# FIBROBLASTS GROWTH FACTOR AND NERVE FRAGMENTS EFFECT ON TIBIAL NERVE REGENERATION IN RATS: A COMPARATIVE STUDY

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## SUMMARY

Objective: To quantitatively compare rats' tibial nerve regeneration stimulation by Fibroblast Growth Factor (FGF) and nerve fragments using silicone tubes. Methods: 18 Wistar rats were employed in this experiment. The experimental surgery consisted of resection of an 8-mm tibial nerve segment, followed by an interposition of silicone tubes. On the right side, the tube was filled with a Fibroblast Growth Factor (FGF) solution, and on the left side, it was filled with 1 mm nerve segments. After three months, the animals were submitted to an additional surgery for exposing tibial nerves to the neuronal marker Fluoro-Gold®. After 48 hours, they were perfused with a paraformaldehyde solution and the medullar segment between L3 and S1 was removed and cut into 40 $\mu$m-thick segments. Results and conclusion: The results of neuronal counts showed a higher amount of neurons on the side where FGF was used compared to the side where nervous fragments were placed, suggesting a superior performance of the fibroblast growth factor over nerve fragments for stimulating nervous regeneration in silicone tubes.

***Keywords:*** Peripheral nerves; Wistar rats; Fibroblast growth factor.

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## INTRODUCTION

Injuries of hand's peripheral nerves constitute a major cause of functional disability, for being a frequent injury in economically active individuals.

Today, the most used repair technique is the epiperineural suture, using microsurgical techniques. However, in injuries with segment loss or in chronic injuries when stump retraction occurs, the use of a graft is required. The obstacle in these cases is that the removal of a peripheral nerve graft implies on injuring a previously normal area; besides, the end result with the use of a graft is inferior to the direct end terminal suture of the stumps.

New alternatives have been studied in order to avoid these complications. Among those studies, we highlight the use of veins[1] and arteries [2,3], which, for being biological and autogenous materials, would cause little reaction and would allow axon growth drive. The difficult part in these cases is that, by interposing large segments, the vein suffers from collabation, making neuronal growth difficult, while the removal of artery segments would cause a reduced irrigation to donator area.

In an attempt to avoid these problems, in recent studies, the use of synthetically-based tubes, particularly silicone tubes, have been researched [4,5,6].

In parallel, much has been studied about neurotrophic substances, where the presence of substances stimulating and guiding axonal stumps' growth is observed [7,8,9,10,11,12].

The objective of the present study is to compare the action of Fibroblasts Growth Factor and nerve fragments on rats' tibial nerves regeneration.

## METHOD

Eighteen adult male Wistar rats, weighting 200 grams in average, were employed.

We used intraperitoneal anesthesia, with anesthetic solution prepared with: chloral hydrate (4.25 g), magnesium sulfate (2.25 g), propylene glycol (4.28 ml), absolute ethyl alcohol (11.5 ml), distilled water (45.7 ml). After weighted, the animals were administered with anesthetic solution at a ratio of 0.4 ml/ 100 g of body weight.

Once anesthetized, the animals were submitted to surgery

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where a posterolateral incision was performed on each thigh, exposing sciatic nerve and its branches - sural nerve, peroneal nerve, and tibial nerve - (Figure 1). We carefully dissected the right tibial nerve, manipulating it as minimally as possible.

We also carefully sectioned an 8-mm segment of the right tibial nerve, leaving the proximal stump of approximately one millimeter proximal and distal. Then, we fixated the nerve's proximal stump inside a silicone tube with nylon 10-0 wires, and infused the 20 Mol Fibroblasts Growth Factor saline solution with a Hamilton's syringe. After filling the whole tube with the solution, we fixated the nerve's distal stump in the tube (Figure 2). On both ends of the tube, we used a fibrin glue (Beriplast, Aventis®) in order to avoid solution leakage.

We performed the dissection and tubing of the left tibial nerve as previously made with the right one, differing only by the insertion of 1-mm nerve segments removed from right leg into the tube.

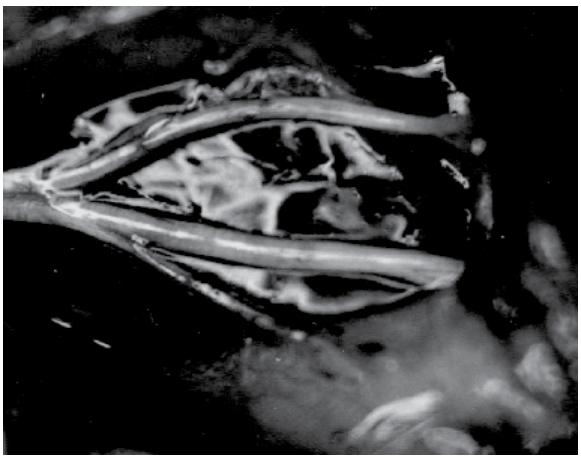

**Figure 1.** *A sciatic nerve with its three branches*

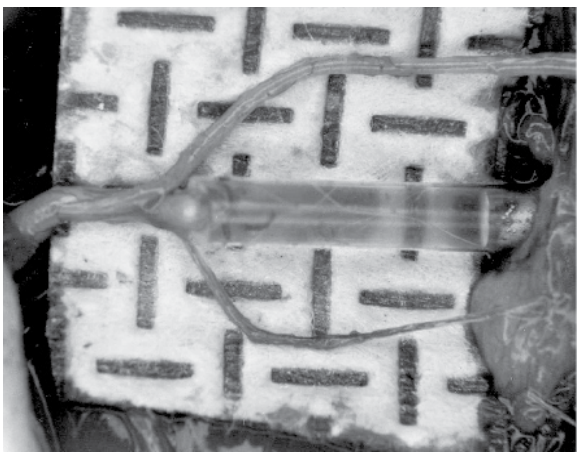

**Figure 2.** *Tibial nerve tubing after resection of 8 mm.*

After 3 months, the animals were re-operated for exposing right and left tibial nerves, and the nerve was sectioned at 1 mm distal to tube port. The proximal stump was kept in contact with the dye Fluoro-Gold® (FG), at a 3% strength and diluted in distilled water for 1h30m.

Forty-eight hours after staining, the animals were again anesthetized via intraperitoneal using 3% Thionembutal 50 mg/kg of body weight. We performed a thoracotomy, exposing the lungs and the heart, followed by the insertion of a catheter into left ventricle, reaching aorta's lumen. With the aid of scissors, we opened a large hole at right atrium, immediately turning infusion pump on, to which the catheter had been previously attached.

By the end of perfusion with paraformaldehyde, we removed the spinal cord, exposing the whole dorsolumbosacral marrow, dried the dura-mater and detected dorsal roots of L3, L4, L5, L6 and S1. We followed roots' path from L3 to S1 up to its insertion on spinal cord, preserving the marrow segment of L4, L5, L6. Without removing the sectioned segment from its bone bed, we cut the dorsal roots of L3, L4, L5, L6 and S1 tangentially to the spinal cord.

The marrow segment was soaked into 4% paraformaldehyde/ phosphate buffer solution ("perfusion solution") for 8 hours.

The piece was then sliced into 40 $\mu$m-thick sections and assembled in slides, which were placed for drying in a refrigerator (4º Celsius) during 24 hours.

When the slides became available, they were studied under a ZEISS-AXIOLAB® microscope with ultraviolet light in order to make marker evident. All sections were examined and the stained motor neurons were counted (Figure 3). Neuron count was made with a magnification of 25x, but magnifications of 50 to 100x were used when the presence of the marker was not clear. All data for all sections were summed up.

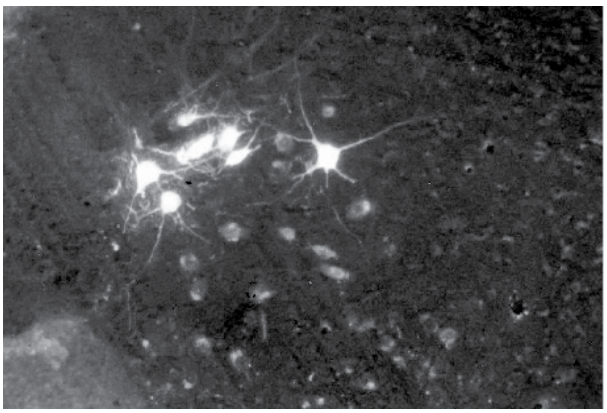

**Figure 3:** *Visualization of neurons by fluorescent microscopy.*

## RESULTS

Neuron count with fluorescent microscopy widely ranged among animals, from 592 to 1920 on the right side (average: 1171), and from 457 to 1556 on the left side (average: 883). However, we noticed that, in all rats, the right side had a higher number of cells (Table 1).

Values on table 1 were adjusted by Abercrombie's criterion [13] (Table 2), where the value found on medullary segments was multiplied by 0.65, because, with 40-$\mu$m fragment sections, we could count a same cell in different slides. Using this criteria, that double-counting would be avoided.

| Rats | Rigth side | Left Side |
|---|---|---|
| 1 | 1150 | 489 |
| 2 | 1920 | 1293 |
| 3 | 1154 | 867 |
| 4 | 1448 | 1144 |
| 5 | 1180 | 915 |
| 6 | 1824 | 1556 |
| 7 | 1180 | 809 |
| 8 | 1690 | 1370 |
| 9 | 1487 | 1183 |
| 10 | 1661 | 1347 |
| 11 | 957 | 941 |
| 12 | 867 | 615 |
| 13 | 1275 | 987 |
| 14 | 1215 | 693 |
| 16 | 840 | 721 |
| 17 | 640 | 509 |
| 18 | 592 | 457 |
| Total | 21080 | 15896 |
| Average | 1171,11 | 883,11 |

**Table 1 -** *Number of absolute cells counted on right and left sides (total amounts and average).*

| Rats | Rigth side | Left side |
|---|---|---|
| 1 | 748 | 318 |
| 2 | 1248 | 841 |
| 3 | 750 | 564 |
| 4 | 941 | 744 |
| 5 | 748 | 595 |
| 6 | 1186 | 1012 |
| 7 | 771 | 526 |
| 8 | 1099 | 891 |
| 9 | 967 | 769 |
| 10 | 1080 | 876 |
| 11 | 622 | 612 |
| 12 | 564 | 400 |
| 13 | 829 | 642 |
| 14 | 790 | 451 |
| 15 | 617 | 438 |
| 16 | 546 | 469 |
| 17 | 416 | 331 |
| 18 | 385 | 297 |
| Total | 14307 | 10776 |
| Average | 794,83 | 598,66 |

**Table 2 -** *Table 1 values adjusted by Abercrombie's criterion for 40 µm section.*

Statistical analysis with signaled Wilcoxon 's non-parametric tests and the parametric Student's *t-test* showed that both groups present statistically significant differences.

## DISCUSSION

In the present study, we used the fibroblasts growth factor, a substance that was first described in 1975 [14], which has shown to play mitogenic, angiogenic and neurotrophic roles [15,16,17,18,19].

FGF's exact mechanism of action is still unknown, although the ability of improving neurons survival after axonal injury has been reported [18,19]. Recent studies have demonstrated the presence of a large amount of FGF receptors in Schwann's cells[18], a fact suggesting that FGF's neurotrophic action occurs mainly by stimulating the growth of the so-called "Büngner's bands"[20]. Those bands are Schwann's cells extensions connected at a longitudinal plane, acting as guides for fibers growth.

Some studies show that the presence of nerve segments inside tubes improves myelinized nervous fibers' regeneration, concluding that these may be an additional source of Schwann's cells and neurotrophic factors [21].

We decided to make a comparative study of the neurotrophic action between nerve fragments and fibroblasts growth factor, once both showed good results in terms of nervous regeneration in previous studies [13,14,15,16,17,19].

Many authors achieved good results using nerve tubing with nerve or artery segments [1,2,3]. The use of synthetic tubes presents some disadvantages compared to biological material because it presents a more intense fibrosis. We chose to use a silicone tube because this is a more inert material, showing less site reaction, less collabation, allowing the accumulation of substances within it, and because it is easily accessible in our environment.

We chose to use a tibial nerve, which is predominantly motive, because studies with mixed nerves, such as the sciatic, may lead to injuries such as ulcers and limb autophagy, in addition to difficult ambulation and feeding. We resected an 8-mm segment, because at the port employed, tibial nerve has an approximate extension of 10 mm; thus, about 1 mm was left on each end. Maintaining this small stump segment was important for us to be able to introduce it into tube's lumen.

The interval between the first and the second surgeries was three months, thus avoiding a more extended time for preventing further animals loss. When assessing the animals before second surgery, we found them presenting with a good limb function and not showing limping as occurred just after the first surgical procedure.

We used the medullary neuronal count technique after staining graft's distal stump with Fluoro-Gold. This technique allows for a more reliable evaluation of the number of cells reaching to graft's distal end, because, in a sectioned axon, a "budding" with various branches occurs. A study of the nerve's distal segment could lead to an overestimate of regenerated axons, because these uncountable branches of the stumps undergo physiological amputation over time [22].

Spinal cords were submitted to 40µm-thick histological sections, enabling a great sensitivity on marked cells counts, but also enhancing the potential of a same neuron to be counted in different sections. Due to this fact, we used the Abercrombie's adjustment method [13], in which the result is multiplied by 0.65, making the result to be as close as possible to the actual number of cells.

By statistical analyses, we found a statistical significant difference between sides, with the side receiving FGF presenting a higher neuron count than the side with nerve segments.

By assessing the results table, we found a significant difference in the number of cells between animals, ranging from 385 to 1248 on the right side, and from 297 to 1012 on the left side. It is important to highlight that the proportions between left and right remained, thus not changing the statistical analysis.

The large variability of rats' number of cells among each other may be explained by each animal's characteristics, which is also reported in previous studies [22]. Due to that variability, we used the paired t-test, which removes an animal's individual effect, testing the averages of differences in each rat. In this test, we saw a Pearson's linear correlation between sides, which can range from zero to one. In our study, it has shown to be quite high (0.942), suggesting that the sides react similarly for each methodology applied.

For results analysis, we also used the Mann-Whitney's test, which assesses each side as independent entities. This study employs the average scores for each group, not removing rats' individual effects. The Wilcoxon's test also assesses the scores, checking differences in each rat. All tests showed no statistically significant difference.

The use of a silicone tube as a peripheral nerve graft has been the object of research by several authors, but the results showed that these cannot be employed as a replacement for an autologous nerve graft. Some good results [6,23] disagree with others, evidencing that no optimal graft is available yet [24].

Other alternatives that seem to provide promising results are the biological materials for tubing, such as arteries and veins.

The combination of a biological material and a growth factor should, theoretically, promote a more appropriate regeneration, but, in this case, we were faced with the technical difficulty of infusing the solution and precluding fluid leakage.

The importance of Schwann's cells was reported in many studies, and the presence of nerve fragments inside the tube

may be an additional source of these cells [21]. Despite that, in our study, this method has proven to be inferior in promoting nervous regeneration.

The fibroblasts growth factor has shown to be quite promising, having shown similar or even superior results compared to traditional nerve graft [24]. In our study, we found better results than with the use of nerve fragments, but when we compare the number of cells counted in each rat to other studies using the same methodology, we can see that the autologous nerve graft actually promotes a better peripheral nerve regeneration [25].

Since neurokins were first reported, new perspectives emerged for improving nervous regeneration; however, to date, none of these factors enabled a fully satisfactory neuronal growth.

The various studies published about nervous regeneration show the level of concern involving this still unclear issue. A better understanding of biochemical phenomena seem to be paramount for developing an optimal treatment method. The use of exogenous neurotrophic substances has already shown exciting results, and maybe a combination with appropriate tubing methods would probably improve the recovery of patients with nervous injuries in the future. This study showed that just the presence of Schwann's cells added as nerve fragments was not enough for a desired regeneration. Based on evidences that the FGF acts especially on Schwann's cells, maybe the combination of both products - FGF and nerve fragments - brings us the desired solution, which is to get an optimal substitute for nerve grafts.

## CONCLUSION

Tubing with fibroblasts growth factor promoted a significant improvement of nervous regeneration when compared to nerve fragments.

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