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Muscle Reinnervation with Nerve-Muscle-Endplate Band Grafting Technique: Correlation Between Force Recovery and Axonal Regeneration

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Abstract

Background—This study was designed to determine the correlation between functional recovery and the extent of axonal regeneration after muscle reinnervation with our recently developed nerve-muscle-endplate band grafting (NMEG) technique in a rat model.

Materials and methods—The right experimentally paralyzed sternomastoid (SM) muscle by nerve transection was immediately reinnervated with a NMEG pedicle harvested from a neighboring sternohyoid (SH) muscle. The NMEG pedicle contained a muscle block (6x6x3 mm), a donor nerve branch with nerve terminals and a motor endplate band. Three months after surgery, the tetanic force of the SM muscle was measured and the regenerated axons in the muscle were detected using neurofilament immunohistochemistry.

Results—The results showed that the maximal tetanic force (a measure of muscle functional recovery) of the NMEG reinnervated SM muscle reached up to 66.0% of the normal control. The wet weight of the reinnervated SM muscle (a measure of muscle mass recovery) was 87.2% of the control. The area fraction of the regenerating axons visualized with neurofilament staining within the NMEG reinnervated SM muscle (a measure of muscle reinnervation) was 42.3%. A positive correlation was revealed between the extent of muscle reinnervation and maximal muscle force.

Conclusions—Our newly developed NMEG technique results in satisfactory functional outcomes and nerve regeneration. Further improvement in the functional recovery after NMEG

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Author's Contributions

SS and LM contributed to the conception and design of experiments. SS and LM did the data acquisition and analysis. SS wrote the first draft of the article and LM made the critical revision of the article.

Disclosure

The authors declare no conflict of interests. The authors report no proprietary or commercial interest in any product mentioned or concepts discussed in the article.

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reinnervation, could be achieved by refining the surgical procedure and creating an ideal environment that favors axon-endplate connections and accelerates axonal growth and sprouting.

Keywords

muscle reinnervation; nerve-muscle-endplate band graft; muscle force; axonal regeneration; rat; nerve transplantation

1. Introduction

Peripheral nerve injuries and resultant muscle paralysis are caused mainly by trauma and surgical intervention which represent a major medical problem [1–4]. Unfortunately, the presently existing nerve repair methods can result in poor functional outcomes.

Based on various situations, peripheral nerve injuries can be treated with nerve end-to-end anastomosis (EEA), nerve grafting, nerve transfer, neurotization, and conduit repair [5]. In general, EEA is commonly used when the two stumps of an injured nerve can be approximated without tension [6,7]. However, only about 50% of patients regain useful function [8,9]. Factors behind poor functional recovery include tension of the anastomosis, neuroma formation, scarring, and loss of the nerve fiber population [10]. Studies have shown that in EEA fewer nerve fibers could pass through the coaptation site and reach the target organ [11]. A significant nerve defect is a common clinical situation. In this case, autologous nerve grafts, nerve transfers, and tubulization may be used. Nerve grafting has been associated with poor functional outcomes when there is a long distance from the level of the injury to the target muscle. The recovery rate of motor function after autogenous nerve grafting is less than 40% [12]. Tubulization methods are feasible only in short nerve gaps [5]. If the gap exceeds 1.0–1.5 cm, regeneration is poor [13]. Longer defects (4.0–6.0 cm) result in useful reinnervation in only 13% of cases with reconstruction of peripheral nerve injuries with conduits [14,15]. For the nerve injuries in which the proximal nerve stump is unavailable, nerve transfer is an option. Nerve transfer is the surgical coaptation of a healthy nerve donor to an injured nerve. A nerve branch that innervates expendable muscle(s) can be repaired to a more functionally important distal stump of an injured nerve. Many nerve-to-nerve transfers have been used to repair the injured nerves in the hand and upper extremity with mixed results [16]. In many cases with trauma, the distal nerve stump might not be available for nerve repair. The only reconstructive option to reinnervate that muscle is direct nerve implantation (i.e., muscular neurotization) [17,18] or nerve-muscle pedicle (NMP) transfer. The NMP technique has been used for laryngeal [19–22] and facial reinnervation [23–25]. However, controversy exists concerning the success rate of the NMP method. Some authors reported satisfactory functional recovery [21,22], whereas others failed to document NMP-induced muscle reinnervation and functional recovery [26,27]. These results emphasize the great need to develop novel treatment strategies for the restoration of paralyzed muscles as the currently used reinnervation methods often result in poor functional recovery.

We recently developed a new reinnervation technique called “nerve-muscle-endplate band grafting (NMEG)” [28] and conducted a series of experiments in a rat model [29–33]. The development of this technique is based on the concept that a healthy motor endplate band

with a nerve branch and terminals that innervates an expendable muscle can be transplanted to a more functionally important denervated muscle for restoring its motor function. The idea is that the implanted NMEG could provide an abundant source of nerve terminals and endplates for nerve regeneration and muscle reinnervation to restore motor function of the target muscle. Our studies showed that NMEG technique yielded satisfactory functional recovery.

The goal of this study was to document NMEG-induced muscle reinnervation and determine the correlation between functional recovery and axonal regeneration following NMEG procedure.

2. Materials and methods

2.1. Animals

The experiments were performed on 10 adult (3.5 months old) Sprague-Dawley female rats (Charles River Laboratories, MA), weighing 350–450 grams. The rats were provided with *ad libitum* access to food and water and housed in standard cages in a 22°C environment with a 12:12-h light-dark cycle. Experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee prior to the onset of our experiments. All animals were handled in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). All efforts were made to minimize the number of animals and their suffering during the experiments.

2.2. NMEG Procedures

Animals used in this study underwent general anesthesia with a mixture of ketamine (80 mg/kg body wt) and xylazine (5 mg/kg body wt) administered intraperitoneally. Microsurgical procedures were performed under aseptic conditions. Under an Olympus SZX12 Stereo zoom surgical microscope (Olympus America Inc, Center Valley, Pennsylvania), a midline cervical incision was made extending from the hyoid bone to the sternum to expose the right sternomastoid (SM) and sternohyoid (SH) muscles and their innervating nerves. The right SM muscle was denervated by removing a 5-mm segment of its innervating nerve. The nerve cut ends were coagulated using a bipolar cautery to prevent nerve regeneration. An NMEG pedicle was harvested from the right SH muscle. The SH nerve branch was identified on the lateral margin of the middle portion of the muscle and traced from the motor point to the motor zone where axon terminals and an endplate band are located. An NMEG harvested from right SH muscle consisted of a block of muscle (~6x6x3 mm), an intact donor nerve branch with numerous nerve terminals, and an endplate band with numerous neuromuscular junctions. The presence of functioning NMEG was confirmed by observing its twitch contractions on nerve stimulation. A muscular defect of the same dimensions as the NMEG was made in an endplate-free region in the caudal portion of the right denervated SM muscle. The NMEG in continuity with its motor nerve branch and feeding vessels was embedded in the SM muscle defect and sutured with four to six 10-0 nylon microsutures (Fig. 1). After surgery, the wound was closed in layers with interrupted simple sutures of 4-0 Prolene.

2.3. Muscle Force Measurement

At the end of the 3-month recovery period, the muscle force of the reinnervated SM was measured by using a stimulation and recording system as described in our publications [28,30–33]. Briefly, the SM muscle was dissected free from surrounding tissue without damaging the SH nerve branch innervating the implanted NMEG. The rostral tendon of the muscle was severed, tied with 5-0 silk suture and connected to a force transducer attached to a servomotor lever arm. The electrical stimulation was provided directly to the SH nerve branch close to the implanted NMEG. Throughout testing, the SM muscle and nerve were regularly bathed with warm mineral oil to maintain a temperature between 35°C and 36°C. Body temperature was maintained at the same level with a closed circuit homeothermic blanket system for rodents (No. 50300; Stoelting Co, Wood Dale, IL).

The recording system consisted of a NI USB board (6251, 16 bit 1.25Ms/s, National Instruments, Austin, TX) connected to a DELL laptop with a custom written program using National Instruments labVIEW 8.2 software. The system generated stimulation pulses, which after isolation from the ground through an optical isolation unit (Analog Stimulus Isolator Model 2200, A-M Systems, Inc, Carlsborg, WA) were used for the current controlled stimulation. The system also provided a position signal, which was used by the servomotor of the Dual-Mode Lever System (305B-LR; Aurora Scientific Inc., Aurora, Canada) to control muscle length. The Acquisition System collected stimulation data and a muscle force signal from the Dual-Mode Lever System. A Grass Amplifier System Model 15LT was available for signal conditioning and recording. Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

During muscle force testing, the SM muscle was stretched by the servomotor lever arm to the optimal length for the development of maximum force. Our preliminary study on the rat's intact SM muscle showed the largest tetanic force when the muscle was stretched with a tension of 0.08N before stimulation. The force-tension data collected in our NMEG study showed the same force-tension relation in the operated muscles as in intact muscles [33]. Therefore the same muscle tension of 0.08N was used in the present study.

Isometric contractions of the SM were obtained with 200 ms trains of biphasic rectangular pulses. The duration of each phase of stimulation pulse was set at 0.2 ms and train frequency was set at 200 pulses/s. The stimulation current was gradually increased from 0 mA to 1 mA. At least one minute breaks separated subsequent tetanic contractions to permit muscle recovery. At the moment of force measurement, the lever arm was stationary. At each stimulation intensity the maximal force produced by a muscle was measured using DIAdem software, and from those measurements the intensity–force plot was made. Maximal force from all stimulation currents was identified. Maximal muscle force from the operated side was calculated as a percentage of maximal muscle force recorded from the control side.

2.4. Measurement of Muscle Weight

Immediately after muscle force measurement, the operated and control SM muscles in each rat were removed and weighed. The muscle weight on the operated side was expressed as the percentage of the control muscle weight on the contralateral side.

2.5. Neurofilament Staining and Assessment of Axonal Regeneration

After muscle weight measurement, the region where the implanted NMEG was located within the SM muscle was cut transversely (10- μ m thick) and immunostained with neurofilament (NF) antibody to label regenerating axons as described previously with some modifications [34]. Briefly, the sections were (1) fixed in Zamboni fixative with 5% sucrose overnight at 4°C; (2) rinsed several times in phosphate-buffered saline (PBS); (3) treated with 0.1 mol/L glycine in PBS at room temperature (RT) for 30 minutes; (4) dipped in 100% methanol at -20°C for 1 hour; (5) incubated in PBS containing 0.3% Triton and 3% bovine serum albumin at RT for 1 hour to inhibit nonspecific protein binding; (6) incubated with primary antibody SMI-31 (dilution 1:800; Covance Research Products, Berkeley, California) in PBS containing 0.1% Triton and 2% bovine serum albumin at 4°C overnight for detecting neurofilaments; (7) rinsed several times in PBS; (8) incubated with a secondary antibody (donkey anti mouse antibody conjugated to Alexa 488; dilution, 1:1000) in PBS containing 0.05% Triton and 2% bovine serum albumin at RT for 2 hours; and (9) rinsed in PBS several times. The stained sections were mounted with Permount.

Stained sections were examined under a Zeiss photomicroscope (Axioplan; Carl Zeiss, Oberkochen, Germany) and photographed with a Sony digital camera (model DXC-5500, Japan) attached to the photomicroscope and connected to a personal computer with image analysis software. The density of the regenerating axons was evaluated by estimating the number and the area fraction (a standard stereological measure, which estimates the total amount of a partition as a fraction of total sample volume) of the NF-positive axons within a section area (1.0 mm²). For each muscle, 3 stained sections at different spatial levels within the motor zone were randomly selected to evaluate regenerating axons. NF-positive axons on each section were measured and the values from the 3 sections were averaged for each muscle. Figure 2 shows the assessment procedure. To the right from two representative NF stained cross sections of the SM muscle (reinnervated with NMEG – upper image and normal control – lower image), two corresponding pictures converted to binary black and white are shown. Areas with NF staining were outlined, measured with the public domain ImageJ software (version 1.45s, National Institutes of Health) and the relation between the operated and control muscles were calculated both for the number and the area fraction of the NF labeled axons.

2.6. Statistical Analysis

We collected data of four parameters of the muscle, including (1) maximal muscle force produced by muscle stimulation with increasing current up to 1mA; (2) muscle weight; (3) number and (4) area fraction of NF-labeled axons. The percentage of the operated to control side was calculated for each of the 4 parameters in each animal. The group mean and standard error were computed for each parameter. Reinnervation levels represented by these 4 parameters were compared with each other with a Kruskal-Wallis test followed with post-hoc analysis with Benferroni corrected multiple comparisons on ranks with Mann-Whitney tests. The Pearson correlation coefficient was calculated to evaluate correlation between muscle force and area of NF-positive axons. Statistical analysis was performed using Statistical Analysis System 9.3 (SAS, Inc., Cary, NC). Statistical significance was set at $p < .05$ with two-tailed test.

3. Results

3.1. Implanted NMEG within the SM Muscle

Figure 1 shows that an NMEG pedicle harvested from the ipsilateral SH muscle was successfully implanted into the dorsal part (endplate-free area) of the denervated SM muscle. Sihler's stain (Fig. 1A) shows the donor nerve branch and nerve terminals within the implanted NMEG, whereas whole-mount acetylcholinesterase and silver staining (AChE-Ag) (Fig. 1B) demonstrates the implanted NMEG with endplate band and its innervating nerve terminals.

3.2. Axonal Regeneration within the NMEG Reinnervated Muscles

NF staining showed that the regenerating axons from the implanted NMEG grew into the recipient SM muscle 3 month after surgery. The density of the regenerating axons in the NMEG reinnervated SM muscle was determined by calculating the number of NF-positive axons within a 1.0-mm² area on the stained muscle sections (Fig. 2). We found that 3 months after NMEG procedure the majority of the regenerating axons innervated the ventral two thirds of the recipient SM muscle, while a few axons reached the distal third of the muscle (Fig. 3). Our data showed that NMEG produced extensive axonal regeneration (Fig. 4). The cumulative area fraction of stained axons after the NMEG procedure was 42.3% of the control.

3.3. Muscle Weight

Three months after NMEG reinnervation, the mean weight of the recipient SM muscle was 87% of the control (Fig. 4). This slight decrease in muscle weight indicates that the reinnervated SM muscle received very good motor reinnervation from the implanted NMEG.

3.4. Force Measurement

Maximum tetanic tension in the NMEG reinnervated SM muscle was evaluated at the end of 3-month recovery period. The degree of functional recovery of the reinnervated SM muscle was determined as compared with that of the contralateral control muscle in each rat. Force measurement showed that NMEG reinnervated SM produced 66.0% of the maximal force of the control (Fig. 4).

3.5. Comparison between 4 Outcome Measures of Muscle Reinnervation

Reductions at the operated side of muscle force, muscle weight, number and cross-section area of stained axons are shown in Fig. 4. These 4 measures of SM muscle reinnervation with the NMEG technique were compared using Kruskal-Wallis test. The test showed statistically significant differences between these measures ($p < .0057$, Chi-Square=12.54, df=3). Post-hoc analysis was made with Mann-Whitney tests with Benferroni correction for multiple comparisons (n=6). This analysis showed significantly smaller reduction of muscle weight in reinnervated muscle (87.2%) than the reduction of cumulative area measure of axonal staining (42.3%, $z=2.99$, $p=.017$) and the count of stained axons (68%, $z=2.76$, $p=.035$). However there were no statistically significant differences between averaged muscle

force (66.0%) and other averaged measures: weight (87.2%), area fraction (42.3%) and number of stained axons (68.0%).

Combined area of NF staining in different operated animals (calculated as a percent of stained area at operated side to control side) was significantly correlated with maximal muscle force produced by the SM muscle to electrical stimulation (calculated as a percent of maximal muscle force at operated to control side; $p < .025$, $r=0.72$, $df=9$, see Fig. 4). This correlation was similar to that we reported previously [32] in the rat SM muscle reinnervated with EEA ($p < .05$, $r=0.77$, $df=6$).

4. Discussion

This animal study was conducted to reveal the association between functional recovery and axonal regeneration 3 months after muscle reinnervation with our newly developed NMEG technique. The results showed that the maximal tetanic force (a measure of muscle functional recovery) of the NMEG reinnervated SM reached up to 66.0% of the control. The NMEG reinnervated SM exhibited good preservation of muscle mass and extensive axonal regeneration. The wet weight of the reinnervated SM (a measure of muscle mass recovery) was 87.2% of the normal control. The area fraction of the regenerating axons visualized with NF staining within the NMEG reinnervated SM (a measure of muscle reinnervation) was 42.3% of the control. A positive correlation was revealed between the extent of muscle reinnervation and maximal muscle force. Numerous regenerating axons from the NMEG were identified to supply the reinnervated SM muscle. The cumulative area fraction of stained axons after NMEG procedure (42.3%) was almost 3 times larger than that after EEA procedure (13.4%) [32]. In addition, the count of the NF-positive axons after the NMEG procedure (68.0% of the control) was more than 2 times larger than that after EEA procedure (31%) [32]. Clearly, NMEG technique yielded better results compared with EEA nerve repair. This is attributed to the fact that the implanted NMEG could provide an abundant source of nerve terminals and endplates for nerve regeneration and muscle reinnervation to restore motor function of the reinnervated muscle.

Nerve injuries may cause permanent denervation with paralysis and disability in humans and represent a challenging problem in microsurgery. Poor outcomes from currently existing reinnervation methods are closely related to inadequate axonal regeneration and to the reestablishment of nerve-muscle connections. Muscle force measurement is widely used to assess the mechanical function and contractile properties of a muscle [35–41]. Our studies [28,30–33] and others [23,25,42,43] have demonstrated that force measurement is a reliable technique in determining the degree of functional recovery of a reinnervated muscle. The denervated muscle is characterized by reduced muscle mass, decreased fiber size, increased connective tissue, and reduced metabolic capacity [44,45] which can be reversed by reinnervation [28,33,46]. Muscle wet weight has been widely used to evaluate muscle reinnervation [28,31–33,47–49]. The present study demonstrated satisfactory recovery of muscle force, good preservation of muscle mass, and extensive axonal regeneration 3 month after NMEG reinnervation. These results can be attributed to several unique technical and neurobiological features of the NMEG that allow this technique to offer substantial advantages over the classic EEA method. First, the NMEG provides an abundant source of

nerve terminals and endplates. Therefore, a large number of the axons are available for muscle reinnervation. Second, the NMEG technique leads to minimal nerve fiber injury and a degenerative reaction. As most of the nerve terminals and endplates within the NMEG remain intact, subsequent nerve degeneration before reinnervation can be prevented. Third, the NMEG has relatively larger pedicle-recipient muscle interfaces which offer more space for axonal regeneration as compared with other reinnervation methods. Finally, the NMEG, rich in fibronectin and laminin from the muscle fibers in the muscle block, provides an excellent medium for axonal sprouting. Evidently, NMEG technique holds great promise for optimal muscle reinnervation.

5. Conclusions

This study further confirms that our newly developed NMEG technique results in satisfactory functional outcomes and nerve regeneration. There is a positive correlation between the extent of regenerating axons and maximal muscle force. Although the results from our NMEG experiments are encouraging, full functional recovery has not been achieved. Incomplete functional recovery following NMEG appears to be related to the fact that the regenerating axons from the implanted NMEG do not reach the distal portion of the recipient muscle. Therefore, further work is needed to improve the functional outcomes of the NMEG. To obtain optimal functional recovery after NMEG reinnervation, it is necessary to refine the surgical procedure and create an ideal environment that favors axon-endplate connections and accelerates axonal growth and sprouting.

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FIGURE 1.

Photographs showing the implanted NMEG in an endplate-free area of the rat SM muscle.

A: Sihler's stained SM muscle reinnervated by NMEG, showing the SM nerve (black arrow) and implanted SH donor nerve branch (blue arrow). **B:** An AChE-Ag stained SM muscle reinnervated by NMEG, showing original endplate band in the SM muscle (black arrow) and the implanted NMEG containing SH nerve branch (blue arrow), an endplate band, and nerve terminals (white arrow). Four microsutures (red arrows) were used to suture the NMEG pedicle in the SM muscle.

FIGURE 2.

NF staining of the rat SM muscle reinnervated with NMEG (**A**) was compared to a normal SM muscle (**B**). NMEG procedure resulted in good axonal regeneration and muscle reinnervation (bright staining). 200x. NF stained muscle sections from the SM muscle were converted to black and white (corresponding pictures in the right column). The number and total area of staining in each section was calculated with public domain ImageJ software (ver 1.46, by Wayne Rasband, <http://imagej.nih.gov/ij>).

FIGURE 3.

NF-labeled axons in SM muscle. **A:** NF staining illustrates progress of axonal reinnervation of rat SM muscle after denervation and immediate repair with NMEG. Three months after implantation of the NMEG to the SM muscle the regenerating axons from the graft innervated ventral two thirds of the recipient SM muscle. **B:** For comparison, NF staining in normal control SM muscle illustrates axonal innervation of both ventral and distal portions of the muscle.

FIGURE 4.

A comparison between 4 attributes of a reinnervated SM muscle: axonal cumulative area (**A**), number of axons (**B**), muscle force (**C**) and muscle weight (**D**). Individual data from all 10 rats, group means (Mean) and standard errors (SE) at 3 months after NMEG procedure are presented. Data were calculated as the percentage of control. The area of axonal staining had the lowest percentage rate (42.3%) whereas the muscle weight was the highest (87.2%). The NMEG procedure produced successful muscle reinnervation (see our recent EEA paper [32] for results comparison).