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Minocycline does not reduce the regenerative capacity of motor and sensory neurons of the sciatic nerve after a conditioning injury

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Udina designed the experiments, funded the project and performed the suture of the nerve grafts. Sanchez-Brualla performed the retrograde labelling, cut, processed the samples and analyzed the data of immunohistochemistry and motoneuron counting. Calls helped with motoneuron counting.

All authors have approved the final article.

1. Abstract

Minocycline has been reported to be both beneficial and detrimental for nerve regeneration after a sciatic nerve injury. It has been proposed that the inhibition of inflammation around motor or sensory neurons may contribute to this effect. However, some experiments have proven the opposite: microglial or macrophage reaction to a peripheral nerve injury (PNI) may not affect or even decrease the regenerative capacity of the injured axons.
Our aim is to determine if the administration of minocycline after a nerve injury inhibits regeneration after a conditioning lesion.
We used two groups of Swiss mice: a control group and a group treated with minocycline (30 mg/kg ip), an inhibitor of microglia and macrophages. We characterized the effects of minocycline in the phenotype of microglia, and we labeled the motor and sensory neurons that had regenerated to a distance of 3 mm in a predegenerated graft, after a conditioning lesion, to test their regenerative capacity in normal circumstances and after microglial and macrophages inhibition.
Our results indicate that minocycline injection is not detrimental to the regenerative capacity of motor and sensory neurons, and even showed a slight, nonsignificant increase in regeneration, both at 7 and 3 days after the suture of the nerve graft. These results indicate that the conditioning effect in peripheral nerves is not completely inhibited by minocycline injection at a dose of 30 mg/kg.

Minocycline is a tetracycline that inhibits microglia and macrophages, and has proven to have positive effects on several pathologies of the CNS (it is neuroprotective in Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, multiple sclerosis, brain ischemia, and it produces analgesia in neuropathic pain) (Keilhoff et al., 2007). However, there has been evidence both supporting and opposing its use to treat inflammatory pain after a nerve injury. Recent research has shown that the inhibition of macrophage infiltration in dorsal root ganglia (DRG) can be detrimental for the conditioning reaction of sensory neurons to an injury (Niemi et al., 2013) and more specifically, the inhibition of macrophage infiltration by minocycline can inhibit neurite outgrowth in *in vitro* cultures of DRG (Kwon et al., 2013). On the other hand, the previous work from the lab of Fansa indicated that minocycline injection delayed nerve regeneration due to its effect in slowering Wallerian degeneration (Keilhoff et al., 2007) and they found increased axonal sprouting in the sciatic nerve when a complete transection was repaired with a predegenerated graft (Keilhoff et al., 2008). However, it has to be noted that an increased axonal sprouting does not necessarily mean an increasing number of regenerating neurons. These authors did not test either what was the effect of minocycline in motor and sensory axons separately.

Given these controversial results, we decided to test if minocycline reduced axonal regeneration of the sciatic nerve after a complete transection followed by the implant of a predegenerated nerve autograft, which was obtained by the in vitro culture of a segment of the sciatic nerve for one week. We did this predegeneration of the autograft to assure that the degeneration of myelin debris and Schwann cells was the same both in control and minocycline-treated animals. We used retrograde dye to determine the number of regenerating motor and sensory neurons that reached the distal tip of the predegenerated autograft.

We chose this model to study the motor and sensory neuron regeneration avoiding the Wallerian degeneration, since we know already that minocycline delays it (Keilhoff, 2007), and this would be decisive to reduce regeneration in minocycline-treated animals. We are interested in studying the effect of minocycline in the regenerative capacity of the motor and sensory neurons.

We used a systemic dose that could reduce pain but were not super strong. Macrophages and microglia are important for the regeneration of motor and sensory neurons, and sometimes anti-inflammatories are given to treat pain, so we wanted to know if a systemic treatment with minocycline affects motor and sensory neurons. This is more relevant from the perspective of the study of motoneurons, and from the perspective of doing an in vivo study. Sensory neurons have generally been more studied, and they have generally been studied in vitro.

2. Experimental procedures

2.1. Animals

We used adult Swiss female mice 10 weeks old (20-30 grams, Janvier Labs). All the procedures were approved by the Ethical Committee of the Universitat Autònoma de Barcelona.

A total of 38 mice were used for the study. 18 of them received i.p. injections of minocycline (Minocycline hydrochloride crystalline. M9511. Sigma-Aldrich) 16 and 1 hours before the first surgery, and then twice daily, for one week, until the second surgery. 20 mice underwent the surgical procedures without any other treatment, and they served as controls.

7 mice (4 controls and 3 minocycline-treated) were used to evaluate the effects of minocycline on microglial reaction in the SC. 19 mice (9 controls and 10 minocycline-treated) were used to evaluate the regeneration of motoneurons and sensory neurons in a conditioning lesion paradigm, 7 days after the test lesion. 12 mice (7 controls and 5 minocycline-treated) were used to evaluate the regeneration of motoneurons in a conditioning lesion paradigm, 3 days after the test lesion.

2.2. Effects of minocycline on microglial activation

2.2.1. Surgeries

7 Mice were anesthesized with ketamine-xylazine (ketamine [Imalgene® Merial Laboratorios, S.A.]: 90 mg/kg, xylazine [Rompun® Bayer]: 10 mg/kg; induction: 2,8 ml/kg; maintenance: 1/3 of the induction dose, every 30 minutes). Right and left sciatic nerves were exposed by a midline posterior incision through the skin, at the popliteal fossa level. After an incision through the gluteus major, nerves were exposed and completely transected with scissors at midthigh. The proximal tip of the nerve was dipped for one hour in a vaseline pool containing a retrograde dye (Hidroxystilbamidine, methanesulfonate. H22845 Life Technologies, -HSA-). After that hour, the pool was retired, the proximal tip of the nerve was washed with saline solution to avoid the retrograde dye to go to close muscles, the wound was closed in layers and the animals were left in their cages to recover.

One week after this surgery, these animals were sacrificed. 4 controls and 3 minocycline-treated animals were transcardially perfused with paraformaldehyde (4% parafolmaldehyde in PB), and SC L4-L5 segments and L4 and L5 DRG were extracted for immunohistochemistry. This surgery is depicted in Figure 1.

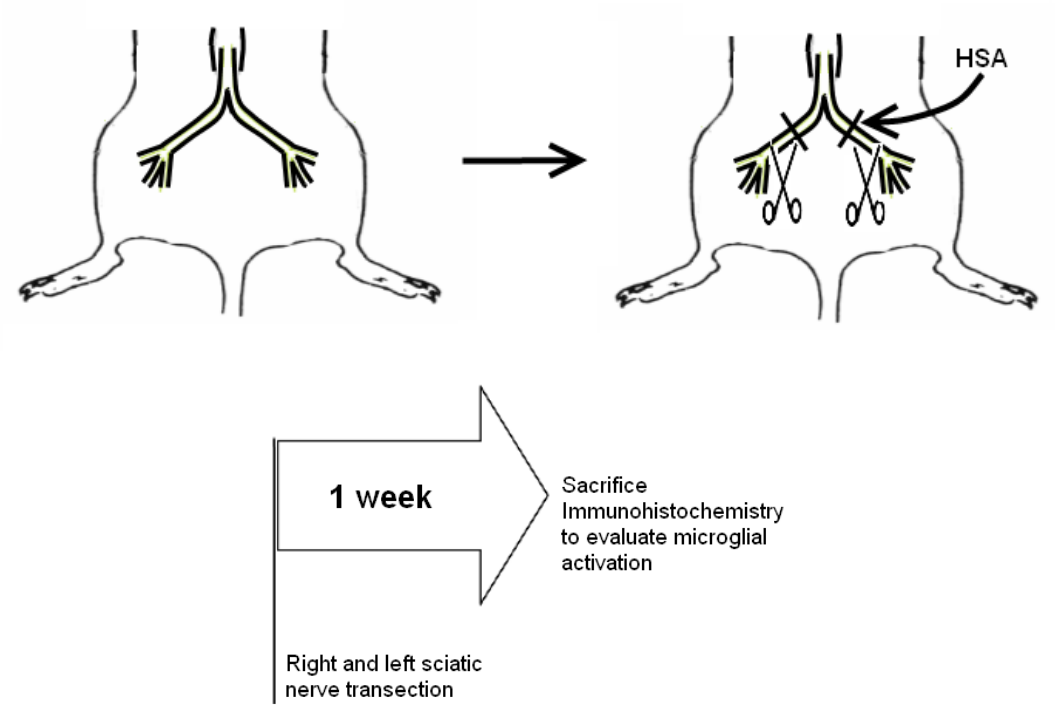


Figure 1. Diagram of the procedure followed. A set of 7 animals underwent a section of both sciatic nerves, and were sacrificed one week later to evaluate the activation of microglia in their spinal cord by immunohistochemistry.

2.2.2. Immunohistochemistry

Transversal **s**pinal cord slices were obtained serially at 20µm thickness on a cryostate (Leica CM1950 cryostat) from the first set of sacrificed mice. The sections were blocked with Fetal Bovine Serum (10 ml/100ml)-Glycine (0,15 g/100 ml)-Gelatine (0,20 g/100 ml)-PBS-Triton (0,03%) to complete a final volume of 100 mL, and incubated overnight with rabbit anti-Iba1 antibody (Iba1, *Wako*. #0*19-19741*. 1:500). After washes, samples were incubated for 2h with Cy3 donkey anti-rabbit as secondary antibody (Donkey anti-Rabbit IgG. Jackson ImmunoResearch. 711-165-152. 1:200), and then mounted with Fluoromount (Fluoromount-G®. SouthernBiotech.). To quantify microglial immunoreactivity, microphotographs of the motoneuron pools were taken at x400 with an Olympus BX51TF Fluorescence Microscope equipped with an Olympus DP50 Camera.

Photos from motoneurons and from microglia were superposed using Adobe Photoshop: the red channel from the photos of motoneurons labelled with HSA was copied and pasted in the blue channel of the final file. The red channel from the photos of microglia was copied

and pasted in the red channel of the final file. The rest of the channels (blue and green channels from the photos of microglia and motoneurons) were deleted. To quantify microglial reactivity, we used a Matlab plugin made at our laboratory.

The microglial reactivity was quantified as the number of red pixels (equivalent to microglial surface) in an area of 10 µm around motoneurons. We established the threshold for considering a red pixel at between 0.9 and 0.95. We quantified the microglial surface around 10 motoneurons per animal, selecting, for each animal, the 10 motoneurons that showed a more intense Iba1 staining around them.

2.3. Effects of minocycline on the conditioning lesion effect on motoneurons

2.3.1. Surgeries

19 Mice were anaesthetised with ketamine-xylazine (same protocol used in the surgery that was previously described). Sciatic nerves were exposed by a midline posterior incision through the skin of the right and left legs, at the popliteal fossa level. After an incision through the gluteus major, the left sciatic nerve was resected and a 6 mm segment was cultured in separated and identified Petri dishes filled with DF10S medium (Life Technologies), and cultured in an incubator, at 37ºC, for one week, to allow Schwann cells to survive, and axons to degenerate. The right sciatic nerve was exposed and completely transected with scissors at midthigh. We applied a retrograde dye to the tip of the right sciatic nerve dying all the neurons from the right sciatic nerve, to evaluate neuronal survival after the lesion (DiI stain. D3911. Invitrogen). The wound was closed in layers and the animals were left in their cages to recover.

One week after the first surgery, the animals were anaesthetised with ketamine-xylazine (same protocol used in the first surgery). Their right sciatic nerve was exposed by the same procedure followed in the first surgery. The conditioning lesion was performed by cutting 1 mm the proximal tip of the right sciatic nerve and the in vitro predegenerated graft from each mouse -to avoid immune rejection-, was sutured to this proximal tip to allow axons to regenerate through these pre-degenerated graft. The wound was closed in layers and the animals were left in their cages to recover.

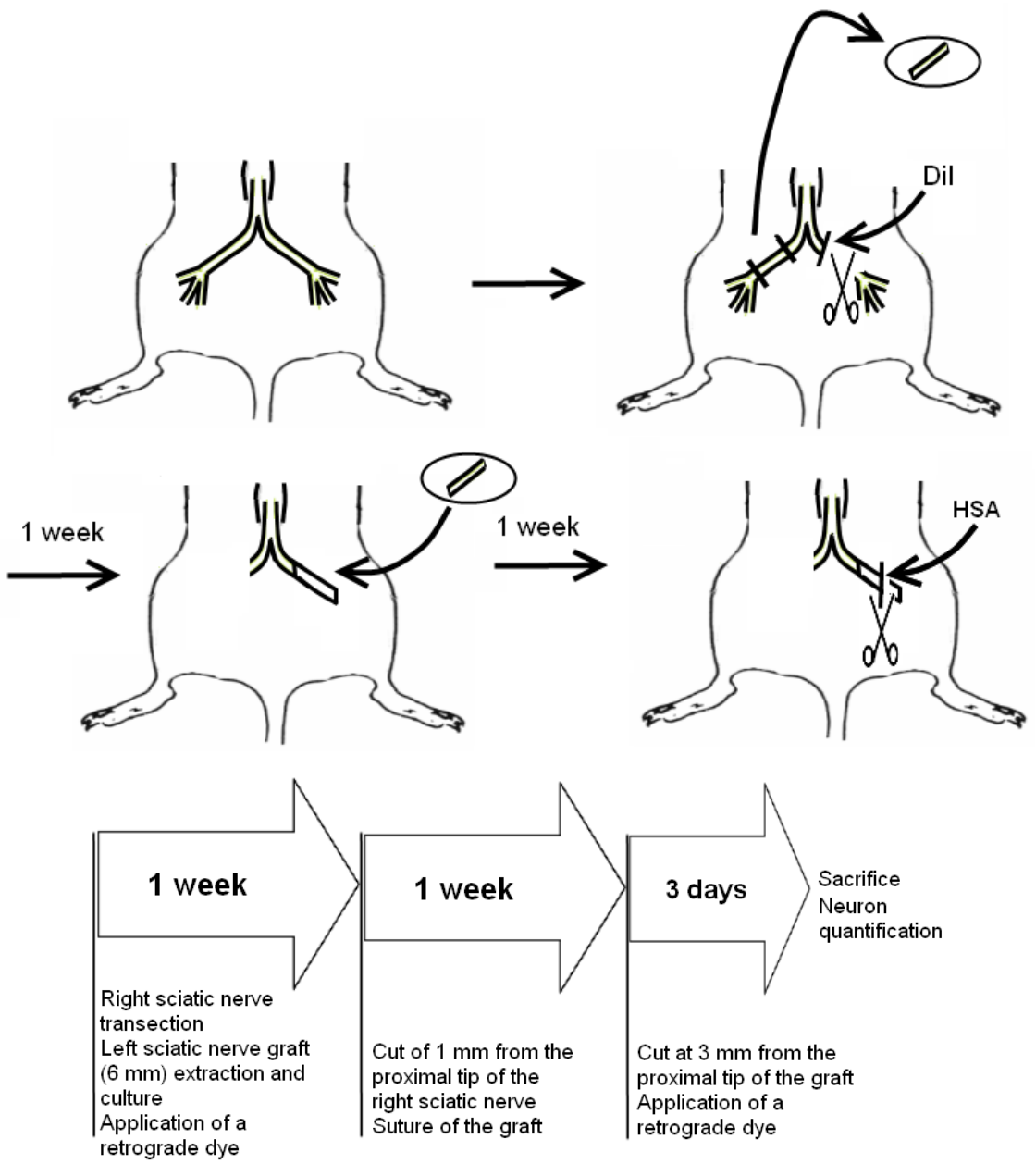


Figure 2. Diagram of the surgeries performed for the second set of experiments. A set of 19 animals underwent a section of the right sciatic nerve (priming or conditioning injury), and we extracted a 6 mm graft from their left sciatic nerve, which was cultured for one week. We also applied a retrograde dye to the tip of the right sciatic nerve to evaluate neuronal survival after the lesion. One week later, we cut 1 mm of the right sciatic nerve (second injury or test lesion) and we sutured the graft. After one week, we cut the graft 3 mm from its proximal tip, and applied a different retrograde dye. 3 days later, we sacrificed the mice, perfused them with PFA 4% in PB, and extracted samples from their spinal cord, right dorsal root ganglia and right sciatic nerves, to evaluate sciatic nerve regeneration by estimating the number of labelled motor and sensory neurons.

One week after the second surgery, the animals went through a third surgery: they were anaesthetised with ketamine-xylazine (same protocol used in the first surgery) and their right sciatic nerve was exposed by the same procedure followed in the first surgery. The graft was cut with scissors at 3 mm from the proximal tip of the graft, and the tip was dipped for one hour in a Vaseline pool containing a retrograde dye (Hidroxystilbamidine, methanesulfonate. H22845 Life Technologies). After 1 hour, the pool was retired, the tip was washed with saline solution to eliminate any remaining dye, the wound was closed in layers and the animals were left in their cages to recover.

Four days after the third surgery, the animals were transcardiacally perfused with 4% paraformaldehyde in PB. SC L4-L5 segments, and L4 and L5 right DRG were extracted for neuron quantification. These surgeries are depicted in Figure 2.

After the quantification of motor and sensory neurons regenerated after 7 days, we evaluated the regeneration of motoneurons 3 days after suturing the graft on 12 mice (7 controls, 5 minocycline-treated). The process was the same described in Figure 2, but instead of adding the retrograde dye 7 days after suturing the graft, we added it 3 days after. These animals were sacrificed and the samples obtained were processed following the same methods than for the animals evaluated 7 days after the test injury.

2.3.2. Motoneuron quantification

Transverse spinal cord slices were obtained serially at 40µm thickness on a cryostate (Leica CM1950 cryostat) and mounted with Fluoromount. Motoneuron regeneration was quantified manually: retrogradely-labelled motoneurons were counted on a fluorescence microscope (Olympus BX40F Fluorescence Microscope) at x400.

One series of each pair was quantified, and the result was multiplied by 2 to obtain the total number of motoneurons, which was corrected by the Abercrombie correction factor (Abercrombie, 1946).

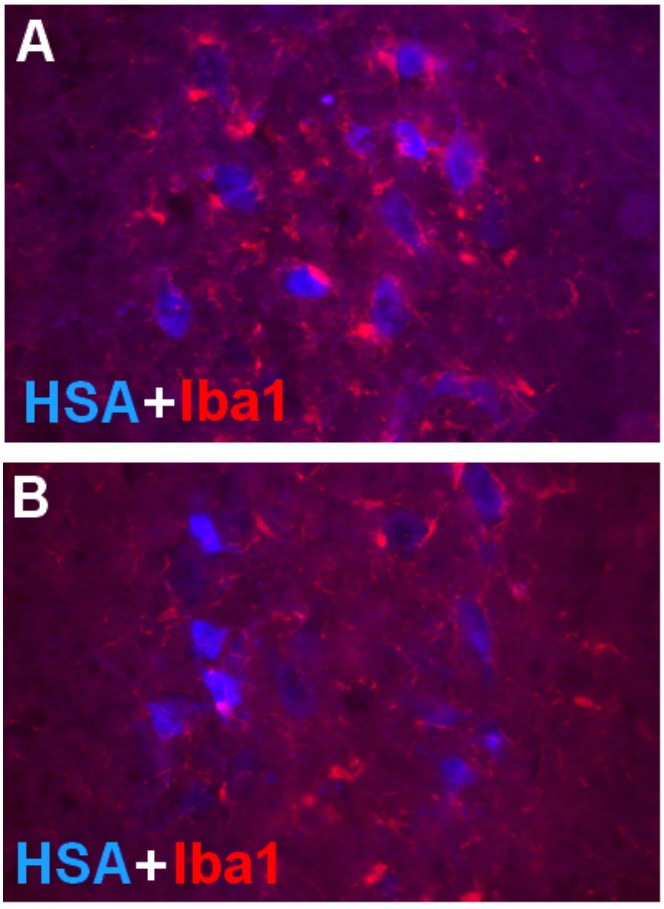
2.3.3. Statistical analysis

All statistical analyses were made using GraphPad Prism 6.01 version. For the analysis of microglial activation, data fit with normality criteria and were analyzed thorough a Fisher's exact test to determine the equality of variances, followed by an unpaired two-tailed t-test. The data for the other analyses did not fit with normality criteria and therefore they were compared using the two-tailed unpaired Mann-Whitney U test. The level of statistical significance was set at 5% (p=0,05) in all the analyses. Data are presented as mean±SEM.

3. Results

3.1. Immunohistochemical analysis of microglial activation

Quantification of the surface occupied by microglia in a 10 µm perimeter around the cell bodies of HSA-labelled motoneurons shown a reduction in Iba1 immunoreactivity around motoneuron cell bodies in minocycline-treated mice, 65.894±13.062 pixels, compared to controls, 124.316±12.971 pixels (p=0,0026 in an unpaired two-tailed Mann-Whitney U test) (Figure 3).



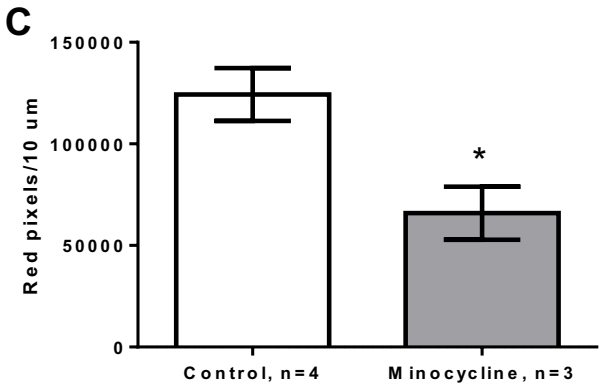


Figure 3. Quantification of microglial immunoreactivity in control and minocycline-treated mice. (A and B) Microglial (Iba1, red) immunoreactivity around motoneurons (HSA, blue) in the spinal cord of a control (A) and a minocycline-treated mouse (B). (C): Quantification of Iba1 immunoreactivity around motoneuron bodies in a a 10um thick-perimeter is decreased in minocycline-treated mice (grey column) compared to control mice (white column) (p=0,0026 in an unpaired two-tailed Mann-Whitney U test). Data are presented as mean±SEM. *p<0,05.

3.2. Quantification of regenerated motoneurons through the pre-degenerated autograft after the conditioning lesion, 7 days after the test injury:

We counted the number of motoneurons labelled by DiI in the SC of 5 control and 4 minocycline-treated animals. The analysis of these data did not show significant differences between the minocycline-treated mice (817,20±64,30 motoneurons) and controls (458,50±111,00 motoneurons) (p=0,0635 in a two-tailed unpaired Mann-Whitney U test). These results are shown in Figure 4.

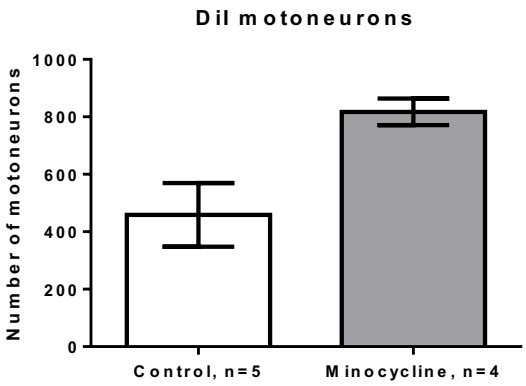


Figure 4. Effect of minocycline administration in motoneuron survival 7 days after a single lesion Motoneuron quantification in control (white columns) and minocycline-treated (grey columns) animals. Mean number of surviving motoneurons is slightly higher in the minocycline-treated group, but this difference is not significant (p=0,0635). Data are presented as mean±SEM.

We counted the number of motoneurons labelled with HSA in the SC of control and minocycline-treated animals. The estimated number of labelled motoneurons in the control group was 483,20±75,96 motoneurons, whereas in minocycline-treated animals was slightly higher, 686,20±87,76 motoneurons, although this difference was not statistically significant (p=0,1318 in a two-tailed unpaired Mann-Whitney U test). These results are shown in Figure 5.

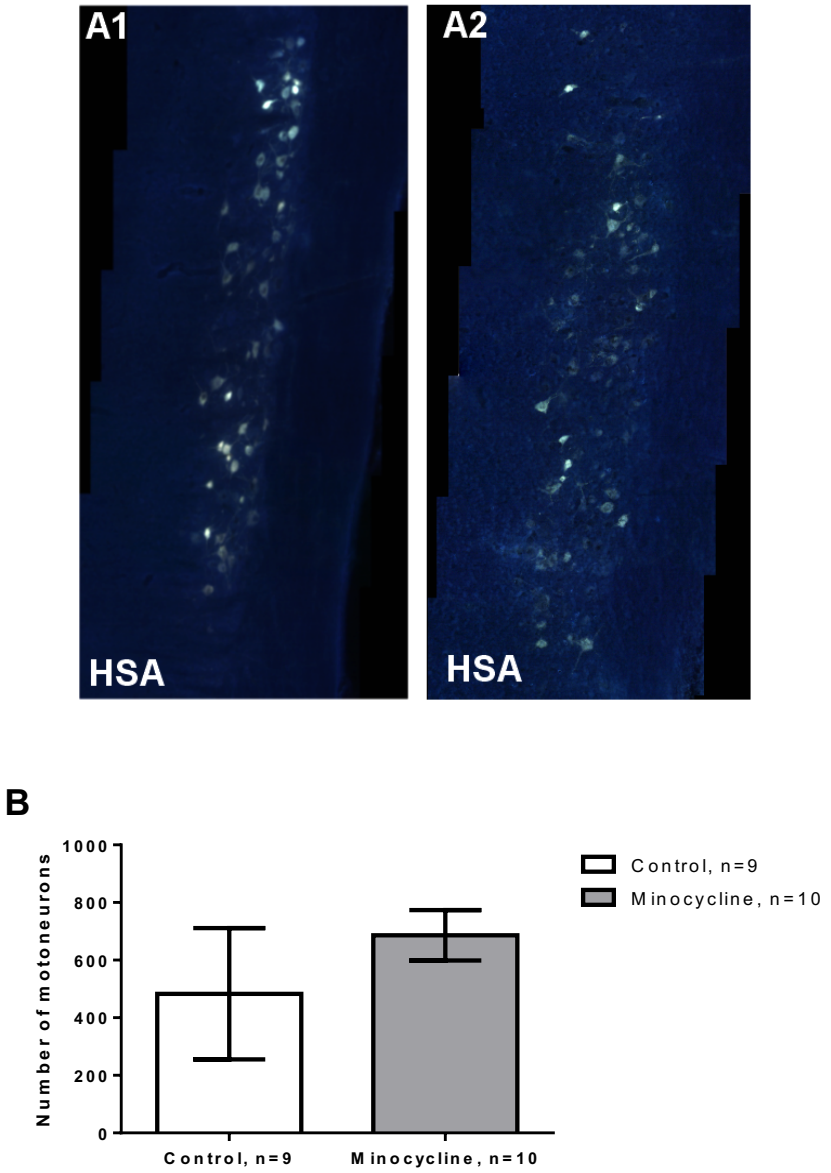


Figure 5. Effect of minocycline administration in motoneuron regeneration after a conditioning lesion, 7 days after the test injury (A) Representative pictures of spinal cord sections, containing motoneurons dyed with the retrograde dye HSA (white) in control (A1) and minocycline-treated (A2) mice. (B) Motoneuron quantification in control (white columns) and minocycline-treated (grey columns) animals. Mean number of regenerated motoneurons is slightly higher in the minocycline-treated group, but this difference is not significant (p=0,1318). Data are presented as mean±SEM.

3.3. ***Quantification of regenerated sensory neurons through a pre-degenerated autograft after the conditioning lesion, 7 days after the test injury:*** The results

of the quantification of sensory neurons followed the same tendency observed for motoneurons. We found a slight increase in labelled neurons in minocycline-treated animals (1.523,00±213,90 sensory neurons) compared to control animals (1.079,00±216,30 sensory neurons), although this difference was not statistically significant (p=0,2079 in a two-tailed unpaired Mann-Whitney U test). These results are shown in Figure 6.

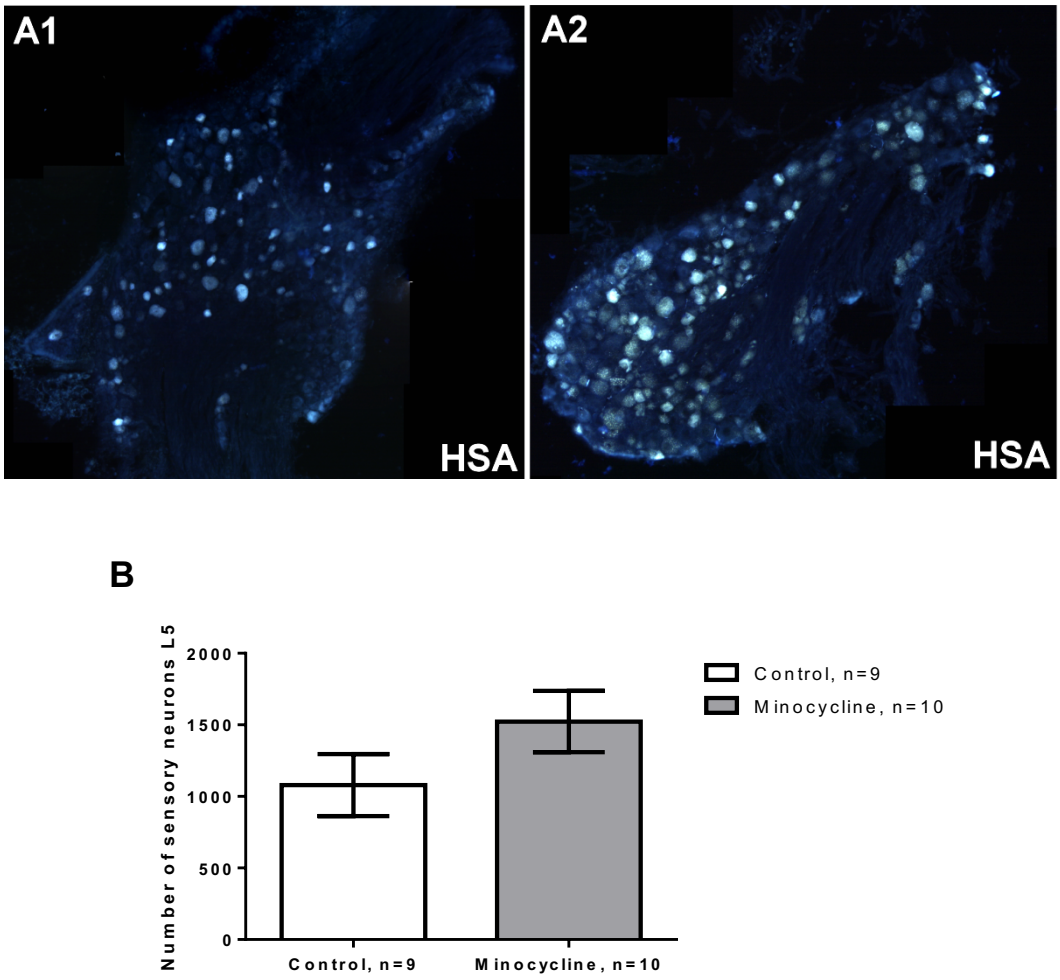


Figure 6. Effect of minocycline administration in the regeneration of sensory neurons after a conditioning lesion, 7 days after the test lesion (A) Representative pictures of a L5 20 µm DRG section from a control (A1) and a minocycline-treated mouse (A2), containing sensory neurons labelled with the retrograde dye HSA (white). (B) Sensory neuron quantification was slightly higher in the minocycline-treated group (grey column) when compared to controls (white column), but this difference was not significant (p=0.2079 in a two-tailed unpaired Mann-Whitney U test). Data are presented as mean±SEM.

3.4. *Quantification of regenerated motoneurons through a pre-degenerated autograft after a conditioning lesion, at 3 days after the test lesion*

The results of the quantification of motor neurons 3 days after the test lesion followed the same tendency observed for motoneurons and sensory neurons at 7 days after the test lesion. We found a slight increase in labelled neurons in minocycline-treated animals (180,80±47,43 motor neurons) compared to control animals (129,00±40,08 motor neurons), although this difference was not statistically significant (p=0,4293 in a two-tailed unpaired Mann-Whitney U test). These results are shown in Figure 7.

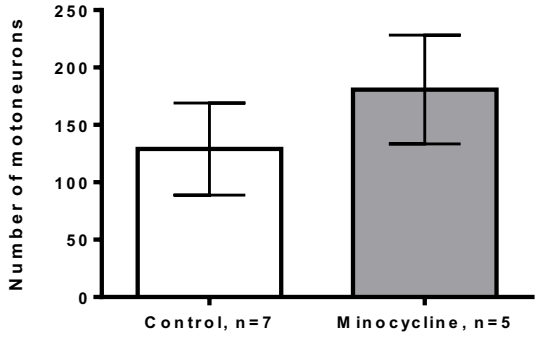


Figure 7. Effect of minocycline administration in motoneuron regeneration after a conditioning lesion, 3 days after the lest lesion. Motoneuron quantification in control (white columns) and minocycline-treated (grey columns) mice. Mean number of regenerated motoneurons is slightly higher in the minocycline-treated group, but this difference is not significant (p=0,4294 in a two-tailed unpaired Mann-Whitney U test). Data are presented as mean±SEM.

4. Discussion

Our results indicate that minocycline injection at a dose that is 5 times bigger than the usual therapeuthical dose for humans (30 mg/kg ip) does not inhibit the intrinsic capacity of motor and sensory neurons to regenerate after a complete transection of the sciatic nerve. Furthermore, it shows a non-significant tendency to increase the number of motor and sensory

neurons that regenerate. This effect is visible at 7 days after the implantation of the predegenerated graft, but it is also present at 3 days after its implantation.

These results indicate that the detrimental effect minocycline could have over neuronal regeneration at therapeuthical doses in humans is very low, if it exists. It is possible that the dose of minocycline needed to reduce axonal regeneration in vivo was much higher than the therapeuthical ranges that are usually considered.

Kwon et al., 2013 showed that if macrophages inhibition is not complete, the effects of minocycline inhibiting neurite elongation in vitro do not happen. Kwon et al shown that intrathecal minocycline infusion inhibited neurite outgrowth from DRG in vitro (Kwon et al., 2013). Probably, the inhibitory effect of minocycline on regeneration happens at much higher doses than the one we used. However, our aim was to determine if minocycline at therapeutical doses could impair regeneration, so we believe there is no reason to increase the dose of minocycline for this study, taking into account that we do not see an aversive effect at a dose that is already 5 times higher than the normally recommended dose for humans. Moreover, we shown by immunohistochemistry that this dose of minocycline reduced significantly the microglial reaction around motoneurons after a sciatic nerve transection. It is possible that we are not achieving a complete inhibition of microglia, but that was not part of our objectives. We used a systemic dose that was on the therapeutical range and therefore are more comparable to the effects of minocycline administration after a nerve injury in humans. Our results are more relevant to the clinical use of minocycline this way.

Niemi et al., 2013 shown similar results to those of Kwon et al., 2013. It is possible that macrophage infiltration is necessary for the activation of sensory neurons from DRG in vitro, but our results show that this process is not so decisive in vivo at a dose of 30 mg/kg ip. It is possible that there were many other factors that are also playing a part on nerve regeneration in vivo, so the absence of macrophage infiltration may be compensated by other processes that are taking place at the same time.

Beyond that, we saw that the effect of minocycline injection was the same in motor and sensory neurons from the sciatic nerve. In both cases, it increased slightly, but not significantly, the number of regenerated neurons compared to the controls group.

Finally, although the injection of minocycline did not affect significantly the survival of the injured neurons, treated animals show a tendency to have less neuronal death after the lesion. This seemingly neuroprotective effect may account for at least part of the tendency of minocycline to increase the regeneration of motoneurons after a treatment during one week.

The absence of significance in the effect of minocycline on the regeneration of motor and sensory neurons in vivo may be due to the great inter-subject variability that we observe in this study. Probably the in vitro studies imply less variability.

The novelty of this study compared with the previous studies about the use of minocycline after a peripheral nerve injury and its effect in the regenerative capacity of neurons is that most of these studies have focused in the regenerative capacity of sensory neurons in vitro. This study uses a systemic administration of minocycline at a dose that is close to the therapeuthical doses used in humans (30mg/kg ip), and we studied the regeneration of both motor and sensory neurons in vivo. This approach is more relevant to the preclinical study of minocycline as a possible drug to treat inflammatory pain after a peripheral nerve injury.

In conclusion, minocycline does not inhibit axonal growth after a sciatic nerve injury, provided that the graft does not require Wallerian degeneration. Therefore we would recommend to continue considering it for its use in the clinic to relieve inflammatory pain after a complete nerve transection.

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