Muscle reinnervation in one or two stages? Experimental study in rats with end-to-side nerve graft[1]

Reinervação muscular em um ou dois estágios? Estudo experimental em ratos com enxerto de nervo término-lateral

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ABSTRACT
PURPOSE: To compare muscle reinnervation in one and two surgical stages using end-to-side neurorrhaphy (ESN) without donor nerve injury.
METHODS: The experiment was performed on four groups of 20 rats. Group 1 (G1), one stage, received the graft which was sutured to the tibial nerve, with ESN, and its free stump was sutured end-to-end to the distal stump of the sectioned peroneal nerve (PN), all in the same operation. In Group 2 (G2), two stages, the nerve graft was sutured to the tibial nerve, with ESN. Two months later the PN was sectioned and its distal stump connected to the distal stump of the graft as in G1. Normal control group (Gn) received the graft only sutured to the tibial nerve, with ESN. Denervated control group (Gd), as well received the graft and had the PN sectioned and its two stumps buried in adjacent musculature, with the aim of denervating the cranial tibial muscle (CTM), the target of this study. The parameters used to evaluate CTM reinnervation were muscle mass, muscle fiber's minimum diameter and area.
RESULTS: The mean CTM mass, the average of the muscular fibers areas and the average of the muscular fiber minimum diameters was higher (all $p<0.0001$) in G2 than in G1. Comparing the four groups, these parameters had their maximum expression in Gn and the minimum in Gd, as expected.
CONCLUSION: The two stages showed better muscle reinnervation than one stage.
Key words: Facial Nerve. Tibial Nerve. Muscle Denervation. Nerve Regeneration. Rats.

RESUMO
OBJETIVO: Comparar a reinervação muscular com enxerto de nervo em um e dois tempos operatórios, utilizando a neurorrafia término-lateral (NTL) sem lesão do nervo doador.
MÉTODOS: Vinte ratos foram distribuídos em quatro grupos. O grupo 1 (G1), um estágio, recebeu o enxerto que foi suturado ao nervo tibial (NT), por meio de NTL, e seu coto livre foi suturado por NTL ao coto distal do nervo peroneal (NP), seccionado a um centímetro do NT, na mesma cirurgia. O grupo 2 (G2), dois estágios, recebeu o enxerto de nervo na primeira cirurgia, como já descrito. Dois meses depois, na segunda cirurgia, o NP foi seccionado e seu coto distal ligado ao coto distal do enxerto como em G1. O grupo controle de normalidade (Gn) recebeu o enxerto da mesma forma, apenas. E o grupo controle de denervação (Gd), além de receber o enxerto, teve o NP seccionado e seus cotos sepultados na musculatura adjacente, com a finalidade de denervar o músculo tibial cranial (MTC), alvo deste estudo. Os parâmetros utilizados para avaliar a reinervação do MTC foram massa muscular, diâmetro mínimo da fibra muscular e área.
RESULTADOS: O grupo G2 apresentou superioridade ($p<0,0001$) em relação ao G1 na massa do MTC, no diâmetro mínimo e na área das fibras musculares. Na comparação entre os quatro grupos, estes mesmos parâmetros tiveram sua expressão máxima em Gn e mínima em Gd, como era esperado.
CONCLUSÃO: A reinervação muscular em dois estágios apresenta melhor resultado quando comparada à técnica em um tempo.
Descritores: Nervo Facial. Nervo Tibial. Denervação Muscular. Regeneração Nervosa. Ratos.

Introduction

Long standing paralysis, such as facial palsies and paralysis caused by brachial plexus avulsion, can be treated by nerve graft associated with muscle transplant. In facial paralysis, this method is known as cross-face nerve graft, in which a nerve graft is used as a bridge between the healthy side and paralyzed side of the face, associated to muscle transplant. One end of the graft is sutured into a sectioned branch of the facial nerve on the healthy side. The other end is sutured to the nerve of the transplanted muscle, generally the gracilis muscle[1-4], the pectoralis minor muscle[5,6], or the latissimus dorsi muscle[7,8], among others[9-13].

This surgical procedure can be carried out in one or in two stages[2,10,14]. When performed in two stages, the first one is the nerve graft. There is a wait for the growth of axons at the distal end of the graft, and then, in a second procedure, the muscle transplant is positioned and reinnervated by the nerve graft previously placed. In a single-stage procedure, the nerve graft and muscle transplant are done in the same surgery. Both methods present advantages and disadvantages. The single-stage method reduces time of hospital stay, provides a shorter recovery time, and presents fewer complications[2]. However, its disadvantage is that muscle transplant remains denervated for a longer time waiting for axon growth through the nerve graft, which can result in atrophy of muscular fibers, jeopardizing the final result[15]. In the two-stage procedure, the muscle remains denervated for less time, because when it is transplanted, the axons had already grown to the distal end of the graft[2,3,5,6,16].

Only two studies[2,15] compared both methods. One clinical study[2] analyzed facial symmetry at rest and when smiling, as well as less need for secondary surgical procedures; found 90 percent for the single-stage and 93 percent for the two-stages. A study[15] in rabbits, comparing muscular reinnervation through nerve grafts in one and two-stage procedures found better results for the two-stage technique.

End-to-end neurorrhaphy is commonly used in the cross-face technique to connect the graft to the donor nerve, damaging some branches of the facial nerve on the healthy side[1-3,5,6,16]. The end-to-side neurorrhaphy without donor nerve lesion, introduced in 1992 by Viterbo *et al*.[17], has been increasingly used[18-32] and has the advantage of not injuring the donor nerve.

The purpose of this experimental study was to compare aspects of muscle reinnervation in one and two surgical stages using end-to-side neurorrhaphy without donor nerve injury.

Methods

The experimental protocol was approved by the Ethic Committee of the Medical School of Sao Paulo State University (UNESP). The rats were kept according to the guidelines of the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and according to the ethical principles of the Brazilian College on Animal Experimentation (COBEA).

Animal housing and anesthesia

Eighty male albino Wistar rats, with average initial body weight of 182.6±14.9g were used. The animals were kept in light-dark cycles (12/12h) with free access to food and water. All surgical procedures and samplings were performed under general anesthesia by intraperitoneal administration of 30mg/kg hydrochloride sodium pentobarbital (Nembutal®) and by using a DFV microscope (MC-M3101) with 16 magnifications.

Experimental groups and surgical procedure

All animals underwent right hind limb surgery by the same surgeon. Initially the sciatic nerve and its branches, the tibial nerve (TN), the caudal cutaneous sural nerve (CCSN), and the peroneal nerve (PN) were dissected in an extension of about 2.5 centimeters. A one-centimeter segment of the CCSN was then removed and sutured to the lateral face of the TN by end-to-side neurorrhaphy (ESN) with 3 simple 10-0 monofilament nylon stitches, without removing the TN epineurium.

Following this phase, four groups of 20 rats each were formed randomly. The normal control group (Gn) had only the graft sutured to the side of the donor nerve by ESN (Figure 1).

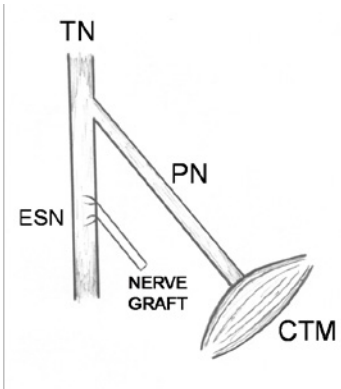

FIGURE 1 – Normal control group (Gn) and first surgery of G2 (two-stage). **TN**: tibial nerve. **PN**: peroneal nerve. **ESN**: end-to-side neurorrhaphy. **CTM**: cranial tibial muscle.

In the denervated control group (Gd), the distal stump of the nerve graft was inverted about 120° and buried in the abductor muscle with a 7-0 polypropylene stitch. Then the PN, which innervates the cranial tibial muscle (CTM), was sectioned 1 centimeter bellow the trifurcation of the sciatic nerve, with its proximal and distal stumps also inverted, but in opposite directions, and also buried in the abductor muscle; this procedure was carried out in order to avoid spontaneous CTM reinnervation (Figure 2).

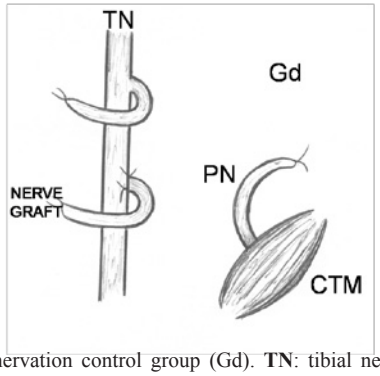

FIGURE 2 – Denervation control group (Gd). **TN**: tibial nerve. **PN**: peroneal nerve. **CTM**: cranial tibial muscle.

Group 1 (G1) underwent the single-stage procedure. After ESN of the TN graft, the PN was sectioned 1 centimeter bellow of the trifurcation of the sciatic nerve, as previously described, and its proximal stump was inverted 120° and buried in the abductor muscle. The distal stump of the graft was sutured to the distal stump of the PN by end-to-end neurorrhaphy (EEN) with three simple 10-0 nylon stitches (Figure 3).

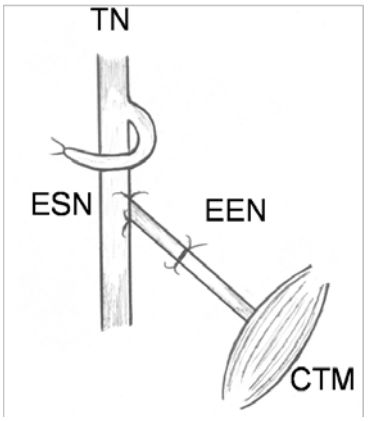

FIGURE 3 – Group 1 (one-stage) and second surgery of group 2 (two-stage). **TN**: tibial nerve. **ESN**: end-to-side neurorrhaphy. **EEN**: end-to-end neurorrhaphy. **CTM**: cranial tibial muscle.

Finally, Group 2 (G2) underwent the two-stage surgical procedure, with the first stage carried out as it was described for Gn (Figure 1). The interval between the first and the second surgeries was two months. In the second surgical stage, 1 millimeter of the free end of the graft was ressected to remove a possible neuroma which could harm the axon growth. This was followed by the sectioning of the PN at 1 centimeter of the trifurcation of the sciatic nerve, continuing the procedure in an identical way as described for G1 (Figure 3).

Collection and analysis of specimen

Six months after the first surgery, all animals were anesthetized for removal of CTM and of nerve segments for the study. The rats were killed by a lethal intraperitoneal anesthetic dose immediately after removal of the tissues.

Removed nerve segments were denominated N1, N2, N3, and N4 (Figure 4). N1 was the extremity of PN proximal stump and it was used to verify whether there was neuroma or occasional reinnervation of the CTM. N2 segment was the joint between the graft and the TN, i.e. the ESN site, while N3 segment contained the distal segment of the PN, after EEN, 10 mm from where it joined the CTM. N4 segment was the distal end of the graft buried in the aductor muscle, present only in Gd.

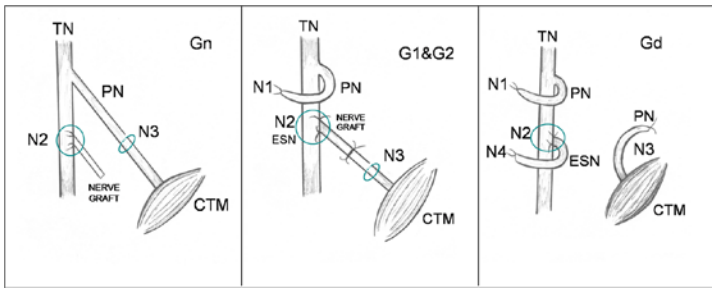

FIGURE 4 – (Left) Gn (normal control group) and G2 (two-stage) first procedure. **(Center)** G1 (one-stage) and G2 after second surgery. **(Right)** Gd (denervated control group). **TN**: tibial nerve. **PN**: peroneal nerve. **CMT**: cranial tibial muscle. N1: proximal stump end of the PN. N2: end-to-side neurorrhaphy between end graft and TN. N3: distal segment of the PN. N4: graft's distal stump buried in the adductor muscle.

N1 and N4 segments were fixed with glutaraldehyde; post-fixed in 1% osmium tetroxide, and set in paraffin. Longitudinal sections were cut at 3 micra thickness, and two slides were made from each segment; one of them stained only with osmium, and the other also stained with toluidine blue. N2 segment was fixed in 10% buffered formalin and set in paraffin. Sections were also longitudinal, with 3 micra thick and two slides from each segment were stained with Masson trichrome, modified by Van de Grieft, and with Bielschowsky silver staining. N3 segment was fixed in glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded in paraffin. Transverse sections were cut at 3 micra, and the two resulting slides were stained as the ones from segments N1 and N4.

Finally, the muscle was frozen in liquid nitrogen for carrying out histological sections which were made in a LEICA cryostat (CM 1800) with five micra thickness, transversal to

the main axis of the muscular fibers in the central part of the muscle, comprising all its thickness. The slides were stained with hematoxylin and eosin. All analyses of all slides were performed by an experienced pathologist who was blinded to the study.

Morphometric analysis

Cranial tibial muscle morphometry included the measurement of the muscular fiber areas in square millimeters and the measurement of their minimum diameters, in millimeters. Fifty muscular fibers were randomly analyzed per animal by using the "SigmaScan Pro Image Analysis", Version 3.00.030, from Jandel Scientific Corporation. The identification of slides was hidden at the moment of image capturing and measuring of attributes. Counts of nerve fibers from N3 segment were performed with the same equipment. A randomized image was taken from each slide; with an area of 0.0192 mm², in which all nerve fibers were counted. For these analyses it was used a 40X COLEMAN microscope (18ST).

Statistical analysis

Comparison between groups regarding initial and final animal mass, CTM mass and number of PN nerve fibers was carried out by variance analysis of one factor complemented by the Bonferroni "*t*" test for multiple comparisons. The comparison of area and minimum muscle fiber diameter was made by Kruskal-Wallis analysis of variance. All analyses were performed using the software program Statistical Package for Social Science (SPSS version 11.0 for Windows, Chicago, IL, USA). Values were considered significant for a confidence interval of 95% ($p<0.05$).

Results

Due to some losses, at the end of the experiment groups G1, G2, Gn, and Gd contained 16, 17, 19, and 16 rats, respectively.

Mass analysis

The initial and final animal body masses did not show statistical differences among the groups. Mean CTM mass, in grams, was higher ($p<0.001$) in G2 (0.603±0.033) than in G1 (0.480±0.066). In Gn (0.918±0.112), this parameter was the highest ($p<0.001$) of all groups, and Gd (0.256±0.013) was the lowest ($p<0.001$) (Table 1).

TABLE 1 – Comparative analysis between all groups and between groups individually.

Parameters	Groups				p-value
	Gn	G1	G2	Gd	
Initial rats body mass (g)	186.3± 22.4	179.4± 12.6	182.4± 16.0	182.3± 8.6	*
Final rats body mass (g)	491.8± 56.1	516.9± 47.4	517.9± 74.5	515.7± 71.5	**
CTM mass (g)	0.918± 0.112	0.480± 0.066	0.603± 0.033	0.256± 0.013	#
CTM fibers area (µm²)	4.634± 0.758	2.102± 0.674	2.613± 0.581	0.433± 0.486	#
CTM fibers minimum diameter (µm)	66.012± 6.347	38.978± 9.114	44.423± 0.768	15.295± 8.610	#
Nerve fibers count (N3)	182.0± 19.4	77.8± 37.7	77.1± 7.4	7.2± 6.3	##

CTM=Cranial tibial muscle; N3=Removed nerve segment; Gn=Normal control group; G1=Group 1; G2=Group 2; Gd=Denervated control group; * P=0.648; ** P=0.545; # P<0.001 the comparison between all groups and the comparison between individual groups (Gn>G2 >G1>Gd); ## P<0.0001 the comparison between all groups and the comparison between individual groups (Gn>G1, G2 and Gd, G1>Gd and G2>Gd), except between G1 and G2 (p=0.4724).

Morphometric analysis

The average of the muscular fibers areas, in µm², and the average of the muscular fiber minimum diameters, in µm, was higher ($p<0.0001$) in G2 (2.613±0.581 and 44.423±0.768, respectively) than in G1 (2.102±0.674 and 38.978±9.114, respectively). Comparing the four groups, these two parameters had their maximum expression in Gn (4.634±0.758 and 66.012±6.347, respectively) and the minimum in Gd (0.433±0.486 and 15.295±8.610, respectively) (Table 1).

The average count of nerve fibers in N3 segment was significantly higher in Gn in relation to the other groups. Gd had the lowest average compared to the other groups ($p<0.0001$). There was no significant difference ($p=0.4724$) in this parameter between the G1 and G2 (Table 1).

Histological features

Cranial tibial muscle histology showed that fibers in Gn had large polygonal fibers, with peripheral nuclei and little connective tissue, characteristics of normal muscular tissue (Figure 5 left, above). There was well defined muscular atrophy in Gd, with a predominance of fiber groups with considerably reduced diameter, with few cells presenting normal diameter and an abundance of connective tissue, compatible with denervated muscle (Figure 5 right, above). G1 (Figure 5 left, below) and G2

(Figure 5 right, below) showed few polygonal cells in the midst of muscle fiber groups with considerably reduced areas, contrasting with others with considerably increased areas and rounded shapes. Some muscle fibers presented a centralized nucleus. In some regions there was an increase of connective tissue. This pattern was not constant in all animals and there was variation in the histological aspect within these two groups.

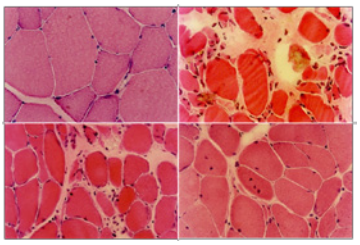

FIGURE 5 – Photomicrographs of the cranial tibial muscle (hematoxylin-eosin, original magnification 540x). **(Left, Above)** Cranial tibial muscle histology in Gn (normal control group) showed large and polygonal muscle fibers, with peripheral nuclei and little conjuctive tissue, characteristic of normal muscle tissue. **(Right, Above)** Gd (denervation control group). There was well defined muscular atrophy, with a predominance of considerably reduced diameter fiber groups, with few cells presenting normal diameter and an abundance of conjunctive tissue, compatible with denervated muscle. **(Left, Below)** G1 (one-stage) and **(Right, Below)** G2 (two-stage) showed few polygonal cells in the midst of muscle fiber groups with considerably reduced area, which contrasted with others with considerably increased area and rounded shape. Some muscle fibers presented a centralized nucleus. Some regions had increased conjunctive tissue.

Nerve histology of N1 revealed characteristics of end neuroma, with fibers growing in different directions, mixed with muscle tissue, on all slides. In N2 segment, site of the ESN between the graft and TN, on the slides stained by the Bielschowsky method it was observed numerous nerve fibers within the graft (85% of 68 slides), which demonstrates the lateral sprouting of nerve fibers (Figure 6 above). N2 stained with trichromic Masson showed epineural discontinuity at the ESN site (88% of 68 slides) (Figure 6 below).

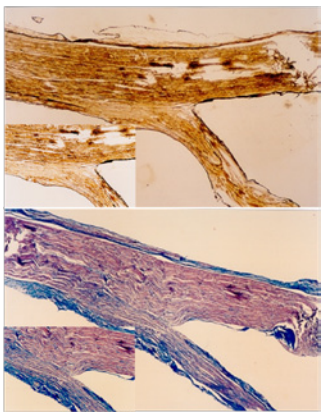

FIGURE 6 – Photomicrographs of the N2 nervous segment (original magnification 37x and 73x). **(Above)** N2, end-to-side neurorrhaphy stained by the Bielschowsky method. Numerous nerve fibers were seen within the graft, which demonstrates nerve fibers sprouting. **(Below)** N2 stained with trichromic Masson showed epineural discontinuity at the end-to-side neurorrhaphy.

Histology of N3 from Gn showed normal nerve characteristics, with myelin sheath, well defined axon and epineural and high density of nerve fibers (Figure 7 left, above). G1 and G2 also showed numerous nerve fibers with a well defined myelin sheath, demonstrating appropriate axonal repopulation, in a similar way (Figures 7 right, above; and left, below).

However, G1 and G2 presented less nerve fibers, smaller diameters, thinner myelin sheaths, and larger amount of connective tissue when compared to Gn.

Gd had very few nerve fibers, with a greatly reduced diameter and a very scarce myelin sheath among substantial fibrosis, what is compatible with nerve degeneration (Figure 7 right, below). Characteristics of end neuroma in N4 segment were observed on all slides, except for one animal in which the graft had almost disappeared.

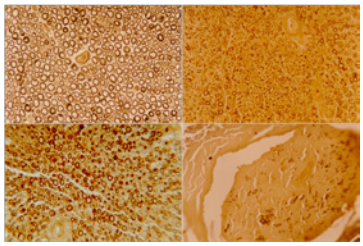

FIGURE 7 – Photomicrographs of the removed nervous segments (osmium, original magnification 540x). **(Left, Above)** N3 histology from Gn (normal control group) showed normal nerve characteristics, with well defined myelin, axon, and epineural sheath and a high density of nerve fibers. **(Right, Above)** G1 (one-stage) and **(Left, Below)** G2 (two-stage) also showed numerous nerve fibers with a well defined myelin sheath, demonstrating adequate axonal repopulation, in a similar form. **(Right, Below)** Gd (denervated control group) showed characteristics compatibles with nerve degeneration.

Discussion

This study used the neuro-muscular tibial-peroneal nerve and the cranial tibial muscle neuromuscular model[33].

Sample homogeneity regarding initial and final animal mass is extremely important since the cranial tibial muscle mass is directly correlated with animal mass[33].

All groups underwent nerve graft with ESN on TN because occasional neurotrophic factors or other factors still unknown could have affected the results[34].

The 2 months chosed time between the first and the second surgeries was enough for axon growth in the grafts[35].

Although in the most studies[1-3,5,6,14,16] on cross-face nerve graft the EEN is the technique used to connect the graft to the donor nerve, some authors[36-38] have shown that the ESN (technique more recently introduced[17]) can also be used successfully. However, to date there is no consensus on the realization of reanimation of the facial paralysis in one or two surgical stages[2,4,9]. In this context,

the main objective of this experimental research was to compare aspects of muscle reinnervation with nerve graft in one and two surgical stages, using the end-to-side neurorrhaphy, because this technique of neurorrhaphy has the great advantage of not injuring the epineurium of donor nerve[17-22] and, consequently, any nerve could potentially be used as a donor[22]. A comprehensive review of pertinent English literature (Medline and Embase databases) found no studies similar to that presented here. To the best of our knowledge, this is the first experimental research comparing muscle reinnervation by means of nerve grafts in one and two-stage procedures, using end-to-side neurorrhaphy of the graft on the donor nerve.

Segments N1 and N4 were sectioned longitudinally to confirm neuroma formation on the end and the non-fiber growth in the direction of the cranial tibial muscle[39], confirming the non occurrence of motor contamination.

N2 segments stained with Masson Trichromic[40] allowed observing the disappearance of the epineurium and perineurium in ESN sites in most cases (88%). Probably these structures had been absorbed. Some studies believe this phenomenon is due to the action of neurotrophic factors[41]; nevertheless, further studies are needed to identify exactly which substances are involved in this process. Bielschowsky technique, which stains nerve fibers in brown showed clearly the lateral sprouting[40].

The nerve fibers counting in the distal portion of PN (N3 segment) showed the highest number in Gn and lowest number in Gd, as expected. There was no difference between G1 and G2, which demonstrated that the axon growth was not affected by the difference in time of the muscle target connection.

The study of CTM was important because all aspects involved in nerve repair are aimed at the target organ, muscle or sensation terminals.

Cranial tibial muscle mass showed normality in Gn and denervation in Gd, but it mainly demonstrated better result in G2 than in G1.

Cranial tibial muscle morphometry measured the area and minimum diameter of 50 fibers from each muscle; this number was shown to be statistically appropriate. Minimum diameter was chosen because any inclination of muscle fibers at the time of sectioning don't influences on it. CTM morphometry also showed a better result in G2 compared to G1. Data were highest in Gn and lowest in Gd, as expected.

The histological aspects of CTM showed normality in Gn and denervation in Gd, as expected. Groups G1 and G2 showed signs of muscular reinnervation.

The muscle mass and muscle fiber morphometry confirmed that the two-stage technique used in G2 provides better muscular reinnervation than the single stage operation (G1), probably due to less denervation time of the target muscle.

Rab *et al*.[15] demonstrated better muscular reinnervation in the two-stage group and this result was attributed to prolonged atrophy in the transplanted muscle with the nerve graft in the single-stage operation. They[15] also found differences in number of nerve fibers after the second neurorrhaphy of nerve graft, which did not occur in our study.

Kumar *et al*.[2] showed the advantages and disadvantages of the cross-face nerve graft with free-muscle transfer for reanimation of the paralyzed side in one and two-stage operations, and despite the fact that muscle reinnervation showed better results in two-stage procedure, they recommend the single-stage due to its advantages. Clinical studies needs large series and generally are difficult to really expose precise results.

Our study also proved the efficacy of muscle reinnervation using ESN, promoting lateral axonal sprouting and muscle reinnervation.

Conclusion

The muscle reinnervation using nerve graft with end-to-side neurorrhaphy in two-stage procedures offers better result than in a single-stage.

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