The work area is completely closed with opaque acrylic plates to prevent the animal from seeing outside. Before entering the work area, the animal is placed in a closed antechamber, where the passage from one to the other is through a drop gate. On the opposite side of the treadmill, another drop gate leads to a dark chamber, to which the animal runs at the end of the test. (Figure 3)

Before the surgical procedure, the animals were trained to walk along this treadmill by means of repeated attempts. When they were able of walk without faltering, we obtained the preoperative record of the footprints, which were captured, identified, stored and processed in the computer, using version CS3 of the Adobe Photoshop® program to adapt the dimensions to the SFI measurement program. The postoperative images were obtained at weekly intervals from the first week after the start of the ultrasonic irradiation (2nd, 3rd, 4th, 5th, 6th and 7th weeks from the operation), and were also identified, stored and processed. The SFI was calculated by the measurement of the PL or print length, TS, or toe spread, transversal distance between the 1st and 5th toes, and IT, or intermediate toes, transversal distance between the 2nd and 4th toes. The reference points of each parameter were simply marked with the mouse, one parameter at a time, and the SFI was calculated automatically by the program developed specifically for this purpose (Functional Analysis of Peripheral Nerves – AFNP in Portuguese), according to the formula proposed by Bain et al.,

\[
\text{SFI} = -38.3 \times \frac{EPL}{NPL} - 109.5 \times \frac{ETS}{NTS} + 13.3 \times \frac{EIT}{NIT} - 8.8
\]

The results of the SFI were compared between the groups using a linear regression model with mixed effects (random and fixed effects), at the significance level of 5% (p≤0.05), employing SAS® 9.0 software and R software.

RESULTS

In general, the animals tolerated well both the anesthesia and the surgical procedure, but two (one per group) were discarded due to balance problems during the postoperative gait analysis, leaving nine animals per group. In both groups, in the first week of ultrasonic irradiation, the animals exhibited difficulty walking, with foot drop, toe adduction, weight bearing on heel and inability to perform push-off in the shifting of weight from one foot to the other, which caused a footprint of more elongated appearance, compatible with severe sciatic nerve dysfunction. In the following weeks, the animals gradually recovered the ability to bear their weight on the operated limb with the toes spread.

126 (7x18) recorded images of gait, including preoperative evaluation (week 0) and from the 1st to the 6th weeks of ultrasonic irradiation, were analyzed and measured. In the preoperative evaluation (week 0), all the animals presented footprints within normal parameters, where the mean value of the SFI was -7.23 (variation: -21.34 to 6.94) in Group 1 and -0.99 (variation: -30.71 to 23.64) in Group 2, and the difference between both was not significant (p>0.05). (Figure 4, Table 1)
DISCUSSION

Post-traumatic regeneration of the peripheral nerves is a well-known spontaneous phenomenon, whose speed is estimated between 1 and 2mm per day, in humans. However, in lesions that are very close to the limb root and far from the effector organs (muscles, sensitive organelles, blood vessels), the regeneration period is very long and, by the time the regenerated axons reach them, an important quota of these is already degenerated, causing deficient functional recovery. Thus the acceleration of the regenerative process by any means would be a major benefit, as the effector organs will be reached by the axons while they are still in a good state, which contributes to a more complete recovery. Such fact justifies the research into resources that can effectively stimulate and accelerate nerve regeneration.

Physical resources such as ultrasound have been investigated, but in spite of encouraging results, it is not yet employed routinely in the rehabilitation process of humans with peripheral nerve lesions, judging by the relative absence of reports in literature. In fact, most investigations into the use of these resources to stimulate injured peripheral nerve regeneration, including ultrasound, involve experiments on animals, with the aggravating factor that there is considerable variability of irradiation parameters (potency, dose, time, etc.), as well as of the anatomical treatment site.

Most investigations target treatment at the lesion site, but spinal cord irradiation is also used. Ultrasonic irradiation on the spinal cord or on the posterior root ganglion is theoretically supported by the fact that these places contain the cell bodies of the motor and sensory neurons, in which the main part of the regenerative process takes place. Indeed, regeneration of the peripheral nerves depends mainly on the neuronal response to trauma or disease, while the neuron prepares for axoplasm production, which includes concentrating the Nissl bodies, rich in RNA, close to the axon exit points, which is the route taken by the axoplasm produced to reach the effector organs. On the other hand, functional regeneration also

After one week of irradiation (week 1, corresponding to the 2nd postoperative week), the mean value of the SFI in Group 1 was -59.12 (variation: -77.65 to -37.23) and in Group 2, -53.31 (variation: -72.12 to -27.73), while the difference between both was not significant (p>0.05).

After the second week of irradiation, the footprint images became clearer and the mean value of the SFI in Group 1 was -28.37 (variation: -51.63 to 2.11), while the difference between both was not significant either (p>0.05). In the 3rd week of irradiation, the mean value of the SFI in Group 1 was -24.96 (variation: -38.47 to -9.43) and in Group 2, -12.13 (variation: -30.23 to -18.4), with a significant difference between the groups (p<0.05).

In Group 1, the SFI increased to -18.3 (variation: -30.23 to -8.48), -13.67 (variation: -28.66 to -1.01) and -12.55 (variation: -33.72 to 6.06) and in Group 2, to -8.82 (variation: -37.46 to -12.64), while the difference between both was now with a significant difference (p<0.05). In the 4th, 5th and 6th weeks of irradiation, respectively. Although the mean values of the SFI remained within normal parameters (0 to -10) in Group 2 and the differences between the periods and the groups were notable, they were not statistically significant (p>0.05).

As a consequence of this, the SFI in Group 2 is not different from normal in the preoperative week, first to sixth weeks of ultrasonic irradiation respectively.

Table 1. Means, standard deviations, estimates, p - value, confidence interval (CI), lower limit (LL) and upper limit (UL), minimum, median and maximum values of the sciatic functional index (SFI ) in the preoperative week, first to sixth weeks of ultrasonic irradiation respectively.

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>Means</th>
<th>Standard Deviation</th>
<th>Estimates</th>
<th>p – value</th>
<th>CI (95%)- LL UL</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
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<td>0.28</td>
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<td></td>
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depends on the morphologic regeneration of the injured axons, which justifies the ultrasonic irradiation and the application of other resources also at this level.3,18,19

In this investigation we tested the theory that ultrasonic irradiation at the height of the neural axis, involving the spinal cord and the sensory ganglia, stimulates or accelerates rat sciatic nerve regeneration after controlled crush injury, evaluating the results by means of the functional analysis of gait and measurement of the sciatic functional index.

The experimental model used was that of the sciatic nerve of the rat, considered suitable for the purposes of the investigation, since this animal is known for its fast nerve regeneration capacity, which favors studies of short duration.34 The crush injury with controlled load is also suitable, since it does not cause rupture of the outermost sheath of the nerve (epineurium), which maintains its basic structure, without the inconvenience, for example, of a section injury followed by suturing, whose functionality depends on variables that are hard to control (microsurgical procedure, surgeon training). In this investigation, the injury was produced with deadweight equipment, developed in our laboratory and employed in previous investigations.28 With this equipment, the applied load does not decrease with time, favoring high reproducibility in controlled crush injuries.

Ultrasonic irradiation was employed with parameters of current use in clinical practice, including lower potency (0.16 W/cm²), which is more adequate for stimulation of nerve regeneration, according to results obtained in investigations in which the irradiation was applied at the lesion site.22,34 The small area irradiated (1.43 cm²), which enabled the concentration of waves on the spinal column, and the low frequency (1 MHz), which is better suited to reach the deepest tissues, as is the case of the spinal cord and of the sensory ganglion, protected by the vertebrae.

In practically all the previous investigations, the animals' tracks were obtained by prints, either using ink on paper, or with liquid developer on unused x-ray film.3,19,20,30,35 In this investigation, the footprint images were obtained by video recording on a mobile treadmill with controlled speed, developed in our laboratory specifically for rat gait analysis. This equipment had already been tested in a previous experiment, having shown greater fidelity in the obtainment of footprint images and greater ease in the measurement of parameters and of the actual SFI, in comparison with the printing methods.36

It is already demonstrated that functional evaluation by the SFI method, as employed in several previous investigations and now in this one, is very reliable, in view of its strong correlation with the morphologic and morphometric evaluation. In actual fact, it is an indirect measure of the animal’s rear foot function, but probably also provides a measurement of the sciatic nerve global function, as the motor function during gait depends at least partly on foot sole sensitivity. The SFI is calculated by means of a relatively complex mathematic formula, but sufficiently tested for years since its introduction and subsequent modifications, correlating very well with the clinical aspect of the animal’s foot and including all the possible permutations.15,27

However, it is already a well-known fact that the SFI is hardly ever equal to zero (0) in normal feet with the sciatic nerve intact, oscillating around 0 over a relatively wide range, which probably indicates that the method is not entirely precise; in this investigation, it oscillated between 0 and -10. On the other hand, the values obtained in the postoperative periods clearly showed that there was functional recovery in the two groups, both in that of the effectively irradiated animals (Group 2), and in the animals with simulated irradiation (Group 1), but slightly better in Group 2, with SFI values within the range of normality (-10 to 0), having the values from the preoperative evaluation as a reference. Nevertheless, the differences between Groups 1 and 2 were only significant in the third week of irradiation, with no difference either before or after, including in the sixth and last week of treatment. The SFI was not measured in the first postoperative week, since all the animals presented gross deformity of the operated foot, which renders the analysis of the necessary parameters unviable and spawns a low rate of reproducibility of results.37

The non-significant differences between Groups 1 and 2 indicate that the effects of the ultrasonic irradiation, though positive, are not pronounced, much less spectacular. This fact had already been observed in a previous investigation, in which the irradiation was initiated early, on the first postoperative day, and targeted the site of the actual injury,19 with results evaluated only until the third week. In fact, the SFI, both in this and in that investigation, was virtually and statistically the same for the two groups in all the weeks, except for the third, but improved more expressively for Group 2, with a difference of 97% between the first and the last postoperative evaluation, by contrast with a difference of 79% for Group 1 in the same period. Although unlikely, it can be speculated that the degree of regeneration and functional recovery was not better with the ultrasonic irradiation, as this was not applied at the lesion site as well, thus not contributing to accelerate the repair of the neural tubes and not favoring better conditions for the growth of the axons up to the effector organs.

The mechanism whereby ultrasonic irradiation can influence the regeneration of an injured peripheral nerve has not yet been elucidated and was not the purpose of this investigation. Although of a merely speculative nature, the mechanism could involve, at least, the thermal effect, with consequent vasodilation, with vascular neoformation and improvement of local irritation, and the release of chemical and chemotactic mediators that stimulate axoplasm production.

CONCLUSION

Despite the limitations of the methodology employed, the authors conclude that therapeutic ultrasound applied on the spinal cord positively influences regeneration of the peripheral nerves. This effect can be useful in the treatment of a variety of pathologies in humans, with the advantage that the low potency is virtually innocuous. More adequate doses for humans still need to be established.
REFERENCES


