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TITLE: Follistatin: A Potential Anabolic Treatment for Re-Innervated Muscle

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## 1. INTRODUCTION:

## 2.

Functional recovery following major peripheral nerve injuries is often suboptimal despite adherence to well accepted nerve repair principles. Though a multifaceted problem, the poor muscle functional recovery often seen following nerve regeneration is in large part due to the progressive catabolic process affecting muscle fibers called "denervation atrophy." While many researchers have approached this issue by attempting to improve axonal regeneration speed, efficiency, and accuracy

(and thereby limiting the degeneration of the muscle), we have sought treatment options aimed at maximizing the potential of the muscle fibers that were able to achieve reinnervation. After experimenting with anabolic steroids (nandrolone), we determined that a more potent but safer anabolic agent would be a better option. Follistatin is a glycoprotein that both blocks the muscle inhibiting peptide myostatin and possesses remarkable independent muscle stimulating properties as well. We hypothesized that the administration of recombinant follistatin delivered to rodent muscles subjected to prolonged but temporary denervation periods (of either 3 or 6 months) would improve final muscle recovery and function. Most published studies have delivered the follistatin as recombinant DNA though some successful administration of recombinant protein has been demonstrated as well leading us to form two wings for our study—one exploring recombinant DNA administration and one exploring protein administration.

## 3. KEYWORDS:

Denervation atrophy, anabolic, follistatin, nerve injury, nerve repair, muscle

**4. 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.

- Specific Aim 1: Utilize an established rodent model of denervation atrophy
- Regulatory Review and Approval Process- complete
- Testing the Protein Stability- complete
- Pilot Study (N=15; Follistatin recombinant DNA, Protein, and Alzet Pump Control Groups). Each group has 5 animals. (100% complete)
- Denervation of hind limb muscles (3 and 6 months) Twelve groups (N=12; total of 144 rodents) were divided into control (sham surgery, sham treatment), sham surgery, sham treatment, and experimental groups (denervation surgery + treatment). Experimental and sham treatment groups underwent left tibial nerve transection to denervate left gastrocnemius muscle. Control and sham surgery groups underwent exposure of the nerve without transection. (100% complete)
- Re-innervation of hind limb muscles. (3 and 6 months) Denervation was reversed by repairing the transected tibial nerve using graft obtained from contralateral tibial nerve. Control rats underwent harvest of graft without repair. (100% complete)
- Specific Aim 2: Treat re-innervated muscle with Follistatin: Recombinant DNA and AAV was provided by Vector BioLabs; BioVision provided the protein.
- Treatment of re-innervated hind limb muscles (3-month and 6-month groups). All rats will undergo either injection of recombinant follistatin DNA packaged in AAV (into gastrocnemius muscle) or implantation of drug delivery reservoir (with either carrier or recombinant follistatin protein + carrier)- (100% complete)
- *Specific Aim 3:* Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing
- Testing of muscle recovery/nerve regeneration (3 months). All rats underwent muscle morphology measurements, nerve conduction, and force generation studies of tibial nerve and gastrocnemius muscle. (100% complete)
- Immunohistology staining and histology of muscle (3 months). Fiber type analysis and satellite cell quantification to be determined for all specimens. (fiber type analysis 100% complete, satellite cell analysis pending)
- Measurement of Follistatin levels in muscle (3 months) immunoassay (100% complete)
- Testing of muscle recovery/nerve regeneration (6 months) All rats to undergo muscle morphology measurements, nerve conduction, and force generation studies of tibial nerve and gastrocnemius muscle. (100% complete)
- Immunohistology staining and histology of muscle (6 months) Fiber type analysis and satellite cell quantification to be determined for all specimens. (fiber type analysis 100% complete, satellite cell analysis pending)
- Measurement of Follistatin levels in muscle (6 months) immunoassay (100% complete)
- Specific Aim 4: Histology (of nerve and muscle), Manuscript preparation, Presentation
- Histology of muscle/nerve (3 and 6 months) Cross sections of muscle specimens will be stained and fiber size, axon numbers, and myelination measured. (100% complete)
- Data Analysis (3 and 6 months) pending
- Manuscript Preparation (3 and 6 months) pending

#### 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.

Observations: Since 2017 Annual Report, the following data analyses have been completed

Protein Treatment: (please see Appendix.2)

- **a.** 3-month Denervation Groups:
  - i. ELISA testing revealed no significant differences in levels of Follistatin protein between repair groups and significantly less Follistatin protein in SHAM groups.
- **b.** 6-month Denervation Groups:
  - **i.** Protein treatment resulted in no significant muscle mass differences in either repair or SHAM groups.
  - **ii.** Protein treatment resulted in significantly more developed muscle force in repair groups and no significant differences in SHAM groups.
  - **iii.** ELISA was unable to identify any Follistatin protein in any of these muscle samples.

Virus Treatment:

**a.** 3-month Denervation Groups:

Virus treatment resulted in no significant differences in Follistatin protein levels in either repair or SHAM groups (though Follistatin levels in Virus treated repair group were clearly higher, but with a large standard deviation prevented statistical significance).

- **b.** 6-month Denervation Groups:
  - iv. Virus treatment resulted in significantly more muscle mass for both repair and SHAM groups.
  - v. Virus treatment resulted in no significant developed muscle force differences in either repair or SHAM groups (though again, there appeared to be a positive difference for the repair group).
  - vi. ELISA testing revealed significantly more Follistatin protein in the virus treated repair group and no significant Follistatin protein difference in SHAM groups.

Muscle Fiber Type Analysis: 3-month Denervation Groups (please see Appendix.3)

- i. The Type 2B muscle fiber area of the muscles that were surgically repaired remained significantly smaller when treated with protein rather than saline.
- **ii.** The type 2A muscle fibers were also significantly smaller in diameter and area than the muscle fibers that were treated with saline after nerve repair.
- **iii.** In contrast, the animals treated with protein had significantly larger diameter type I muscle fibers than if they were treated with saline after nerve repair.
- iv. There is significantly greater proportion of muscle fibers expressing type 2B isoforms when the muscle is treated with <u>FS virus</u> after sham surgery
- v. In contrast, there is a significantly larger proportion of muscle fibers expressing Type I MHC when the muscle is treated with <u>FS protein</u> after sham surgery

Muscle Fiber Type Analysis: 6-month Denervation Groups (please see Appendix.4)

We can see that the longer denervation period led to smaller muscle fibers but also just confinement to the cages may have contributed.

The only significant differences in muscle fiber size refer to sham operated animals.

- i. In Sham operated muscle, the type 2B muscle fiber area is significantly larger when treated with FS protein vs saline.
- ii. There is significantly greater proportion of 2B muscle fiber types when the muscle is treated with FS protein after sham surgery.
- iii. In contrast, there is significantly greater proportion of 2A muscle fiber types when the muscle is treated with FS <u>virus</u> after sham surgery.

#### 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.

Noting 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.

- Finalize axon count, and satellite cell data analysis
  - 3-month denervation groups (1-6):
    - Nerve Histology Finalize analysis of axon counts
  - 6-month denervation groups (7-12):
    - Nerve Histology Finalize analysis of axon counts
- Interpret final data analyses and Prepare Manuscript and Presentations
- *4.* **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

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

Nothing to Report.

## What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

Nothing to Report.

## What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

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

Nothing to Report.

## What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

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

Nothing to Report.

**5.** CHANGES/PROBLEMS: The 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:

See below.

## Actual or anticipated problems or delays and actions or plans to resolve them

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

#### 3 Month Data:

-Only ~70% of the data was usable – the short recording distance of 20mm resulted in signal overlap between tissue artifact and nerve action potential such that the action potential was not a clear entity in some trials limiting our ability to process those nerve conduction studies. -The only difference was a higher peak amplitude in repair+viral treatment vs repair+sham viral treatment. However, this analysis was based on 4 rats per group as the other files were not usable limiting the utility of this specific finding.

6 Month Data:

-None of the data is usable. In an attempt to correct the issue we encountered with the 3mo data, we increased the sampling frequency. The goal of this was to provide better resolution between the tissue artifact and the desired nerve action potential. Unfortunately, when the sampling frequency was increased to get better resolution at the recording electrodes, the pulses per second of the stimulus was simultaneously increased. This resulted in a double tissue artifact that completely obscured the nerve action potential, preventing us from being able to analyze any of this specific data.

## 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.

**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).

- One Abstract will be presented in American Society for Peripheral Nerve 2019 Annual Conference: (please see Appendix.6) *Recovery of Chronically Denervated Muscle Enhanced with Follistatin Treatment*
- One Abstract will be presented in Society for Neuroscience 2018 Conference: (please see Appendix.5) Does time make a difference? The effect of the administration of follistatin on re-innervated skeletal muscle fiber recovery after 3 vs 6 months of denervation

**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".

Name: Jonathan Isaacs, M.D. Project Role: PI Nearest person month worked: 2 Contribution to project: Regulatory process, supervising the study. Funding support: VCU salary, MCV physicians salary for clinical work, protocol no. ANG-CP-007, Cook Biotech, Inc. industry grant, Flow through funding from NIH: 1R34NS097113-01

Name: Satya Mallu, M.D. Project Role: Co-investigator Nearest person month worked: 4 Contribution to project: Assisted with regulatory process, performed main study surgeries. Funding support: VCU salary, Cook Biotech, Inc. industry grant, Flow-through funding from NIH: 1R34NS097113-01, AFSH grant

Name: Gaurangkumar Patel, B.S. Project Role: Lab technician Nearest person month worked: 7.5 Contribution to project: Main study, assisted with main study surgeries. Funding support: VCU salary

Name: Mary Shall, PhD Project Role: Co-PI Nearest person month worked: 2.5 Contribution to project: ELISA and Muscle Fiber Type Analysis Funding support: VCU salary

Name: Jeffery Dupree, PhD Project Role: Co-investigator Nearest person month worked: 0.25 Contribution to project: Pilot study Funding support: VCU salary and NIH grants

# Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

#### What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership: <u>Organization Name:</u> <u>Location of Organization: (if foreign location list country)</u> <u>Partner's contribution to the project</u> (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: N/A

## **QUAD CHARTS:**

Attached. (see appendix 1)

## 9. APPENDICES:

- 1. Quad Chart
- 2. Follistatin Main Study Results
   3. Follistatin Histology-Muscle-3-month Group Results
- 4. Follistatin Histology-Muscle-6-month Group Results
- 5. SFN-2018 Poster
- 6. ASPN-2019 Abstract

# Follistatin: A Potential Anabolic Treatment for Re-Innervated Muscle Proposal #11231008



**PI:** Jonathan Isaacs, MD

Org: Virginia Commonwealth University

Award Amount: \$705,041

#### **Study Aims**

•To utilize an established animal model of denervation atrophy to determine if Follistatin treatment (administered either as a recombinant protein or as a recombinant DNA) will improve muscle recovery following re-innervation after prolonged periods of denervation.

•To determine Follistatin effects on nerve regeneration and intramuscular fibrosis (in re-innervated tissue).

## Approach

Based on the pilot study result, rodents will undergo transection of one tibial nerve to denervate the hind limb muscles (including gastrocnemius). After a delay (of either 3 or 6 months) the nerve will be repaired and the muscles re-innervated. The re-innervated muscle will be treated with either recombinant follistatin protein (delivered thru an implantable drug delivery system) or recombinant follistatin DNA (delivered thru an adeno viral vectors injected into the reinnervated gastrocnemius muscle). After 8 weeks recovery, the effects of the follistatin treatment will be determined utilizing strength testing, muscle morphology, muscle histology, and muscle immunohistology (to determine muscle fiber type distribution and satellite, or regenerative cell, population pools). Nerve conduction testing will be performed to differentiate follistatin effects on nerve regeneration and function; muscle staining for collagen will determine effects on succle fibrosis; and follistatin levels will be measured in treated muscle to confirm effective dosing and delivery of follistatin. Test results will be compared with sham surgery (plus FS treatment), re-innervation (without treatment), and controlgroups.

## **Timeline and Cost**



#### Follistatin Project Experiment Timeline



1. Recombinant Follistatin Protein (FS-288)	EXP	7. Recombinant Follistatin Protein (FS-288)
2. Recombinant Follistatin DNA (FS-288)	EXP	8. Recombinant Follistatin DNA (FS-288)
3. Recombinant Follistatin Protein (FS-288)	SHAM SURGERY	9. Recombinant Follistatin Protein (FS-288)
4. Recombinant Follistatin DNA (FS-288)	SHAM SURGERY	10. Recombinant Follistatin DNA (FS-288)
5. Only Carrier (Buffer Saline)	SHAM TREATMENT	11. Only Carrier (Buffer Saline)
6. Only Carrier (Buffer Saline)	CTRL	12. Only Carrier (Buffer Saline)

Note: EXP and SHAM TREATMENT undergo DENERVATION Surgeries; SHAM SURGERY and CTRI, have "NO" DENERVATION Surgeries.

#### Goals/ Milestones

#### CY15 Goal - Utilize an established rodent model of denervation atrophy

Regulatory Process – Received ACURO approved on Jan 19, 2016

CY16 Goals – Treat re-innervated muscle with Follistatin, Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing

Pilot Project Surgeries Completed

Denervation of hind limb muscles

- CY17-18 Goals Treat re-innervated muscle with Follistatin, Determine treatment effects utilizing strength testing, muscle morphology, electrophysiology nerve testing; Histology (of nerve and muscle), Manuscript preparation, Presentation
- √ Reinnervation of hind limb muscles
- Synthesis of Recombinant Follistatin DNA/Protein
- J Treatment with Follistatin
- , Testing of muscle recovery/nerve regeneration
- ☑ Immunohistology staining and histology of muscle
- Measurement of Follistatin levels in muscle
- Histology of muscle/nerve
- D Data Analysis-ONGOING D

Manuscript Preparation-ONGOING

#### Comments/Challenges/Issues/Concerns

 Pilot Project is added after consulting with GOR. Pilot project started with Follistatin DNA (with Adeno Virus) and Control Groups. FS-DNA with AV did not show any Follistatin Protein in muscle. We worked on Follistatin DNA group with Adeno Associated Virus (AAV) vector delivery and the data analysis is complete. Some of the goals moved from CY16 to CY17 reflecting the delays occurred.

Budget Expenditure to Date

Projected Expenditure: \$705,041

## Follistatin Main Study Results

## **<u>3-month Denervation Groups:</u>**

Treatment	Group #	n	Group Name	Group Description
	Group 1	12	Repair + Treatment	Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then protein treatment (surgery 3) twelve weeks post-surgery 2.
Group 3 Protein Group 5a Group 6a	Group 3	12	SHAM + Treatment	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then protein treatment (surgery 3) twelve weeks post-surgery 2.
	Group 5a	6 Repair + 6 SHAM Treatment		Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
	Group 6a	6	SHAM + SHAM Treatment	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
Group 2		12	Repair + Treatment	Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then virus treatment (surgery 3) twelve weeks post-surgery 2.
Virus -	Group 4	12	SHAM + Treatment	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then virus treatment (surgery 3) twelve weeks post-surgery 2.
	Group 5b	Group 5b 6		Three-month denervation (surgery 1) followed by nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
	Group 6b	6 SHAM + 6 SHAM Treatment		Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.

Table 1: 3-month Denervation Experimental Groups

	Pr	otein Treatment	Virus Treatment	
	n	Value	n	Value
Repair + Treatment	11	$0.64 \pm 0.18$	12	$0.99 \pm 0.37$
<b>Repair + SHAM Treatment</b>	6	$0.93 \pm 0.17$	6	$1.08 \pm 0.24$
SHAM + Treatment	12	$2.22 \pm 0.17$	12	$2.43 \pm 0.20$
SHAM + SHAM Treatment	6	$2.42 \pm 0.19$	6	$2.20 \pm 0.26$

#### 3-month Denervation Groups: Muscle Weight (grams):

 Table 2: 3-month Denervation Groups Muscle Weight Descriptive Statistics (value = average ± standard deviation, units = grams)



Figure 1: 3-month Denervation Groups Muscle Weight (error bars = standard deviation)

Comparison	p-value	Interpretation
Group 1 vg Group 50	0.005	Protein treatment leads to significantly less muscle mass
Gloup I vs Gloup Sa	0.005	growth in repair groups.
Group 2 vg Group 60	0.040	Protein treatment leads to significantly less muscle mass
Gloup 5 vs Gloup da	0.040	growth in SHAM groups.
Group 2 vg Group 5h	0.624	Virus treatment leads to no significant muscle mass
010up 2 vs 010up 30	0.024	difference in repair groups.
Group A ve Group 6h	0.050	Virus treatment leads to no significant muscle mass
Gloup 4 vs Gloup ob	0.039	difference in SHAM groups.
		Virus treatment leads to significant more muscle mass
Group 1 vs Group 2	0.009	growth in repair group in comparison with protein
		treatment.
	0.013	Virus treatment leads to significant more muscle mass
Group 3 vs Group 4		growth in SHAM group in comparison with protein
		treatment.

 Table 3: 3-month Denervation Groups Muscle Weight Statistical Analysis Results (student t-test used for each of the comparison)

	Pr	otein Treatment	Virus Treatment	
	n	Value	n	Value
Repair + Treatment	11	$0.373 \pm 0.187$	12	$0.495 \pm 0.285$
Repair + SHAM Treatment	6	$0.451 \pm 0.248$	6	$0.487 \pm 0.302$
SHAM + Treatment	12	$0.979 \pm 0.467$	12	$1.088 \pm 0.438$
SHAM + SHAM Treatment	6	$1.745 \pm 0.597$	6	$1.070 \pm 0.380$

## **3-month Denervation Groups: Muscle Developed Force (Newtons):**

Table 4: 3-month Denervation Groups Muscle Developed Force Descriptive Statistics (value =average ± standard deviation, units = Newtons)



Figure 2: 3-month Denervation Groups Muscle Developed Force (error bars = standard deviation)

Comparison	p-value	Interpretation
Group 1 vs Group 5a	0.474	Protein treatment leads to no significant muscle
· ·		developed force difference in repair groups.
Group 3 vs Group 69	0.008	Protein treatment leads to significantly less muscle
	0.008	developed force in SHAM groups.
Group 2 vs Group 5h	0.050	Virus treatment leads to no significant muscle
Group 2 vs Group 30	0.939	developed force difference in repair groups.
Group 4 vs Group 6b	0.020	Virus treatment leads to no significant muscle
	0.930	developed force difference in SHAM groups.
		No significant difference in muscle developed force
Group 1 vs Group 2	0.245	when comparing between protein and virus treatment
		repair groups.
		No significant difference in muscle developed force
Group 3 vs Group 4	0.558	when comparing between protein and virus treatment
		SHAM groups.

 Table 5: 3-month Denervation Groups Muscle Developed Force Statistical Analysis Results (student t-test used for each of the comparison)

	Pr	otein Treatment	Virus Treatment	
	n	Value	n	Value
<b>Repair + Treatment</b>	11	$2679 \pm 2184$	11	$10994 \pm 9789$
<b>Repair + SHAM Treatment</b>	6	$2650\pm609$	5	$2897 \pm 1830$
SHAM + Treatment	10	$1924\pm995$	11	$2135\pm737$
SHAM + SHAM Treatment	6	$2576 \pm 715$	5	$1470 \pm 561$

<u>3-month Denervation Groups: Follistatin Protein using ELISA (pg Follistatin/mg protein):</u>

 Table 6: 3-month Denervation Groups Follistatin Protein using ELISA Descriptive Statistics (value

 = average ± standard deviation, units = pg Follistatin/mg protein)



**Figure 3: 3-month Denervation Groups Follistatin Protein using ELISA** (error bars = standard deviation)

Comparison	p-value	Interpretation
Group 1 vs Group 5a	0.075	Protein treatment leads to no significant Follistatin
Gloup I vs Gloup 3a	0.975	protein difference in repair groups.
Group 2 vg Group 60	0.011	Protein treatment leads to significantly less Follistatin
Gloup 5 vs Gloup da	0.011	protein in SHAM groups.
Group 2 vs Group 5h	0.003	Virus treatment leads to no significant Follistatin protein
Gloup 2 vs Gloup 30	0.095	difference in repair groups.
Group A va Group 6h	0.006	Virus treatment leads to no significant Follistatin protein
Group 4 vs Group 60	0.090	difference in SHAM groups.
		Virus treatment leads to significant more Follistatin
Group 1 vs Group 2	0.012	protein in repair group in comparison with protein
		treatment.
		No significant difference in Follistatin protein when
Group 3 vs Group 4	0.585	comparing between protein and virus treatment SHAM
		groups.

 Table 7: 3-month Denervation Groups Follistatin Protein using ELISA Statistical Analysis Results (student t-test used for each of the comparison)

## 6-month Denervation Groups:

Treatment	Group	n	Group Name	Group Description
	Group 7 12		Repair + Treatment	Six-month denervation (surgery 1) followed by nerve repair (surgery 2) and then protein treatment (surgery 3) twelve weeks post-surgery 2.
Group 9 Protein Group 11a Group 12a	Group 9	12	SHAM + Treatment	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then protein treatment (surgery 3) twelve weeks post-surgery 2.
	Group 11a	6	Repair + SHAM Treatment	Six-month denervation (surgery 1) followed by nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
	Group 12a	Group 12a 6 SHAM + SHAM Treatment		Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
	Group 8	12	Repair + Treatment	Six-month denervation (surgery 1) followed by nerve repair (surgery 2) and then virus treatment (surgery 3) twelve weeks post-surgery 2.
Virus -	Group 10	12	SHAM + Treatment	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then virus treatment (surgery 3) twelve weeks post-surgery 2.
	Group 11b	Group 11b 6 Repair + SHAM Treatment		Six-month denervation (surgery 1) followed by nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.
	Group 12b	6	SHAM + SHAM Treatment	Sham denervation (surgery 1) followed by sham nerve repair (surgery 2) and then saline treatment (surgery 3) twelve weeks post-surgery 2.

				-	reatment			
Table 8:	6-month	n Dener	vatior	n Ex	periment	al (	Grou	os

	Pr	otein Treatment	Virus Treatment			
	n	Value	n	Value		
Repair + Treatment	11	$1.01 \pm 0.53$	11	$0.91 \pm 0.47$		
Repair + SHAM Treatment	6	$0.81 \pm 0.33$	5	$0.40 \pm 0.07$		
SHAM + Treatment	10	$2.23 \pm 0.12$	12	$2.47 \pm 0.15$		
SHAM + SHAM Treatment	6	$2.17 \pm 0.49$	6	$2.26 \pm 0.12$		

#### 6-month Denervation Groups: Muscle Weight (grams):

 Table 9: 6-month Denervation Groups Muscle Weight Descriptive Statistics (value = average ± standard deviation, units = grams)



Figure 4: 6-month Denervation Groups Muscle Weight (error bars = standard deviation)

Comparison p val		Interpretation			
Group 7 vs Group 11a	0.441	Protein treatment leads to no significant muscle mass difference in repair groups.			
Group 9 vs Group 12a	0.692	Protein treatment leads to no significant muscle mass difference in SHAM groups.			
Group 8 vs Group 11b 0.03		Virus treatment leads to significantly more muscle mass growth in repair groups.			
Group 10 vs Group 12b	0.010	Virus treatment leads to significantly more muscle mass growth in SHAM groups.			
Group 7 vs Group 8	0.659	No significant difference in muscle mass when comparing between protein and virus treatment repair groups.			
Group 9 vs Group 10	0.0007	Virus treatment leads to significant more muscle mass growth in SHAM group in comparison with protein treatment.			

 Table 10: 6-month Denervation Groups Muscle Weight Statistical Analysis Results (student t-test used for each of the comparison)

	Pr	otein Treatment	Virus Treatment		
	n	Value	n	Value	
<b>Repair + Treatment</b>	10	$0.630 \pm 0.448$	10	$0.328 \pm 0.267$	
<b>Repair + SHAM Treatment</b>	5	$0.201 \pm 0.180$	5	$0.194 \pm 0.135$	
SHAM + Treatment	11	$1.034 \pm 0.395$	12	$1.179 \pm 0.509$	
SHAM + SHAM Treatment	6	$1.290 \pm 0.632$	6	$1.492 \pm 0.425$	

## 6-month Denervation Groups: Muscle Developed Force (Newtons):

**Table 11: 6-month Denervation Groups Muscle Developed Force Descriptive Statistics** (value = average ± standard deviation, units = Newtons)



Figure 5: 6-month Denervation Groups Muscle Developed Force (error bars = standard deviation)

Comparison p-va		Interpretation				
Group 7 vs Group 11a	0.063	Protein treatment leads to significantly more muscle				
Group 9 vs Group 12a	0.316	Protein treatment leads to no significant muscle				
Gloup 7 v3 Gloup 12a	0.510	developed force in SHAM groups.				
	0.215	Virus treatment leads to no significant muscle				
Group 8 vs Group 11b	0.315	developed force difference in repair groups.				
Course 10 and Course 12h	0.215	Virus treatment leads to no significant muscle				
Group 10 vs Group 126	0.215	developed force difference in SHAM groups.				
		No significant difference in muscle developed force				
Group 7 vs Group 8	0.083	when comparing between protein and virus treatment				
		repair groups.				
		No significant difference in muscle developed force				
Group 9 vs Group 10	0.457	when comparing between protein and virus treatment				
		SHAM groups.				

 Table 12: 6-month Denervation Groups Muscle Developed Force Statistical Analysis Results

 (student t-test used for each of the comparison)

	Pr	otein Treatment	Virus Treatment			
	n	Value	n	Value		
Repair + Treatment	11	$0\pm 0$	11	$2772 \pm 2762$		
<b>Repair + SHAM Treatment</b>	6	$0\pm 0$	6	$108 \pm 37$		
SHAM + Treatment	11	$0\pm 0$	10	$168 \pm 66$		
SHAM + SHAM Treatment	6	$0\pm 0$	6	$130 \pm 61$		

6-month Denervation Groups: Follistatin Protein using ELISA (pg Follistatin/mg protein):

 Table 13: 6-month Denervation Groups Follistatin Protein using ELISA Descriptive Statistics

 (value = average ± standard deviation, units = pg Follistatin/mg protein)



**Figure 6: 6-month Denervation Groups Follistatin Protein using ELISA** (error bars = standard deviation)

Comparison	p-value	Interpretation				
Group 7 vs Group 11a	NA	None				
Group 9 vs Group 12a	NA	None				
Group 8 vs Group 11h	0.034	Virus treatment leads to significantly more Follistatin				
	0.034	protein in repair groups.				
Group 10 vs Group 12h	0.291	Virus treatment leads to no significant Follistatin				
Gloup 10 vs Gloup 120	0.201	protein difference in SHAM groups.				
		Virus treatment leads to significant more Follistatin				
Group 7 vs Group 8	0.003	protein in repair group in comparison with protein				
		treatment.				
		Virus treatment leads to significant more Follistatin				
Group 9 vs Group 10	0.0000007	protein in SHAM group in comparison with protein				
		treatment.				

 Table 14: 6-month Denervation Groups Follistatin Protein using ELISA Statistical Analysis

 Results (student t-test used for each of the comparison)

## 3-month denervation groups: 2B diameter



	protein treatment			is treatment
Repair + treatment	Ν	value	Ν	Value
Repair + Treatment	11	$37.35\pm10.75$	12	$40.93 \pm 10.04$
Repair + Sham treatment	6	$44.38\pm3.47$	6	$47.48 \pm 9.51$
Sham + treatment	12	$67.22 \pm 6.2$	12	$68.8 \pm 7.2$
Sham + sham treatment	6	$62.6 \pm 7.25$	6	$64.88 \pm 6.45$

## 2B diameters

Comparison	p-value	Interpretation
Group 1 vs Group 5a	.773	Protein treatment does not enhance the 2B muscle fiber diameter
		of muscles that have been denervated and repaired.
Group 3 vs Group 6a	.969	Protein treatment does not enhance the 2B muscle fiber diameter
		of muscles that have been denervated and repaired.
Group 2 vs Group 5b	.861	Protein treatment does not enhance the 2B muscle fiber diameter
		of muscles that have been denervated and repaired.
Group 4 vs Group 6b	1.0	Virus treatment has no effect on the 2B muscle fiber diameter that
		have had only sham denervation
Group 1 vs Group 2	.978	There is no difference in 2B muscle fiber diameter when treated
		with protein vs virus.
Group 3 vs Group 4	1.0	There is no difference in 2B muscle fiber diameter when treated
		with protein vs virus even if there has only been sham surgery

## <u>3-month denervation groups:</u> <u>2B areas</u>



	protein treatment				virı	as treatment	
	<u>n</u>	Va	ılue		<u>n</u>	value	
Repair + Treatment	11	$1979.49 \pm 1008.91$			<u>11</u>	$2295.8 \pm 1042.97$	
Repair + Sham treatment	6	6 2891 ± 659.23			<u>5</u>	$3066.0 \pm 1226.21$	
Sham + treatment	12	65	$556.66 \pm 11$	153.42	<u>11</u>	$7124.74 \pm 1339.29$	
Sham + sham treatment	6	6 5309.57 ± 1720.33			5	$6316.48 \pm 815.16$	
Comparison			р-	Interpretation			
			value				
Group 1 vs Group 5a			.0001	The Typ	e 2B	muscle fiber area of the muscles that were	
				surgically repaired remained significantly smaller when			
				treated with protein.			
Group 3 vs Group 6a			.996	There is no difference of Type 2B muscle fiber area			
Group 2 vs Group 5b			.926	There is no difference of Type 2B muscle fiber area			
Group 4 vs Group 6b			.738	There is no difference of Type 2B muscle fiber area			
Group 1 vs Group 2	.610	There is no difference of Type 2B muscle fiber area					
Group 3 vs Group 4			1.0	There is no difference of Type 2B muscle fiber area			

## <u>3-month denervation groups:</u>



	protein treatment				virus treatment			
	<u>n</u>		value		<u>n</u>	value		
Repair + Treatment 11			29.	$.24 \pm 7.48$	<u>11</u>	$35.04 \pm 9.4$		
Repair + Sham treatment	6		48.	$.54 \pm 6.0$	<u>5</u>	$39.67 \pm 8.0$		
Sham + treatment	12		55.	$.54 \pm 5.81$	<u>11</u>	$55.13 \pm 6.74$		
Sham + sham treatment	6		58.	$.32 \pm 3.71$	<u>5</u>	$48.54 \pm 12.52$		
Comparison		p-		Interpreta	tion			
		valu	e					
Group 1 vs Group 5a		.000	.0001 The type		be 2A muscle fibers were significantly smaller			
				in diameter than the muscle fibers that were treated				
		with saline			after nerve repair.			
Group 3 vs Group 6a		.996 There is no			statistical difference of type 2A muscle			
				fiber diameters between groups				
Group 2 vs Group 5b		.926 There is no		There is no	statistical difference of type 2A muscle			
			fiber diame		eters between groups			
Group 4 vs Group 6b				There is no statistical difference of type 2A muscle				
			fiber diame		eters between groups			
Group 1 vs Group 2				There is no statistical difference of type 2A muscle				
			fiber diame		eters between groups			
Group 3 vs Group 4			1.0 There is no		statistical difference of type 2A muscle			
				fiber diameters between groups				

## <u>3-month denervation groups:</u>





	protein treatment				viru	virus treatment		
	<u>n</u>	Va	lue		<u>n</u>	value		
Repair + Treatment	11	13	$69.21 \pm 56$	50.3	<u>11</u>	$1807.84 \pm 938.82$		
Repair + Sham treatment	6	32	$37.23 \pm 77$	79.11	<u>5</u>	$2335.35 \pm 872.82$		
Sham + treatment	12	43	$89.45 \pm 64$	16.85	<u>11</u>	$4499.88 \pm 912.16$		
Sham + sham treatment	6	46	$73.85 \pm 40$	)3.98	<u>5</u>	$3594.77 \pm 1328.5$		
Comparison			р-	Interpretat	ion			
	value							
Group 1 vs Group 5a			.001	The type 2A muscle fibers are significantly smaller in				
				area than the muscles that were treated with saline				
				after nerve repair				
Group 3 vs Group 6a			.997	There is no difference in the areas of type 2A muscle				
				fibers between groups				
Group 2 vs Group 5b			.900	There is no difference in the areas of type 2A muscle				
				fibers between groups				
Group 4 vs Group 6b			.441	There is no statistically significant difference in the				
				areas of type 2A muscle fibers between groups				
Group 1 vs Group 2			.901	There is no difference in the areas of type 2A muscle				
				fibers between groups				
Group 3 vs Group 4	1.0	There is no difference in the areas of type 2A muscle						
				fibers between groups				

## <u>3-month denervation groups:</u> <u>I diameter</u>



	protein trea	atmen	t		virus treatment				
	<u>n</u>		val	lue	<u>n</u>	value			
Repair + Treatment	11		37	$.42 \pm 10.07$	<u>11</u>	37.41 ± 5.03			
Repair + Sham treatment	6		$48.8\pm8.45$		<u>5</u>	$41.85 \pm 7.3$			
Sham + treatment	12		54	$.53 \pm 6.41$	<u>11</u>	$54.39 \pm 5.0$			
Sham + sham treatment	6		57	.9 ± 5.2	<u>5</u>	$51.34 \pm 10.29$			
		1		1					
Comparison		p-	•	Interpretation					
Group 1 vs Group 5a		.05	e	The animals significantly fibers after	The animals treated with protein had significantly larger diameter type I muscle fibers after denervation				
Group 3 vs Group 6a		.983		The differen	The difference in muscle fiber diameter is not significantly different				
Group 2 vs Group 5b		.934		The difference in muscle fiber diameter is not significantly different					
Group 4 vs Group 6b				The differen	nce in muscle fiber diameter is antly different				
Group 1 vs Group 2			1.0 The differen		nce in muscle fiber diameter is antly different				
Group 3 vs Group 4		1.0		The differen	The difference in muscle fiber diameter not significantly different				

## <u>3-month denervation groups:</u>



	prot	tein tr	reatment		virı	virus treatment		
	<u>n</u>	valı	<u>1e</u>		<u>n</u>	value		
Repair + Treatment	11	$2298.89 \pm 772.61$				$2019.07 \pm 602.36$		
Repair + Sham treatment	6	322	$2.52 \pm 1068$	3.88	<u>5</u>	$2501.93 \pm 743.56$		
Sham + treatment	12	433	$5.33 \pm 726.$	24	<u>11</u>	4225.34 ± 521.33		
Sham + sham treatment	6	443	$4.22 \pm 750.$	21	<u>5</u>	$4202.2 \pm 1646.11$		
Comparison			p-value	Interpretat	Interpretation			
Group 1 vs Group 5a			.355	The difference in muscle fiber diameter is not				
				significantly different though there is a tendency toward larger fibers if treated with protein				
Group 3 vs Group 6a			1.0	The differen	The difference in muscle fiber diameter is not			
				significantly	y diff	ferent		
Group 2 vs Group 5b			.945	The different significantly	The difference in muscle fiber diameter is not significantly different			
Group 4 vs Group 6b			1.0	The differen	nce in	n muscle fiber diameter is not		
				significantly	y diff	ferent		
Group 1 vs Group 2			.933	The differen	nce in	n muscle fiber diameter is not		
				significantly	significantly different			
Group 3 vs Group 4			1.0	The differen	The difference in muscle fiber diameter is not			
				significantly different				

I area



	protein	treatmen	t		virus treatment				
	<u>n</u>	<u>n</u>		lue	<u>n</u>	value	-		
Repair + Treatment	11		50	$0.84 \pm 9.05$	11	$58.42 \pm 11.33$			
Repair + Sham treatment	6		47	$1.4 \pm 8.34$	5	$48.27 \pm 5.44$			
Sham + treatment	12		30	$.33 \pm 13.71$	11	$53.98 \pm 10.45$			
Sham + sham treatment	6		38	$.06 \pm 12.6$	5	$53.68 \pm 13.65$			
Comparison		p-valu	le	Interpretati	Interpretation				
Group 1 vs Group 5a		.999		There is no s	There is no significant difference in the				
				proportion of	proportion of type 2B muscle fibers				
Group 3 vs Group 6a		.855		There is no c	lifference in the	proportion of type			
				2B muscle fibers					
Group 2 vs Group 5b		.600 There is		There is no s	s no significant difference in the proportion				
				of type 2B muscle fibers					
Group 4 vs Group 6b		1.0		There is no difference in the proportion of type 2B muscle fibers					
Group 1 vs Group 2 .749				There is no difference in the proportion of type 2B muscle fibers					
Group 3 vs Group 4		.0001		There is sign	ificantly greater	r proportion of 2B			
- •				muscle fiber types when the muscle is treated with					
				FS virus after sham surgery					



	protein treatment	protein treatment			virus treatment			
	N		va	lue	<u>n</u>	value		
Repair + Treatment	11		28	$.88 \pm 7.62$	11	$29.89 \pm 8.04$		
Repair + Sham treatment	6		31	$.99 \pm 12.34$	5	$35.65 \pm 9.33$		
Sham + treatment	12		41	$.56 \pm 7.0$	11	$31.74\pm9.48$		
Sham + sham treatment	6		40	$.23 \pm 11.95$	5	$35.25 \pm 10.33$		
Comparison	p-		e	Interpretat	ion			
Group 1 vs Group 5a	.998		<u> </u>	There is no s proportion o	significant difference in the of type 2A muscle fibers			
Group 3 vs Group 6a		1.0		There is no o 2A muscle f	There is no difference in the proportion of type 2A muscle fibers			
Group 2 vs Group 5b		.91		There is no significant difference in the proportion				
Group 4 vs Group 6b	broup 4 vs Group 6b .996			There is no difference in the proportion of type 2B muscle fibers				
Group 1 vs Group 2	1.0			There is no difference in the proportion of type 2B muscle fibers				
Group 3 vs Group 4		.186		There is a tendency toward a greater proportion of 2A muscle fiber types when the muscle is treated with FS protein after sham surgery				



	protein	treatment	t		virus treatment			
	N	N		lue	<u>n</u>	value		
Repair + Treatment	11		20	$.32 \pm 6.29$	11	$11.63 \pm 5.57$		
Repair + Sham treatment	6		23	$.27 \pm 18.73$	5	$16.44 \pm 4.14$		
Sham + treatment	12		29	$.2 \pm 16.77$	11	$13.98 \pm 8.11$		
Sham + sham treatment	6		21	$.68 \pm 11.41$	5	$13.27 \pm 5.91$		
Comparison		p-valu	e	Interpretat	ion			
Group 1 vs Group 5a	.998			There is no s proportion o	s no significant difference in the ion of type I muscle fibers			
Group 3 vs Group 6a	.8610			There is no o I muscle fibe	e is no difference in the proportion of type scle fibers			
Group 2 vs Group 5b		.986		There is no difference in the proportion of type I muscle fibers				
Group 4 vs Group 6b		1.0		There is no difference in the proportion of type I muscle fibers				
Group 1 vs Group 2		.578		There is no s	significant dif	ference in the prop	ortion	
				of type I mu	scle fibers tho	ugh there tends to	be	
				more type I	when the mus	cle is treated with		
				protein.				
Group 3 vs Group 4		.028		There is a si	here is a significantly larger proportion of muscle			
				fibers expres	ssing Type I N	IHC when the mus	cle is	
				treated with	FS protein aft	er sham surgery		

<u>3-month denervation groups:</u>

## **Conclusions:**

## The significant differences at three months:

- 1. The Type 2B muscle fiber area of the muscles that were surgically repaired remained significantly smaller when treated with protein rather than saline.
- 2. The type 2A muscle fibers were also significantly smaller in diameter and area than the muscle fibers that were treated with saline after nerve repair.
- 3. In contrast, the animals treated with protein had significantly larger diameter type I muscle fibers than if they were treated with saline after nerve repair.
- 4. There is significantly greater proportion of muscle fibers expressing type 2B isoforms when the muscle is treated with <u>FS virus</u> after sham surgery
- 5. In contrast, there is a significantly larger proportion of muscle fibers expressing Type I MHC when the muscle is treated with <u>FS protein</u> after sham surgery

## **Tendencies:**

- 1. There is a tendency that the virus treatment (rather than saline) leads to larger 2B muscle fiber diameter of muscles that have been denervated and repaired
- 2. The animals treated with protein (rather than saline) had significantly larger diameter type I muscle fibers after denervation.
- 3. There is a tendency toward a greater proportion of 2A muscle fiber types when the muscle is treated with FS protein after sham surgery
- 4. There is no significant difference in the proportion of repaired type I muscle fibers when denervated though there tends to be more type I when the muscle is treated with protein.

## <u>6-month denervation groups:</u> 2B diameter



	prote	ein treatment	viru	virus treatment			
Repair + treatment	Ν	value	Ν	Value			
Repair + Treatment	11	$37.74 \pm 12.12$	12	$37.47 \pm 13.60$			
Repair + Sham treatment	6	$32.24 \pm 11.60$	6	$24.48 \pm 12.29$			
Sham + treatment	12	$2 56.76 \pm 8.51$		$65.4 \pm 10.07$			
Sham + sham treatment	6	$42.27 \pm 2.91$	6	$60.42 \pm 6.08$			

## 2B diameters

Comparison	p-value	Interpretation
Group 7 vs Group 11a	.969	Protein treatment does not enhance the 2B muscle fiber diameter
		of muscles that have been denervated and repaired.
Group 8 vs Group 11b	.251	There was a tendency that the FS virus treatment (rather than
		saline) led to larger 2B muscle fiber diameter of muscles that have
		been denervated and repaired.
Group 9 vs Group 12a	.140	After sham denervation, there was a tendency that protein
		treatment led to larger 2B muscle fiber diameter of muscles than
		muscles that had saline injected.
Group 10 vs Group 12b	.981	Virus treatment has no effect on the 2B muscle fiber diameter that
		have had only sham denervation
Group 7 vs Group 8	1.0	There is no difference in 2B muscle fiber diameter when treated
		with protein vs virus.
Group 9 vs Group 10	.520	The difference in muscle fiber diameter is not significantly
		different.

## 6-month denervation groups:





	protei	rotein treatment			virus treatment			
	<u>n</u>	V	value		<u>n</u>	value		
Repair + Treatment	11	2	$106.1 \pm 112$	20.75	<u>11</u>	$2148.88 \pm 1456.93$		
Repair + Sham treatment	6	1'	$702.41 \pm 10$	010.38	<u>5</u>	$971.18 \pm 1049.17$		
Sham + treatment	12	4	$836.35 \pm 13$	322.75	<u>11</u>	$5909.49 \pm 1680.28$		
Sham + sham treatment	6	20	$566.76 \pm 32$	26.57	<u>5</u>	$5280.64 \pm 1075.16$		
Comparison		p- Interp			etation			
			value					
Group 7 vs Group 11a			.988	There is no difference of Type 2B muscle fiber area if				
				treated with FS protein				
Group 9 vs Group 12a			.028	In Sham operated muscle, the type 2B muscle fiber area				
				is significantly larger when treated with FS protein rather				
thar					than saline.			
Group 8 vs Group 11b .611 Ther				There is	There is no difference of Type 2B muscle fiber area			
Group 10 vs Group 12b	.975 There i				There is no difference of Type 2B muscle fiber area			
Group 7 vs Group 8			.993	There is	here is no difference of Type 2B muscle fiber area			
Group 9 vs Group 10			.480	There is	e is no difference of Type 2B muscle fiber area			



	protein trea	atmen	t		virus treatment			
	<u>n</u>		value		<u>n</u>	value		
Repair + Treatment	11		$35.55 \pm 12.1$		<u>11</u>	$28.65 \pm 9.4$		
Repair + Sham treatment	6		29	$.47 \pm 7.89$	<u>5</u>	$22.85\pm6.37$		
Sham + treatment	12		45	$.79 \pm 12.1$	<u>11</u>	$46.46 \pm 5.33$		
Sham + sham treatment	6		39	$.54 \pm 3.95$	<u>5</u>	$46.62 \pm 6.74$		
		-						
Comparison		p-		Interpreta	tion			
		valu	e					
Group 7 vs Group 11a		.799		There is no statistical difference of type 2				
				muscle fiber diameters between groups				
Group 9 vs Group 12a		.766		There is no statistical difference of type 2A				
				muscle fiber diameters between groups				
Group 8 vs Group 11b		.835		There is no statistical difference of type 2A				
				muscle fibe	er diameters betw	een groups		
Group 10 vs Group 12b		1.0	0 There is no statistical difference of type 2					
				muscle fibe	er diameters betw	een groups		
Group 7 vs Group 8		.463		There is no statistical difference of type 2A				
			muscle fibe		er diameters between groups			
Group 9 vs Group 10		1.0		There is no statistical difference of type 2A				
				muscle fibe	er diameters between groups			

## 6-month denervation groups:





	protei	n tre	eatment		viru	s treatment		
	<u>n</u>	value			<u>n</u>	value		
Repair + Treatment	11	19	$1942.72 \pm 1200.2$			$1323.4 \pm 713.62$		
Repair + Sham treatment	6	14	$19.89 \pm 59$	90.36	<u>5</u>	881.17 ±489.15		
Sham + treatment	12	31	$78.64 \pm 67$	75.59	<u>11</u>	$3109.89 \pm 582.37$		
Sham + sham treatment	6	24	19.09 ±43	5.49	<u>5</u>	$3133.63 \pm 740.93$		
Comparison			р-	Interpretat	Interpretation			
			value					
Group 7 vs Group 11a .			.867	There is no statistically significant difference in the				
				areas of type	e 2A 1	muscle fibers between groups		
Group 9 vs Group 12a			.495	There is no statistically significant difference in the				
				areas of type 2A muscle fibers between groups				
				though the protein tended to enhance the area.				
Group 8 vs Group 11b			.940	There is no statistically significant difference in the				
				areas of type 2A muscle fibers between groups				
Group 10 vs Group 12b			1.0	There is no a	There is no statistically significant difference in the			
				areas of type	areas of type 2A muscle fibers between groups			
Group 7 vs Group 8	p 8 .534			There is no statistically significant difference in the				
				areas of type	areas of type 2A muscle fibers between groups			
Group 9 vs Group 10	1.0			There is no statistically significant difference in the				
				areas of type 2A muscle fibers between groups				

## I diameter



	protein t	reatmen	t		virus tre	virus treatment		
	<u>n</u>		val	lue	<u>n</u>	value		
Repair + Treatment	11	11		$.02 \pm 14.16$	<u>11</u>	$32.2 \pm 10.17$		
Repair + Sham treatment	6	6		$.58 \pm 12.21$	<u>5</u>	$26.35 \pm 9.35$		
Sham + treatment	12	12		$.63 \pm 9.94$	<u>11</u>	$45.26 \pm 3.75$		
Sham + sham treatment	6	6		.82 ± 4.17	<u>5</u>	48.06 <u>+</u> 3.79		
Comparison	p-			Interpretation				
Group 7 vs Group 11a		.389		The difference in muscle fiber diameter is not significantly different though the tendency is larger type I muscle fibers when treated with protein				
Group 9 vs Group 12a		.469		The difference in muscle fiber diameter is not				
Group 8 vs Group 11b		.926		The difference in muscle fiber diameter is not significantly different				
Group 10 vs Group 12b		.999		The difference in muscle fiber diameter is not significantly different				
Group 7 vs Group 8		.766		The different significantly	nce in mus y different	cle fiber diameter is not		
Group 9 vs Group 10		.172		There is a tendency toward larger type 1 muscle fiber in sham operated muscle when using protein treatment.				



	prot	tein tı	reatment		viru	virus treatment		
	<u>n</u>	valu	<u>1e</u>		<u>n</u>	value		
Repair + Treatment	11	226	8.92 ± 1410	0.07	<u>11</u>	$1663.84 \pm 927.58$		
Repair + Sham treatment	6	131	$2.04 \pm 953.$	18	<u>5</u>	$1206.44 \pm 815.15$		
Sham + treatment	12	420	$7.26 \pm 1112$	2.04	<u>11</u>	3213.39 ± 633.86		
Sham + sham treatment	6	3130.18 ±426.87				3364.22 ± 621.66		
			•	<b>T 1 1 1</b>	•			
Comparison			p-value					
Group 7 vs Group 11a			.511	The difference in muscle fiber diameter is not significantly different				
Group 9 vs Group 12a			.357	The difference in muscle fiber diameter is not significantly different				
Group 8 vs Group 11b			.981	The different significantly	The difference in muscle fiber diameter is not significantly different			
Group 10 vs Group 12b	Group 10 vs Group 12b 1.0			The difference in muscle fiber diameter is not significantly different				
Group 7 vs Group 8	.814			The different significantly	nce in v diff	n muscle fiber diameter is not ferent		
Group 9 vs Group 10			.221	There is a te	There is a tendency toward a difference between a			
r · · · · · · · · · · · · · · ·				larger type	1 mu	scle fiber in sham operated muscle		
				when using virus treatment.				



	protein treatment				virus treatment		
	<u>n</u>		value		<u>n</u>	value	
Repair + Treatment	11		$65.87 \pm 3.76$		11	$61.55 \pm 8.27$	
Repair + Sham treatment	6		$67.3 \pm 2.25$		5	$64.88\pm3.42$	
Sham + treatment	12		$69.2 \pm 5.23$		11	$59.18 \pm 7.33$	
Sham + sham treatment	6		$67.72 \pm 2.89$		5	$65.82 \pm 1.79$	
Comparison		p-va	lue	Interpret	ation		
Group 7 vs Group 11a	Group 7 vs Group 11a			There is no difference in the proportion of			
				type 2B muscle fibers			
Group 9 vs Group 12a		.999		There is no difference in the proportion of			
				type 2B muscle fibers			
Group 8 vs Group 11b		.930		There is no difference in the proportion of			
				type 2B muscle fibers			
Group 10 vs Group 12b		.250		There is no significant difference in the			
				proportion of type 2B muscle fibers			
Group 7 vs Group 8		.591		There is no difference in the proportion of			
				type 2B muscle fibers			
Group 9 vs Group 10		.001		There is significantly greater proportion of			
		type 2B m		uscle fiber types when the muscle			
				is treated with FS protein after sham surgery			



	protein treatment		virus treatment				
	N		value		<u>n</u>	value	
Repair + Treatment	11		24.94 ±4.74		11	$27.37\pm4.6$	
Repair + Sham treatment	6		$20.47\pm3.46$		5	$27.75\pm4.15$	
Sham + treatment	12		$20.86\pm3.23$		11	$29.64\pm7.0$	
Sham + sham treatment	6		$24.1 \pm 3.27$		5	$25.62 \pm 1.28$	
Comparison		p- I		Interpretation			
		value					
Group 7 vs Group 11a		.545		There is no difference in the proportion of			
				type 2A muscle fibers			
Group 9 vs Group 12a		.857		There is no difference in the proportion of			
				type 2A muscle fibers			
Group 8 vs Group 11b		1.0		There is no difference in the proportion of			
				type 2A muscle fibers			
Group 10 vs Group 12b		.654		There is no difference in the proportion of			
				type 2A muscle fibers			
Group 7 vs Group 8		.915		There is no difference in the proportion of			
				type 2A muscle fibers			
Group 9 vs Group 10		.001		There is sign	There is significantly greater proportion of		
			2A muscle t		iber types when the muscle is		
				treated with FS virus after sham surgery			



	protein treatment				virus treatment		
	N		value		<u>n</u>	value	
Repair + Treatment	11		$9.18\pm2.9$		11	$11.07\pm5.04$	
Repair + Sham treatment	6		$12.12\pm2.19$		5	$7.37\pm2.06$	
Sham + treatment	12		$9.95\pm3.27$		11	$11.18\pm4.15$	
Sham + sham treatment	6		$8.18\pm4.35$		5	$8.57 \pm 1.48$	
Comparison	Comparison		Interpretat		ion		
		value					
Group 7 vs Group 11a		.741		There is no difference in the proportion of			
				type I muscle fibers			
Group 9 vs Group 12a		.977		There is no difference in the proportion of			
				type I muscle fibers			
Group 8 vs Group 11b		.467		There is no difference in the proportion of			
				type I muscle fibers			
Group 10 vs Group 12b		.828		There is no difference in the proportion of			
				type I muscl	e fibers		
Group 7 vs Group 8		.918		There is no difference in the proportion of			
		type I musc		type I muscl	le fibers		
Group 9 vs Group 10		.991 Th		There is no o	There is no difference in the proportion of		
			type I mu		ele fibers		

## 6-month denervation groups:

## **Conclusions:**

We can see that the longer denervation period led to smaller muscle fibers but also just confinement to the cages may have contributed.

## The only significant differences in muscle fiber size refer to sham operated animals.

- 1. In Sham operated muscle, the type 2B muscle fiber area is significantly larger when treated with FS protein vs saline.
- 2. There is significantly greater proportion of 2B muscle fiber types when the muscle is treated with FS <u>protein</u> after sham surgery.
- 3. In contrast, there is significantly greater proportion of 2A muscle fiber types when the muscle is treated with FS <u>virus</u> after sham surgery.

## Tendencies

- 1. There is a tendency that the virus treatment (rather than saline) leads to larger 2B muscle fiber diameter of muscles that have been denervated and repaired.
- 2. After sham denervation, there was a tendency that <u>protein treatment</u> led to larger 2B muscle fiber diameter and larger type I muscle fiber diameters than muscles that had saline injected.

Does time make a difference? The effect of the administration of follistatin on re-innervated skeletal muscle fiber recovery after 3 vs 6 months of denervation

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#### Abstract:

The objective is to evaluate the effect of Follistatin on the recovery of skeletal muscle strength and skeletal muscle fiber diameter after different periods of denervation time and re-innervation. Rationale: Functional recovery following traumatic peripheral nerve injury is often suboptimal despite appropriate treatment. Due to the slow rate of axonal regeneration (1-3 mm/d), the target muscle may undergo significant atrophy before the axon attempts reinnervation. Follistatin influences muscle regeneration at several levels including directly inhibiting myostatin, a signal transduction protein that regulates muscle mass by inhibiting muscle regeneration. As a result, Follistatin stimulates muscle fiber hypertrophy and hyperplasia in normal animal models.

Methods: Transection of the tibial nerve in the hindlimb of Sprague-Dawley rats, followed by delayed (3 or 6 month) repair (utilizing microsurgical nerve suturing with nerve graft) induced partial recovery of the muscle with mild or moderate residual strength deficits due to irreversible atrophy.

Recombinant protein and recombinant DNA were synthesized (and the DNA packaged in adenoassociated viral vectors) in the Virginia Commonwealth University (VCU) Biological Macromolecule Core Facility. The Follistatin protein was delivered, after reinnervation, to the gastrocnemius muscle utilizing an Alzet (Cupertino, CA, USA) implantable drug delivery system. Treatment effects on the muscle were evaluated by cryosectioning the muscles after evaluation of muscle force. Muscle fiber types identified with Immunohistochemistry allowed differential evaluation of three primary muscle fiber types.

Results: The muscles of the animals that were denervated 3 months and repaired, followed by Follistatin treatment, exerted about the same force as experimental animals without Follistatin treatment and had about the same size muscle fibers. Muscles that were denervated 6 months before reinnervation and treatment with Follistatin protein developed significantly more force than denervated animals without treatment. The muscle fibers expressing type IIa and IIb myosin heavy chains were larger in diameter in the experimental animals that were treated with either type of Follistatin than control animals that received no Follistatin.

Conclusions: In this rodent model of delayed peripheral nerve repair, we found that Follistatin, administered 1 month after reinnervation had a greater facilitating effect after 6 months than 3 months of denervation on the recovery of type IIa and IIb muscle fibers and muscle force.

## Recovery of Chronically Denervated Muscle Enhanced with Follistatin Treatment

Introduction: Poor functional recovery following major peripheral nerve injury is largely due to the progressive catabolic process (denervation atrophy) affecting muscle fibers. Follistatin is a glycoprotein that blocks the muscle growth inhibiting peptide myostatin and also possesses remarkable independent muscle stimulating properties. We hypothesized that the administration of follistatin to rodent muscles subjected to prolonged but temporary denervation (3 or 6 months) would improve final muscle recovery and function.

#### Materials and methods:

One hundred forty-four (three-month old female) Sprague-Dawley rats were divided into 8 groups comprising animal muscles with or without temporary denervation and subsequent repair (3 or 6 months) and with or without follistatin treatment (delivered via Adenovirus viral vector containing recombinant DNA or direct delivery of recombinant protein via subcutaneous osmotic pumps).

After final recovery, muscle weight and force were recorded and statistically compared between groups with an alpha level of .05.

#### Results:

For 3-month denervation groups, there was no improvement in muscle weight or force generation following either protein or recombinant DNA treatment. For 6-month group, virus administration resulted in higher final muscle weights in both denervation and sham denervation groups. Protein treatment resulted in greater muscle force generation in the denervation group.

Conclusion: Six month chronically denervated muscle showed modest improvements in muscle mass and strength recovery following follistatin treatment. The effect was not consistent and further study will be necessary to elucidate any future role of this novel treatment strategy.



