Lasers Med Sci (2016) 31:965–972
DOI 10.1007/s10103-016-1939-2

ORIGINAL ARTICLE

The new heterologous fibrin sealant in combination with low-level laser therapy (LLLT) in the repair of the buccal branch of the facial nerve

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Received: 5 November 2015 / Accepted: 11 April 2016 / Published online: 25 April 2016

Abstract This study aimed to evaluate the effects of low-level laser therapy (LLLT) in the repair of the buccal branch of the facial nerve with two surgical techniques: end-to-end epineural suture and coaptation with heterologous fibrin sealant. Forty-two male Wistar rats were randomly divided into five groups: control group (CG) in which the buccal branch of the facial nerve was collected without injury; (2) experimental group with suture (EGS) and experimental group with fibrin (EGF): The buccal branch of the facial nerve was transected on both sides of the face. End-to-end suture was performed on the right side and fibrin sealant on the left side; (3) Experimental group with suture and laser (EGSL) and experimental group with fibrin and laser (EGFL). All animals underwent the same surgical procedures in the EGS and EGF groups, in combination with the application of LLLT (wavelength of 830 nm, 30 mW optical power output of potency, and energy density of 6 J/cm^2). The animals of the five groups were euthanized at 5 weeks post-surgery and 10 weeks post-surgery. Axonal sprouting was observed in the distal stump of the facial nerve in all experimental groups. The observed morphology was similar to the fibers of the control group, with a predominance of myelinated fibers. In the final period of the experiment, the EGSL presented the closest results to the CG, in all variables measured, except in the axon area. Both surgical techniques analyzed were effective in the treatment of peripheral nerve injuries, where the use of fibrin sealant allowed the manipulation of the nerve stumps without trauma. LLLT exhibited satisfactory results on facial nerve regeneration, being therefore a useful technique to stimulate axonal regeneration process.

Keywords Facial nerve · Fibrin sealant · Low-level laser therapy · Nerve regeneration · Peripheral nerve injury

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Introduction

Facial palsy, with the loss of facial expression, is a social problem that leads to significant deterioration in quality of life [1, 2]. It can compromise any verbal communication conveyed by facial expressions which is essential to social relationships, for example, a spontaneous and dynamic smile that is essential in community interaction [3, 4].

Facial traumas are common and can result from various factors such as motor vehicle accidents, falls and accidental injuries, injuries from sharp objects, and firearms. They can also result from iatrogenic causes and postoperative chronic injuries that lead to fractures and lacerations of the face, resulting in facial nerve injuries that compromise the tone and dynamics of facial expression [4–6].

Lesions involving peripheral nerves are classified according to the degree of nerve impairment and structures involved

in neuropraxia, axonotmesis, and neurotmesis [7]. Peripheral nerves, when damaged, are capable of regenerating by themselves or with the aid of surgical techniques depending on the type of lesion. Thus, in the case of neurological injuries of the neurotmesis lesions, where the nerve underwent complete section of the axon and its surroundings, surgical repair becomes necessary [8–10].

End-to-end epineural neurorrhaphy is the technique of choice in lesions characterized by complete section of the nerve or when the continuous injury has a minor extent. In these types of injuries where there is tissue loss, the stumps can be approximated without exaggerated tension and then sutured, presenting a greater chance of recovery [11, 12].

However, despite the end-to-end epineural suture technique being a widely used method in neurological injuries of neurotmesis lesions of minor extent, it requires microsurgery techniques, as well as excessive manipulation of the tissues for the approximation of the stumps [13, 14]. The fibrin sealant is being used in the recovery of peripheral nerve injuries, with the purpose of minimizing the disadvantages of the epineural suture technique [15].

The new heterologous fibrin sealant, derived from snake venom, was developed with the primary objective of producing a sealant without the use of human blood components, hence preventing the transmission of infectious diseases [16]. It is a biological and biodegradable plasma-derived concentrate for topical use, whose mechanism of action is similar to the last stage of physiological coagulation. The clot formed by the sealant is a physiological compound found in tissue repair, and this makes it different from other types of sealants which present toxicity and high fibrin formation [17]. Fibrin sealant presents its own biological properties, being versatile in different clinical settings, including in combination with conventional suture techniques [16, 18].

Low-level laser therapy (LLLT) is an effective ally in the treatment of peripheral nerve regeneration. Its use started in the 1980s and is the subject of many recent studies, for it has shown an immediate protective effect, reducing the formation of scar tissue on the wound and significantly increasing the axonal outgrowth and myelination [19–21]. Moreover, it has the ability to reduce the migration of mononuclear cells, leading to a decrease in the edema area, analgesia, and anti-inflammatory action, promoting a more rapid regeneration [22].

Considering its frequency and the functional and social unease that facial nerve injury produces, the objective of this study was to evaluate the effects of LLLT in the repair of lesions of buccal branch of the facial nerve by two surgical techniques: end-to-end epineural suture and coaptation with heterologous fibrin sealant.

Materials and methods

Fibrin sealant

Heterologous fibrin sealant derived from snake venom was kindly supplied by the State University of São Paulo (UNESP), more specifically its Center for the Study of Venoms and Venomous Animals (CEVAP); its constituents and instructions for use are stated in its patents (registration numbers BR1020140114327 and BR1020140114360). At the time of use, the components were previously thawed, reconstituted, mixed, and applied according to the following protocol: The first bottle had fibrinogen obtained from buffalo blood (5 μl), the second contained calcium chloride (2 μl), and the last bottle had a thrombin-like fraction (1 μl), totaling 8 μl [16, 23–26].

Study design

The experimental procedures performed were approved by the Ethics Committee on Teaching and Research in animals of the Bauru School of Dentistry, University of São Paulo (São Paulo, Brazil), by way of Protocol 034/2011.

A total of 42 male Wistar rats (*Rattus norvegicus*), 60 days in age, weighing an average of 250 g were used. All animals were kept in appropriate boxes and received water and food "*ad libitum*, " with no movement restrictions, a 12-h regime of light and dark, obtained with the use of a "timer" in the animal maintenance room, as well as exposure to an approximate temperature of 22 °C.

The animals were randomly divided into a control group and four experimental groups, as follows:

1. Control group (CG): Consisting of 10 animals, where the buccal branch of the facial nerve was collected at 95 and 130 days of life (five animals in each period), which were the euthanasia periods of the experimental groups.
2. Experimental group with suture (EGS) and experimental group with fibrin (EGF): Consisting of 16 animals in which the buccal branch of the facial nerve was transected on both sides of the face, but on the right side, end-to-end epineural suture was performed, whereas on the left side, fibrin sealant was used for coaptation of the extremities. Eight animals, in each period, were euthanized at 95 days of life (5 weeks post-surgery) and at 130 days of life (10 weeks post-surgery).
3. Experimental group with suture and laser (EGSL) and experimental group with fibrin and laser (EGFL) associated with the low-level laser therapy: Consisting of 16 animals that underwent the same surgical procedures as EGS and EGF, in combination with low-level laser application. Eight animals, in each period, were euthanized at 95 days of life (5 weeks post-surgery) and at 130 days of life (10 weeks post-surgery).

Surgical procedures

For the surgical procedure, at 60 days of age, in experimental groups (EGS, EGF, EGSL, and EGFL), all animals were weighed and underwent general anesthesia through an intramuscular injection of the anesthetic Zoletil 50® (Virbac of Brazil), which contains a 1:1 ratio (125:125 mg) of tiletamine hydrochloride and zolazepam hydrochloride (0.15 ml/kg/IM). After the adoption of antisepsis techniques for the procedure, trichotomy was performed on all animals, and they were placed in lateral decubitus position.

An incision in the face with a scalpel blade no. 15 (approximately from the tragus of the ear toward the labial commissure) was performed with subsequent dessication up to the exposure of the buccal branch of the facial nerve, which was cut with straight tip scissors, without removing fragments displayed on the surgical microscope (DFV, Brazil).

In the EGS group, end-to-end epineural suture with nylon 10-0 monofilament (Ethicon®, Johnson & Johnson, Brazil) was performed on the right side of the face. In the EGF group, after the same procedures described, the nerve stumps were approximated and coaptated with fibrin sealant. To end the procedure, the skin was sutured using 4-0 silk thread (Ethicon®, Johnson & Johnson, Brazil).

In the EGSL and EGFL groups, the animals went through the same surgical procedures, but the application of continuous pulse laser was applied.

Laser irradiation

In the EGSL and EGFL groups, laser therapy with continuous pulse via gallium-aluminum-arsenide laser (GaAlAs, Laserpulse IBRAMED®, Brazil) was applied on the animals. The protocol was applied using a laser pen at three different points in the surgical area, on the skin surface, with a wavelength of 830 nm, with 30 mW optical power output of potency, energy density of 6 J/cm^2, beam area of 0.116 cm^2, power density of 258.6 mW/cm^2, and exposure time of 24 s (per point). The laser source had been previously tested to certify the dose. The laser therapy applications were performed 1 day after surgery, and in the postoperative period, three times a week for a total of 5 weeks [27–29].

Processing for optical microscopy and transmission electron microscopy

After 5 and 10 weeks of the surgical procedure [30, 31], in each period, five animals in the control group and eight animals from each experimental group were euthanized with an "*overdose*" of anesthesia to collect the intact buccal branch of the facial nerve from the control group and the distal stump from the buccal branch of the facial nerve in animals from the experimental groups.

The samples were set in Karnovsky's fixative for 24 h. For the optical microscopy (OM) and transmission electron microscopy (TEM) processing, routine laboratory procedures including HistoResin (Leica Mycrosistems®, Germany) mounting media were used. After inclusion, ultrathin cross sections (5 μm) of the fascicles were obtained and stained with 1 % toluidine blue, combined with 1 % sodium borate.

Morphometric and statistical analysis

The following measurements were undertaken: area and diameter of the fibers, area and diameter of the axon, and area and thickness of the myelin sheath.

The measurements were obtained through images captured using an Olympus® microscope (Japan), and photographs were taken with a coupled digital camera (Olympus® DP 71, Japan). Quantitative analysis was performed using the Image Pro-Plus 6.0 program (Media Cybernetics®, USA). Statistical analysis was performed using variance analysis (ANOVA) with Tukey's post-test. The level of significance for the comparisons was predetermined at $p < 0.05$.

Results

Morphological evaluation (optical microscopy)

In animals from the CG at 95 days of age, myelinated fibers with regular placement and diameters were observed. In the experimental groups, the nerve fibers presented irregular placement, with myelinated fibers of heterogeneous diameters. In all groups, unmyelinated fibers were also observed (Fig. 1).

In animals at 130 days of age from the CG, myelinic fibers with regular placement and diameters were observed. In the experimental groups, myelin fibers were heterogeneous and had an irregular placement (Fig. 2).

Morphological evaluation (electron microscopy)

The TEM confirmed, in both periods analyzed, the presence of regenerated axons, mostly myelinic, consisting of regular features in the CG and irregular in experimental groups (Figs. 3 and 4). The presence of collagen fibers and Schwann cell nuclei were observed (Fig. 4).

Morphometric and statistical evaluation

In animals at 95 days of age, there was a significant difference between the CG and all experimental groups, as well as between EGF and EGSL, with relation to the area and diameter of the fibers. In the variable area of the axon, there was a significant difference between all groups. There was a

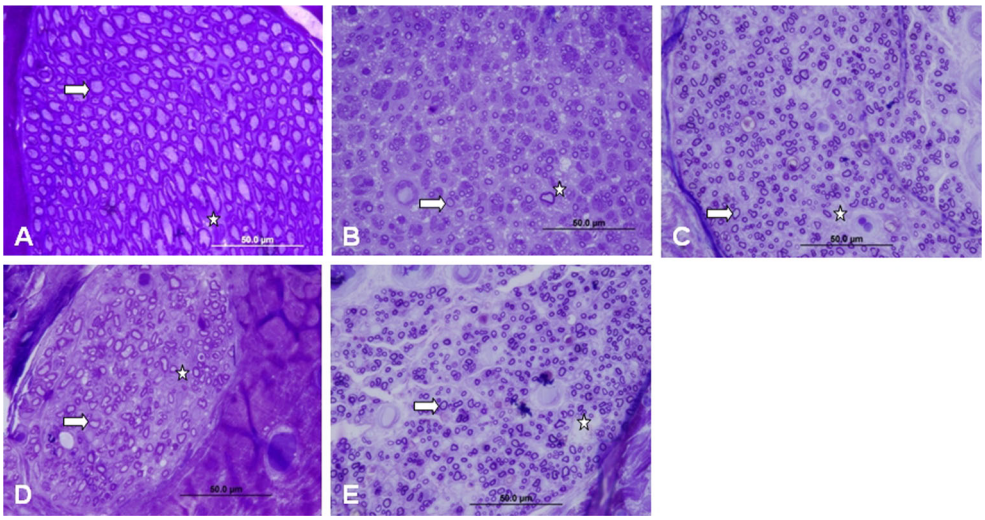

Fig. 1 Animals with 95 days. Microscopic aspect of the buccal branch of the facial nerve in groups: **a** CG, **b** EGS, **c** EGF, **d** EGSL, and **e** EGFL. Presence of regenerated myelinic (*white arrow*) and unmyelinic (*white star*) nerve fibers

significant difference in the diameter of the axon between the CG and all experimental groups and between the EGF and all other groups. A significant difference was observed between the CG and all experimental groups with respect to the area of the myelin sheath. A significant difference in the myelin sheath thickness was observed when comparing the CG and EGF groups, as well as the CG and EGFL groups (Table 1).

In animals at 130 days of age, there was a significant difference between the CG and EGS and between CG and EGF and also between CG and EGFL with regard to the fiber area. There were significant differences between the CG and EGF as well as between CG and EGFL and also between EGF and EGSL with respect to the diameter of the fiber. In the variable area of the axon, there was no significant difference between the CG and the experimental groups. A significant difference in the diameter of the axon was observed between the CG and the EGS, EGF and EGFL groups (Table 2).

Discussion

The aim of the present study was to compare the repair of the buccal branch of the facial nerve by means of two techniques: end-to-end suture, considered a gold standard for the recovery of peripheral nerve injuries, and the coaptation technique of joining the injured stumps through the use of new heterologous fibrin sealant. In addition, it sought to verify the influence of low-level laser in the repair process.

End-to-end suture is described as an effective technique (gold standard) for axonal regeneration in cases of the neurotmesis lesions which do not exhibit tissue loss and where the stumps can be approximated without exaggerated pressure [11, 12]. In the two repair techniques which were used in this experiment, surgery was performed visually amplification in a surgical microscope to improve coaptation of the fragments. Failure to correct adaptation of the nerve stumps leads to the

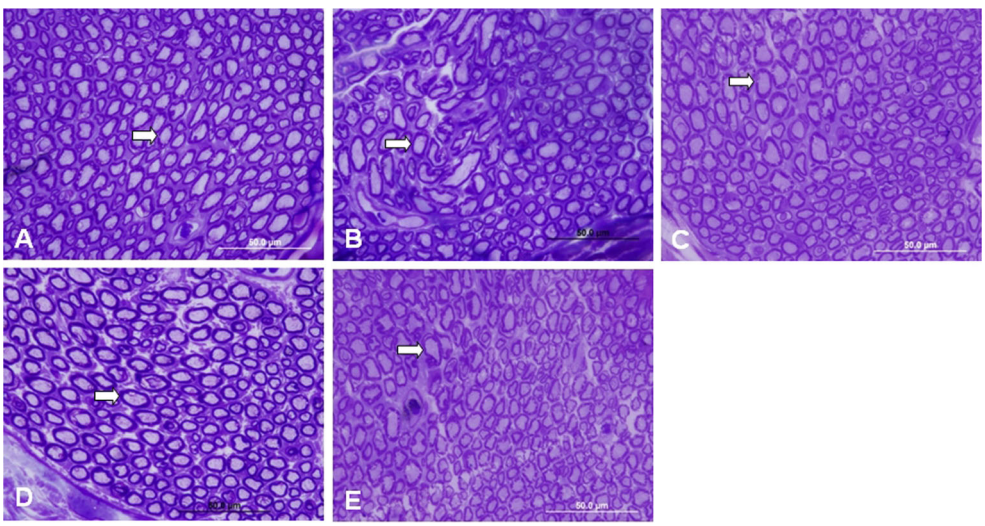

Fig. 2 Animals with 130 days. Microscopic aspect of the buccal branch of the facial nerve in groups: **a** CG, **b** EGS, **c** EGF, **d** EGSL, and **e** EGFL. Presence of regenerated myelinic nerve fibers (*white arrow*)

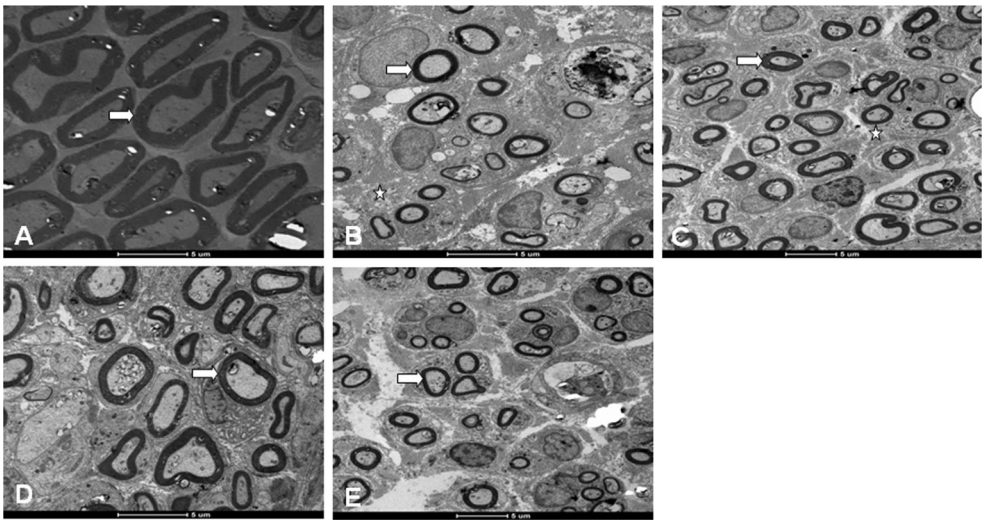

Fig. 3 Animals with 95 days. Electron microscopy of the buccal branch of the facial nerve in groups: **a** CG, **b** EGS, **c** EGF, **d** EGSL, and **e** EGFL. Presence of regenerated myelinic (*white arrow*) and unmyelinic (*white star*) nerve fibers

formation of granulation tissue blocking axonal regeneration. Microsurgical magnification and more precise knowledge of anatomy have improved the results, decreasing unwanted fascicular disorientation [32].

In this study, the buccal branch of the facial nerve was sectioned without removing fragments, allowing the joining of the stumps and the performance of end-to-end suture with satisfactory results, given that this technique is mostly used in cases of injuries with minor extension that do not present tissue loss. In search of an alternative technique, or one that can be combined with suture, fibrin sealants have been shown to be effective in repair, with easier implementation, and also resulting in less time for surgery [33, 34].

Comparing the use of epineural suture (EGS) with fibrin sealant (EGF), at 130 days of the experiment, there was no significant difference between the variables measured, indicating that fibrin sealant is an effective method in the repair process of peripheral nerves and that during the experimental surgical procedures, it presented flexibility, versatility, and good adhesive property [16, 26, 29, 35].

In the morphological analysis of the experimental groups in general, myelin fibers occurred with predominance, with irregular placement and diameters in all groups, features that are characteristic of the nerve regeneration process. In the period of 10 weeks, the myelin fibers showed better organization of the fascicle in relation to the prior 5-week period, in which an evolutionary stage in the experimental groups was observed [36, 37]. There was no evidence of inflammatory response to the sealant or foreign body reaction [38, 39].

In the morphometric analysis, within 10 weeks after surgery, taking the CG as reference (nerve control), the EGSL presented the closest results (no significant difference) in all variables measured, except in axon area. The speed of propagated nerve impulses is directly related to the nerve fiber diameter and myelin sheath. The fastest nerve fibers have larger diameters and are myelinated [40]. The fact that the CG and

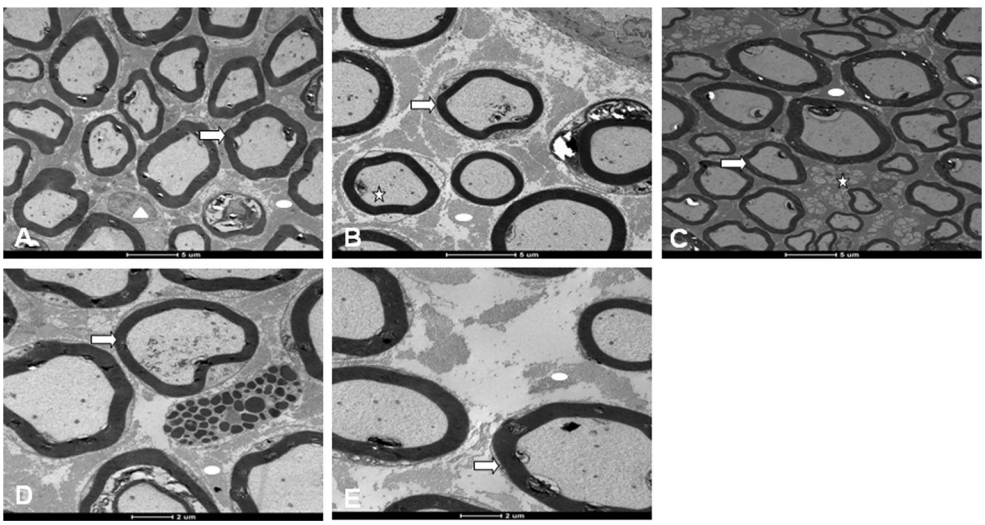

Fig. 4 Animals with 130 days. Electron microscopy of the buccal branch of the facial nerve in groups: **a** CG, **b** EGS, **c** EGF, **d** EGSL, and **e** EGFL. Presence of regenerated myelinic (*white arrow*) and unmyelinic (*white star*) nerve fibers, Schwann cell (*white triangle*), and collagen fibers (*white ellipse*)

Table 1 Animals with 95 days of age

	Fiber area (μm^2)	Axon area (μm^2)	Fiber diameter (μm)	Axon diameter (μm)	Myelin sheath area (μm^2)	Myelin sheath thinkness (μm)
CG	21.83 ± 1.90a	7.36 ± 0.48a	5.50 ± 0.24a	3.29 ± 0.15a	14.47 ± 1.84a	2.20 ± 0.39a
EGS	12.91 ± 1.00b	5.06 ± 0.31b	4.25 ± 0.23b	2.57 ± 0.16b	7.85 ± 0.81b	1.68 ± 0.34ac
EGF	11.94 ± 0.83bc	3.89 ± 0.24c	3.94 ± 0.18bc	2.32 ± 0.14c	8.05 ± 0.83b	1.61 ± 0.16bc
EGSL	13.98 ± 0.80bd	5.60 ± 0.36d	4.43 ± 0.27bd	2.70 ± 0.11b	8.38 ± 0.80b	1.73 ± 0.37ac
EGFL	13.22 ± 0.66b	4.43 ± 0.30e	4.14 ± 0.22b	2.61 ± 0.11b	8.79 ± 0.78b	1.53 ± 0.32bc

The morphometry of the nerve fibers in terms of the different measurements (mean and standard deviation) was performed comparing the control group (CG), experimental group with suture (EGS), experimental group with fibrin (EGF), experimental group with suture and laser (EGSL), and experimental group with fibrin and laser (EGFL). Different lowercase letters indicate significant differences among the groups by means of the analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$)

the experimental groups had statistically similar results in the cited measures showed that the two repair techniques analyzed are efficient, with satisfactory results in relation to the degree of maturation of fibers [13].

In the transmission electron microscopy, within a 10-week period, the myelinated fibers showed better organization in the nerve fascicle in relation to the 5-week period. The microscope also showed the presence of oblique and longitudinal collagen fibers, indicating a random orientation of the fibers in the regenerating tissue. Collagen fibers are important in the regeneration process because they provide biological support for cell growth, increasing differentiation of various cell types [41, 42].

Regarding the use of LLLT, the most effective laser application with regard to wavelength, energy density, exposure time, and pulsed or continuous wave is still controversial, but its use is suggested to facilitate the nerve regeneration [27, 43]. Based in this study, on the statistical data, similar results were observed in most measured variables, when comparing EGS to EGSL and EGF to EGFL in both periods, but with mean values almost always favorable with laser therapy.

This finding suggests that despite the satisfactory results obtained in clinical and experimental research, there is a need to carry out further studies to obtain the confirmation of the effects of laser therapy on the repair of peripheral nerves, as well as the standardization of the applied protocols and evaluation parameters [19, 20, 22, 29, 44].

Conclusion

Both techniques analyzed were effective in the treatment of peripheral nerve injuries, because they allow for a sprouting of the myelinated nerve fibers in the distal stump, where the sealant allowed the coaptation of the stumps without trauma to the nerve fibers. Low-level laser therapy exhibited satisfactory results on facial nerve regeneration, being therefore a useful technique to stimulate axonal regeneration process.

Table 2 Animals with 130 days of age

	Fiber area (μm^2)	Axon area (μm^2)	Fiber diameter (μm)	Axon diameter (μm)	Myelin sheath area (μm^2)	Myelin sheath thinkness (μm)
CG	50.19 ± 2.66a	18.92 ± 1.12a	11.34 ± 1.31a	6.84 ± 0.42a	31.27 ± 3.55a	4.50 ± 1.09a
EGS	45.43 ± 3.17bc	14.93 ± 0.91b	10.02 ± 0.80ac	6.06 ± 0.45bc	30.50 ± 2.94a	3.96 ± 0.95a
EGF	42.92 ± 1.99bc	14.36 ± 1.25b	8.91 ± 0.52bc	5.58 ± 0.44bc	28.56 ± 3.03a	3.33 ± 0.35a
EGSL	46.21 ± 2.17ac	15.05 ± 0.91b	10.23 ± 0.70ad	6.12 ± 0.33ac	31.15 ± 2.25a	4.11 ± 0.66a
EGFL	44.01 ± 2.38bc	14.71 ± 1.62b	9.48 ± 0.85bc	5.84 ± 0.51bc	29.29 ± 3.72a	3.63 ± 0.79a

The morphometry of the nerve fibers in terms of the different measurements (mean and standard deviation) was performed comparing the control group (CG), experimental group with suture (EGS), experimental group with fibrin (EGF), experimental group with suture and laser (EGSL), and experimental group with fibrin and laser (EGFL). Different lowercase letters indicate significant differences among the groups by means of the analysis of variance (ANOVA), followed by Tukey's test ($p < 0.05$)

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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