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14. ABSTRACT
Loss of nerve function occurs when a nerve is severed. Any significant loss of nerve tissue requires a graft to restore continuity and promote nerve regeneration and recovery of function. Presently, there is no acceptable nerve grafting method for the repair of extensive nerve damage. An acellular nerve allograft product is available for the reconstruction of short to moderate length nerve defects, however, in its present from the allograft is not suitable for the repair of lengthy nerve defects. The aims of this project are to evaluate several long (7cm) nerve allograft technologies. All experimental tasks scheduled for years 1 and 2 were completed. A 6-month no-cost extension was approved to complete the extensive data analyses and documentation. An overview of findings indicates that the SIS-graft provided for nerve regeneration and meaningful recovering of nerve function that, in several cases was better than autografting. Other decellularized allografts tested did not perform well in this repair model. Additional evaluations and data analyses are ongoing to determine the efficacy and failures observed.

15. SUBJECT TERMS
peripheral nerve, nerve graft, allograft, nerve regeneration, rehabilitation

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INTRODUCTION

Loss of nerve function is complete when a nerve is severed and significant recovery without surgical intervention is rare. Any significant loss of nerve tissue requires a graft to restore continuity and promote nerve regeneration. Presently, there is no acceptable nerve grafting method for the repair of extensive nerve damage. Recently, an acellular nerve allograft product was brought to market that is now used widely for the reconstruction of short to moderate length nerve defects. However, this allograft, in its present from, is not suitable for the repair of lengthy nerve defects, like those often encountered with battlefield trauma. This proposal aims to greatly expand the application of nerve allografts for the reconstruction of extensive nerve damage. One approach to apply an acellular allograft in the repair of long nerve defects is the stepping-stone graft. Stepping-stone grafts combine two moderate length allografts with a small, interposed autologous nerve segment. A second approach is to apply a biodegradable gel coating to protect and prolong the efficacy of the acellular graft once implanted.

Specific Aims are to: 1) Evaluate 7 cm acellular allograft prototypes 4 weeks after implantation in rabbits. Key aspects of graft viability and integration will be assessed to finalize grafting protocols. 2) Conduct a 26-week preclinical evaluation of 7 cm stepping-stone allografts (SS-grafts) and 7 cm hydrogel-coated allografts in the reconstruction and rehabilitation of extensive peripheral nerve injury in rabbits. Control conditions include a standard acellular allograft and cellular autograft (the gold standard in nerve grafting). Outcomes will be evaluated by translational neurological testing.

BODY

Tasks 1a, b, c were scheduled for Year 1 and were completed successfully (see Annual Report for Year 1).

Task 2. Evaluate nerve grafts in 26-week study (months 7-24):
Task 2a. Collect, process and prepare 7 cm acellular allografts (18 rabbits)(months 7-10).
Task 2b. Implant nerve grafts in rabbits (48 rabbits) (months 8-14).

Task 2 (months 7-24) evaluated nerve grafts in a long-term study. This first involved the preparation and implantation of the nerve grafts (Tasks 2a and 2b), which were completed successfully (see Annual Report for Year 1). The experimental design and list of outcome evaluations are shown in the Table 1.

Table 1. 26-week evaluation of nerve graft technologies (revised)

Nerve graft (7 cm)	Sample	End-point	Evaluations
Autograft	n=12	26 weeks	• Toe-spread function • Trans-graft electrophysiology • Axon morphometrics • Graft integrity, cellularity, viability
DCI-graft	n=12		
DCI-graft + SIS tube	n=12		
SS-graft (4-1-2)	n=12		

This Progress Report for Year-2 will address the Tasks 2c-f.
The SOW and chronology were revised according to a 6-month no-cost extension.

Task 2c. Assess progressive recovery of function after nerve repair (months 12-20).

i) Beginning 16 weeks after nerve grafting, perform weekly function testing and collect video documentation of all rabbits (n=48).
ii) Extract still frames (n=4) from each test session.
iii) Perform image analysis to measure toe-spread function and calculate index for each test session.
iv) Plot and perform statistical analyses of function data.

Progress:

A rabbit's ability to spread its toes is lost after transection of the peroneal nerve. In this study transected peroneal nerves were repaired with various nerve graft constructs. The loss of toe-spread function persists until the cut nerve fibers regrow through the graft and distal nerve and reinnervate the muscles that control movements of the toes. The methods used to test toe-spread function are described in Figure 1.

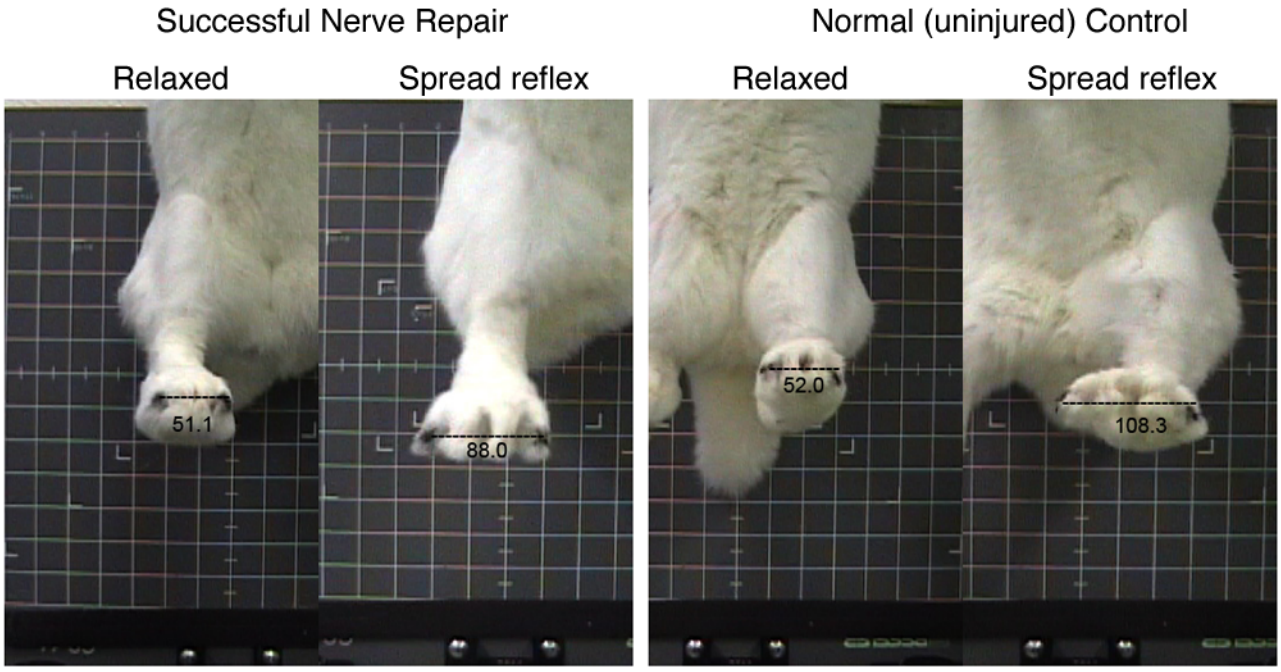

Figure 1. Measuring the toe-spread reflex in rabbit. The rabbit was lifted by the tuft of the neck. When fully relaxed, the rabbit was suddenly lowered a few inches to simulate the feeling of falling and induce the toe-spread reflex. The toe-spread behavior was recorded by digital video photography and still frame images were captured from 6-8 trials per test session. The distances from digits II-V were measured by image analysis software. The Toe-spread Index (TSI) was calculated as the mean of the Spread measurements divided by the mean of the Relaxed measurements. A TSI indicative of no function was 0.9-1.1. A normal TSI was 1.5-2.1. Shown above are measurements after a highly successful nerve graft reconstruction (left panels; TSI, 88.0÷51.1=1.72) and a normal rabbit response (right panels; TSI, 108.3÷52.0=2.08).

Task 2c was completed. All animals were assessed weekly until termination 26 weeks after receiving the nerve graft. Videos were taken and numerous still photos (single frames) were extracted. We have completed calculating the toe spread function scores at all time points. After

termination the engrafted nerves were examined for nerve-graft continuity. Animals with a loss of continuity due to failure of sutures at the coaptation were excluded from the analyses. Based on this criterion, two or three animals were excluded from each group.

Outcomes of recovery of function are shown in Table 2. The autograft condition gave the best recovery of function with 9/10 animals displaying some degree of toe-spread. Five of nine rabbits in the DCI-graft condition showed some degree of toe-spread function recovery. Seven of nine rabbits in the SS-graft condition showed toe-spread. No recovery of function was observed for animals in the DCI-graft+SIS tube condition. The mean TSI score for all the graft conditions were well below normal (1.5-2.1). The highest individual TSI score was 1.4 and there were more individual animals with high scores in the SS-graft condition than in the other groups.

Table 2. Recovery of toe-spread function 26 weeks after nerve grafting

Graft condition	Animals with TS function	Mean TSI score
Autograft	9/10	1.27
DCI-graft	5/9	1.25
DCI-graft + SIS tube	0/10	1.09
SS-graft (4-1-2)	7/9	1.24

The Toe-spread Index (TSI) was calculated as described in Figure 1.

2d. Perform trans-graft electrophysiology 26 weeks after grafting (months 14-20).

i) Obtain single stimulus-generated compound action potentials through the implant at stimulation intensities for large diameter myelinated A-fibers and small diameter myelinated and unmyelinated fibers.
ii) Measure conduction latency and velocity for each fiber group.
iii) Obtain paired stimulus compound potentials and determine alterations in conduction through the graft.
iv) Evaluate progression of incomplete regeneration by stimulation at different sites within and distal to the graft.

Progress

Immediately prior to termination of the animal subjects, the nerve grafts were exposed surgically and trans-graft electrophysiology performed to determine the conduction of signals through the grafted nerve. All electrophysiological recordings were performed. The recordings were analyzed and data extracted to determine nerve conduction velocities and the extent of muscle reinnervation. Findings are shown in Table 3.

Table 3. Trans-graft nerve conduction 26 weeks after nerve grafting

Graft condition	Animals with conduction	Mean conduction (m/sec)
Autograft	9/10	34.9
DCI-graft	2/9	8.7
DCI-graft + SIS tube	0/10	0.0
SS-graft (4-1-2)	4/9	13.6

Electrophysiological recordings were best in the autograft condition and 9/10 animals had meaningful conduction of action potential. Only 2/9 rabbits in the DCI-graft condition and 4/9 rabbits in the SS-graft condition met criteria for measurable conduction. None of the animals in the DCI-graft+SIS tube condition showed trans-graft conduction. The best individual conduction velocities were observed in the autograft condition (37.2 - 54.9 m/sec) and were mostly half that recorded for normal nerves (mean= 100.4 m/sec).

Task 2e. Perform histological/immunocytochemical examination on grafts (months 19-27).
i) Axon morphometrics (scoring neurofilament immunopositive axons)
ii) Graft integrity, cellularity and viability within the graft (H&E staining)
iii) Schwann cell migration from autologous nerve segment (S100 immunolabeling).
Task 2f. Perform histomorphometric scoring for nerve regeneration (months 23-29).

Progress

All rabbits survived the 26-week survival period in good health. All animals were terminated, the engrafted nerves were removed and processed for histological and immunocytochemical examinations. Key points in the nerves were sectioned and stained. Image analysis to score regenerated axons that grew through the grafts and into the host nerve (indicative of successful nerve repair) was completed. A summary of results is shown in Table 4.

Table 4. Trans-graft axonal growth 26 weeks after nerve grafting

Graft condition	Animals with distal axons	Mean axon count (labeled pixels)
Autograft	9/10	1,129,171
DCI-graft	4/9	219,220
DCI-graft + SIS tube	0/10	0
SS-graft (4-1-2)	7/9	410,588

Scoring axonal regeneration at other points (e.g., within the grafts) was also completed to determine the temporal progression of nerve regeneration. Other features of nerve growth within the grafts have been examined. Representative observations are shown in Figure 2. Nerve regeneration in some graft conditions was poor. Numerous additional tissue sections of the graft content were examined to better understand the dynamics of nerve regeneration within the grafts. Representative observations of a failed nerve graft are shown in Figure 3. Deterioration of the distal aspect of the graft was a conspicuous feature of failure. Successful processed allografts were thoroughly compared to the most successful autographs. Representative evaluations are shown in Figure 4. Detailed analyses of these data are ongoing as we prepare written reports of our findings.

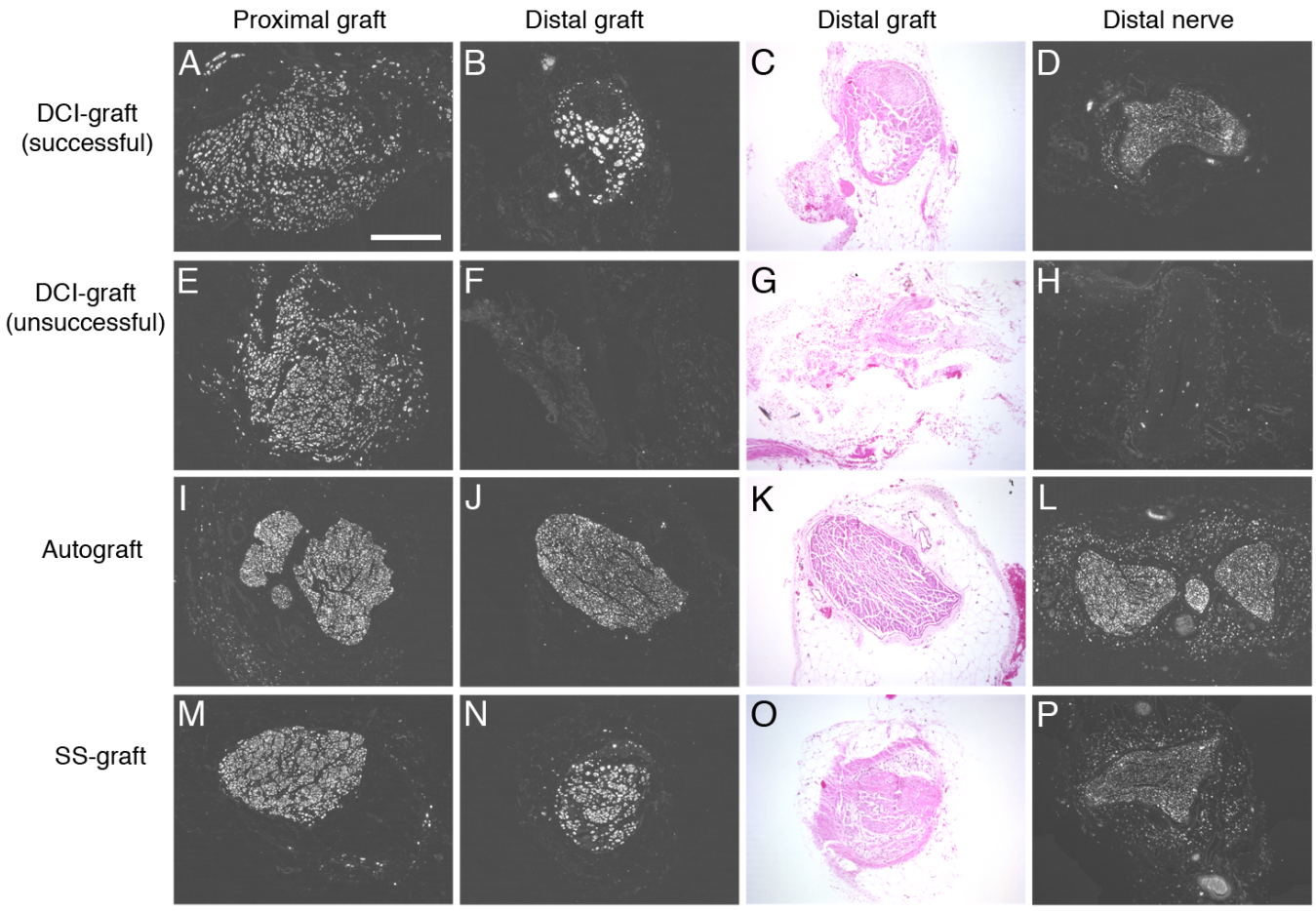

Figure 2. Regeneration within and distal to 7 cm grafts at 26 weeks post-implantation.

A: Successful Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
B: Successful Processed Allograft, neurofilament immunolabeled axons at 1.5 proximal to the distal coaptation
C: Successful Processed Allograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in B)
D: Successful Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve

E: Failed Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
F: Failed Processed Allograft, neurofilament immunolabeled axons at 1.5 cm proximal to the distal coaptation
G: Failed Processed Allograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in F)
H: Failed Processed Allograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve

I: Autograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
J: Autograft, neurofilament immunolabeled axons at 1.5 cm proximal to the distal coaptation
K: Autograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in J)
L: Autograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve

M: Acellular Allograft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft
N: Acellular Allograft, neurofilament immunolabeled axons at 1.5 cm proximal to the distal coaptation
O: Acellular Allograft, H&E stain at 1.5 cm proximal to the distal coaptation (as in N)
P: Acellular Allograft, neurofilament immunolabeled axons at 0.5 cm into the distal nerve
Scale bar A-P, 360 μm

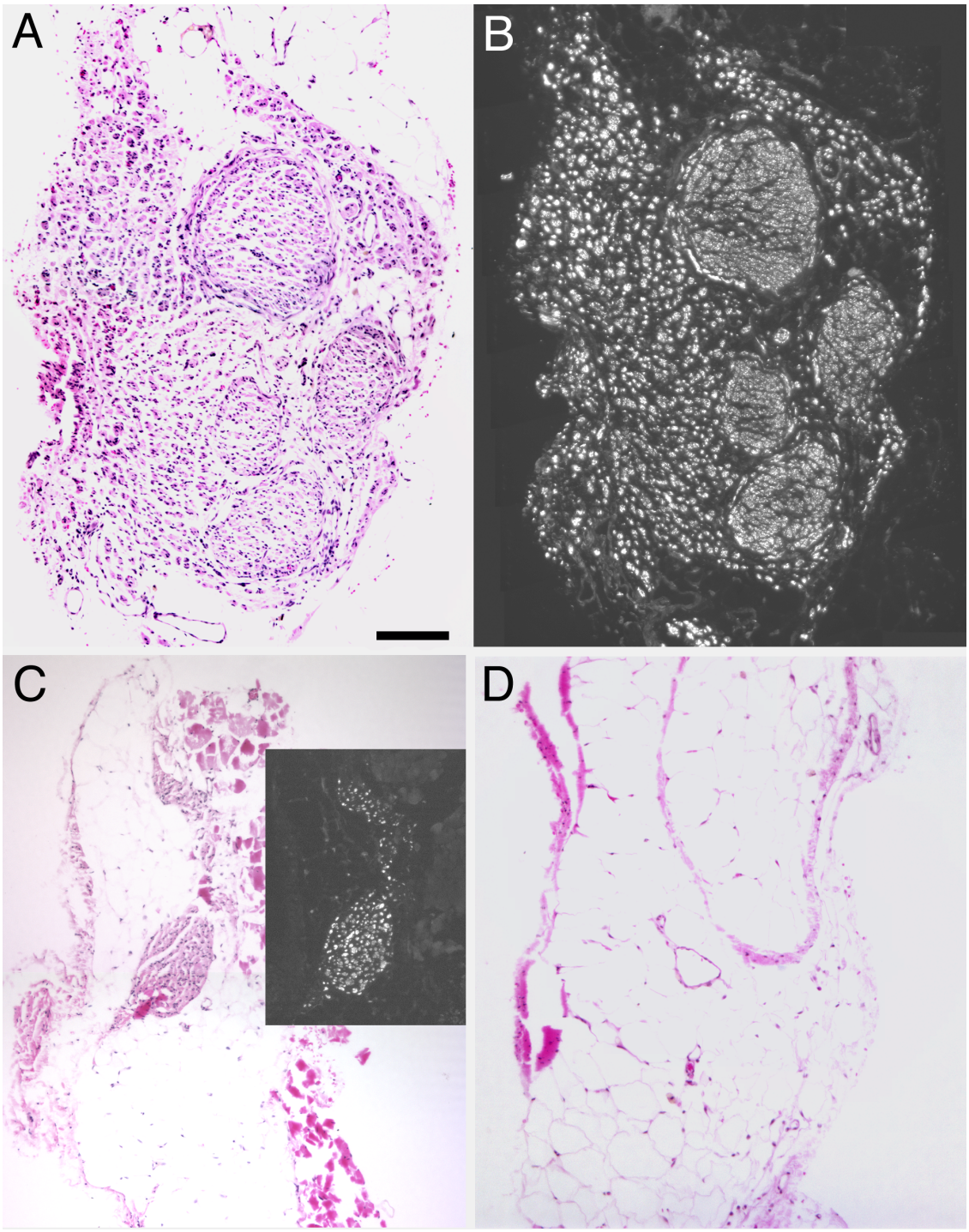

Figure 3. Failed regeneration within 7 cm rabbit nerve Processed Allografts at 26 weeks post-implantation.

A: Proximal graft, H&E stain at 0.5 cm into the graft.

B: Proximal graft, neurofilament immunolabeled axons at 0.5 cm into the proximal graft

C: Mid-graft, H&E stain at 2.5 cm into the proximal graft. Inset, neurofilament immunolabeling.

D: Distal graft, H&E stain at 1.5 cm from the distal coaptation.

(Scale bar, 200 μm)

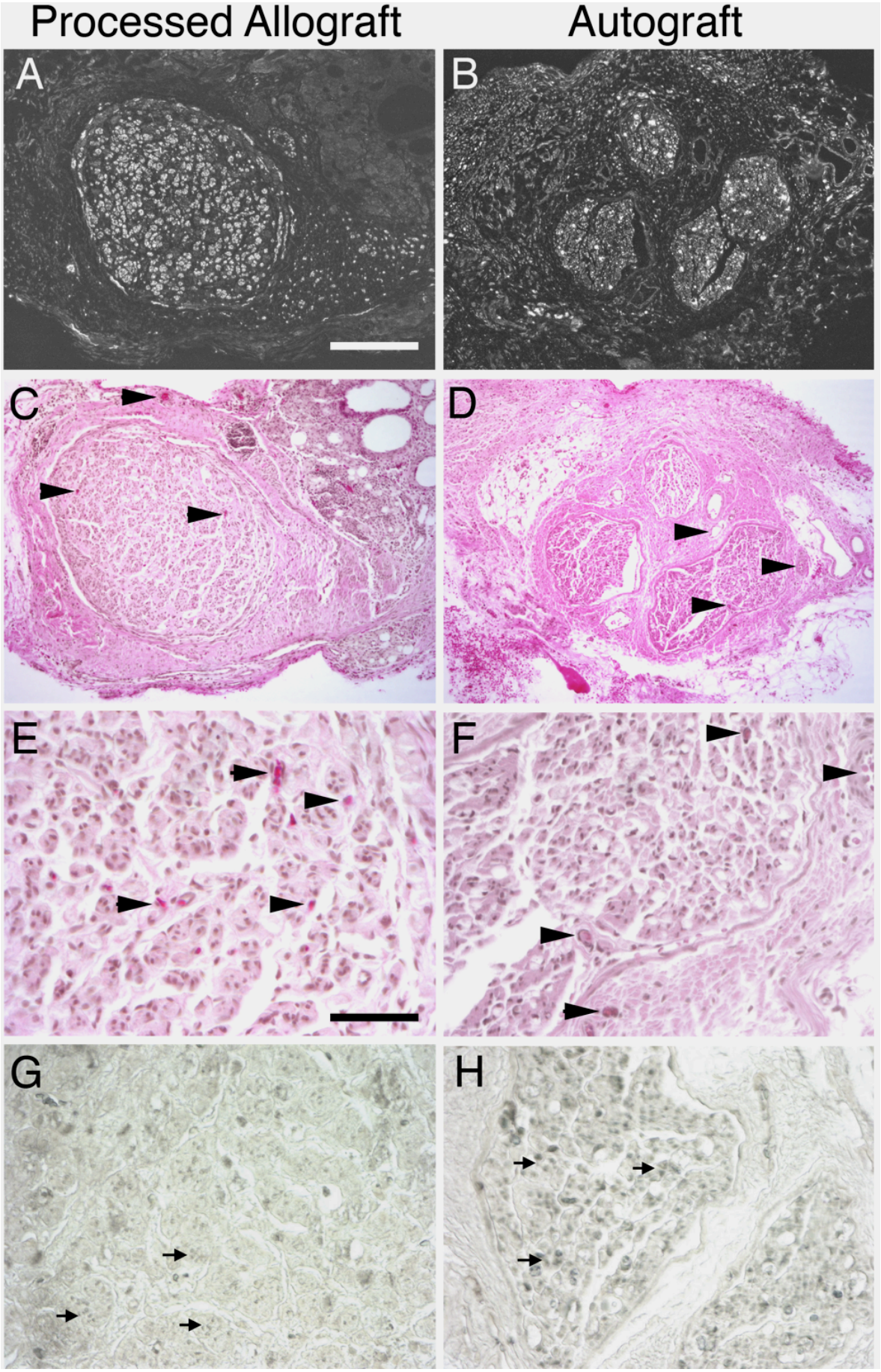
Processed Allograft
Autograft
A
B
C
D
E
F
G
H

Figure 4. Representative photomicrographs of the proximal aspect of 7 cm grafts at 4 weeks post-implantation.

A: Processed Allograft, neurofilament immunolabeled axons.
B: Autograft, neurofilament immunolabeled axons.
C: Processed Allograft, H&E stain. Arrowheads: blood vessels.
D: Autograft, H&E stain. Arrowheads: blood vessels.
E: Processed Allograft, H&E stain. Arrowheads: blood vessels.
F: Autograft, H&E stain. Arrowheads: blood vessels.
G: Processed Allograft, Sudan black stain. Arrowheads: clusters of myelinated axons.
H: Autograft, Sudan black stain. Arrowheads: clusters of myelinated axons.

Scale bar A-D, 360 μm
Scale bar E-H, 90 μm

Future Work

Task 2g. Analyze and document results (months 24-30).

KEY RESEARCH ACCOMPLISHMENTS:

- Forty-eight rabbits received nerve grafts, survived the recovery period in good health and nerve regeneration was evaluated successfully.
- Determined that the DCI grafts were marginally effective.
- Determined an autograft insert into a DCI graft (the SS-graft) improved the outcome significantly.
- Determined that an SIS tube around the distal aspect of a DCI graft was detrimental and all grafts in this condition failed.

CONCLUSION: The Tasks completed in years 1 and 2 established a novel decellularized nerve allograft model in rabbit. Several nerve allograft prototypes were examined and protocols established for long-term in vivo evaluation. The goal is to discover ways to increase the effective length of decellularized nerve allografts. Presently nerve allografting is limited to the repair of moderate (1-4 cm) nerve gaps. This project tested four grafting technologies involving 7 cm nerve allografts in a small diameter rabbit nerve model. All nerve grafting procedures and initial evaluations were completed on schedule. Analyses of the extensive data collected are ongoing and a 6-month no-cost extension was approved for this purpose. An overview of the data indicates that the SIS-graft (a hybrid of decellularized and autograft segments) provided significant recovery of function that, in several cases, was better than autografting. With proper technique the SIS-graft may offer an effective alternative to conventional autografting using off-the-shelf nerve that overcome the length limitations of conventional nerve allografting.

REPORTABLE OUTCOMES: None

REFERENCES: None

APPENDICES: None