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PRINCIPAL INVESTIGATOR: Liancai Mu, M.D., Ph.D.,

RECIPIENT: Hackensack University Medical Center
Hackensack, NJ 07610

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Liancai Mu, MD, PhD; Stanislaw Sobotka, PhD; Jingming Chen, MD

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14. ABSTRACT

Our previous studies showed that NMEG-NMZ reinnervation technique resulted in functional recovery (82% of the control) of the target muscle. In order to promote the efficacy of NME-NMZ, we investigated the effects of focal administration of exogenous neurotrophic factors (ENF) and intra-operative nerve electrical stimulation (ES) on the outcomes of NMEG-NMZ (NN) technique. At 3 months after treatment, we analyzed the muscle force, muscle mass, myofiber morphology, regenerated axons, and endplate reinnervation. The results showed that NN/ENF and NN/ES cotreatments resulted in more optimal functional recovery (91% and 90% of the control, respectively) as compared with NMEG-NMZ surgery alone (82%). Histological and immunohistochemical studies showed that NN/ENF treated muscles exhibited excellent muscle mass recovery (92% of the control), abundant regenerated axons (84% of the control), and a high percentage (86%) of reinnervated motor endplates. Our findings suggest that NN/ENF or NN/ES improves the outcomes of NMEG-NMZ reinnervation technique.

15. SUBJECT TERMS Peripheral nerve injury, muscle reinnervation, nerve-muscle-endplate band grafting, nerve regeneration, motor endplate band, native motor zone, muscle force measurement, functional recovery

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TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Traumatic peripheral nerve injury to the head/neck and extremity is a significant cause of morbidity and disability in both military and civil circumstances today. Despite advances in microsurgical techniques for nerve repair, functional recovery is rarely complete. We developed a new surgical method called nerve-muscle-endplate band grafting (NMEG) for muscle reinnervation [Neurosurgery 69(Suppl. 2):208-224, 2011]. The development of the NMEG reinnervation technique is based on the concept that a paralyzed muscle could be reinnervated by transplanting an NMEG from a neighboring donor muscle. A healthy nerve branch with its terminals that innervates an expendable muscle can be transplanted to a more functionally important denervated muscle for restoring its motor function. In a rat model, a NMEG pedicle containing a nerve branch and a muscle block with nerve terminals and motor endplates was harvested from the sternohyoid muscle and transplanted into the ipsilateral experimentally denervated sternomastoid muscle. Several lines of evidence demonstrated that the NMEG procedure results in encouraging functional recovery (67% of the control). The major goal of the proposed work funded by DOD is to augment the efficacy of the NMEG technique by creating an ideal environment that physically facilitates axon-endplate connections and biologically enhances nerve regeneration. As motor endplate reinnervation is critical for restoring motor function of the denervated muscle, we modified the surgical procedure. Specifically, the NMEG pedicle harvested from the sternohyoid muscle was implanted into the native motor zone (NMZ) of the experimentally denervated sternomastoid muscle, instead of an endplate-free area in the target muscle as originally designed procedure. At 3 months after surgery, maximal tetanic force measurement, muscle mass and myofiber morphology, motoneurons, regenerated axons, and axon-endplate connections of the muscles were analyzed and compared with those of the contralateral control muscle. Our studies (Brain and Behavious, 2016 under review) showed that NMEG-NMZ technique resulted in significant muscle force recovery (82% of the control). The reinnervated muscles exhibited good preservation of muscle mass (89% of the control) and myofiber morphology. In the treated muscles, the mean count and area of the axons reached up to 76.8% and 75.6% of the controls, respectively and the majority (80%) of the denervated motor endplates regained motor innervation. These results should be attributed to the unique advantages of the NMEG-NMZ technique. Specifically, a transferred NMEG could provide an abundant source of nerve terminals and motor endplates for nerve regeneration and muscle reinnervation. NMEG has sufficient pedicle-recipient muscle interfaces, which provide enough space for axonal regeneration. The axons could start to regenerate at multiple points in the implanted NMEG and grow across the pedicle-recipient muscle interfaces to reach the recipient muscle fibers. Thus, the NMEG-NMZ procedure provides a favorable environment that physically shortens regeneration distances and facilitates rapid axon-endplate connections between regenerating axons from the NMEG pedicle and denervated endplates in the NMZ of the target muscle. Here, we reported the beneficial effects of local administration of exogenous neurotrophic factors (ENF) and intraoperative 1-hour of electrical stimulation (ES) on nerve regeneration and functional recovery. We found that a combination of NMEG-NMZ with ENF (NN/ENF) or with ES (NN/ES) resulted in more optional functional recovery (91% and 90% of the control, respectively) as compared with NN surgery alone (82%). Our ongoing studies are to determine if the NMEG-NMZ, NN/ENF and NN/ES have the potential for delayed reinnervation that is not uncommon in clinical practice. The results will be reported during the following year.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Peripheral nerve injury, muscle reinnervation, nerve-muscle-endplate band grafting, nerve regeneration, motor endplate band, native motor zone, muscle force, functional recovery

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals and objectives of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Goal 1: To evaluate functional recovery of the paralyzed muscles treated by the NMEG-NMZ technique with/without ES and ENF.
Goal 2: To determine the extent of neural regeneration and axon-endplate connections in the treated muscles.
Goal 3: To document histological and immunohistochemical alterations in the treated muscles.
Milestones: • Surgical procedures for immediate reinnervation will be accomplished in year 1 and those for delayed reinnervation will be completed in year 3.
• Electrophysiological studies in Goal 1 will be accomplished in year 3.
• The timetable for conducting the studies on neural and muscular tissues in Goals 2 and 3 is in years 2-4.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

• **Major Activities**

For this reporting period, we have performed the following major activities.

Animal surgeries

We performed 135 operations on animals in 2 immediate (Imm-) reinnervation groups (Imm-NN/ES and Imm-NN/ES/ENF) and 3 delayed reinnervation groups (3-mon-Del-NN, 3-mon-Del-NN/ES, and 3-mon-Del-NN/ENF) for neurosurgery and force measurement (15 rats/each group). Each rat in the immediate reinnervation groups underwent 2 operations (NMEG surgery and force measurement), while each animal in the delayed reinnervation groups was subjected to 3 operations (muscle denervation, NMEG surgery, and force measurement). All the surgical procedures were successfully performed (**Fig. 1**).

NN = NMEG-NMZ transplantation; ES = intraoperative 1-hour electrical stimulation;
ENF = focal administration of exogenous neurotrophic factors; 3-mon-Del = 3-month-delay.

Muscle force measurements and data analyses

Three months after surgery, muscle force measurements were performed on 90 SM muscles of 45 rats. Specifically, the rats in Imm-NN/ES, Imm-NN/ES/ENF, and 3-mon-Del-NN groups (15 rats/per group) underwent force measurements (**Fig. 2**). For each rat, muscle force was measured from the target and contralateral control muscles. The force data from the SM muscles (n=90) of the rats (n=45) in Imm-NN/ENF, Imm-NN/ES, and Imm-NN/ES/ENF groups were analyzed and major findings are summarized below (see Key Outcomes).

Tissue studies

At the end of experiments, the right experimental and left control muscles for each animal were removed, measured, and prepared for tissue studies. The muscle samples (n = 180; 4 samples/ each animal) from animals (n = 45) in Imm-NN/ENF, Imm-NN/ES, and Imm-NN/ES/ENF groups were sectioned and stained using histochemical and immunohistochemical techniques to analyze muscle structure, myofiber morphology, axonal regeneration, and motor endplate reinnervation (**Figs. 3-5**). For each rat, the data from the target muscle were prepared with those from the contralateral control.

• **Specific Objectives**

For this reporting period, data from the animals in Imm-NN/ENF and Imm-NN/ES groups have been collected and analyzed to determine the effects of nerve stimulation and neurotrophic factors on the outcomes of NMEG-NMZ reinnervation technique.

***Objective 1*:** To determine the degree of functional recovery of the paralyzed muscles treated by Imm-NN/ES and Imm-NN/ENF.

***Objective 2*:** To determine the extent of nerve regeneration and endplate reinnervation in the muscles treated by Imm-NN/ES and Imm-NN/ENF.

***Objective 3*:** To document morphological and histological changes in the muscles treated by Imm-NN/ES and Imm-NN/ENF.

• **Major Procedures**

NMEG-NMZ implantation

The details regarding the surgical procedures for NMEG-NMZ technique have been described in our paper (*Brain and Behavior, 2016 under review*). Briefly, the right sternomastoid (SM) muscle in the rat was exposed and denervated by resecting its innervating nerve (~5 mm). The native motor zone (NMZ) of the right SM was outlined in the middle segment of the muscle (**Fig. 1A**) and a muscular defect with the same dimensions as the NMEG was made in the NMZ of the denervated SM muscle (**Fig. 1B**). An NMEG pedicle was harvested from the NMZ of the right sternohyoid (SH) donor muscle (**Fig. 1B**). An NMEG pedicle contained a block of muscle (~6 x 6 x 3 mm), a nerve branch, intramuscular nerve terminals, and a motor endplate (MEP) band with numerous neuromuscular junctions. The superficial muscle fibers on the ventral aspect of the NMEG pedicle were removed to create a denuded surface for better axon regeneration. The well-prepared NMEG was embedded in the muscle defect of the right SM and sutured with four to six 10-0 nylon microsutures (**Fig. 1C**). Thus, the experimentally denervated SM muscle was immediately reinnervated with NMEG-NMZ technique.

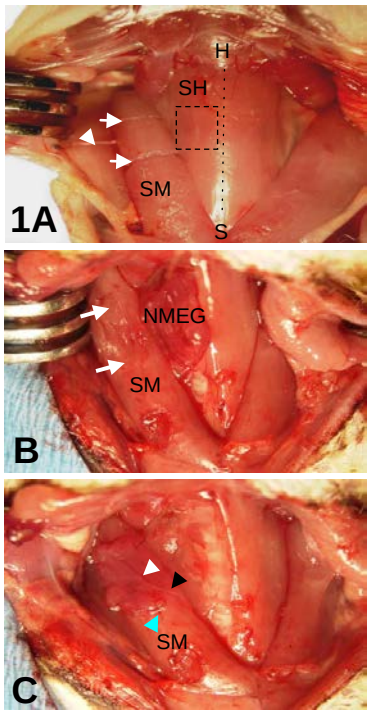


Fig. 1. Photographs from a rat, showing surgical procedures for NMEG-NMZ reinnervation technique. **A:** surgically outlined motor zones in the recipient SM (between fiber cuts as indicated by arrows) and in the donor SH (boxed region). The arrowhead indicates the SM nerve. The dashed line indicates the midline between both SH muscles. H, hyoid bone; S, sternum. **B:** an NMEG pedicle was harvested from the ipsilateral SH muscle and a muscular defect (recipient bed) of the same dimensions as the NMEG (between arrows) was made in the denervated motor zone of the SM muscle. **C:** The prepared NMEG was embedded in the recipient bed and sutured with 10-0 nylon microsutures (green arrowhead). Note that the implanted NMEG contained a nerve branch (black arrowhead) and blood vessels (white arrowhead).

NMEG-NMZ plus exogenous neurotrophic factors (NN/ENF)

The SM muscles of the rats (n = 30) in Imm-NN/ENF and Imm-NN/ES/ENF groups underwent NMEG-NMZ implantation and a single focal administration of exogenous nerve growth factor (NGF) and basic fibroblast growth factor (FGF-2) to optimize the neural environment for enhancing nerve regeneration. Fibrin sealant containing NGF and FGF-2 was used for the slow continual release of the factors directly to the target. A single local administration of fibrin sealant containing a mixture of NGF and FGF-2 was performed during surgery. Specifically, the muscular defect created in the SM muscle for NMEG implantation (**Fig. 1B**) was covered with 0.5 mL fibrin sealant containing 50 μg recombinant rat NGF and FGF-2 (R&D Systems). Then, the NMEG pedicle was adhered by setting TISSEEL to the recipient bed and sutured.

NMEG-NMZ plus intra-operative 1-hour electrical stimulation (NN/ES)

The animals in Imm-NN/ES and Imm-NN/ES/ENF groups (n = 30) were subjected to an intra-operative 1-hour of ES prior to NMEG-NMZ implantation. The SH nerve branch supplying the NMEG was placed on bipolar stainless steel hook electrodes and stimulated. ES with supramaximal pulses (0.1 msec; 3V) was delivered in a continuous 20 Hz train to the nerve branch for 1 hour prior to NMEG implantation. Throughout the ES procedure, the muscle pedicle and its innervating SH nerve branch stimulated were regularly bathed with warm mineral oil. Immediately after stimulation, the harvested NMEG pedicle was implanted into the NMZ in the recipient SM muscle. Intra-operative 1-hour continuous ES (20 Hz) has been demonstrated to have the potential for enhancing axon outgrowth across the site of injury and facilitate long-term axon regeneration, thereby leading to significant functional recovery.

Muscle force measurement

Three months after treatment, the degree of functional recovery of the treated and contralateral SM muscles (n = 90) of the rats (n = 45) in Imm-NN/ES, Imm-NN/ES/ENF, and 3-mon-Del-NN groups were evaluated using muscle force measurement (**Fig. 2**). The details regarding muscle force measurement have been given in our previous publications and annual report. Briefly, the distal tendon of the SM muscle was severed, tied with 2-0 silk suture, and connected to a servomotor lever arm with a force transducer. The nerve branch supplying the NMEG was stimulated. 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 until the tetanic force reached a plateau. Muscle force was measured when muscle was stretched at optimal tension of 0.8 N. Maximal muscle force was calculated as average muscle contraction to 5 stimulation currents, ranging from 0.6 to 1.0 mA (**Fig. 2**).

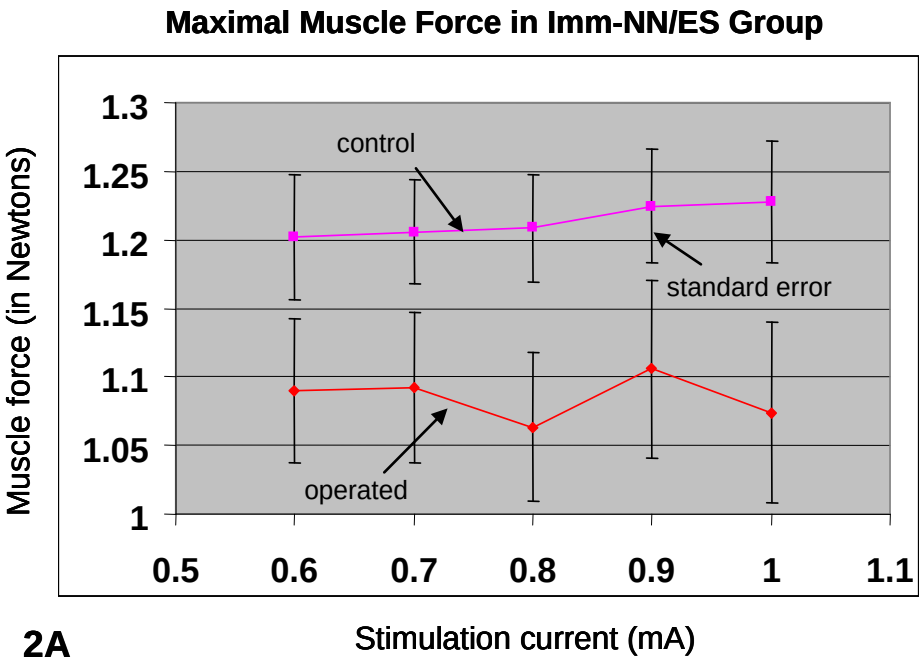
Tissue studies

Tissue samples (n = 180; 2 samples/per muscle) from the SM muscles (n = 90) of the rats (n = 45) in the Imm-NN/ENF, Imm-NN/ES, and Imm-NN/ES/ENF groups have been sectioned and stained using various histological and immunohistochemical methods. Cross sections from the rostral or caudal portion of the SM muscle were used for routine hematoxylin and eosin staining to examine alterations in the muscle structure and myofiber morphology (**Fig. 3**). Sagittal sections from the middle SM containing the NMEG implant were immunostained with neurofilament staining to count regenerated axons (**Fig. 4**) and double fluorescence staining to identify innervated and non-innervated motor endplates (**Fig. 5**). The density of the regenerated axons on a stained section was measured with public domain ImageJ software (v. 1.45s; NIH, Bethesda, Maryland) (**Fig. 4**). The innervated and non-innervated motor endplates in each muscle were computed.

• **Key Outcomes**

Functional recovery

Muscle force measurement was used to evaluate functional outcome of the treated muscles. The percentage of functional recovery of the treated SM muscle was determined as compared with that of the contralateral control muscle in each rat. Post-operative evaluations demonstrated that the outcomes of NMEG-NMZ technique were improved by additional use of ENF (i.e., NGF and FGF-2) and intra-operative 1-hour ES. The combination of NMEG-NMZ (NN) with the use of a mixture of NGF and FGF-2 (i.e., NN/ENF) or with electrical stimulation (i.e., NN/ES) resulted in optimal functional recovery. Specifically, the mean force of the muscles treated with Imm-NN/ENF was measured to be 91% of the control. Similar results were obtained from the rats in the Imm-NN/ES group (90% of the control) (**Fig. 2A**). Clearly, NN/ENF or NN/ES cotreatment resulted in better functional recovery as compared with NN surgery alone (82%). These findings suggest that ENF and ES have similar beneficial effect on the NMEG-NMZ surgery. ENF and ES play an important role in muscle reinnervation and functional recovery by enhancing nerve regeneration. Successful restoration of muscle function following NN/ENF or NN/ES cotreatment was consistent with recovery of muscle mass (**Fig. 3**), the number of regenerated axons that enter the native motor zone of the denervated muscle (**Fig. 4**), and the percentage of reinnervated motor endplates in the target muscle (**Fig. 5**). These findings suggest that the efficacy of NMEG-NMZ reinnervation technique can be promoted by additional use of ENF or ES.



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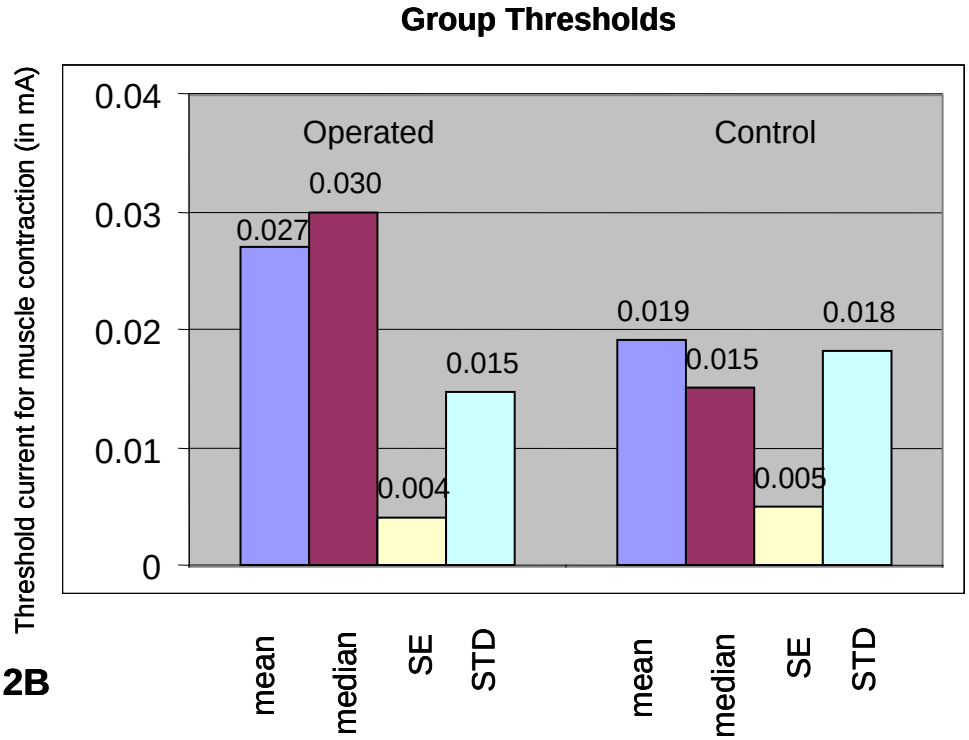


2B

Fig. 2. Force data from Imm-NN-ES group. **A:** Group mean muscle force. Note that the mean force of the treated muscles is 90% of the control. **B:** Group thresholds. Note that average threshold stimulation current of SM nerve, which produces visible muscle contraction, is 0.029 mA at the operated side, whereas that at the contralateral control side is 0.019 mA.

Muscle weight and myofiber morphology

Three months after treatment, both SM muscles in each rat were removed and weighed. There was no significant difference in muscle wet weight between muscles treated with NN/ENF (92% of the control) and NN/ES (90% of the control). Gross appearance and size of the NN/ENF- and NN/ES-treated SM muscles were similar to those of the contralateral control muscles (**Fig. 3A**). The treated muscles were larger in size than 3-month-denervated SM (44% of the control) (**Fig. 3B**). These findings indicate that Imm-NN/ENF and Imm-NN/ES resulted in excellent muscle mass recovery.

Hematoxylin and eosin-stained cross muscle sections showed that the SM muscles treated with Imm-NN/ENF or Imm-NN/ES exhibited better preservation of muscle structure and myofiber morphology (**Fig. 3C**) as compared with denervated muscles (**Fig. 3E**).

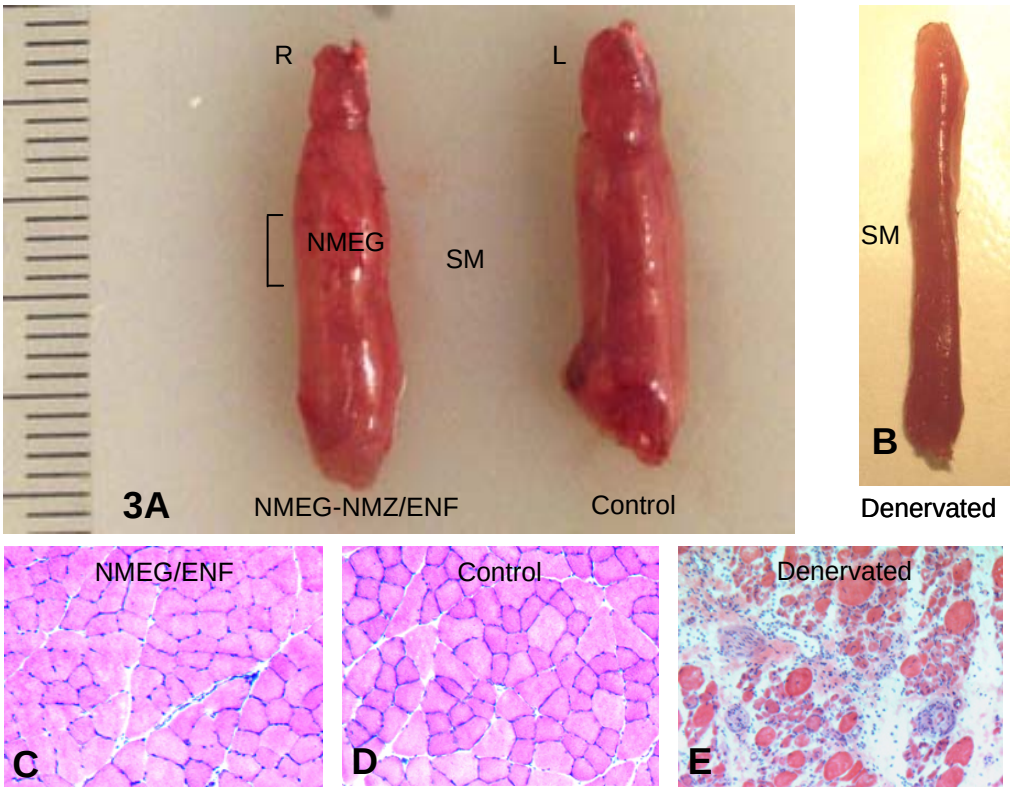


Fig. 3. Gross appearance, muscle mass, and myofiber morphology of the NMEG-NMZ/ENF treated, normal, and denervated sternomastoid (SM) muscles in the rats. **A:** A pair of SM muscles removed from a rat 3 months after NMEG-NMZ/ENF treatment. Note that the mass of the right (R) treated SM muscle was close to that of the left (L) control muscle. The outlined region in the right SM is the location of the implanted NMEG. **B:** A 3-month completely denervated SM muscle. Note that the denervated SM showed a more significant loss of muscle mass as compared with the treated and normal SM muscles. **C-E:** Hematoxylin and eosin-stained cross sections from the NMEG-NMZ/ENF treated SM (**C**), contralateral control (**D**), and denervated (**E**) SM muscles. Note that the treated SM exhibited very good preservation of muscle structure and myofiber morphology with less fiber atrophy as compared with the normal and denervated muscles. The SM denervated for 3 months exhibited significant myofiber atrophy. x 200 for C-E.

Nerve regeneration

Three months after treatment with Imm-NN/ENF and Imm-NN/ES, the muscle sections immunostained for neurofilaments (NF) showed that Imm-NN/ENF resulted in more extensive axonal regeneration as compared with Imm-NN/ES. By counting NF-immunoreactive (NF-ir) axons per field at 200× magnification, we determined that the mean number of the NF-ir axons in the NN/ENF treated muscles was 84% of the contralateral control and that in the NN/ES treated muscles was 81% of the control. The regenerated axons in the NN/ENF and NN/ES treated muscles are more abundant than those in the muscles reinnervated by Imm-NN surgery alone (76.8% of the control). No NF-ir axons were identified in 3-month denervated muscles. These findings indicate that the use of neurotrophic factors (i.e., NGF and FGF-2) or electrical stimulation had significant beneficial effects on axonal regeneration and improved outcomes of NMEG-NMZ surgery for muscle reinnervation. Figure 4 gives an example to show the regenerated axons in the right SM treated with Imm-NN/ENF and left control SM in a rat. Note that the regenerated axons from the implanted NMEG supply the denervated native motor zone within the target muscle.

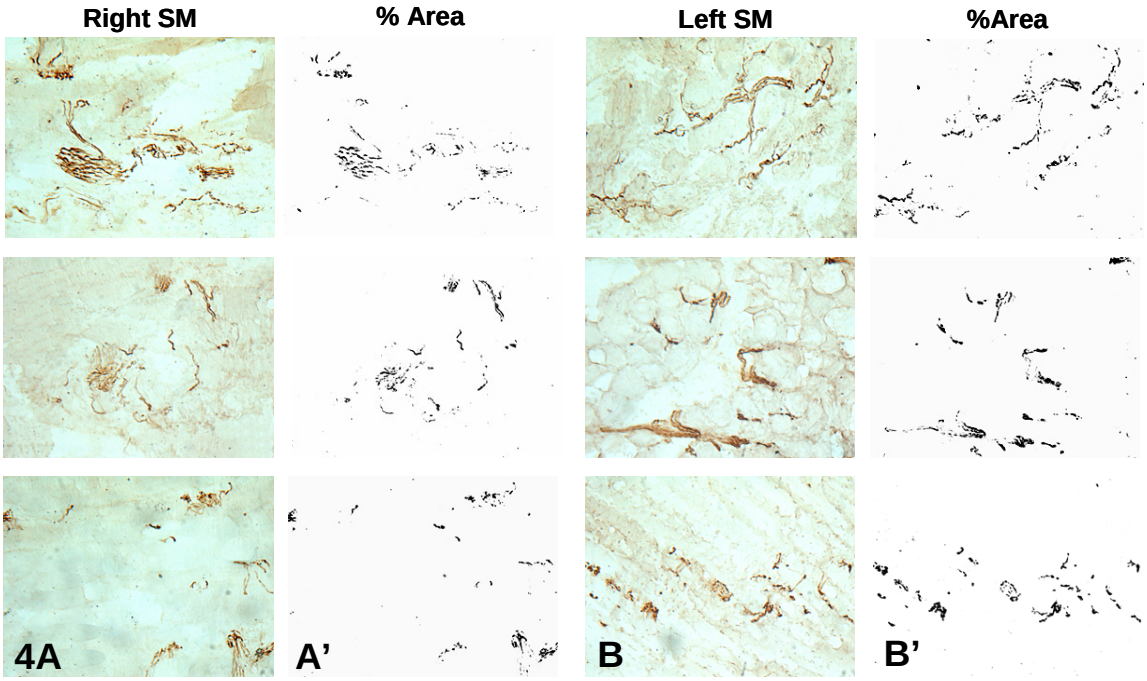


Fig. 4. Images of the immunostained sections of right Imm-NN/ENF treated (**A**) and left control (**B**) SM muscles of a rat. **A-B:** The sections were immunostained with antibody against neurofilament (NF) and photographed from ventral (top) to dorsal (bottom) aspects of the muscle. Note that the nerve fascicles and axons (darkly stained threads and dots) in the right Imm-NN/ENF treated SM are distributed throughout the thickness of the muscle. x200. **A'-B':** The stained sections in A and B were opened using ImageJ software and converted to 8-bit (binary) images, color thresholded, and particle analyzed for nerve morphometry. The density of the axons was evaluated by estimating the number and area fraction of the NF-positive axons within a section area (1.0 mm^2). For this animal (rat #13), as compared with the left control SM (mean axon count: 902; mean area: 0.899) the right SM exhibited very good muscle reinnervation as indicated by the mean axon count (779; 86% of the control) and the mean area (0.824; 92% of the control).

Motor endplate reinnervation

The muscle sections immunostained with double fluorescence staining showed the innervated and non-innervated motor endplates (MEPs). In the SM muscles treated with Imm-NN/ENF, the regenerating axons grew across the NMZ to innervate the denervated MEPs (**Fig. 5**). The majority of the denervated MEPs in the muscles treated with Imm-NN/ENF (86%) and with Imm-NN/ES (83%) were reinnervated by regenerating axons. In the treated muscles, axonal sprouts and newly formed small MEPs were also identified. In contrast, no axons were found in the 3-month-denervated muscles.

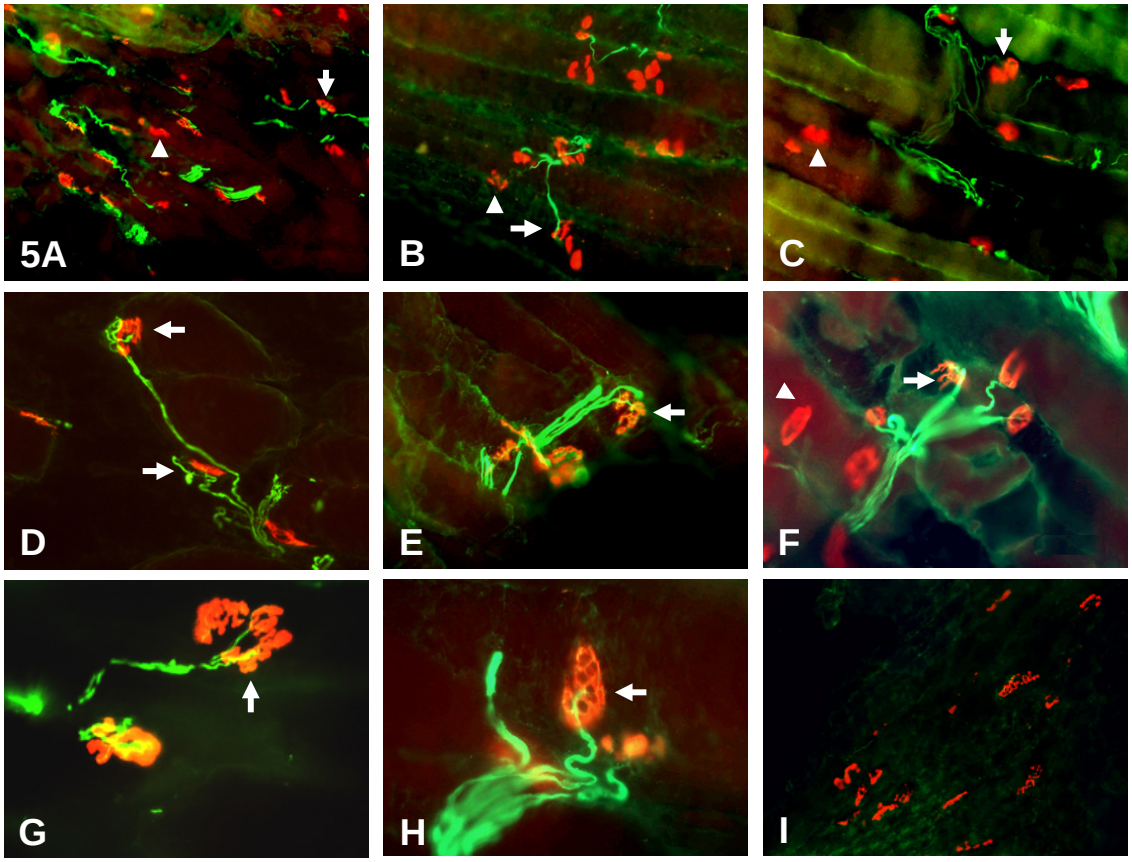


Fig. 5. Images of sagittal sections from right SM muscle of a rat treated with Imm-NN/ENF (**A-H**) and from a denervated SM muscle (**I**). The sections were taken from middle portion of the treated SM where the implanted NMEG was located. The sections were immunostained with double fluorescence staining showing axon-endplate connections. Regenerated axons (green) were detected with SMI-31 monoclonal against neurofilaments, while motor endplates (MEPs; red) were labeled with α -bungarotoxin. **A-C:** Low-power view of the stained sections of the treated SM (x100), showing the distribution of the MEPs and axon-MEP connections. Note that the regenerated axons branched extensively into fields of MEPs in the native motor zone of the target muscle and that the majority of the MEPs in the treated SM muscle were reinnervated by regenerated axons (arrows), while some MEPs in the same muscle were unoccupied by regenerated axons (arrowheads). **D-F:** High-power view of the stained sections, showing innervated (arrows) and non-innervated (arrowheads) MEPs (x200). **G-H:** High-power view of the treated SM, showing how the regenerated axons innervate the MEPs (x400). **I:** An image from a rat SM muscle denervated for 3 months (x200). Double fluorescence staining showed that the denervated MEPs were still present, but became fragmented. No axons were identified in the denervated muscle.

• **Summary**

Our studies performed in year 2 allow us to make the following conclusions.

First, the native motor zone (NMZ) of the target muscle is an ideal site for implantation of NMEG and for the development of novel strategies to treat muscle paralysis. For example, NMEG-NMZ resulted in better functional recovery (82% of the control; Brain and Behaviors, 2016 submitted) as compared with our originally designed NMEG [67% of the control; Neurosurgery 69(Suppl. 2):208-224, 2011]. In our originally designed surgery, the NMEG was implanted into an endplate-free area in the target muscle. More recently, we reported that direct nerve implantation into the NMZ of the denervated muscle also resulted in encouraging outcomes (J Reconstr Microsurg, 2016 in press).

Second, motor endplate (MEP) reinnervation is critical for motor functional recovery after peripheral nerve injury and nerve repair. We found that in the muscles reinnervated with NMEG-NMZ technique 80% of denervated MEPs regained motor innervation. This is most likely due to such a fact that the NMEG-NMZ procedure physically shortens regeneration distances and facilitates rapid MEP reinnervation, thereby avoiding irreversible loss of the denervated MEPs in the target muscle.

Third, the outcomes of NMEG-NMZ technique can be further improved by a combination of NMEG-NMZ technique with focal administration of exogenous neurotrophic factors (i.e., NN/ENF) and/or intraoperative 1-hour electrical stimulation (i.e., NN/ES). Our observations from the experiments performed in this reporting period demonstrated that NN/ENF or NN/ES cotreatment resulted in more optimal functional recovery as compared with Imm-NN alone. The excellent functional outcome should be attributed to the beneficial effects of ENF or ES on axonal regeneration and a high percentage of innervated MEPs.

Finally, our ongoing studies focus on determining if the NMEG-NMZ technique has the potential for delayed reinnervation that is not uncommon in clinical practice. For this purpose, it would be important to know the decreasing rate of the MEPs in the completely denervated muscle and determine the time point when all the denervated MEPs cannot be detectable in the target muscle. This information is useful for determining the best time point to perform NMEG-NMZ transplantation for delayed muscle reinnervation.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The following experiments will be performed during the next reporting period.

Force data analysis (3-mon-Del-NN group) – The collected force data from 3-mon-Del-NN group (30 muscles) will be analyzed.

Muscle force measurement (3-mon-Del-NN/ES group) – The animals in 3-mon-Del-NN/ES group (n = 15) will undergo force measurement (30 muscles) during the next reporting period.

Animal surgery (3-mon-Del-NN/ENF group) – The rats in 3-mon-Del-NN/ENF group (n=15) will undergo NMEG-NMZ transplantation and ENF injections (second surgery).

Tissue studies (3-mon-Del-NN group) – The muscle samples (n = 60; 4 samples/each rat) obtained from the animals in the 3-mon-Del-NN group (n = 15 rats) will be sectioned and stained to assess the extent of nerve regeneration after a combination of NMEG-NMZ transplantation with 1-hour electrical stimulation of the nerve branch supplying the NMEG pedicle.

***Manuscript preparation*:** Two manuscripts will be prepared for publication.

1. *Outcome of nerve-muscle-endplate band grafting technique for muscle reinnervation is improved by the use of nerve growth factor and FGF-2.*
2. *One-hour electrical stimulation during surgery enhances outcome of nerve-muscle-endplate band grafting technique for muscle reinnervation.*

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our findings demonstrated that native motor zone (NMZ) in the skeletal muscle is the best site for NMEG transplantation and for the development of other new reinnervation methods. The efficacy of the NMEG-NMZ reinnervation technique was enhanced by combined use of either local administration of exogenous neurotrophic factors or intra-operative 1-hour electrical stimulation. We believe that NMEG-NMZ will become a useful method in the near future to treat our patients with muscle paralysis.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Two papers resulting from the work under this award have been submitted to scientific journals for publication. One paper has been accepted for publication and another is under review. 1) Sobotka S, Chen J, Nyirenda T, Mu L. Outcomes of muscle reinnervation with direct nerve implantation into the native motor zone of the target muscle. Journal of Reconstructive Microsurgery (in press), 2016. Acknowledgement of federal support (yes). 2) Mu L, Sobotka S, Chen J, Nyirenda T. Reinnervation of denervated muscle by implantation of nerve-muscle-endplate band graft to the native motor zone of the target muscle. Brain and Behavior (under review), 2016. Acknowledgement of federal support (yes).

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

* We participated in a conference and presented part of our data from this research.
1) “Muscle reinnervation: Modified nerve-muscle-endplate band grafting technique” by Mu L, Sobotka S, Chen J, Nyirenda T. Presented at the Gorge Perez Research Colloquium at the Seton Hall University, South Orange, NJ, April 22, 2016.

2) “Outcomes of muscle reinnervation with direct implantation into the native motor zone of the target muscle” by Sobotka S, Chen J, Nyirenda T, and Mu L. Presented at the Gorge Perez Research Colloquium at the Seton Hall University, South Orange, NJ, April 22, 2016.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*

- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Liancai Mu	no change
Stanislaw Sobotka	no change
Jingming Chen	no change
Themba Nyirenda	no change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report." If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported

previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

A quad chart has been updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

In Appendices, one paper accepted for publication in Journal of Reconstructive Microsurgery has been attached.

Sobotka S, Chen J, Nyirenda T, Mu L. Outcomes of muscle reinnervation with direct nerve implantation into the native motor zone of the target muscle. Journal of Surgical Research (accepted), 2016. Acknowledgement of federal support (yes).

Reinnervation of Paralyzed Muscle by Nerve-Muscle-Endplate Band Grafting

ERMS Number 12223004
W81XWH-14-1-0442

PI: Liancai Mu, MD, PhD **Org:** Hackensack University Medical Center **Award Amount:** $800,000

Study/Product Aim(s)

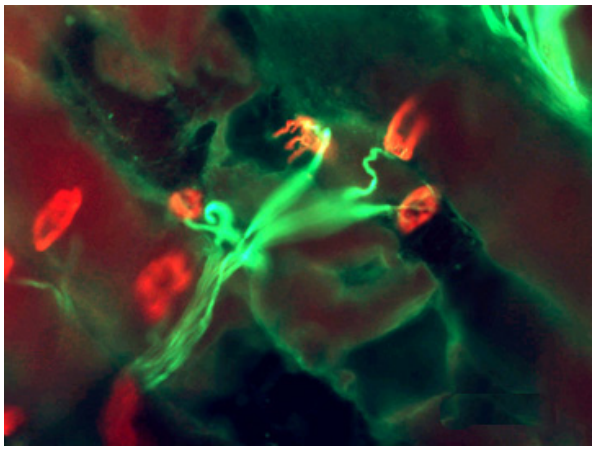
Aim 1: To evaluate functional recovery of the paralyzed muscles treated by NMEG-NMZ technique with/without ES and ENF.

Aim 2: To determine the extent of neural regeneration and axon-endplate connections in the treated muscles.

Aim 3: To document histological and immunohistochemical alterations in the treated muscles.

Approach

- Microsurgical procedures (NMEG-NMZ and DNI techniques).
- Intra-operative 1-hour electrical stimulation (ES).
- Focal administration of exogenous NGF and FGF-2.
- EMG and muscle force measurements.
- Various staining methods to label regenerating axons, MEPs.
- Analyze muscle fiber types and myosin heavy chains.



Innervated and non-innervated motor endplates (red) in the muscles treated with NMEG-NMZ plus local administration of ENF

Timeline and Cost

Activities CY	1	2	3	4
Surgery				
Physiological evaluations				
Neural studies				
Muscle studies & data analyses				
Estimated Budget ($K)	**$200k**	**$200k**	**$200k**	**$200k**

Updated: (10/10/2016)

Goals/Milestones

CY1 Goal – Microsurgery,functional evaluations, and neural studies.
☐ Perform surgeries, muscle force measurement, and nerve staining.
CY2 Goal – Microsurgery, functional evaluations, and tissue studies.
☐ Perform surgeries, force and tissue studies.
CY3 Goal – Functional evaluation and tissue studies.
☐ Complete surgeries and perform force and tissue studies.
CY4 Goal – Tissue studies, data collection, and data analyses.
☐ Investigate nerve regeneration and muscle fiber type and myosin heavy chain composition.

Comments/Challenges/Issues/Concerns

If timelines change, comment here.
If off by more than one quarter in spending, comment here.

Budget Expenditure to Date

Projected Expenditure: $50,000
Actual Expenditure: **$35,521**

Outcomes of Muscle Reinnervation with Direct Nerve Implantation into the Native Motor Zone of the Target Muscle

Stanislaw Sobotka, PhD, DSc[1,2] Jingming Chen, MD[1] Themba Nyirenda, PhD[1] Liancai Mu, MD, PhD[1]

[1] Department of Research, Hackensack University Medical Center, Upper Airway Research Laboratory, Hackensack, New Jersey
[2] Department of Neurosurgery, Icahn School of Medicine at Mount Sinai, New York, New York

Address for correspondence Stanislaw Sobotka, PhD, DSc, Department of Research, Hackensack University Medical Center, 30 Prospect Ave, Hackensack, NJ 07601 (e-mail: stanislaw.sobotka@mountsinai.org).

J Reconstr Microsurg

Abstract

Background Our recent work has demonstrated that the native motor zone (NMZ) within a given skeletal muscle is the best site for muscle reinnervation. This study was designed to explore the outcomes of direct nerve implantation (DNI) into the NMZ of denervated sternomastoid (SM) muscle in a rat model.

Methods The right SM muscle was experimentally denervated by transecting its innervating nerve. The proximal stump of the severed SM nerve was immediately implanted into a small muscle slit made in the NMZ of the muscle where denervated motor endplates were concentrated. The outcomes of DNI-NMZ reinnervation were evaluated 3 months after surgery. Specifically, the degree of functional recovery was examined with muscle force measurement. The extent of nerve regeneration and endplate reinnervation was assessed using histological and immunohistochemical methods.

Results This study showed that the mean muscle force of the treated muscles was 64% of the contralateral control. Reinnervated SM muscles weighed 71% of the weight of the control muscles. Abundant regenerated axons were identified in the NMZ of the target muscle. The mean number and area of the regenerated axons in the treated muscles was computed to be 62% and 51% of the control muscles, respectively. On average, 66% of the denervated endplates in the treated muscles were reinnervated by regenerated axons.

Conclusion Our results suggest that the NMZ within a muscle is an ideal site for endplate reinnervation and satisfactory functional recovery. Further studies are needed to promote the efficacy of DNI-NMZ technique for muscle reinnervation.

Keywords
- direct nerve implantation
- nerve regeneration
- muscle force measurement

Peripheral nerve injuries (PNIs) are very common in both military[1] and civil[2,3] circumstances. Current nerve repair methods include nerve end-to-end anastomosis, end-to-side neurorrhaphy, nerve grafting, nerve transfer, muscular neurotization, tubulization techniques, and many others.[4–9] Unfortunately, the currently used methods result in poor muscle reinnervation and functional recovery. Therefore, there is a great need to seek new strategies for the treatment of PNI-related muscle paralysis.

Direct nerve implantation (DNI) or muscular neurotization is commonly performed when the distal stump of the injured nerve is not available for nerve repair.[8,10] The proximal stump

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of the original nerve or a healthy but less valuable foreign motor nerve can be implanted into a denervated muscle to restore its motor function. DNI has been used for selective reinnervation of paralyzed laryngeal and facial muscles[11,12] as well as the extremities.[8,10] However, further studies are needed to determine the potential of DNI in muscle reinnervation and functional recovery.

Our recent study has demonstrated that the native motor zone (NMZ) of the target muscle is the best site for muscle reinnervation.[13] The concept is that the NMZ in a muscle contains numerous motor endplates which are preferentially reinnervated. Previous studies showed that after nerve injury regenerating axons preferentially grow into and reinnervate the regions of the original endplates.[14–18] Using DNI model, some investigators observed preferential reinnervation of the native endplates in the target muscle by abundant regenerating axons and sprouts.[19,20] However, little is known whether DNI-NMZ reinnervation could result in satisfactory functional recovery.

This study was designed to test our hypothesis that better functional outcome could be achieved by implanting a nerve stump into the NMZ of the target muscle. The reinnervated muscles were assessed using morphological, immunohistochemical, and electrophysiological techniques to determine the extent of muscle reinnervation and functional recovery.

Materials and Methods

experiments were performed on 3-month-old female Sprague-Dawley rats (Taconic Laboratories, Cranbury, NJ) with body masses ranging from 200 to 250 g at initial operation. The experiments and procedures were ethically reviewed and approved by the Institutional Animal Care and Use Committee prior to the onset of experiments. All animals were handled in accordance with the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication no. 85–23, revised 1996). The animals were housed at a constant temperature (22°C) on a 12 hour light–dark cycle and were provided with food and water in the state of the art animal housing facilities of Hackensack University Medical Center.

Direct Nerve Implantation-Native Motor Zone Procedures

A total of 15 animals were used to perform the DNI-NMZ procedure. The right sternomastoid (SM) muscle was experimentally denervated and reinnervated with DNI-NMZ. As SM muscle model was used in our previous muscle reinnervation studies,[21–27] there is a solid database regarding its innervation pattern and contractile properties available for comparison.

Surgical procedures were conducted under aseptic conditions. Animals underwent general anesthesia with a mixture of ketamine (80 mg/kg body weight) and xylazine (5 mg/kg body weight) administered intraperitoneally. A midline cervical incision was made extending from the hyoid bone to the sternum to expose the right SM muscle and its innervating nerve under an operating microscope. The right SM muscle was denervated by transecting its innervating nerve at its entrance to the muscle. The proximal stump of the severed nerve was immediately buried into a small slit made in the NMZ of the denervated SM muscle and secured in position with an epineurial suture of 10–0 nylon (►**Fig. 1**). After surgery, the wound was closed.

Additional control study was run on 17 denervated rats in an identical experimental setup to that described earlier. The only difference between the surgery in the control and the DNI-NMZ groups was that in the control group after denervation rats were not subjected to the DNI-NMZ reinervation. The right SM muscle was denervated by resecting a 5 mm segment of its innervating nerve and the cut ends of the nerve were then coagulated with a bipolar cautery to prevent nerve regeneration.

At the end of the 3-month recovery period, all experimental animals underwent postoperative evaluations to assess functional recovery, nerve regeneration, and muscle reinnervation. For each animal, contralateral intact SM served as a control.

Maximal Tetanic Force Measurement

The degree of functional recovery of the reinnervated SM was detected using muscle force measurement as previously reported in our previous publications[21,24–27] and others.[12,28,29] Briefly, SM was exposed and dissected. The rostral tendon of the muscle was severed close to the insertion, tied with a 2–0 suture, and connected to a servomotor lever arm (model 305B Dual-Mode Lever Arm System; Aurora Scientific Inc, Aurora, Ontario, Canada). The SM nerve on each side was stimulated using a bipolar stimulating electrode.

A stimulation and recording system (National Instruments Corp, Austin, TX) controlled by user-written LabVIEW software (National Instruments Corp) was used to deliver biphasic rectangular pulses to the nerve stimulated. Isometric contraction of the SM muscle was produced with 200-millisecond trains of biphasic rectangular pulses. The duration of each phase of stimulation pulse was set at 0.2 milliseconds and the train frequency was set at 200 pulses per second. The stimulation current was gradually increased until the tetanic force reached a plateau. A break of at least 1 minute was taken before the next measurement was attempted. The maximum value of muscle force during the 200-millisecond contraction was identified, as well as initial passive tension before stimulation. The difference between the maximal active force and the preloaded passive force was used as the muscle force measurement. The force generated by the contraction of the SM muscle was transduced with the servomotor of a 305B lever system and displayed on a computer screen. At the moment of force measurement, the lever arm was stationary, and the muscle was adjusted to the optimal length for the development of maximum force. During the experiment, the rat was placed supine on a heating pad, and the core body temperature was monitored with a rectal thermistor and maintained at 36°C. The muscle and nerve examined were bathed regularly with mineral oil warmed to 35°C.

The force data were obtained and processed by an acquisition system built from a multifunction I/O National Instruments Acquisition Board (NI USB 6251; 16 bit, 1.25 Ms/s;

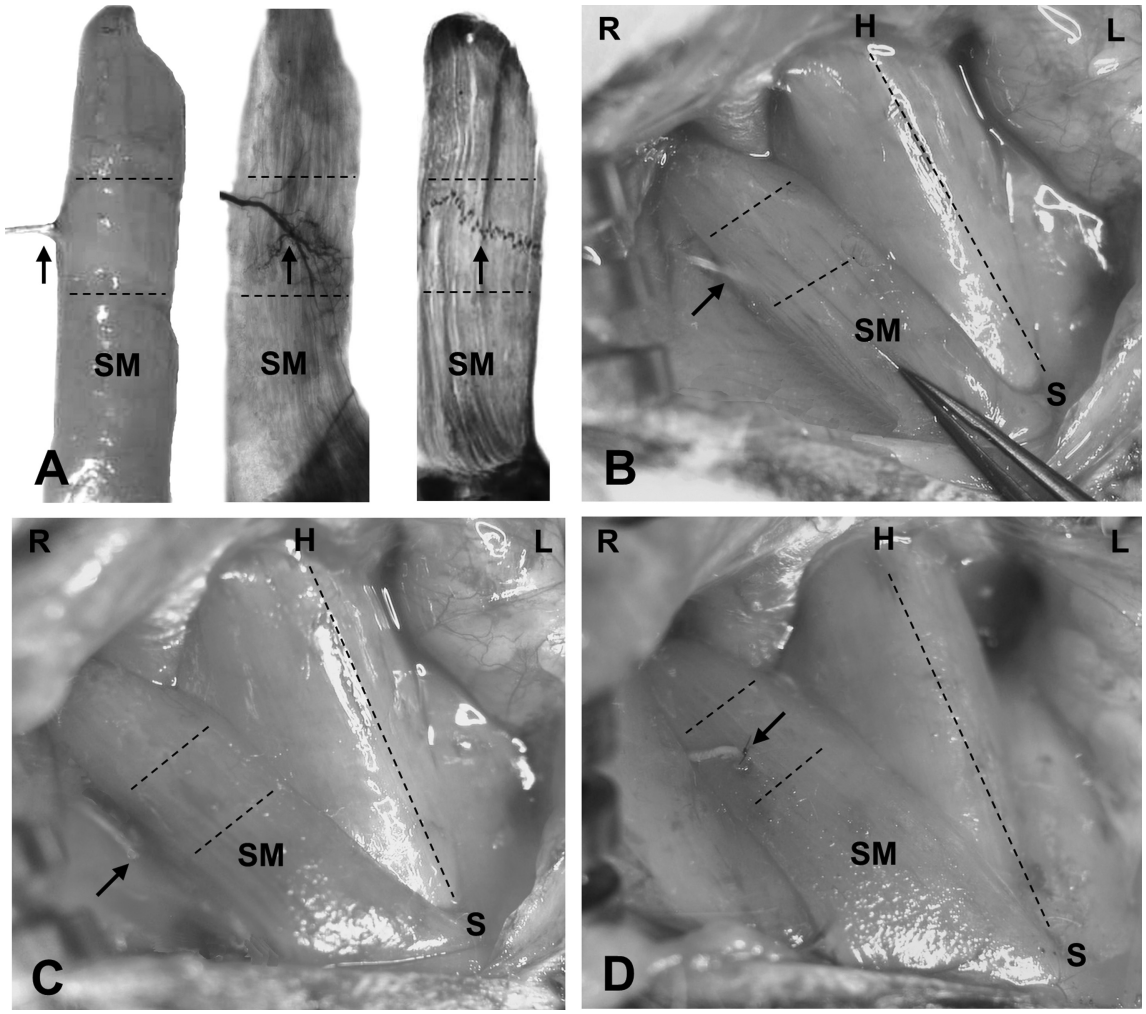

Fig. 1 Native motor zone (NMZ) of sternomastoid (SM) muscle and surgical procedures for direct nerve implantation-native motor zone (DNI-NMZ) in the rat. (**A**) NMZ of the rat right SM in the middle portion of the muscle between dashed lines in fresh (left), Sihler's stained (middle), and acetylcholinesterase(right) stained muscles. Note that SM nerve enters the NMZ on the lateral margin of the muscle (arrow in the left image). The NMZ contains numerous intramuscular twigs and nerve terminals (middle image) and a motor endplate band with numerous neuromuscular junctions (right image). (**B–D**) Photographs from a rat during DNI-NMZ surgery, showing the entrance point (arrow) of the SM nerve into the muscle (**B**), transected and isolated SM nerve (**C**), and implantation of the proximal stump of the severed SM nerve into the NMZ of the target muscle (**D**). The oblique dashed line in B–D indicates the midline. Note: H, hyoid bone; L, left; R, right; S, sternum.

National Instruments) connected to a Dell laptop with a custom-written program using LabVIEW 8.2 software. The system produced stimulation pulses, which, after isolation from the ground through an optical isolation unit (Analog Stimulus Isolator model 2200; AM Systems, Inc, Carlsborg, WA), were used for the current controlled nerve stimulation. The acquisition system was also used to control muscle length and to collect a muscle force signal from the 305B Dual-Mode Lever System. Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

Muscle Tissue Preparation

Immediately after force measurement, SM muscles on both sides in each animal were removed and weighed. Each muscle was divided into three segments: rostral, middle, and caudal. The muscle segments were frozen in melting isopentane cooled with dry ice and cut in a cryostat (Reichert-Jung 1800; Mannheim, Germany) at –25°C. For each muscle, the caudal and rostral segments were cut transversely (10 µm). The cross sections were stained with routine hematoxylin and eosin staining to examine muscle structure and myofiber morphology. The middle muscle segment was cut sagittally (60 µm) and immunostained to document nerve regeneration and reinnervation of the denervated endplates in the target muscle. For comparison, five right SM muscles denervated for 3 months by resecting a 5-mm segment of its innervating nerve in five additional rats were also prepared as described earlier and processed together with the experimental muscles.

Assessments of Regenerated Axons and Reinnervated Endplates

Neurofilament Immunostaining

Some sagittal sections were immunostained with a monoclonal antibody against phosphorylated neurofilament (NF)

(SMI-31, Covance Research Products, Berkeley, CA) as a marker for all axons as described in our previous publications.[13,30] Briefly, the sections were: (1) treated in 2% bovine serum albumin for 30 minutes, (2) incubated with primary antibody SMI-31 (dilution 1:800) in PBS containing 0.03% triton at 4°C overnight, (3) incubated for 2 hours with the biotinylated secondary antibody (anti-mouse, 1:1000, Vector, Burlingame, CA), (4) treated with avidin–biotin complex method with a Vectastain ABC kit (1:1000 ABC Elite, Vector), and (5) treated with diaminobenzidine-nickel as chromogen to visualize peroxidase labeling. Control sections were stained as described except that the incubation with the primary antibody was omitted.

The stained sections were examined under a Zeiss photomicroscope (Axiophot-2; Carl Zeiss, Gottingen, Germany) and photographed using a digital camera (Spot-32; Diagnostic Instruments, Keene, NH). The intramuscular axonal density was assessed by estimating the number of the NF-immunoreactive (NF-ir) axons and the area fraction of the axons within a section area (1.0-mm^2) as described in our previous publications.[13,25,27] For a given muscle, three sections stained for NF were selected at different spatial levels to count NF-ir axons. Areas with NF-positive staining were outlined, measured with public domain ImageJ software (v. 1.45*s*; NIH, Bethesda, MD).

Double Fluorescence Staining

Endplate reinnervation in the treated SM was detected using double fluorescence staining as described.[13] Briefly, some sagittal muscle sections were: (1) placed in Zamboni fixative with 5% sucrose for 20 minutes at 4°C, (2) treated with 0.1 mol/L of glycine in 1.5 T buffer for 30 minutes and dipped in 100% methanol at –20°C, (3) blocked in 1.5 T buffer containing 4% normal goat serum for 30 minutes, (4) incubated overnight at 4°C with primary antibodies (SMI-31 to detect neurofilaments and SMI-81 to label thinner axons; Covance Research Products Inc, Berkeley, CA), (5) incubated at room temperature for 2 hours both with a secondary antibody (goat anti-mouse antibody conjugated to Alexa 488) and with α-bungarotoxin conjugated with Alexa 596 (Invitrogen Corporation, Carlsbad, CA), and (6) coverslipped.

The stained sections were photographed. SMI-31 and SMI-81 detected axons (green), while α-bungarotoxin-labeled postsynaptic acetylcholine receptor site in the endplates (red). For each muscle sample, 50 labeled endplates were randomly selected to determine the percentages of the innervated (visible axon attachment) and noninnervated (no visible axon attachment) endplates.

Statistical Analysis

Muscle weights, force values, the number and the area fraction of NF-ir axons, and innervated and noninnervated endplates of the operated and unoperated SM muscles in each rat were computed. The variables of the reinnervated SM muscles were expressed as the percentages of the values of the contralateral control muscles. All data were presented as mean ± standard deviation. The Student *t* test (paired, 2-tailed) was used to compare differences in the mean values of the variables examined between operated and unoperated SM muscles. A difference was considered statistically significant at $p < 0.05$.

Results

Muscle Force Recovery

Muscle force data were successfully collected from both sides in 14 rats as the nerve on the operated side in one rat was damaged during muscle force recording. The degree of functional recovery of the reinnervated SM muscle was determined as compared with the force value of the contralateral control muscle in each rat. The DNI-NMZ reinnervated SM muscles produced 63.6% of the maximal tetanic tension of the contralateral control muscles (►**Fig. 2**). Averaged maximal muscle force at the operated side was 0.763 N, whereas 1.200 N at the contralateral control side. The difference between these averages (0.437 N) was statistically significant ($p = 0.0013$, $t = 4.08$, $df = 13$, two-sided t test). The average rate (calculated between operated and control muscles) was 0.640. This rate was statistically different from 1 ($p = 0.0022$, $t = 3.80$, two-sided single sample t test).

Muscle Mass and Morphology

At the end of experiment, muscle examination showed that the mass of the DNI-NMZ reinnervated muscle was smaller than that of the contralateral control (►**Fig. 3A** and **B**). The mean value of the wet weight of the reinnervated SM muscles (0.251 g) was smaller compared with that of the control muscles (0.352 g), but larger than that of the denervated SM muscles (0.199 g). Specifically, DNI-NMZ reinnervated SM muscles weighed 71% of the weight of contralateral control muscles (►**Table 1**). The SM muscles reinnervated with the DNI-NMZ technique and studied 3 months later were greater in size than the SM muscles left with complete denervation for 3 months. The mean percent rate of wet weight of DNI-NMZ reinnervated SM muscles in relation to normal contralateral side (71%, with standard deviation [STD] = 0.11, $n = 15$) was much higher than the rate of the denervated SM muscles in relation to normal contralateral side (44%, with STD = 0.20, $n = 17$; $t = 4.72$, $df = 30$, $p < 0.00001$). Man–Whitney U test confirmed strong significant difference in percentage rates between the two groups ($U_A = 223.5$, $U_B = 31.5$, $p = 0.00012$).

Stained histological sections showed that the DNI-NMZ reinnervated muscles exhibited slight-to-moderate fiber atrophy as compared with the controls (►**Fig. 3C** and **D**). In contrast, denervation resulted in significant myofiber atrophy as indicated by a reduction in fiber size and an increase in connective tissue (data not shown).

Nerve Regeneration and Endplate Reinnervation

Three months after surgery, muscle sections immunostained for NF exhibited abundant axons in the NMZ of the target muscle. The regenerating axons were derived from the implanted SM nerve and supplied the denervated NMZ of the treated muscle (►**Fig. 4A**). The density of the regenerated axons in the reinnervated muscles as indicated by the number

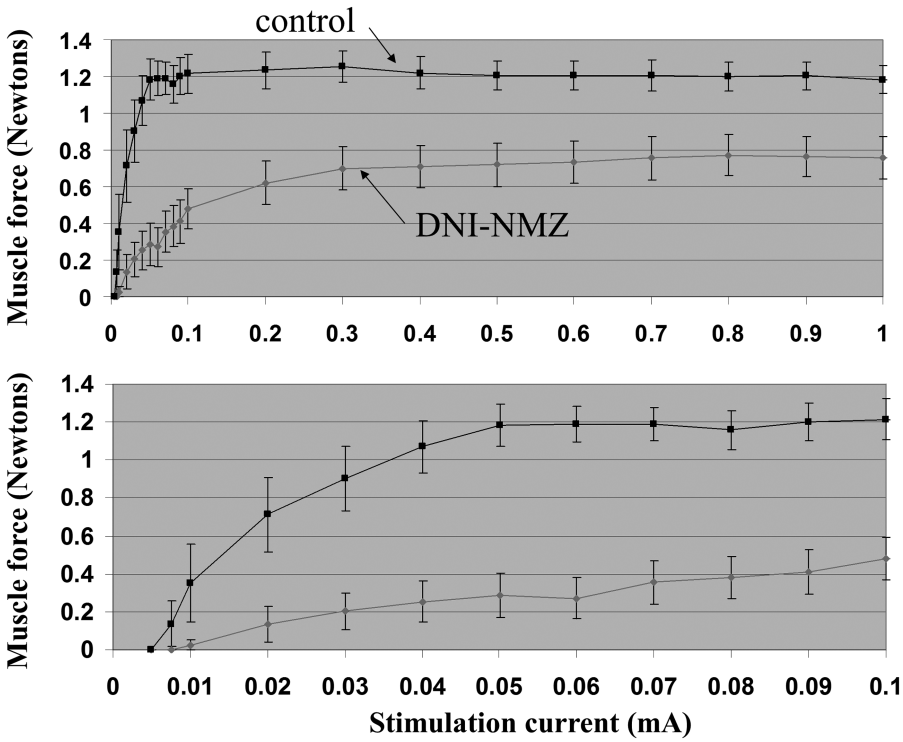

Fig. 2 Muscle force as a function of stimulation current in operated and control sternomastoid (SM) muscles. The passive tension was set at a moderate level (0.08 N). Stimulation was made with a 0.2-second train of 0.2-millisecond-wide biphasic pulses at frequency of 200 Hz. The lower graph shows, in expanded scale, the early portion of the upper graph. Note that operated SM muscle (with DNI-NMZ label) when compared with control muscle at the opposite side (with control label) has larger stimulation threshold (0.02 vs 0.0075 mA), reach the level of maximal force with larger stimulation current (0.3 vs 0.05 mA), and has smaller maximal force. Maximal muscle force level was calculated as the average muscle force to stimulation currents from 0.6–1 mA. Average maximal muscle force level at the operated side (0.76 N) was 63.6% of muscle force at the control side (1.20 N). Vertical bars represent the standard deviation of the mean.

and the area fraction of labeled axons was summarized in ►**Table 2**. The mean number and area of the NF-ir axons in the reinnervated SM muscles (►**Fig. 4A** and **4A'**) was computed to be 62% and 51% of the contralateral control muscles (►**Fig. 4B** and **4B'**), respectively.

Double fluorescence staining showed the innervated and noninnervated endplates. In the treated SM, the regenerated axons were identified in the NMZ of the target muscle to innervate the denervated endplates (►**Fig. 5**). On average, 66% of the denervated endplates in the treated muscles were reinnervated by regenerated axons and 34% of the endplates were unoccupied by axons. In addition, axonal sprouts and newly formed small endplates were identified in the DNI-NMZ reinnervated muscles.

Discussion

To our knowledge, this is the first study to explore whether DNI into the NMZ of the denervated muscle could restore better motor function in a rat model. There are several key findings. First, DNI-NMZ resulted in satisfactory functional recovery as indicated by muscle force measurement. Specifically, DNI-NMZ reinnervated SM muscles produced 64% of the maximal tetanic force of the contralateral control muscles 3 months after surgery. Second, the muscle mass (71% of the control) and myofiber morphology of the DNI-NMZ reinnervated muscles were preserved well. Third, reinnervated muscles gained abundant regenerated axons as indicated by the mean number (62% of the control) and area (51% of the control) of the NF-ir axons in the target muscle. Finally, 66% of the denervated endplates in the treated muscles were reinnervated by regenerated axons.

DNI into the denervated muscle (neurotization) has been used in animal studies and clinical practice to reinnervate denervated muscles.[8,10–12,31–36] Preclinical and clinical studies have demonstrated that the outcome of DNI is associated with the chronicity of denervation,[36] regeneration distance,[32,34,35] and surgical techniques.[35,37] However, little is known whether DNI-NMZ reinnervation results in satisfactory functional recovery.

The NMZ is generally located in the middle region of a skeletal muscle and contains an endplate band with numerous neuromuscular junctions and their innervating nerve terminals. As endplate reinnervation is critical for motor recovery, we implanted a nerve stump into the NMZ of the target muscle to determine the outcomes of DNI-NMZ procedure. The data from this study showed that DNI-NMZ resulted in better functional recovery (64% of the control) as compared with the method reported in other studies (50%), in which DNI was not specifically performed in the NMZ of the denervated muscles.[20,31,38] Our encouraging results should be attributed to such a fact that DNI-NMZ procedure shortens

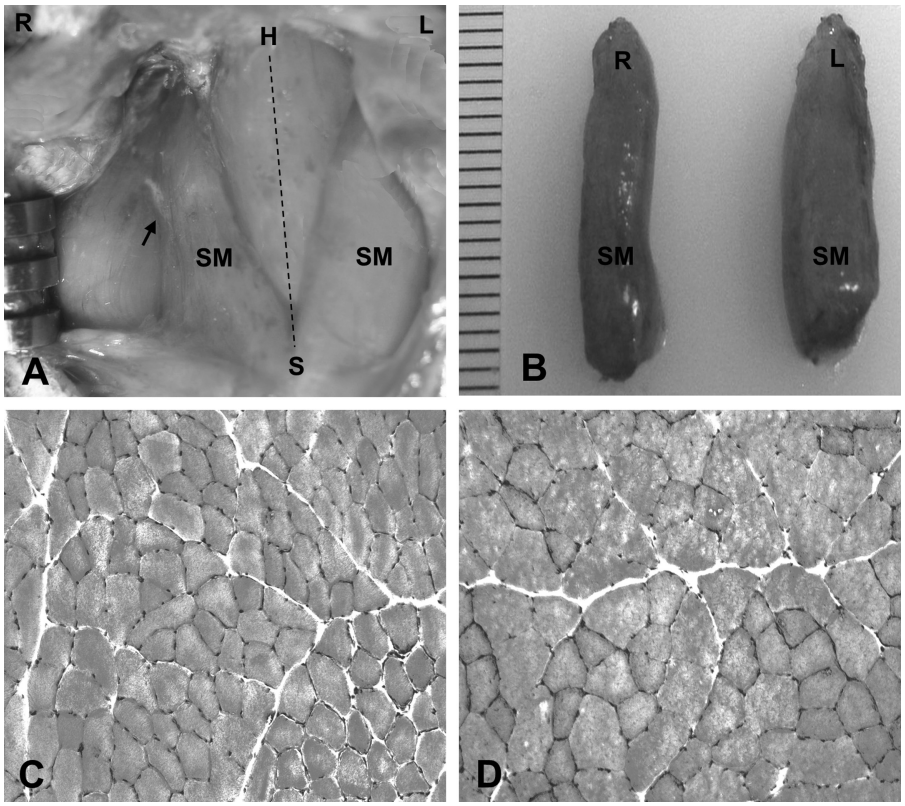

Fig. 3 Photographs of a pair of sternomastoid (SM) muscles removed from a rat 3 months after surgery, showing the morphological difference between the reinnervated and contralateral control muscles. (**A**) An image taken before removal of the SM muscles, showing the implanted SM nerve (arrow). (**B**) Removed SM muscles from the same animal in A. Note that the mass of the right (R) operated SM muscle (0.312 g) was smaller as compared with that of the left (L) control muscle (0.390 g). (**C–D**) Hematoxylin and eosin -stained cross sections from the SM muscles in B. Note that the right reinnervated muscle (**C**) exhibited mild to moderate myofiber atrophy as compared with the control (**D**). Initial magnification 200× for C and D.

Table 1 Comparison of wet muscle weight between right operated and left control sternomastoid (SM) muscles in rats ($n = 15$)

Animal (No.)	Body weight (g)	Right reinnervated SM (g)	Left intact SM (g)
1	353	0.234	0.352
2	347	0.250	0.392
3	343	0.220	0.312
4	340	0.286	0.382
5	327	0.275	0.298
6	322	0.300	0.417
7	355	0.277	0.355
8	328	0.212	0.390
9	316	0.160	0.282
10	332	0.229	0.310
11	355	0.210	0.361
12	378	0.253	0.356
13	349	0.299	0.341
14	407	0.312	0.390
15	380	0.255	0.336
Average	349	0.251	0.352
Ratio, %		71	100

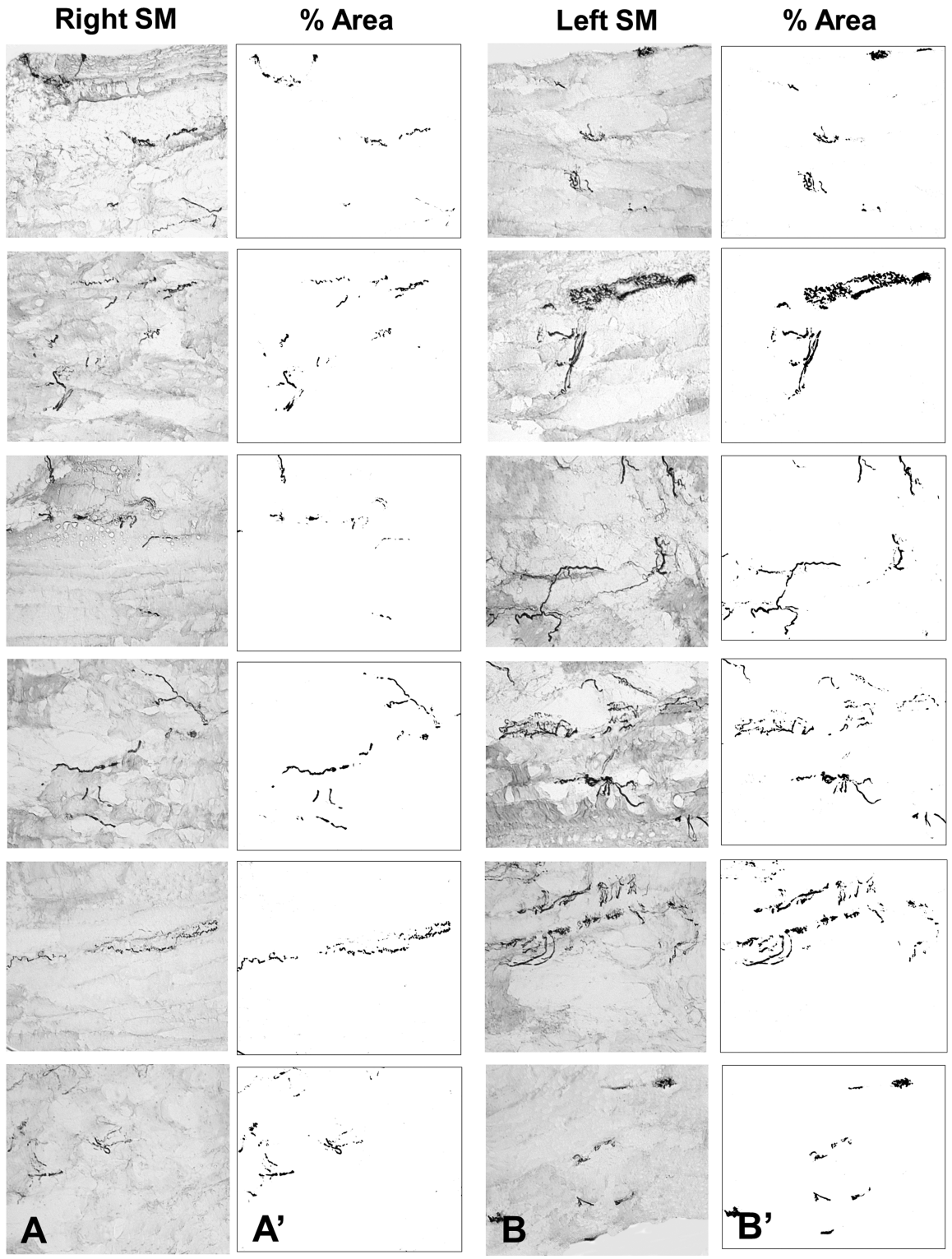

Fig. 4 Images of sagittal sections immunostained for neurofilament (NF) from the operated and unoperated sternomastoid (SM) muscles of the rat number 15. (**A**) Six microscopic fields in a stained section from the right operated SM (first column), showing the NF-positive axons (dark staining) in the native motor zone of the target muscle. Magnification 200×. (**A'**) The images in the first column were converted to black and white (second column) by use of ImageJ software to calculate the number and percent area of staining in each section (mean axon count, 533; 56% of the control; mean area, 0.636; 43% of the control). (**B–B'**) Images from the left unoperated SM (mean axon count, 974; mean area, 1.479). Magnification 200×.

the distance between the nerve implantation site and the denervated endplates in the target muscle. Therefore, the regenerating axons from the implanted nerve could easily reach and reinnervate the denervated endplates in the NMZ of the treated muscle. In contrast, if a nerve cut end is implanted into an area outside the NMZ it will take time to form new motor endplates and synapses. As reported, functional recovery may not be achieved for a long period after

Table 2 Comparison of count and percent area of neurofilament-positive axons between right operated and left control sternomastoid (SM) muscles in rats ($n = 15$)

Animal (No.)	Right SM		Left SM		Ratio (R/L)	
	Count	Percent area	Count	Percent area	Count	Percent area
1	511	0.412	1640	1.336	0.312	0.308
2	668	0.430	678	0.626	0.985	0.687
3	420	0.552	1160	1.145	0.362	0.482
4	300	0.456	720	0.566	0.417	0.806
5	473	0.762	510	0.908	0.927	0.839
6	441	0.560	1074	1.358	0.411	0.412
7	312	0.370	600	1.100	0.520	0.336
8	340	0.247	649	0.931	0.524	0.265
9	624	0.623	861	1.295	0.725	0.481
10	671	0.660	752	1.303	0.892	0.507
11	499	0.640	623	1.557	0.801	0.411
12	435	0.511	814	1.496	0.534	0.342
13	319	0.509	893	1.280	0.357	0.398
14	748	0.630	751	0.716	0.996	0.880
15	533	0.636	947	1.479	0.563	0.430
Average	486	0.533	845	1.140	0.622	0.506

Abbreviations: L, left; R, right.

DNI surgery.[35] Studies have demonstrated that after nerve injury, functional motor recovery is primarily determined by endplate reinnervation and the absolute number of regenerated motor axons that reach target.[39]

Although our experiments showed the potential of DNI-NMZ in immediate muscle reinnervation, this study also has some limitations. For example, postoperative evaluations were performed at the end of 3 months after surgery. Three-month recovery period may be not enough to provide a complete picture of what occurs after DNI-NMZ. Further studies are needed to determine morphological and functional alterations at different time points after DNI-NMZ reinnervation. This information would be helpful for better understanding the time-related changes of muscle reinnervation and functional recovery. In addition, it remains unknown if the DNI-NMZ has the potential for delayed reinnervation that is not uncommon in clinical practice. One of our ongoing studies is to determine the efficacy of DNI-NMZ for chronic muscle denervation. For this purpose, it is important to know the decreasing rate of the endplates in the completely denervated muscle and to determine the time point when all the endplates cannot be detected.

For future clinical application of this technique, our ongoing work is to promote the efficacy of DNI-NMZ reinnervation by further refining surgical procedure and accelerating

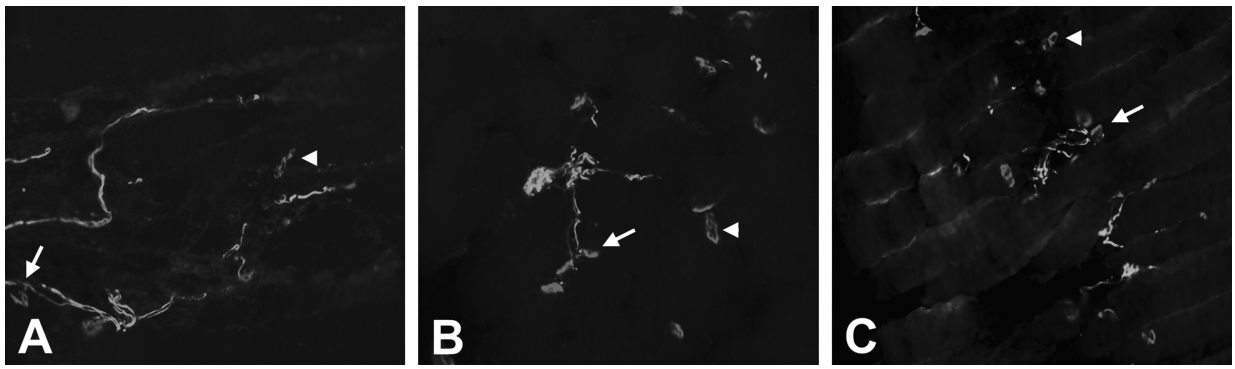

Fig. 5 Sagittal sections immunostained with double fluorescence staining of the right sternomastoid (SM) muscle reinnervated by direct nerve implantation-native motor zone in a rat, showing reinnervated and nonreinnervated motor endplates. Note that some of the endplates were reinnervated by the regenerated axons (arrows in **A–C**), while others in the same muscle were unoccupied by regenerated axons (arrowheads in **A–C**). Original magnification 200× for A through C.

axonal regeneration. For example, the nerve stump can be divided into two or more fascicles before implantation. Direct implantation of the divided nerve fascicles into the target muscle has shown to enhance end results.[8,20] This procedure can be applied to DNI-NMZ for better results. For accelerating axonal regeneration, a very brief 1-hour period of low-frequency (20 Hz) continuous electrical stimulation of the proximal nerve stump at the time of operation[40,41] and local administration of neurotrophic factors[42] could be combined with DNI-NMZ technique to enhance axonal regeneration. We believe that these approaches would promote the outcomes of the DNI-NMZ technique.

Conclusion

In summary, DNI-NMZ technique appears to be a promising reconstructive option for muscle reinnervation. DNI-NMZ has the potential for functional motor restoration of denervated muscles in certain conditions. For optimal outcome, further studies are needed to promote the efficacy of this technique.

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