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TITLE: Reinnervation of Paralyzed Limb Muscle by Nerve-Muscle-Endplate Grafting Technique

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14. ABSTRACT

In our previous annual report, we presented outcomes of the NMEG-NMZ technique and standard nerve-end-to-end anastomosis (EEA; technique control) for limb reinnervation. Overall, the NMEG-NMZ (NN) resulted in better functional recovery (79% of the control) compared to the EEA (51%). Here, we summarize the data obtained from animals in the Imm-NN/ENF, Imm-NN/ES, and Imm-ANG (technique control) groups. Three months after treatment, outcomes were evaluated by measuring toe spread distances and muscle force, quantifying the extent of axonal regeneration in the target muscle, determining the percentage of reinnervated motor endplates (MEPs), measuring muscle weight and examining fiber-type distribution. Our results showed that Imm-NN/ENF and Imm-NN/ES resulted in more optimal functional recovery (90% and 85%, respectively) as compared with NMEG-NMZ alone (79%) and Imm-ANG (46%). The results confirm that the adjunctive therapies ENF and ES have the potential for improving the outcomes of the NMEG-NMZ surgery in the rat *immediate limb reinnervation* model.

15. SUBJECT TERMS Nerve-muscle-endplate band grafting, peripheral nerve injury, limb reinnervation, axonal regeneration, motor endplate band, native motor zone, 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.*

Peripheral nerve injury (PNI) to the extremities is a significant cause of morbidity and disability in both military and civil circumstances. Unfortunately, currently available nerve repair methods result in poor functional recovery. This is due primarily to insufficient axonal regeneration and failure to reinnervate the denervated motor endplates (MEPs) in the target muscle. In response to this, we developed a new muscle reinnervation technique called nerve-muscle-endplate grafting (NMEG) (*Neurosurgery 69(Suppl. 2):208-224, 2011*). The underlying concept is that a paralyzed muscle could be reinnervated by transplanting an NMEG from a neighboring donor muscle. Using a rat neck muscle model, we performed a series of studies to investigate the NMEG technique. An NMEG pedicle containing a nerve branch and a muscle block with nerve terminals and motor endplates (MEPs) was harvested from the sternohyoid muscle and transplanted into the ipsilateral experimentally denervated sternomastoid muscle. Our subsequent studies have demonstrated that implantation of the NMEG pedicle to the native motor zone (NMZ) in the denervated muscle (*Brain Behav 7(6):e00668. doi:10.1002/brb3.668, 2017*) resulted in better muscle reinnervation and functional recovery than implantation of the NMEG pedicle to a MEP-free area (*Neurosurgery 69(Suppl. 2):208-224, 2011*). These findings indicate that denervated MEPs in the NMZ are preferential sites for reinnervation and that NMEG-NMZ technique would be useful for restoring motor function following PNI. An NMEG pedicle contains an abundant source of nerve terminals that favor axonal regeneration. Implantation of the NMEG pedicle to the NMZ of the target muscle facilitates rapid axon-MEP connections, thereby resulting in optimal functional recovery. In this research, we use the NMEG-NMZ technique to treat denervated tibialis anterior muscle for determining if this surgical procedure is effective for limb reinnervation.

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

Nerve-muscle-endplate grafting (NMEG), native motor zone (NMZ), peripheral nerve injury, limb reinnervation, axonal regeneration, axon-endplate connection, functional recovery

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

What were the major goals 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.

Aim 1: Evaluate the efficacy of NMEG-NMZ for immediate reinnervation of denervated limb muscle, and determine the beneficial effects of nerve growth-stimulating methods (i.e., intraoperative brief ES and local administration of the ENFs NGF and FGF-2) on axonal regeneration and outcomes of NMEG-NMZ surgery (100% completed).

Aim 2: Evaluate the efficacy of NMEG-NMZ for reinnervation of chronically denervated limb muscle, and assess the potential of specific therapeutic strategies for preservation of muscle mass and MEPs prior to NMEG-NMZ.

Major Task 2.1: Perform TA muscle denervation surgeries, 3-month delayed (Del) reinnervation surgeries, and execute techniques with potential to preserve muscle mass and MEPs.

Subtask 2.1.1: Perform defect surgeries in rats to denervate the tibialis anterior (TA) muscles and postpone rennervation for three months before treating defects using NMEG-NMZ and two technique controls. Groups: Del-NN, Del-EEA, and Del-ANG [n=45 total rats, 15 per group]. (50% completed)

Subtask 2.1.2: Conduct denervation control surgeries, ligating both nerve ends to avoid regeneration for 6 months [n=15 total rats]. (30% completed)

The proposed work is to test our hypothesis that NMEG-NMZ technique would be effective for reinnervation of denervated limb muscle, and that the outcomes of NMEG-NMZ could be augmented by incorporating specific therapeutic strategies that enhance nerve regeneration as well as preserve muscle mass and MEPs in the target muscle. For NMEG-NMZ, we harvested an NMEG pedicle from lateral belly of the gastrocnemius muscle (GM-l) to reinnervate the experimentally denervated ipsilateral tibialis anterior (TA) muscle. Adjunctive therapies used in this research include intraoperative brief electrical stimulation (ES) and local application of exogenous neurotrophic factors (ENF). For comparison, we used standard nerve end-to-end anastomosis (EEA) and autologous nerve graft (ANG) as technique controls. We also use 3-month and 6-month denervation as controls.

We use two reinnervation models: immediate and 3-mo delayed. Immediate reinnervation is performed immediately after TA nerve transection, while delayed reinnervation is carried out at the end of 3 months after TA nerve transection. The animals are randomly assigned to 12 groups (15 rats/per group), 6 immediate and 6 delayed reinnervation groups.

Immediate (Imm) reinnervation groups: (1) Imm-NMEG-NMZ (Imm-NN); (2) Imm-NN/ES; (3) Imm-NN/ENF; (4) Imm-EEA; (5) Imm-ANG; and (6) 3-month denervation.

Delyed (Del) reinnervation groups: (1) Del-NN; (2) Del-ENF/NN; (3) Del-sensory protection/NN (SP/NN); (4) Del-EEA; (5) Del-ANG; and (6) 6-month denervation.

The experimental animals undergo post-operative evaluations after a 3-month recovery period. The post-operative evaluations include functional assessment (i.e., static toe spread analysis and muscle force measurement), neural studies (i.e., axonal regeneration and axon-MEP connections), and muscle studies (i.e., muscle mass, fiber size, fiber type and myosin heavy chain composition). The data from this research are expected to provide evidence for the effectiveness of the NMEG-NMZ and adjunctive therapies for limb reinnervation.

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**

The following major activities were carried out over the past year.

Animal surgeries

We performed 120 operations on 45 rats in 3 delayed reinnervation groups (15 rats/per group) that include: (1) 6-month denervation (6-mon-Den); (2) Del-NN; and (3) Del-ENF/NN. Each rat in the 6-mon-Den group received 2 operations (total: 30 operations), while each animal in the Del-NN and Del-ENF/NN groups had 3 operations (total: 90 operations). These surgical procedures were successfully performed.

Static toe spread analysis

All the experimental rats underwent static toe spread analysis (STSA) to assess behavior motor functional recovery of the hind limbs. In this study, STSA was performed just before muscle force measurement at the end of the 3-month recovery period. For STSA, the rat was placed in an acrylic 40×20×20 cm container on a transparent base plate for observing footprints on the plantar view. A camera was positioned underneath the base plate to photograph the plantar surface of the rat hind limb paws. Images from the operated (O) and non-operated (N) footprints were randomly selected to measure the distance (mm) between the first and fifth toe spread (TS) and between the second and fourth intermediate toe spread (ITS). The mean values of TS and ITS in each group were computed and used for determining the TS and ITS factors (TSF and ITSF): TSF = (OTS – NTS)/NTS and ITSF = (OITS – NITS)/NITS. The ratios of the static sciatic index (SSI) were calculated using the following equation: SSI = (108.44 × TSF) + (31.85 × ITSF)–5.49. An index score of 0 is defined as normal and an index score of −100 indicates a complete functional loss.

Muscle force measurements

All animals in the Del-NN and Del-ENF/NN groups (n = 30 rats) were subjected to muscle force measurement 3 months after muscle reinnervation to evaluate functional recovery.

The force data from animals in Del-NN and Del-ENF/NN groups have been collected and will be analyzed in the following year.

The force data from the animals in Imm-NN and Imm-EEA have been given in Y1 annual report and a manuscript was submitted to *NEUROSUERY* for publication (*see Item 6. Products-Journal Publications*).

The force data from the animals in Imm-NN/ENF, Imm-NN/ES, and Imm-ANG groups have been analyzed and the key findings are summarized below (*see Key Outcomes*).

Tissue studies

After muscle force measurement, the left experimental TA and right control for each rat were removed, measured, and prepared for tissue studies. The muscle samples were sectioned and stained using histological and immunohistochemical techniques.

In this report, the major findings from the animals in Imm-NN/ENF, Imm-NN/ES, and Imm-ANG groups are summarized below (*see Key Outcomes*). The tissue samples from other groups will be processed and analyzed in the following year.

● **Specific Objectives**

The results from the animals in Imm-NN/ENF, Imm-NN/ES, and Imm-ANG groups have been analyzed to determine the differences in the outcomes among these groups and the major findings are presented in the Key Outcomes.

Objective 1: Determine the degree of functional recovery of the denervated TA muscles treated by Imm-NN/ENF, Imm-NN/ES, and Imm-ANG techniques.
Objective 2: Determine the extent of axonal regeneration and axon-MEP connections in the TA muscles treated by Imm-NN/ENF, Imm-NN/ES, and Imm-ANG techniques.
Objective 3: Document morphological and histological changes (i.e., muscle mass, fiber size, and fiber type and myosin heavy chain composition) in the treated TA muscles.

● **Major Procedures**

In the past year, animals in Del-NN, Del-ENF/NN, and 6-mon-Den groups (n = 45) underwent surgical procedures as described below. In the delayed (Del) reinnervation groups, all treatments were given three months after denervation of the left TA.

Del-NN

Animals in Del-NN group (n = 15) underwent NMEG-NMZ 3 months after denervation of the left TA by excising a 10-mm segment of its nerve. The details regarding NMEG-NMZ procedures have been given in our Y1 annual report. Briefly, a muscular defect, with the same dimensions as the NMEG pedicle, was made in the NMZ of the left denervated TA. An NMEG pedicle containing a block of muscle, axon terminals and a MEP band was harvested from the NMZ of GM-l in continuity with its nerve branch. Finally, the NMEG pedicle was embedded into the TA defect and sutured with 10-0 nylon.

Del-ENF/NN

The rats in Del-ENF/NN group (n =15) received local injection of ENFs (i.e., NGF and FGF-2). Specifically, immediately after left TA denervation by resecting a 10-mm segment of its nerve, the NMZ of the denervated TA were injected with 0.5 ml of a mixture of NGF (100 ng/ml) and FGF-2 (100 μg/ml) biweekly for 3 months (**Fig. 1**), followed by NMEG-NMZ.

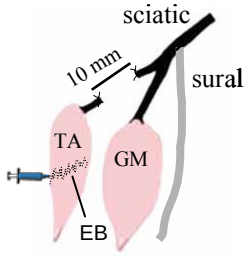


Fig. 1. Schematic showing injection of NGF/FGF-2 into the endplate band (EB) in the denervated TA muscle.

6-Mon-Den (control)

The animals in 6-mon-Den group (n = 15) underwent denervation of the left TA by excising a 10-mm segment of its nerve for six months without any treatments. At the end of experiment, the TA muscles on both sides were removed, weighed, photographed, and prepared for tissue studies.

The data from the three groups (i.e., Del-NN, Del-ENF/NN, and 6-mon-Den) are being collected and analyzed. The results will be reported in Y3 annual report, followed by a manuscript submission to academic journals for publication.

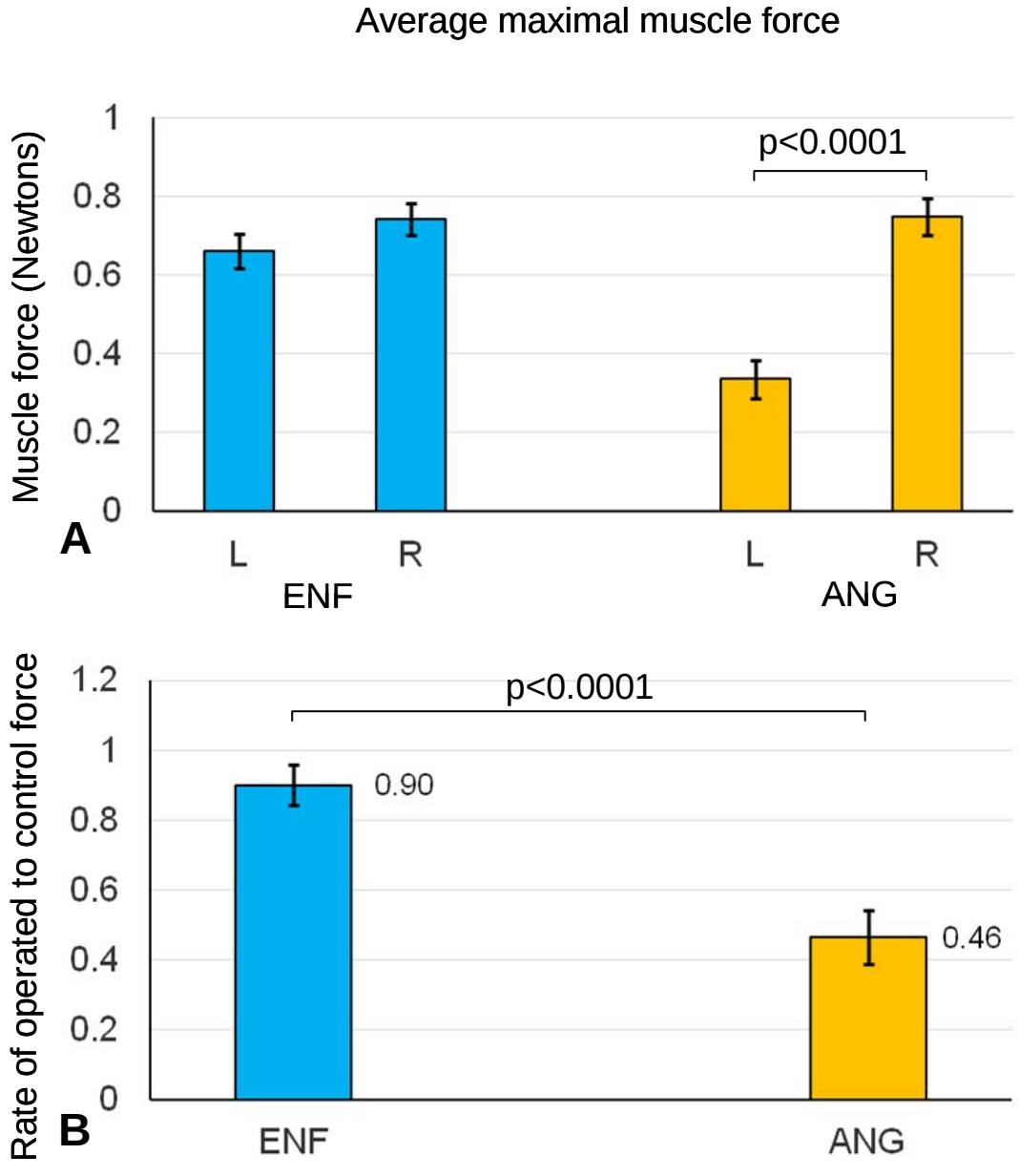
● **Key Outcomes**

The major findings from the rats in Imm-NN, Imm-EEA, and 3-mon-Den groups have been presented in Y1 annual report and a manuscript has been submitted to *NEUROSURGERY* for publication (*see Item 6. Products-Journal Publications*).

The data from the rats in Imm-NN/ENF, Imm-NN/ES, and Imm-ANG groups have been collected, analyzed, and summarized below. A manuscript will be submitted to an academic journal for publication in the following year.

- Functional Recovery

i) Maximal muscle force: The force data from the animals in Imm-NN/ENF, Imm-NN/ES, and Imm-ANG groups showed that Imm-NN/ENF and Imm-NN/ES resulted in better outcomes as compared with Imm-ANG. We found that ENFs and ES improved the surgical outcomes of NMEG-NMZ. Three months after NN/ENF or NN/ES treatment, the muscle force of the treated TA was improved. Specifically, the mean muscle force of the NN/ENF treated TA (90% of the control) was similar to that of the NN/ES treated TA (85%), but much better than that of the ANG repaired muscle (46%) (**Fig. 2**). The NN/ENF and NN/ES treated muscles resulted in better functional recovery as compared with NMEG-NMZ alone (79%) and standard EEA (51%) (*Sobotka S, Mu L, Chen J, Li J, Nyirenda T. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. Neurosurgery (under review, 2022.*).



ii) Static toe spread analysis: Static toe spread analysis (STSA) was used for assessing limb function after treatments. Our data showed that toe spread of the rats in Imm-NN/ENF and Imm-NN/ES groups recovered almost up to normal level (**Fig. 3A-B**). However, Imm-ANG resulted in poor toe spread (**Fig. 3C**).

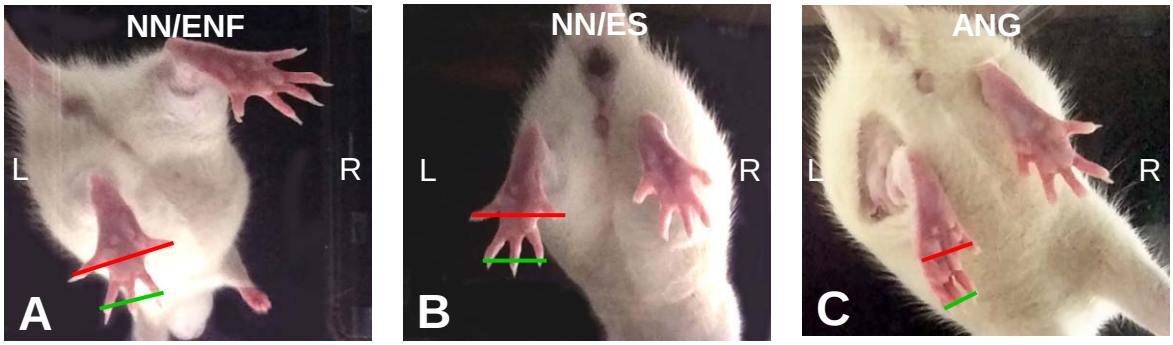


Fig. 3. Comparison of hind limb footprints between left (L) operated and right (R) non-operated sides in the rats with Imm-NN/ENF (**A**), Imm-NN/ES (**B**), and Imm-ANG (**C**). Note that NN/ENF and NN/ES resulted in better toe spread recovery as compared with ANG.

- Muscle Weight and Myofiber Morphology

For each rat, the TA muscles on both sides were removed and weighed at the end of experiments. Here, muscle weights from the animals in Imm-NN/ENF group are given in **Table 1**, while those from the animals in Imm-NN/ES and Imm-ANG groups are not shown.

Our results showed that the average muscle weight recovered up to 87% of the control for Imm-NN/ENF group (**Table 1**), 85% for Imm-NN/ES group, and 52% for Imm-ANG group (individual values not shown). Gross appearance and muscle volumes of the treated TA muscles and contralateral controls are shown in **Figure 4A-C**. H&E stained cross-sections showed that the TA muscles treated with NN/ENF and NN/ES exhibited very good preservation of muscle structure and myofiber morphology with less fiber atrophy and connective tissue hyperplasia (**Fig. 4A'-B'**) as compared with the ANG treated TA (**Fig. 4C'**). These findings demonstrated that the combination of the NMEG-NMZ surgery with the ENF or electrical nerve stimulation (ES) resulted in better preservation of the muscle mass as compared with ANG.

Table 1. Wet muscle weight measurement for the left reinnervated with NN/ENF and right control tibialis anterior (TA) muscles in rats (n = 15)

Animal no.	Body weight, g	Left TA g	Right TA g	Ratio L/R
1	331	0.52	0.63	0.83
2	401	0.61	0.64	0.95
3	385	0.47	0.61	0.77
4	400	0.64	0.71	0.90
5	312	0.54	0.61	0.89
6	297	0.45	0.56	0.80
7	331	0.46	0.54	0.85
8	294	0.54	0.56	0.96
9	360	0.55	0.61	0.90
10	345	0.52	0.65	0.80
11	348	0.45	0.55	0.82
12	345	0.60	0.67	0.90
13	305	0.57	0.59	0.97
14	307	0.46	0.52	0.88
15	283	0.40	0.48	0.83
Average	336	0.52	0.60	0.87

L, left; R, right.

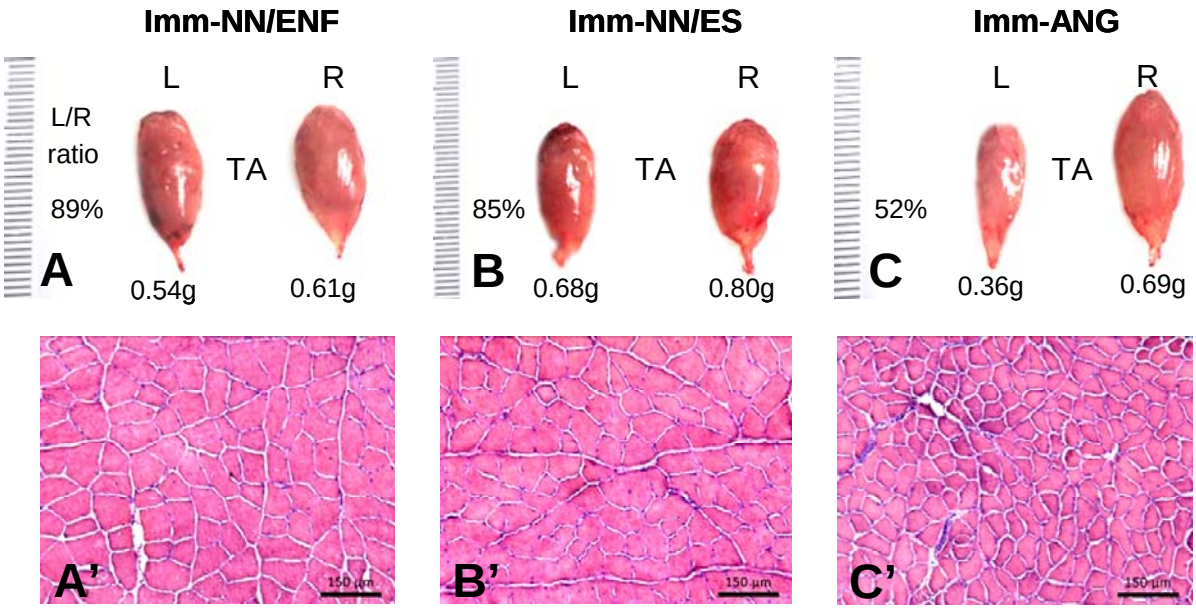


Fig. 4. Gross appearance, muscle mass, and myofiber morphology of the rat left (L) TA muscles treated with imm-NN/ENF (**A**), Imm-NN/ES (**B**), and Imm-ANG (**C**) and right (R) control muscles. Note that the muscle masses of the left TA muscles treated with NN/ENF and NN/ES are larger as compared with that of the ANG treated muscle. H&E stained cross sections from the left treated muscles (**A', B',C'**) showed that NN/ENF and NN/ES treated muscle exhibited very good preservation of muscle structure with less fiber atrophy as compared with ANG treated muscle. Bar = 150 µm for A' through C'.

- Nerve Regeneration

Muscle sections immunostained for neurofilament (NF) showed that Imm-NN/ENF (**Fig. 5A**) and Imm-NN/ES (data not shown) resulted in extensive nerve regeneration in the treated muscles. In contrast, Imm-ANG resulted in poor nerve regeneration (**Fig. 5B**). These findings are consistent with force data, indicating that the number of the regenerating axons correlates with force recovery. 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) (**Fig. 5A', B'**). Areas with NF-positive staining were outlined, measured with public domain ImageJ software (v. 1.45s; NIH, Bethesda, Maryland). For each rat, the number and the area fraction of the NF-ir axons in the operated muscle were compared with those in the contralateral control.

Table 2 below summarizes the mean count and %area of regenerated axons in the TA muscles treated with Imm-NN/ENF. Note that the mean axon count in the Imm-NN/ENF treated muscles was measured to be 88% of the contralateral control. This is similar to that in the Imm-NN/ES treated muscles (83%), but is much higher than that in the Imm-ANG treated muscles (39%).

In addition, the mean percentage of the regenerated axons in the Imm-NN-ENF group or Imm-NN-ES group was higher compared to that seen in the Imm-NN alone group (76%) or in the Imm-EEA group (46%) as reported in our paper (*Sobotka S, Mu L, Chen J, Li J, Nyirenda T. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. Neurosurgery (under review, 2022.*). Clearly, ENF and ES have the potential for enhancing axonal regeneration and for improving outcomes of NMEG-NMZ (NN) surgery in the rat model.

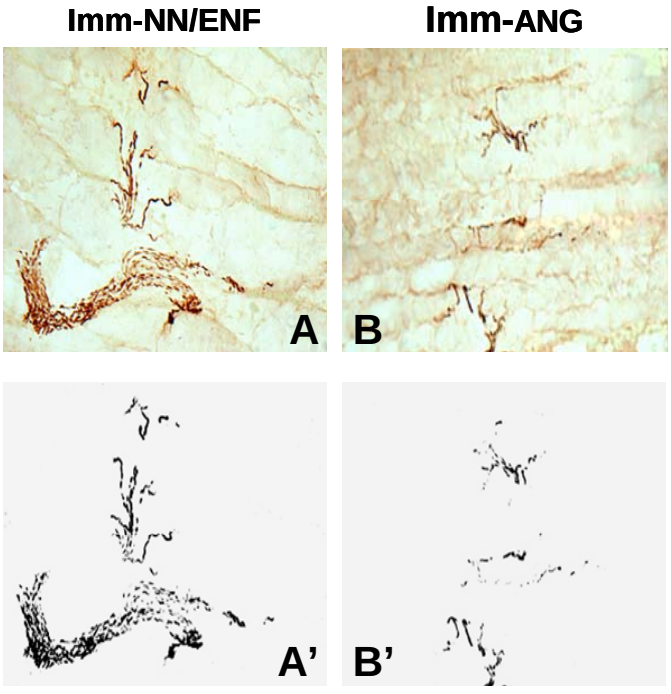


Fig. 5. Muscle sections immunostained with NF staining, showing intramuscular regenerated axons (darkly stained threads and dots) in the Imm-NN/ENF treated TA (**A**) and Imm-ANG treated muscle (**B**) three months after treatment in rats. The stained sections in A and B were opened using ImageJ software and converted to 8-bit (binary) images, color thresholded, and particle analyzed for nerve morphometry (**A', B'**). 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). Note that the Imm-NN/ENF treated TA had much more regenerated axons as compared with the Imm-ANG treated muscle. 200x.

Table 2. Count and %Area of Regenerated Axons in the Left Treated and Right Control Tibialis Anterior (TA) Muscles of the Rats in Imm-NN/ENF Group (n = 15)

Animal no.	Left TA		Right TA		Ratio (L/R)	
	Count	%Area	Count	%Area	Count	%Area
1	826	0.812	937	1.023	0.882	0.794
2	738	0.756	848	0.836	0.870	0.904
3	886	0.844	966	1.132	0.917	0.746
4	699	0.703	835	0.812	0.837	0.866
5	799	0.696	902	0.975	0.886	0.714
6	787	0.721	899	0.837	0.875	0.861
7	903	0.899	993	1.134	0.909	0.793
8	768	0.696	912	0.767	0.842	0.907
9	847	0.812	938	0.938	0.903	0.866
10	924	0.884	996	1.126	0.928	0.785
11	699	0.689	801	0.792	0.873	0.870
12	783	0.782	924	0.915	0.847	0.855
13	864	0.804	1046	0.997	0.826	0.806
14	889	0.762	973	1.055	0.914	0.722
15	919	0.831	1028	0.993	0.894	0.837
Average	822	0.779	933	0.955	0.880	0.822

L, left; R, right.

- Motor Endplate (MEP) Reinnervation

Horizontal muscle sections immunostained with double fluorescence staining showed innervated (with visible axon attachments) and non-innervated (without visible axon attachments) MEPs. Our results showed that the average percentage of the reinnervated MEPs in the Imm-NN/ENF (89%) and Imm-NN/ES (85%) treated TA muscles was higher as compared with that in the Imm-ANG treated muscles (48%) (**Fig. 6**), as well as with those seen in Imm-NN (83%) and in Imm-EEA (59%) (*Sobotka S, Mu L, Chen J, Li J, Nyirenda T. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. Neurosurgery (under review, 2022*.).

These findings are consistent with the extent of functional recovery, suggesting that reinnervation of the denervated MEPs is important for functional restoration.

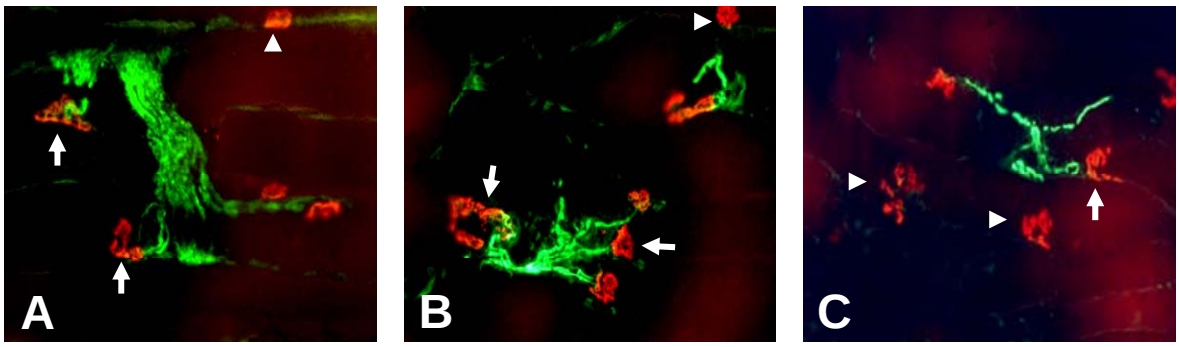


Fig. 6. Immunostained sections of the TA muscles treated with Imm-NN/ENF (**A**), Imm-NN/ES (**B**), and Imm-ANG (**C**), showing MEPs (red) and their innervating axons (green). Arrows indicate innervated MEPs with visible axon attachments, while arrowheads indicate non-innervated MEPs without visible axon attachments. 200x.

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.

During the next reporting period, the following experiments will be performed.

i) Animals in the 3 remaining delayed reinnervation groups (i.e., Del-SP/NN, Del-ANG, and Del-EEA) will undergo surgical and related procedures.

ii) The collected toe spread images and muscle force recordings from the TA muscles in the delayed reinnervation groups will be analyzed.

iii) The muscle samples obtained from the animals in the delayed reinnervation groups will be cut and stained using various histochemical and immunohistochemical techniques to assess nerve regeneration, axon-MEP connections, and fiber type-distribution.

iv) Submit a manuscript entitled "Outcomes of NMEG-NMZ technique in 3-month delayed limb muscle reinnervation" for publication.

4. **IMPACT:** D*escribe 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).

Analyses of the data obtained from animals with Imm-NN/ENF, Imm-NN/ES, and Imm-ANG groups showed that ENF and ES promote outcomes of NMEG-NMZ (NN) surgery. Specifically, Imm-NN/ENF and Imm-NN/ES resulted in better functional recovery (90% and 85% of the control, respectively) compared to ANG (46%) as well as NMEG-NMZ alone (79%) and standard EEA (51%) as reported in our publications. Clearly, ENF or ES have potential to augment the outcomes of the NMEG-NMZ in limb reinnervation.

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 PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official 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.

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).*

Three papers resulting from the work under this award have been published or submitted to academic journals for publication as shown below.

1) Mu L, Chen J, Li J, Sobotka S, Nyirenda T. Limb muscle reinnervation with nerve-muscle-endplate grafting technique: An anatomical feasibility study. Neurology Research International 2021, Article ID 6009342, 7 pages, https://doi.org/10.1155/2021/6009342 Acknowledgement of federal support (yes).

2) Sobotka S, Mu L, Chen J, Li J, Nyirenda T. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. Neurosurgery (under review), 2022. Acknowledgement of federal support (yes).

3) Mu L, Chen J, Sobotka S, Li J, Nyirenda T. Focal application of neurotrophic factors augments outcomes of nerve-muscle-endplate grafting technique for limb muscle reinnervation. Journal of Neurosurgery (under review), 2022. 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.*

Nothing to report.

- **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. Describe the technologies or techniques were 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. 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;*
  - *physical 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
Jingming Chen	no change
Jing Li	no change
Stanislaw Sobotka	no change
Themba Nyirenda	no change

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 Principal Investigator (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.*

Publications

1) Mu L, Chen J, Li J, Sobotka S, Nyirenda T. Limb muscle reinnervation with nerve-muscle-endplate grafting technique: An anatomical feasibility study. Neurology Research International 2021, Article ID 6009342, 7 pages, https://doi.org/10.1155/2021/6009342

2) Sobotka S, Mu L, Chen J, Li J, Nyirenda T. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. Neurosurgery (under review), 2022.

3) Mu L, Chen J, Sobotka S, Li J, Nyirenda T. Focal application of neurotrophic factors augments outcomes of nerve-muscle-endplate grafting technique for limb muscle reinnervation. Journal of Neurosurgery (under review), 2022.

Reinnervation of Paralyzed Limb muscle by Nerve-Muscle-Endplate Grafting Technique

W81XWH2010195

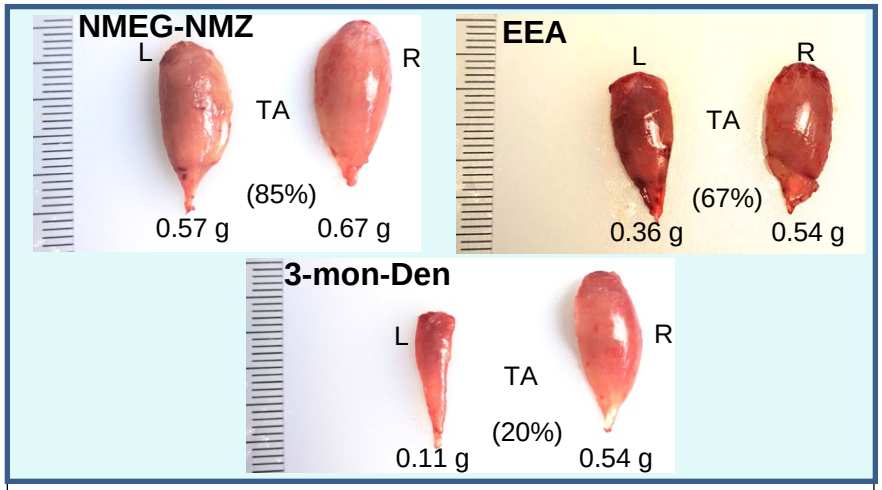
PI: Liancai Mu, MD, Ph.D **Org:** Hackensack Meridian Health - CDI **Award Amount: $1,686,264**

Study/Product Aim(s)

- **Aim 1:** Evaluate the efficacy of NMEG-NMZ technique for immediate reinnervation of denervated limb muscle, and determine the beneficial effects of nerve growth stimulation methods (i.e., brief nerve stimulation and ENF) on axonal regeneration and outcomes of NMEG-NMZ.
- **Aim 2:** Evaluate the efficacy of NMEG-NMZ technique for reinnervation of chronically denervated limb muscle, and assess the potential of specific therapies (i.e., sensory protection, and intramuscular injection of neurotrophic factors) for preservation of muscle mass and endplates prior to NMEG-NMZ surgery.

Approach

- Microsurgical procedures (NMEG-NMZ; sensory protection; nerve end-to-end anastomosis control; and denervation control).
- Physiological evaluation (muscle force measurement).
- Neural studies (nerve regeneration and axon-endplate connections).
- Muscle studies (fiber types, fiber size, muscle mass).



Photographs, showing the differences in wet weights of the treated tibialis anterior (TA) muscles of rats between NMEG-NMZ, EEA and 3-mon-Den groups.

Timeline and Cost

Activities	CY 1	2	3
Microsurgical procedures			
Physiological evaluation			
Neural studies			
Muscle studies & data analyses			
Estimated Budget ($K)	**$515k**	**$586k**	**$585k**

Updated: (0426/2022)

Goals/Milestones

CY1 Goal – Microsurgery and functional evaluations.

☑ Perform surgeries and some muscle force measurement.

CY2 Goals – Microsurgery, functional evaluations, and tissue studies.

☑ Perform surgeries and physiological testing.

☑ Perform neural and muscle studies.

CY3 Goal – Tissue studies, data collection, and data analyses.

☐ Examine intramuscular axonal regeneration and axon-endplate connections.

☐ Investigate myosin heavy chain-based fiber types, fiber size and muscle mass.

Comments/Challenges/Issues/Concerns

None.

Budget Expenditure to Date

Projected Expenditure:

Actual Expenditure: **$1,040,995**

Hindawi
Neurology Research International
Volume 2021, Article ID 6009342, 7 pages
https://doi.org/10.1155/2021/6009342

Research Article

Limb Muscle Reinnervation with the Nerve-Muscle-Endplate Grafting Technique: An Anatomical Feasibility Study

Liancai Mu,[1] **Jingming Chen**,[1] **Jing Li**,[1] **Stanislaw Sobotka**,[1,2] **and Themba Nyirenda**[1]

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Academic Editor: Mamede de Carvalho

Background. Peroneal nerve injuries results in tibialis anterior (TA) muscle paralysis. TA paralysis could cause "foot drop," a disabling condition that can make walking difficult. As current treatment methods result in poor functional recovery, novel treatment approaches need to be studied. The aim of this study was to explore anatomical feasibility of limb reinnervation with our recently developed nerve-muscle-endplate grafting (NMEG) in the native motor zone (NMZ). *Methods*. As the NMEG-NMZ technique involves in nerves and motor endplates (MEPs), the nerve supply patterns and locations of the MEP bands within the gastrocnemius (GM) and TA muscles of rats were investigated using Sihler's stain and whole-mount acetylcholinesterase (AChE) staining, respectively. Five adult rats underwent TA nerve transaction. The denervated TA was reinnervated by transferring an NMEG pedicle from the ipsilateral lateral GM. At the end of a 3-month recovery period, maximal muscle force was measured to document functional recovery. *Results*. The results showed that the TA was innervated by the deep peroneal nerve. A single MEP band was located obliquely in the middle of the TA. The GM was composed of two neuromuscular compartments, lateral (GM-l) and medial (GM-m), each of which was innervated by a separate nerve branch derived from the tibial nerve and had a vertically positioned MEP band. The locations of MEP bands in the GM and TA muscles and nerve supply patterns demonstrated that an NMEG pedicle can be harvested from the GM-l and implanted into the NMZ within the TA muscle. The NMEG-NMZ pilot study showed that this technique resulted in optimal muscle force recovery. *Conclusion*. NMEG-NMZ surgery is feasible for limb reinnervation. Specifically, the denervated TA caused by peroneal nerve injuries can be reinnervated with a NMEG from the GM-l.

1. Introduction

Peripheral nerve injuries (PNIs) to the extremities and resultant muscle paralysis are a major source of chronic disabilities which limit the opportunities to work and diminish quality of life [1]. Although a number of surgical procedures have been used to restore motor function following PNIs [2], the currently available nerve repair surgeries result in poor functional recovery [2–4] due primarily to insufficient axonal regeneration and a failure to reinnervate the denervated motor endplates (MEPs) in the target muscle [5–7]. Therefore, there is a pressing need for new methods to improve outcomes.

We developed a novel surgical technique called the nerve-muscle-endplate grafting (NMEG) technique for muscle reinnervation [8]. The ideal is that a denervated muscle could be reinnervated by transplanting an NMEG pedicle from a neighboring donor muscle. An NMEG pedicle is composed of a donor nerve branch and a block of muscle that contains numerous MEPs and nerve terminals. In our neck muscle model, an NMEG was harvested from sternohyoid muscle and implanted to an MEP-free area in the ipsilateral denervated sternomastoid muscle [8]. As MEP reinnervation of a denervated muscle is critical for motor recovery [6, 7], we modified the procedures by implanting the NMEG pedicle to the native motor zone (NMZ) of the target muscle that contains an MEP band and nerve terminals. This NMEG-NMZ is based on the rationale that denervated MEPs in the NMZ are preferential sites for reinnervation. Studies showed that, after nerve injury and/or

direct nerve implantation, regenerating axons preferentially make synaptic contact at the original MEPs [9–15]. Unlike other nerve repair methods, NMEG-NMZ provides an abundant source of nerve terminals that favor axonal regeneration. As the NMEG pedicle is implanted to the NMZ of the target muscle, this facilitates rapid axon-MEP connections. We have demonstrated that NMEG-NMZ results in better functional recovery (82% of the control) [16] than NMEG implantation to an MEP-free area in the target muscle (67%) [8]. However, it remains unknown if the NMEG-NMZ technique is effective for limb reinnervation.

The purpose of this study was to determine the anatomical feasibility of transferring an NMEG from the gastrocnemius muscle (GM) to reinnervate the ipsilateral denervated tibialis anterior (TA) muscle in a rat model.

2. Materials and Methods

2.1. Animals. In this study, ten hind limbs of adult female Sprague Dawley rats (Charles River Laboratories, MA) were obtained after completion of other experiments. The nerve supply patterns and the locations of MEP bands in the GM and TA muscles were studied. In addition, five rats were used in our pilot study to determine the surgical feasibility and functional outcome. These animal studies 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 US National Institutes of Health (NIH Publication no. 85–23, revised 1996).

2.2. Sihler's Stain. Five fresh left legs of rats were removed and processed with Sihler's stain, a whole-mount nerve staining technique, to map out branching and distribution patterns of the sciatic nerve and its branches. The details regarding Shiner's stain have been given in our previous publications [17, 18]. In brief, the legs were fixed for 3 weeks in 10% unneutralized formalin; macerated and depigmented for 2 weeks in 3% potassium hydroxide (KOH) solution; decalcified for 2 week in Sihler's solution I (one part glacial acetic acid, one part glycerin, and six parts 1% aqueous chloral hydrate) with several changes; stained for 3 weeks in Sihler's solution II (one part stock Ehrlich's hematoxylin, one part glycerin, and six parts 1% aqueous chloral hydrate); and destained for 3 hr in Sihler's solution I. The legs were washed in running tap water for 1 hr between the aforementioned staining steps. The stained legs were then rinsed for 1 hr in 0.05% lithium carbonate solution to darken the nerves, cleared for 3 days in 50% glycerin, and finally, preserved for 4 weeks before microdissection in 100% glycerin with a few thymol crystals for transparency. After transillumination by a xenon light source (model 610; Karl Storz, Endoscopy-America, Culver City, CA), the stained limb muscles were dissected under a dissecting microscope (TYP 3555110; Wild, Heerbrugg, Switzerland) with 10–30x magnification using microsurgical instruments. The nerves supplying the calf muscles were traced from the main trunk of the sciatic nerve to its major branches and terminations within individual calf muscles. Finally, the dissected specimens were photographed with a Nikon camera (model D5300; Nikon, Japan) under transillumination from a xenon light source (P-Frame A-5A, Taiwan).

2.3. Whole-mount AChE Staining. Five entire GM and TA muscles on the left side were removed from rat legs. The muscles were treated with whole-mount AChE staining to locate the MEP band as described in our previous publications [18, 19]. Briefly, the entire TA and GM muscles were fixed for 2 hr in 10% phosphate-buffered formalin; washed in 0.1 M phosphate buffer (PB) at pH 7.4 and pH 6.0 for 15 min in each; incubated in stock solution (cupric sulfate 150 mg, glycerin 190 mg, magnesium chloride 500 mg, maleic acid 900 mg, 4% sodium hydroxide 15 ml, 40% sodium sulfate (anhydrous) 85 ml, and acetylthiocholine iodide 100 mg) at pH 6.0 and 37°C for 2 hr; rinsed in 40% sodium sulfate (anhydrous) for 15 min; washed for 15 min in distilled water (DW); immersed for 15 min in 20% potassium ferricyanide; washed in DW for 60 min; and preserved in 50% glycerin for 3 days. The stained muscles were transilluminated by a xenon light source, dissected under a dissecting microscope (TYP 3555110, Wild), and photographed with a Nikon camera (model D5300; Nikon) under transillumination from a xenon light source (P-Frame A-5A).

2.4. Surgical Feasibility Pilot Study. After determining the nerve supply patterns and the location of the MEP bands within the TA and GM muscles, we performed NMEG-NMZ surgery in five rats under general anesthesia as described in our previous publications [8, 16]. First, the left TA was denervated by excising a 10 mm segment of its nerve. Both ends of the nerve were ligated to prevent nerve regeneration. Second, an NMEG pedicle containing a block of muscle ($\sim 8 \times 6 \times 4$ mm), axon terminals, and a MEP band with neuromuscular junctions was harvested from the NMZ of the left lateral GM in continuity with its nerve branch. Third, a muscular defect, with dimensions similar to the NMEG pedicle, was made in the NMZ of the left denervated TA. Finally, the NMEG pedicle was embedded into the TA defect and sutured with 10–0 nylon. At the end of the 3-month recovery period, the maximal muscle force of the TA muscles on both sides was measured as described [8, 16] to document functional recovery.

3. Results

3.1. Calf Muscles. Figure 1 shows the calf muscles and major branches of the sciatic nerve in the rat hind limb. The calf muscles include GM, soleus, flexor hallucis longus (FHL), flexor digitorum longus (FDL), tibialis posterior (TP), TA, and extensor digitorum longus (EDL). The GM is composed of two neuromuscular compartments (NMCs), lateral (GM-l) and medial (GM-m).

The TA is a fusiform muscle located in the anterior part of the leg. It arises from the lateral tibia, and its tendon inserts on the medial foot. Along with fibularis tertius, EDL and EHL, it comprises the anterior (extensor) compartment of the leg. TA lies medial to EDL, which makes it the most medial muscle in the anterior compartment of the leg.

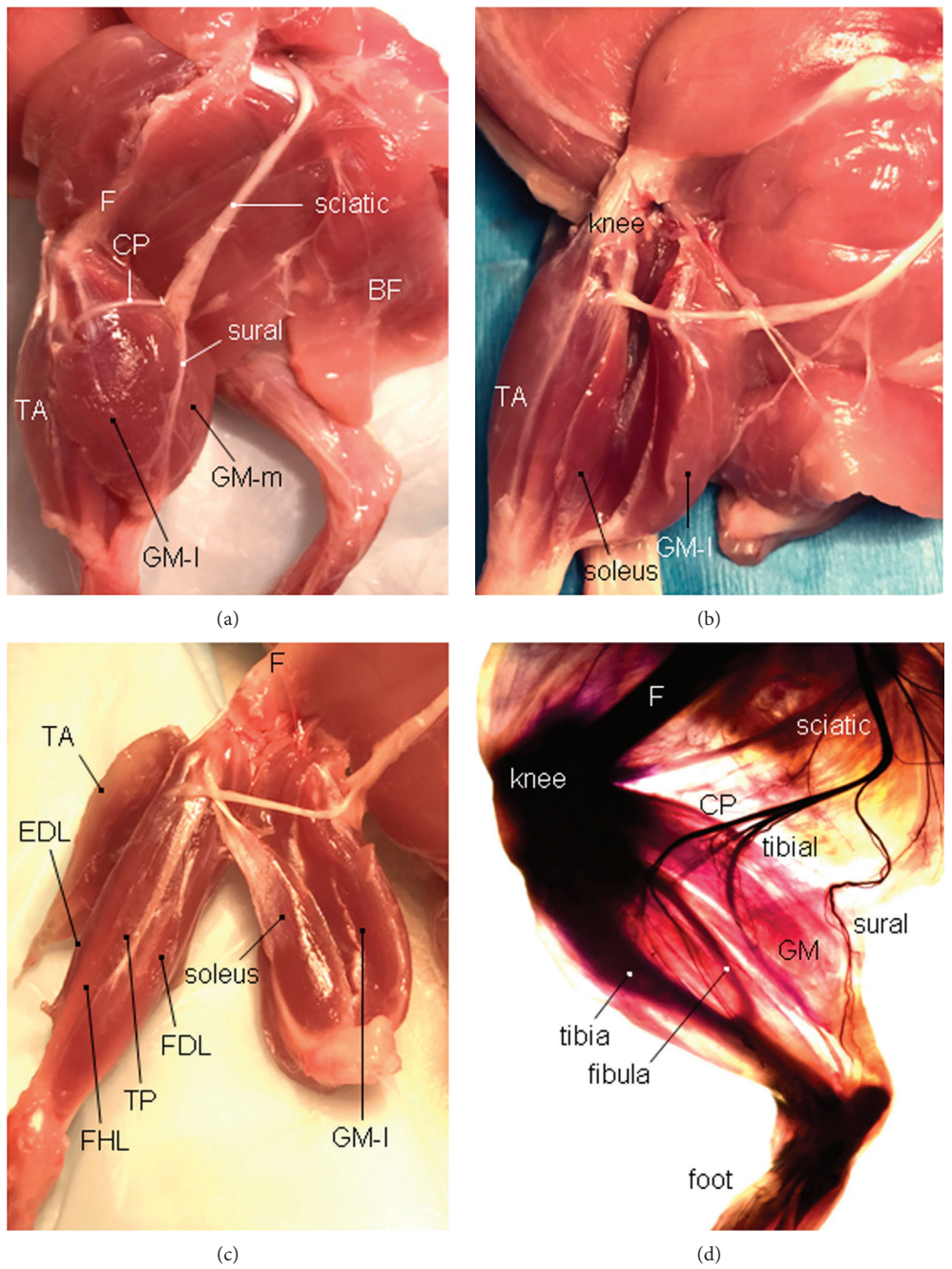


Figure 1: Calf muscles and their innervation of the rat left hind limb. (a) The calf muscle group of the left hind limb is exposed after the biceps femoris (BF) is reflected laterally. CP, common peroneal nerve; F, femur; TA, tibialis anterior muscle; GM-l, lateral compartment of the GM; and GM-m, medial compartment of the GM. (b) Left GM is detached and separated from the soleus muscle. (c) The separated calf muscles, including GM, soleus, flexor digitorum longus (FDL), flexor hallucis longus (FHL), and tibialis posterior (TP) muscle innervated by the tibial nerve, as well as the TA and extensor digitorum longus (EDL) innervated by the deep peroneal nerve. F, femur. (d) A rat left hind limb processed with Sihler's stain without microdissection, showing anatomical relationships among calf muscles, innervating nerves, and bone structures.

3.2. Branching and Distribution of Sciatic Nerve. Sihler's stain (Figures 1(d) and 2) showed that the sciatic nerve is divided into three major branches: common peroneal nerve (CP), tibial nerve, and sural nerve (sensory). The CP winds around the neck of the fibula and divides into a superficial and a deep branch. The deep peroneal nerve (DPN) innervates the TA and EDL in the anterior compartment of the leg.

The tibial nerve innerves the GM, soleus, FHL, FDL, and muscles in the foot (Figure 2). Specifically, tibial nerve gives off three branches, the first branch (the thinnest one) to the GM

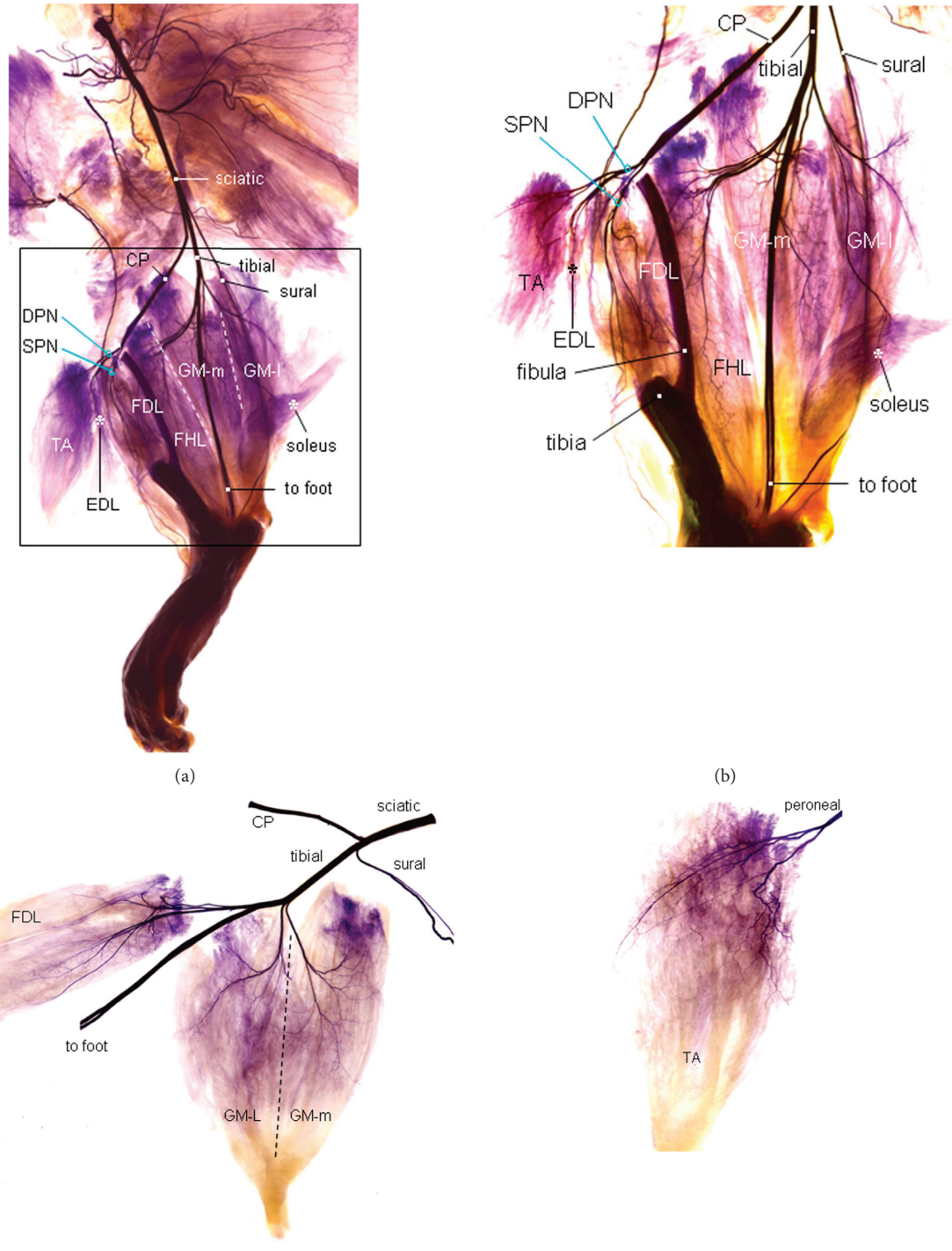
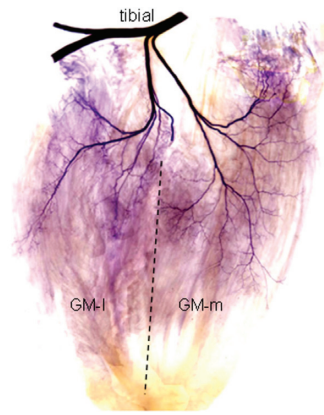


Figure 2: Continued.



(e)

Figure 2: A Sihler's stained rat hind limb, showing the major branches of the sciatic nerve and their distribution in the calf muscles. (a) Low-power view of the Sihler's stained left hind limb after microdissection, 4x. The sciatic nerve is divided into three major branches: CP, tibial, and sural (sensory). The peroneal nerve gives off branches to innervate TA and EDL. The tibial nerve gives off branches to supply the GM, soleus, FHL, FDL, and muscles in the foot. The GM-l and GM-m are outlined by white dotted lines. (b) Magnification of the boxed region in A showing the intramuscular branching and distribution patterns of the peroneal and tibial nerves. The upper part of the tibia was removed. The deep peroneal nerve (DPN) supplies branches to the TA and EDL. The tibial nerve gives off three branches, first branch to the GM, second branch to the foot, and third branch to the FHL and FDL. Also, the GM nerve branch gives off two secondary branches, one innervating the GM-l and soleus and another supplying the GM-m. SPN, superficial peroneal nerve, 12x. (c–e) High-power view of the branching of the sciatic nerve and intramuscular innervation of the tibial and peroneal nerves, 16x.

and soleus, the second branch (the thickest one) to the foot, and the third branch to the FHL and FDL. GM-l and GM-m are innervated by separate nerve branches derived from the tibial nerve. The nerve branch to the GM-l gives off a branch to innervate the soleus muscle (Figures 2(a) and 2(b)).

3.3. MEP Bands within the TA and GM Muscles. The MEP band is formed by numerous neuromuscular junctions. Figure 3 shows the MEP bands within the TA and GM muscles. The MEP band within the TA is located obliquely in the middle of the muscle (Figure 3(a)). In the GM, each of the GM-l and GM-m compartments has its own MEP band which is vertically located (Figure 3(b)).

3.4. Surgical Feasibility of NMEG-NMZ in Limb Reinnervation. The NMZs within the GM and TA muscles were delineated based on the locations of MEP bands and their innervating nerve terminals (Figure 4(a)). Our NMEG-NMZ pilot study showed that an NMEG pedicle can be harvested from the NMZ of the GM-l and transplanted to the NMZ of the TA (Figure 4(b)). In the rats with NMEG-NMZ surgery ($n = 5$), the average muscle force of the reinnervated TA recovered up to 81% of the contralateral control. These findings suggest that if the TA is denervated following peroneal nerve injury, the NMEG-NMZ technique could be an option to treat "foot drop" caused by TA paralysis.

4. Discussion

We investigated the branching and distribution of the sciatic nerve and NMZs within the TA and GM muscles in the rat. This anatomical study on the nerve supply patterns and locations of MEP bands in the TA and GM muscles allows us to identify their NMZs for NMEG-NMZ surgery. Since the GM-l lies adjacent to the TA, an NMEG pedicle from the NMZ of the GM-l could reach to the NMZ of the TA without difficulty. Our pilot study showed that NMEG-NMZ resulted in promising functional recovery three months after limb muscle reinnervation.

TA is the dorsiflexor of the foot and plays a critical role in walking. Paralysis of the TA caused by CP or DPN injuries or lesions results in foot drop, a disabling condition that can make walking difficult and lead to frequent falls.

Traditional treatment modalities include use of an ankle-foot orthosis, tendon surgery, and nerve repair. Tendon transfer surgery is often used to treat foot drop with mixed results [20, 21]. For example, all or a part of the healthy posterior tibial tendon is transferred to the dorsum of the foot for restoring foot dorsiflexion. However, the foot drop tendon transfer surgery results in weak ankle dorsiflexion [22].

Nerve repair [23], nerve grafting [24], and nerve transfer [25–28] are commonly used to manage sciatic and peroneal nerve injuries and lesions. Unfortunately, 64% of repair and grafting of the sciatic nerve [29] and 46–54% of the common peroneal nerve palsies [23, 24, 29] fail to restore functional dorsiflexion. Nerve transfer procedures such as a tibial nerve branch to the deep peroneal nerve [26–28] or a bundle of nerves supplying the soleus and lateral GM to the deep peroneal nerve [25] have been used to treat TA paralysis after peroneal and/or sciatic nerve injuries, which have had mixed results. Therefore, there is a great need to develop new approaches for foot drop treatment.

Poor motor recovery after PNIs and nerve repair is due primarily to insufficient axonal regeneration and failure to reinnervate the denervated MEPs in the target muscle. In

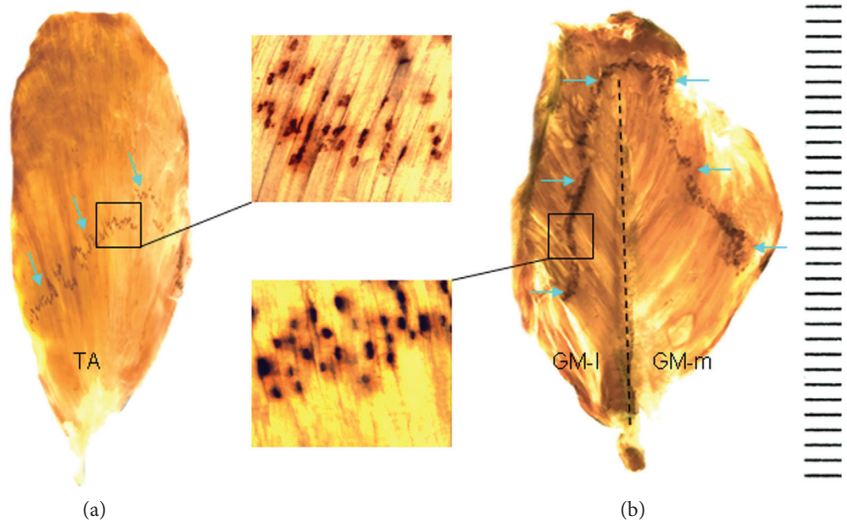


Figure 3: Locations of the MEP bands within the TA and GM as demonstrated by whole-mount AChE staining. (a) The MEP band (arrows) with numerous neuromuscular junctions (black dots) within the TA is located obliquely in the middle of the muscle. (b) Each of the GM-l and GM-m compartments in the GM has its own MEP band (arrows). The vertical dished line in the GM indicates midline.

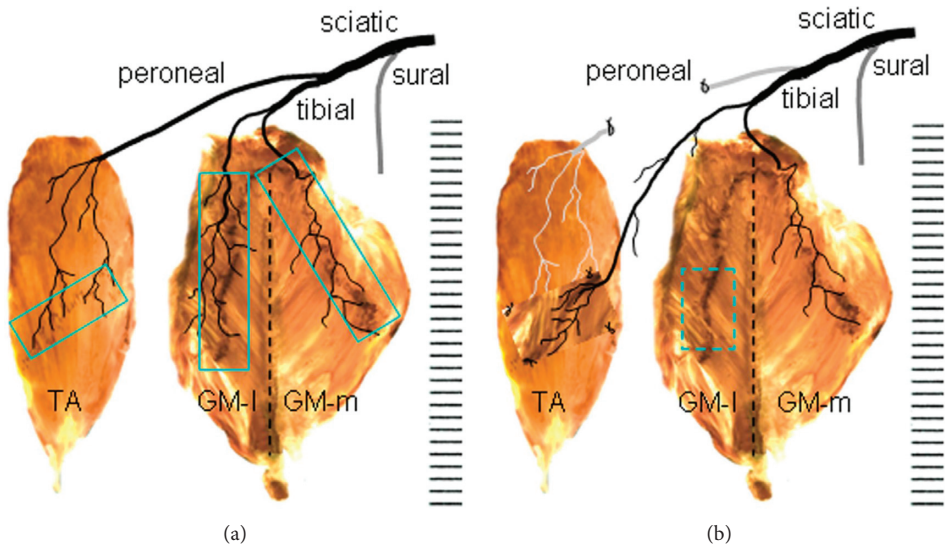


Figure 4: (a) Native motor zones (NMZs) within the TA and GM muscles (boxed regions) as demonstrated by Sihler's stain and AChE staining. (b) TA denervation and NMEG-NMZ transplantation. The TA is denervated by resecting a segment of the peroneal nerve. The denervated TA is treated with the NMEG-NMZ technique. An NMEG pedicle with a nerve branch is harvested from the NMZ of the GM-l (boxed region) and implanted to the NMZ of the denervated TA muscle.

response to this, we developed the NMEG. NMEG-NMZ is a recently developed novel surgical technique that targets NMZ for rapid MEP reinnervation, thereby leading to favorable functional recovery. Transplanting an NMEG from GM-l to the NMZ of the TA muscle is anatomically and surgically feasible and could offer several advantages to current treatment options. First, NMEG-NMZ provides an abundant source of nerve terminals that favor axonal regeneration. Second, as an NMEG pedicle is implanted directly to the MEP zone, NMEG-NMZ physically shortens regeneration distances and favors rapid axon-MEP connections. Finally, NMEG has ample pedicle-recipient muscle interfaces, which provide enough space for axonal regeneration at multiple points in the implanted NMEG pedicle and grow across the interfaces to reach the target.

This study showed that transferring an NMEG pedicle from GM-l to the NMZ of the TA can be used to treat TA paralysis caused by CP or DPN injuries. Further experimental studies are needed to evaluate the efficacy of the NMEG-NMZ technique for limb muscle reinnervation.

Data Availability

All data of this study are available upon request from the first author Liancai Mu.

Ethical Approval

In conducting research using animals, the investigators adhered to the laws of the United States and regulations of the Department of Agriculture. This protocol was approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of rats.

Disclosure

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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References

[1] M. Wiberg and G. Terenghi, "Will it be possible to produce peripheral nerves?" *Surgical Technolology International*, vol. 11, pp. 303–310, 2003.

[2] M. Siemionow and G. Brzezicki, "Chapter 8: current techniques and concepts in peripheral nerve repair," *International Review of Neurobiology*, vol. 87, pp. 141–172, 2009.

[3] B. J. F. Wong and R. L. Crumley, "Nerve wound healing. An overview," *Otolaryngologic Clinics of North America*, vol. 28, no. 5, pp. 881–895, 1995.

[4] S. K. Lee and S. W. Wolfe, "Peripheral nerve injury and repair," *Journal of the American Academy of Orthopaedic Surgeons*, vol. 8, no. 4, pp. 243–252, 2000.

[5] D. C. Green, G. S. Berke, and M. C. Graves, "A functional evaluation of ansa cervicalis nerve transfer for unilateral vocal cord paralysis: future directions for laryngeal reinnervation," *Otolaryngology-Head and Neck Surgery*, vol. 104, no. 4, pp. 453–466, 1991.

[6] C. H. E. Ma, T. Omura, E. J. Cobos et al., "Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice," *Journal of Clinical Investigation*, vol. 121, no. 11, pp. 4332–4347, 2011.

[7] J. Barbour, A. Yee, L. C. Kahn, and S. E. Mackinnon, "Supercharged end-to-side anterior interosseous to ulnar motor nerve transfer for intrinsic musculature reinnervation," *The Journal of Hand Surgery*, vol. 37, no. 10, pp. 2150–2159, 2012.

[8] L. Mu, S. Sobotka, and H. Su, "Nerve-muscle-endplate band grafting: a new technique for muscle reinnervation," *Neurosurgery*, vol. 69, no. ONS Suppl. 2, pp. ons208–ons224, 2011.

[9] T. Iwayama, "Relation of regenerating nerve terminals to original endplates," *Nature*, vol. 224, no. 5214, pp. 81-82, 1969.

[10] H. T. Sakellarides, C. Sorbie, and L. James, "Reinnervation of denervated muscles by nerve transplantation," *Clinical Orthopaedics and Related Research*, vol. 83, pp. 195–201, 1972.

[11] M. R. Bennett, E. M. McLachlan, and R. S. Taylor, "The formation of synapses in reinnervated mammalian striated muscle," *The Journal of Physiology*, vol. 233, no. 3, pp. 481–500, 1973.

[12] A. Gorio, G. Carmignoto, M. Finesso, P. Polato, and M. G. Nunzi, "Muscle reinnervation. II. Sprouting, synapse formation and repression," *Neuroscience*, vol. 8, no. 3, pp. 403–416, 1983.

[13] J. R. Sanes and J. Covault, "Axon guidance during reinnervation of skeletal muscle," *Trends in Neurosciences*, vol. 8, pp. 523–528, 1985.

[14] J. Covault, J. M. Cunningham, and J. R. Sanes, "Neurite outgrowth on cryostat sections of innervated and denervated skeletal muscle," *The Journal of Cell Biology*, vol. 105, no. 6 Pt 1, pp. 2479–2488, 1987.

[15] U. J. McMahan and B. G. Wallace, "Molecules in basal lamina that direct the formation of synaptic specializations at neuromuscular junctions," *Developmental Neuroscience*, vol. 11, no. 4-5, pp. 227–247, 1989.

[16] L. Mu, S. Sobotka, J. Chen, and T. Nyirenda, "Reinnervation of denervated muscle by implantation of nerve-muscle-endplate band graft to the native motor zone of the target muscle," *Brain and Behavior*, vol. 7, no. 6, Article ID e00668, 2017.

[17] L. Mu and I. Sanders, "Sihler's whole mount nerve staining technique: a review," *Biotechnic & Histochemistry*, vol. 85, no. 1, pp. 19–42, 2010.

[18] L. Mu and I. Sanders, "Human tongue neuroanatomy: nerve supply and motor endplates," *Clinical Anatomy*, vol. 23, no. 7, pp. 777–791, 2010.

[19] M. Ren and L. Mu, "Intrinsic properties of the adult human mylohyoid muscle: neural organization, fiber-type distribution, and myosin heavy chain expression," *Dysphagia*, vol. 20, no. 3, pp. 182–194, 2005.

[20] F. De Marchi, F. Malerba, U. Montrasio Alfieri, M. Ferrarin, and M. Rabuffetti, "Tibialis posterior tendon transfer through the interosseal membrane in paralysis of the common peroneal nerve," *Foot and Ankle Surgery*, vol. 6, no. 1, pp. 19–25, 2000.

[21] J. S. Yeap, R. Birch, and D. Singh, "Long-term results of tibialis posterior tendon transfer for drop-foot," *International Orthopaedics*, vol. 25, no. 2, pp. 114–118, 2001.

[22] J. R. Prahinski, K. A. McHale, H. T. Temple, and J. P. Jackson, "Bridle transfer for paresis of the anterior and lateral compartment musculature," *Foot & Ankle International*, vol. 17, no. 10, pp. 615–619, 1996.

[23] M. B. Wood, "Peroneal nerve repair. Surgical results," *Clinical Orthopaedics and Related Research*, vol. 267, pp. 206–210, 1991.

[24] D. H. Kim and D. G. Kline, "Management and results of peroneal nerve lesions," *Neurosurgery*, vol. 39, no. 2, pp. 312–320, 1996.

[25] J. Gousheh and A. Babaei, "A new surgical technique for the treatment of high common peroneal nerve palsy," *Plastic and Reconstructive Surgery*, vol. 109, no. 3, pp. 994–998, 2002.

[26] R. K. Nath, A. B. Lyons, and M. Paizi, "Successful management of foot drop by nerve transfers to the deep peroneal nerve," *Journal of Reconstructive Microsurgery*, vol. 24, no. 6, pp. 419–427, 2008.

[27] J. L. Giuffre, A. T. Bishop, R. J. Spinner, B. A. Levy, and A. Y. Shin, "Partial tibial nerve transfer to the tibialis anterior motor branch to treat peroneal nerve injury after knee trauma," *Clinical Orthopaedics and Related Research*, vol. 470, no. 3, pp. 779–790, 2012.

[28] R. K. Nath and C. Somasundaram, "Gait improvements after peroneal or tibial nerve transfer in patients with foot drop: a retrospective study," *Eplasty*, vol. 17, p. e31, 2017.

[29] D. K. Clawson and H. J. Seddon, "The results of repair of the sciatic nerve," *The Journal of Bone and Joint Surgery. British volume*, vol. 42-B, no. 2, pp. 205–212, 1960.

Neurosurgery

Reinnervation of Paralyzed Limb Muscle by Nerve-Muscle-Endplate Grafting Technique

--Manuscript Draft--

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Abstract:	BACKGROUND: We have developed a novel reinnervation technique called nerve-muscle- endplate grafting in the native motor zone (NMEG-NMZ). However, it remains unknown if the NMEG-NMZ is effective for limb reinnervation. OBJECTIVE: To evaluate the efficacy of the NMEG-NMZ in limb muscle reinnervation. METHODS: Forty-five adult rats were divided into three groups: NMEG, end-to-end anastomosis (EEA, technique control), and denervation control (DC). The left tibialis anterior (TA) muscle was denervated by resecting its nerve. For NMEG-NMZ, the denervated TA was reinnervated by transferring a NMEG pedicle from the lateral gastrocnemius muscle. Three months after surgery, static toe spread analysis was formed for all rats and muscle force was measured for the rats treated with NMEG and EEA. Muscle weight, myofiber morphology, regenerated axons, and reinnervated motor endplates (MEPs) in the treated muscles were also quantified and compared with those in the DC group. RESULTS: NMEG-NMZ technique resulted in better muscle force recovery (79% of the control) compared to EEA (51% of the control). The average weight of the NMEG-NMZ reinnervated muscles (86%) was greater than those of the EEA treated (71%) and DC (26%) muscles. The mean count of the regenerated axons in the muscles with NMEG-NMZ was 76% of the non-operated side, which was larger than that in the muscles with EEA (46%). The proportion of the reinnervated MEPs in the NMEG-NMZ muscles (83%) was larger than that in the EEA muscles (59%). CONCLUSION: NMEG-NMZ technique has unique advantages and is superior to EEA for muscle reinnervation and functional recovery.
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Question	Response
Significance of the Work: Please include a brief statement summarizing the significance of the work and in particular how it differs from and advances existing literature.	There is unsatisfactory motor recovery after peripheral nerve injury (PNI) and subsequent nerve repair. Therefore, a great need exists to develop novel treatment strategies for improving surgical outcomes. More recently, we developed a novel nerve-muscle-endplate grafting (NMEG) technique for muscle reinnervation and performed a series of related studies in a rat neck muscle model. The underlying concept is that a denervated muscle following PNIs can be reinnervated by transferring an NMEG pedicle from a neighboring intact muscle (native motor zone in the recipient muscle is an ideal region for muscle reinnervation). However, it remains unknown if our NMEG technique is effective for limb reinnervation. We hypothesized that NMEG technique may be a useful treatment option to restore motor function of the denervated tibialis anterior (TA) muscle. To our knowledge, this is the first study in which an NMEG pedicle obtained from the gastrocnemius muscle muscle (donor) was transferred to the denervated TA muscle (recipient) for restoring its motor function. All experimental animals were subjected to a series of postoperative evaluations at the end of 3-month recovery period. The present study showed that our NMEG technique resulted in much better outcomes for limb muscle reinnervation as compared with standard end-to-end anastomosis.
Compliance with Research Reporting Guidelines: *Neurosurgery* endorses several reporting guidelines and requires authors to submit their research articles in accordance with the appropriate guideline statement(s) and checklist(s). Completed applicable checklists and flow diagrams must be included with submissions. Research articles that must be submitted according to the appropriate reporting guideline(s) include, but are not limited to: randomized trials, systematic reviews, meta-analyses of interventions, meta-analyses of observational studies, diagnostic accuracy studies, and observational epidemiological studies (eg, case series, cohort, case-control, and cross-sectional studies). Consult the EQUATOR Network, which maintains a useful, up-to-date list of guidelines as they are published, with links to articles and checklists: http://www.equator-network.org. Please confirm below that information is	Not Applicable - Submission Does Not Report Research That Requires Adherence to Reporting Guideline(s)

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December 16, 2021

Nelson M. Oyesiku, MD, PhD, FACS,
Editor-in-Chief, NEUROSURGERY
1510 Clifton Road, NE
Suite G65
Atlanta, GA 30322

Dear Dr. Oyesiku:

We would like to submit our manuscript entitled "**Reinnervation of Paralyzed Limb Muscle by Nerve-Muscle-Endplate Grafting Technique**" for publication in *Neurosurgery*.

The work reported in this manuscript has not been, and is not intended to be, published anywhere except in *Neurosurgery*.

Authorship:
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Liancai Mu, M.D., Ph.D.
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Disclosure:
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Ethical experimentation:
The experimental protocols are approved by the Institutional Animal Care and Use Committee and are 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).

Thank you for your consideration.

Sincerely,

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Reinnervation of Paralyzed Limb Muscle by Nerve-Muscle-Endplate Grafting Technique

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Abbreviated title: LIMB REINNERVATION BY NERVE-MUSCLE-ENDPLATE GRAFTING

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ABSTRACT

BACKGROUND: We have developed a novel reinnervation technique called nerve-muscle-endplate grafting in the native motor zone (NMEG-NMZ). However, it remains unknown if the NMEG-NMZ is effective for limb reinnervation.

OBJECTIVE: To evaluate the efficacy of the NMEG-NMZ in limb muscle reinnervation.

METHODS: Forty-five adult rats were divided into three groups: NMEG, end-to-end anastomosis (EEA, technique control), and denervation control (DC). The left tibialis anterior (TA) muscle was denervated by resecting its nerve. For NMEG-NMZ, the denervated TA was reinnervated by transferring a NMEG pedicle from the lateral gastrocnemius muscle. Three months after surgery, static toe spread analysis was formed for all rats and muscle force was measured for the rats treated with NMEG and EEA. Muscle weight, myofiber morphology, regenerated axons, and reinnervated motor endplates (MEPs) in the treated muscles were also quantified and compared with those in the DC group.

RESULTS: NMEG-NMZ technique resulted in better muscle force recovery (79% of the control) compared to EEA (51% of the control). The average weight of the NMEG-NMZ reinnervated muscles (86%) was greater than those of the EEA treated (71%) and DC (26%) muscles. The mean count of the regenerated axons in the muscles with NMEG-NMZ was 76% of the non-operated side, which was larger than that in the muscles with EEA (46%). The proportion of the reinnervated MEPs in the NMEG-NMZ muscles (83%) was larger than that in the EEA muscles (59%).

CONCLUSION: NMEG-NMZ technique has unique advantages and is superior to EEA for muscle reinnervation and functional recovery.

KEY WORDS: nerve-muscle-endplate grafting, muscle reinnervation, peripheral nerve injury, anterior tibialis muscle, gastrocnemius muscle, tetanic force measurement

ABBREVIATIONS: ABC, avidin-biotin complex; **AChE,** acetylcholinesterase; **AChR,** acetylcholine receptor; **BSA,** bovine serum albumin; **DC,** denervation control; **EEA,** end-to-end anastomosis; **GM,** gastrocnemius muscle; **GM-l,** lateral compartment of GM; **GM-m,** medial compartment of GM; **H&E,** hematoxylin and eosin; **IACUC,** Institutional Animal Care and Use Committee; **ITS,** intermediary toe spread; **ITSF,** ITS factor; **MEP,** motor endplate; **NF,**

neurofilament; **NF-ir,** NF immunoreactive; **NITS,** non-operated side intermediate toe spread; **NMEG,** nerve-muscle-endplate grafting; **NMZ,** native motor zone; **NN,** NMEG-NMZ; **NTS,** non-operated side toe spread; **OITS,** operated side intermediate toe spread; **OTS,** operated side toe spread; **PBS,** phosphate-buffered saline; **PNI,** peripheral nerve injury; **SSI,** static sciatic index; **STSA,** static toe spread analysis; **TA,** tibialis anterior; **TS,** toe spread; **TSF,** TS factor

SHORT TITLE: LIMB REINNERVATION BY NERVE-MUSCLE-ENDPLATE GRAFTING

INTRODUCTION

Traumatic injuries to the sciatic nerve and/or its major motor divisions (i.e., peroneal and tibial nerves) result in leg muscle paralysis that is a predominant source of chronic disabilities. In the United States, there are 200,000 cases of peripheral nerve injuries (PNIs) annually,[1] and an estimated $300 billion is spent annually on care for disabled Americans.[2,3] At present, multiple surgical interventions have been used for restoring motor function following PNIs.[4] Unfortunately, only about 50% of patients regain useful function after nerve repair surgery,[5,6] as fewer nerve fibers are able to pass through the coaptation site and reach the target muscle.[7,8] Unsatisfactory motor recovery after PNIs and nerve repair has been attributed mainly to insufficient axonal regeneration and poor reinnervation of the denervated motor endplates (MEPs) in the target muscle.[9,10] Therefore, a great need exists to develop novel treatment strategies for improving surgical outcomes.

More recently, we developed nerve-muscle-endplate grafting (NMEG) technique for muscle reinnervation[11] and performed a series of related studies[12-14] in a rat neck muscle model. The underlying concept is that a denervated muscle following PNIs can be reinnervated by transferring an NMEG pedicle from a neighboring intact muscle. Our studies have demonstrated that native motor zone (NMZ) in the recipient muscle is an ideal region for muscle reinnervation. The NMZ within a muscle contains a MEP band with numerous neuromuscular junctions and their innervating nerve terminals.[14] Our studies showed that implantation of a NMEG from a donor muscle to the NMZ within a recipient denervated muscle[14] results in better outcomes as compared with NMEG implantation to a MEP-free area.[11] However, it remains unknown if the NMEG-NMZ technique is effective for limb reinnervation.

Clinically, paralysis of tibialis anterior (TA) muscle caused by peroneal nerve injuries or lesions results in foot drop, a disabling condition. Foot drop is usually treated with tendon transfer with mixed results.[15-17] Nerve repair,[18] nerve grafting,[19] and nerve transfer[20-23] are commonly used to manage sciatic and peroneal nerve injuries and lesions. Unfortunately, about half of the patients with sciatic nerve repair and grafting[24] and peroneal nerve repare[18,19] fail to restore functional dorsiflexion.

We hypothesized that NMEG-NMZ technique may be a useful treatment option to restore motor function of the denervated TA muscle. We performed anatomical feasibility study on the limb muscle reinnervation by the NMEG-NMZ and showed that an NMEG pedicle can be harvested from the lateral gastrocnemius muscle (GM-l) to reinnervate TA muscle.[25]

The purpose of this study was to assess the efficacy of the NMEG-NMZ technique for TA reinnervation following PNIs in a rat model.

MATERIALS AND METHODS

Animals and Experimental Groups

In this study, fourty five 3-month-old female Sprague-Dawley rats (Charles River Laboratories, MA) were used. The body weight of the animals ranged from 250 to 300 g at the time of initial operation. The animals were assigned to three groups (15 rats/per group): NMEG-NMZ (NN group), end-to-end anastomosis (EEA group), and denervation control (DC group). These animal studies were ethically reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) 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 US National Institutes of Health (NIH Publication no. 85-23, revised 1996). The animals were provided with food and water *ad libitum* in a 22°C environment with a 12:12-h light-dark cycle and housed in standard cages in the state of the art animal housing facilities of Center for Discovery and Innovation. All efforts were made to minimize the number of animals and their suffering in the experiments.

Surgical Procedures

Animals were anesthetized via an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg). Microsurgical procedures for NMEG-NMZ were performed under an Olympus SZX12 Stereo zoom surgical microscope (Olympus America Inc., Center Valley, PA). Under aseptic conditions, an incision was made in the posterior thigh and calf of the hind limb to expose the TA and GM and the three major branches of the sciatic nerve (i.e., common peroneal, tibial and sural nerves).

The NMEG-NMZ procedure includes several steps. First, the left TA muscle was denervated by resecting a 10-mm segment of its innervating nerve (i.e., deep peroneal nerve). The nerve cut ends were then ligated to prevent nerve regeneration. Second, the NMZs in the left TA and GM were outlined according to the locations of the MEP band and muscular nerve branches (Figure 1A) as described.[25] An NMEG containing a block of muscle (~8×6×4 mm), axon terminals, and a MEP band with neuromuscular junctions was outlined and harvested from the NMZ of the GM-l in continuity with its motor nerve branch. Third, a muscular defect, with the same dimensions as the NMEG pedicle, was made in the NMZ of the left denervated TA. Finally, the prepared NMEG was embedded into the TA defect and sutured with four 10-0 nylon microsutures (Figure 1B). After surgery, the wound was closed in layers with interrupted simple sutures of 4-0 prolene.

For nerve repair with EEA (technique control), the left TA nerve (deep peroneal nerve) was transected approximately 5 mm proximal to the TA and both nerve cut ends were anastomosed with two interrupted 10-0 nylon microsutures.

For denervation control (DC), the rats underwent muscle denervation by excising a 10-mm segment of the TA nerve and ligating both ends to prevent nerve regeneration.

Postoperative Evaluations

All experimental animals were subjected to a series of postoperative evaluations at the end of 3-month recovery period. Specifically, we evaluated (a) functional recovery of the treated muscles as demonstrated by static toe spread analysis and muscle force measurement; (b) muscle weight measurement and myofiber morphology; and (c) regenerated axons and reinnervated MEPs in the target muscles.

Static Toe Spread Analysis

Static toe spread analysis (STSA) described by Bervar (2000)[26] was used to assess limb function in rats. In this study, STSA was performed just before muscle force measurement at the end of the 3-month recovery period. Bervar (2000)[26] proposed a static sciatic index (SSI) for toe spread analysis. Subsequent studies by others[27-30] have demonstrated that STSA is a useful method for evaluating limb motor function. For STSA, the rat was placed in an acrylic 40×20×20 cm container on a transparent base plate for observing footprints on the plantar view. A camera was positioned underneath the base plate to photograph the plantar surface of the rat hind limb paws. Three separate images from the operated (O) and non-operated (N) footprints were randomly selected to measure the distance (mm) between the first and fifth toe spread (TS) and between the second and fourth intermediate toe spread (ITS). The mean values of TS and ITS in each group were computed and used for determining the TS and ITS factors (TSF and ITSF): TSF = (OTS – NTS)/NTS and ITSF = (OITS – NITS)/NITS. The ratios of the SSI were calculated using the following equation: SSI = (108.44 × TSF) + (31.85 × ITSF)–5.49.[26] An index score of 0 is defined as normal and an index score of −100 indicates a complete functional loss.[26]

TSF: toe spread factor; ITSF: intermediate toe spread factor; OTS: operated side toe spread; NTS: non-operated side toe spread; OITS: operated side intermediate toe spread; NITS: non-operated side intermediate toe spread.

Maximal Tetanic Force Measurement

At the end of the 3-month recovery period, the rats treated with NMEG-NMZ and EEA underwent muscle force measurement. For each rat, the TA muscles on both sides were examined using a stimulation and recording system as described in our publications.[11-14] The anesthetized rat was placed in prone position on a platform, the distal tendon of the TA was severed and the muscle was dissected proximally up to the level of its origin. The transected TA tendon was tied with a 4-0 suture and connected to force transducer attached to a servomotor lever arm of the Dual-Mode Lever System (305B-LR; Aurora Scientific Inc., Aurora, Canada). The TA muscle was stretched by the servomotor lever arm at increasing muscle tension to establish the optimal muscle tension for the development of maximum force. Finally, electrical stimulation (0.2

s train of 0.2 ms bipolar pulses at 200 pulses/s with current 0.01-5 mA) was provided directly to the repaired peroneal nerve (EEA group) or the transplanted tibial nerve branch supplying the NMEG (NN group). At least one-minute breaks separated subsequent tetanic contractions to permit muscle recovery. Throughout testing, the TA 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). Collected data were analyzed offline with DIAdem 11.0 software (National Instruments).

Muscle Sample Preparation

At the end of the experiments, the operated and non-operated TA muscles in each rat were removed, weighed, and examined using histological and immunohistochemical techniques to examine myofiber morphology, regenerated axons and reinnervated MEPs. Each removed TA muscle was divided into two segments: superior one-third and inferior two-thirds. The muscle samples were frozen in melting isopentane cooled with dry ice. The superior muscle segments were cut transversly (10-μm thick) and stained for routine hematoxylin and eosin (H&E) staining to examine myofiber morphology. The inferior muscle segments that contain NMZ and/or implanted NMEG were cut horizontally (50-μm thick) and immunostained for documenting nerve regeneration and MEP reinnervation.

Neurofilament (NF) Staining

Some horizontal sections were immunostained with a monoclonal antibody against phosphorylated NF (SMI-31; Covance Research Products, Berkeley, CA, USA) as a marker for all axons as described in our previous publication.[14] Briefly, the sections were treated in phosphate-buffered saline (PBS) containing 0.3% Triton and 2% bovine serum albumin (BSA) for 30 minutes. The treated sections were incubated with primary antibody SMI-31 (dilution 1:800) in PBS containing 0.03% Triton at 4ºC overnight and then incubated for 2 hours with the biotinylated secondary antibody (anti-mouse, 1:1000, Vector, Burlingame, CA, USA). The incubated sections were treated with avidin-biotin complex method with a Vectastain ABC kit (1:1000 ABC Elite, Vector). Finally, the sections were 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 density of the intramuscular axons was evaluated 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). For a give muscle, three sections stained for NF were selected at different spatial levels to count NF-ir axons. For each section, two microscopic fields with NF-ir axons were identified and photographed. Areas with NF-positive staining were outlined and measured with public domain ImageJ software (v. 1.45s; NIH, Bethesda, Maryland). For each rat, the number and the area fraction of the NF-ir axons in the operated muscle were compared with those in the contralateral control.

Double Fluorescence Staining

Intramuscular axons and MEPs in the TA muscles were labled with double fluorescence staining as described in our publications.[14] Briefly, some horizontal sections were placed in Zamboni fixative with 5% sucrose for 20 minutes at 4ºC, washed several times with 1.5-T buffer with 0.05% Tween-20 and treated with 0.1 mol/L of glycine in 1.5 T buffer for 30 minutes, and dipped in 100% methanol at –20ºC. The sections were then blocked in 1.5 T buffer containing 4% normal goat serum for 30 minutes and incubated overnight at 4ºC with primary antibodies (SMI-31 to detect neurofilaments and SMI-81 to label thinner axons; Covance Research Products Inc). The sections were then 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, USA). The stained sections were washed in 1.5 T buffer with 0.05% Tween-20 and coverslipped.

The stained sections were viewed under a Zeiss photomicroscope (Axiophot-1; Carl Zeiss, Goettingen, Germany) equipped with epifluorescence optics. Sections were photographed using a USB 3.0 digital microscope camera (Infinity 3-3URC; Lumenera Corp., Ottawa, Ontario, Canada). SMI-31 and SMI-81 detected axons (green), while α-bungarotoxin labeled postsynaptic acetylcholine receptor (AChR) site in the MEPs (red). For each muscle sample, at least 100 labeled MEPs were randomly selected to determine the percentages of the innervated (visible axon attachment) and noninnervated (no visible axon attachment) MEPs.

RESULTS

Motor functional recovery following TA reinnervation with NMEG-NMZ and EEA was demonstrated by static toe spread analysis and muscle force measurement.

Toe Spread Recovery

Static toe spread analysis showed that the animals in the NN group had better toe spread recovery as compared with those in the EEA and DC groups (Figure 2). The mean SSI score was calculated to be -16.84 (std=4.16) for the NN group and -41.7 (std=13.46) for the EEA group. In other word, toe spead recovered up to 83% and 58% of the control for NN and EEA groups, respectively. The difference in SSI scores between NN and EEA groups were strongly significant ($p<0.0001$, unpaired *t*-test). Clearly, NMEG-NMZ improved limb motor recovery more significantly as compared with EEA.

Muscle Force Recovery

The threshold current was established as the minimal current, which produce visible muscle contraction (between 0.01 and 0.1mA). Increasing stimulation current produced increased muscle contraction until muscle force reached plateau of maximal muscle force (between 0.1 and 0.2mA).

Maximal muscle force was recorded at large stimulation current of 0.5mA. The boxplot in Figure 3A shows the comparison between the distribution of maximal muscle force on the operated and non-operated sides in NN and EEA groups. Group averages of maximal muscle force are shown in Figure 3B. In both NN and EEA groups, the mean maximal muscle force of the reinnervated TA was significantly lower than that of the contralateral side (both paired *t*-tests, $p<0.025$).

The comparison between rates of muscle force at experimental to control sides in NN and EEA groups is shown in Figure 3C. The mean rates of experimental to control side were 0.791 for NN group and 0.505 for EEA group. The median rate of experimental to control side in EEA group (0.353) was significantly smaller the rate in NN group (0.716) (Mann-Whitney U test, $z=1.98$, $p=0.048$).

The stimulation threshold defined as minimal stimulation current which produced visible muscle contraction is shown in Figure 3D-F. The boxplot in Figure 3D shows the comparison between the distribution of the stimulation thresholds on both sides. Group averages of stimulation threshold in these 4 conditions are shown in Figure 3E.

In NN group, the stimulation threshold was not significantly different at the operated side (mean=0.052, median=0.050 as compared to contralateral side (mean=0.047, median=0.050, mean difference=0.005, median difference=0, Wilcoxon Sign-Rank test, $p>0.05$). The average rate of experimental to contralateral side was 1.333.

In EEA group, the stimulation threshold was significantly larger at the operated side (mean=0.139, median=0.075) as compared to contralateral side (mean=0.078, median=0.050, mean difference=0.061, median difference=0.025, Wilcoxon Sign-Rank test, $p=0.0238$). The average rate of experimental to contralateral side was 2.15.

The comparison between rates of stimulation threshold at experimental to control sides in EEA and NN groups is shown in Figure 3F. The median rates of experimental to control side were: 1.50 in EEA group and 1.00 in NN group. However, this rate difference did not reach statistical significance (Mann-Whitney U test, z=1,244, $p=0.215$). As the distribution of rates data in EEA and NN groups were not normal (Shapiro-Wilk test, $p= 0.000031, 0.0045$), nonparametric test was used for this rate comparison.

Muscle Mass, Wet Weight and Myofiber Morphology

The size of the TA reinnervated with NMEG-NMZ was similar to that of the contralateral control muscle and larger than that of the TA treated with EEA and denervated TA (Figure 4A-C). H&E stained cross-sections showed that NMEG-NMZ reinnervated TA exhibited less fiber atrophy compared to the EEA treated and denervated TA muscles (Figure 4A'-C').

Muscle weights of the TA muscles on both sides in NN group are shown in Table 1. Group average at the operated side was 0.52g, and 0.61g at control non-operated side. The mean weight of the muscle on operated side was significantly lower than that on control side ($p<0.0001$, paired *t*-test). The average weight ratio of the operated to control side was 0.859 (std=0.041).

Muscle weights of TA muscles on both sides in EEA group are given in Table 2. Group average at the operated side was 0.43g (std=0.088), and 0.60g (std=0.068) at control non-operated side. The mean weight of the muscle on operated side was significantly lower than that on control side ($p<0.0001$, paired t-test). The average weight ratio of the operated to control side was 0.717 (std=0.092).

Comparison of average weight ratios of operated to control side between NN (0.859 (std=0.041)) and EEA (0.717 (std=0.092)) groups shows that NMEG-NMZ technique resulted in significantly better muscle weight recovery as compared to EEA ($p<0.0001$, unpaired t-test).

In the DC group, the mean muscle weight of the TA on the left operated side (0.15g) was lower than that on the right non-operated side (0.57g). The average ratio (left/right) was 0.260 (individual values no shown).

Axonal Regeneration and MEP Reinnervation

NF staining showed that the TA muscles reinnervated with NMEG-NMZ exhibited more extensive axon regeneration as compared with EEA reinnervated muscles (Figure 5A, B, D and E). The mean number and area fraction of the NF-ir axons for both TA muscles of each rat in NN and EEA groups are summarized in Tables 3 and 4, respectively. On average, the regenerated axons were calculated to be 76% of the controls in the NN group and 45.7% of the control in the EEA group. The axon counts in the NMEG-NMZ reinnervated TA were significantly higher than those in the EEA treated TA ($p<0.0001$, unpaired ttest). Similarly strong statistical difference was was observed for area fractions ($p<0.0001$, unpaired *t*-test).

The sections immunostained with double fluorescence staining showed the innervated and noninnervated MEPs (Figure 5C and F). The proportion of the reinnervated MEPs in the NMEG-NMZ reinnervated muscles (83%) were larger as compared with that in the EEA reinnervated muscles (59%). Axonal sprouts and newly formed small MEPs were also identified in both the NME-NMZ and EEA reinnervated muscles.

DISCUSSION

This study investigated the efficacy of NMEG-NMZ technique for limb muscle reinnervation after peripheral nerve injury. To our knowledge, this is the first study in which an

NMEG-NMZ pedicle obtained from the GM muscle (donor) was transferred to the denervated TA muscle (recipient) for restoring its motor function. There are several key findings. First of all, NMEG-NMZ surgery is surgically feasible for limb muscle reinnervation in the rat. Specifically, the denervated TA caused by peroneal nerve injuries can be reinnervated with GM-to-TA transplantation of an NMEG pedicle. Second, 3 months after surgery, NMEG-NMZ technique resulted in better functional recovery (toe spread and maximal muscle force recovered up to 83% and 79% of the control, respectively) as compared with standard EEA (58% and 51%, respectively). Third, NMEG-NMZ resulted in a greater percentage (76% of the control) of the regenerated axons than the EEA (46%). Fourth, the proportion of the reinnervated MEPs in the NMEG-NMZ treated muscles (83%) was larger than that in the EEA treated muscles (59%). Finally, NMEG-NMZ reinnervated muscles had a larger wet weight (86% of the control) as compared with EEA treated (71%) and denervated (26%) muscles. Clearly, NMEG-NMZ technique has unique advantages and is superior to EEA for muscle reinnervation and functional recovery.

The NMEG-NMZ technique was developed on the basis of the concept that the NMZ in the skeletal muscle containing numerous axon terminals and MEPs is an ideal target region for muscle reinnervation. Therefore, an NMEG pedicle harvested from the NMZ in the donor muscle provides an abundant source of nerve terminals and MEPs for nerve regeneration, whereas an NMEG pedicle implanted directely to the NMZ in the recipient muscle physically shortens regeneration distances and favors rapid axon-MEP connections. The regenerating axons from the implanted NMEG pedicle could easily reinnervate the denervated MEPs in the NMZ of the target muscle and form functional synapses, thereby avoiding irreversible loss of the denervated MEPs. This concept gains support from studies reported by others[31-37] and this research team.[11,14] Previous studies showed that after nerve injury and/or direct nerve implantation, regenerating axons preferentially make synaptic contact at the original MEPs[31-37] as a consequence of an as-yet poorly understood property of the muscle fibers within the MEP zone. Our previous study showed that implantation of the NMEG pedicle to a MEP-free area outside of the NMZ in the receipient muscle resulted in unfavorable functional recovery (67% of the control).[11] This suboptimal result was related, at least in part, to the fact that the regenerating axons from the NMEG pedicle did not reach the distal part of the reinnervated muscle[13] and failed to reinnervate

the denervated MEPs in the NMZ of the target muscle.[11,13] As MEP reinnervation is critical for motor recovery,[9,10] we changed the implantation site from MEP-free area[11] to the NMZ of the target muscle and showed that the NMZ in a muscle is the best site for NMEG implantation.[14]

The present study showed that NMEG-NMZ technique resulted in much better outcomes for limb muscle reinnervation as compared with standard EEA. One of the major causes leading to poor functional recovery after EEA surgery is a drop in the number of nerve fibers, which could pass through the coaptation site to reinnervate the denervated muscle.[7,8] This notion gains support from our findings which showed that the mean number of the intramuscular axons in the EEA treated TA (46% of the control) was much lower than that in the NMEG-NMZ reinnervated TA (76% of the control). In addition to the decreased regenerated axons, we also found that the mean number of reinnervated MEPs in the EEA treated muscles (59%) was significantly lower than that in the NMEG-NMZ reinnervated muscles (83%). We believe that both the decreased regenerated axons and innervated MEPs are attributed to the reduced muscle mass, fiber size, and muscle force generation.

Our recent[25] and present studies showed that transferring an NMEG pedicle from the NMZ in the lateral compartment of the GM muscle to the NMZ of the ipsilateral TA muscle is anatomically and surgically feasible. Here, we documented that the NMEG-NMZ technique is effective for immediate limb muscle reinnervation following peripheral nerve injuries. However, more work is needed to seek novel therapies for further improving nerve regeneration, MEP reinnervation, and functional recovery following NMEG-NMZ surgery. To this end, focal application of exogenous neurotrophic factors and/or introoperative brief electrical nerve stimulation would be useful approaches. Studies have demonstrated that nerve growth factor (NGF) and basic fibroblast growth factor (FGF-2) and many others have the potential for enhancing nerve regeneration and motor recovery.[38-41] Introoperative brief (one hour) low-frequency (20 Hz) continuous electrical nerve stimulation of the proximal nerve stump just after nerve repair has been shown to have the potential for enhancing axonal regeneration and promoting functional recovery.[42-45] Our previous animal studies have demonstrated that the focal application of NGF/FGF-2[46] and brief electrical stimulation[47] resulted in better axonal regeneration and functional recovery than NMEG-NMZ surgery alone in a rat neck muscle

reinnervation model. These findings encourage us to further investigate the beneficial effects of these nerve stimulating methods on limb reinnervation with NMEG-NMZ technique.

CONCLUSION

The NMEG-NMZ surgery resulted in more successful reinnervation of denervated leg muscle, when compared to EEA. With NMEG-NMZ technique, axonal regeneration and MEP reinnervation as well as musle preservation were substantially better as compared with EEA. There was also more successful functional recovery as assessed by musle force measurement and toe spread analysis. Clearly, the NMEG-NMZ transplantation is a promising technique for treating muscle paralysis caused by peripheral nerve injuries. NMEG-NMZ technique would be a useful surgical procedure to treat foot drop caused by paralyzed TA muscle following peripheral nerve injuries or lesions.

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Disclosures

The authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article.

REFERENCES

1. Wiberg M, Terenghi G. Will it be possible to produce peripheral nerves? *Surg Technol Int.* 2003;11:303-310.

2. Fern KT, Smith JT, Zee B, Lee A, Borschneck D, Pichora DR. Trauma patients with multiple extremity injuries: resource utilization and long-term outcome in relation to injury severity scores. *J Trauma.* 1998;45:489-494.

3. Dischinger PC, Read KM, Kufera JA, et al. Consequences and costs of lower extremity injuries. *Annu Proc Assoc Adv Automot Med.* 2004;48:339-353.

4. Siemionow M, Brzezicki G. Chapter 8: Current techniques and concepts in peripheral nerve repair. *Int Rev Neurobiol.* 2009;87:141-172.

5. Wong BJ, Crumley RL. Nerve wound healing. An overview. *Otolaryngol Clin North Am.* 1995; 28:881-895.

6. Lee SK, Wolfe SW. Peripheral nerve injury and repair. *J Am Acad Orthop Surg.* 2000;8:243-252.

7. Green DC, Berke GS, Graves MC. A functional evaluation of ansa cervicalis nerve transfer for unilateral vocal cord paralysis: future directions for laryngeal reinnervation. *Otolaryngol Head Neck Surg.* 1991;104:453-466.

8. Myckatyn TM, Mackinnon SE. A review of research endeavors to optimize peripheral nerve reconstruction. *Neurol Res.* 2004;26:124-138.

9. Ma CHE, Omura T, Cobos EJ, et al. Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. *J Clin Invest.* 2011;121:4332-4347.

10. Barbour J, Yee A, Kahn LC, Mackinnon SE. Supercharged end-to-side anterior interosseous to ulnar motor nerve transfer for intrinsic musculature reinnervation. *J Hand Surg Am.* 2012;37:2150-2159.

11. Mu L, Sobotka S, Su H. Nerve-muscle-endplate band grafting: a new technique for muscle reinnervation. *Neurosurgery.* 2011;69(2 Suppl Operative):ons208-ons224.

12. Sobotka S, Mu L. Comparison of muscle force after immediate and delayed reinnervation using nerve-muscle-endplate band grafting. *J Surg Res.* 2013;179:E117-E126.

13. Sobotka S, Mu L. Muscle reinnervation with nerve-muscle-endplate band grafting technique: correlation between force recovery and axonal regeneration. *J Surg Res.* 2015;195:144-151.

14. 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 Behav.* 2017;7(6):e00668. doi:10.1002/brb3.668.

15. Prahinski JR, McHale KA, Temple HT, Jackson JP. Bridle transfer for paresis of the anterior and lateral compartment musculature. *Foot Ankle Int.* 1996;17:615-619.
16. De Marchi F, Malerba F, Montrasio Alfieri U, Ferrarin M, Rabufetti M. Tibialis posterior tendon transfer through the interosseal membrane in paralysis of the common peroneal nerve. *Foot Ankle Surg.* 2000;6:19-25.
17. Yeap JS, Birch R, Singh D. Long-term results of tibialis posterior tendon transfer for drop-foot. *Int Orthop.* 2001;25:114-118.
18. Wood MB. Peroneal nerve repair. Surgical results. *Clin Orthop.* 1991;267:206-210.
19. Kim DH, Kline DG. Management and results of peroneal nerve lesions. *Neurosurgery.* 1996;39:312-320.
20. Gousheh J, Babaei A. A new surgical technique for the treatment of high common peroneal nerve palsy. *Plast Reconstr Surg.* 2002;109:994-998.
21. Nath RK, Lyons AB, Paizi M. Successful management of foot drop by nerve transfers to the deep peroneal nerve. *J Reconstr Microsurg.* 2008;24:419-427.
22. Giuffre JL, Bishop AT, Spinner RJ, Levy BA, Shin AY. Partial tibial nerve transfer to the tibialis anterior motor branch to treat peroneal nerve injury after knee trauma. *Clin Orthop Relat Res.* 2012;470:779-790.
23. Nath RK, Somasundaram C. Gait improvements after peroneal or tibial nerve transfer in patients with foot drop: A retrospective Study. *Eplasty.* 2017;17:e31.
24. Clawson DK, Seddon HJ. The results of repair of the sciatic nerve. *J Bone Joint Surg [Br]*. 1960;42:205-212.
25. Mu L, Chen J, Li J, Sobotka S, Nyirenda T. Limb muscle reinnervation with nerve-muscle-endplate grafting technique: An anatomical feasibility study. *Neurol Res Int.* 2021;2021: Article ID 6009342, 7 pages, https://doi.org/10.1155/2021/6009342
26. Bervar M. Video analysis of standing–an alternative footprint analysis to assess functional loss following injury to the rat sciatic nerve. *J Neurosci Methods* 2000;102:109-116.
27. Smit X, van Neck JW, Ebeli MJ, Hovius SER. Static footprint analysis: A time-saving functional evaluation of nerve repair in rats. *Scand J Plast Reconstr Surg Hand Surg.* 2004;38:321-325.

28. Bozkurt A, Scheffel J, Brook GA, et al. Aspects of static and dynamic motor function in peripheral nerve regeneration: SSI and CatWalk gait analysis. *Behav Brain Res.* 2011;219:55-62.
29. Hosseinian MA, Gharibi Loron A, Nemati B, Khandaghy M. Comparison of a distal end-to-side neurorrhaphy with a proximaldistal end-to-side neurorrhaphy: in a rat model. *Eur J Orthop Surg Traumatol.* 2015;25:1261-1264.
30. Hosseinian MA, Shirian S, Loron AG, Ebrahimy AA, Hayatolah GH. Distal sensory to distal motor nerve anastomosis can protect lower extremity muscle atrophy in a murine model. *Eur J Plast Surg.* 2018;41:9-14.
31. Sakellarides HT, Sorbie C, James L. Reinnervation of denervated muscles by nerve transplantation. *Clin Orthop Relat Res.* 1972;83:194-201.
32. Iwayama T. Relation of regenerating nerve terminals to original endplates. *Nature.* 1969;224:81-82.
33. Bennett MR, McLachlan EM, Taylor RS. The formation of synapses in reinnervated mammalian striated muscle. *J Physiol (Lond.).* 1973;233:481-500.
34. Gorio A, Carmignoto G, Finesso M, Polato P, Nunzi MG. Muscle reinnervation. II. Sprouting, synapse formation, and repression. *Neurosci.* 1983;8:403-416.
35. Sanes JR, Covault J. Axon guidance during reinnervation of skeletal muscle. *Trends Neuro Sci.* 1985;8:523-528.
36. Covault J, Cunningham JM, Sanes JR. Neurite outgrowth on cryostat sections of innervated and denervated skeletal muscle. *J Cell Biol.* 1987;105:2479-2488.
37. McMahan UJ, Wallace BG. Molecules in basal lamina that direct the formation of synaptic specializations at neuromuscular junctions. *Dev Neurosci.* 1989;11:227-247.
38. Derby A, Engleman VW, Frierdich GE, Neises G, Rapp SR, Roufa DG. Nerve growth factor facilitates regeneration across nerve gaps: morphological and behavioral studies in rat sciatic nerve. *Exp Neurol.* 1993;119:176-191.
39. Jubran M, Widenfalk J. Repair of peripheral nerve transections with fibrin sealant containing neurotrophic factors. *Exp Neurol.* 2003;181:204-212.
40. Lee AC, Yu VM, Lowe JB, et al. Controlled release of nerve growth factor enhances sciatic nerve regeneration. Exp Neurol. 2003;184:295-303.

41. Grothe C, Nikkhah G. The role of basic fibroblast growth factor in peripheral nerve regeneration. *Anat Embryol (Berl).* 2001;204:171-177.
42. Al-Majed AA, Neumann CM, Brushart TM, Gordon T. Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. *J Neurosci.* 2000;20:2602-2608.
43. Gordon T, Sulaiman O, Boyd JG. Experimental strategies to promote functional recovery after peripheral nerve injuries. *J Peripher Nerv Syst.* 2003;8:236-250.
44. Gordon T, Brushart TM, Amirjani N, Chan KM. The potential of electrical stimulation to promote functional recovery after peripheral nerve injury-comparisons between rats and humans. *Acta Neurochir Suppl (Wien).* 2007;100:3-11.
45. Gordon T, Amirjani N, Edwards DC, Chan KM. Brief post-surgical electrical stimulation accelerates axon regeneration and muscle reinnervation without affecting the functional measures in carpal tunnel syndrome patients. *Exp Neurol.* 2010;223:192-202.
46. Mu L, Sobotka S, Chen J, Nyirenda T. Nerve growth factor and basic fibroblast growth factor promote reinnervation by nerve-muscle-endplate grafting. *Muscle Nerve.* 2018;57:449-459.
47. Sobotka S, Chen J, Nyirenda T, Mu L. Intraoperative 1-hour electrical nerve stimulation enhances outcomes of nerve-muscle-endplate band grafting technique for muscle reinnervation. *J Reconstr Microsurg.* 2017;33:533-543.

Figure Legends

FIGURE 1. A, native motor zones (NMZs, boxed regions) within the left rat tibialis anterior (TA) muscle and gastrocnemius muscle (GM) as demonstrated by Sihler's stain that shows nerve supply patterns and wholemount AChE staining which localizes motor endplate (MEP) bands with numerous neuromuscular junctions (black dots). **B**, TA denervation and NMEG-NMZ transplantation. The TA is denervated by resecting a segment of peroneal nerve. The denervated TA is reinnervated with NMEG-NMZ technique. An NMEG pedicle containing a block of muscle tissue and a nerve branch is harvested from the NMZ of the lateral compartment of GM muscle (GM-l) (boxed region) and implanted to the NMZ of the denervated TA muscle.

FIGURE 2. Comparison of hind limb footprints between left (L) operated and right (R) non-operated sides in the rats with NMEG-NMZ (**A**), EEA (**B**), and 3-month denervation (**C**). Note that NMEG-NMZ resulted in more optimal motor recovery of the operated side as compared with EEA.

FIGURE 3. Analyses of muscle force and stimulation threshold data obtained from operated and non-operated TA muscles in NN and EEA groups. **A**, maximal muscle force. **B**, group averages of maximal muscle force. **C**, experimental/control muscle force. **D**, stimulation threshold. **E**, group averages of stimulation threshold. **F**, experimental/control threshold.

FIGURE 4. A-C, representative images of the left (L) reinnervated and denervated tibialis anterior (TA) muscles and right (R) contralateral controls from rats in NN (rat #1), EEA (rat #15) and denervation control (rat #6) groups, showing gross appearance and muscle mass. **A'-C'**, hematoxylin and eosin-stained cross-sections of the TA muscles reinnervated with NMEG-NMZ (**A'**) and EEA (**B'**) as well as denervated TA (**C'**). Note that NMEG-NMZ reinnervated TA exhibited very good preservation of myofiber morphology with less fiber atrophy as compared with EEA reinnervated and denervated muscles. Bar = 50 μm for A' through C'.

FIGURE 5. Immunostained horizontal sections of the rat tibialis anterior (TA) muscles reinnervated with NMEG-NMZ (**A-C**) and EEA (**D-F**), showing intramuscular regenerated axons (**A, B, D** and **E**) and innervated (arrows in **C** and **F**) and non-innervated (arrowheads in **C** and **F**) motor endplates (MEPs). Regenerated axons (darkly stained threads and dots in **A** and **D**; and green in **C** and **F**) were detected with SMI-31monoclonal against neurofilaments, while MEPs (red) were labeled with α-bungarotoxin. The stained sections in **A** and **D** were opened using ImageJ software and converted to binary images (**B, E**). The density of the regenerated axons was determined by estimating the number and area fraction of the positively stained axons within a section area (1.0 mm2). For the rat with NMEG-NMZ surgery in A (rat # 10), the operated TA had a very good muscle reinnervation as indicated by the mean axon count (813; 80.1% of the control) and the mean area (0.758; 76.3% of the control). For the rat with EEA nerve repair in B (rat # 2), the treated TA had less regenerated axons (421; 51% of the control) and area fraction

(0.298; 47.8% of the control). Also note that the NMEG-NMZ treated TA (**C**) had more reinnervated MEPs (red; arrows in **C**) as compared with EEA treated muscle (F). Arrowheads indicate non-reinnervated MEPs. Bar = 100 μm for A through F.

Figure 1

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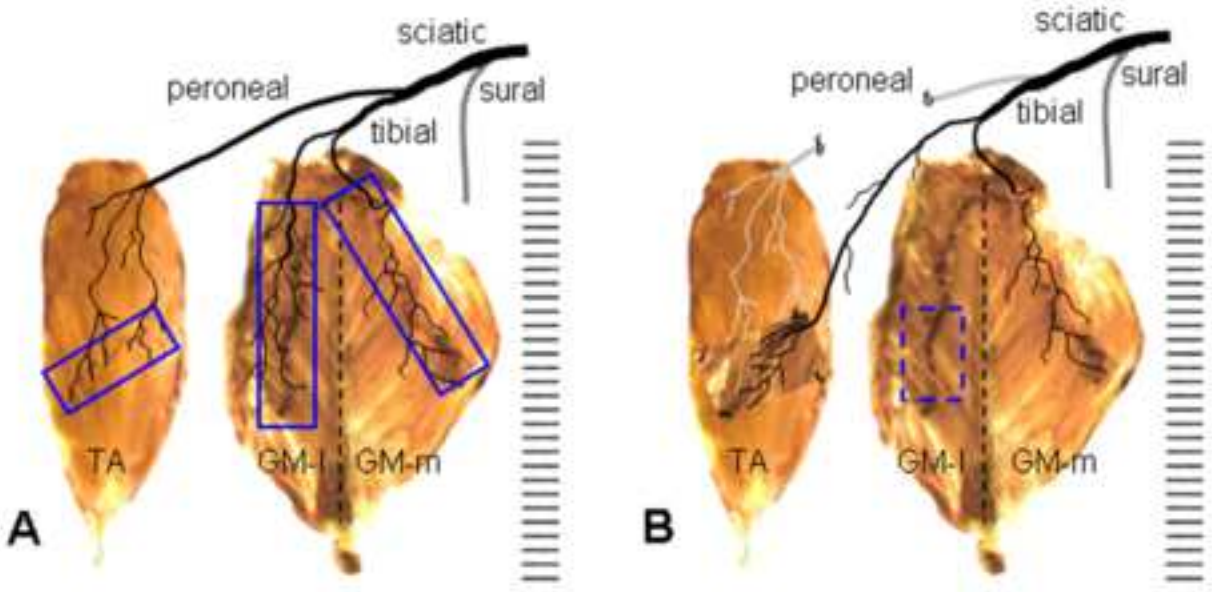

Figure 2

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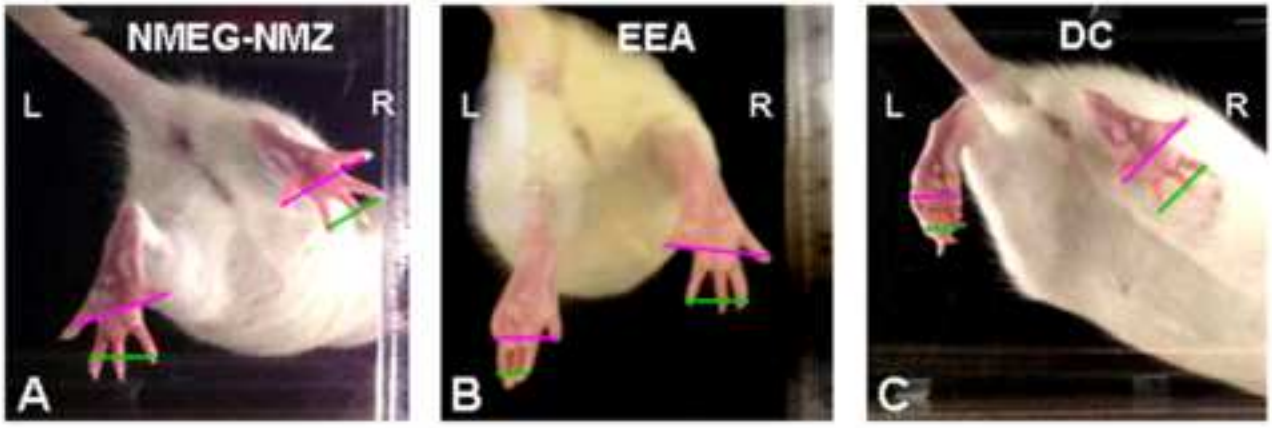

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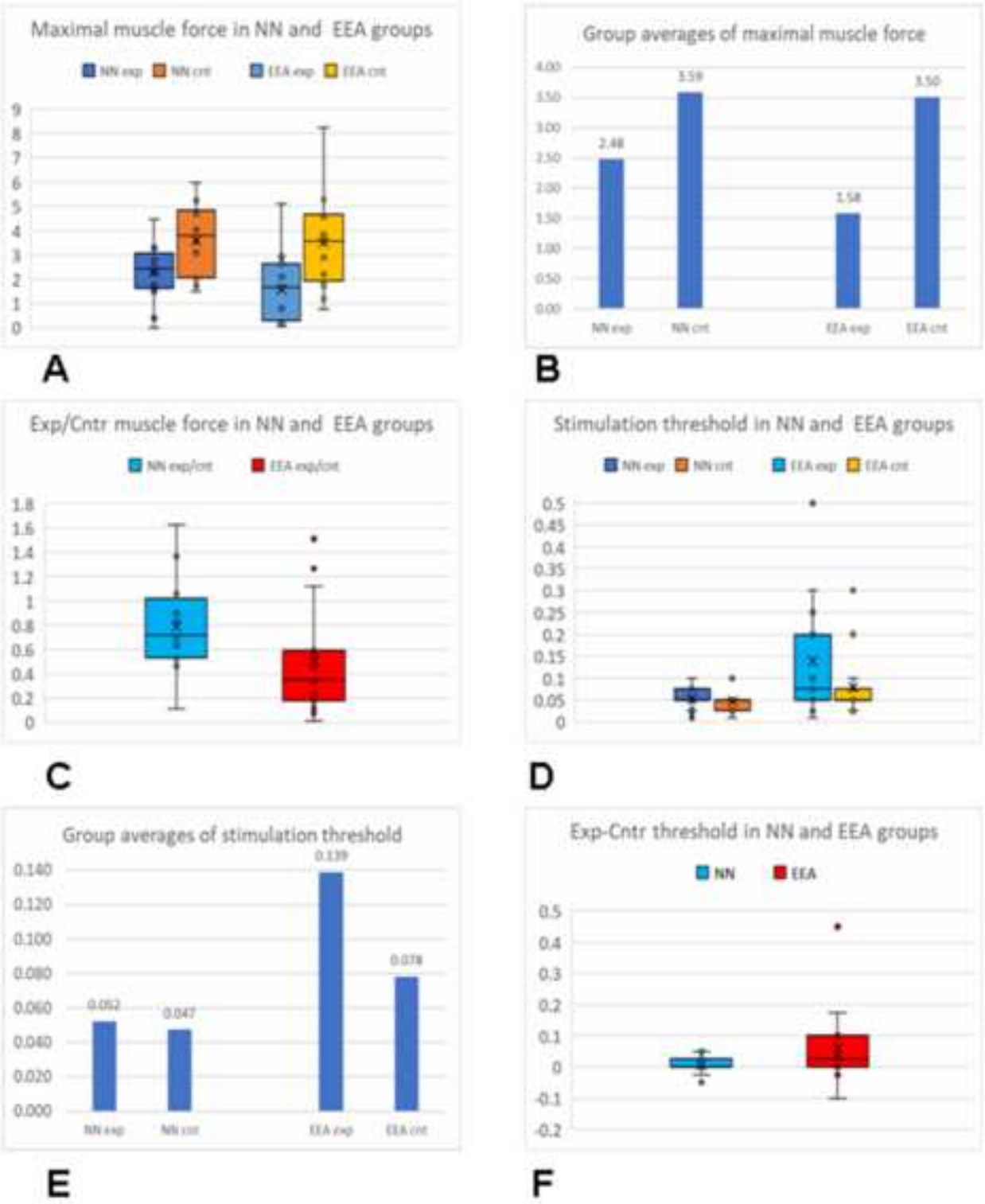

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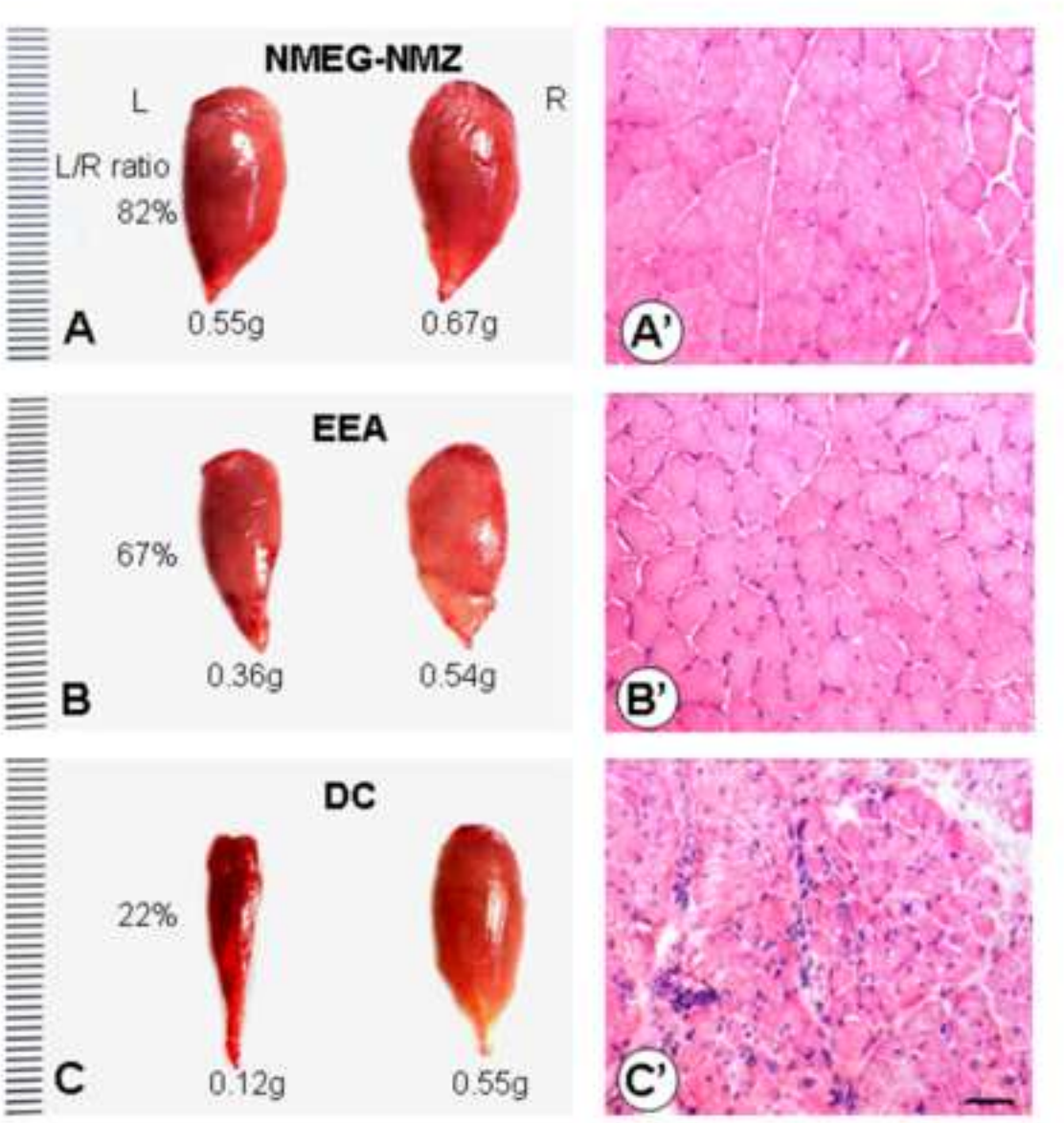

Figure 5

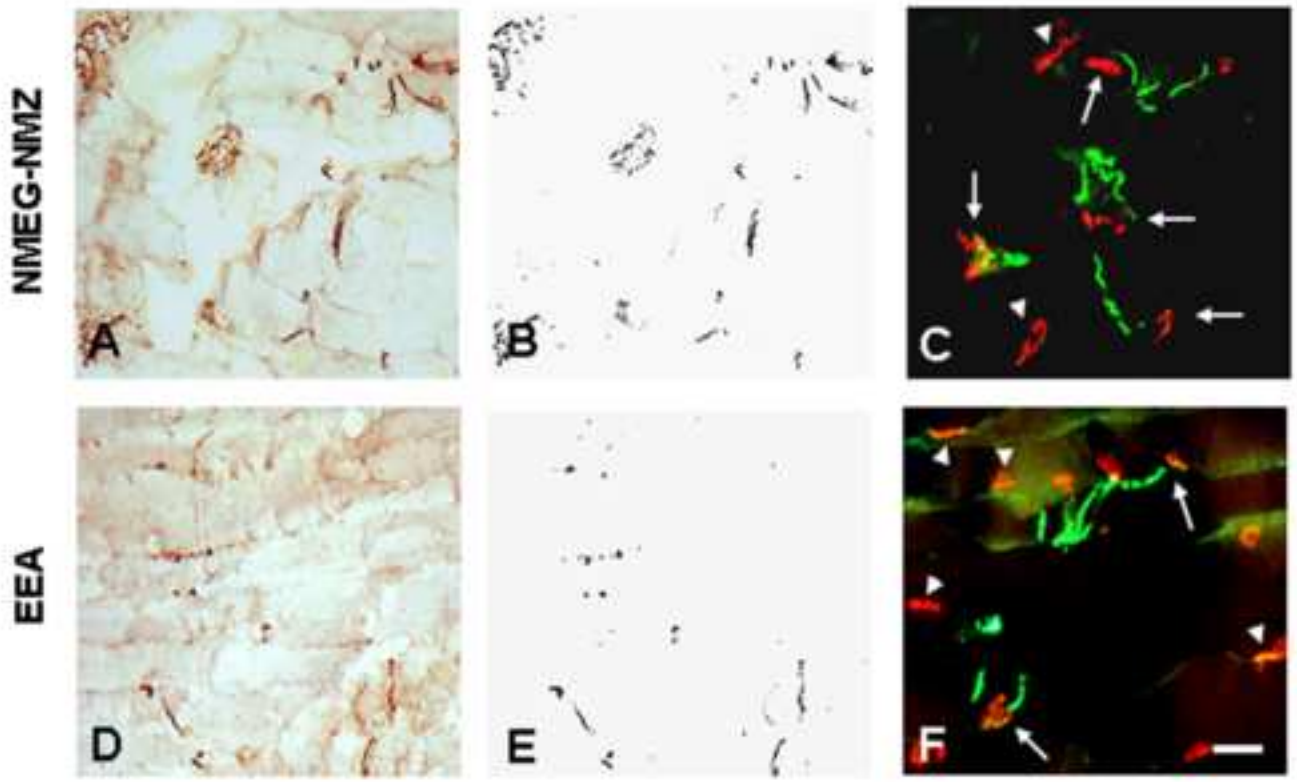

Table 1

TABLE 1. Wet Muscle Weight Measurement for the Left Tibialis Anterior (TA) Muscles Reinnervated with NMEG-NMZ and Right Controls in Rats (n = 15)

Animal no.	Body weight, g	Left TA g	Right TA g	Ratio L/R
1	349	0.55	0.67	0.82
2	334	0.52	0.61	0.85
3	314	0.51	0.60	0.85
4	316	0.50	0.65	0.77
5	340	0.52	0.63	0.83
6	315	0.53	0.59	0.90
7	348	0.52	0.59	0.88
8	350	0.51	0.62	0.82
9	324	0.55	0.61	0.90
10	320	0.54	0.58	0.93
11	305	0.52	0.62	0.84
12	333	0.49	0.58	0.84
13	321	0.52	0.59	0.88
14	441	0.57	0.65	0.88
15	325	0.48	0.54	0.89
Average	336	0.52	0.61	0.86
STD	32.3	0.024	0.033	0.041

L, left; R, right.

Table 2

TABLE 2. Wet Muscle Weight Measurement for the Left Tibialis Anterior (TA) Muscles Reinnervated with EEA and Right Controls in Rats (n = 15)

Animal no.	Body weight, g	Left TA g	Right TA g	Ratio L/R
1	279	0.33	0.54	0.61
2	303	0.48	0.60	0.80
3	302	0.51	0.66	0.77
4	310	0.57	0.67	0.85
5	300	0.44	0.71	0.62
6	315	0.50	0.61	0.82
7	330	0.53	0.70	0.76
8	315	0.40	0.62	0.65
9	315	0.52	0.61	0.85
10	327	0.39	0.57	0.68
11	337	0.34	0.54	0.63
12	335	0.45	0.65	0.69
13	265	0.34	0.51	0.67
14	252	0.28	0.49	0.57
15	280	0.36	0.54	0.67
Average	304	0.43	0.60	0.71
STD	25.5	0.088	0.068	0.092

L, left; R, right.

TABLE 3. Count and %Area of Regenerated Axons in the Left NMEG-NMZ Reinnervated and Right Control Tibialis Anterior (TA) Muscles in Rats (n = 15)

Animal no.	Left TA		Right TA		Ratio (L/R)	
	Count	%Area	Count	%Area	Count	%Area
1	632	0.426	836	0.628	0.756	0.678
2	695	0.399	877	0.699	0.792	0.571
3	801	0.529	984	0.738	0.814	0.717
4	597	0.602	933	0.805	0.640	0.748
5	510	0.435	723	0.899	0.705	0.484
6	683	0.615	894	0.973	0.764	0.632
7	804	0.586	993	0.941	0.810	0.623
8	721	0.533	924	0.745	0.780	0.715
9	669	0.601	822	0.869	0.814	0.692
10	813	0.758	1015	0.993	0.801	0.763
11	497	0.401	677	0.551	0.734	0.728
12	622	0.587	738	0.725	0.843	0.810
13	599	0.473	892	0.694	0.672	0.682
14	714	0.651	965	0.986	0.740	0.660
15	588	0.549	799	0.789	0.736	0.696
Average	663	0.543	871	0.802	0.760	0.680
STD	98.8	0.102	103.8	0.137	0.057	0.080

L, left; R, right.

Table 4

TABLE 4. Count and %Area of Regenerated Axons in the Left EEA Reinnervated and Right Control Tibialis Anterior (TA) Muscles in Rats (n = 15)

Animal no.	Left TA		Right TA		Ratio (L/R)	
	Count	%Area	Count	%Area	Count	%Area
1	352	0.311	759	0.613	0.464	0.507
2	421	0.298	825	0.623	0.510	0.478
3	298	0.307	794	0.712	0.375	0.431
4	306	0.332	698	0.679	0.438	0.489
5	420	0.481	913	0.927	0.460	0.519
6	439	0.502	1102	0.983	0.398	0.511
7	288	0.413	699	0.865	0.412	0.477
8	367	0.298	635	0.701	0.578	0.425
9	508	0.501	886	1.028	0.573	0.487
10	396	0.364	769	0.772	0.515	0.472
11	380	0.321	877	0.699	0.433	0.459
12	401	0.406	873	0.793	0.459	0.512
13	399	0.508	1225	0.978	0.326	0.519
14	515	0.486	983	0.883	0.524	0.550
15	330	0.523	847	0.946	0.390	0.553
Average	388	0.403	859	0.813	0.457	0.493
STD	68.4	0.089	155.3	0.140	0.073	0.037

L, left; R, right.

Focal application of neurotrophic factors augments outcomes of nerve-muscle-endplate grafting technique for limb muscle reinnervation

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Abstract

OBJECTIVE Our recently developed nerve-muscle-endplate grafting in the native motor zone (NMEG-NMZ) technique has been used for limb reinnervation. This study aimed to augment the outcomes of the NMEG-NMZ by focal administration of neurotrophic factors.

METHODS Thirty adult rats were randomly assigned into two groups (n=15 per group): NMEG-NMZ (NN) plus exogenous neurotrophic factors (NN/ENF) and autologous nerve grafting (ANG, technique control). The left tibialis anterior (TA) muscle was denervated by resecting its nerve and immediately treated with NN/ENF or ANG, respectively. For NMEG-NMZ, an NMEG pedicle was harvested from the lateral gastrocnemius muscle and implanted to the NMZ of the denervated TA. For ANG, the nerve gap was bridged with sural nerve. Three months after treatment, the animals underwent static toe spread analysis and muscle force measurement. Muscle weight, regenerated axons, and reinnervated motor endplates (MEPs) were quantified.

RESULTS NN/ENF resulted in better muscle force recovery (90% of the control) as compared with ANG (46%) and NMEG-NMZ surgery alone (79%) as reported elsewhere. The average muscle weight was 87% and 52% of the control for NN/ENF and ANG treated muscles, respectively. The mean count of the regenerated axons was measured to be 88% of the control for the muscles treated with NN/ENF, which was significantly larger than that in the ANG repaired muscles (39%). The average percentage of the reinnervated MEPs in the NN/ENF treated TA (89%) was higher as compared with that in the ANG repaired TA (48%).

CONCLUSIONS ENF enhances nerve regeneration and MEP reinnervation that further augment outcomes of NMEG-NMZ technique for limb muscle reinnervation.

KEYWORDS nerve-muscle-endplate grafting; exogenous neurotrophic factors; peripheral nerve injury; tibialis anterior; gastrocnemius

ABBREVIATIONS ABC = avidin-biotin complex; AChE = acetylcholinesterase; AChR = acetylcholine receptor; ANG = autologous nerve graft; BSA = bovine serum albumin; DAB = 3,3'-diaminobenzidine; EEA = end-to-end anastomosis; ENF = exogenous neurotrophic factors; FGF-2 = basic fibroblast growth factor; GM = gastrocnemius muscle; GM-l = lateral compartment of GM; GM-m = medial compartment of GM; H&E = hematoxylin and eosin; IACUC = Institutional Animal Care and Use Committee; ITS = intermediary toe spread; ITSF = ITS factor; MEP = motor endplate; N-CAM = neural cell adhesion molecule; NF = neurofilament; NF-ir = NF immunoreactive; NGF = nerve grouth factor; NITS = non-operated side intermediate toe spread; NMEG = nerve-muscle-endplate grafting; NMZ = native motor zone; NN = NMEG-NMZ; NN/ENF = NMEG-NMZ plus ENF; NTS = non-operated side toe spread; OITS = operated side intermediate toe spread; OTS = operated side toe spread; PBS = phosphate-buffered saline; PNI = peripheral nerve injury; RT = room temperature; SSI = static sciatic index; STSA = static toe spread analysis; TA = tibialis anterior; TS = toe spread; TSF = TS factor

Introduction

Peripheral nerve injuries (PNIs) to the extremities and resultant muscle paralysis represent a significant cause of morbidity and disability in both military and civilian populations.[1] Among soldiers with extremity injuries, 37% had PNIs.[2] More than one-third of soldiers with extremity injuries were not fit to return to active duty due to disability.[3] In the civilian population, PNIs are mostly caused by vehicle accidents[4] and surgical intervention.[5] It is estimated that 20 million Americans suffer from PNIs,[6] with a health care burden of $150 billion annually.[7] Severe PNI has a potentially devastating impact on patients' quality of life.

A number of surgical procedures have been used to restore motor function following PNIs, including nerve end-to-end anastomosis (EEA), end-to-side neurorrhaphy, autologous nerve grafting (ANG), nerve transfer, muscular neurotization, and tubulization techniques.[8] Unfortunately, these surgical procedures result in unsatisfactory functional recovery. Only about half of patients treated with EEA regain useful function.[9] Our animal studies showed that maximal force of the muscles treated with EEA recovered only 51-57% and average number of the regenerated axons in the treated muscles was reduced to 31-46% of the control in the rat.[10,11] The unfavorable outcome of the EEA is associated with insufficient regenerating axons that pass through the coaptation site to reach the target muscle.[10-12]

PNIs with a long segmental gap present a difficult clinical problem for peripheral nerve surgeons. Nerve gap defects (segmental nerve injuries) are generally repaired with ANG, nerve conduits, or nerve transfer. However, the recovery rate of motor function for ANG, the gold-standard treatment for nerve gap injuries, is only about 40%.[13] The outcomes are even poorer with nerve conduits, resulting in useful reinnervation in only 13% of cases with larger nerve defects.[14,15] If the proximal nerve stump of an injured nerve is unavailable, nerve transfer is often used to repair the injured nerves, with mixed results.[16] Despite intensive research and plentiful published procedures, none have achieved optimal outcomes.

As poor motor recovery after PNIs and nerve repair is due mainly to insufficient axonal regeneration and failure to reinnervate the denervated motor endplates (MEPs) in the target muscle,[17] we developed a novel surgical technique called nerve-muscle-endplate grafting (NMEG) technique.[18] The idea is that a more functionally important denervated muscle can be reinnervated by transferring an NMEG pedicle from a neighboring expendable muscle. We harvested an NMEG pedicle from the native motor zone (NMZ) of the donor muscle and implanted it into the NMZ in the recipient muscle. Our studies showed that NMEG-NMZ resulted in promissing functional recovery of the reinnervated neck (82%)[19] and limb (79%)[11] muscles in the rat.

This study aimed to further augment the outcomes of NMEG-NMZ by focal application of exogenous neurotrophic factors (ENFs) to promote nerve regeneration and functional recovery.[20-23]

Methods

Experiment Design and Groups

Thirty 3-month-old female Sprague-Dawley rats (Charles River Laboratories, MA) were used in this study. Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of our institution. All animals had ad libutum access to food and water and were housed according to the NIH *Guide for the Care and Use of Laboratory Animals* in the veterinary facilities at our institution. The animals were randomly assigned into two groups (15 rats/per group): NMEG-NMZ plus ENF (NN/ENF) group and autologous nerve grafting (ANG; technique control).

Surgical Procedures and Focal Administration of ENFs

Under general anesthesia with an intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (5 mg/kg), animals had surgical procedures on the left legs. Under aseptic conditions, an incision was made in the posterior thigh and calf of the hind limb to expose the TA and GM and the three major branches of the sciatic nerve (Fig. 1).

Animals in NN/ENF group underwent NMEG-NMZ and focal administration of ENFs [a mixture of nerve growth factor (NGF) and basic fibroblast growth factor (FGF-2)] as described.[11,24] Briefly, the left TA muscle was denervated by resecting a 10-mm segment of its innervating nerve. The denervated TA was treated with NMEG-NMZ and ENFs. For NMEG-NMZ, the NMZs in the left TA and GM were outlined according to the locations of the MEP bands and muscular nerve branches (Fig. 1) as described.[11,24] An NMEG pedicle containing a block of muscle (~8×6×4 mm), axon terminals and MEPs was harvested from the NMZ of the lateral GM (GM-l) in continuity with its motor nerve branch. Then, a muscular defect with the same dimensions as the NMEG pedicle was made in the NMZ of the left denervated TA muscle (Fig. 1). Figure 2 illustrates steps for NN/ENF procedures. During NMEG-NMZ, the muscular defect created on the TA was filled with 0.5 ml of fibrin sealant (TISSEEL Kit®; Baxter

Healthcare, Westlake Village, CA) containing recombinant rat NGF (100 ng/ml) and FGF-2 (100 μg/ml) (R&D Systems, Minneapolis, MN). As reported, focal application of both neurotrophic factors at these concentrations resulted in better functional recovery.[25,26] Then, the NMEG pedicle was placed on the fibrin sealant and sutured with four 10-0 microsutures. After surgery, the wound was closed.

For ANG nerve repair, a gap defect was made by resecting a 10-mm segment of the TA nerve 5 mm proximal to the motor point. The gap defect was bridged with the same length of sural nerve (sensory) using 10-0 nylon microsutures.

Postoperative evaluations were performed on both sides at the end of the 3-month recovery period.

Evaluating Functional Recovery

Static Toe Spread Analysis

Static toe spread analysis (STSA) proposed by Bervar (2000)[27] has been demonstrated to be a useful method for assessing limb motor function.[28,29] In this study, STSA was performed just before muscle force measurement as described.[11] In brief, the rat was placed in an acrylic 40×20×20 cm container on a transparent base plate. A camera was positioned underneath the base plate to photograph the plantar surface of the rat hind limb paws. The distance (mm) between the 1-5 toe spread (TS) and between the 2-4 intermediate toe spread (ITS) on the operated (O) and non-operated (N) sides were measured. The mean values of TS and ITS in each group were used to determine the TS and ITS factors (TSF and ITSF): TSF = (OTS – NTS)/NTS and ITSF = (OITS – NITS)/NITS. Bervar (2000)[27] proposed a static sciatic index (SSI) for toe spread analysis. The SSI is calculated using the static TSF and ITSF in the equation: SSI = (108.44 × TSF) + (31.85 × ITSF)–5.49. An index score of 0 is defined as normal and an index score of −100 indicates a complete functional loss.

Maximal Muscle Force Measurement

Under general anesthesia, animals underwent maximal muscle force measurement to quantify the degree of functional recovery of the left TA as described.[11] Briefly, the transplanted tibial nerve branch supplying the NMEG (NN/ENF group) or the repaired peroneal nerve (ANG group) was identified, isolated, and placed on a bipolar stimulating electrode for nerve stimulation.

The distal tendon of the TA was severed and the muscle was dissected proximally up to the level of its origin. The transected TA tendon was tied with a 4-0 suture, and connected to force transducer attached to a servomotor lever arm of the Dual-Mode Lever System (305B-LR; Aurora Scientific Inc, Aurora, Ontario, Canada). A stimulation and recording system (National Instruments Corp, Austin, Texas) controlled by user-written LabVIEW 8.2 software (National Instruments Corporation) was used to deliver biphasic rectangular pulses to the nerve stimulated. The nerve was placed on hook-shaped stimulating electrodes and stimulated with 200 ms trains of biphasic rectangular pulses at the optimal muscle length. 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 5 mA. A break of at least 1.0 minute was taken between two stimulations to permit muscle recovery.

Collected data were analyzed offline with DIAdem 11.0 software (National Instruments). Maximal muscle force from the operated side was calculated as a percentage of muscle force recorded from the control side. During force measurement, the TA muscle and nerve were bathed regularly with warmed mineral oil to maintain muscle temperature between 35°C and 36°C and the core body temperature was maintained at the same level with a closed circuit homeothermic blanket system for rodents.

Examining Muscle Weight, Fiber Morphology, Denervated Myofibers, and Fiber Type-Grouping

At the end of experiment, the TA muscles on both sides were removed, weighed using a microscale, photographed, and prepared for tissue studies. Each of TA muscles was divided into two segments: superior one-third and inferior two-thirds, which were frozen and cut on a cryostat (Reichert-Jung 1800; Mannheim, Germany) at –25ºC. The superior segments were cut transversly (10-µm thick) and stained with hematoxylin and eosin (H&E) and monoclonal antibodies to examine fiber morphology,

fiber type-grouping, and denervated and atrophied myofibers. The inferior segments were cut horizontally (50-µm thick) and immunostained to examine regenerated axons and MEPs.

Immunohistochemistry for Detecting Fiber Type-Grouping

Anti-myosin heavy chain monoclonal antibody NOQ7-5-4D was used to label slow type I muscle fibers for detecting fiber type-grouping as described.[30] Some cross-sections were: (1) fixed in 4% paraformaldehyde for 10 minutes; (2) blocked in a blocking solution composed of 2% bovine serum albumin (BSA) and 0.1% Triton X-100 at room temperature (RT) for 20 minutes; (3) incubated monoclonal antibody NOQ7-5-4D (1:1000; Sigma, St. Louis, MO) for 1 hour at RT; (4) processed with a Vectastain antimouse IgG (ATCC, Rockville, MD) for 1 hour at RT; (5) reacted for 1 hour at RT in ABC reagent; and (6) reacted for 10 minutes at RT with DAB substrate kit (SK-4100; Vector Labs, Burlingame, CA). Control sections were treated using the aforementioned procedures but without incubation with a primary antibody.

The stained sections were viewed on a Zeiss photomicroscope (Axiophot-1; Carl Zeiss, Goettingen, Germany) and photographed with a USB 3.0 digital microscope camera (Infinity 3-3URC; Lumenera Corp., Ottawa, Ontario, Canada). For a given muscle, 3 stained cross-sections were randomly selected at different spatial levels through the muscle to identify fiber type-grouping that is indicative of partial denervation and reinnervation.

Neural Cell Adhesion Molecule (N-CAM) Immunohistochemistry for Detecting Denervated Myofibers

N-CAM is abundant on the surface of early embryonic myotubes, declines in level as development proceeds, nearly disappears in the adult muscle, reappears when adult muscles are denervated, and is lost after reinnervation.[31] Therefore, N-CAM is a molecular marker of muscle fiber denervation. In this study, some cross-sections were immunostained to detect denervated myofibers as described.[30] Briefly, the sections were: (1) fixed with methanol at –20°C for 20 minutes; (2) blocked with 5% goat serum (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) for 30 minutes; (3) incubated for 2 hours with a

primary monoclonal rabbit antirat N-CAM antibody (Chemicon, Temecula, CA); and (4) incubated with a secondary CY3-conjugated goat antirabbit IgG (Jackson ImmunoResearch laboratories, West Grove, PA) at RT for 1 hour. Control sections were stained without the incubation with the primary antibody. The stained sections were mounted with Vectashield mounting medium (Vector) and photographed.

For a given muscle, 3 stained cross-sections were randomly selected at different spatial levels through the muscle to identify N-CAM positive fibers. The mean proportion of the denervated fibers for each group was computed.

Quantifying Regenerated Axons and Reinnervated MEPs

Neurofilament (NF) Staining to Label Regenerated Axons

Some horizontal sections were immunostained with monoclonal antibody SMI-31 (Covance Research Products, Berkeley, CA) as a marker for all axons as described.[11,19] Briefly, the sections were: (1) blocked in 2% BSA for 30 minutes; (2) incubated with primary antibody SMI-31 (1:800) in PBS containing 0.03% Triton at 4ºC overnight; (3) incubated with anti-mouse biotinylated secondary antibody (1:1000; Vector) for 2 hours; (4) processed with the avidin-biotin complex method using a VectaStain ABC kit (1:1,000; 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 photographed. The density of the intramuscular axons was determined by computing the neurofilament immunoreactive (NF-ir) axons and the area fraction of the axons within a section area (1.0 mm^2) as described.[11] For a given muscle 3 stained sections at different spatial levels through the muscle were randomly selected to count NF-ir axons with public domain ImageJ software v1.45s (National Institutes of Health, Bethesda).

Double-Fluorescence Staining to Identify MEPs

MEPs were examined using double-fluorescence staining as described. [11,19] In brief, some horizontal sections were: (1) dried and then placed in Zamboni fixative at 4ºC for 20 minutes; (2) blocked in 4% normal goat serum for 30 minutes; (3) incubated overnight at 4°C with primary antibody SMI-31 to label axons (1:1000, Covance Research Products Inc); and (3) incubated at RT for 2 hours both with Alexa Fluor 488 goat antimouse IgG secondary antibody (1:500; Invitrogen Corp., Carlsbad, CA) to label axons and with Alexa Fluor 596–conjugated α-bungarotoxin (1:500; Invitrogen) to visualize postsynaptic acetylcholine receptors (AChRs) at MEPs. Control sections were stained without the incubation with the primary antibody.

The stained sections were viewed under a photomicroscope equipped with epifluorescence optics and photographed. SMI-31 detected axons (green), while α-bungarotoxin labeled postsynaptic AChR site in the MEPs (red). For each muscle sample, at least 100 labeled MEPs were randomly selected from three stained sections at different spatial levels through the muscle to determine the percentages of the innervated (visible axon attachment) and noninnervated (no visible axon attachment) MEPs.

Data Analysis

STSA measures, force values, wet muscle weights, NF-ir axon counts, and innervated and noninnervated MEPs of the TA muscles in each rat were computed. The Student *t*-test (paired or unpaired as appropriate) was used for comparison of normally distributed data. Mann-Whitney U test (for unpaired comparisons) or Wilcoxon signed rank test (for paired comparisons) was used for comparison of nonparametric data. Type I error at $p<0.05$ (two tailed *t*-test) was treated as statistically significant. All comparisons were made with SAS 9.4 software or with online statistics calculators.

Results

Static Toe Spread

The rats in the NN/ENF group (Fig. 3A) group had better toe spread recovery as compared with those in the ANG group (Fig. 3B). The mean SSI score was calculated to be –11.3 and –50.9 for the NN/ENF and ANG groups, respectively (Fig. 3C). In other word, the toe spread recovered up to 89% of the control for the NN/ENF group and 49% for the ANG group ($p<0.0001$).

Maximal Tetanic Muscle Force

Figure 4A shows the average threshold current that produced visible muscle contraction at the left (operated) and right (contralateral control) sides. Figure 4B shows the rate of operated to control current. The thresholds currents from the treated muscles were significantly higher than those from the contralateral controls (in ANG, $p = 0.003$, but not in NN/ENF, $p>0.05$). On average, ANG repaired TA had a higher threshold rate of nerve stimulation currents at the operated to control sides (7.20) as compared with NN/ENF (1.90, $p=0.03$).

Figure 4C shows the average maximal muscle force of the left treated and right control TA muscles and Figure 4D shows the rate of operated to control muscle force. Increasing stimulation current was accompanied by increasing muscle force until muscle force reached horizontal asymptote at the current of about 0.1 mA. Maximal muscle force was calculated in response to larger stimulation currents from 0.5 mA to 0.75 mA. Maximal muscle force was significantly smaller at the treated side compared to the control in ANG group ($p<0.0001$), but the difference did not reach statistical significance in NN/ENF group ($p>0.05$). NN/ENF resulted in much better muscle force recovery (90%) as compared with ANG (46%). The rate of operated to control force was significanly higher ($p<0.0001$) in NN/ENF as compared to ANG group.

Muscle Weight and Muscle Fiber Morphology

Wet muscle weight, size and fiber morphology of the left treated and right control TA muscles in each group are displayed in Figure 5. Mean group data on muscle weights of the left treated and right control TA muscles (Fig. 5A) and left (L)/right (R) ratio (Fig. 5B) showed that NN/ENF resulted in better muscle weight recovery than ANG ($p < 0.0001$). The mean muscle weignts of the left treated TA and right control TA muscles were 0.52 g and 0.60 g for NN/ENF group and 0.34 g and 0.62 g for ANG group (Fig. 5A). The TA muscle weights were recovered to 87% and 52% for the NN/ENF and ANG groups, respectively (Fig. 5B).

Figure 5C shows paired TA muscles of some individual rats selected from NN/ENF (left) and ANG (right) groups. Note that the size of the TA treated with NN/ENF was close to that of the right control, whereas the ANG treated TA had a significant loss of muscle mass. H&E stained cross-sections (Fig. 5D) showed that NN/ENF (left) treated muscle exhibited less fiber atrophy compared to the ANG treated TA (right).

Fiber-Type Grouping and Denervated Muscle Fibers

Type I fiber type grouping was observed frequently in the ANG repaired TA muscles (Fig. 6A) and occasionally identified in the NN/ENF treated muscles (data not shown).

Denervated and atrophied muscle fibers in the rat TA were labeled with N-CAM immunohistochemistry. N-CAM was expressed around muscle fibers and/or muscle fibers displayed sarcoplasmic expression. The N-CAM-positive fibers were identified more frequently in the ANG treated TA muscles (Fig. 6B) than in the NN/ENF treated muscles. The N-CAM-positive fibers accounted for 32% of the total fiber population in the ANG treated TA, 8% in the NN/ENF treated TA.

Regenerated Axons and Reinnervated MEPs

Figure 7 summarizes regenerated axons and reinnervated MEPs in the treated TA muscles. The profiles of the regenerated axons (Fig. 7A, D) and converted images for computing the numbers of the

regenerated axons (Fig. 7B, E) are presented. The TA muscles treated with NN/ENF (Fig.7A-C) exhibited more axonal regeneration compared to the ANG treated muscles (Fig. 7D-F). Figure 7G shows group mean data on the left/right (L/R) ratio of the regenerated axons. The mean number of the regenerated axons on the left treated TA was measured to be 88% of the right control for the NN/ENF, which was significantly higher than that for the ANG treated muscles (39%) ($p<0.0001$). The innervated and noninnervated MEPs were lebeled with double-fluorescence staining (Fig. 7C, F). The average percentage of the innervated MEPs in the NN/ENF group (89%) was higher as compared with that in the ANG group (48%).

Discussion

To the best of our knowledge, this is the first study to use fibrin-based neurotrophic factor-delivery system for augment outcomes of NMEG-NMZ in limb reinnervation. Our experiments documented that NGF/FGF-2 could promote the efficacy of NMEG-NMZ for limb muscle reinnervation. The extent of functional recovery is positively correlated to the extent of muscle atrophy, axonal regeneration, and MEP reinnervation.

Previous studies have demonstrated that NGF enhances nerve regeneration and motor recovery.[21,33] FGF-2 promotes nerve regeneration,[34] increases the number of proliferating Schwann cells and regenerating axons[34] and MEPs,[25] and improves functional recovery.[25] The delivery of biologically active molecules locally during regeneration has been extensively pursued in animal models for decades. Various ENFs have been used in nerve repair[21] and tubulization.[35] They can be administrated locally via direct injection into the target nerve[36] or muscle,[25] or by using a surgically implanted osmotic pump.[37] More recently, focal administration of a fibrin sealant containing ENFs has been used in PNI and repair models. This approach permits slow continual release of ENFs directly to the damaged and/or repaired nerve in order to accelerate axonal regeneration and subsequent functional recovery.[20,21,38,39] *In* vitro, neurotrophic factors in fibrin sealant can be released locally over periods of 2-4 weeks.[20,39] Studies

have demonstrated that combinations of neurotrophic factors yield better results than single factors alone.[37,40] Thus, the fibrin-based drug-delivery system for ENF release is a promising method for slow continual release of neurotrophic factors that favors accelerating axonal regeneration, thereby improving innervation surgical outcomes. Our findings indicate that the combination of NGF and FGF-2 have the ability to improve outcomes of NMEG-NMZ surgery and may become a useful adjunct therapy for muscle reinnervation.

Clearly, focal administration of neurotrophic factors has the potential to accelerate axonal regeneration, MEP reinnervation, and muscle mass preservation, thereby promoting functional recovery of the treated muscle after PNIs. However, further work is needed to assess the efficacy of the NMEG-NMZ technique and nerve growth stimulation methods for delayed muscle reinnervation. Clinically, some injured nerves cannot be repaired in the acute phase of PNI due to concomitant injuries. In this case, nerve reconstruction is often performed after three to six months' delay if there is no evidence of reinnervation. Unfortunately, by three months after PNI, the number of distal axons are reduced by about 80-90%,[41] and chronic denervation or delayed muscle reinnervation results in insufficient axonal regeneration and very poor functional recovery.[17] Our recent studies showed that 3-, 6-, and 9-month denervation of the rat sternomastoid muscle resulted in a progressive reduction in muscle weight (38%, 31%, and 19% of the control, respectively), fiber diameter (52%, 40%, and 28%), and MEP count (79%, 65%, and 43%).[32] Thus, muscle loss, reduced axons and MEPs would be major factors leading to poor functional recovery. At present, however, there is a lack of effective methods to delay or prevent muscle atrophy and MEP degradation after denervation. There is a pressing need to develop specific therapeutic strategies to minimize these complications of delayed reinnervation.

Conclusions

First, the NMEG-NMZ could be an alternative option to treat denervated limb muscles caused by PNIs, especially segmental nerve injuries.

Second, the outcomes of NMEG-NMZ can be augmented by a combination of NMEG-NMZ surgery with focal application of neurotrophic factors.

Finally, more work is warranted to determine the effectiveness of this combined approach for delayed limb reinnervation.

Aknowledgments

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Mu, Sobotka, Chen.

Acquisition of data: Chen, Sobotka, Li, Mu.

Analysis and interpretation of data: All authors.

Drafting the article: Mu, Sobotka.

Critically revising the article: Mu, Sobotka.

Reviewed submitted version of manuscript: all authors.

Approved the final version of the manuscript on behalf of all authors: Mu.

Statistical analysis: Sobotka, Nyirenda.

Administrative/technical/material support: Chen, Li, Sobotka, Mu.

Study supervision: Mu, Sobotka.

References

1. Ecklund JM, Ling GS. From the battlefront: peripheral nerve surgery in modern day warfare. *Neurosurg Clin N Am.* 2009;20(1):107-110.

2. Rivera JC, Glebus GP, Cho MS. Disability following combat-sustained nerve injury of the upper limb. *Bone Joint J.* 2014;96-B(2):254-258.

3. Masini BD, Waterman SM, Wenke JC, Owens BD, Hsu JR, Ficke JR. Resource utilization and disability outcome assessment of combat casualties from Operation Iraqi Freedom and Operation Enduring Freedom. *J Orthop Trauma.* 2009;23(4):261-266.

4. Eser F, Aktekin LA, Bodur H, Atan C. Etiological factors of traumatic peripheral nerve injuries. *Neurol India.* 2009;57(4):434-437.

5. Kretschmer T, Antoniadis G, Braun V, Rath SA, Richter HP. Evaluation of iatrogenic lesions in 722 surgically treated cases of peripheral nerve trauma. *J Neurosurg.* 2001;94(6):905-912.

6. Lundborg G. Nerve injury and repair – a challenge to the plastic brain. *J Peripher Nerv Syst.* 2003;8(4):209-226.

7. Taylor CA, Braza D, Rice JB, Dillingham T. The incidence of peripheral nerve injury in extremity trauma. *Am J Phys Med Rehabil.* 2008;87(5):381-385.

8. Siemionow M, Brzezicki G. Chapter 8: Current techniques and concepts in peripheral nerve repair. *Int Rev Neurobiol.* 2009;87:141-172.

9. Lee SK, Wolfe SW. Peripheral nerve injury and repair. *J Am Acad Orthop Surg.* 2000;8(4):243-252.

10. Sobotka S, Mu L. Force recovery and axonal regeneration of the sternomastoid muscle reinnervated with the end-to-end nerve anastomosis. *J Surg Res.* 2013;182(2):e51-e59.

11. Sobotka S, Mu L, Chen J, Li J, Nyirenda T. Reinnervation of paralyzed limb muscle by nerve-muscle-endplate grafting technique. *Neurosurgery.* 2022;(under review).

12. Green DC, Berke GS, Graves MC. A functional evaluation of ansa cervicalis nerve transfer for unilateral vocal cord paralysis: future directions for laryngeal reinnervation. *Otolaryngol Head Neck Surg.* 1991;104(4):453-466.

13. Moneim M, Omer G. Clinical outcome following acute nerve repair, In: Omeg G, Spinner M, Van Beek A, eds. *Management of Peripheral Nerve Problems.* Philadelphia; Saunders, 1998:414-419.

14. Stanec S, Stanec Z. Reconstruction of upper-extremity peripheral-nerve injuries with ePTFE conduits. *J Reconstr Microsurg.* 1998;14(4):227-232.

15. Strauch B. Use of nerve conduits in peripheral nerve repair. *Hand Clin.* 2000;16(1):123-130.

16. Lee SK, Wolfe SW. Nerve transfers for the upper extremity: new horizons in nerve reconstruction. *J Am Acad Orthop Surg.* 2012;20(8):506-517.

17. Ma CHE, Omura T, Cobos EJ, Latremoliere A, Ghasemlou N, Brenner GJ, et al. Accelerating axonal growth promotes motor recovery after peripheral nerve injury in mice. *J Clin Invest.* 2011;121(11):4332-4347.

18. Mu L, Sobotka S, Su H. Nerve-muscle-endplate band grafting: a new technique for muscle reinnervation. *Neurosurgery.* 2011;69(ONS Suppl 2):ons208-ons224.

19. 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 Behav.* 2017;7(6):e00668.

20. Zeng L, Worseg A, Redl H, Schlag G. Peripheral nerve repair with nerve growth factor and fibrin matrix. *Eur J Plast Surg.* 1994;17:228-232.

21. Jubran M, Widenfalk J. Repair of peripheral nerve transections with fibrin sealant containing neurotrophic factors. *Exp Neurol.* 2003;181(2):204-212.

22. Lee AC, Yu VM, Lowe JB, Brenner MJ, Hunter DA, Mackinnon SE, et al. Controlled release of nerve growth factor enhances sciatic nerve regeneration. *Exp Neurol.* 2003;184(1):295-303.

23. Aebischer P, Salessiotis AN, Winn SR. Basic fibroblast growth factor released from synthetic guidance channels facilitates peripheral nerve regeneration across long nerve gaps. *J Neurosci Res.* 1989;23:282-289.

24. Mu L, Chen J, Li J, Sobotka S, Nyirenda T. Limb muscle reinnervation with nerve-muscle-endplate grafting technique: An anatomical feasibility study. *Neurol Res Int.* vol. 2021; Article ID 6009342, 7 pages, 2021.

25. Iwata Y, Ozaki N, Hirata H, et al. Fibroblast growth factor-2 enhances functional recovery of reinnervated muscle. *Muscle Nerve.* 2006;34(5):623-630.

26. Mu L, Sobotka S, Chen J, Nyirenda T. Nerve growth factor and basic fibroblast growth factor promote reinnervation by nerve-muscle-endplate grafting. *Muscle Nerve.* 2018;57(3):449-459.

27. Bervar M. Video analysis of standing – an alternative footprint analysis to assess functional loss following injury to the rat sciatic nerve. *J Neurosci Methods.* 2000;102(2):109-116.

28. Smit X, van Neck JW, Ebeli MJ, Hovius SER. Static footprint analysis: a time-saving functional evaluation of nerve repair in rats. *Scand J Plast Reconstr Surg Hand Surg.* 2004;38(6):321-325.

29. Hosseinian MA, Shirian S, Loron AG, Ebrahimy AA, Hayatolah GH. Distal sensory to distal motor nerve anastomosis can protect lower extremity muscle atrophy in a murine model. *Eur J Plast Surg.* 2018;41:9-14.

30. Mu L, Sobotka S, Chen J, et al. Alpha-synuclein pathology and axonal degeneration of the peripheral motor nerves innervating pharyngeal muscles in Parkinson disease. *J Neuropathol Exp Neurol.* 2013;72(2):119-129.

31. Cashman NR, Covault J, Wollman RL, Sanes JR. Neural cell adhesion molecule in normal, denervated, and myopathic human muscle. *Ann Neurol.* 1987;21(5):481-489.

32. Mu L, Chen J, Li J, Nyirenda T, Fowkes M, Sobotka S. Immunohistochemical detection of motor endplates in the long-term denervated muscle. *J Reconstr Microsurg.* 2018;34(5):348-358.

33. Derby A, Engleman VW, Frierdich GE, Neises G, Rapp SR, Roufa DG. Nerve growth factor facilitates regeneration across nerve gaps: morphological and behavioral studies in rat sciatic nerve. *Exp Neurol.* 1993;119(2):176-191.

34. Grothe C, Nikkhah G. The role of basic fibroblast growth factor in peripheral nerve regeneration. *Anat Embryol (Berl).* 2001;204:171-177.

35. Wang S, Cai Q, Hou J, et al. Acceleration effect of basic fibroblast growth factor on the regeneration of peripheral nerve through a 15-mm gap. *J Biomed Mater Res.* 2003;66A(3):522-531.

36. Casella GTB, Almeida VW, Grumbles RM, Liu Y, Thomas CK. Neurotrophic factors improve muscle reinnervation from embryonic neurons. *Muscle Nerve.* 2010;42(5):788-797.

37. Lewin SL, Utley DS, Cheng ET, Verity AN, Terris DJ. Simultaneous treatment with BDNF and CNTF after peripheral nerve transection and repair enhances rate of functional recovery compared with BDNF treatment alone. *Laryngoscope.* 1997;107(7):992-999.

38. Cheng H, Fraidakis M, Blomback B, Lapchak P, Hoffer B, Olson L. Characterization of a fibrin glue-GDNF slow-release preparation. *Cell Transplant.* 1998;7:53-61.

39. Tajdaran K, Gordon T, Wood MD, Shoichet MS, Borschel GH. A glia cell line-derived neurotrophic factor delivery system enhances nerve regeneration across acellular nerve allografts. *Acta Biomaterialia.* 2016;29:62-70.

40. Ho PR, Coan GM, Cheng ET, et al. Repair with collagen tubules linked with brain-derived neurotrophic factor and ciliary neurotrophic factor in a rat sciatic nerve injury model. *Arch Otolaryngol Head Neck Surg.* 1998;124(7):761-766.

41. Jonsson S, Wiberg R, McGrath AM, et al. Effect of delayed peripheral nerve repair on nerve regeneration, Schwann cell function and target muscle recovery. *PLoS ONE.* 2013;8(2):e56484.

Figure Legends

FIG. 1. Neuromuscular organization of the rat tibialis anterior (TA) and gastrocnemius (GM) muscles and implantation of NMEG-NMZ. (**A**) Lateral view of a rat left fresh hind limb, showing the anatomical relationship between the TA and GM as well as sciatic nerve and its branches (i.e., peroneal, tibial and sural nerves). Note that the GM is composed of tow compartments, lateral (GM-l) and medial (GM-m). The tibial nerve enters the GM on the top between GM-l and GM-m. F, femur. (**B**) Locations of motor endplate (MEP) bands with numerous neuromuscular junctions (black dots) within the rat TA, GM-l and GM-m as demonstrated by wholemount acetylcolinesterase (AChE) staining. Vertical dished line in the GM indicates midline. (**C**) Native motor zones (NMZs) containing MEP bands and their innervating nerve terminals (boxed regions) within the TA, GM-l and GM-m. Nerve branching and supply patterns

are determined by Sihler's stain, a wholemount nerve staining technique. (**D**) Images showing that the TA muscle denervated by resecting a segment of its nerve is reinnervated with NMEG-NMZ technique. An NMEG pedicle with a nerve branch is harvested from the NMZ (boxed region) of the GM-l and implanted to the NMZ of the denervated TA muscle.

FIG. 2. Images showing Imm-NN/ENF procedures. (**A**) Lateral view of the TA and GM-l in the rat left hind limb. (**B**) An NMEG pedicle is harvested from the GM-l. (**C**) A muscle defect is created on the surface of the NMZ of the TA. (**D**) A fibrin sealant, containing exogenous neurotrophic factors (ENF) NGF and FGF-2, is applied locally to the TA muscle defect. (**E**) The fibrin sealant is covered by the prepared NMEG pedicle. (**F**) The transferred NMEG is sutured with 10-0 nylon microsutures as indicated by arrowheads.

FIG. 3. Toe spread recovery. Comparison of hind limb footprints between left (L) operated and right (R) non-operated sides in the rats with Imm-NN/ENF (**A**) and Imm-ANG (**B**). Note that NN/ENF resulted in better toe spread recovery as compared with ANG. (**C**) Average group data, showing that the differences in calculated static sciatic index (SSI) are statistically significant between both groups ($p < 0.0001$).

FIG. 4. Comparison of muscle force from rat TA muscles treated with NN/ENF and ANG. (**A**) Average current thresholds for muscle contraction. (**B**) Rate of operated to control current. (**C**) Average maximal muscle force. (**D**) Rate of operated to control force.

FIG. 5. (A) Mean group data on muscle weights of the left (L) treated and right (R) control TA muscles. Note that the mean weight of the left TA treated with NN/ENF is higher than that of the ANG repaired TA. (**B**) Left/right ratio of the muscle weight in the NN/ENF and ANG groups ($p<0.0001$). Bars represent standard error. (**C**) Comparisons of the muscle masses of the left treated and right control TA

muscles from individual rats in the NN/ENF (left pair, rat #5) and ANG (right pair, rat #3) groups. (**D**) H&E stained cross-sections from the left TA muscles treated with NN/ENF (left) and ANG (right), showing differences in fiber size and structure between the treatments. Note that NN/ENF treated muscle exhibited very good preservation of muscle structure with less fiber atrophy as compared with ANG treated muscle. Bar = 150 μm.

FIG. 6. Cross-sections of the rat ANG treated TA muscles, showing fiber type-grouping and denervated and atrophied fibers. (**A**) A section stained with monoclonal antibody NOQ7-5-4D specific for slow type I fibers (dark staining), showing type I fiber grouping (arrows). (**B**) A section immunostained for N-CAM, showing denervated and atrophied (bright staining) fibers. Bar = 50 μm for A and B.

FIG. 7. Immunostained horizontal sections showing regenerated axons and reinnervated MEPs in the TA muscles treated with NN/ENF (**A-C**) and ANG (**D-F**). Muscle sections immunostained with NF staining (**A, D**), showing intramuscular regenerated axons (darkly stained threads and dots). The NF stained sections were opened using ImageJ software and converted to 8-bit (binary) images, color thresholded, and particle analyzed for nerve morphometry (**B, E**). The density of the axons was evaluated by estimating the number of the NF-positive axons within a section area (1.0 mm^2). The muscle sections labeled with double fluorescence staining **(C, F)** showed MEPs (red) and their innervating axons (green). Arrows indicate innervated MEPs with visible axon attachments, whereas arrowheads indicate non-innervated MEPs without visible axon attachments. Note that the TA muscles treated with NN/ENF (**A-C**) exhibited much more regenerated axons and reinnervated MEPs as compared with those treated with ANG (**D-F**). Bar = 50 μm for A through F. (**G**) Mean group data on the left/right (L/R) ratio of the regenerated axons. The mean number of the regenerated axons on the left treated TA was measured to be 88% of the right control for the NN/ENF, which was significantly higher than that for the ANG treated muscle (39%) ($p<0.0001$).

Focal application of neurotrophic factors augments outcomes of nerve-muscle-endplate grafting technique for limb muscle reinnervation

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Number of videos: 0

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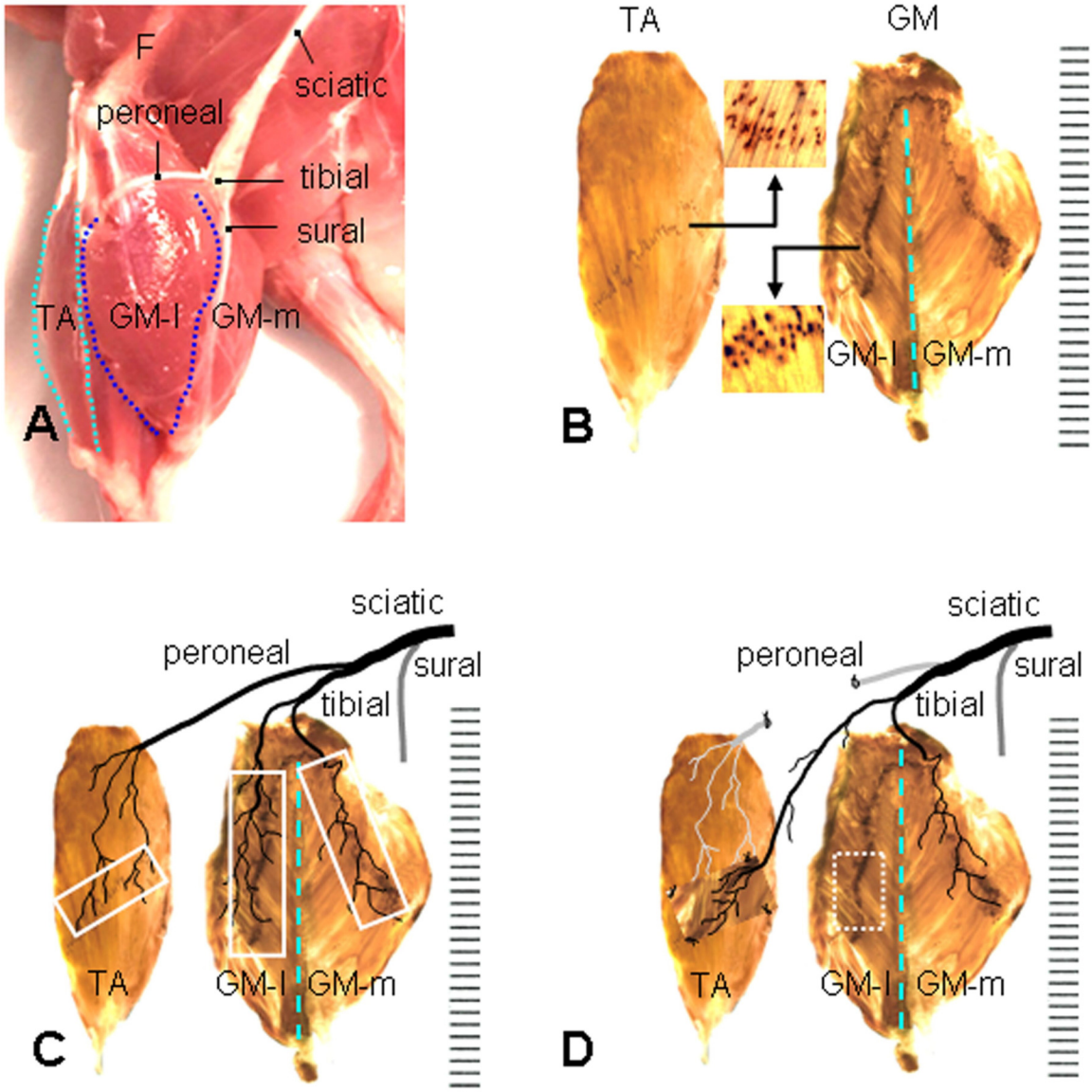
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sciatic
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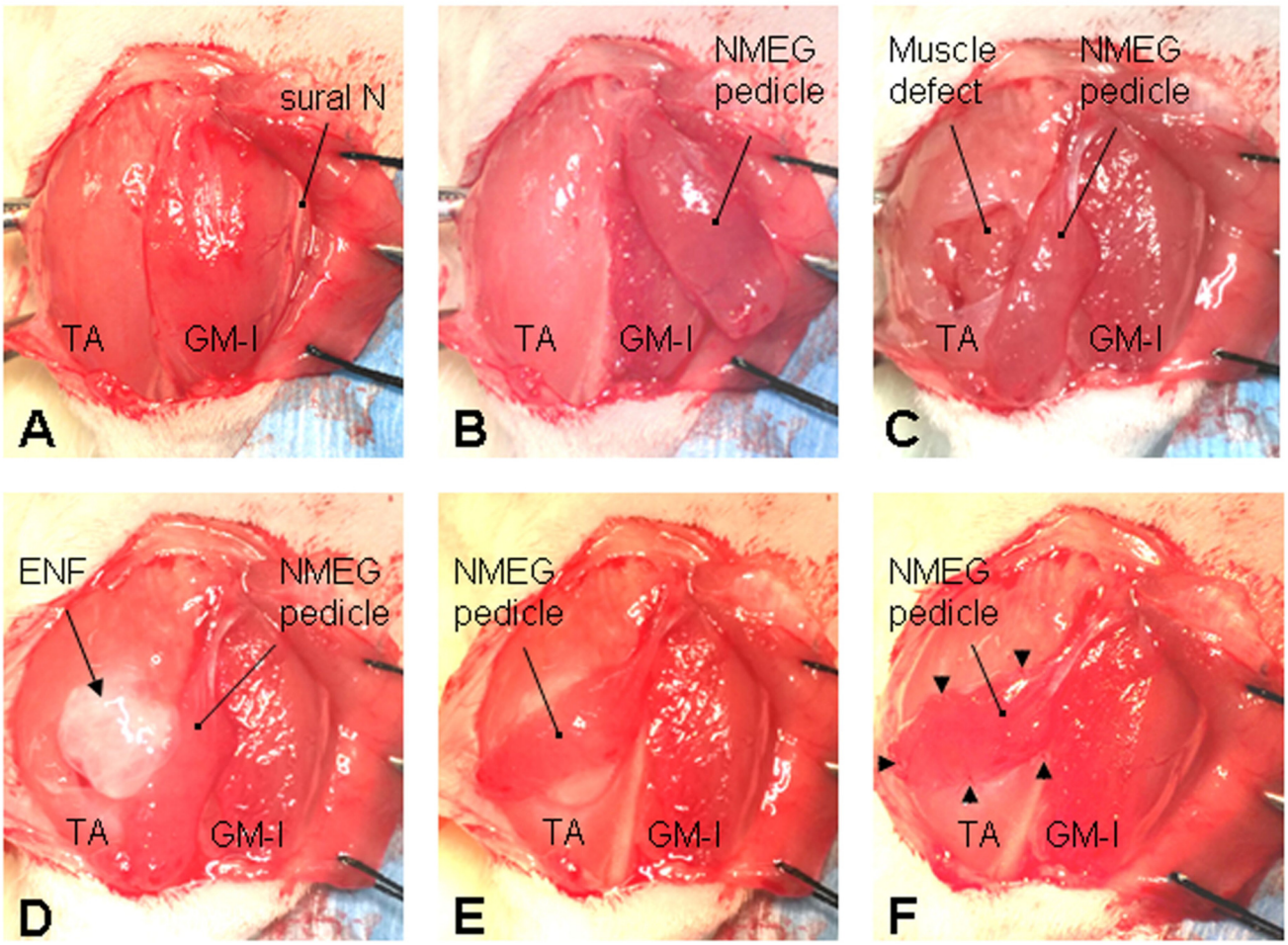
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pedicle
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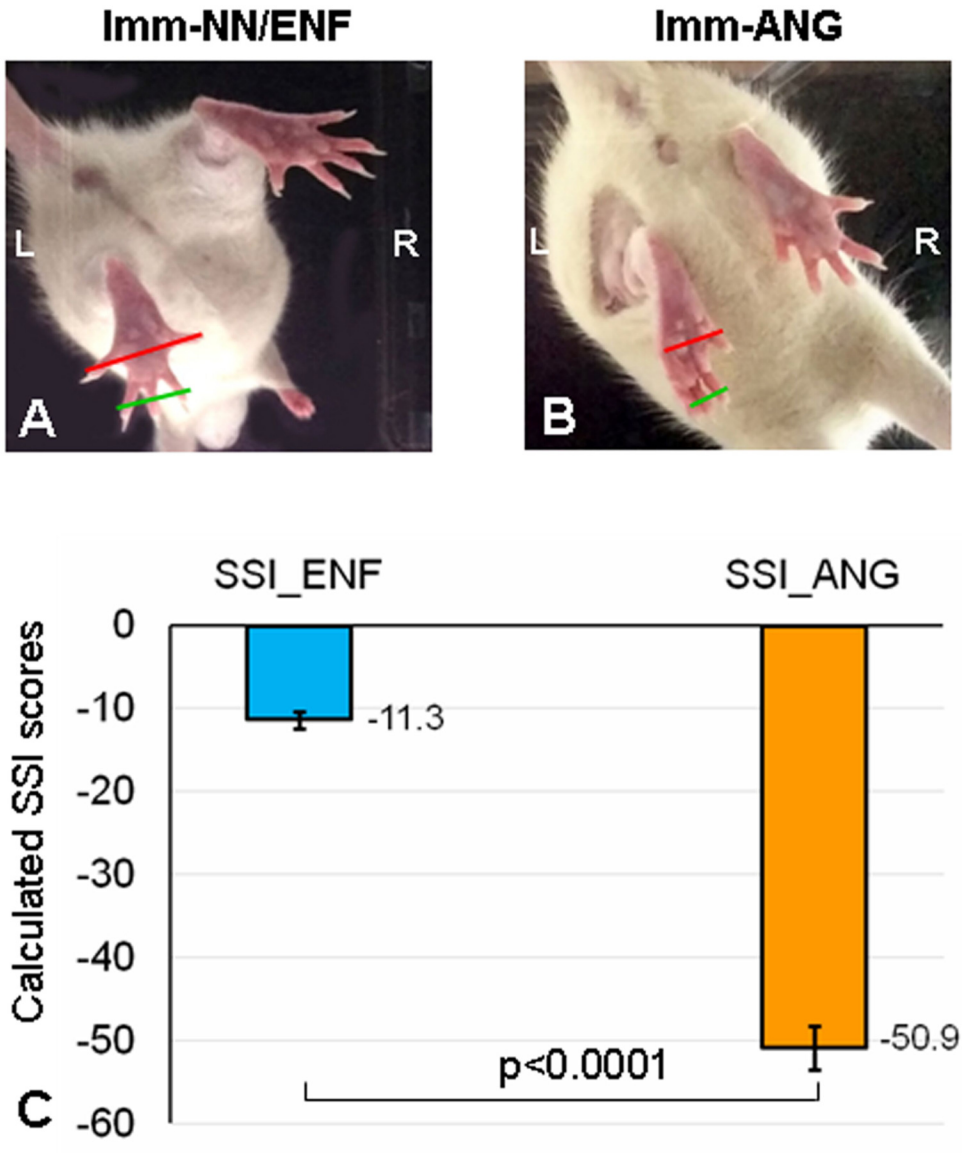
Fig. 3.. FIG. 3. Toe spread recovery. Comparison of hind limb footprints between left (L) operated and right (R) non-operated sides in the rats with Imm-NN/ENF (A) and Imm-ANG (B). Note that NN/ENF resulted in better toe spread recovery as compared with ANG. (C) Average group data, showing that the differences in calculated static sciatic index (SSI) are statistically significant between both groups ($p < 0.0001$).


Imm-NN/ENF
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Calculated SSI scores
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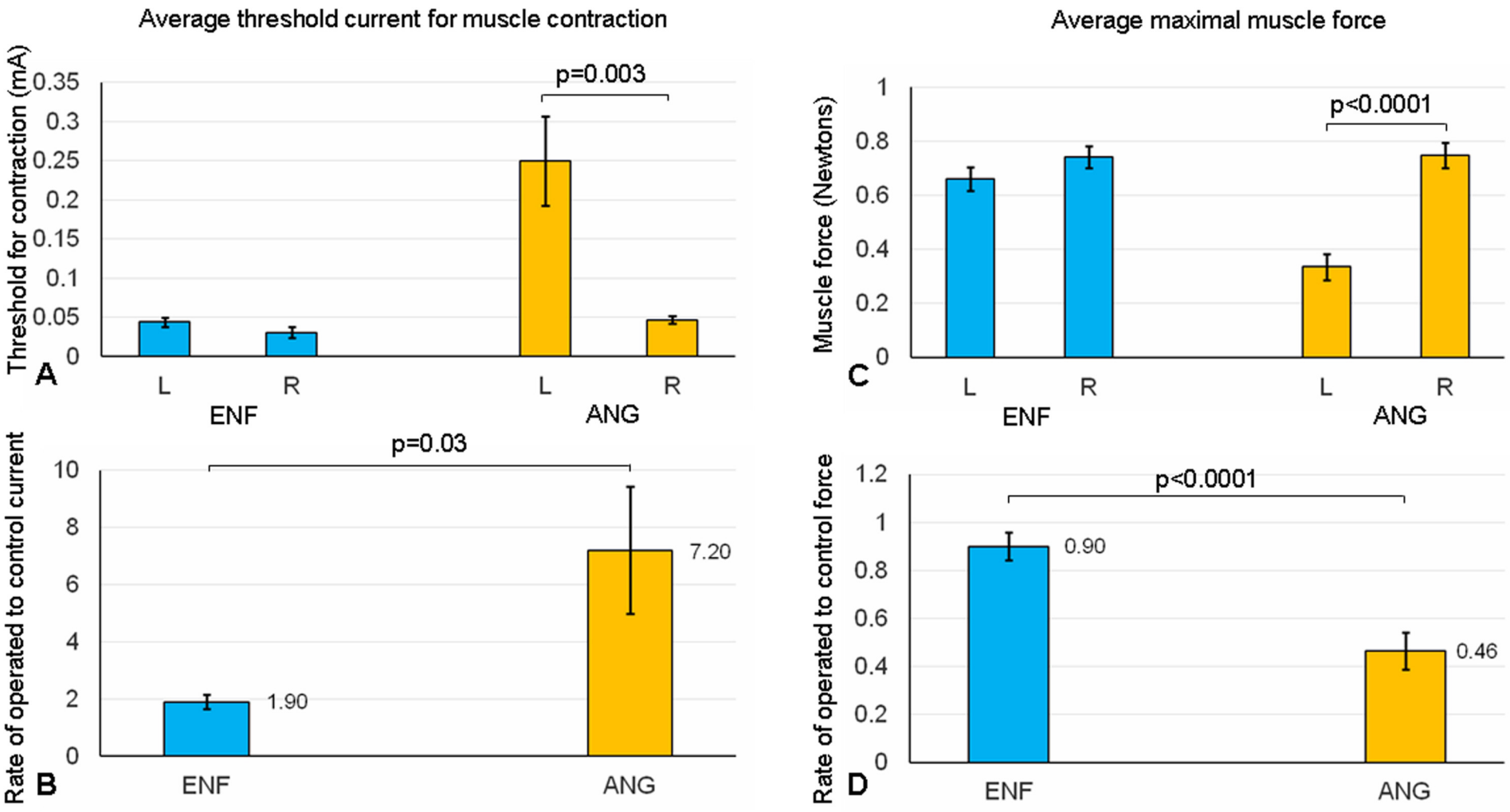
-10
-20
-30
-40
-50
-60
-11.3
-50.9
p<0.0001
C

Fig. 4.. FIG. 4. Comparison of muscle force from rat TA muscles treated with NN/ENF and ANG. (A) Average current thresholds for muscle contraction. (B) Rate of operated to control current. (C) Average maximal muscle force. (D) Rate of operated to control force.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Threshold for contraction (mA)
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0.25
0.2
0.15
0.1
0.05
0
p=0.003
A
L
R
L
R
ENF
ANG
Average maximal muscle force
Muscle force (Newtons)
1
0.8
0.6
0.4
0.2
0
p<0.0001
C
L
R
L
R
ENF
ANG
Rate of operated to control current
10
8
6
4
2
0
p=0.03
1.90
7.20
B
ENF
ANG
Rate of operated to control force
1.2
1
0.8
0.6
0.4
0.2
0
p<0.0001
0.90
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ENF
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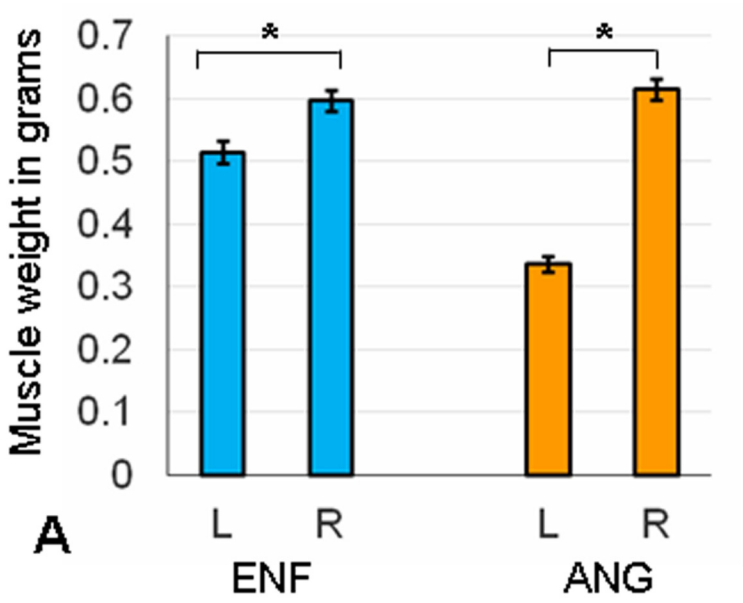
Fig. 5.. FIG. 5. (A) Mean group data on muscle weights of the left (L) treated and right (R) control TA muscles. Note that the mean weight of the left TA treated with NN/ENF is higher than that of the ANG repaired TA. (B) Left/right ratio of the muscle weight in the NN/ENF and ANG groups ($p<0.0001$). Bars represent standard error. (C) Comparisons of the muscle masses of the left treated and right control TA muscles from individual rats in the NN/ENF (left pair, rat #5) and ANG (right pair, rat #3) groups. (D) H&E stained cross-sections from the left TA muscles treated with NN/ENF (left) and ANG (right), showing differences in fiber size and structure between the treatments. Note that NN/ENF treated muscle exhibited very good preservation of muscle structure with less fiber atrophy as compared with ANG treated muscle. Bar = 150 ?m.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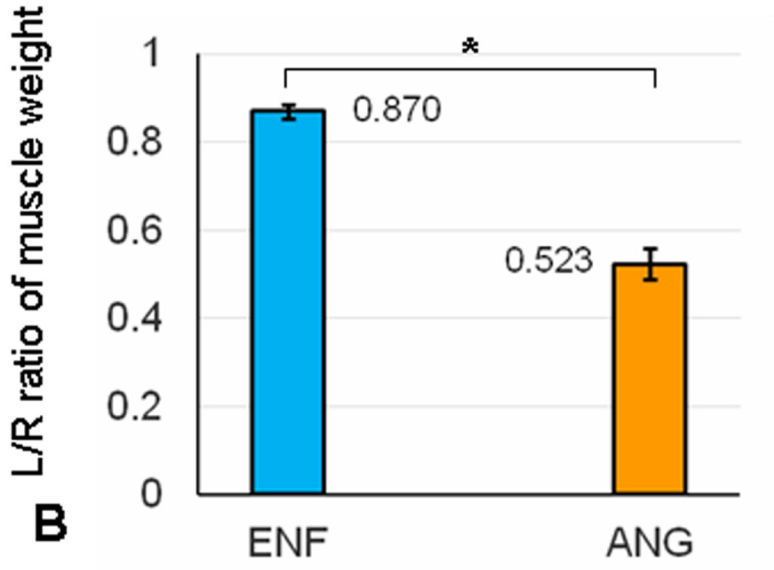
L/R ratio of muscle weight
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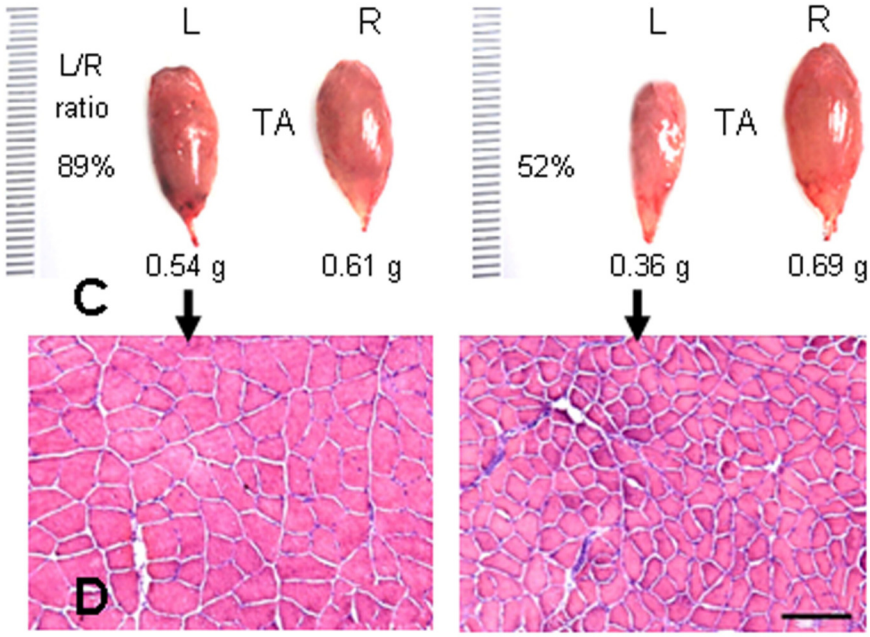
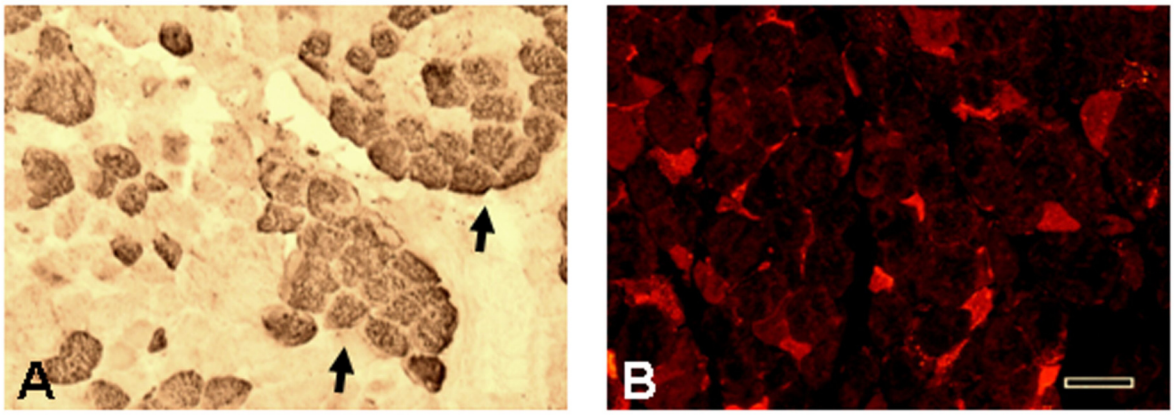

L
R
L/R ratio
89%
TA
0.54 g
0.61 g
52%
TA
0.36 g
0.69 g
C
D

Fig. 6.. FIG. 6. Cross-sections of the rat ANG treated TA muscles, showing fiber type-grouping and denervated and atrophied fibers. (A) A section stained with monoclonal antibody NOQ7-5-4D specific for slow type I fibers (dark staining), showing type I fiber grouping (arrows). (B) A section immunostained for N-CAM, showing denervated and atrophied (bright staining) fibers. Bar = 50 ?m for A and B.


A
B

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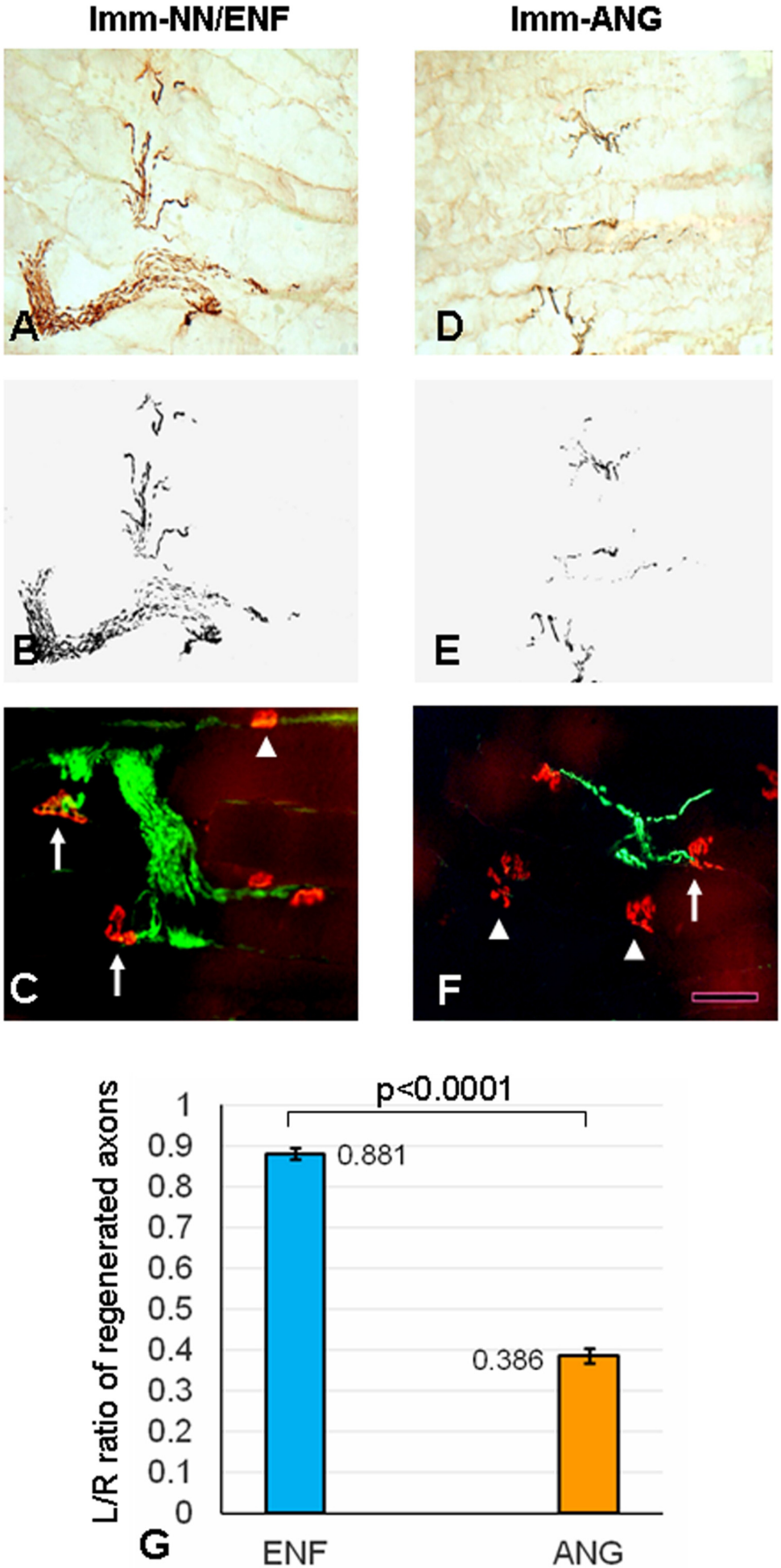
Imm-NN/ENF
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A
D
B
E
C
F
p<0.0001
0.881
0.386
L/R ratio of regenerated axons
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ENF
ANG
G