

Nerve growth factor with fibrin glue in end-to-side nerve repair in rats¹

Fator de crescimento nervoso em cola de fibrina no reparo término-lateral de nervos em ratos

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ABSTRACT

PURPOSE: To determine the effects of end-to-side nerve repair performed only with fibrin glue containing nerve growth in rats.

METHODS: Seventy two Wistar rats were divided into six equal groups: group A was not submitted to nerve section; group B was submitted to nerve fibular section only. The others groups had the nerve fibular sectioned and then repaired in the lateral surface of an intact tibial nerve, with different procedures: group C: ETS with sutures; group D: ETS with sutures and NGF; group E: ETS with FG only; group F: ETS with FG containing NGF. The motor function was accompanied and the tibial muscle mass, the number and diameter of muscular fibers and regenerated axons were measured.

RESULTS: All the analyzed variables did not show any differences among the four operated groups ($p>0.05$), which were statistically superior to group B ($p<0.05$), but inferior to group A ($p>0.05$).

CONCLUSION: The end-to-side nerve repair presented the same recovery pattern, independent from the repair used, showing that the addition of nerve growth factor in fibrin glue was not enough for the results potentiating.

Key words: Suture Techniques. Fibrin Tissue Adhesive. Microsurgery. Rats.

RESUMO

OBJETIVO: Determinar os efeitos do reparo nervoso término-lateral realizado apenas com cola de fibrina contendo fator de crescimento nervoso em ratos.

MÉTODOS: Setenta e dois ratos Wistar foram distribuídos em seis grupos: A - não submetido à secção nervosa; B - secção do nervo fibular (sem reparo); Os outros grupos tiveram o nervo fibular seccionado e então reparado na superfície lateral do nervo tibial intacto, com diferentes procedimentos: C - RNTL com suturas; D - RNTL com suturas e FCN; E - RNTL apenas com CF; F - RNTL com CF contendo FCN. A função motora foi acompanhada e a massa do músculo tibial, o número e o diâmetro das fibras musculares e axônios regenerados foram medidos.

RESULTADOS: Não houve diferença entre as variáveis avaliadas nos quatro grupos operados ($p>0,05$), os quais foram superiores ao grupo B ($p<0,05$), mas inferiores ao grupo A ($p>0,05$).

CONCLUSÕES: O reparo nervoso término-lateral mostrou o mesmo padrão de recuperação, independente do tipo de reparo utilizado, evidenciando que a adição de fator de crescimento nervoso na cola de fibrina não foi suficiente para a potencialização dos resultados.

Descritores: Técnicas de Sutura. Adesivo Tecidual de Fibrina. Microcirurgia. Ratos.

Introduction

The repair of peripheral nerves even under ideal conditions has frequently presented non-satisfactory results. The axonal regeneration rarely reaches the previous levels and the sequelae are very frequent. The need for the discovery of alternatives for improving results of nerve repairs is a challenge reported by many authors for years¹⁻⁴.

Nerve Growth Factor (NGF) has been highlighted in literature as an important neuroprotector and neurostimulator, facilitating the nerve post trauma regeneration and consequently obtaining better post-operative results⁵.

Despite being highly desirable the NGF application in nerve repair is difficult and complex⁵. Its bioavailability is very short and it is quickly inactivated *in vivo*³. The already described alternatives for applying NGF are of high cost, difficult clinical applicability and uncertain efficiency^{1,2}. The search for the ideal method still persists what hampers its usage in large scale^{3,5}.

Many authors have recently enhanced the results of end-to-end nerve repair (ETE) by using fibrin glue associated with NGF¹⁻³. The use of fibrin glue as a carrier of diverse elements is not new in literature however the discovery that it would be able to keep the NGF active longer, gradually liberating it in the nerve repair was considered a great advance in microsurgery⁶.

The successful association of fibrin glue containing NGF in ETE has boosted its usage in other kinds of nerve repair¹⁻³. The end-to-side nerve repair (ETS) with sutures as described by Viterbo *et al.*⁷ has become an excellent alternative for the cases in which ETE cannot be performed (when the proximal nerve stump is not available or when a nerve gap is present) or when the nerve grafting is not the best option. However there are some authors who still believe that the ETS with sutures could harm the donating nerve, especially small dimension nerves^{8,9}.

Therefore the fibrin glue has been recently studied in ETS with the main purpose of preservation of the donating nerve, keeping it completely untouched after the repair¹⁰. In 2010 we confirmed the effectiveness of this technique through functional and morphometric analysis. However, as already expected, the regeneration of axons despite being satisfactory did not reach normal levels pointing out the need for more studies in order to enhance axon regeneration with this technique¹⁰.

The ETS with fibrin glue containing NGF has not been tested yet and the potential benefit of such association justified this research. Therefore the purpose of this study was to determine the effects of an ETS performed only with fibrin glue containing NGF in rats.

Methods

The study was approved by the Experimental Research Ethics Commission of the Federal University of Mato Grosso do Sul (UFMS) - Brazil. The study was performed at the Laboratory of Experimental Surgery Techniques of UFMS from January to December 2010.

The experiment was performed with 72 adult male Wistar rats (*Rattus norvegicus albinos*) ranging from 130 to 150 days old, supplied by the UFMS vivarium.

The animals were randomized into six equal groups (A to F) with 12 animals each. Immediately after the distribution of the groups each animal was weighed and submitted to walking track tests. The animals were placed on a walking down corridor (11.5 x 50 cm) with access to a dark environment. A cardboard sheet was placed on top of the corridor. The animals had their hind paws painted and, as they walked, they left prints.

Following the procedure, the animals were anesthetized with quetamin (50mg/kg) and xylazine (50mg/kg) by intramuscular injection.

The left fibular nerve was exposed in all animals under sterile conditions through a muscle-splitting incision in the lateral surface of the left pelvic member.

The animals in groups B to F had the fibular nerve sectioned with micro-scissors at 3 mm from its origin in the sciatic nerve. The proximal stump was bent in an approximately 100° angle and introduced in the abductor musculature with a single polyamide 9-0 suture.

The animals in group B were not submitted to the nerve repair. The distal stump was sutured in the abductor musculature far away from the proximal stump.

The animals in group C were submitted to an ETS with sutures, with epineurium preservation, according to the technique described by Viterbo *et al.*⁷. The distal stump of the fibular nerve was sutured to the lateral portion of the tibial nerve at 5 mm from its origin, with two polyamide 10-0 sutures.

The animals in group D were submitted to the same ETS⁷, but now in association with 2.5µg NGF as described by Jubran and Widenfalk² (Recombinant Rat Beta-NGF – Peptrotech, Rocky Hill, NJ).

The animals in group E were submitted to an ETS only with fibrin glue – without sutures¹⁰. The distal stump of the fibular nerve suffered nerve repair to the lateral face of the tibial nerve at 5 mm from its origin. It was used 20 µl of fibrin glue (Baxter AG, Vienna, Austria).

The animals in group F were submitted to the same

ETS with fibrin glue¹⁰, but now in association with 2.5µg NGF² (Recombinant Rat Beta-NGF – Peprotech, Rocky Hill, NJ).

Figures 1 and 2 show intraoperative aspects of the study groups.

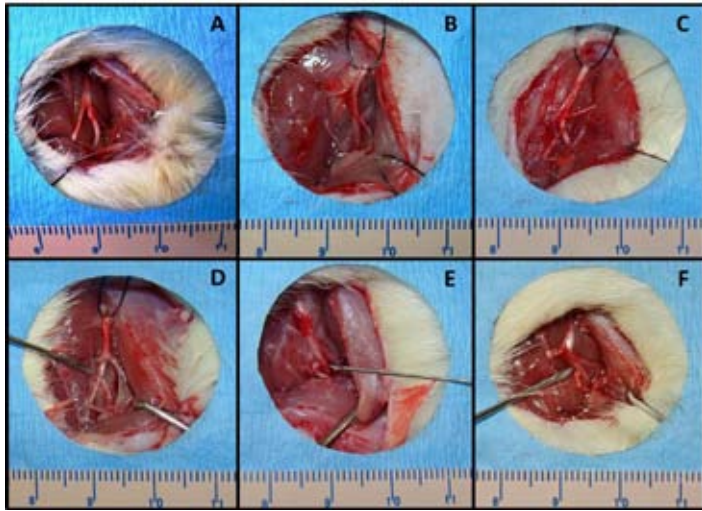


FIGURE 1 - Study groups: **A.** Control group; **B.** Fibular nerve section without repair; **C.** ETS with sutures; **D.** ETS with sutures associated to NGF; **E.** ETS with fibrin glue; **F.** ETS with fibrin glue containing NGF.

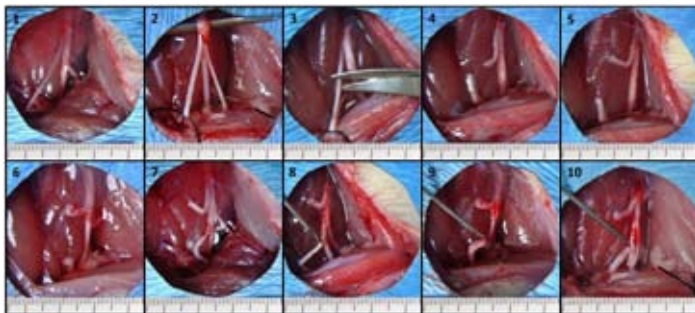


FIGURE 2 - Intraoperative photographs of the animals submitted to the experiment identifying the surgical sequence – **1.** Anatomy of the nerves under study; **2.** Identification of the fibular nerve (black suture) and the sural nerve (blue suture); **3.** Section of the fibular nerve with microscissors; **4.** Separate nerve stumps after fibular nerve section; **5.** Anchorage of the proximal nerve stump; **6.** Anchorage of the distal nerve stump – group B; **7.** ETS with sutures – group C; **8.** ETS with sutures associated with NGF – group D; **9.** ETS with fibrin glue only – group E; **10.** ETS with fibrin glue containing NGF – group F.

The skin was then sutured with continuous 4-0 polyamide monofilament suture. The right pelvic member (RPM) was not submitted to surgery.

The analysis of the motor function by the walking track analysis was repeated after 30, 60 and 90 days. Four consecutive prints per animal were analyzed. The measurements used to calculate the function recovery were: distance between the talus and the third distal phalange (PL= print length); distance between

the first and the fifth distal phalange (TS= toe spreading) and distance between the second and the fourth distal phalange (ITS= intermediary toe spreading)¹⁰ (Figure 3).

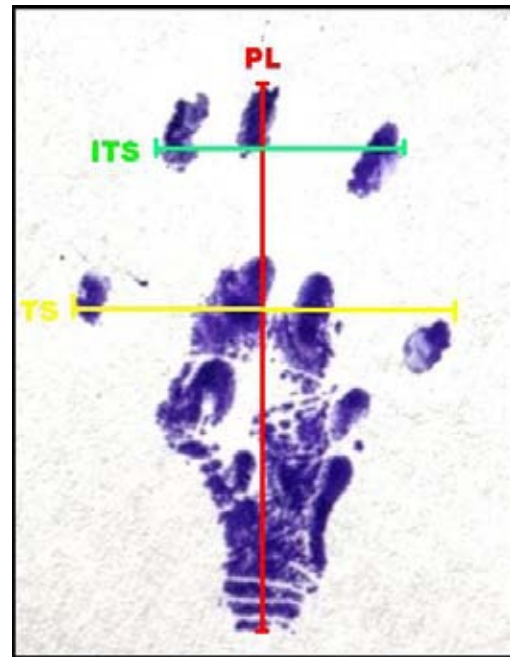


FIGURE 3 - Animal print showing the measurements taken: PL= print length; TS = toe spreading; ITS = intermediary toe spreading.

According to the measurements taken, the Sciatic Function Index (SFI)¹¹ was calculated by the formula:

$$SFI = -38.3 \frac{(PL \text{ LPM} - PL \text{ RPM})}{PL \text{ RPM}} + 109.5 \frac{(TS \text{ LPM} - TS \text{ RPM})}{TS \text{ RPM}} + 13.3 \frac{(ITS \text{ LPM} - ITS \text{ RPM})}{ITS \text{ RPM}} - 8.8$$

According to SFI interpretation values close to zero are considered normal and values close to -100 indicate complete lesion of the nerve or absent nervous regeneration¹¹.

The animals were sacrificed with lethal dose of sodic pentobarbital (75mg/kg) by intraperitoneal injection in the ninetieth day. Following the methodology used in the first procedure a new incision was performed with disruption of the structures up to identification of the study area.

A nervous segment with the extremity of the sciatic nerve was removed for histological analysis as well as a 2 cm long segment of the tibial nerve and the fibular nerve. The left and right cranial tibial muscles (CTM) were also removed and weighed. The left CTM was sent to histological analysis.

The microscopic slides were produced by means of fibular nerve trans-sections at 8 mm from its origin in group A, 5 mm from its distal stump in group B and at 5 mm from ETS in the others groups. A central fragment of the left CTM was removed and used for the histological analysis.

The samples were included in paraffin and cut with microtome – 5 µm thick. It was obtained a slide with one sectional cut from each anatomical piece, dyed with hematoxylin and eosine (HE).

The quantitative analysis of the recovery was performed by means of digitizing four fields of each slide (enlarged 400 times). Each field delimited by computer presented 97.98 square micrometers. The morphometric analysis was performed by means of Image ProPlus 4.5® program and the number and the diameter of the myelinated axons and muscle fibers were evaluated per field.

The obtained data was analyzed with the BioEstat 4.0 program. ANOVA and Kruskal Wallis (*a posteriori* Student

Newman Keuls) tests were used to compare groups A to F. The comparison between the walking track tests was performed by means of Friedman test. The significance level adopted was 5%.

Results

It was not identified any infection in the operatory wound neither animal deaths. The statistical analysis comparing the studied groups is presented on tables 1-5 in average and standard deviation (SD). Figures 4-7 present aspects of the analyzed histology.

TABLE 1 – Animal mass (g) analysis on the first and the last day of the study.

DAY	GROUPS						p (A X B X C X D X E X F)
	A	B	C	D	E	F	
	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	
01	301.8±07.8	299.2±08.2	299.2±06.2	300.8±06.9	298.6±08.9	298.5±09.1	0.886
93	549.9±12.3	533.9±10.1	547.2±13.9	543.7±16.9	545.4±14.1	544.2±11.8	0.089

Note: if $p \leq 0.05$ – statistically significant difference. ANOVA.

TABLE 2 – Sciatic Function Index (SFI) obtained on the first, second, third and fourth walking track tests (x-1).

Test	GROUPS						p ⁽¹⁾ (A X B X C X D X E X F)
	A	B	C	D	E	F	
	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	
SFI 1°	9.1±2.9	8.9±2.8	8.7±4.0	8.3±3.4	7.7±3.9	8.8±1.9	0.976
SFI 2°	8.3±2.6	63.6±13.2	64.8±13.3	65.3±14.1	67.1±15.1	71.0±11.6	<0.001 A ≠ all
SFI 3°	7.8±3.5	66.2±6.3	66.7±6.0	67.1±7.0	67.0±7.7	58.3±29.7	<0.001 A ≠ all
SFI 4°	8.7±2.8	74.3±8.5	32.7±27.1	31.5±28.5	37.9±38.9	25.3±34.7	<0.001 A ≠ all; B ≠ all
p ⁽²⁾ (1°X2°X3°X4°)	0.960	<0.001 1°≠(2°,3°,4°)	<0.001 1°≠(2°,3°)	<0.001 1°≠(2°,3°)	<0.001 1°≠(2°,3°)	<0.001 1°≠(2°,3°)	

Note: if $p \leq 0.05$ – statistically significant difference. (1) Kruskal Wallis and a posteriori Student Newman Keuls for comparison among groups (AxBxCxDxExF). (2) Friedman test for comparing between the evaluated tests (1°x2°x3°x4°) in each group.

TABLE 3 – Right and left CTM mass (g).

CTM Mass	GROUPS						p (A X B X C X D X E X F)
	A	B	C	D	E	F	
	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	
Right	1.56±0.13	1.57±0.14	1.58±0.12	1.54±0.16	1.53±0.10	1.55±0.14	(1) 0.938
Left	1.55±0.11	0.44±0.23	0.85±0.22	0.88±0.29	0.96±0.27	1.03±0.42	(2) < 0.001 A ≠ all; B ≠ all

Note: if $p \leq 0.05$ – statistically significant difference. (1) ANOVA. (2) Kruskal Wallis and a posteriori Student Newman Keuls.

TABLE 4 – Number and diameter of the axons, per field.

Axons	GROUPS						p (A X B X C X D X E X F)
	A	B	C	D	E	F	
	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	AVERAGE±SD	
Number	122.23±12.51	2.98±1.09	68.58±36.78	81.67±35.88	82.48±44.78	86.04±47.08	< 0.001 A ≠ all; B ≠ all
Diameter	10.01±0.46	3.44±1.82	9.33±1.91	9.13±2.46	8.10±2.42	9.23±1.80	< 0.001 B ≠ all

Note: if $p \leq 0.05$ – statistically significant difference. (1) Kruskal Wallis and a posteriori Student Newman Keuls.

TABLE 5 – Number and diameter of the muscle fibers of the left CTM, per field.

Muscle Fibers	GROUPS						p (A X B X C X D X E X F)
	A	B	C	D	E	F	
	AVERAGE±DP	AVERAGE±DP	AVERAGE±DP	AVERAGE±DP	AVERAGE±DP	AVERAGE±DP	
Number	19.79±2.03	18.48±3.66	17.73±3.09	19.08±3.86	19.19±2.40	20.02±3.13	0.452
Diameter	44.42±8.28	22.80±4.21	38.46±9.29	40.41±8.33	40.70±10.79	38.32±11.87	< 0.001 B ≠ all

Note: if $p \leq 0.05$ – statistically significant difference. (1) Kruskal Wallis and a posteriori Student Newman Keuls.

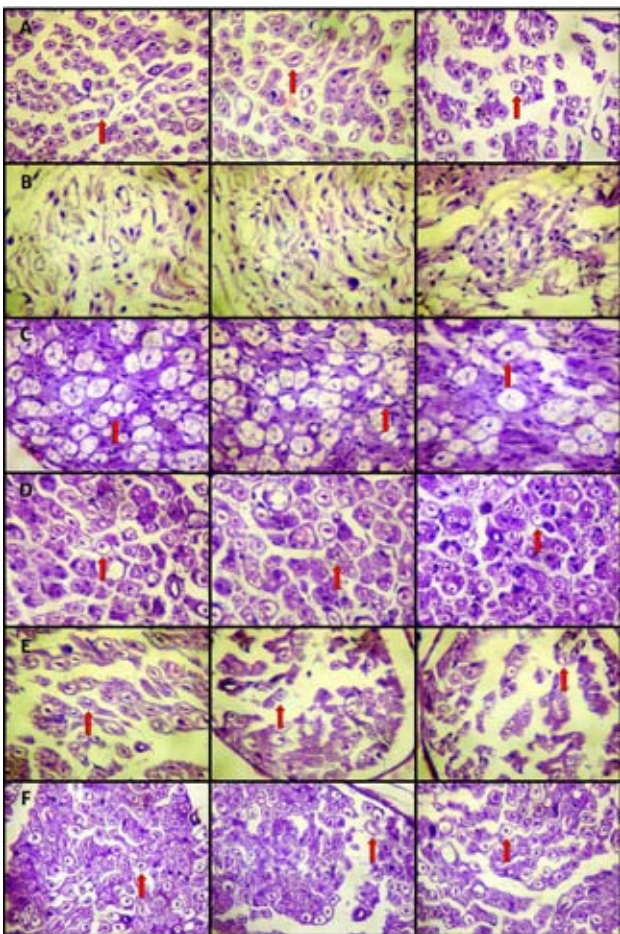


FIGURE 4 - Fibular nerve histology photomicrographs of groups under study. The red arrows represent examples of myelinated axons (HE; 400x).

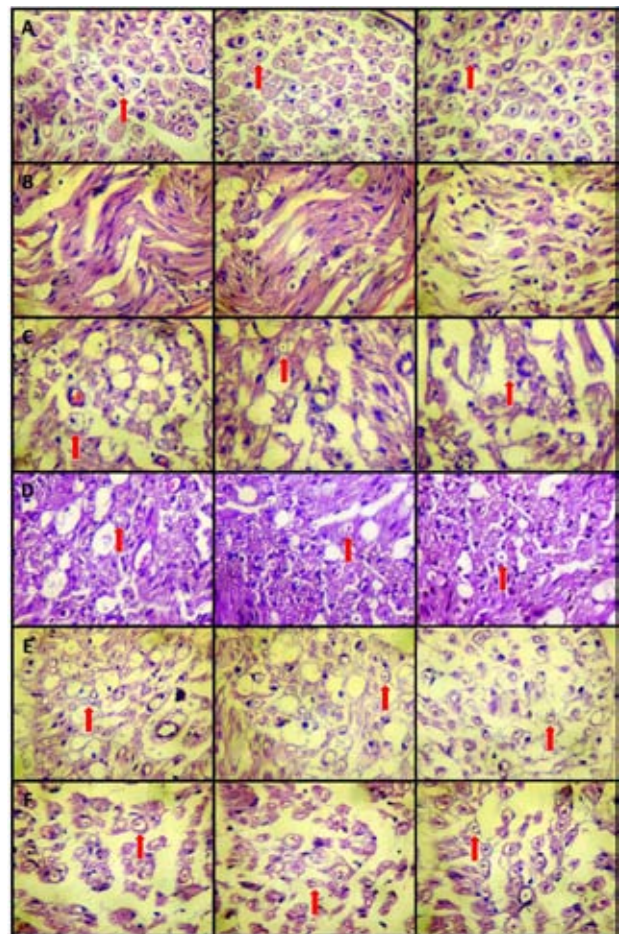


FIGURE 5 - Fibular nerve histology photomicrographs of other animals of groups under study. The red arrows represent examples of myelinated axons (HE; 400x).

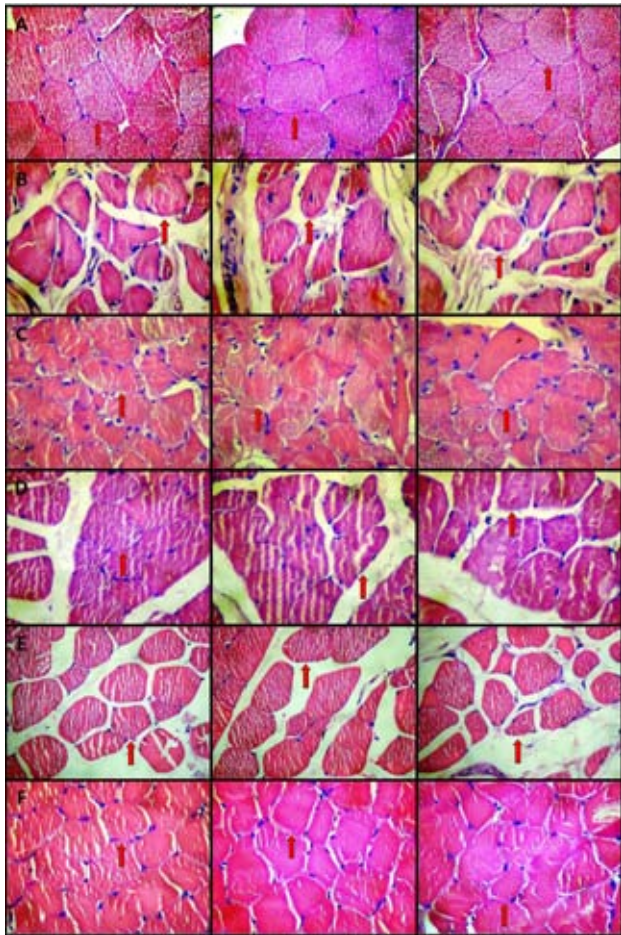


FIGURE 6 - Left CTM histology photomicrographs of groups under study. The red arrows represent examples of muscle fibers (HE; 400x).

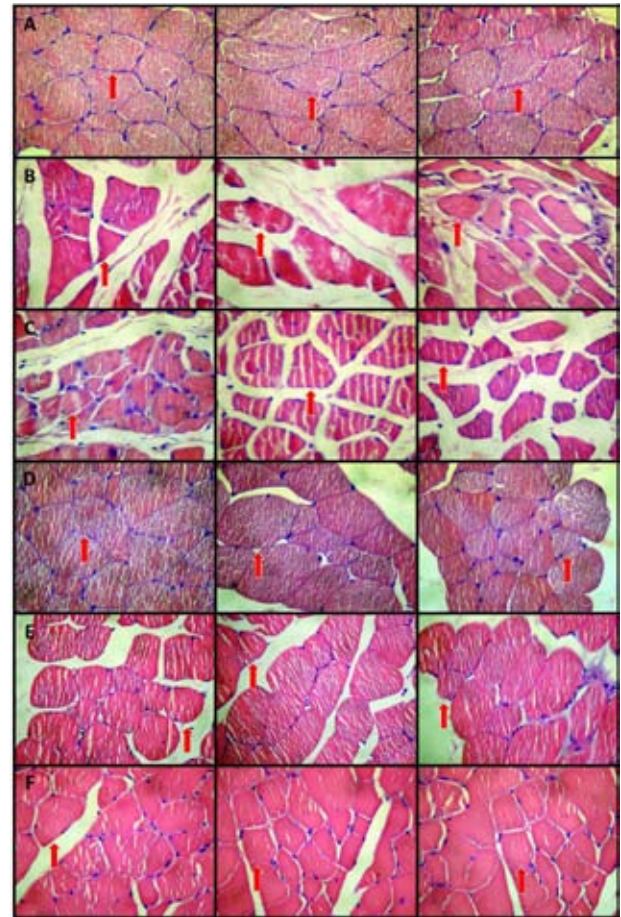


FIGURE 7 - Left CTM histology photomicrographs of other animals of groups under study. The red arrows represent examples of muscle fibers (HE; 400x).

Discussion

Seventeen years after their first description Viterbo *et al.*¹² referred that it is necessary to obtain better results with ETS in order to develop the technique. The authors also emphasized that such evolution will depend on a better comprehension of the role of neurotrophic factors in this process¹².

Following the goods reports related to the usage of NGF at ETE it was considered to evaluate its role at ETS. As in ETE the main difficulty in evaluating the NGF at the ETS was in developing the right way to present it at the repair point with the right dosage and for a longer time – preventing its fast *in vivo* degradation¹³.

Many questionable models for the local administration were then reported^{1,2}. According to Santos *et al.*¹³ systemic administration was not a good option because of the large amounts of substance needed and the impossibility of knowing the concentration of the agent at the nerve lesion site. In the same way implantable infusion pumps, among other options, despite presenting excellent results, were difficult to be applied at the day clinic.

The studies by Chunzheng *et al.*¹, Jubran and Widenfalk², and Zeng *et al.*³, performed in ETE, raised interest in the usage of fibrin glue as a carrier of NGF at ETS. The lack of previous studies related to the presented technique (ETS with fibrin glue containing NGF) made the comparison of our results difficult.

There was no difference among the mass of the animals, in all groups, in the first or in the last measurements, what presented the homogeneity of the sample and indicated that the surgery did not interfere in the animals eating habits¹⁴. In addition to that, such results allowed us to make comparisons among others varieties studied such as for example, the CTM mass, that is proportional to the animal mass⁴.

The analysis of the walking track test results showed that groups C to F presented on the 30th and 60th day walking patterns similar to group B – loss of function, which was already expected after the nerve lesion inflicted to the animals. However, on the 90th day, these groups presented walking pattern recovery, indicating reestablishment of the motor function after ETS, proving the repair success¹⁰. There was no difference between the groups regardless the repair (suture X fibrin glue) and the use of NGF.

so quickly stimulated when compared to ETE or ETS with sutures. Such specific characteristics of ETS performed with fibrin glue might require that in order to obtain the potentialization of axonal regeneration, the NGF had to be kept active for a longer time at the repair site.

Nevertheless, the role of fibrin glue as a carrier and stabilizer of NGF is recent in literature, stimulating the debate over the subject⁶. For example, the period during which the fibrin glue would keep NGF active in the repair still has to be defined. According to Bhang *et al.*⁵ the liberation of NGF is stable and kept for two weeks. However Zeng *et al.*³ described a liberation peak in 18 hours which is progressively reduced until its complete extinction in 14 days. Differently Currie *et al.*⁶ referred that the fibrin glue is no longer found in the repair site 10 days after its application, limiting the NGF action for a longer period of time.

Another aspect to be defined is the ideal liberation gradient. Its definition would depend on many aspects such as the cross-linking fibrin gel density, the concentration of fibrin in the glue, its intrinsic degradation rate and its heparin dissociation ratio⁵.

Such varieties could be modified according to the characteristics and needs of the case (as in ETS with fibrin glue), delaying or not the NGF liberation and altering the technique success rates⁵. Trying to prolong the NGF liberation, Yu *et al.*¹⁴ have recently involved it in polymerized microspheres which were fixed in the fibrin glue. Alternatives using, for example, particulate technology, stent technology and coated cells, have also been published with promising results^{1,5}.

This work created a new experimental model which has to be improved in order to define the real role of the NGF carried by the fibrin glue in ETS. The development of slower degradation glue or the association of another carrier method which kept the NGF active for a longer period of time could, for example, contribute for better procedure results. The disseminated clinical usage of NGF is still to be established and the possibilities brought to microsurgery justify new studies related to the technique¹⁵.

Conclusion

The fibrin glue containing nerve growth factor did not demonstrate superior results in end-to-side nerve repair.

The superiority of group A was already expected in the animal model utilized⁴.

The evaluation of the CTM mass took us to similar conclusions. The mass of the left CTM of group B was inferior to the other groups indicating expected nerve post-lesion muscle atrophy¹⁴. The animals in groups C to F had similar muscle mass values among them, superior to group B, showing that the performed repair – independent from the used technique – determined favorable repercussion in the muscle. Once again, the addition of NGF in fibrin glue did not improve the recovery of group F. As expected again, the animals in groups C to F presented muscle mass values inferior to the animals in group A, showing that the repair success was not enough for complete muscle mass recovery⁴.

The histological evaluation of the operated nerve showed differences between groups C to F when compared to group B, indicating that there was axonal regeneration after ETS. One more time, there was no difference in relation to the repair method (suture x fibrin glue) and to the use of NGF. Group A presented superior results in relation to groups C to F, showing that the axonal regeneration, occurred after ETS, was not enough to reach the normal levels⁴. According to Santos *et al.*¹³, with current techniques, it is expected that axonal regeneration never reach normal values even after a successful nerve repair.

The histological analysis of the left CTM indicated important reduction in fibers diameter of group B – confirming the expected muscle atrophy after the nerve fibular section⁴. However the analyses of the other groups show that there was no difference among them, indicating that the axonal regeneration obtained in the groups submitted to different ETS was enough to determine the recovery of the muscle fibers in a similar way the control group⁴. Once again, the addition of NGF to fibrin glue did not determine any difference in the result in relation to the other animals.

The results presented here confirm the effectiveness of ETS with sutures or only with fibrin glue. However, differently from others authors who obtained in ETE¹⁻³, the NGF positive influence was not verified in ETS.

According to Akeda *et al.*⁸, the process of axonal sprouting in an intact nerve, such as ETS with fibrin glue, is slower and in smaller scale than in a nerve with violated epineurium. In an ETE, for example, the axons are identified in the repair point in about two days; in an ETS, with sutures, such event is only detected from the fifteenth day on. As it does not include axonotmesis, the ETS with fibrin glue stimulates the growth factors liberation later, only after the natural donating nerve epineurial lysis⁸. In such way the chemotactic gradient is reduced and the axonal sprouting is not

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Received: November 23, 2011

Review: January 24, 2012

Accepted: February 20, 2012

Conflict of interest: none

Financial source: none

¹Research performed at Laboratory of Experimental Surgery Techniques, Federal University of Mato Grosso do Sul (UFMS), Brazil.