RESEARCH REPORT

High- and low-frequency transcutaneous electrical nerve stimulation delay sciatic nerve regeneration after crush lesion in the mouse

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Abstract The stimulation of peripheral nerve regeneration has been studied in different ways, including the use of electrical fields. The capacity of this modality to enhance nerve regeneration is influenced by the parameters used, including current type, frequency, intensity, and means of administration. Transcutaneous electrical nerve stimulation (TENS) is a frequently used form of administering electrical current to the body, but its effects on peripheral nerve regeneration are not known. This study assessed the influence of TENS on sciatic nerve regeneration, using a model of crush lesion in the mouse. Mice were stimulated 30 min a day, 5 days a week, for 5 weeks with both high- (100 Hz) and low- (4 Hz) frequency TENS. Control animals had the sciatic nerve crushed but were not stimulated. Assessment was performed weekly by functional analysis using the Static Sciatic Index for the mouse and at the end of the experiment by light and electron microscopy. The results showed that although there were no differences between the groups regarding the Static Sciatic Index values, TENS led to nerves with morphological signs of impaired regeneration. At light microscopy level, TENS nerves presented more axons with dark axoplasm, signs of edema, and a less organized cytoarchitecture. Electronmicrographs showed fewer and thinner thick myelinated fibers and increased number of Schwann cell nuclei. Myelinated axon diameters and density and diameter of nonmyelinated fibers were not affected by TENS, leading to the conclusion that this regimen of electrical stimulation leads to a delayed regeneration after a crush lesion of the sciatic nerve in the mouse. All these effects were more pronounced on high-frequency TENS nerves.

Key words: function recovery, histology, mice, nerve, regeneration, sciatic, transcutaneous electric nerve stimulation

Introduction

Degeneration and regeneration of a peripheral nerve involve a complex sequence of events initiated by Wallerian degeneration (WD) of the distal stump, which may or may not favor target-organ reinnervation *(Ide, 1996)*. The time course of degeneration and

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regeneration varies among species, site of lesion, nerve, and fiber type *(Griffin et al., 1995)*. Also, different types of lesions have different prognoses. Crush lesions maintain the basal lamina, generating a propitious environment for regeneration, which is not observed in a transection injury *(Stoll and Müller, 1999)*. Successful regeneration may be impeded by the distance to be covered by the growing nerve fibers. When the lesion occurs far from the target organ (e.g., brachial plexus injury), physiological regeneration speed in humans (1 mm/day) can be insufficient to promote functional reinnervation because of prolonged denervation and atrophy *(Gordon et al., 2003)*. Therefore, to promote adequate function recovery, strategies to enhance regeneration are important.

Many studies have investigated the influence of electrical fields on peripheral nerve regeneration using laboratory animals. Most of them have used implanted or percutaneous electrodes, and positive effects were achieved with different stimulus parameters (weak constant, DC and AC pulsed electrical fields) *(Nix and Hopf, 1983; Pomeranz et al., 1984; McDevitt et al., 1987; Beveridge and Politis, 1988; Politis et al., 1988; Kerns et al., 1991; Pomeranz and Campbell, 1993; Chen et al., 2001; Inoue et al., 2003; Mendonça et al., 2003)*. Transcutaneous electrical nerve stimulation (TENS) is a very simple, practical, low-cost and non-invasive means to provide therapeutic electrical stimulation *(Sluka and Walsh, 2003)*. It uses biphasic and balanced pulses, with short width (about 150 μs) and frequencies ranging from 2 to 150 Hz. Biphasic pulses are not commonly used to induce peripheral nerve regeneration because they do not have electrophoretic effects, as opposed to monophasic currents. The electrophoretic effects attributed to monophasic currents are thought to have a beneficial effect because they can provide orientation to cell membrane proteins toward the cathode, creating an "electric cue" for regeneration *(McCaig et al., 1994)*. Nevertheless, positive results have been demonstrated with the use of biphasic currents, such as spreading of fibers in the saphenous nerve *(Pomeranz et al., 1984)*, increases in regeneration speed and precision, expression of regeneration-associated genes, brain-derived neurotrophic factor, tyrosine kinase B receptor, Tα1-tubulin and growth-associated protein-43, and reduction in neurofilament expression *(Al-Majed et al., 2000a; 2000b; 2004; Brushart et al., 2002)*.

Previous studies have assessed the influence of TENS on regeneration of tissues such as tendons *(Burssens et al., 2003; 2005)*, skin *(Kaada and Emru, 1988; Kjartansson et al., 1988; Khalil and Merhi, 2000; Liebano et al., 2003)*, and bone *(Kahn, 1982)*, with varied results. The effects of TENS on nervous system regeneration, however, remain to be unequivocally demonstrated. This modality has been associated with improvement of blood flow *(de Vries et al., 2007; Sandberg et al., 2007)* or collagen synthesis *(Burssens et al., 2005)*. However, electrical current amplitudes above 1 mA are frequently used to overcome skin impedance and reach deep tissues. These amplitudes may be associated with decreased ATP concentrations *(Cheng et al., 1982)* and could lead to inhibition of regeneration.

Also, TENS is usually employed to control neuropathic pain *(Leem et al., 1995; Hanai, 2000; Nam et al., 2001; Cheing and Luk, 2005; Somers and Clemente, 1998; 2003; 2006)*, which is often associated with peripheral nerve lesions and sometimes WD *(Latinovic et al., 2006)*. TENS analgesic effects are related to the release of endogenous opioids *(Sluka and Walsh, 2003)*; this is related to another controversy on its use to improve nerve regeneration because chronic opioid exposure may be associated with tolerance and inhibition of various steps of the peripheral nerve restoration process *(Smith and Hui, 1973; Sinatra and Ford, 1979; Sinatra et al., 1979; Zeng et al., 2007)*.

Because we could find no reports on the influence of TENS on peripheral nerve regeneration, this study was performed as an exploratory approach, to assess the influence of two different TENS protocols on peripheral nerve regeneration in mice, using the sciatic crush model. Animals were treated 30 min a day, 5 days a week, for 5 weeks, and nerve regeneration was assessed by functional, morphological, and histomorphometric analyses.

Materials and Methods

We used 15 male Swiss mice (*Mus musculus*) from our animal facility, weighing 35–45 g. All procedures were approved by the Committee for Ethics in Animal Experimentation of the Fundação Bahiana para Desenvolvimento das Ciências, Salvador, Brazil. The animals were housed in individual cages with food and water *ad libitum* and a 12:12 h light/dark cycle.

Surgical procedures

Animals were deeply anesthetized with ketamine (0.1 mL/mg) and xylazine (0.2 mL/mg) and then submitted to asepsis and trichotomy of the right gluteus region. After a longitudinal incision, the sciatic nerve was exposed, isolated from the adjacent tissues, and crushed 1 cm from the spinal cord using a nonserrated needle holder, maintained on the first lock for 30 s. Muscle and skin were then sutured by layers using 4.0 suture (Ethicon).

Electrical stimulation

Electrical stimulation was initiated 4 days after surgery to avoid interference of the electrodes in preventing initial scar tissue formation. For the stimulation, the animals were lightly anesthetized with halotane and immobilized on a special apparatus. Electrical stimulation was provided using a clinical biphasic pulse generator (TENS vif 962, QUARK Medical), previously calibrated for the study. As the interface between the apparatus and the skin, we used two square silicone rubber electrodes, soaked with a special gel for electrostimulation, 1.5 cm^2 in area, positioned longitudinally to the incision. The distance between them was 2 cm. Electrostimulation parameters were based on those reported to generate analgesia *(Low and Reed, 2001; Nelson et al., 2003)*.

The animals were divided into three groups according to the procedure to be used:

(1) high-frequency TENS (high TENS): 100 Hz frequency, 80-μs pulse width, in a continuous pattern, with amplitude immediately below motor threshold ($n = 5$);
(2) low-frequency TENS (low TENS): 4 Hz frequency, 240-μs pulse width, modulated in 2 Hz bursts, with amplitude exactly on the motor threshold, where visible contractions should be observed ($n = 5$);
(3) control: animals submitted to the same anesthesia protocol but without stimulation ($n = 5$).

Animals were stimulated for 5 weeks, as this period of stimulation is sufficient to allow recovery from crush lesions in mice *(Baptista et al., 2007)*.

Functional assessment

On the previous day and on days 7, 14, 21, 28, and 35 post-lesion, the animals were evaluated to obtain two footprint parameters: the distance between the first and the fifth toes, or toe spread (TS) and the distance between the tip of the third toe and the most posterior part of the foot in contact with the ground, or print length (PL). These values were used to obtain the Static Sciatic Index for the mouse (SSIm).

The SSIm was obtained with a previously described protocol *(Baptista et al., 2007)*. Briefly, animals were placed in an acrylic corridor (48 × 4.5 cm) with a mirror placed underneath the apparatus at an angle of 45°. A 50W lamp, placed longitudinally to the floor, illuminated the corridor. The animal's cage was placed at the end of the corridor to induce it to walk in this direction, and before they entered the cage, the corridor was closed. When the animals stopped, three photos were taken with a Web Cam (Drop Cam 100, Go Tec), positioned 2.5 cm from the mirror. The means for the footprint parameters were assessed with image analysis software (Image J, National Institutes of Health), calibrated with a scanned caliper. The TS and PL were estimated and generated the TS factor (TSF) and PL factor (PLF) (factor = experimental value − normal value/normal value) that were used in the formula:

$$SSIm = 101.3 \times TSF - 54.03 \times PLF - 9.5$$

In the SSIm, a value of 0 corresponds to normal function and a value of −100 corresponds to complete loss of function.

Histomorphometric assessment

One day after the last functional analysis, on day 35 post-lesion, the animals were deeply anesthetized and euthanized by transcardiac perfusion with fixative solution (4% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, 25 mL/animal). The sciatic nerves ipsilateral to the lesion were harvested, and a 2-mm segment, 3 mm distal from the lesion site was dissected. Contralateral nerves were also dissected, and a 2-mm segment collected from the equivalent portion of the lesioned nerve.

The segments were post-fixed, dehydrated in increasing concentrations of acetone (30–100%), osmicated, infiltrated, and plastic embedded. Transverse sections of 0.5 μm (semithin) and 70 nm (ultrathin) were obtained using an ultramicrotome (Ultracut, Reichert-Jung). The semithin sections were stained with 1% toluidine blue. Images were acquired on a light microscope (Olympus CX41) connected to a digital camera (Evolution Color PL 1642, Media Cybernetics). A 1,000× magnification was used to assess the general morphological condition of the nerves and the number and area of blood vessels.

For the ultrastructural analysis, 70-nm sections were obtained and contrasted with 1% uranyl acetate and lead citrate and observed through a transmission electron microscope (Zeiss EM 109) equipped with an image acquisition system (MegaView II, Analysis-Imaging-System). A magnification of 7,000× was used for morphological assessment and measurement of the densities and diameters of myelinated and nonmyelinated fibers, as well as the number of Schwann cell nuclei. Densities were calculated by dividing the number of cells by the total area of 10 systematically chosen fields. Using these measurements, G ratios (axon diameter/fiber diameter) were also obtained.

Statistical analysis

The independent variable for all groups was the use of high- and low TENS. Dependent variables were

derived from functional analysis and histomorphometry. In functional analysis using the SSIm, paired (intragroup, between days) and unpaired (between groups on each day) comparisons were made. For histomorphometric analysis, we considered as dependent variables the number and area of blood vessels (obtained at 1,000× magnification), density and diameter of myelinated fibers stratified by diameter on 0–2, 2–6, and 6–12 μm, density and diameter of nonmyelinated fibers, density of Schwann cell nuclei and G ratio (obtained at 7,000× magnification), stratified in ranges of 0.1–0.4, 0.4–0.5, 0.5–0.6, 0.6–0.7, and 0.7–0.9. When ranges were used, the lowest portions were always included and the highest portions excluded (e.g., the 0–2 range includes 0 through 1.99, excluding 2). Paired inferences were done by the Friedman nonparametric test and unpaired inferences by the Kruskal-Wallis nonparametric test, associated with the Student-Newmann-Keuls post-hoc analysis, when necessary. The CI was 95%, with an accepted alpha value of 5% ($p < 0.05$). The analyses were carried out using the BioEstat 4.0 statistical software (Mamirauá).

Results

Functional assessment

There were no signs of motor impairment on the last day of the study. The three groups exhibited a similar pattern of function throughout the assessment period, as shown by the SSIm. The first week represented the functional nadir, with gradual recovery in all groups (control, high-, and low TENS). Paired analysis with Friedman nonparametric test showed significant differences among weeks for the control ($Fr = 15.63$, $p < 0.01$), high TENS ($Fr = 18.49$, $p < 0.005$), and low TENS ($Fr = 19.06$, $p < 0.005$) (Fig. 1).

Unpaired analysis comparing the three groups weekly by the Kruskal-Wallis nonparametric test showed no differences.

Morphological assessment

Morphological changes were assessed using images acquired by both light and electron microscopy. Viewed by light microscopy, crushed and electrically stimulated nerves generally showed larger endoneural spaces and smaller fibers compared with normal nerves. Fibers with dark axoplasm could also be observed in these nerves (Fig. 2A). Only one nerve stimulated with low TENS showed signs of edema (Fig. 2B). Two of the control nerves appeared similar to normal nerves, where fibers were more compacted, with larger diameters and less endoneural space (Figs. 2C and 2D).

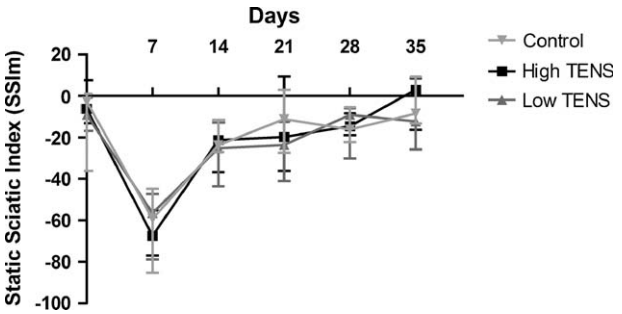

Figure 1. Static Sciatic Index for the mouse. Graph shows a similar pattern of locomotor function for the control (crush lesion, without stimulation), high-TENS, and low-TENS (crush lesion and stimulation) groups during the 35-day experimental period. Locomotor function was worse in the first week post-lesion on the three groups and came back to normal on the second week. Data are presented as median and 25–75 quartiles.

Qualitative analysis of electromicrographs was consistent with the results previously described for the normal nerves as seen by light microscopy. The nerves treated with high TENS had less myelin in their fibers, but the normal axonal aspect was maintained. In these nerves, the regenerative clusters were more easily identifiable (Fig. 3A). Low-TENS and control nerves occasionally contained lipid inclusions that could not be seen in high-TENS or normal nerves (Figs. 3B and 3C). Fig. 3C shows one of the control nerves, with a normal pattern of fiber morphology. Normal nerves always had less endoneural space and larger and more myelinated fibers (Fig. 3D).

Histomorphometry

Morphometric analysis was performed using both light and electron microscopy. The number and area of blood vessels assessed at 1,000× magnification did not differ between groups ($p > 0.05$). Myelinated fibers, assessed at 7,000× magnification, were stratified by diameter in 0–2, 2–6, and 6–12 mm, and the majority of them were in the 2–6 mm diameter range in all groups. In the 6–12 mm range, stimulated nerves had significantly fewer fibers than the normal or control nerves. Schwann cell nuclei density was greater in high- and low-TENS nerves (Table 1).

Regarding fiber diameter, high-TENS and control nerves showed significantly smaller fibers than normal nerves, again in the 6–12 mm range (Table 2). This pattern was not observed for axon diameters (Table 3), indicating less myelin content in the high-TENS and control groups compared with normal nerves. Low TENS reached normal values of fiber and axon diameters, indicating normal myelination (Tables 2 and 3).

G ratio distribution was also different between groups. Normal, control, and low-TENS nerves had

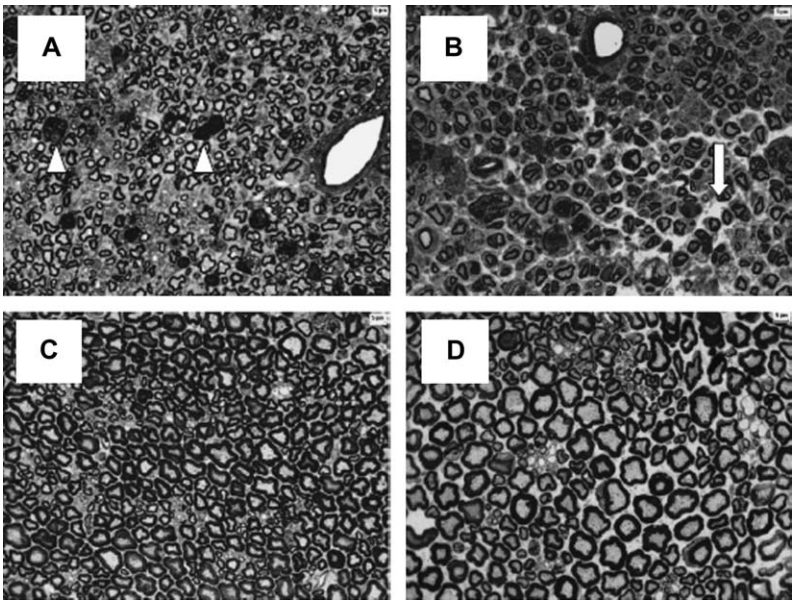

Figure 2. Semithin transverse sciatic nerve section. (A) High-TENS nerve (crush and 100 Hz stimulation) presenting small diameter myelinated fibers. Some of them present dark axoplasm (arrow heads), may be representing fibers which are still in the process of axon degeneration. (B) Note the edema (arrow) found in a low TENS (nerve crush lesion and 2 Hz stimulation) that was not seen on the others. Stimulated nerves (A and B) always presented smaller fibers than control (crush lesion, without stimulation) or normal nerves (no lesion or stimulation) (C and D). (C) A control nerve with some fibers with normal morphological aspect. (D) Normal nerve with less endoneurial space and fibers with a more compacted distribution. Magnification 1,000×.

the majority of fibers in the range of 0.5–0.6. High-TENS nerves differed from the others showing a rightward deviation with its peak in the 0.6–0.7 range. This confirms the supposition that high TENS led to less myelin content. Control nerves also showed an altered pattern, with a significantly higher concentration of fibers in the 0.7–0.9 range (Fig. 4).

Discussion

In our study, the morphological aspect of stimulated nerve groups was generally worse than crushed but not stimulated, and normal groups. Electrically stimulated nerves showed signs of compromised regeneration such as edema, reduced fiber compaction, more regenerative clusters, and the presence of lipid inclusions (Figs. 2 and 3). The reduction in number of large myelinated fibers (6–12 μm diameter) and increasing in the number of Schwann cell nuclei (Table 1), associated to both types of electrical stimulation, can be related to a delay in the regeneration process. The G quotient indicates the ratio between axon diameter and fiber diameter, and high G ratios are normally associated with low myelin content. High-TENS nerves presented fibers with a G ratio peak in 0.6–0.7 (Fig. 4), differing from the other groups, that had the peak in the 0.5–0.6 range, probably indicating inhibited myelin formation. Despite the negative effect of low TENS on the number of large fibers, myelination reached normal levels (Tables 2 and 3). Additionally, electrical stimulation did not interfere significantly on the area or number of blood vessels, showing no influence on structural revascularization (data not shown). Although electrical stimulation was associated with morphological and morphometric abnormalities, these did not significantly impact gait (Fig. 1). In summary, our findings point to a negative influence of TENS on peripheral nerve regeneration, most prominently of high TENS.

The lack of a direct relationship between morphological data and functional studies in laboratory animals, as observed in this study, has also been reported by others *(de Medinaceli, 1995)*. Morphological or nerve conduction assessment cannot be as specific as gait analysis to assess proper reinnervation after traumatic nerve lesions *(de Medinaceli et al., 1983; Goldberg et al., 1984)* because these kinds of assessment do not address overall nerve function. Our study showed no significant differences in paw function between groups (Fig. 1), indicating that despite the worse morphological aspect of nerve regeneration after electrical stimulation, there were no negative effects on sensory-motor function.

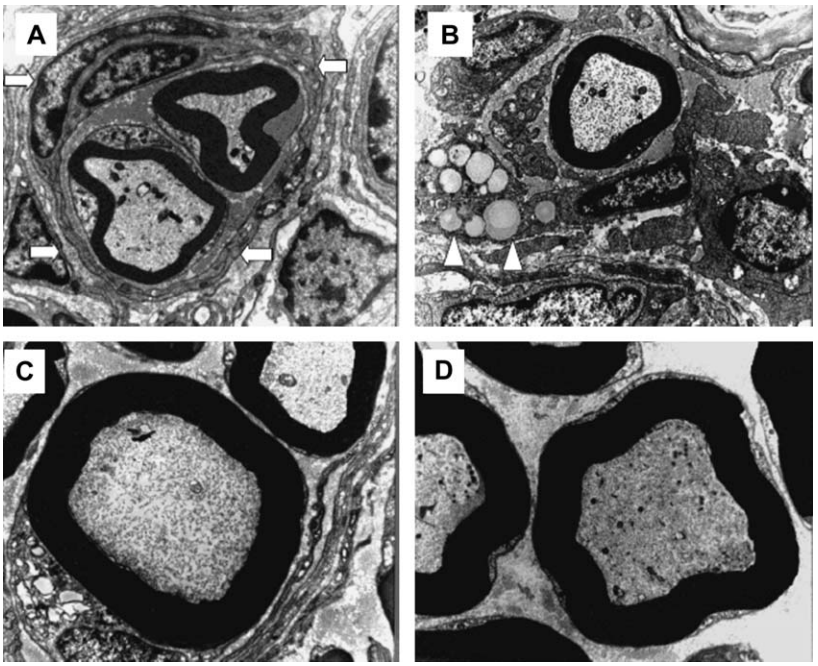

Figure 3. Ultrathin transverse sciatic nerve sections. (A) High-TENS nerve (crush lesion and 100 Hz stimulation) showing a regenerative cluster (arrows) and small diameter myelinated fibers. (B) Low-TENS nerve (crush lesion and 2 Hz stimulation), with an aspect similar to high-TENS nerves, also presenting regenerative clusters and some lipidic inclusions (arrow head). (C) Control nerve (crush lesion, without stimulation) where larger and more myelinated fiber than stimulated nerves could be observed. (D) Normal nerve (no lesion or stimulation) presenting larger and more myelinated fibers. Magnification 7,000×.

Transcutaneous electrical stimulation has to overcome the high cutaneous electrical impedance, frequently using amplitudes exceeding by 10× to 10,000× those used with percutaneous or implanted electrodes. In our study, the combination of pulse amplitude of 5 mA or above and pulse width higher than 80 μs may be an important factor causing delay in regeneration. Cheng *et al. (1982)* found that at lower levels (1 mA), electric current inhibited ATP synthesis in cell cultures. Thus, low ATP concentrations may have led to impairment of the normal regeneration process. Aydin *et al. (2006)* submitted rats with a peroneal crush lesion to a whole-body exposure of a 50-Hz biphasic electrical field with an estimated induced electrical field of 10 mV/m and found a weak inhibition of regeneration expressed by morphological defects that did not interfere with gait function. It is difficult to estimate the strength of the electrical field when using the mouse model because the dielectric constant of mice is not yet known. However, considering that a field magnitude of 10 mV/m would be the minimal induced field necessary to overcome the basic endogenous noise in cellular membranes and produce physical and biological effects *(Astumian et al., 1995; Aydin et al., 2006)*, even very low electrical field intensities could cause negative biological effects on regeneration. Chen *et al. (2001)* also found adverse detrimental consequences of electrical fields on peripheral nerve regeneration using percutaneous electrodes and a 1-mA monophasic current. In their

Table 1. Fiber and Schwann cell nuclei density.

			Density (mm^2)			
Group	*n*	NMF	MF (0–2 μm)	MF (2–6 μm)	MF (6–12 μm)	SCN
Normal	15	2,590 (1,192–3,372)	117.6 (58.8–147.1)	1,529.4 (1,235.3–1,735.3)	529.4 (441.2–588.2)	0 (0–0)
Control	5	1,564 (1,205–1,615)	58.8 (58.8–117.6)	1,411.8 (1,235.3–1,470.6)	352.9 (294.1–588.2)	58.8 (0–58.8)
HighTENS	5	1,513 (1,282–2,538)	117.6 (117.6–117.6)	1,705.9 (1,470.6–1,823.5)	117.6 (58.8–176.5) a*b**	58.8 (58.8–58.8) a**
LowTENS	5	1,179 (1,103–1,205)	117.6 (0–235.3)	1,647.1 (1,529.4–1,882.4)	58.8 (58.8–176.5) c*d***	58.8 (29.4–58.8) c*

Data expressed as median and 25–75 quartiles.
High TENS × control (a); High TENS × normal (b); Low TENS × control (c); Low TENS × normal (d)
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Kruskal-Wallis + Student-Newmann-Keuls).
NMF, non-myelinated fibers; MF, myelinated fibers; SCN, Schwann cell nuclei.

Table 2. Fiber diameter.

Group	*n*	Fiber diameter			
		NMF	MF (0–2 μm)	MF (2–6 μm)	MF (6–12 μm)
Normal	15	0.59 (0.56–0.61)	1.71 (1.48–1.83)	3.84 (3.63–3.97)	7.43 (7.08–7.72)
Control	5	0.61 (0.54–0.63)	1.80 (1.63–1.84)	4.12 (3.24–4.16)	6.50 (6.31–7.01) b*
High TENS	5	0.58 (0.554–0.62)	1.58 (1.42–1.68)	3.66 (3.62–3.72)	6.57 (6.35–6.78) a*
Low TENS	5	0.63 (0.53–0.66)	1.86 (1.69–1.91)	3.59 (3.46–3.82)	6.88 (6.24–7.06)

Data expressed as median and 25–75 quartiles.
High TENS × normal (a); Control × normal (b)
*$p < 0.05$ (Kruskal-Wallis + Student-Newmann-Keuls).
NMF, non myelinated fibers; MF, myelinated fibers.

experiment, electrical stimulation inhibited the number of regenerating fibers crossing an experimental 10-mm gap, although the fibers that successfully crossed this barrier were morphologically more normal. These results suggest that electrical currents above 1 mA can have weak, but significant, effects on peripheral nerve regeneration, generally leading to inhibition of axon number and area or myelin fiber content, but insufficient to affect the function.

The effects of electrical fields on blood flow were reported to be associated with improvement in axon sprouting and nerve regeneration *(McCaig et al., 2005)*. Low frequency can selectively stimulate sensory C fibers and enhance the expression of neuropeptides such as substance-P (SP), which in turn generates vasodilatation *(Kjartansson et al., 1988; Kashiba and Ueda, 1991; Burssens et al., 2005)*. Low TENS is usually associated to rhythmic muscle contraction, which may also have positive circulatory effects *(Dobsák et al., 2006)*. However, there is a recent evidence supporting effects of high-frequency TENS on blood flow as well. de Vries *et al. (2007)* demonstrated an improvement in the coronary circulation, attributed to an enhancement in coronary perfusion. In contrast, in our study, it was not possible to observe effects on intraneural blood flow, assessed by number and area of blood vessels, indicating perhaps a different behavior concerning peripheral nerves. Although this method is not very sensitive, if one accepts the common statement that improvement in blood circulation is associated to enhancement of regeneration, TENS should have had positive and not negative effects, as shown. In addition, it should be considered that the exposure to electric fields and consequent angiogenesis are not always positive, as they have also been associated with increased concentrations of reactive oxygen species (ROS) *(Rosenspire et al., 2001; Sauer and Wartenberg, 2005)* and DNA damage *(Delimaris et al., 2006)*. These mechanisms may also explain the delay of regeneration observed in TENS-stimulated nerves.

The majority of TENS effects studied are related to pain control, with high-TENS analgesia being mediated by the activation of δ-opioid receptors and low TENS by μ-opioid receptors *(Kalra et al., 2001; Sluka and Walsh, 2003)*. Sinatra and Ford *(1979a, b)* demonstrated that chronic morphine use for 14 days led to delay of peripheral nerve regeneration, expressed by fewer axon profiles, decreased myelin debris removal, Schwann cell hypertrophy, and proliferation. Zeng *et al. (2007)* also showed that chronic morphine exposure, acting via μ-opioid receptors, improves regeneration of nonmyelinated fibers but inhibits regeneration of myelinated fibers after a sciatic crush lesion. This is in accordance with our results, given that nonmyelinated fibers were not affected by TENS (Table 1), but large diameter fibers were fewer and myelinated, especially in the high-TENS group. The poorer progress of high-TENS nerves, however, may not be explained by δ-opioid activation because it is associated with neurogenesis and neuroprotection in the mouse central nervous system *(Zhang et al., 2002; Narita et al., 2006)* and probably could have the same function on the peripheral nervous system. The

Table 3. Myelinated axon diameter.

Group	*n*	Axon diameter		
		MA (0–2 μm)	MA (2–6 μm)	MA (6–12 μm)
Normal	15	0.96 (0.77–1.00)	2.03 (1.91–2.14)	4.12 (3.98–4.45)
Control	5	1.07 (0.95–1.14)	1.94 (1.94–2.19)	4.48 (4.36–4.61)
EENT C	5	0.97 (0.87–1.00)	2.27 (2.19–2.33)	4.67 (4.31–4.85)
EENT A	5	0.73 (0.70–0.91)	2.1 (2.01–2.18)	4.00 (3.82–4.46)

Data expressed as median and 25–75 quartiles.
MA, myelinated axon.

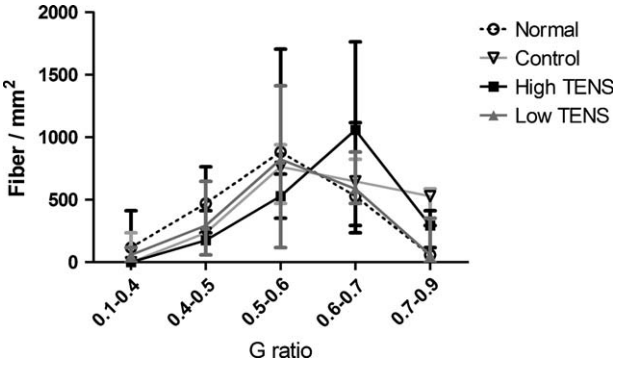

Figure 4. G ratio stratified by ranges. G ratio was obtained dividing axon diameter by fiber diameter. Higher portions of the G ratio ranges are included and lower portions excluded. Note that only high-TENS nerves (crush lesion and stimulation with 100 Hz) presented a peak of fiber density on the 0.6–0.7 range. The other nerves presented the peak on the 0.5–0.6 range. Data are presented as median and 25–75 quartiles.

key difference between our study and those showing that opioid receptor activation was successful in improving nerve tissue regeneration was the prolonged time of use, which can lead to opioid tolerance. Chandran and Sluka *(2003)* showed that after repeated administration of high- and low TENS, 20 min a day, rats developed opioid tolerance on the fourth day of stimulation. Mao *et al. (2002)* demonstrated that morphine tolerance is mediated by the N-methyD-aspartate-caspase pathway and leads to spinal neural apoptosis. Therefore, prolonged use of TENS may have led to morphine tolerance and neurotoxic consequences for the cells involved in regeneration.

Curiously, drugs commonly used to inhibit opioid tolerance include SP, calcitonin gene-related peptide (CGRP), cholecystokinin (CCK), and NMDA selective antagonists *(King et al., 2005)*. After a peripheral nerve lesion, α and β CGRP and CCK are overexpressed *(Saika et al., 1991)*. CCK is upregulated with sustained administration of morphine *(Zhou et al., 1993; Stanfa et al., 1994)* or electroacupuncture *(Fukazawa et al., 2007)*, antagonizing morphine effects. It is also related to nerve regeneration through the improvement of nerve growth factor synthesis and CGRP concentrations *(Manni et al., 2000; 2001; Sanchez et al., 2001)*. Consequently, overexpression of CCK, possibly associated with 35 days of TENS, could have had beneficial effects on nerve regeneration, balancing opioid tolerance neurotoxic effects. It remains to be demonstrated if TENS can really enhance these peptide concentrations and what parameters should be used to evoke its potential neuroprotection against opioid tolerance.

The present study was the first to assess the influence of TENS on peripheral nerve regeneration. The indication that TENS can be associated with a delay in this process, although not sufficient to influence function, is very important in the clinical setting because this modality of electrical stimulation is widely used and often associated to no harmful or side effects. Future research should assess the influence of TENS on ATP concentrations, peripheral nerve blood circulation, and expression of ROS, as these factors may directly influence peripheral nerve regeneration. Also, the possibility that opioid tolerance developed by TENS can lead to delayed peripheral nerve regeneration can open a broad field of research on its appropriate use. TENS may have very different effects if used briefly or with frequency and amplitude variations, preventing opioid tolerance effects. Also, further studies can be performed on animal models to assess the consequences of opioid or colecystokinine pharmacological blockade on regeneration. Case-control studies can be performed including patients where nerve conduction tests are available and addressing the influence of different TENS modalities on peripheral nerve regeneration. Meanwhile, the use of TENS in patients with these characteristics should be reevaluated.

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