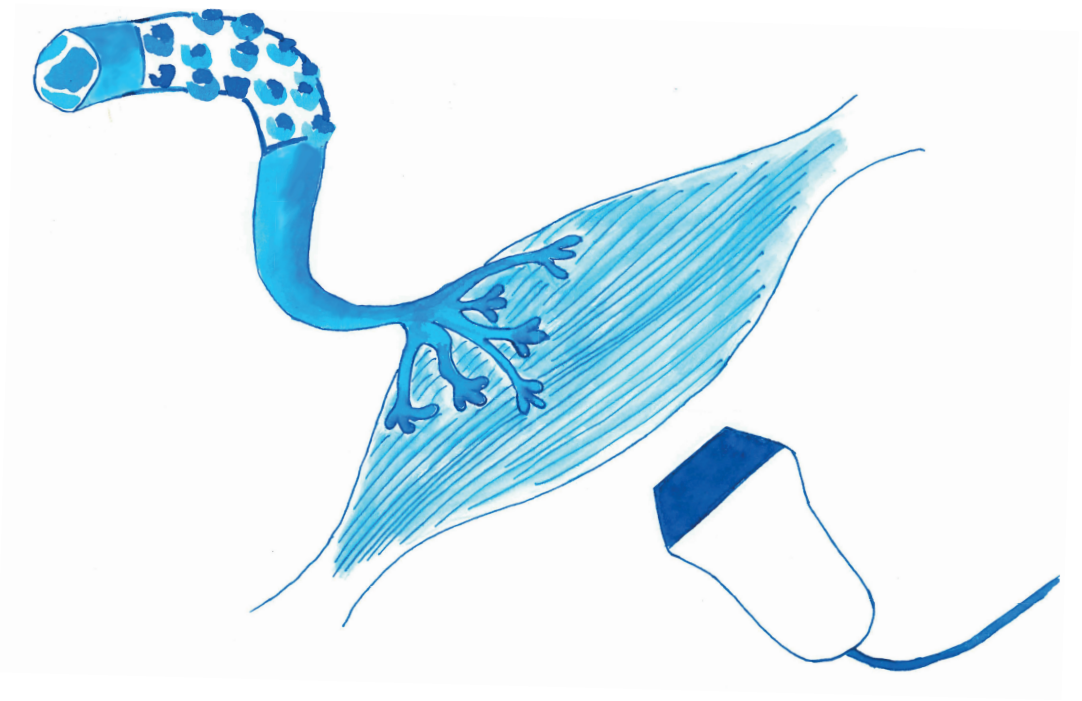
NERVE RECONSTRUCTION

Improving outcome using allografts and stem cells in motor nerve repair



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Nerve Reconstruction:

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Nerve Reconstruction:
Improving outcome using allografts and stem cells in motor nerve repair

Zenuw reconstructie: het verbeteren van uitkomsten met behulp van allografts en stamcellen voor motorisch zenuw herstel

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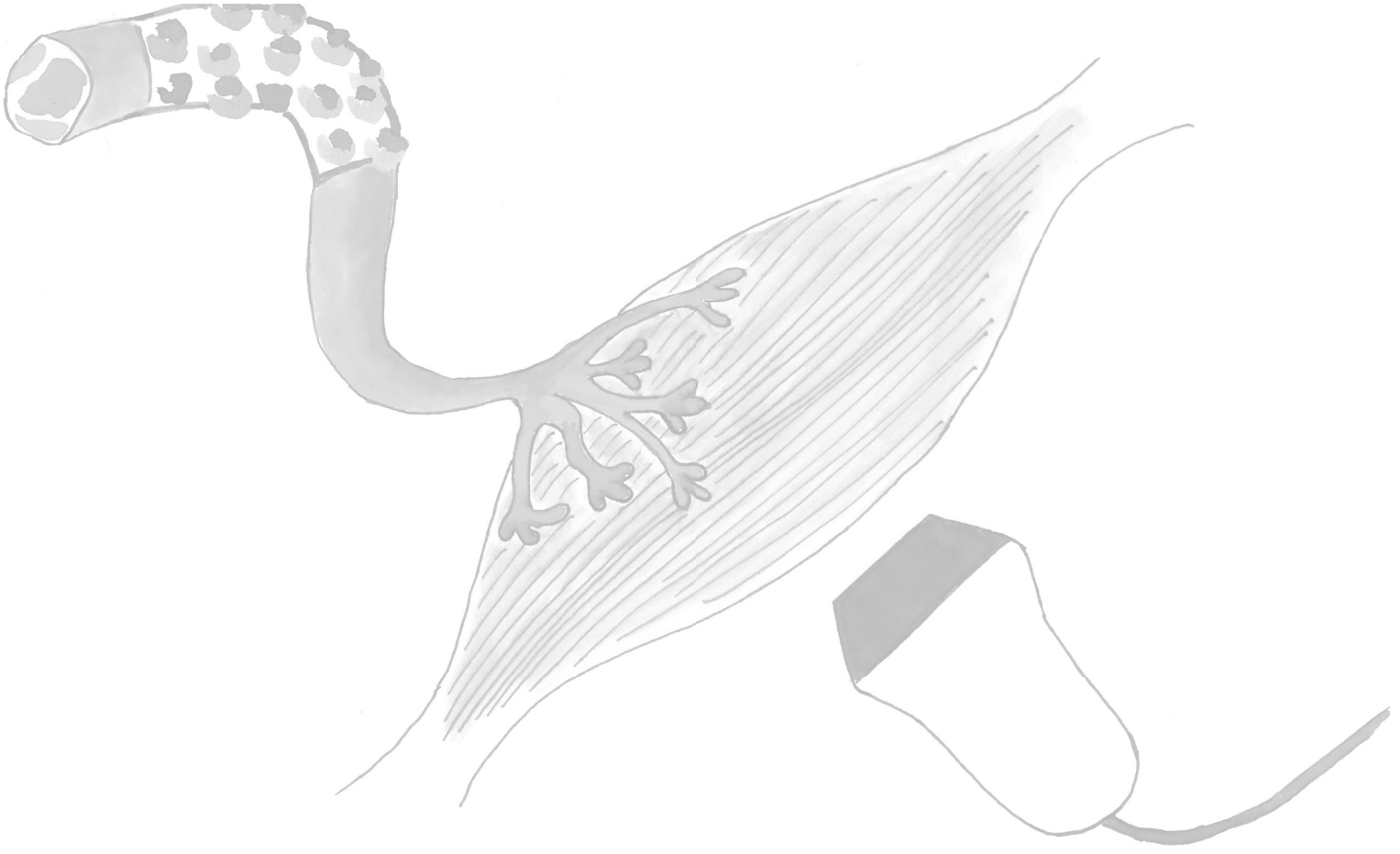

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General introduction and outline of this thesis

Introduction of the clinical problem

Peripheral nerves enable communication between central nervous system (CNS) and peripheral organs including muscles and skin (sensation). Peripheral nerve injuries occur in up to 3% of patients suffering extremity trauma and can have devastating impact on patients' daily functions and quality of life.[1-3]

A special group of often large and very proximal nerve injuries is formed by brachial plexus injuries. The brachial plexus is a complex network of nerves that arises from the neck and innervates the muscles and skin of the upper extremities. An injury to the brachial plexus, commonly caused by motor vehicle or snow mobile accidents, can thus result in a severely impaired or even completely flail arm. Timely and adequate reconstruction of the nerve damage is the main priority. When direct coaptation is not possible either an interposition graft (a segment from a different nerve to bridge the gap) can be used or nerve transfers (diverting a less important nerve), with or without additional nerve grafts, can be performed. These techniques are further explained below. An important challenge in the reconstruction of extensive nerve gaps is the limited amount of available autologous nerve graft. Due to a shortage of donor nerve material to reconstruct the large gaps, surgeons are frequently constrained to choose which defects to repair comprising functional outcome.[4] Despite improvement of treatment options over the past decades, patients treated for upper extremity peripheral nerve injury still report substantial disability.[5]

A possible solution would be the use of readily available processed nerve allografts that provide similar results as the gold standard nerve autografts. A nerve allograft is a nerve that is obtained from a deceased donor and processed so that it does not elicit an immune response in the acceptor. Use of nerve allografts also avoids donor site morbidity caused by harvesting autologous nerve grafts. Besides implementation of optimized nerve allografts in animal models, this thesis also looks into the possibility of enhancing nerve reconstruction by adding stem cells. The overarching aim of this thesis is to improve nerve reconstruction using an off the shelf peripheral nerve allograft that is unlimited in supply and can be individualized to each patient using stem cells, providing functional recovery comparable to autograft nerve.

In this introduction, we will first discuss the basic anatomy of the brachial plexus and peripheral nerves. Secondly, the basic principles of nerve injury and regeneration will be discussed followed by an overview of reconstructive options and their current clinical challenges. Thirdly, we will explain several measurements of functional recovery that are currently used in animal models to evaluate new experimental treatment strategies. Finally, the general aims and outline of this thesis are provided.

Anatomy

The brachial plexus is a network of nerves innervating both muscles and skin of the upper extremity. The network arises from the spinal nerves at level C5 – T1 and is typically divided into the following anatomic sections: nerves (C5-T1), trunks (upper, middle and lower), divisions (an anterior and posterior division for each trunk), cords (posterior, lateral and

medial) and finally the terminal branches that flow forth into multiple peripheral nerves. An overview of the brachial plexus anatomy and the peripheral nerves that originate from it is depicted in Figure 1.[6,7]

Each spinal nerve is composed of a ventral and dorsal root that arises from the spinal cord. The cell bodies of the ventral roots (anterior horn cells), providing motor information, are located in the gray matter of the spinal cord, which is part of the central nervous system. These motor neurons provide axons that end in the motor endplates of muscles. Bundles of axons from these anterior horn cells form the ventral roots. The cell bodies that process sensory information are located in the dorsal root ganglia just outside the spinal cord.

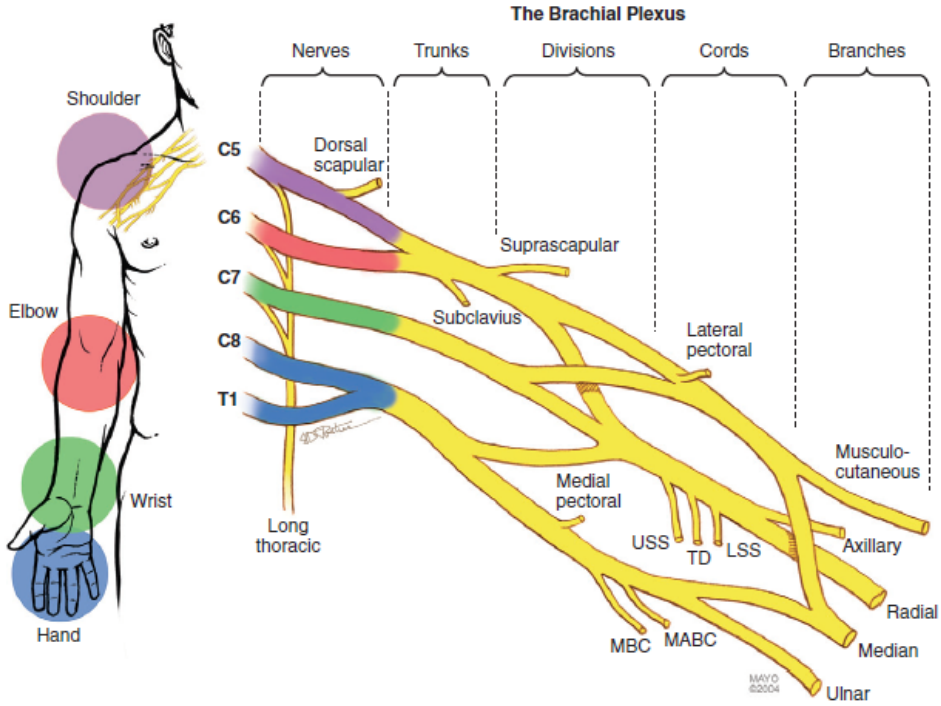


Figure 1. Overview of the anatomy of the brachial plexus from the spinal nerves onto the terminal branches that flow forth into multiple peripheral nerves. The diagram on the left presents a simplified indication of the different parts of the brachial plexus and related shoulder-, elbow-, wrist- and finger movement. This may be used for patient education.

LSS, lower subscapular nerve; MABC, medial antebrachial cutaneous nerve; MBC, medial brachial cutaneous nerve; TD, thoracodorsal nerve; USS, upper subscapular nerve. (Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved.)

The nerves distal to the plexus are referred to as peripheral nerves. Peripheral nerves can be divided into three main categories: motor nerves (containing mostly motor fibers), sensory nerves (containing mostly sensory fibers) and mixed nerves that contain both motor and sensory fibers. Peripheral nerves are composed of nerve fibers and connective tissue, organized in several layers. Individual axons can be either myelinated or unmyelinated. In

unmyelinated axons, a group of multiple axons is enclosed by chained Schwann cells without additional myelin sheaths. Myelinated axons are separately sheathed by myelin, produced by Schwann cells, which increases conduction velocity. The thicker the myelin sheath, the faster the conduction speed. Most peripheral nerves consist of myelinated fibers. Multiple axons and their Schwann cells are held together by the endoneurium and surrounded by the perineurium to form fascicles. Groups of fascicles are surrounded by epineurium to form a peripheral nerve. This is schematically depicted in Figure 2. For blood supply, peripheral nerves have two systems. The vasa nervosum is a capillary plexus that runs through the epineurium and perineurium. Secondly, the endoneurium contains longitudinally running microvessels. Both systems are connected.[8,9]

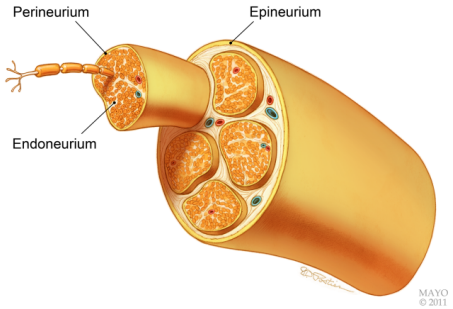


Figure 2. Schematic overview of the anatomy of peripheral nerves. (Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved.)

Injury to peripheral nerves and the brachial plexus

A commonly used classification system for peripheral nerve injury was first introduced by Seddon[10] in 1943 and later expanded by Sunderland[11] and is based on the anatomy of peripheral nerves. This classification with a general description is presented in Table 1. Causes of nerve injury are numerous including trauma, tumors and iatrogenic injury.[12-14]

Table 1. Description of various types of nerve injury.

Type	Description	Recovery
I First-degree (neurapraxia)	- focal conduction block - preservation of continuity of nerve components	- spontaneous recovery within weeks to months
II Second-degree (axonotmesis)	- disruption of axons, endo-, peri- and epineurium remain intact	- spontaneous recovery expected, but may take months to years
III Third-degree (axonotmesis)	- disruption of axons and endoneurium. Peri- and epineurium remain intact.	- spontaneous recovery may (partially) occur but is uncertain
IV Fourth-degree (axonotmesis)	- disruption of axons, endo- and perineurium. Epineurium intact. - neuroma in continuity	- spontaneous recovery is not expected
V Fifth-degree (neurotmesis)	- complete nerve disruption	- no spontaneous recovery possible

In brachial plexus injury, the extent of the lesions is highly dependent on the mechanism of trauma. The several forms of nerve injury as described in Table 1 can occur at any location in the brachial plexus. Specifically in brachial plexus injury a nerve root avulsion can occur. This typically happens after high energy trauma, such as motor vehicle accidents (downward traction of the arm) or grasping onto something during a fall from height (upward traction of the arm) causing one or more nerve roots to get avulsed from the spinal cord. Neurotmetic lesions (fifth-degree) in brachial plexus injury include postganglionic ruptures and preganglionic root avulsions.[7,13,14]
Within an individual patient, different degrees of injury at different nerve levels may occur. This makes the clinical presentation of patients suffering from brachial plexus injury variable and challenging to assess in the emergency department.

Nerve regeneration
Following second-degree nerve injury or worse, a process called Wallerian degeneration will occur distal to the site of injury. In this process of Wallerian degeneration, the nerve distal to the site of injury is cleared from myelin and cellular debris, creating room for axons and Schwann cells to elongate and grow towards the target muscle to establish reinnervation.
To guide this process, local Schwann cells change from a myelinating phenotype to a growth supporting phenotype. Subsequently, Schwann cells gain several features to support nerve regeneration. Several days after the nerve injury, degeneration of the detached axons occurs and the permeability of the blood-nerve barrier decreases to allow for influx of immune cells and growth factors. A local immune response is triggered to clear myelin from the distal Schwann cells. In the cleared basal lamina tubes, endoneurial tubes align to form the so-called Bands of Bungner that support and guide the growing axons. This is followed by the formation of new myelin. Furthermore, there is an increased production of growth factors that are known to stimulate nerve regeneration, including glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), pleiotrophin, vascular endothelial growth factor (VEGF). These growth factors support axonal elongation, neuronal survival and formation of new blood vessels. Extracellular matrix (ECM) proteins, especially laminin, seem to play an important role in nerve regeneration, although the exact role of the ECM in nerve regeneration is not fully understood yet.[15-18]
In the whole process of nerve regeneration, the supporting Schwann cells and the extracellular matrix (ECM) are crucial to guide axons in the right direction. Depending on the severity of the injury, regeneration may occur at a rate of approximately 1 mm/day. Regeneration may be only partial or there can be no regeneration at all in second- to fifth-degree injury, so that surgical intervention is necessary.
In this thesis, the emphasis is on motor nerve regeneration. In motor nerve regeneration, when it takes too long for the axons to reach their target, degeneration of the motor endplates will occur. Reinnervation of the target muscle will then no longer be possible. Motor endplate degeneration becomes mostly irreversible after approximate 12-18 months in adult patients. When making a reconstructive plan for peripheral nerve injuries, it is

therefore important to take into account the distance between the injury site and target muscle and the time that has already elapsed since the injury.[19-21]

Reconstructive options

When possible, the preferred technique is direct tension free coaptation of the nerve ends. When this is not possible, there are roughly two techniques to reinnervate the target organs distal from the injury site: (1) transfer of one or more healthy but expandable nerves or nerve fascicles to the injured nerve, distal to the site of injury (nerve transfers) or (2) use of an interposition conduit or graft to reconnect the two nerve ends (bridging the gap).[19,22]

Nerve transfers

The principle of nerve transfers can be defined as the coaptation of one or more healthy (at least Medical Research Council [MRC] grade 4) but expandable nerves or nerve fascicles to the injured nerve that is deemed more important, at a level distal to the site of injury. Indications for nerve transfers include nerve root avulsions, proximal nerve injuries and patients with a delayed presentation where nerve grafting is not expected to provide timely reinnervation to prevent motor-endplate atrophy. Furthermore, nerve transfers can be indicated in multiple level nerve injuries or for large neuromas in continuity. Nerve transfers can also be used to provide innervation for free-functioning muscle transfers. When the length of the donor nerve is insufficient to reach the target nerve, a bridging nerve graft can be interposed. However, this may pose a clinical challenge in multiple level nerve injuries where donor nerves are already necessary for grafting of several nerve lesions and there may be a shortage of donor material.[4,23-27]

Bridging the gap

Nerve autograft

The current gold standard to bridge a nerve gap, is the use of a nerve autograft. Autologous nerves are immunologically inert and naturally contain a viable source of Schwann cells to support nerve regeneration. The most commonly used source for autologous donor material is the sural nerve, but other options have been described as well. Unfortunately, the nerve autograft has some important disadvantages including the chance of formation of painful neuromas, donor site morbidity and the limited amount of autologous tissue that is available. The limited amount of available nerve graft is especially a problem in large defects such as traumatic brachial plexus injuries. The shortage of donor material may require the surgeon to prioritize reconstructive options, impeding optimal results. Furthermore, the additional procedure to harvest the sural nerve may lengthen operative time, especially in obese patients.[28,29]

Alternatives: conduits and decellularized nerve allografts

Over the past decades, many alternatives for the nerve autograft have been proposed and used, including biological and synthetic conduits and nerve allografts. Conduits fabricated out

of many different synthetic materials have been evaluated with varying results. An important lesson learned is that synthetic conduits that are not bioabsorbable frequently cause irritation and need to be removed.[30] Synthetic conduits have shown to only provide acceptable results when used for strict indications. Currently, these indications only include small-diameter, noncritical nerve gaps of less than 3 cm length. In other cases, decellularized nerve allografts are considered a possible alternative to the autograft.[12,30-32]

Nerve allografts are nerve segments derived from deceased donors, that can potentially provide an unlimited supply. Fresh allograft nerves contain cellular components that elicit an immune response when directly transplanted to a patient.[33,34] To suppress this response, one solution can be the use of immunosuppressive drugs. However, these drugs have major disadvantages such as a high risk at several diseases including opportunistic infections, diabetes mellitus and malignant skin changes. They should therefore best be avoided when possible.[35] Another solution to avoid an immune response in the recipient, is to remove all cellular material of the nerve allograft prior to transplantation. The key to success herein is to remove the potentially immunogenic cellular material as much as possible, while maintaining the ultrastructure formed by the allografts extracellular matrix and basal lamina tubes, providing adequate guidance for regeneration axons. Many different processing techniques, including freeze-thawing, cold preservation, irradiation and chemical detergent based techniques have been developed and implemented in research setting, with varying success.[36] Up to date only one processed nerve allograft has been FDA approved for use in clinical setting (AxoGen).[37]

Although some successful recovery using decellularized nerve allografts in the clinical setting is reported for sensory nerve gaps, comparative studies that show satisfactory results for mixed and motor nerve defects are lacking. In an observational study, Brooks et al.[37] reconstructed 5-50mm gaps in sensory, motor and mixed nerves. Unfortunately, specific gap lengths for the mixed and motor nerves are not provided. They report outcomes of MRC grade ≥3 in 6/7 motor nerves and in 10/13 mixed nerves. However, any correlations between gap length and outcome, and specific clinical implications remain unknown.

Despite promising results of allograft reconstruction in mixed/motor nerve defects, there is definitely room for further improvement in this field. Previous animal studies found that autograft reconstruction still yielded better functional outcome than the nerve allograft that was processed according to the protocol of the commercially available AxoGen graft.[38] Therefore, the processing technique to decellularize nerve allografts was recently optimized. The enzyme elastase was incorporated in the processing technique to more efficiently diminish cellular debris (and thereby diminish possible immune responses). To protect and better preserve the carefully decellularized ECM ultrastructure prior to use, different storage techniques were compared. It was shown in vitro that storage at 4°C does not significantly alter the ECM ultrastructure and storage at -80°C may need to be avoided for optimal guidance of regenerating axons.[39] However, further in vivo studies were needed and are the basis of this thesis.

Improvement of nerve regeneration after acellular allograft reconstruction

Since the decellularized allograft is devoid of any cells, local Schwann cells to support nerve regeneration are lacking. Alternative sources of neurotrophic growth factors may be the key to boost nerve regeneration across acellular nerve allografts. Multiple studies have previously shown the beneficial effect of mesenchymal stem cells (MSCs) on nerve regeneration. Mesenchymal stem cells can be obtained from various sources including adipose tissue, bone marrow, skin, dental pulp and hair follicles. Adipose derived mesenchymal stem cells are easily accessible with low donor morbidity and proliferate rapidly with high yield, providing a preferred stem cell source. Furthermore, adipose derived MSCs can produce growth factors (including NGF, BDNF, VEGF). Adipose derived MSCs may therefore enhance nerve regeneration after acellular allograft reconstruction. However, the exact role and fate of MSCs in nerve regeneration have not been fully elucidated yet.[38-44]

Evaluation of functional recovery in animal models

To study alternative treatment strategies for peripheral nerve injury, animal models are frequently used. To evaluate recovery after various treatment strategies, reliable measurement techniques are of paramount importance. Many different outcome measurements have been used in literature. Currently, the most frequently used outcome measurements in both the rat and the rabbit model are: Electrophysiology (CMAP), isometric tetanic force, muscle mass and histomorphometry.

Electrophysiology

Electrophysiology measurement can be used to measure nerve conduction capacities. Compound muscle action potential (CMAP) amplitude is frequently reported in nerve reconstruction studies in animals. A reduction of amplitude (in comparison to the contralateral side) generally indicates a lower number of axons in the reconstructed nerve.[45]

Isometric tetanic force

Many frequently used outcome measures, including CMAP, muscle mass and histomorphometry, are indirect measures of reinnervation; i.e. they do not measure actual muscle function. As a more direct measurement of muscle function, the isometric tetanic force measurement was developed. While stimulating the reconstructed nerve, maximum contraction force produced by the target muscle is measured. The technique was validated for use in both the rat and the rabbit model and is believed to more accurately measures function of the muscle.

Muscle mass

At the end of the sacrifice procedure, the reinnervated target muscle can be excised. After nerve reconstruction, the target muscle will first decrease in size due to atrophy. Once the axons reach the muscle's motor end plates, the muscle becomes again innervated and will

slowly increase in size. Successful reinnervation will yield a target muscle mass comparable to that of the contralateral, healthy, side.

Histomorphometry

Histomorphometric measurement techniques can be used for quantification of nerve regeneration. Nerve segments are excised and ultrathin sections are stained to highlight different components of the peripheral nerve including axons and myelin. Results can be expressed in various parameters including nerve cross sectional area, myelinated fiber area and number of axons. It is considered most useful to evaluate nerve segments distal to the reconstructed part to evaluate the quality of nerve fibers that crossed the repair site and hopefully reached the target muscle. However, it is not possible to differentiate between axonal sprouting (which is of no benefit) and successful axonal regeneration based on these thin slides. Therefore, multiple outcome measurements should always be used to correlate.

Development of new, non-invasive, techniques

All these evaluation techniques require the animals to be sacrificed. A non-invasive method that does not require sacrifice of the animal and that can reliably measure muscle function would be highly desirable. The non-invasive nature would allow for multiple measurements over time in the same animal. This could decrease inter-animal variation which will theoretically increase power, and diminish the number of test animals required in future studies.

Walking track analysis has been used in the rat as a non-invasive alternative. However, it was shown that the walking track analysis does not correlate well with other functional outcome measurements.[46] Furthermore, it can only be done in the rat model and not in the rabbit model.

In the validation of a new evaluation technique, two main aspects are taken into consideration. First a high inter- and intra-rater reliability is desired to guarantee good reproducibility. Second, the new technique needs to correlate with established measurements of functional recovery.

Furthermore, as we believe it is important to evaluate new strategies in both the rat and the rabbit model prior to use in humans, the technique can ideally be used in both animals. For the rabbit model, a technique that does not require anesthesia would be highly favorable, as rabbits are known to be sensitive to anesthesia. Ultrasound is a non-invasive technique that has previously proven useful for evaluation of muscle recovery after nerve injury.[47,48] In this thesis we evaluated the use of ultrasound for evaluation of tibialis anterior muscle recovery after nerve reconstruction in both the rat and the rabbit model.

General aim and outline of the thesis

The overarching aim of this thesis is to improve nerve reconstruction using an off the shelf peripheral nerve allograft that is unlimited in supply and can be individualized to each patient using stem cells, providing functional recovery comparable to autograft nerve.

In the first part, studies on the current clinical use of nerve grafts are presented. The clinical focus of this part is on brachial plexus injury, as these injuries typically require large amounts of donor nerve material that is frequently not available. The availability of an off the shelf alternative would be very beneficial in this field. **Chapter 2** provides an overview of different techniques that are currently used to reconstruct elbow function after brachial plexus injury. In **chapter 3** we present a new technique that combines a nerve transfer with a long autologous nerve graft to reconstruct elbow extension after brachial plexus injury.

Before implementing new nerve reconstruction strategies in vivo in animal models, we recognized there was a need for non-invasive follow-up methods. Allowing for multiple measurements over time in the same animal, this could decrease inter-animal variation, diminish the number of test animals required in future studies and provide information on early nerve regeneration.

Therefore, in the second part of this thesis, we aimed to develop a non-invasive, ultrasound based, evaluation technique to measure muscle recovery after nerve reconstruction in both the small rat model **(chapter 4)** as well as the larger rabbit model **(chapter 5)**.

In the third part of this thesis, studies are presented that focus on the implementation and further improvement of the processed nerve allograft. To this end, we first implemented our previously optimized nerve allograft in a small gap in the rat model **(chapter 6)**. Success in the small rat model is an important first step, but does not guarantee success in larger animals, that are of paramount importance prior to human implementation. Therefore, in **chapter 7**, we subsequently implemented a similar study set up in a larger animal model, the rabbit. This model not only allows for larger nerve gaps to reconstruct, but also more closely mimics human nerve regeneration speed and immune response.

Results in rabbit were promising, but did show there was further room for improvement. We hypothesized the need for a source of supportive and stimulating growth factors. Allografts are devoid of cells that would provide this stimulus in autografts. We hypothesized that patient own adipose derived stem cells (MSCs) added to the nerve allograft could potentially produce these growth factors to further improve outcomes. First, we developed a simple technique to deliver stem cells to the nerve allograft **(chapter 8)**. In **chapter 9**, we aimed to clarify the potential role of the MSCs in combination with our nerve allograft. We evaluated the interaction between MSCs and our optimized nerve allograft to evaluate their behavior and growth factor production using qPCR

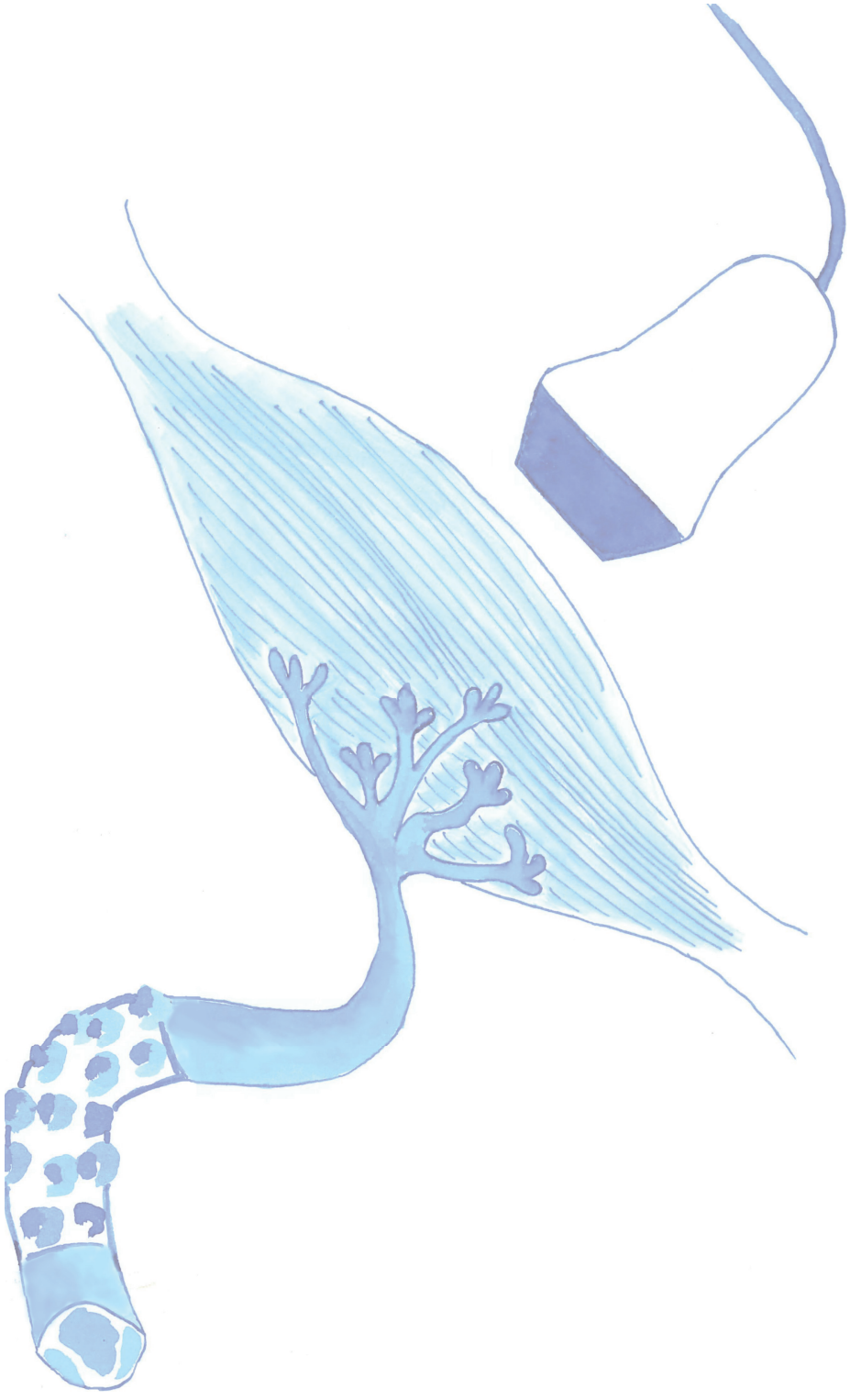
In **Chapter 10** we would like to address our points of discussion, the conclusions, future perspectives and recommendations that have arisen during the production of this thesis.

REFERENCES

1. Wojtkiewicz DM, Saunders J, Domeshek L, Novak CB, Kaskutas V, Mackinnon SE. Social impact of peripheral nerve injuries. Hand (N Y). 2015;10(2):161-167.
2. 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.
3. Noble J, Munro CA, Prasad VS, Midha R. Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. J Trauma. 1998;45(1):116-122.
4. Shin AY, Spinner RJ, Steinmann SP, Bishop AT. Adult traumatic brachial plexus injuries. J Am Acad Orthop Surg. 2005;13(6):382-396.
5. Novak CB, Anastakis DJ, Beaton DE, Mackinnon SE, Katz J. Biomedical and psychosocial factors associated with disability after peripheral nerve injury. J Bone Joint Surg Am. 2011;93(10):929-936.
6. Kattan AE, Borschel GH. Anatomy of the brachial plexus. J Pediatr Rehabil Med. 2011;4(2):107-111.
7. Wolfe SW, Hotchkiss RN, Pederson WC, Kozin SH, Cohen MS, Green DP. Green's Operative Hand Surgery E-Book. Vol Seventh edition. Philadelphia, PA: Elsevier; 2017.
8. Hijdra A, Koudstaal PJ, Roos RAC. Neurologie. Houten: Bohn Stafleu van Loghum; 2016.
9. Standring S. Gray's Anatomy E-Book : The Anatomical Basis of Clinical Practice. Vol Forty-first edition. New York: Elsevier; 2016.
10. Seddon HJ. THREE TYPES OF NERVE INJURY. Brain. 1943;66(4):237-288.
11. Sunderland S. A classification of peripheral nerve injuries producing loss of function. Brain. 1951;74(4):491-516.
12. Ray WZ, Mackinnon SE. Management of nerve gaps: autografts, allografts, nerve transfers, and end-to-side neurorrhaphy. Exp Neurol. 2010;223(1):77-85.
13. Kim DH, Murovic JA, Tiel RL, Kline DG. Mechanisms of injury in operative brachial plexus lesions. Neurosurg Focus. 2004;16(5):E2.
14. Coene LN. Mechanisms of brachial plexus lesions. Clin Neurol Neurosurg. 1993;95 Suppl:S24-29.
15. Stoll G, Muller HW. Nerve injury, axonal degeneration and neural regeneration: basic insights. Brain Pathol. 1999;9(2):313-325.
16. Jessen KR, Mirsky R. The repair Schwann cell and its function in regenerating nerves. J Physiol. 2016;594(13):3521-3531.
17. Gomez-Sanchez JA, Carty L, Iruarrizaga-Lejarreta M, et al. Schwann cell autophagy, myelinophagy, initiates myelin clearance from injured nerves. J Cell Biol. 2015;210(1):153-168.
18. Gaudet AD, Popovich PG, Ramer MS. Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. J Neuroinflammation. 2011;8:110.
19. Boyd KU, Nimigan AS, Mackinnon SE. Nerve reconstruction in the hand and upper extremity. Clin Plast Surg. 2011;38(4):643-660.
20. Campbell WW. Evaluation and management of peripheral nerve injury. Clin Neurophysiol. 2008;119(9):1951-1965.
21. Moore AM, Wagner IJ, Fox IK. Principles of nerve repair in complex wounds of the upper extremity. Semin Plast Surg. 2015;29(1):40-47.
22. Bassilios Habre S, Bond G, Jing XL, Kostopoulos E, Wallace RD, Konofaos P. The Surgical Management of Nerve Gaps: Present and Future. Ann Plast Surg. 2017.
23. Carlsen BT, Bishop AT, Shin AY. Late reconstruction for brachial plexus injury. Neurosurg Clin N Am. 2009;20(1):51-64, vi.
24. Giuffre JL, Bishop AT, Spinner RJ, Shin AY. The best of tendon and nerve transfers in the upper extremity. Plast Reconstr Surg. 2015;135(3):617e-630e.
25. Houdek MT, Shin AY. Management and complications of traumatic peripheral nerve injuries. Hand Clin. 2015;31(2):151-163.

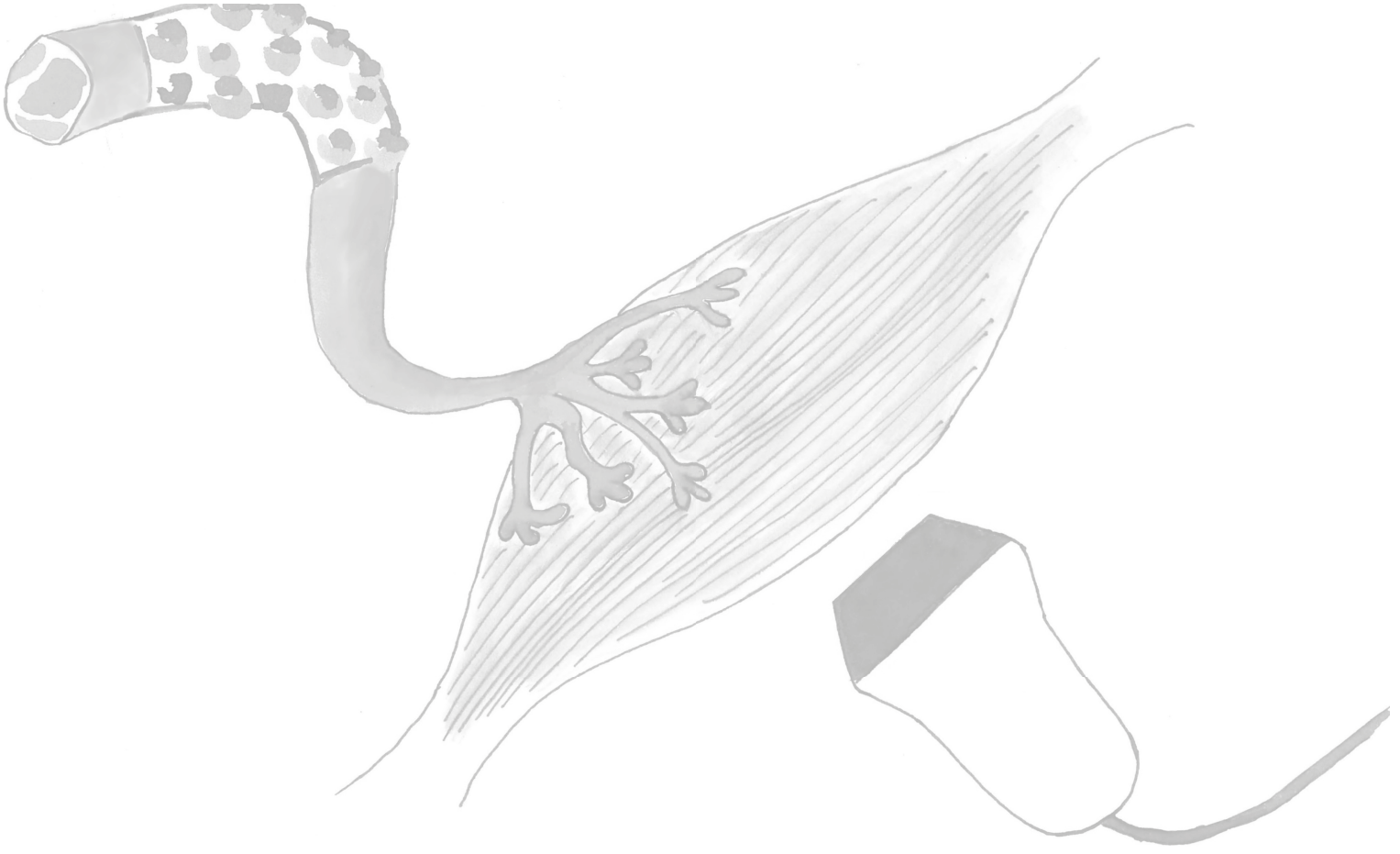
26. Merrell GA, Barrie KA, Katz DL, Wolfe SW. Results of nerve transfer techniques for restoration of shoulder and elbow function in the context of a meta-analysis of the English literature. J Hand Surg Am. 2001;26(2):303-314.
27. Tung TH, Mackinnon SE. Nerve transfers: indications, techniques, and outcomes. J Hand Surg Am. 2010;35(2):332-341.
28. Poppler LH, Davidge K, Lu JC, Armstrong J, Fox IK, Mackinnon SE. Alternatives to sural nerve grafts in the upper extremity. Hand (N Y). 2015;10(1):68-75.
29. Ehretsman RL, Novak CB, Mackinnon SE. Subjective recovery of nerve graft donor site. Ann Plast Surg. 1999;43(6):606-612.
30. Meek MF, Coert JH. Clinical use of nerve conduits in peripheral-nerve repair: review of the literature. J Reconstr Microsurg. 2002;18(2):97-109.
31. Moore AM, Kasukurthi R, Magill CK, Farhadi HF, Borschel GH, Mackinnon SE. Limitations of conduits in peripheral nerve repairs. Hand (N Y). 2009;4(2):180-186.
32. Meek MF, Coert JH. US Food and Drug Administration/Conformit Europe-approved absorbable nerve conduits for clinical repair of peripheral and cranial nerves. Ann Plast Surg. 2008;60(1):110-116.
33. Brenner MJ, Jensen JN, Lowe JB, 3rd, et al. Anti-CD40 ligand antibody permits regeneration through peripheral nerve allografts in a nonhuman primate model. Plast Reconstr Surg. 2004;114(7):1802-1814; discussion 1815-1807.
34. Mackinnon SE, Doolabh VB, Novak CB, Trulock EP. Clinical outcome following nerve allograft transplantation. Plast Reconstr Surg. 2001;107(6):1419-1429.
35. Reske A, Reske A, Metze M. Complications of immunosuppressive agents therapy in transplant patients. Minerva Anestesiol. 2015;81(11):1244-1261.
36. Szynkaruk M, Kemp SW, Wood MD, Gordon T, Borschel GH. Experimental and clinical evidence for use of decellularized nerve allografts in peripheral nerve gap reconstruction. Tissue Eng Part B Rev. 2013;19(1):83-96.
37. Brooks DN, Weber RV, Chao JD, et al. Processed nerve allografts for peripheral nerve reconstruction: a multicenter study of utilization and outcomes in sensory, mixed, and motor nerve reconstructions. Microsurgery. 2012;32(1):1-14.
38. Giusti G, Willems WF, Kremer T, Friedrich PF, Bishop AT, Shin AY. Return of motor function after segmental nerve loss in a rat model: comparison of autogenous nerve graft, collagen conduit, and processed allograft (AxoGen). J Bone Joint Surg Am. 2012;94(5):410-417.
39. Hundepool CA, Nijhuis TH, Kotsougiani D, Friedrich PF, Bishop AT, Shin AY. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. Neurosurg Focus. 2017;42(3):E4.
40. di Summa PG, Kingham PJ, Raffoul W, Wiberg M, Terenghi G, Kalbermatten DF. Adipose-derived stem cells enhance peripheral nerve regeneration. J Plast Reconstr Aesthet Surg. 2010;63(9):1544-1552.
41. Hundepool CA, Nijhuis TH, Mohseny B, Selles RW, Hovius SE. The effect of stem cells in bridging peripheral nerve defects: a meta-analysis. J Neurosurg. 2014;121(1):195-209.
42. Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G. Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Exp Neurol. 2007;207(2):267-274.
43. Kingham PJ, Kolar MK, Novikova LN, Novikov LN, Wiberg M. Stimulating the neurotrophic and angiogenic properties of human adipose-derived stem cells enhances nerve repair. Stem Cells Dev. 2014;23(7):741-754.
44. Nijhuis TH, Brzezicki G, Klimczak A, Siemionow M. Isogenic venous graft supported with bone marrow stromal cells as a natural conduit for bridging a 20 mm nerve gap. Microsurgery. 2010;30(8):639-645.
45. Orbay H, Uysal AC, Hyakusoku H, Mizuno H. Differentiated and undifferentiated adipose-derived stem cells improve function in rats with peripheral nerve gaps. J Plast Reconstr Aesthet Surg. 2012;65(5):657-664.

46. Tomita K, Madura T, Sakai Y, Yano K, Terenghi G, Hosokawa K. Glial differentiation of human adipose-derived stem cells: implications for cell-based transplantation therapy. Neuroscience. 2013;236:55-65.
47. Calvert GT, Doolabh VB, Grand AG, Hunter DA, Mackinnon SE. Rat-strain differences in recovery following peripheral-nerve allotransplantation. J Reconstr Microsurg. 2001;17(3):185-191.
48. Amillo S, Yanez R, Barrios RH. Nerve regeneration in different types of grafts: experimental study in rabbits. Microsurgery. 1995;16(9):621-630.
49. Deumens R, Bozkurt A, Meek MF, et al. Repairing injured peripheral nerves: Bridging the gap. Prog Neurobiol. 2010;92(3):245-276.
50. Gordon T, Borschel GH. The use of the rat as a model for studying peripheral nerve regeneration and sprouting after complete and partial nerve injuries. Exp Neurol. 2017;287(Pt 3):331-347.
51. Lee JY, Giusti G, Wang H, Friedrich PF, Bishop AT, Shin AY. Functional evaluation in the rat sciatic nerve defect model: a comparison of the sciatic functional index, ankle angles, and isometric tetanic force. Plast Reconstr Surg. 2013;132(5):1173-1180.
52. Mohseny B, Nijhuis TH, Hundepool CA, Janssen WG, Selles RW, Coert JH. Ultrasonographic quantification of intrinsic hand muscle cross-sectional area; reliability and validity for predicting muscle strength. Arch Phys Med Rehabil. 2015;96(5):845-853.
53. Nijhuis TH, de Boer SA, Wahegaonkar AL, et al. A new approach to assess the gastrocnemius muscle volume in rodents using ultrasound; comparison with the gastrocnemius muscle index. PLoS One. 2013;8(1):e54041.



PART I

Current clinical use of nerve grafts



2

Nerve transfers to restore elbow function

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ABSTRACT

The purpose of this article is to provide an overview of the various nerve transfer options for restoration of elbow function. This article describes nerve transfer strategies for elbow flexion and extension including the indications, limitations, and expected outcomes based on current literature.

INTRODUCTION

Elbow flexion is considered the most important function to restore in the paralyzed upper extremity as it provides positioning of the hand in a useful position for daily activities. Elbow extension is necessary to stabilize the elbow, to achieve a stabile grasp, and for any activity that requires the arm to be lifted above horizontal position. Moreover, in cases of insufficient (M0 to M2) recovery of elbow flexion with primary surgery, the reinnervated triceps could be transferred to provide this function.[1]

Causes of loss of elbow function include traumatic brachial plexus injury, spinal cord injuries, and injury to the nerves innervating the elbow flexors and/or extensors such as stab wounds, gunshot injuries, radiation-induced neuropathies, and brachial plexus birth palsy.[2,3] When direct nerve repair is not possible, and nerve grafting is not expected to provide satisfactory results, nerve transfers are a viable option. The role of nerve transfers has expanded drastically in the past 15 years, providing a commonly used treatment strategy for the restoration of elbow function, being used for direct neurotization of target muscles as well as neurotization of free-functioning muscle transfers (FFMTs).[4,5]

HISTORICAL PERSPECTIVE

Nerve transfers were described in the early 20th century by Harris[6] and Tuttle,[7] who in 1913 reported

coaptation of the anterior terminal branch of C4 to the split upper trunk. Unfortunately, results were not well recorded in these early cases.

A seminal advancement was the application of intercostal nerves for the neurotization of the musculocutaneous nerve described by Seddon[8] in 1961. The unsatisfactory results were secondary to the interposition nerve grafts, and subsequently Tsuyama and Hara[9] modified the technique such that direct intercostal to motor biceps branch could be performed. The use of intercostal nerves to innervate the musculocutaneous nerve was further popularized by Narakas,[10] and good results have been reported by multiple groups.[11–15] The challenge of restoration of elbow flexion worldwide resulted in many groups exploring the potential of different donor nerves, expanding the options that were already described by Lurje[16] in 1948, including the long thoracic nerve, medial and lateral pectoral nerve, radial nerve, and thoracodorsal and subscapular nerve.[17–19] More recent developments in nerve transfers for elbow function, the ulnar nerve fascicle transfer as described by Oberlin,[20] and transfer of fascicles of the median nerve as described by Mackinnon and colleagues[21] successfully moved the coaptation site more distal, further improving functional outcome. Over the past decade, many of these options have been modified and refined resulting in more reliable restoration of elbow function.

INDICATIONS AND CONTRAINDICATIONS

Nerve transfers are indicated in patients with no hope for spontaneous recovery or improvement of their nerve injury. Possible indications and contraindications are described in Table 1.[22,23]

It is important to note that strategies of reconstruction have evolved and changed and will continue to do so. Some authors prefer not to explore the brachial plexus, stating that the surgery is difficult, especially in a scarred bed, and proceed to nerve transfers, while others always explore the brachial plexus to evaluate for viable nerve roots that can be used with nerve grafts to critical targets in lieu of nerve transfers. Although this controversy will remain until better outcome studies are performed, it is the authors' philosophy to explore every acute (<6–7 months after injury) brachial plexus injury and evaluate the nerve roots. If a viable root exists, it will be used with nerve grafts to target shoulder girdle musculature. If a second viable root exists, it will be used with nerve grafts to target elbow flexion or extension.

Table 1. Indications and contraindications for nerve transfers

Indications	Contraindications
• Preganglionic injury (i.e. nerve root avulsions) • High peripheral nerve injuries • Multiple level nerve injuries • Delayed presentation (between 6-12 months) • Large neuromas in continuity • Innervation for free functioning muscle transfer	• Absolute contraindications ○ Less invasive options possible ○ Spontaneous recovery expected ○ Irreversible damage of atrophy of recipient muscles (>12 mo from injury) ○ Unmotivated patient for invasive procedure and intensive rehabilitation • Relative contraindications ○ Joint stiffness and contractures, patient age, comorbidities, traumatic brain injury or spinal cord injury

PRINCIPLES OF NERVE TRANSFERS

The principle of nerve transfers is the coaptation of one or more healthy (Medical Research Council
[MRC]>4) but expandable nerves or nerve fascicles to an injured more important nerve, distal to the site of injury (Box 1).

TIMING

The timing of the surgery should be carefully considered based on multiple factors including mechanism of injury, physical examination, and imaging results, as well as the surgeon's preference. Because of the degeneration of motor endplates that becomes mostly irreversible after approximately 12 to 18 months in adult patients, the observation period prior to surgery should be limited, and nerve transfers should be performed within 6 to 9 months after injury.[5,12,18,24–28] Some authors have expanded the time to surgery up to 12 months. Ideally, nerve surgery should be performed prior to 6 months when and if possible.

Box 1. General principles of nerve transfers
• Nerve transfers allow for conversion of a very proximal nerve injury to a more distal injury, decreased time to reinnervation, preventing motor end-plate degeneration. • A large number of "pure" axons can be delivered • Extensive dissection in a scarred wound bed can be avoided • Aim to avoid the use of nerve grafts and therefore an extra anastomosis, considering the better functional outcomes. • The use of donor nerves with a synergistic function as the recipient is preferred but not necessary.

DESCRIPTION OF NERVE TRANSFER OPTIONS

For elbow flexion, the goal is to innervate the musculocutaneous nerve (MCN), or specifically the biceps (and/or brachialis) branch.[29] For elbow extension, either the whole radial nerve or branches to the long head of the triceps (BLHT) can be targeted.[30] Intraplexal donors are preferred when available.[31] Different nerve transfers may be combined to yield best functional outcome. In selecting the most appropriate donor, the number of motor axons, the distance between donor and recipient
nerve and the size match with the recipient nerve should be considered. Different neurotization techniques for restoration of both elbow flexion and extension will be described in more detail.

Ulnar Fascicular Transfer (Oberlin)

Since its description by Oberlin and colleagues,[20] this technique has become widely accepted. A fascicle of the ulnar nerve to the Flexor Carpi Ulnaris (FCU) is divided and transferred to the biceps motor branch, as shown in Fig. 1.[20,32] This technique is only suitable for patients with preserved C8-T1 function and is most frequently used for C5-6 injury, but success for C5-7 injuries has also been reported.[33] Important advantages of this technique are the relative technical ease and quick reinnervation, as the nerve coaptation site is close to the motor endplates. The first recovery of biceps contraction usually begins several months after the procedure. Adequate grip strength (more than 10 kg) should be present prior to considering this technique.[32]

The Oberlin procedure is one of the most successful techniques to restore useful elbow flexion in adult upper trunk brachial plexus injury, achieving MRC grade 3 strength or better in the vast majority of patients with no evidence of donor site morbidity.[20,34–37] Although early reinnervation is preferred, Sedain and colleagues[38] performed this procedure in 9 patients with brachial plexus injury 7 to 24 months after injury and found useful biceps recovery (MRC3) in almost 80%. Flores[30] and Pet and colleagues[39] transferred a motor fascicle of the ulnar nerve to the BLHT, obtaining M4 or better elbow extension in 4 cases.

Double Fascicular Transfer

As described by Tung and colleagues[40] and Ray and colleagues,[41] in addition to neurotization of the biceps, the brachialis muscle can be neurotized to improve functional outcome. Local

donor nerve options are provided by median nerve fascicles to the flexor carpi radialis (FCR), flexor digitorum superficialis (FDS), or palmaris longus.[40] A schematic overview of this technique is shown in Fig. 1. For patients with weakness and sensory deficit in the median or ulnar nerve distribution regions preoperatively, other strategies should be considered.

This combined nerve transfer has shown an over 80% recovery of ≥M4 elbow flexion strength with minimal donor morbidity in the outcomes from Tung and Ray.[5,21,40–42] In contradistinction, Carlsen and colleagues[33] found that there was not a statistical improvement in elbow flexion strength using the double versus the single nerve transfer and recommended sparing the median nerve for another use. Martins and colleagues[43] prospectively compared outcomes and morbidity of the single and double fascicular nerve transfer. This study showed no significant differences between the 2 procedures regarding elbow flexion strength. Donor site morbidity including detoriation of grip strength and sensibility were observed in a small number of patients and resolved during followup. No differences were observed between the groups, supporting the findings of Carlsen and colleagues.[33]

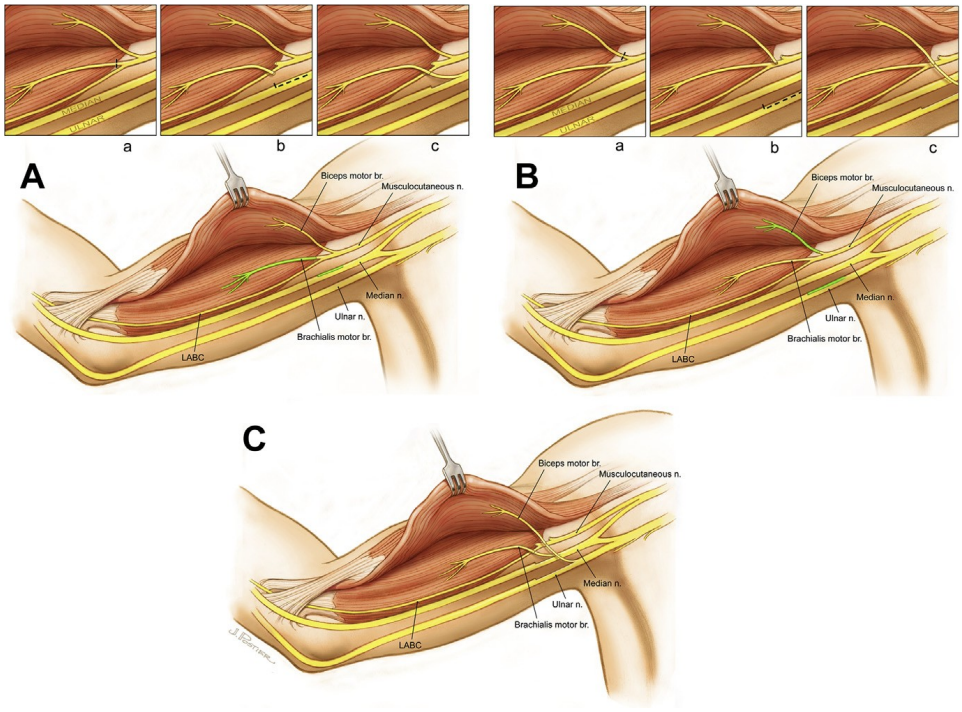


Figure 1. Schematic overview of the double fascicular nerve transfer. (A) The brachialis motor branch and suitable median nerve fascicle are identified (details in left upper panes). (B) The biceps motor branch and ulnar nerve fascicle to the FCU are identified (details in right upper panes). (C) The median nerve fascicle is coapted to the brachialis motor branch and the ulnar nerve fascicle to the biceps motor branch. (Courtesy of Mayo Foundation, Rochester, MN; with permission.)

Medial Pectoral Nerve

In patients with upper (C5-6) or upper extended (C5-7) brachial plexus palsy, collateral motor branches of the plexus are available as regional donor.[44,45] When using the medial pectoral nerve (MPN), selecting only one of its multiple branches can preserve innervation of the pectoralis sternal head.[46] The number of motor fibers in the MPN ranges from 1170 to 2140 in the main trunk and 400 to 600 fibers in a muscular branch.[45] An important disadvantage of this nerve is the large discrepancy in nerve diameter with the MCN and the limited length of most MPNs.[40]

Multiple series have been published regarding MPN to MCN transfer reporting variable but encouraging results with useful recovery (≥M3) in 60% to 100% of the cases. Of the studies reporting M4 recovery, the average M4 recovery was 71%.[40,44–50] The variability in outcome may be explained by the different solutions chosen by different groups to overcome the diameter mismatch, including combination with other nerve transfers (intercostals, spinal accessory).

As shown by Flores,[51] the MPN can also be used for triceps reinnervation in patients sustaining C5-7 brachial plexus palsies. Targeting either the radial nerve or the BLHT, M3 was found in 5 and M4 in 7 of 12 cases (58%).

Thoracodorsal Nerve

The thoracodorsal nerve (TDN) receives nerve fibers fromC7 (more than 52%), C8, and sometimes C6 nerve roots and innervates the latissimus dorsi muscle.[45,52] The mean surgically useable length of the nerve is 12.3 cm, with an average of 2409 myelinated fibers.[53,54] An important advantage of the TDN in comparison to the MPN is its sufficient length for direct coaptation to the biceps nerve.

Different studies found consistently good MRC grade 3 or higher biceps recovery in 85% to 100% of patients, with an average reported grade 4 or higher in 50% of patients.[45,48,50,55–59] Using the thoracodorsal nerve, Soldado and colleagues[60] found MRC 4 triceps recovery in 7 out of their 8 patients and MRC 3 in 1 patient. None of the studies found serious adverse effects of the (partial) denervation of the latissimus dorsi muscle.

Intercostal Nerves

If donor nerves from within the brachial plexus are not available, extraplexal nerves such as intercostal nerves (ICNs) can be transferred to reinnervate the biceps or triceps. Generally 3 to 4 ICNs provide a good size match for musculocutaneous nerve innervation or 2 to 3 ICNs for biceps motor branch or triceps reinnervation.[61–63] The third to sixth ICN often allows for direct coaptation to the MCN. The sensory and motor components, which contain 1300 axons, of the ICN can be separated for the delivery of more pure motor axons.[64] ICN to MCN transfer is shown in Fig. 2. The lateral antebrachial cutaneous nerve can be separated from the biceps branch of the MCN to allow a maximum number of motor axons to reach the target muscle. Pulmonary function can be temporarily impaired but normalizes by 3 months.[65] When chest wall trauma is present, ICN may be damaged, although this is rare.

ICNs are also used for the innervation of free functioning muscle transfers (FFMT) for late brachial (pan)plexus injuries (presenting 7–8months or later after injury). This procedure can be combined with innervation of the native biceps with ICN, as shown in Fig. 3.[66,67]

For biceps flexion, it takes at least 12 months for patients to learn to flex the joint voluntarily.[61] Coughing and sneezing may continue to cause involuntary muscle contractions.[68]

The influence of interposition nerve grafts for ICN transfers is controversial in the literature. Although nerve grafts allow for the use of more proximal parts of the ICN to avoid loss of motor axons, having an extra coaptation site could impair recovery.[62] A meta-analysis by Merrell and colleagues[5] describes restoration of elbow flexion MRC grade 3 strength or better in 72% of patients when a direct coaptation was performed compared to 47% of the patients with an interposition nerve graft. This significant difference advocates avoiding interposition grafts when possible. More recent studies show more variable results of elbow flexion after ICN to MCN transfer, ranging from 33% to 89% of patients reaching functional recovery.[50,69–72]

For elbow extension, an advantage is that the radial nerve is spared, which gives a chance of spontaneous triceps recovery by reinnervation from the C8 root. Results of triceps reinnervation with ICN are strongly variable.[30,73–76]

One of the main drawbacks of intercostal nerve transfer is the need to prevent abduction and external rotation of the shoulder greater than 90° and 90° respectively. Such motion can potentially disrupt the nerve coaptation. Thus in patients who have good potential for shoulder function recovery, an alternative nerve transfer should be considered. Intercostal nerve transfers are typically used in patients with panplexus avulsive injuries when no other options exist.

Spinal Accessory Nerve (Cranial Nerve XI)

When using the spinal accessory nerve for neurotization, a distal branch of this nerve is used to spare the innervation of the trapezius muscle. In brachial plexus injury, the spinal accessory nerve (with approximately 1700 axons) is frequently preferred for neurotization of the suprascapular nerve but is also successfully used to restore elbow flexion.[5,28,64,77] If used for elbow flexion, an intervening nerve graft is typically necessary as shown in Fig. 4. Similar to the ICN, the spinal accessory can also be used for the innervation of FFMT.[4]

The first signs of recovery of biceps function may be seen after 6 to 15 months.[50] A metaanalysis described a recovery rate of 77% ≥M3 and 29% ≥M4 for biceps strength.[5] Later series confirmed the effectiveness of the spinal accessory to MCN transfer, reporting a functional recovery rate of 65% and 66%.[50,69] Grafts less than 12 cm and operation within 6 months after injury show significantly better results.[78] When compared, ICN transfers show a better ability to achieve ≥M4 elbow strength than the spinal accessory nerve (41% vs 29%, P<.001).[5]

Reports on the use of the spinal accessory nerve for triceps reinnervation (with an interpositional graft) are not sufficient to draw any conclusions about the usefulness of this transfer for elbow extension.[30] Contralateral C7 The use of the contralateral C7 (cC7) was first described by Gu and colleagues64 in 1991, providing a new extraplexal donor. Either the complete cC7 or partial C7 can be used, providing a sufficient amount of nerve fibers (approx. 24,000 for the whole C7).[9,64,79]

During the past 15 years, several groups implemented cC7 transfer for reconstruction of elbow function. Functional (≥M3) biceps recovery varies strongly among groups, ranging from 33% to 85%.[9,79–82] Functional triceps recovery is achieved in only 20% to 66% of patients.[79,81,82] Immediately after surgery, temporary motor and sensory deficits always occur, usually recovering within 6 months due to cross-innervation by other spinal nerves. An important disadvantage is the need for extremely long nerve grafts despite alternative proposed techniques.[83,84] Regarding functional outcome, the most important issue is that independent function after this transfer is often not achieved. This means that patients need to activate muscles previously innervated by the C7 in their normal arm to bend their injured arm, which significantly impairs the function.[19] Altogether, the outcome of the (hemi-)cC7 does not seem to justify the risk of donor site morbidity of this procedure, and the authors have abandoned the procedure in adult patients.

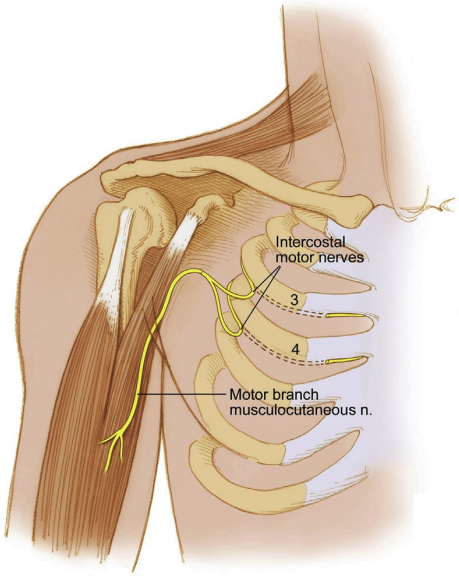


Figure 2. Transfer of the third and fourth Intercostal motor nerves to the musculocutaneous nerve for restoration of elbow flexion. (Courtesy of Mayo Foundation, Rochester, MN; with permission.)

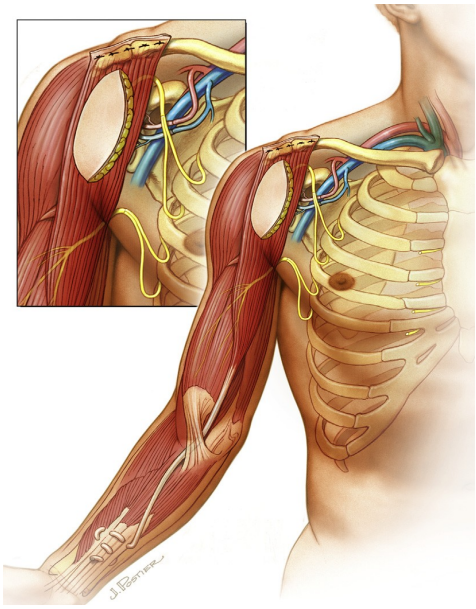


Figure 3. Combined neurotization of both the native biceps muscle and free functioning muscle transfers (FFMT) with intercostal nerves. A skin paddle is used for monitoring of the FFMT. (Courtesy of Mayo Foundation, Rochester, MN; with permission.)

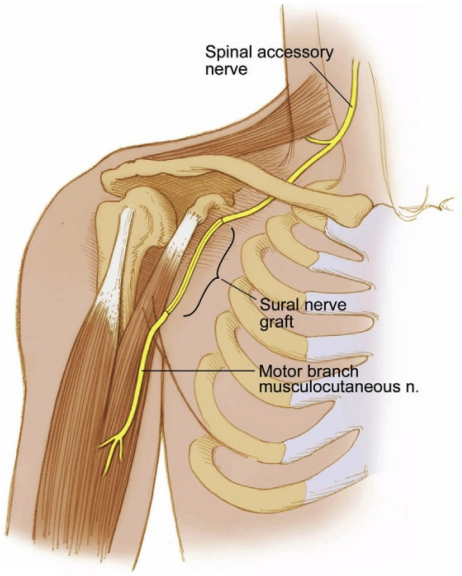


Figure 4. Neurotization of the spinal accessory nerve to the musculocutaneous nerve with an interpositional sural nerve graft. (Courtesy of Mayo Foundation, Rochester, MN; with permission.)

Phrenic Nerve

The phrenic nerve (approximately 800 myelinated axons) is often functioning in cases of complete brachial plexus avulsion injuries as a result of its major contributions from C3 and C4, providing an extraplexal nerve donor.[66,85,86] When the phrenic nerve is harvested in a supraclavicular fashion, it always needs an intervening nerve graft to reach the MCN; to reach the anterior division of the upper trunk (ADUT) nerve grafts are typically not required, yielding similar results.[87] An alternative harvesting route using video-assisted thoracic surgery (VATS) has been proposed by Xu and colleagues,[88] providing direct coaptation to the MCN and significantly faster recovery compared with the traditional technique.

Biceps contractions can be expected 8 to 9 months after surgery, initiated by a deep breath.[89] Functional biceps recovery is by most groups achieved in 70% to 85% of the patients.[65,75,87–93] Phrenic and intercostal nerve transfers provide similar results in global brachial plexus injury.[94] A drawback of the phrenic nerve transfer is that long-term effects on pulmonary function remain unknown.[65,75,93] Flores[30] used the phrenic nerve for triceps recovery. Out of 7 patients, 5 showed meaningful recovery. It seems that coaptation to the BLHT may be more successful than coaptation to the whole radial nerve, but larger numbers need to be studied.

Hypoglossal Nerve

Two studies report a case of biceps neurotization using part of the hypoglossal nerve. Although both Bertelli and colleagues[95] and Malessy and colleagues[96] report good functional recovery of M4 and M5 respectively, significant adverse effects have been reported. Even after 5 years follow-up, tongue movement still provoked biceps contraction, and patients did not become able to regain volitional control over the target muscle. [95,96] Therefore, the hypoglossal nerve does not seem to provide a suitable nerve for reconstruction of elbow function.

Posterior Branch of the Axillary Nerve

For restoration of elbow extension, a nerve transfer to the triceps using the posterior branch of the axillary nerve was recently described.[97] Fig. 5 describes the technique that was used.

The authors report improvement of elbow extension from M0 to M4 in an 18-year-old woman with C7-T1 injury who was operated 6 months after injury. Triceps contraction was first noted 6 months after surgery, recovering up to M4 by 12 months. Good deltoid function is imperative when considering this technique.

Although the number of cases is still limited, this so called “reversed Leechavengvong procedure” seems a promising technique to restore triceps function without significant donor site morbidity in lower brachial plexus injuries.

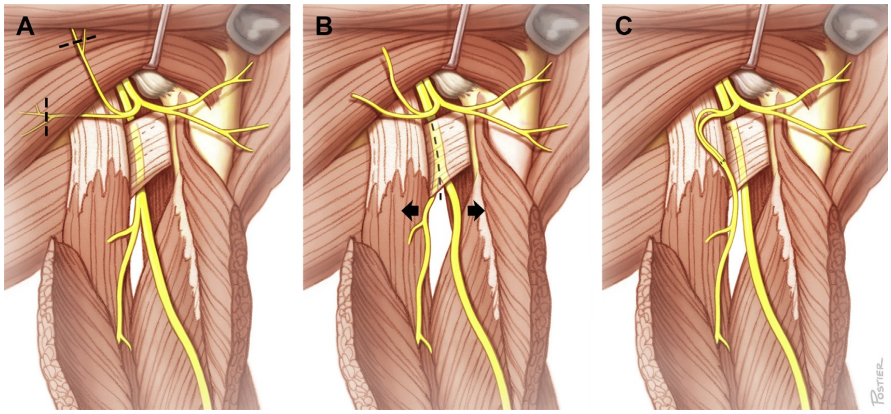


Figure 5. Transfer of the posterior branch of the axillary nerve branches to the triceps branch. (A) The posterior axillary nerve branches to the teres minor and posterior deltoid are identified and separated. (B) The branch to the triceps is separated from the posterior cord to obtain additional length (black arrows). (C) Both posterior axillary nerve branches are coapted to the triceps branch. (Courtesy of Mayo Foundation, Rochester, MN; with permission.)

SUMMARY

Depending on the extent of the injury, different nerve transfer options are available for reconstruction of elbow function in the flail arm. If hand function is preserved, the double fascicular has proven good and consistent results. In total brachial plexus injuries, where only extraplexal donors are available, ICNs are most widely used for both direct target muscle neurotization and innervation of a free-functioning muscle transfer. Although outcomes using the contralateral C7 nerve root seem promising in some hands, independent function is often not achieved.

REFERENCES

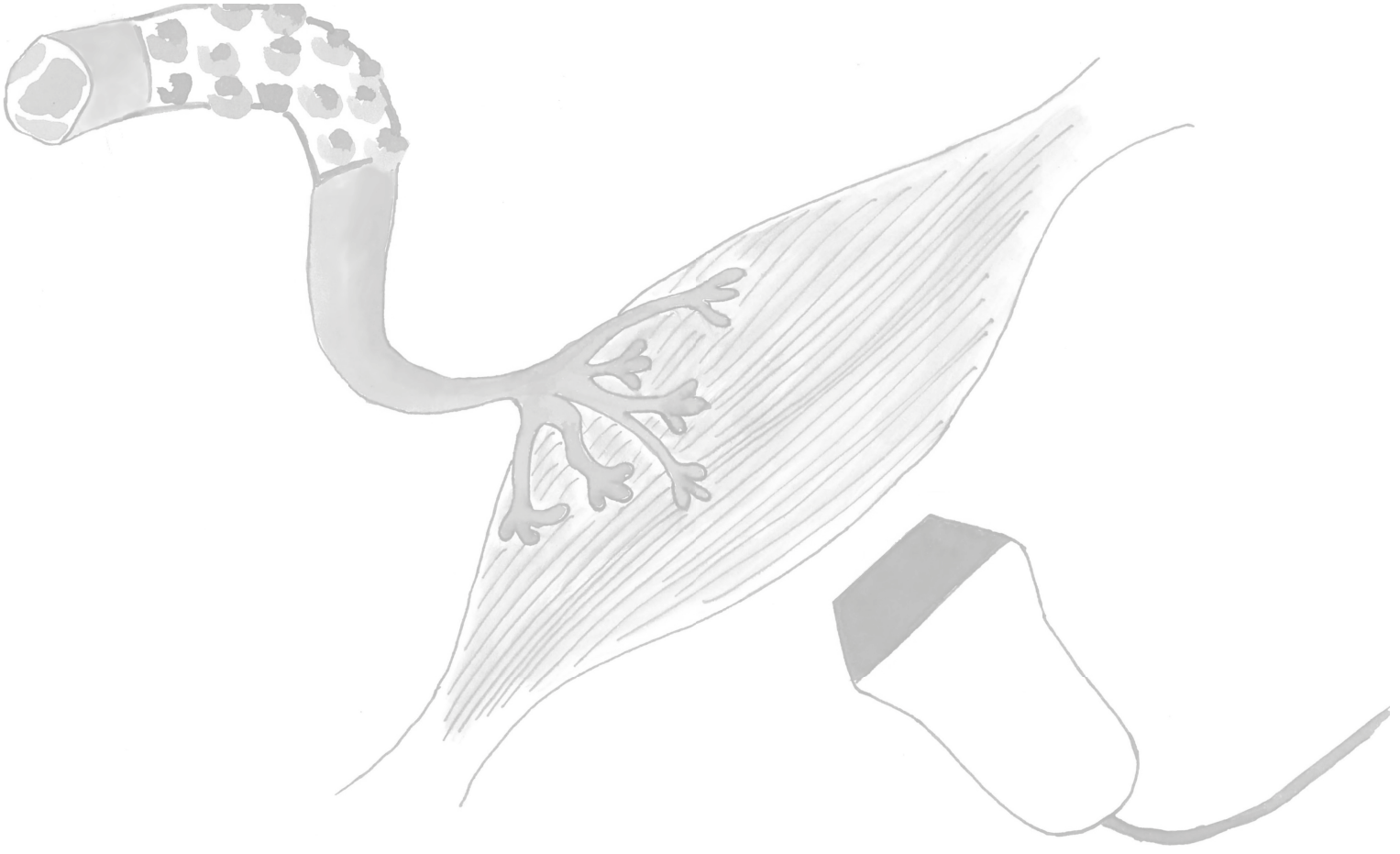
1. Haninec P, Szeder V. Reconstruction of elbow flexion by transposition of pedicled long head of triceps brachii muscle. Acta Chir Plast 1999;41(3):82–6.
2. Ruhmann O, Schmolke S, Gosse F, et al. Transposition of local muscles to restore elbow flexion in brachial plexus palsy. Injury 2002;33(7):597–609.
3. Tung TH, Liu DZ, Mackinnon SE. Nerve transfer for elbow flexion in radiation-induced brachial plexopathy: a case report. Hand 2009;4(2):123–8.
4. Chim H, Kircher MF, Spinner RJ, et al. Free functioning gracilis transfer for traumatic brachial plexus injuries in children. J Hand Surg 2014; 39(10):1959–66.
5. Merrell GA, Barrie KA, Katz DL, et al. Results of nerve transfer techniques for restoration of shoulder and elbow function in the context of a meta-analysis of the English literature. J Hand Surg Am 2001;26(2): 303–14.
6. Harris W, Low VW. On the Importance of accurate muscular analysis in lesions of the brachial plexus; and the treatment of erb's palsy and infantile paralysis of the upper extremity by cross-union of the nerve roots. Br Med J 1903;2(2234):1035–8.
7. Tuttle HK. Exposure of the brachial plexus with nerve-transplantation. JAMA 1913;61(1):15–7
8. Seddon HJ. Nerve grafting. J Bone Joint Surg Br 1963;45:447–61.
9. Tsuyama N, Hara T. Intercostal nerve transfer in the treatment of brachial plexus injury of root avulsion type. Exerpta Medica 1972;29(1):35 I.
10. Narakas A. Surgical treatment of traction injuries of the brachial plexus. Clin Orthop Relat Res 1978;(133):71–90.
11. Nagano A, Ochiai N, Okinaga S. Restoration of elbow flexion in root lesions of brachial plexus injuries. J Hand Surg Am 1992;17(5):815–21.
12. Krakauer JD, Wood MB. Intercostal nerve transfer for brachial plexopathy. J Hand Surg Am 994; 19(5):829–35.
13. Nagano A, Yamamoto S, Mikami Y. Intercostal nerve transfer to restore upper extremity functions after brachial plexus injury. Ann Acad Med Singapore 1995;24(4 Suppl):42–5.
14. Ogino T, Naito T. Intercostal nerve crossing to restore elbow flexion and sensibility of the hand for a root avulsion type of brachial plexus injury. Microsurgery 1995;16(8):571–7.
15. Kotani PT, Matsuda H, Suzuki T. Trial surgical procedures of nerve transfers to avulsion injuries of plexus brachialis. Exerpta Medica 1972;29(1):348.
16. Lurje A. Concerning surgical treatment of traumatic injury to the upper division of the brachial plexus (Erb's type). Ann Surg 1948;127(2):317–26.
17. Allieu Y, Privat JM, Bonnel F. Paralysis in root avulsion of the brachial plexus. Neurotization by the spinal accessory nerve. Clin Plast Surg 1984;11(1): 133–6.
18. Allieu Y, Cenac P. Neurotization via the spinal accessory nerve in complete paralysis due to multiple avulsion injuries of the brachial plexus. Clin Orthop Relat Res 1988;(237):67–74.
19. Sammer DM, Kircher MF, Bishop AT, et al. Hemicontralateral C7 transfer in traumatic brachial plexus injuries: outcomes and complications. J Bone Joint Surg Am 2012;94(2):131–7.
20. Oberlin C, Beal D, Leechavengvongs S, et al. Nerve transfer to biceps muscle using a part of ulnar nerve for C5-C6 avulsion of the brachial plexus: anatomical study and report of four cases. J Hand Surg Am 1994;19(2):232–7.
21. Mackinnon SE, Novak CB, Myckatyn TM, et al. Results of reinnervation of the biceps and brachialis muscles with a double fascicular transfer for elbow flexion. J Hand Surg Am 2005;30(5):978–85.
22. Tung TH, Mackinnon SE. Nerve transfers: indications, techniques, and outcomes. J Hand Surg Am 2010;35(2):332–41.
23. Boyd KU, Nimigan AS, Mackinnon SE. Nerve reconstruction in the hand and upper extremity. Clin Plast Surg 2011;38(4):643–60.

24. Gorio A, Carmignoto G. Reformation, maturation and stabilization of neuromuscular junctions in peripheral nerve regeneration. In: Gorio A, Millesi H, Mingrino S, editors. Posttraumatic peripheral nerve regeneration. New York: Raven Press; 1981. p. 481–92.
25. Bishop AT. Functioning free-muscle transfer for brachial plexus injury. Hand Clin 2005;21(1):91–102.
26. Narakas AO, Hentz VR. Neurotization in brachial plexus injuries. Indication and results. Clin Orthop Relat Res 1988;(237):43–56.
27. Ruch DS, Friedman A, Nunley JA. The restoration of elbow flexion with intercostal nerve transfers. Clin Orthop Relat Res 1995;(314):95–103.
28. Songcharoen P, Mahaisavariya B, Chotigavanich C. Spinal accessory neurotization for restoration of elbow flexion in avulsion injuries of the brachial plexus. J Hand Surg Am 1996;21(3):387–90.
29. Terzis JK, Kostopoulos VK. The surgical treatment of brachial plexus injuries in adults. Plast Reconstr Surg 2007;119(4):73e–92e.
30. Flores LP. Triceps brachii reinnervation in primary reconstruction of the adult brachial plexus: experience in 25 cases. Acta Neurochir (Wien) 2011; 153(10):1999–2007.
31. Samardzic M, Grujicic D, Antunovic V. Nerve transfer in brachial plexus traction injuries. J Neurosurg 1992;76(2):191–7.
32. Oberlin C, Ameur NE, Teboul F, et al. Restoration of elbow flexion in brachial plexus injury by transfer of ulnar nerve fascicles to the nerve to the biceps muscle. Tech Hand Up Extrem Surg 2002;6(2):86–90.
33. Carlsen BT, Kircher MF, Spinner RJ, et al. Comparison of single versus double nerve transfers for elbow flexion after brachial plexus injury. Plast Reconstr Surg 2011;127(1):269–76.
34. Leechavengvongs S, Witoonchart K, Uerpairojkit C, et al. Combined nerve transfers for C5 and C6 brachial plexus avulsion injury. J Hand Surg Am 2006;31(2):183–9.
35. Leechavengvongs S, Witoonchart K, Uerpairojkit C, et al. Nerve transfer to biceps muscle using a part of the ulnar nerve in brachial plexus injury (upper arm type): a report of 32 cases. J Hand Surg Am 1998;23(4):711–6.
36. Teboul F, Kakkar R, Ameur N, et al. Transfer of fascicles from the ulnar nerve to the nerve to the biceps in the treatment of upper brachial plexus palsy. J Bone Joint Surg Am 2004;86–A(7):1485–90.
37. Ali ZS, Heuer GG, Faught RW, et al. Upper brachial plexus injury in adults: comparative effectiveness of different repair techniques. J Neurosurg 2015; 122(1):195–201.
38. Sedain G, Sharma MS, Sharma BS, et al. Outcome after delayed oberlin transfer in brachial plexus injury. Neurosurgery 2011;69(4):822–7.
39. Pet MA, Ray WZ, Yee A, et al. Nerve transfer to the triceps after brachial plexus injury: report of four cases. J Hand Surg Am 2011;36(3):398–405.
40. Tung TH, Novak CB, Mackinnon SE. Nerve transfers to the biceps and brachialis branches to improve elbow flexion strength after brachial plexus injuries. J Neurosurg 2003;98(2):313–8.
41. Ray WZ, Pet MA, Yee A, et al. Double fascicular nerve transfer to the biceps and brachialis muscles after brachial plexus injury: clinical outcomes in a series of 29 cases. J Neurosurg 2011;114(6):1520–8.
42. Liverneaux PA, Diaz LC, Beaulieu JY, et al. Preliminary results of double nerve transfer to restore elbow flexion in upper type brachial plexus palsies. Plast Reconstr Surg 2006;117(3):915–9.
43. Martins RS, Siqueira MG, Heise CO, et al. A prospective study comparing single and double fascicular transfer to restore elbow flexion after brachial plexus injury. Neurosurgery 2013;72(5): 709–14 [discussion: 714–5; quiz: 715].
44. Samardzic M, Grujicic D, Rasulic L, et al. Transfer of the medial pectoral nerve: myth or reality? Neurosurgery 2002;50(6):1277–82.
45. Samardzic M, Rasulic LG, Grujicic DM, et al. Nerve transfers using collateral branches of the brachial plexus as donors in patients with upper palsy–thirty years' experience. Acta Neurochir (Wien) 2011; 153(10):2009–19 [discussion: 2019].
46. Chuang D. Neurotization procedures for brachial plexus injuries. Hand Clin 1995;11(4):633–45.

47. Hems T. Nerve transfers for traumatic brachial plexus injury: advantages and problems. J Hand Microsurg 2011;3(1):6–10.
48. Haninec P, Samal F, Tomas R, et al. Direct repair (nerve grafting), neurotization, and end-to-side neurorrhaphy in the treatment of brachial plexus injury. J Neurosurg 2007;106(3):391–9.
49. Sulaiman OA, Kim DD, Burkett C, et al. Nerve transfer surgery for adult brachial plexus injury: a 10-year experience at Louisiana State University. Neurosurgery 2009;65(4 Suppl):A55–62.
50. Samardzic M, Rasulic L, Grujicic D, et al. Results of nerve transfers to the musculocutaneous and axillary nerves. Neurosurgery 2000;46(1):93–101 [discussion: 101–3].
51. Flores LP. Reanimation of elbow extension withmedial pectoral nerve transfer in partial injuries to the brachial plexus. J Neurosurg 2013;118(3):588–93.
52. Lu W, Xu JG, Wang DP, et al. Microanatomical study on the functional origin and direction of the thoracodorsal nerve from the trunks of brachial plexus. Clin Anat 2008;21(6):509–13.
53. Shin AY, Spinner RJ, Steinmann SP, et al. Adult traumatic brachial plexus injuries. J Am Acad Orthop Surg 2005;13(6):382–96.
54. Schreiber JJ, Byun DJ, Khair MM, et al. Optimal axon counts for brachial plexus nerve transfers to restore elbow flexion. Plast Reconstr Surg 2015; 135(1):135e–41e.
55. Soldado F, Ghizoni MF, Bertelli J. Thoracodorsal nerve transfer for elbow flexion reconstruction in infraclavicular brachial plexus injuries. J Hand Surg Am 2014;39(9):1766–70.
56. Samardzic M, Rasulic L, Lakicevic N, et al. Collateral branches of the brachial plexus as donors in nerve transfers. Vojnosanit Pregl 2012;69(7):594–603.
57. Samardzic MM, Grujicic DM, Rasulic LG, et al. The use of thoracodorsal nerve transfer in restoration of irreparable C5 and C6 spinal nerve lesions. Br J Plast Surg 2005;58(4):541–6.
58. Novak CB, Mackinnon SE, Tung TH. Patient outcome following a thoracodorsal to musculocutaneous nerve transfer for reconstruction of elbow flexion. Br J Plast Surg 2002;55(5):416–9.
59. Richardson PM. Recovery of biceps function after delayed repair for brachial plexus injury. J Trauma 1997;42(5):791–2.
60. Soldado F, GhizoniMF, Bertelli J. Thoracodorsal nerve transfer for triceps reinnervation in partial brachial plexus injuries. Microsurgery 2015 [Epub ahead of print].
61. Chuang DC, Yeh MC, Wei FC. Intercostal nerve transfer of the musculocutaneous nerve in avulsed brachial plexus injuries: evaluation of 66 patients. J Hand Surg Am 1992;17(5):822–8.
62. Malessy MJ, Thomeer RT. Evaluation of intercostal to musculocutaneous nerve transfer in reconstructive brachial plexus surgery. J Neurosurg 1998;88(2): 266–71.
63. Xiao C, Lao J, Wang T, et al. Intercostal nerve transfer to neurotize the musculocutaneous nerve after traumatic brachial plexus avulsion: a comparison of two, three, and four nerve transfers. J Reconstr Microsurg 2014;30(5):297–304.
64. Gu YD, Zhang GM, Chen DS, et al. Cervical nerve root transfer from contralateral normal side for treatment of brachial plexus root avulsions. Chin Med J (Engl) 1991;104(3):208–11.
65. Chalidapong P, Sananpanich K, Kraisarin J, et al. Pulmonary and biceps function after intercostal and phrenic nerve transfer for brachial plexus injuries. J Hand Surg Br 2004;29(1):8–11.
66. Brandt KE, Mackinnon SE. A technique for maximizing biceps recovery in brachial plexus reconstruction. J Hand Surg Am 1993;18(4):726–33.
67. Carlsen BT, Bishop AT, Shin AY. Late reconstruction for brachial plexus injury. Neurosurg Clin N Am 2009;20(1):51–64, vi.
68. Nagano A, Tsuyama N, Ochiai N, et al. Direct nerve crossing with the intercostal nerve to treat avulsion injuries of the brachial plexus. J Hand Surg Am 1989;14(6):980–5.
69. Bhatia A, Shyam AK, Doshi P, et al. Nerve reconstruction: a cohort study of 93 cases of global brachial plexus palsy. Indian J Orthop 2011;45(2):153–60.
70. Moiyadi AV, Devi BI, Nair KP. Brachial plexus injuries: outcome following neurotization with intercostal nerve. J Neurosurg 2007;107(2):308–13.

71. Terzis JK, Barbitsioti A. Primary restoration of elbow flexion in adult post-traumatic plexopathy patients. J Plast Reconstr Aesthet Surg 2012;65(1):72–84.
72. El-Gammal TA, Fathi NA. Outcomes of surgical treatment of brachial plexus injuries using nerve grafting and nerve transfers. J Reconstr Microsurg 2002; 18(1):7–15.
73. Goubier JN, Teboul F, Khalifa H. Reanimation of elbow extension with intercostal nerves transfers in total brachial plexus palsies. Microsurgery 2011; 31(1):7–11.
74. Gao K, Lao J, Zhao X, et al. Outcome after transfer of intercostal nerves to the nerve of triceps long head in 25 adult patients with total brachial plexus root avulsion injury. J Neurosurg 2013; 118(3):606–10.
75. Zheng MX, Xu WD, Qiu YQ, et al. Phrenic nerve transfer for elbow flexion and intercostal nerve transfer for elbow extension. J Hand Surg Am 2010;35(8): 1304–9.
76. Terzis JK, Barmpitsioti A. Our experience with triceps nerve reconstruction in patients with brachial plexus injury. J Plast Reconstr Aesthet Surg 2012; 65(5):590–600.
77. Giuffre JL, Kakar S, Bishop AT, et al. Current concepts of the treatment of adult brachial plexus injuries. J Hand Surg 2010;35(4):678–88.
78. Samii A, Carvalho GA, Samii M. Brachial plexus injury: factors affecting functional outcome in spinal accessory nerve transfer for the restoration of elbow flexion. J Neurosurg 2003;98(2):307–12.
79. Gao K, Lao J, Zhao X, et al. Outcome of contralateral C7 transfer to two recipient nerves in 22 patients with the total brachial plexus avulsion injury. Microsurgery 2013;33(8):605–11.
80. Chuang DC, Hernon C. Minimum 4-year follow-up on contralateral C7 nerve transfers for brachial plexus injuries. J Hand Surg Am 2012;37(2):270–6.
81. Terzis JK, Kokkalis ZT. Selective contralateral C7 transfer in posttraumatic brachial plexus injuries: a report of 56 cases. Plast Reconstr Surg 2009; 123(3):927–38.
82. Gu Y, Xu J, Chen L, et al. Long term outcome of contralateral C7 transfer: a report of 32 cases. Chin Med J (Engl) 2002;115(6):866–8.
83. Xu L, Gu Y, Xu J, et al. Contralateral C7 transfer via the prespinal and retropharyngeal route to repair brachial plexus root avulsion: a preliminary report. Neurosurgery 2008;63(3):553–8 [discussion: 558–9].
84. Wang S, Yiu HW, Li P, et al. Contralateral C7 nerve root transfer to neurotize the upper trunk via a modified prespinal route in repair of brachial plexus avulsion injury. Microsurgery 2012;32(3):183–8.
85. Gu YD, Wu MM, Zhen YL, et al. Phrenic nerve transfer for treatment of root avulsion of the brachial plexus. Chin Med J (Engl) 1990;103(4):267–70.
86. Gu YD, Wu MM, Zhen YL, et al. Phrenic nerve transfer for brachial plexus motor neurotization. Microsurgery 1989;10(4):287–9.
87. Liu Y, Lao J, Gao K, et al. Comparative study of phrenic nerve transfers with and without nerve graft for elbow flexion after global brachial plexus injury. Injury 2014;45(1):227–31.
88. Xu WD, Gu YD, Xu JG, et al. Full-length phrenic nerve transfer by means of video-assisted thoracic surgery in treating brachial plexus avulsion injury. Plast Reconstr Surg 2002;110(1):104–9 [discussion: 110–1].
89. Dong Z, Zhang CG, Gu YD. Surgical outcome of phrenic nerve transfer to the anterior division of the upper trunk in treating brachial plexus avulsion. J Neurosurg 2010;112(2):383–5.
90. Bertelli JA, Ghizoni MF. Contralateral motor rootlets and ipsilateral nerve transfers in brachial plexus reconstruction. J Neurosurg 2004;101(5):770–8.
91. Gu YD, Ma MK. Use of the phrenic nerve for brachial plexus reconstruction. Clin Orthop Relat Res 1996;(323):119–21.
92. Monreal R. Restoration of elbow flexion by transfer of the phrenic nerve to musculocutaneous nerve after brachial plexus injuries. Hand 2007;2(4):206–11.
93. Siqueira MG, Martins RS. Phrenic nerve transfer in the restoration of elbow flexion in brachial plexus avulsion injuries: how effective and safe is it? Neurosurgery 2009;65(4 Suppl):A125–31.
94. Liu Y, Lao J, Zhao X. Comparative study of phrenic and intercostal nerve transfers for elbow flexion after global brachial plexus injury. Injury 2015;46(4):671–5.

95. Bertelli JA. Platysma motor branch transfer in brachial plexus repair: report of the first case. J Brachial Plex Peripher Nerve Inj 2007;2:12.
96. Malessy MJ, Hoffmann CF, Thomeer RT. Initial report on the limited value of hypoglossal nerve transfer to treat brachial plexus root avulsions. Journal of Neurosurgery 1999;91(4):601–4.
97. Klika BJ, Spinner RJ, Bishop AT, et al. Posterior branch of the axillary nerve transfer to the lateral triceps branch for restoration of elbow extension: case report. J Hand Surg Am 2013;38(6):1145–9.



3

Spinal accessory nerve to triceps muscle transfer using long autologous nerve grafts for recovery of elbow extension in traumatic brachial plexus injuries

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ABSTRACT

Objective

Reconstructive options for brachial plexus lesions continue to expand and improve. The purpose of this study was to evaluate the prevalence and quality of restored elbow extension in patients with brachial plexus injuries who underwent transfer of the spinal accessory nerve to the motor branch of the radial nerve to the long head of the triceps muscle with an intervening autologous nerve graft and to identify patient and injury factors that influence functional triceps outcome.

Methods

A total of 42 patients were included in this retrospective review. All patients underwent transfer of the spinal accessory nerve to the motor branch of the radial nerve to the long head of the triceps muscle as part of their reconstruction plan after brachial plexus injury. The primary outcome was elbow extension strength according to the modified Medical Research Council muscle grading scale, and signs of triceps muscle recovery were recorded using electromyography.

Results

When evaluating the entire study population (follow-up range 12–45 months, mean 24.3 months), 52.4% of patients achieved meaningful recovery. More specifically, 45.2% reached Grade 0 or 1 recovery, 19.1% obtained Grade 2, and 35.7% improved to Grade 3 or better. The presence of a vascular injury impaired functional outcome. In the subgroup with a minimum follow-up of 20 months (n = 26), meaningful recovery was obtained by 69.5%. In this subgroup, 7.7% had no recovery (Grade 0), 19.2% had recovery to Grade 1, and 23.1% had recovery to Grade 2. Grade 3 or better was reached by 50% of patients, of whom 34.5% obtained Grade 4 elbow extension.

Conclusions

Transfer of the spinal accessory nerve to the radial nerve branch to the long head of the triceps muscle with an interposition nerve graft is an adequate option for restoration of elbow extension, despite the relatively long time required for reinnervation. The presence of vascular injury impairs functional recovery of the triceps muscle, and the use of shorter nerve grafts is recommended when and if possible.

INTRODUCTION

Traumatic brachial plexus injuries are devastating injuries, resulting in severely impaired or complete flail upper extremities. Elbow flexion is traditionally considered to be the most important function to restore and is always prioritized, followed by restoration of shoulder function.[23] Over the past few decades, reconstruction options have expanded, and reconstruction of other upper-limb functions, such as grasp and elbow extension, are becoming more important. Elbow extension is critical for stabilization of the elbow without having to use the healthy opposite arm in order to achieve a stable grasp and for any activity that requires the arm to be lifted above the horizontal position.[4,7,8,13] Double and single free-functioning muscle transfers (FFMTs) have taken on an important role in the reconstruction of prehensile function in complete brachial plexus injury.[1,7] When restoration of prehensile function is attempted with FFMTs, elbow extension is imperative to act as an agonist to the free functional muscle transfer, which crosses the anterior elbow joint.[8] Previous studies have shown that patients who undergo a double FFMT for combined elbow flexion and finger flexion and extension need at least British Medical Research Council (MRC)[19] Grade 2 elbow extension power to sufficiently stabilize their elbow while attempting finger movement.[5,8] Moreover, in cases of insufficient (Grade 0–2) recovery of elbow flexion with primary surgery, a sufficiently reinnervated triceps muscle (Grade 4 or better) could be transferred to the biceps tendon to provide elbow flexion function.[15]

Successful use of the spinal accessory nerve transfer has been described for restoration of elbow flexion and shoulder function.[2,9,20,24] However, existing reports on the use of the spinal accessory nerve for triceps reinnervation (with an interpositional graft) are not sufficient to draw any conclusions about the usefulness of this transfer for elbow extension. The purpose of this study was to evaluate prevalence and quality of restored elbow extension in patients with traumatic brachial plexus injury who underwent transfer of the spinal accessory nerve to the radial nerve branch to the long head of the triceps muscle and to identify patient and injury-related factors that might influence functional outcome.

METHODS

Patient Population

After approval from the institutional review board was granted, all patients who underwent a spinal accessory nerve transfer for triceps reinnervation after traumatic brachial plexus injury between 2001 and 2015 were identified. Patients without signs of spontaneous recovery who were at least 15 years old and with a minimum of 12 months of follow-up after surgery composed the cohort of the study.

Demographic data (including age, sex, body mass index [BMI], comorbidities, and smoking status) and injury-related factors (including mechanism of injury, extent of injury, dominant side affected, interval between injury and surgery, type and length of the used nerve graft,

concomitant injuries, and concomitant surgeries) were retrieved from the medical charts. CT myelography, intraoperative electrical stimulation (somatosensory and motor evoked potentials) and radiographs were used to determine the extent of the injury. Patients also underwent MR angiography to evaluate for associated vascular injuries.

Outcome Measures

All patients underwent clinical evaluation performed by the senior authors (A.Y.S., A.T.B., and R.J.S.) pre- and postoperatively. Elbow extension muscle strength was evaluated and agreed on by the senior authors using the modified British MRC grading system.[19] Furthermore, signs of triceps muscle reinnervation on electromyography (EMG) were recorded. For the EMG measurements, reinnervation success was defined as measurable voluntary motor unit potentials in the reinnervated long head of the triceps muscle.

For patients who underwent an FFMT for reconstruction of finger flexion or combined finger and elbow flexion as part of their reconstructive plan, an MRC grade of 2 or higher in the triceps muscle was considered sufficient to stabilize the elbow.[8] Therefore, in these cases an MRC grade ≥ 2 was considered as meaningful recovery. For all other cases an MRC grade of 3 or higher was considered as meaningful recovery.

Surgical Technique

All patients underwent a transfer of the ipsilateral spinal accessory nerve to the motor branch of the radial nerve to the long head of the triceps muscle in combination with various reconstructive interventions to reconstruct upper limb functions in addition to elbow extension. These other interventions included reconstruction of elbow flexion (e.g., transfer of intercostal nerves to the biceps motor branch, gracilis FFMT innervated by intercostal nerves), shoulder stabilization (e.g., reinnervation of suprascapular and/or axillary nerve with viable nerve roots when available or nerve transfers such as a [hemi]contralateral C-7), and prehensile function (e.g., tendon transfer of the gracilis FFMT to the finger flexors). In this cohort, the spinal accessory nerve was only used for triceps muscle reinnervation. While shoulder function is important, triceps muscle function in the setting of an FFMT for finger flexion is necessary to act as an agonist. In patients who underwent FFMT for finger flexion, triceps muscle function thus became a priority, and shoulder function was restored by tendon transfers or arthrodesis and was included in the reconstructive plan. Details on the FFMT procedure were previously reported.[3,17]

Through a supraclavicular approach, the supraclavicular plexus was explored. The spinal accessory nerve was identified on the medial border of the trapezius muscle and traced distally. A handheld nerve stimulator was used to verify the contraction of the trapezius muscle. The first motor ramus was identified, and the spinal accessory nerve was divided distal to this branch.

A deltopectoral incision was used to explore the infraclavicular plexus and expose the radial nerve and the branches that innervated the long head of the triceps muscle. These were dissected, mobilized proximally, and divided. An autologous nerve graft was harvested. The source of nerve graft depended on the other reconstructions being performed and included sural nerves, ipsilateral denervated superficial branch of the radial nerve, medial antebrachial cutaneous nerve, and lateral antebrachial cutaneous nerve. The nerve ends were sutured with the aid of an operative microscope by using two or three 9-0 nylon sutures, the nerve coaptation was wrapped with a split collagen nerve conduit (NeuraGen tube, Integra LifeSciences), and glued using fibrin glue (Tisseel, Baxter Healthcare Corp.). The procedure is schematically depicted in Fig. 1.

Statistical Analysis

Continuous variables (e.g., age, follow-up) are described as the mean with standard deviation for parametric data and median and range for nonparametric data. Categorical data are described using frequencies, median, and range.

For comparisons between the groups with and without meaningful recovery, various tests were used, including the Student t-test or Mann-Whitney U-test for continuous variables and chi-square or Fisher's exact test for categorical variables as appropriate. The Wilcoxon signed-rank test was used to compare pre- and postoperative MRC scores. Univariate logistic regression analysis was used to evaluate the effect of individual clinical and demographic factors. All statistical analyses were performed using SPSS Statistics (version 21, IBM Corp). Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Patient Demographics

A total of 42 patients met the inclusion criteria. The demographic and clinical factors of these patients are outlined in Table 1. All patients had an absence of detectable triceps muscle function (Grade 0). The most common mechanism of injury was motorcycle accidents (42.9%), followed by motor vehicle accidents (21.9%), snowmobile accidents (11.9%), bicycling injuries (4.8%), and football accidents (4.8%). Other mechanisms of injury (total 14.3%) were all-terrain vehicle accidents, an earthquake, fall, snowboard injury, jet ski injury, and a pedestrian versus car accident (n = 1 for each).

There were 7 patients (16.7%) with incomplete injuries. These patients sustained the following injuries: C5–7 (n = 2), C7–T1 (n = 2), C6–8 (n = 1), C5–8 (n = 1), and C6–T1 (n = 1). Sural nerve grafts were used in 10 patients. Within the group of patients who received an ipsilateral denervated donor nerve, the superficial branch of the radial nerve was used in 29 patients, the lateral antebrachial cutaneous nerve in 2 patients, and the medial antebrachial cutaneous nerve in 1 patient. Eight patients (19%) had a BMI of ≥ 30 kg/m2 and were categorized as obese. None of the patients had diabetes, and 5 patients (11.9%) were reported as current smokers. No complications related to the spinal accessory nerve–triceps

muscle transfer were reported, and all patients maintained upper trapezius muscle function (secondary to sparing of the first motor branch of the spinal accessory nerve). Regarding vascular injury, a dissection of the subclavian artery was found in 3 patients, stenosis of the axillary artery in 1 patient, and occlusion of the subclavian artery in 1 patient.

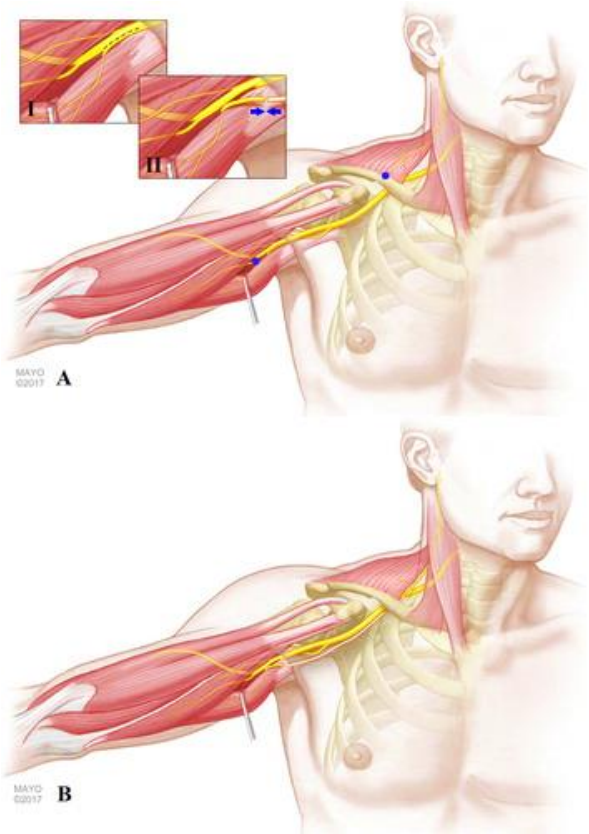


Figure 1. Schematic overview of the transfer of the spinal accessory nerve to the radial nerve motor branch to the long head of the triceps muscle. **A**: The spinal accessory nerve is identified on the medial border of the trapezius muscle, traced distally, and divided distal to the first motor ramus (*proximal blue dot*). The radial nerve is exposed (*distal blue dot*), and its branches that innervate the long head of the triceps muscle are mobilized proximally and divided (**inset I**). The mobilized branch to the long head of the triceps muscle is connected to an autologous nerve graft (*blue arrows*, **inset II**). **B**: The nerve graft is proximally connected to the spinal accessory nerve, allowing for a tension-free coaptation. Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved.)

Thirty-one patients underwent an FFMT for finger flexion/prehension. The decision to perform an FFMT for prehension was made based on a combination of factors, including injury type, associated concomitant injuries, and patient preferences. The decision whether to use an FFMT was made a priori.

Overall Results

Meaningful recovery was reached by 52.4% of the 42 patients. The median follow-up of the overall cohort was 22.9 months (range 12.3–45.0 months). Meaningful recovery, as previously stated, was defined as a strength of Grade 2 or higher for FFMTs for prehension and Grade 3 or higher for triceps muscle function alone. The median postoperative MRC grade was 2 (range 0–4). Nine patients (21.4%) regained no triceps muscle function (Grade 0). Ten patients (23.8%) regained Grade 1 triceps muscle strength, and Grade 2 was reached in 8 patients (19.1%). MRC grades of 3 and 4 were reached in 6 (14.3%) and 9 (21.4%) patients, respectively. Elbow extension MRC grades were significantly improved after surgery ($p < 0.001$). Signs of recovery on EMG were detected in 47.6% of the patients. Meaningful recovery of triceps muscle function was not associated with demographic or clinical differences between groups, with the exception of follow-up time and the presence of vascular injury (Table 2).

To evaluate whether reconstruction of elbow extension is effective, we compared patients who received an FFMT for reconstruction of finger flexion or combined finger and elbow flexion with patients who did not receive an FFMT. Meaningful recovery (≥ Grade 2) was reached by 54.8% of the patients who received an FFMT (n = 31). For the patients who did not receive an FFMT (n = 11), 45.5% regained meaningful recovery (≥ Grade 3). The difference between these groups was not statistically significant ($p = 0.59$) with regard to triceps muscle function.

Table 1. Summary of patient demographics in 42 patients undergoing triceps muscle reinnervation

Variable	Value
Mean age at op ± SD in yrs	27.7 ± 10.1
Sex, n (%)	
Male	34 (81)
Female	8 (19)
Median BMI in kg/m^2 (range)	25.2 (18.1–41.3)
Extent of injury, n (%)	
Complete	35 (83.3)
Incomplete	7 (16.7)
Dominant side affected, n (%)	20 (47.6)
Median time to op in mos (range)	5.3 (2.4–9.1)
Nerve graft source, n (%)	
Sural nerve	10 (23.8)
Ipsilateral denervated nerve	32 (76.2)
Median graft length in cm (range)	15.0 (10.0–25.0)
FFMT for finger flexion, n (%)	31 (73.8)
Vascular injury, n (%)	5 (11.9)
Tobacco use, n (%)	5 (11.9)
Median follow-up in mos (range)	22.9 (12.3–45.0)

Subgroup Analysis of Patients With At Least 20 Months of Follow-Up

The follow-up time was significantly different between the groups with and without meaningful recovery (p = 0.003, Table 2). A subgroup analysis was conducted among patients with a minimum of 20 months of follow-up (n = 26). In this subgroup analysis, follow-up time was no longer significantly different between the groups with and without meaningful recovery (Table 3). Within this subgroup, 4 patients (15.4%) sustained incomplete brachial plexus injury, of whom 2 patients had a C5–7 level injury and 2 patients a C7–T1 level injury. Nine patients (34.6%) received a sural nerve graft. Of the other 17 patients (65.4%), a superficial branch of the radial nerve graft was used in 16 patients, and a medial antebrachial cutaneous nerve graft was used in 1 patient.

The median MRC score was 2.5 (range 0–4). Two patients (7.7%) did not regain any triceps muscle function. Five patients (19.2%) reached Grade 1, and 6 patients (23.1%) had Grade 2 function. Fifty percent of this subgroup reached Group 3 or better, with 34.5% achieving Grade 4. We found that after a mean follow-up of 30 months (range 20.85–44.89 months) meaningful recovery was obtained by 69.5%, and 57.7% of patients showed signs of reinnervation on EMG.

Meaningful recovery of triceps muscle function was not associated with demographic or clinical differences between the groups (Table 3). The influence of vascular injury and smoking could not be evaluated in this subgroup due to the small number of cases (3 patients were reported smokers and only 1 patient had sustained vascular injury). Meaningful recovery was reached by 73.7% of the 19 patients who received an FFMT for elbow and/or finger flexion. Of the patients who did not receive an FFMT (n = 7), 57.1% regained meaningful recovery. The difference between these groups was not statistically significant (p = 0.64).

Table 2. Comparison between patients with and without meaningful recovery

Variable	Meaningful Recovery (n = 22)	No Meaningful Recovery (n = 20)	p Value
Age in yrs	27.8 ± 11.39	27.6 ± 8.87	0.67
Time to op in mos	5.2 ± 1.44	5.2 ± 1.56	0.95
Follow-up in mos	28.4 ± 9.1	19.7 ± 7.57	0.003*
Graft length in cm	15.8 ± 3.59	17.1 ± 4.26	0.35
BMI in kg/m^2	26.8 ± 6.14	25.8 ± 4.6	0.69
Male sex, n (%)	18 (81.8)	16 (80)	1
Complete injury, n (%)	17 (77.3)	18 (90)	0.41
Sural nerve graft, n (%)	6 (27.3)	4 (20)	0.72
Dominant side, n (%)	9 (40.9)	11 (55)	0.36
Smoking, n (%)	3 (13.6)	2 (10)	1
Vascular injury, n (%)	0 (0)	5 (25)	0.018*

Data are presented as the mean ± SD, unless otherwise noted. *Statistically significant ($p < 0.05$).

To further evaluate the influence of clinically important factors, we performed a univariate logistic regression analysis within this subgroup (Table 4). Although not reaching significance (p = 0.058), functional recovery seems to be impaired when longer grafts are used (OR 0.785, 95% CI 0.611–1.008). Age, time to surgery, graft length, and BMI were not found to significantly influence functional outcome.

Table 3. Comparison of recovery outcomes in the 26 patients with at least 20 months of follow-up

Variable	Meaningful Recovery (n = 18)	No Meaningful Recovery (n = 8)	p Value
Age in yrs	29.7 ± 11.7	27.5 ± 9.8	0.644
Time to op in mos	5.3 ± 1.49	5.1 ± 1.59	0.849
Follow-up in mos	31.1 ± 7.6	27.1 ± 6.5	0.338
Graft length in cm	15.6 ± 3.67	19.4 ± 4.5	0.103
BMI in kg/m^2	27.05 ± 6.3	26.3 ± 2.9	0.932
Male sex, n (%)	14 (77.8)	6 (75)	1
Complete injury, n (%)	15 (83.3)	7 (87.5)	1
Sural nerve graft, n (%)	6 (33.3)	3 (37.5)	1
Dominant side, n (%)	7 (38.9)	3 (37.5)	1

Data are presented as the mean ± SD, unless otherwise noted.

Table 4. Univariate logistic regression analysis in the subgroup with at least 20 months of follow-up

Variable	OR	95% CI	p Value
Age	1.02	0.942–1.104	0.630
Time to op	1.056	0.594–1.876	0.853
Follow-up	1.089	0.954–1.244	0.207
Graft length	0.785	0.611–1.008	0.058
BMI	1.028	0.871–1.214	0.741

DISCUSSION

Patients with partial or complete loss of their upper-extremity function due to brachial plexus injury are severely disabled. Traditionally, reconstruction of elbow flexion and shoulder function are considered the main priorities in brachial plexus reconstruction.[12,23] More recently, reconstructive goals have expanded, including reconstruction of elbow extension. Especially when reconstruction of prehensile function is aimed for or in cases of brachial plexus injury in which hand function is spared, reconstruction of elbow extension is imperative for obtaining meaningful use of the hand and should be considered a priority.[4,8] This study aimed to evaluate the prevalence and quality of restored elbow extension in patients with traumatic brachial plexus injury who underwent transfer of the spinal accessory nerve to the radial nerve branch to the long head of the triceps muscle and to identify patient and injury-related factors that may influence functional outcome.

Dodakundi et al.[5] and Doi et al.[8] found that triceps muscle strength of at least Grade 2 provided sufficient power to achieve a stable elbow while attempting finger movement in patients who received a double FFMT for combined elbow flexion and finger flexion and extension. Thus, we classified triceps function of at least Grade 2 as meaningful recovery in patients who also underwent FFMT for finger and elbow flexion (31 of 42) and at least Grade 3 for patients without this FFMT.

In our cohort of 42 patients, 52.4% obtained meaningful recovery. The percentage of meaningful recovery was not statistically significantly different (p = 0.59) when we compared patients with and without an FFMT; thus, the nerve transfer performed in this study (spinal accessory nerve–to–branch to the long head of the triceps muscle) seems to be equally useful in both groups.

Although the minimum follow-up for inclusion was 12 months, a number of patients may not have reached maximum clinical recovery at the time of their last follow-up. Given the fact that follow-up time was significantly different between the groups with and without meaningful recovery, 12 months may be too short for patients who undergo this specific transfer to reach their final recovery. To minimize the influence of patients who had not reached their final outcome, we performed a subgroup analysis of patients with a minimum of 20 months of follow-up (Table 3). After a mean follow-up of 30 months (range 20.85–44.89 months), the percentage of patients who reached meaningful recovery was considerably higher at 69.5%.

Association of Long Nerve Grafts and Longer Time to Reinnervation

Although the influence of an increase in graft length did not reach the arbitrary significance level (p = 0.058), possibly due to the sample size, the OR of 0.785 (95% CI 0.611–1.008) does indicate an important effect. In line with our findings and current concepts in literature, it is advisable to use shorter grafts when and if possible.[26] This is supported by the findings of Baltzer et al.[2] who reported an average of 10 months before EMG evidence of infraspinatus reinnervation was detected after spinal accessory nerve–to–infraspinatus muscle transfer without the use of an interposition graft. Thus, the knowledge that this transfer requires a relatively long time before functional recovery is to be expected must be relayed to patients.

With the growing interest in restoration of elbow extension in total or partial brachial plexus injury, several other surgical techniques have been proposed. Articles specifically reporting triceps muscle recovery after reinnervation with the intercostal transfer demonstrate widely varying results, with a reported recovery of Grade 3 or better varying from 0% to 90%. All studies have a long follow-up of at least 24 months.[6,11,14,18,30] The worst recovery (all ≤ Grade 2) was reported by Zheng et al.[30] who combined an intercostal nerve transfer for elbow extension with a phrenic nerve transfer for elbow flexion. When we compare our group of patients who had an FFMT and a minimum of 20 months of follow-up with the study of Doi

et al.[6] consisting of 25 patients who underwent the double free-muscle technique with a minimum of 24 months of follow-up, the percentage of Grade 2 or better recovery is similar (68% for Doi et al.[6] vs 73.7% in our cohort). However the percentage of patients attaining Grade 3 or better triceps muscle function is higher in our cohort (50%) than that of 28% in the study of Doi et al.[6] Furthermore, the intercostal nerve is typically used for reconstruction of elbow flexion at our institution.[23] Yang et al.[28] conducted a review on a contralateral C-7 transfer for the treatment of traumatic brachial plexus injury and concluded that outcomes of contralateral C-7 transfer for triceps muscle recovery vary strongly, with an average of 50% elbow extension recovery of Grade 3 or better. Besides the unpredictable results, lack of independent function and donor site morbidity to the uninjured contralateral extremity are important disadvantages of this technique.[29]

We recognize the limitations that are inherent to a retrospective study with a relatively small cohort of patients. Due to its subjective nature, the modified MRC scale for grading manual muscle strength is controversial. In future studies, it is therefore advised that more objective measurement techniques are used.[22] The discrepancy in the percentage of recovery measured by means of manual strength testing and EMG may be due to the fact that the exact location of the needles for the EMG was not always reported. It is possible that in some cases, parts other than the long head of the triceps muscle were measured, giving a false-negative result.

Ideally, a larger cohort of patients would be studied to identify factors predictive of functional outcome, allowing for more advanced multivariable regression analysis. In our series, we found that the presence of vascular injury seems to impair the chance of reaching meaningful recovery. Contrary to other studies, we did not find a significant effect of BMI or smoking on the functional recovery in this study.[16] This can probably be explained by the distribution and the small number of patients who smoked (n = 5) or were obese (n = 8), causing a lack of power to detect any effects.

Furthermore, there are presumably factors that influence results that were not evaluated in this study. A potentially important factor is the amount of physical therapy patients received. As antagonistic functions (elbow flexion and extension) are reconstructed using several nerve and muscle transfers, this requires complicated motor relearning and cortical reorganization. Unfortunately, we were not able to adequately evaluate this in this retrospective study, but it should be addressed in future research.

Reinnervation of the radial nerve branch to the long head of the triceps muscle has shown favorable results in comparison with targeting the radial nerve.[10] The ratio between myelinated fibers in the donor spinal accessory nerve (> 800 at the terminal end) and myelinated fibers in the receiving radial nerve branch to the long head of the triceps (~ 1200) is believed to be sufficient.[21,26,27] However, due to variations in the radial nerve anatomy, it is

never certain how many axons will actually reach the target organ, and in some less likely cases, another nerve branch of the radial nerve might have been targeted.[25]

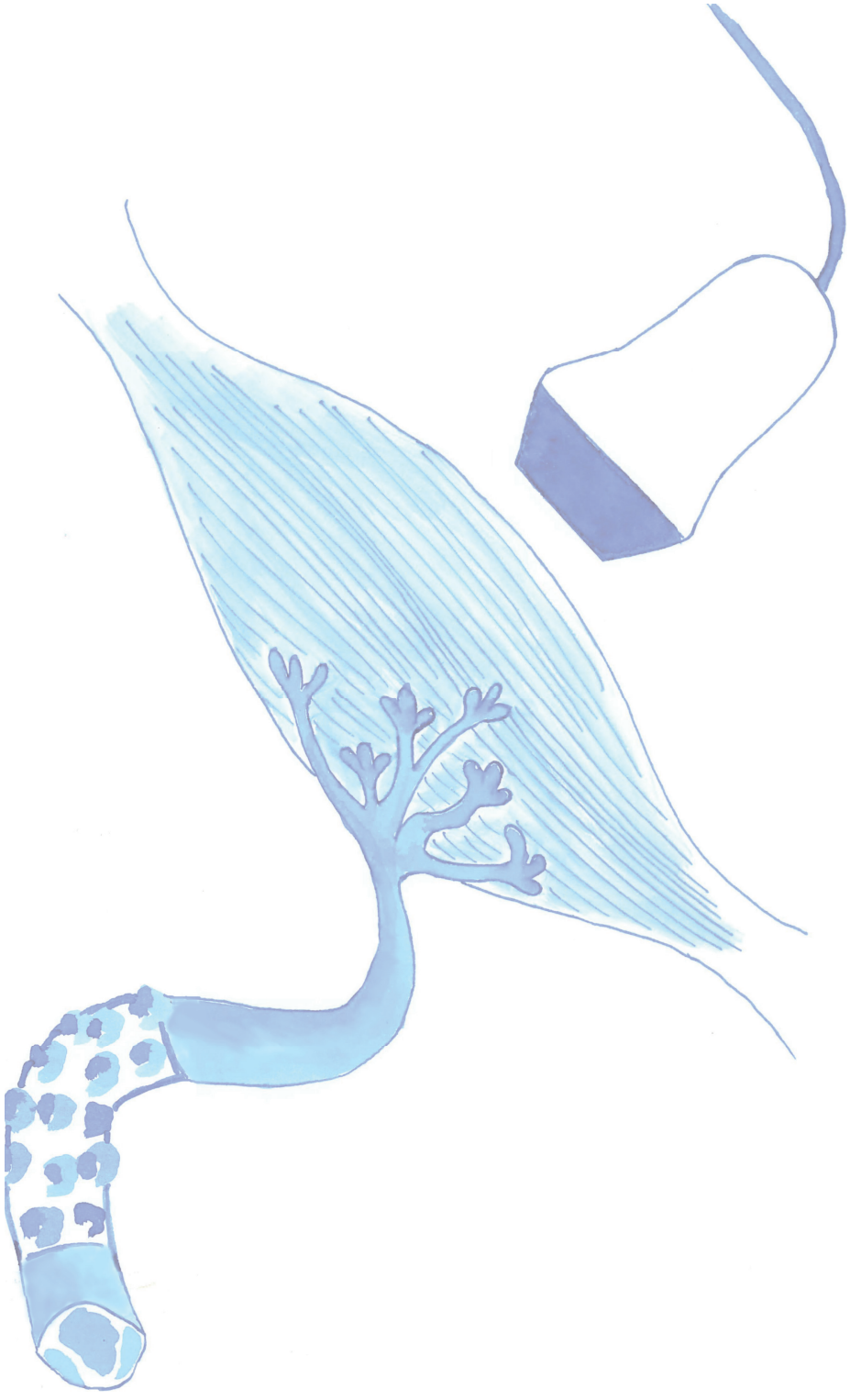
CONCLUSIONS

Especially in cases of brachial plexus injury in which prehensile function is restored or preserved, reinnervation of the triceps muscle is recommended. In our series, we found that transfer of the spinal accessory nerve to the radial nerve branch to the long head of the triceps is an adequate option for restoration of elbow extension, despite the relatively long time required for reinnervation. The presence of vascular injury impairs functional recovery of the triceps muscle, and the use of shorter nerve grafts is recommended when possible.

REFERENCES

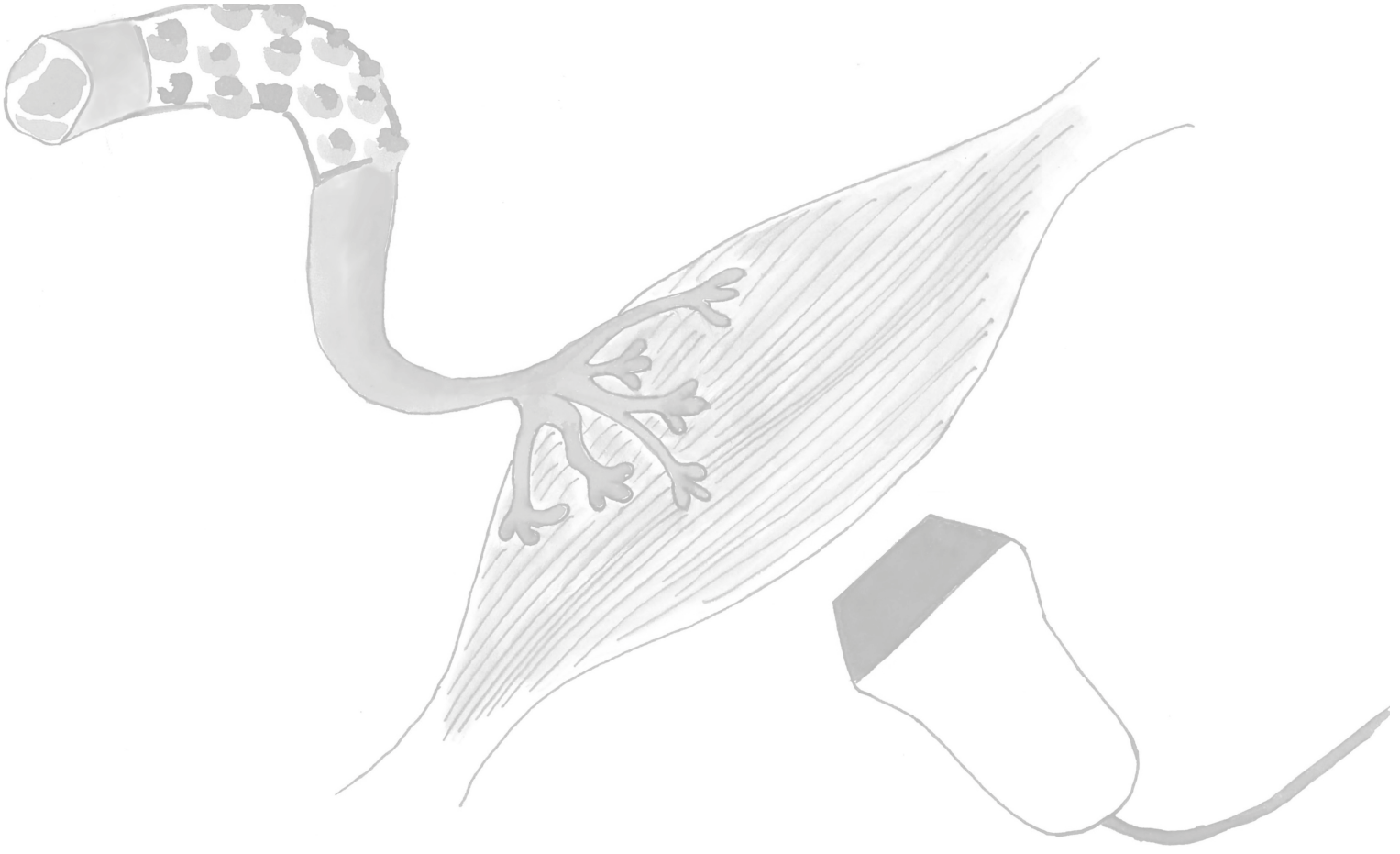
1. Adams JE, Kircher MF, Spinner RJ, Torchia ME, Bishop AT, Shin AY: Complications and outcomes of functional free gracilis transfer in brachial plexus palsy. Acta Orthop Belg 75:8–13, 2009
2. Baltzer HL, Wagner ER, Kircher MF, Spinner RJ, Bishop AT, Shin AY: Evaluation of infraspinatus reinnervation and function following spinal accessory nerve to suprascapular nerve transfer in adult traumatic brachial plexus injuries. Microsurgery 37:365–370, 2017
3. Barrie KA, Steinmann SP, Shin AY, Spinner RJ, Bishop AT: Gracilis free muscle transfer for restoration of function after complete brachial plexus avulsion. Neurosurg Focus 16(5):E8, 2004
4. Bertelli JA: Lower trapezius muscle transfer for reconstruction of elbow extension in brachial plexus injuries. J Hand Surg Eur Vol. 34:459–464, 2009
5. Dodakundi C, Doi K, Hattori Y, Sakamoto S, Fujihara Y, Takagi T, et al.: Outcome of surgical reconstruction after traumatic total brachial plexus palsy. J Bone Joint Surg Am 95:1505–1512, 2013
6. Doi K, Muramatsu K, Hattori Y, Otsuka K, Tan SH, Nanda V, et al.: Restoration of prehension with the double free muscle technique following complete avulsion of the brachial plexus. Indications and long-term results. J Bone Joint Surg Am 82:652–666, 2000
7. Doi K, Sakai K, Kuwata N, Ihara K, Kawai S: Double free-muscle transfer to restore prehension following complete brachial plexus avulsion. J Hand Surg Am 20:408–414, 1995
8. Doi K, Shigetomi M, Kaneko K, Soo-Heong T, Hiura Y, Hattori Y, et al.: Significance of elbow extension in reconstruction of prehension with reinnervated free-muscle transfer following complete brachial plexus avulsion. Plast Reconstr Surg 100:364–374, 1997
9. Emamhadi M, Alijani B, Andalib S: Long-term clinical outcomes of spinal accessory nerve transfer to the suprascapular nerve in patients with brachial plexus palsy. Acta Neurochir (Wien) 158:1801–1806, 2016
10. Flores LP, Socolovsky M: Phrenic nerve transfer for reconstruction of elbow extension in severe brachial plexus injuries. J Reconstr Microsurg 32:546–550, 2016
11. Gao K, Lao J, Zhao X, Gu Y: Outcome after transfer of intercostal nerves to the nerve of triceps long head in 25 adult patients with total brachial plexus root avulsion injury. J Neurosurg118:606–610, 2013
12. Giuffre JL, Kakar S, Bishop AT, Spinner RJ, Shin AY: Current concepts of the treatment of adult brachial plexus injuries. J Hand Surg Am 35:678–688, 2010 (Erratum in J Hand Surg Am35:1226, 2010)
13. Goubier JN, Teboul F: Transfer of the intercostal nerves to the nerve of the long head of the triceps to recover elbow extension in brachial plexus palsy. Tech Hand Up Extrem Surg11:139–141, 2007
14. Goubier JN, Teboul F, Khalifa H: Reanimation of elbow extension with intercostal nerves transfers in total brachial plexus palsies. Microsurgery 31:7–11, 2011
15. Hoang PH, Mills C, Burke FD: Triceps to biceps transfer for established brachial plexus palsy. J Bone Joint Surg Br 71:268–271, 1989
16. Lee JY, Kircher MF, Spinner RJ, Bishop AT, Shin AY: Factors affecting outcome of triceps motor branch transfer for isolated axillary nerve injury. J Hand Surg Am 37:2350–2356, 2012
17. Maldonado AA, Kircher MF, Spinner RJ, Bishop AT, Shin AY: Free functioning gracilis muscle transfer with and without simultaneous intercostal nerve transfer to musculocutaneous nerve for restoration of elbow flexion after traumatic adult brachial pan-plexus injury. J Hand Surg Am42:293.e1–293.e7, 2017
18. Malungpaishrope K, Leechavengvongs S, Witoonchart K, Uerpairojkit C, Boonyalapa A, Janesaksrisakul D: Simultaneous intercostal nerve transfers to deltoid and triceps muscle through the posterior approach. J Hand Surg Am 37:677–682, 2012
19. Mendell JR, Florence J: Manual muscle testing. Muscle Nerve 13 Suppl:S16–S20, 1990
20. Merrell GA, Barrie KA, Katz DL, Wolfe SW: Results of nerve transfer techniques for restoration of shoulder and elbow function in the context of a meta-analysis of the English literature. J Hand Surg Am 26:303–314, 2001
21. Schreiber JJ, Byun DJ, Khair MM, Rosenblatt L, Lee SK, Wolfe SW: Optimal axon counts for brachial plexus nerve transfers to restore elbow flexion. Plast Reconstr Surg 135:135e–141e, 2015

22. Shahgholi L, Bengtson KA, Bishop AT, Shin AY, Spinner RJ, Basford JR, et al.: A comparison of manual and quantitative elbow strength testing. Am J Phys Med Rehabil 91:856–862, 2012
23. Shin AY, Spinner RJ, Steinmann SP, Bishop AT: Adult traumatic brachial plexus injuries. J Am Acad Orthop Surg 13:382–396, 2005
24. Songcharoen P, Mahaisavariya B, Chotigavanich C: Spinal accessory neurotization for restoration of elbow flexion in avulsion injuries of the brachial plexus. J Hand Surg Am 21:387–390, 1996
25. Uerpairojkit C, Ketwongwiriya S, Leechavengvongs S, Malungpaishrope K, Witoonchart K, Mekrungcharas N, et al.: Surgical anatomy of the radial nerve branches to triceps muscle. Clin Anat 26:386–391, 2013
26. Vathana T, Larsen M, de Ruiter GC, Bishop AT, Spinner RJ, Shin AY: An anatomic study of the spinal accessory nerve: extended harvest permits direct nerve transfer to distal plexus targets. Clin Anat 20:899–904, 2007
27. Witoonchart K, Leechavengvongs S, Uerpairojkit C, Thuvasethakul P, Wongnopsuwan V: Nerve transfer to deltoid muscle using the nerve to the long head of the triceps, part I: an anatomic feasibility study. J Hand Surg Am 28:628–632, 2003
28. Yang G, Chang KW, Chung KC: A systematic review of contralateral C7 transfer for the treatment of traumatic brachial plexus injury: part 1. Overall outcomes. Plast Reconstr Surg136:794–809, 2015
29. Yang G, Chang KW, Chung KC: A systematic review of outcomes of contralateral C7 transfer for the treatment of traumatic brachial plexus injury: part 2. Donor-site morbidity. Plast Reconstr Surg 136:480e–489e, 2015
30. Zheng MX, Xu WD, Qiu YQ, Xu JG, Gu YD: Phrenic nerve transfer for elbow flexion and intercostal nerve transfer for elbow extension. J Hand Surg Am 35:1304–1309, 2010



Part II

Evaluation techniques



4

Noninvasive ultrasound of the tibial muscle for longitudinal analysis of nerve regeneration in rats.

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ABSTRACT

Background

Today's golden standard to measure functional recovery after nerve trauma in experimental studies is the muscle mass ratio and the isometric tetanic force, both of which are invasive and require a sacrificial procedure. We propose ultrasound as a non-invasive method to obtain the amount of muscle atrophy and determine its validity and reliability by comparing it to muscle mass ratio, isometric tetanic force and histology.

Methods

Fifty rats sustained a 10 mm autograft sciatic nerve reconstruction. With a two-week interval, 5 animals were tested with a total follow-up of 20 weeks. The functional recovery of the hindlimb muscles were measured with ultrasound, muscle mass ratio and isometric tetanic force. Additionally, neuromuscular junctions were histologically analysed. The different evaluation techniques were compared and the reliability of the ultrasound was determined.

Results

Four weeks after denervation extensive muscle atrophy resulted in a decrease of muscle mass up to 30%. Ultrasound showed good correlations with muscle mass ratio for both tibial (r=0.85) and gastrocnemic muscles (r=0.89). The intra- and inter-rater reliability of the ultrasound were high (r=0.97 and 0.88). The correlation with force was lower (0.62), although still statistically significant.

Conclusions

Ultrasound measurement of muscle atrophy was highly correlated with the golden standard muscle mass ratio and was also significantly correlated with isometric tetanic force. Histology confirmed the regeneration pattern observed with ultrasound. We propose that ultrasound can be used as a valid alternative to muscle mass ratio to study muscle atrophy after nerve injury in a less-invasive and more animal-friendly manner.

INTRODUCTION

To study peripheral nerve injury, experimental studies are required that are valid and reliable in measuring successful restoration of nerve function. A wide variety of tests assess the peripheral motor nerve outcome after nerve reconstruction: Electrophysiology, muscle mass ratio, ankle angle, histomorphometry and functional tests as the walking-track analysis and isometric tetanic force.[1-4] Walking track analysis is only used in rats and was previously commonly used as a measure of motor outcome. It is reported to be fraught with inaccuracies stemming from autotomy of the feet and contractures of the ankle.[5] The most commonly-used test is presently the post-sacrifice muscle mass ratio. The main reason for the popularity of this test is its simplicity. However, the isometric tetanic force measurement may be the most valid assessment of muscle strength, as it directly evaluates the actual strength capacity and not the indirect muscle mass measure.[2] Several studies already confirmed the lack of association between the different tests available.[5-8]

Recently we introduced the use of ultrasound as a valid, non-invasive, and repeatable tool to measure the gastrocnemic muscle thickness and thus quantify muscle atrophy following nerve injury.[9] While we found a high correlation with muscle mass, a number of questions still remained. For example, it is unclear how the ultrasound measurement would compare to a measure of true muscle force, such as the isometric tetanic force measurement. In addition, the method was only validated for a single muscle, the gastrocnemius muscle; the reliability and validity of the visualization and measurements of the tibial muscle has not yet been described. Since many nerve regeneration studies use the tibial muscle, a much smaller muscle, as a reference, an ultrasound protocol to allow assessment of this muscle would be very valuable.[2, 10, 11]

The aim of this study is to determine if ultrasound is a valid tool to visualize muscle atrophy in the tibial muscle by comparing the ultrasound assessment to muscle weight and to a more true measure of muscle force, i.e. isometric tetanic force testing. The ultrasound recordings and muscle measurements were also obtained of the bigger, gastrocnemic muscle. In addition, the neuromuscular junction (NMJ) in the muscle was histologically examined by visualizing the denervation and re-innervation of the motor endplates of the tibial and gastrocnemic muscle, and were related to the other outcome assessments. We hypothesize that the early reinnervation, before return of strength can be visualized with this method. The overall goal of this study is thus to create a valid, repeatable, and, most importantly, non-invasive method to analyze functional outcome after nerve injury over time using ultrasound.

METHODS

Animals

The experimental protocol was approved by the institutional animal care and use committee. Fifty adult female Lewis rats were used, weighing 175-200 grams (Charles River Laboratories Inc.). Animals were pair-housed in hooded cages on a 12-hour light/dark schedule at room temperature. The rats were given food and water ad libitum. Surgical and sacrificial

procedures were performed under general anaesthesia (Isoflurane, 1-2 % in a mixture of O_2/N_2O).

Surgical Technique

For this experiment, a reversed nerve autograft reconstruction was made. The surgical procedure was performed by one surgeon and assistant using standard aseptic microsurgical techniques under the operating microscope (Zeiss OP-MI 6-SD; Carl Zeiss, Goettingen, Germany) on sciatic nerves of the hind limbs. The operated leg (left or right side) was randomly chosen. The sciatic nerve was exposed by an oblique skin incision. A 10 mm segment of the sciatic nerve was dissected, reversed and sutured back using 10/0 Ethilon sutures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands). The muscle was approximated using 2 6/0 Vicryl Rapide sutures after which the skin was closed with the same sutures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands). Buprenorphine (0.5 mL/kg BW) was injected submuscular as an analgesic.

Evaluation of muscle atrophy

Every other week, functional measurements were performed on both legs on a total of five animals per time period. The total follow-up duration was 20 weeks with a total of 100 legs examined.

Ultrasound

After initial isoflurane anesthesia, ultrasound images of the muscles were recorded. At first, for better conduction of ultrasound waves, hair of the leg was removed by shaving and application of depilation cream. For analysis of the tibial muscle, the knee and ankle joint where held in a 90° angle. Transducer gel was applied and the probe was placed in a 45° angle without applying pressure on the muscle. The sagittal plane of the tibial muscle was visualized. The experimental ultrasound set-up is depicted in Figure 1. To perform the ultrasound measurements of the gastrocnemic muscle, the rat hind paw was placed in a water basin, as previously described by our group[9]. In this position, images of the sagittal plane of the gastrocnemic muscle were recorded. All measurements were conducted bilaterally. The ultrasound recordings were performed with the use of the SonoSite Titan Ultrasound (SonoSite Inc., Bothell, USA), with a 5-10 MHz linear array probe. In order to obtain an inter- and intrarater reliability of the ultrasound measurements of the tibial muscle, two independent and blinded investigators performed each evaluation twice. Images were analyzed to determine the muscle thickness for the gastrocnemic- and the cross-sectional area of the tibial muscle using Adobe Photoshop CS6 Extended (Adobe Systems Incorporated, USA).

Isometric tetanic force measurement

The isometric tetanic force measurement was determined for the tibial anterior muscles of

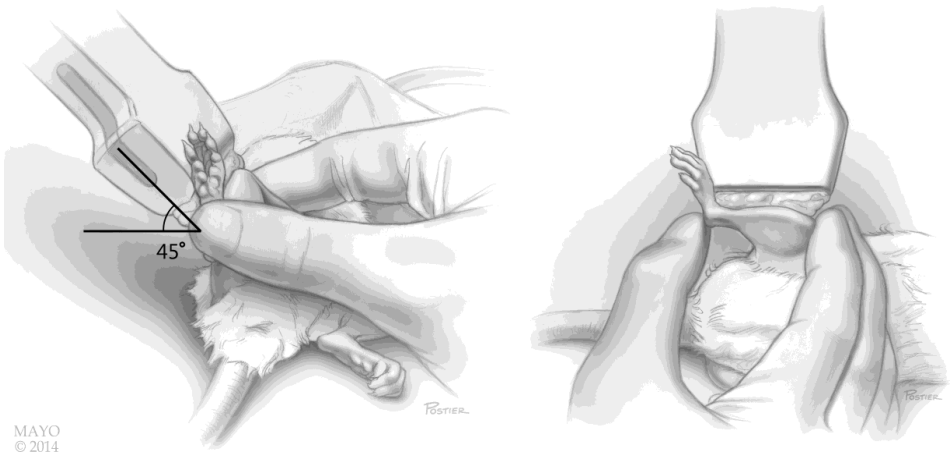


Figure 1. Experimental set-up of the ultrasound recording of the tibial muscle. (Published with permission of the Mayo Foundation for Medical Education and Research. All rights reserved, copyright 2015.)

both legs as described by Shin et al.[2] The sciatic nerve was exposed by an oblique skin incision. The tendon of the tibial muscle was exposed via a second skin incision and released at its insertion. The leg was fixed to a platform with Kirschner wires, which prevents the leg from moving during muscle stimulation. The Kirschner wires were placed through the knee- and ankle joint. The tendon of the tibial muscle was connected to the force transducer (MDB-50, Transducer Techniques, Temecula, CA) and placed in a horizontal position. A miniature bipolar stimulating electrode (Harvard Apparatus) was placed around the exposed sciatic nerve. A bipolar stimulator (Medtronic, NL) was used to generate the stimulus and the signal acquired from the force transducer was processed on a PC using WinTest (Testometric, Hartech, NL). During testing, body temperature was maintained with a heating pad and the muscles were kept wet and warm with a constant warm saline drip (37˚C). Prior to the maximum muscle force measurements, the optimal values of initial muscle tension (pre load) needed to be determined. This is the muscle tension in which the muscle can generate its maximum muscle force. With this optimal preload, the isometric muscle contraction force was measured with the predetermined frequencies of 100, 120 and 140 Hz. Stimulation was applied for a maximum of 2 seconds or until a force peak was clearly observed.

Muscle mass

After the muscle testing, the animal was euthanized using an overdose pentobarbital (1 mL/kg BW) that was injected intracardially. The tibial and gastrocnemic muscles of both the operated and control leg were carefully dissected. The muscle weight was obtained directly after dissection to obtain the wet muscle weight.

Histology

For histological analysis, tibial and gastrocnemic muscles where fast frozen at -40 °C and serial 50-µm frozen sections were cut using a cryostat. A combination stain for the demonstration of motor nerve terminals and cholinesterase at the neuromuscular junctions was used as previously described by Pestronk et al.[12] This method uses bromoindoxyl acetate staining for cholinesterase and silver-gold impregnation for nerve terminals. The silver stain reveals individual myelinated axons as black filaments, while the acetylcholinesterase stain labels motor endplates containing the enzyme acetylcholinesterase as well-demarcated transparent blue zones. Unlike acetylcholine receptors, acetylcholinesterase stays permanently after denervation.

Statistical Analysis

All collected data was expressed as a ratio of the operated leg divided by the healthy leg to allow comparison of the percentage of recovery between techniques. Obtained values were compared between the different evaluation techniques. Two comparisons were made: the isometric tetanic force method with the ultrasound and the muscle weight with the ultrasound. A Pearson correlation coefficient was calculated between the different measurements of the same muscles and the associated confidence intervals were determined. A Bland Altman plot was used to visualize the difference between the ultrasound and muscle mass ratio of the tibial muscle. To assess the validity and reliability of these measurements, the inter-rater and intra-rater reliability of this method was calculated using the inter-observer correlation (ICC). A p-value < 0.05 was considered statistically significant.

RESULTS

After a short learning curve of approximately two weeks, tibial muscle images could easily be obtained using ultrasound by both examiners. The procedure did not take more than 10 minutes per animal. Images of the ultrasound recordings are depicted in Figure 2.

Tibial muscle

Muscle atrophy obtained with the golden standard, muscle mass ratio, showed a steep decrease in the fourth week (32 ± 9% (SD)), followed by an increase of muscle mass with a ratio of 72 ± 6% in the 14th week and 78 ± 5% in the last follow-up week (Fig.3) The recovery of the muscle force, depicted in Figure 4, showed recovery rates of 40 ± 4% in week 10, reaching up to 76 ± 8% in the 20th follow-up week. As the force was not powerful enough to produce measures in the first three follow-up weeks, no recovery rates of these weeks could be obtained. When relating ultrasound to muscle weight and muscle force, we found that ultrasound images from the tibial muscle showed the same pattern of atrophy followed by recovery of the muscle. Similar to muscle mass ratio, with ultrasound most atrophy was observed at week 4 (44% ± 9%), followed by recovery reaching 76 ± 2 percent in the last follow-up week. The correlations between the ultrasound and the muscle mass and muscle force are depicted in Table 1. Ultrasound showed strong significant correlations with muscle

weight for the tibial muscle (0.854 ± 0.06). The correlation between ultrasound and muscle force was lower (0.624 ± 0.17) but still highly significant (p<0.001).

Gastrocnemic muscle

The recovery rates of the gastrocnemic muscle measured with ultrasound and muscle weight are displayed in Figure 5. Ultrasound recordings of the gastrocnemic muscle showed slightly lower values than for muscle weight. The lowest recovery rate was 40 ± 2% in week 4 with the ultrasound and a muscle mass ratio of 28 ±4 percent. The correlation between the ultrasound and muscle weight measurements of the muscle atrophy was high (0.893 ± 0.04), as depicted in Table 1.

Table 1. Correlation between Ultrasound, Muscle Weight and Isometric Tetanic Force for both muscles

	Ultrasound	
Tibial muscle	**Correlation (95% CI)**	***p***
Muscle weight	0.854 (0.789-0.899)	< 0.001*
Isometric Tetanic Force	0.624 (0.428-0.759)	< 0.001*
Gastrocnemic muscle		
Muscle weight	0.893 (0.844-0.927)	< 0.001*

*Statistically significant correlation (*p*<0.05)

Neuromuscular junctions

Staining of the motor endplates and myelinated axons visualized the denervation and re-innervation of the muscles, as shown in Figure 6. Where the ultrasound and muscle mass ratio still shows high values in the second post-operative week, histology shows few axons and free endplates. Also, clubbing was observed, that is axonal sprouting which occurs first as an outgrowth of fine-beaded fibers ending as an axonal dilatation. This is a sign of nerve regrowth.

This phenomenon was seen up till week 8. Free endplates were visible up till week 4, in accordance with ultrasound where we observed an increase in the muscle mass starting in week 4. Motor endings formed by several motor arborizations were observed in week 6 and week 8 after nerve injury with reconstruction. Sprouting of the axons and poly- innervated motor endplates could be seen between the 6^{th} and 16^{th} week. Starting from week 14, the innervation of the muscle showed a more normalized pattern. This is also in accordance with our ultrasound finding where we saw a stabilization starting at the 16^{th} week. Up till 20 weeks, poly- innervated motor endplates were observed. The muscle of the control side was also examined end showed no pathological changes in the innervation pattern.

Reliability

The reliability of the ultrasound recordings of the tibial muscle are depicted in Table 2. Both

the inter- rater and intra-rater reliability were high. The Bland Altman plot (Figure 7) was created to visualize the comparison between the two methods i.e. the ultrasound and muscle weight. The two techniques generally did not differ more than 10%, which shows a good overall reliability of these measurements.

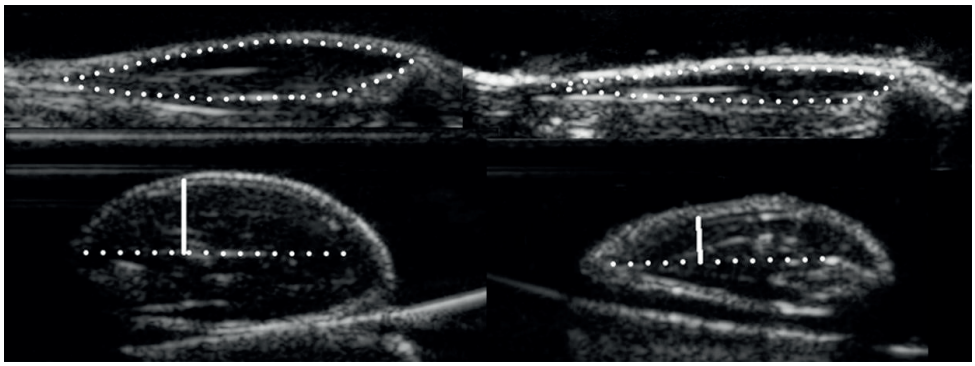


Figure 2. Ultrasonographic images of the tibial muscle. The dotted line indicates the area of the muscle used to calculate the area of the tibial muscle of the healthy (*Above, left*) and operated leg (*Above, right*). The operated legs show a smaller surface area of the muscle than the healthy leg. The tibial muscle was measured 6 weeks postoperatively. At the bottom, the solid line indicates the muscle thickness measured in the gastrocnemic muscle at the healthy (*Below, left*) and operated side (*Below, right*).

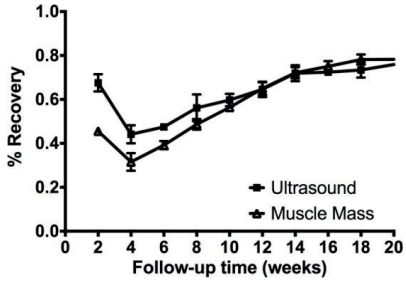


Figure 3. The recovery of the tibial muscle measured with muscle mass ratio and ultrasound. The recovery of the muscle shows the same trend in both evaluation methods, error bars = SEM.

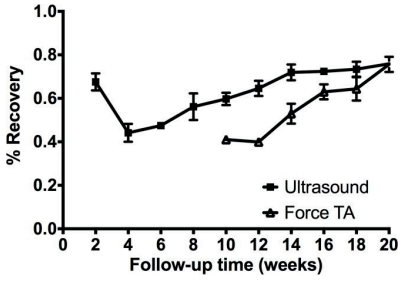


Figure 4. The recovery of the tibial muscle measured with ultrasound and force. The correlation between ultrasound and force was lower than ultrasound and weight. However, their significant correlation is 0.624, error bars = SEM.

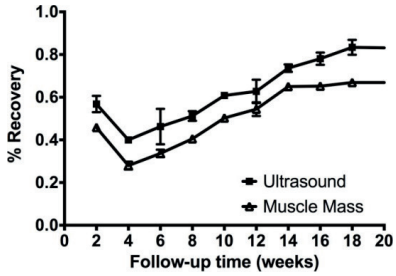


Figure 5. The recovery of the gastrocnemic muscle measured with ultrasound and weight. The recovery of the muscle shows the same trend in both evaluation methods, error bars = SEM.

Table 2. Validity and reliability of ultrasound recordings of the tibial muscle

	ICC (95% CI)	*p*
Intrarater reliability	0.974 (0.961-0.983)	< 0.001*
Interrater reliability	0.882 (0.825-0.921)	< 0.001*

*Statistically significant correlation (p<0.05)

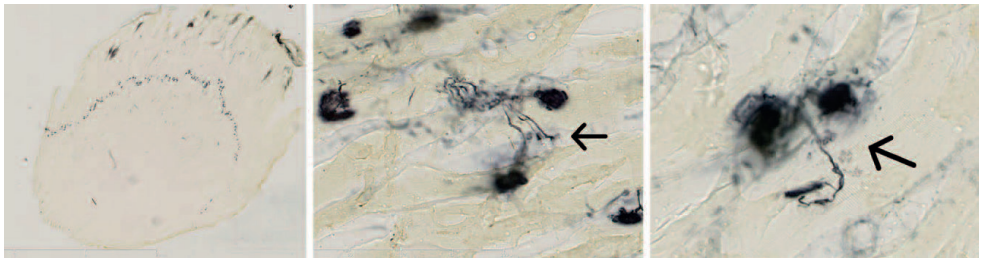


Figure 6. Acetylcholinesterase staining to visualize the neuromuscular junctions. (Left) Overview; whole-mount acetylcholinesterase stained rat tibial muscle, showing that this muscle is composed of one belly (original magnification, × 1.25). Note that the muscle has a single motor endplate band that is located in the middle portion of the muscle. (Center) Clubbing; reinnervated tibial muscle (week 8), showing two endplates on atrophic muscle fibers (original magnification, × 40) and two growing nerve fibers ending as growth clubs (arrow). (Right) Sprouting; reinnervated tibial muscle (week 14), showing a branching axon forming a double motor arborization, assumed to supply one muscle fiber (original magnification, × 40). The thickness of the sections (50 µm) causes part of the material to be out of the plane of focus at high magnification.

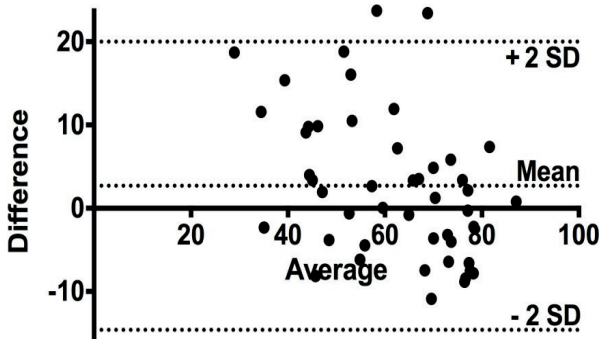


Figure 7. A Bland Altman plot of the two methods, muscle mass ratio and ultrasound. The average value of the methods is displayed on the x-axis where the difference between the two methods is displayed on the y-axis. The plot shows a good overall reliability of the measurements and reasonably small limits of agreement. SD=standard deviation.

DISCUSSION

New applications for the use of ultrasound are studied and introduced in different research fields for various indications and implications[13,14], as ultrasound is a non-invasive and an easy to use method. This study investigated the relation between muscle force, muscle weight and muscle diameter obtained with ultrasound to assess the functional motor nerve recovery in a rodent model after nerve injury with reconstruction. Ultrasonographic recordings in this study showed similar recovery of the muscle as shown by the muscle weight; the correlation between both measurements was 0.854 and 0.893, indicating strong relations between the ultrasound and muscle mass. A Bland Altman plot further confirmed that ultrasound and muscle weight have small limits of agreement. The muscle force and ultrasound findings showed lower but still highly-significant correlations (r=0.624). One possible explanation is that force does not relate as directly with ultrasound as muscle weight (i.e. more fibers, more force). This theory is further strengthened by the fact that during the first weeks of recovery the muscle weight gradually increased with delayed response intensity with the isometric tetanic force evaluation. Staining of the neuromuscular junctions shows the same denervation and re-innervation patterns of the muscles as observed with ultrasound, weight and force. Sprouting of the axons and poly- innervated motor endplates were visible until the sixteenth post-operative week, after which the pattern was normalized.

A systematic review of Wood et al[5] in 2011 explicitly stated the importance of the comparison of different outcome measures of peripheral nerve regeneration. They found an extensive battery of tests but poor correlations between most of them. Today, muscle mass ratio is generally accepted as the golden standard for the evaluation of nerve regeneration in experimental animal studies. Although the assessment is easy, we would like to emphasize that muscle force is closer to the real functional recovery. Shin et al. found that the sciatic foot index (SFI) as a non-invasive assessment did not significantly correlate with the isometric tetanic force or muscle weight.[7] These findings were also confirmed by Urbancheck et al.[8] Several other authors confirmed the lack of specificity of the SFI.[4-6] To our knowledge, no other methods than ultrasound have proven to be non-invasive, making it possible to obtain serial measurements and significantly correlate with other valid outcome measurements.

There are various ways to analyze the functional recovery after nerve trauma in animal experiments, which all have their advantages and disadvantages. This new non-invasive ultrasound method allows analysing functional motor recovery after nerve trauma in the same animal over time. The assessment is easy and could be performed in less than ten minutes. In future studies, ultrasound assessment can be executed without sacrificing the animal, leading to a reduction in the number of animals required for an experiment. Since the regeneration will be evaluated in the same animal, variation within a group will decrease. This will further reduce the number of animals needed in experimental studies on nerve reconstruction and simultaneously increase the power of the study. A combination can be made with other non-invasive measurements as the sensory pinprick test, to obtain an overall indication of nerve recovery.

A limitation of this present study is that animals where not measured over time to prove that this can also be reliable. In striving to achieve not only a reduction in the required number of animals needed, but also a reduction in variation, animal tests in nerve regeneration studies should preferably be non- invasive. These limitations notwithstanding, this study is the next step in creating non-invasive tests for the total analysis of the animal after nerve trauma with or without reconstruction.

CONCLUSIONS

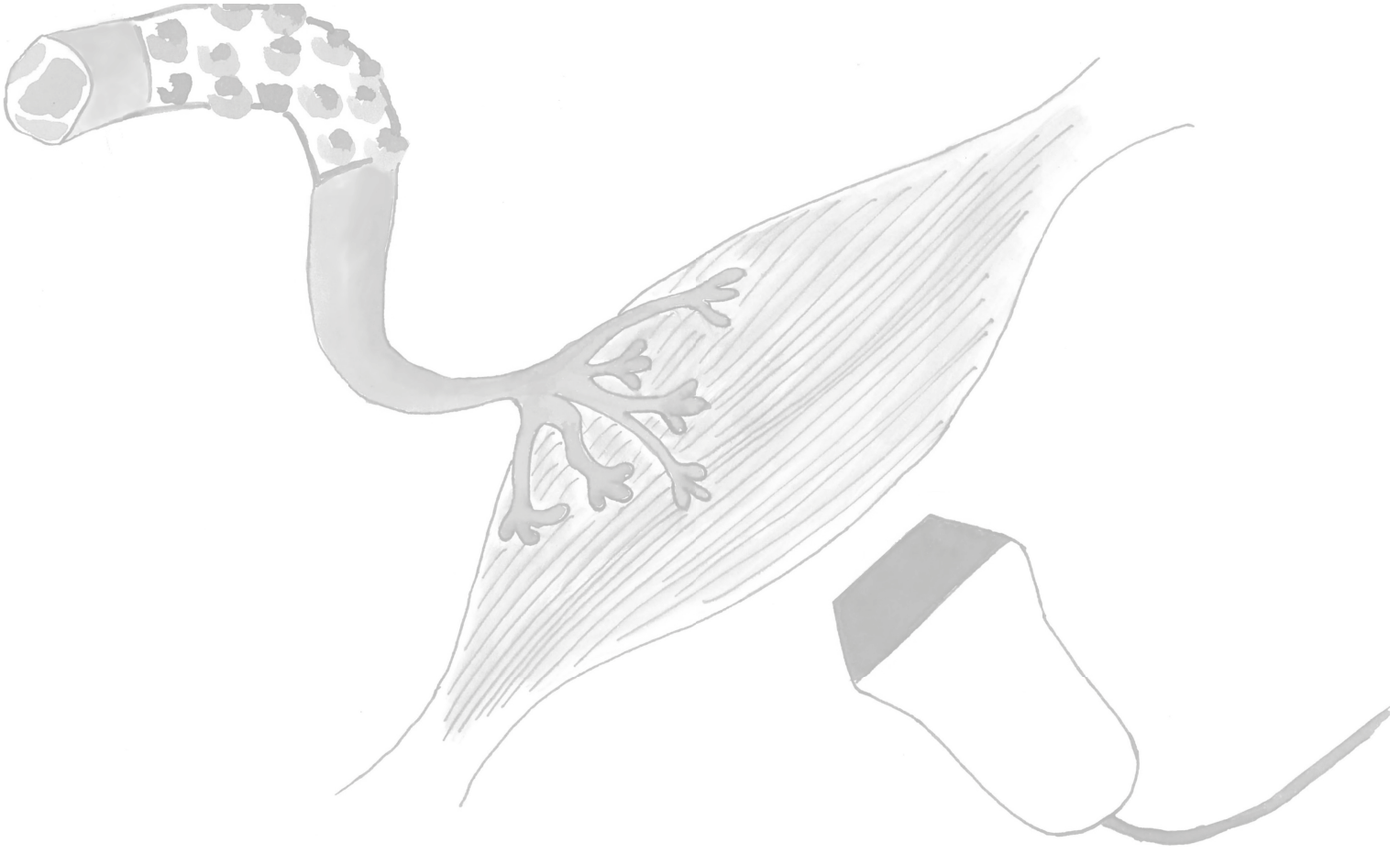
In conclusion, this study illustrates the purpose of the new, non-invasive method to analyze the functional recovery of the muscle after nerve reconstruction. This method proves to be a valid and reliable method to obtain the muscle atrophy and recovery of both the gastrocnemic and tibial muscles. Non-invasive evaluation techniques will reduce the required number of animals for experiments and will also reduce the variation within the study groups.

ACKNOWLEDGEMENTS

The authors would like to thank dr. A.Y. Shin for generously providing the figure of the experimental set- up.

REFERENCES

1. Bain JR, Mackinnon SE, Hunter DA. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. Plast Reconstr Surg. 1989;83:129–138.
2. Shin RH, Vathana T, Giessler GA, Friedrich PF, Bishop AT, Shin AY. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery 2008;28:452–457.
3. Hunter DA, Moradzadeh A, Whitlock EL, et al. Binary imaging analysis for comprehensive quantitative histomorphometry of peripheral nerve. J Neurosci Methods 2007;166:116–124.
4. Kanaya F, Firrell JC, Breidenbach WC. Sciatic function index, nerve conduction tests, muscle contraction, and axon morphometry as indicators of regeneration. Plast Reconstr Surg. 1996;98:1264–1271, discussion 1272.
5. Wood MD, Kemp SW, Weber C, Borschel GH, Gordon T. Outcome measures of peripheral nerve regeneration. Ann Anat. 2011;193:321–333.
6. Munro CA, Szalai JP, Mackinnon SE, Midha R. Lack of association between outcome measures of nerve regeneration. Muscle Nerve 1998;21:1095–1097.
7. Lee JY, Giusti G, Wang H, Friedrich PF, Bishop AT, Shin AY. Functional evaluation in the rat sciatic nerve defect model: A comparison of the sciatic functional index, ankle angles, and isometric tetanic force. Plast Reconstr Surg. 2013;132:1173–1180.
8. Urbanchek MS, Chung KC, Asato H, Washington LN, Kuzon WM Jr. Rat walking tracks do not reflect maximal muscle force capacity. J Reconstr Microsurg. 1999;15:143–149.
9. Nijhuis TH, de Boer SA, Wahegaonkar AL, et al. A new approach to assess the gastrocnemius muscle volume in rodents using ultrasound; comparison with the gastrocnemius muscle index. PLoS One 2013;8:e54041.
10. Brenner MJ, Hess JR, Myckatyn TM, Hayashi A, Hunter DA, Mackinnon SE. Repair of motor nerve gaps with sensory nerve inhibits regeneration in rats. Laryngoscope 2006;116:1685–1692.
11. Lee JY, Giusti G, Friedrich PF, et al. The effect of collagen nerve conduits filled with collagen-glycosaminoglycan matrix on peripheral motor nerve regeneration in a rat model. J Bone Joint Surg Am. 2012;94:2084–2091.
12. Pestronk A, Drachman DB. A new stain for quantitative measurement of sprouting at neuromuscular junctions. Muscle Nerve 1978;1:70–74.
13. Wittenberg M. Will ultrasound scanners replace the stethoscope? BMJ 2014;348:g3463.
14. Solomon SD, Saldana F. Point-of-care ultrasound in medical education: Stop listening and look. N Engl J Med. 2014;370:1083–1085.



5

Motor nerve recovery in a rabbit model: description and validation of a noninvasive ultrasound technique.

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ABSTRACT

Purpose

To develop and validate a noninvasive ultrasound technique for the longitudinal analysis of functional recovery after segmental peroneal nerve reconstruction in a rabbit model.

Methods

Twelve male New Zealand White rabbits underwent a 1-cm peroneal nerve autograft reconstruction. Ultrasound measurements were performed before surgery and at 1, 2, 4, 8, 12, and 16 weeks postoperatively. All rabbits were managed with manual restraint for the ultrasound procedure, avoiding the risks of anesthetics. At 12 and 16 weeks, we evaluated functional recovery using compound muscle action potential, isometric tetanic force measurements, wet muscle weight, and nerve histomorphometry. Data were compared with ultrasound measurements by calculating the Pearson correlation coefficient. We determined intra-rater and inter-rater reliability of the ultrasound measurements.

Results

Ultrasound demonstrated good correlation with isometric tetanic force measurements and wet muscle weight, good correlation with nerve histomorphometry, and moderate correlation with compound muscle action potential. Both intra-rater and inter-rater reliability of the ultrasound technique was excellent.

Conclusions

Ultrasound analysis of the tibialis anterior muscle provided a reliable method for analysis of functional recovery in a rabbit peroneal nerve reconstruction model. The noninvasive nature allowed for longitudinal follow-up within the same animal and measurement of early recovery without the use of anesthesia.

Clinical relevance

Application of this noninvasive technique can reduce the variability and sample size necessary in peripheral nerve reconstruction studies and may provide an ideal tool for comparative studies in larger animal models.

INTRODUCTION

The treatment of peripheral nerve injury represents a clinical challenge.[1] To study alternative treatment strategies for peripheral nerve repair, animal models are used.[2] Rats are frequently used and are especially useful as a first *in vivo step* to study short-term nerve regeneration because of their fast neuroregenerative capacity. However, important disadvantages impair the clinical relevance of such studies because of the rat's much faster nerve regeneration compared with humans and the limited length of nerve gaps that can be created in rats.[3] Those limitations can be overcome using rabbits, in which nerve gaps up to 8 cm are feasible.[4] Furthermore, the rabbit's neuroregenerative and immunological properties more closely mimic the human situation, providing an important step before clinical research.[5,6]

To evaluate motor nerve recovery after nerve reconstruction in both rat and rabbit, the easily obtainable wet muscle weight is frequently used. Other commonly used and established outcome measurements include isometric tetanic force measurement (ITF), a more accurate measurement of functional recovery, electrophysiological testing, and nerve histomorphometry.[7–9] However, all of those techniques require the animal to be killed for assessment, which restricts analysis of outcome over time. A commonly used noninvasive test is the sciatic function index. Unfortunately, this technique correlates poorly with other functional outcome measurements and is feasible only in the rat model.[10]

The use of ultrasound in a rat model has been shown to be both reliable and valid.[7,9]Allowing for multiple measurements over time within the same animal, ultrasound can reduce variability within the groups studied. This would ultimately reduce the required sample size and costs of these studies. We hypothesized that a similar technique could be used in the rabbit model as a noninvasive method for analyzing nerve reconstruction results. Therefore, the aim of this study was to investigate the reproducibility, reliability, and validity of a noninvasive ultrasound technique to measure the tibialis anterior muscle cross-sectional area to analyze functional recovery in a rabbit peroneal nerve injury model.

MATERIALS AND METHODS

After our institutional animal care and use committee granted approval, 12 male New Zealand White rabbits (Harlan Laboratories, Inc, Indianapolis, IN) (weighing 3–4 kg) underwent a 1-cm peroneal nerve autograft reconstruction on the left side. Animals were housed individually with a 12-hour light–dark cycle. Food and water were provided *ad libitum*.

Ultrasound measurements

The optimal ultrasound protocol was determined in a pilot study. Based on previous studies, we compared different probe positions to obtain the muscle cross-sectional area employing ultrasound using cadaveric rabbit legs (data not shown).[7,9,11] The protocol that both observers rated as most easily obtainable showed an SD of less than 5% for measurements within and between observers, and this protocol was chosen. All rabbits tolerated the ultrasound

procedure with manual restraint in a Bunny Snuggle jacket (Lomir Biomedical, Inc, Malone, NY) and thereby avoided the risks of anesthetics. Ultrasound measurements were obtained before surgery and 1, 2, 4, 8 weeks after surgery and at the time of death, either 12 or 16 weeks after surgery. The hind limbs were shaved and remaining hair was removed using hair removal cream (Surgi-Prep; Miltex, York, PA). The experimental setup of the ultrasound measurements of the tibialis anterior muscle in the rabbit is depicted in Figure 1. Video 1 is an instructional video of the ultrasound protocol (available on the *Journal's* Web site at www.jhandsurg.org). With the ankle joint of the rabbit at 90°, the patella and lateral malleolus were identified. The ultrasound probe was placed perpendicular to the muscle one third of the way between those landmarks, and cross-sectional images of the muscle were obtained using the Philips CX50 ultrasound system with a 12- to 3-MHz linear transducer (Philips Healthcare, Eindhoven, The Netherlands) and ultrasound gel (Aquasonic 100; Parker Laboratories, Fairfield, NJ). Following the American College of Laboratory Animal Medicine and the Institutional Animal Care and Use Committee guidelines, animals were monitored for signs of discomfort.[12] At all time points multiple measurements were obtained by 2 similarly trained observers independently of each other. The cross-sectional area of the tibialis anterior muscle was analyzed in Adobe Photoshop CS5 Extended (Adobe Systems, Inc, San Jose, CA).

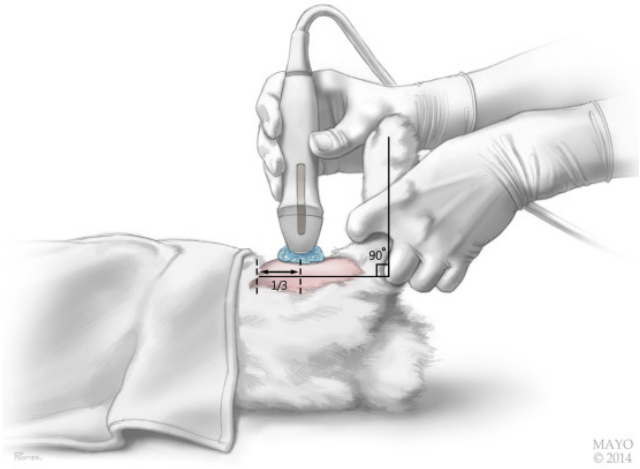

Figure 1. Experimental setup of the ultrasound recording showing the position of the probe and leg.

Surgical procedure

We induced anesthesia using 35 mg/kg ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and 5 mg/kg xylazine (Vettek, Bluesprings, MO) administered intramuscularly. After a subcutaneous injection of 0.18 mg/kg buprenorphine (Buprinex; Reckitt Benckiser Pharmaceuticals, Inc, Richmond, VA), animals were intubated and maintained at spontaneous ventilation with the Bain ventilation system (Harvard Apparatus, Holliston, MA) under 1% to 2% isoflurane. Animals received intravenous lactated Ringer solution, and animal temperature was maintained at 37°C. All surgical procedures were performed under standard

aseptic conditions. Through a longitudinal incision on the posterolateral aspect of the left thigh, the sciatic nerve and its peroneal branch were exposed by a muscle-splitting approach. Under a surgical microscope, a 1-cm segment of the peroneal nerve was dissected at a level 4 cm distal to the border of the external obturator muscle. The autograft was reversed and sutured using 6 10-0 nylon epineural sutures (Ethicon, Inc., Somerville, NJ) on each side. All nerves had a similar diameter. Wounds were closed in layers using 4-0 Vicryl rapid bioabsorbable sutures (Ethicon, Inc., Somerville, NJ). After surgery, animals received 5 mg/kg enrofloxacin (Baytril; Bayer Animal Health, Shawnee Mission, KS) and 4 mg/kg carprofen (Rimadyl; Pfizer Animal Health, New York, NY) subcutaneously once daily for 5 days. Elizabethan collars were worn for 2 weeks after surgery. Rabbits were observed daily until the end of the study.

Nonsurvival procedure

At 12 and 16 weeks, 6 animals per time point underwent a nonsurvival procedure. All measurements were conducted bilaterally. Similar to the surgical procedure, anesthesia was induced and animals were intubated. Anesthesia was maintained using 0.6 mg/kg/min propofol (Fresenius Kabi, Lake Zurich, IL) intravenously. Infusion rate was adjusted based on corneal reflex and rates of respiration and pulse. Propofol was used in lieu of isoflurane, which has been shown to interfere with skeletal muscle contraction.[4,13] Dextrose 5% was administered intravenously to prevent muscle fatigue.

Electrophysiology

On an anesthetized rabbit, we clamped a miniature bipolar electrode (Harvard Apparatus, Holliston, MA) around the exposed sciatic nerve proximal to the autograft and placed a ground electrode in the surrounding musculature. Two recording electrodes were placed on the tibialis anterior muscle surface, one proximally near the motor end plates and the other distally.[14] Using a VikingQuest portable electromyelogram (Nicolet Biomedical, Madison, WI), the compound muscle action potential (CMAP) was measured. We used a stimulation duration of 0.02 ms and minimum intensity to elicit a maximum CMAP signal. Responses were analyzed for CMAP amplitude.

Isometric tetanic force measurement

Isometric tetanic force (ITF) measurement was performed as described by Giusti et al.[4] Briefly, the tibialis anterior tendon was connected to a force transducer (MDB-50; Transducer Techniques, Temecula, CA) using a custom clamp. Using Kirschner wires, the knee and ankle joints were secured on a custom-made platform. A bipolar stimulator (Grass SD9; Grass Instrument Co, Quincy, MA) was used to stimulate the peroneal nerve distal to the graft, and the signal acquired from the force transducer was processed using LabVIEW software (National Instruments, Austin, TX). After determining the optimal initial muscle tension, the isometric tetanic muscle force was measured at 100, 125, and 150 Hz.

Histomorphometry

Animals were killed with an intravenous overdose of Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI) and the lower body was perfused with 10% formalin through the exposed aorta. A 2.5-mm segment of the peroneal nerve distal from the graft was harvested 2 cm proximal to where the nerve entered the tibial muscle and was fixed in Trump fixative solution. Samples were cut into 1-µm sections, stained with toluidine blue (Fisher Scientific, Pittsburgh, PA), and analyzed using Image-Pro software (Image-Pro Plus 7.0; Media Cybernetics, Bethesda, MD). The N-ratio was calculated as the total myelinated fiber area divided by the total tissue cable area.[15]

Wet muscle weight

The tibialis anterior muscles on both sides were carefully dissected and the tendons were removed. Muscles were directly weighed to determine the wet muscle weight.

Statistical analysis

The sample size of this study was based on data from the pilot study, which showed that the highest SD of the ultrasound recordings was 5%. The variability of the other outcome measurements was based on previous studies.[4,15] Assuming the same variability would occur in this study, the number of animals needed for 80% power to detect a correlation of at least 0.65 reliably was calculated to be 12.

To determine the validity, reliability, and reproducibility of the ultrasound technique, the inter-rater and intra-rater reliability (intra-class correlation coefficient) (2-way mixed model, absolute agreement, single measure) were calculated.[16] In addition, Pearson correlation coefficients between ultrasound recordings and CMAP, ITF, muscle weight, and histomorphometry were calculated. To this end, data were expressed as percent recovery by dividing the data of the grafted side by the data of the control side of the same animal. To confirm the correlation further between ultrasound and wet muscle weight of the tibial muscle, a Bland–Altman plot was created. $P < .05$ was considered statistically significant.

RESULTS

All rabbits survived surgery and follow-up, and no major complications occurred. Minor complications were superficial licking wounds on the dorsum of the foot of the operated leg (n = 4) and a superficial skin inflammation on the leg (n = 1). Topical silver sulfadiazine cream 1% (Thermazene; Ascend Laboratories LLC, Montvale, NJ) was applied until wound healing was complete.

Ultrasound imaging showed the smallest muscle cross-sectional area index at 8 weeks (mean ± SD, 51% ± 6%) increasing to a mean recovery rate of 60% ± 8% at 12 weeks and 91% ± 10% at 16 weeks. Figure 2 depicts images of the ultrasound recordings. The reliability and reproducibility of the ultrasound technique are demonstrated in Figure 3 as the mean

recovery rates found by both observers at each time point. Both the intra-rater and inter-rater reliability were excellent, as shown in Table 1.

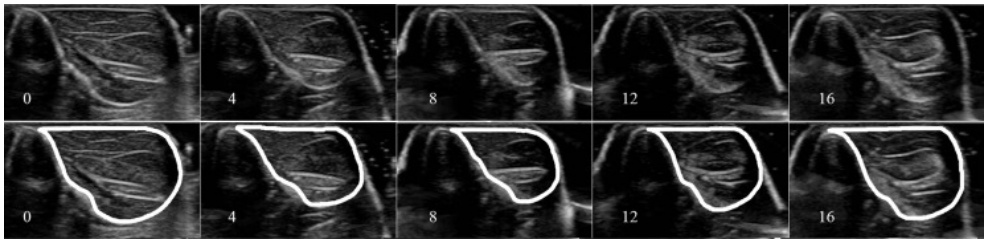

Figure 2. Ultrasound recordings of the same animal over time. In the bottom row, the tibialis anterior muscle is delineated with a white line. The number in each picture indicates the follow-up time point in weeks.

Functional outcome measurements

Compound muscle action potential measurements indicated a mean recovery rate of 44% ± 8% (SD) at 12 weeks and 67% ± 9% at 16 weeks. In 3 animals (n = 1 in the 12-week group and n = 2 in the 16-week group), CMAP could not be obtained reliably. In one animal (week 16), the ITF test could not be performed owing to mechanical failure of the force transducer. The ITF showed 39% ± 24% recovery at 12 weeks and 104% ± 37% at 16 weeks. The wet muscle weight of the tibial muscle showed a mean recovery of 45% ± 8% at 12 weeks and 86% ± 13% at 16 weeks. Finally, calculation of the N-ratio revealed a mean recovery at 12 weeks of 61% ± 14% and 83% ± 10% at 16 weeks.

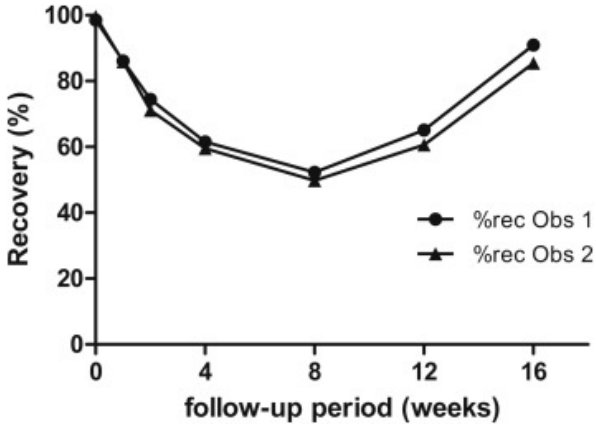

Figure 3. Muscle cross-sectional area recovery rate over time after nerve reconstruction. The muscle cross-sectional area of the operated leg is expressed as a percentage of the non-operated side as measured by the 2 independent observers (Obs 1 and Obs 2) at the different time points.

Table 1. Validity and Reliability of Ultrasound Recordings of Tibial Muscle		
	Intra-class Correlation (95% Confidence Interval)	***P* Value**
Intra-rater reliability	0.994 (0.992–0.996)	< .001*
Inter-rater reliability	0.989 (0.984–0.992)	< .001*

*Statistically significant at $P < .05$.

Correlations with ultrasound measurements

Correlations between ultrasound measurements and other outcome measurements at 12 and 16 weeks are shown in Table 2. All correlations were moderate to excellent and were statistically significant (P < .01). To test if the correlation between two variables is significant, Pearson's r is tested against the null hypothesis: ρ = 0 (no correlation). A significant result (P < .05) means that the correlation is significantly different from 0 (eg, there is a significant correlation).

A Bland–Altman plot of ultrasound measurements and wet muscle weight is shown in Figure 4. The narrow 95% limits of agreement indicate statistical equivalence of the methods.

Table 2. Correlations Between Ultrasound and 4 Established Outcome Measurements		
	Ultrasound **Correlation (95% Confidence Interval)**	***P* Value**
Compound muscle action potential	0.59 (0.18–0.83)	< .010*
Isometric tetanic force	0.83 (0.62–0.93)	< .001*
Muscle weight	0.97 (0.94–0.99)	< .001*
Histomorphometry	0.69 (0.41–0.86)	< .001*

*Statistically significant at $P < .05$.

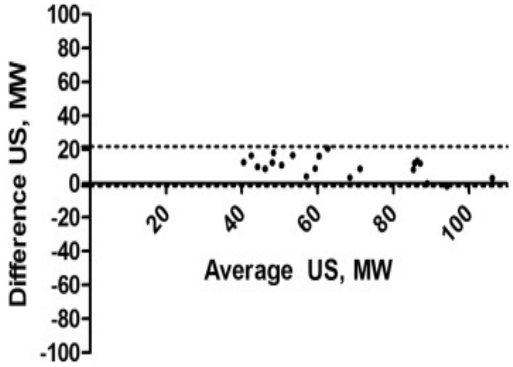

Figure 4. Bland–Altman plot of ultrasound and muscle weight measurements. To visualize the difference between the recovery rates calculated using ultrasound (US) measurement and muscle weight (MW), a Bland–Altman plot was created. The average of the 2 measurements is plotted on the x axis and the difference on the y axis. Dotted lines indicate 95% limits of agreement.

DISCUSSION

Ultrasound provides a promising noninvasive tool for functional evaluation of motor recovery after peripheral nerve reconstruction in this rabbit model. The noninvasive nature allows animals to be used as their own controls over time. This minimizes inter-animal variability, yielding higher power with the same number of animals.

Ultrasound and the conventional wet muscle weight share the same rationale for the measurement of motor nerve regeneration. Denervation will lead to atrophy in the target muscle, causing a decrease in muscle mass and muscle cross-sectional area. Both will increase when reinnervation occurs.[17]

Ultrasound measurements were obtained at several time points after surgery to investigate the reliability and reproducibility of the technique for muscles in different stages of recovery. Results depicted in Figures 2 and 3 show that early onset of muscle recovery can be detected using ultrasound.

This study found high inter-rater and intra-rater reliability (r = 0.989 and 0.994), indicating both high reliability and reproducibility of this method, which is in line with previous research.[7,9]

The excellent correlation between ultrasound and magnetic resonance imaging to measure muscle cross-sectional area has been extensively reported and therefore was not repeated in this study.[18–21] Furthermore, because this study showed a strong correlation between a noninvasive technique and the current reference standard muscle weight, additional correlation to more invasive techniques such as magnetic resonance imaging may be obsolete.

We chose 2 different end points, 12 and 16 weeks, to study the validity of the technique in a model with varying stages of muscle reinnervation. The validity of this technique to quantify functional recovery was shown by the strong correlations between ultrasound and both ITF and muscle weight (r = 0.83 and 0.97, respectively) and good and moderate correlations with histomorphometry (r = 0.69) and CMAP (r = 0.59). The lower correlation between ultrasound and CMAP could be explained by the higher variability in CMAP values. This is in line with previous studies that used CMAP in rabbits.[4,15]

Previous studies validated muscle cross-sectional area measurement by ultrasound for both the gastrocnemius and tibial muscle in rats.[7,9] In the rabbit, typically only the peroneal branch, innervating the tibialis anterior muscle, instead of the entire sciatic nerve, is grafted.[15] Therefore, measurement of the gastrocnemius muscle, which Nijhuis et al[7]proposed, would not be suitable. In both rat ultrasound studies, the animals were killed at various intervals. In our study, ultrasound images were obtained for each animal at multiple time points. In addition to the previous studies, this study showed a correlation between ultrasound measurements and CMAP and nerve histomorphometry.

A review of the literature for noninvasive evaluation of functional recovery after nerve reconstruction in the rabbit peroneal nerve model yielded few techniques, all with limitations. The toe spread reflex described by Schmitz and Beer[22] indicates the onset of motor recovery but does not discriminate in the quality of the muscle function.

Meffert et al[23]described transcutaneous muscle strength quantification that requires deep sedation. With a 7% risk of death in rabbits as a result of to anesthetics, techniques that can avoid anesthesia are highly favorable.[24] Our ultrasound procedure can be performed in less than 15 minutes with no form of anesthesia, which therefore limits animal stress.

When implementing this technique, a well-defined protocol will allow repeated measurements of the same muscle section. In this study, the learning curve of both observers was diminished by experience gained in the pilot study.

That ultrasound measurement of the muscle cross-sectional area was an indirect measure of motor nerve recovery could be considered a limitation of this study. However, this is similar for the muscle weight index that is commonly used and accepted.

Furthermore, we found a good correlation between ultrasound measurements and ITF measurements, a direct measurement for functional motor recovery.[4,15]

Recovery of more than 100% ITF is in line with previously reported data and may be explained by a side preference in the rabbit.[4,9,15,25]

This method can be used for other nerves and innervated muscles including brachial plexus injury models. The noninvasive nature allows for longitudinal follow-up and measurement of early recovery, and provides a useful tool for comparative studies of nerve reconstruction strategies.

ACKNOWLEDGMENTS

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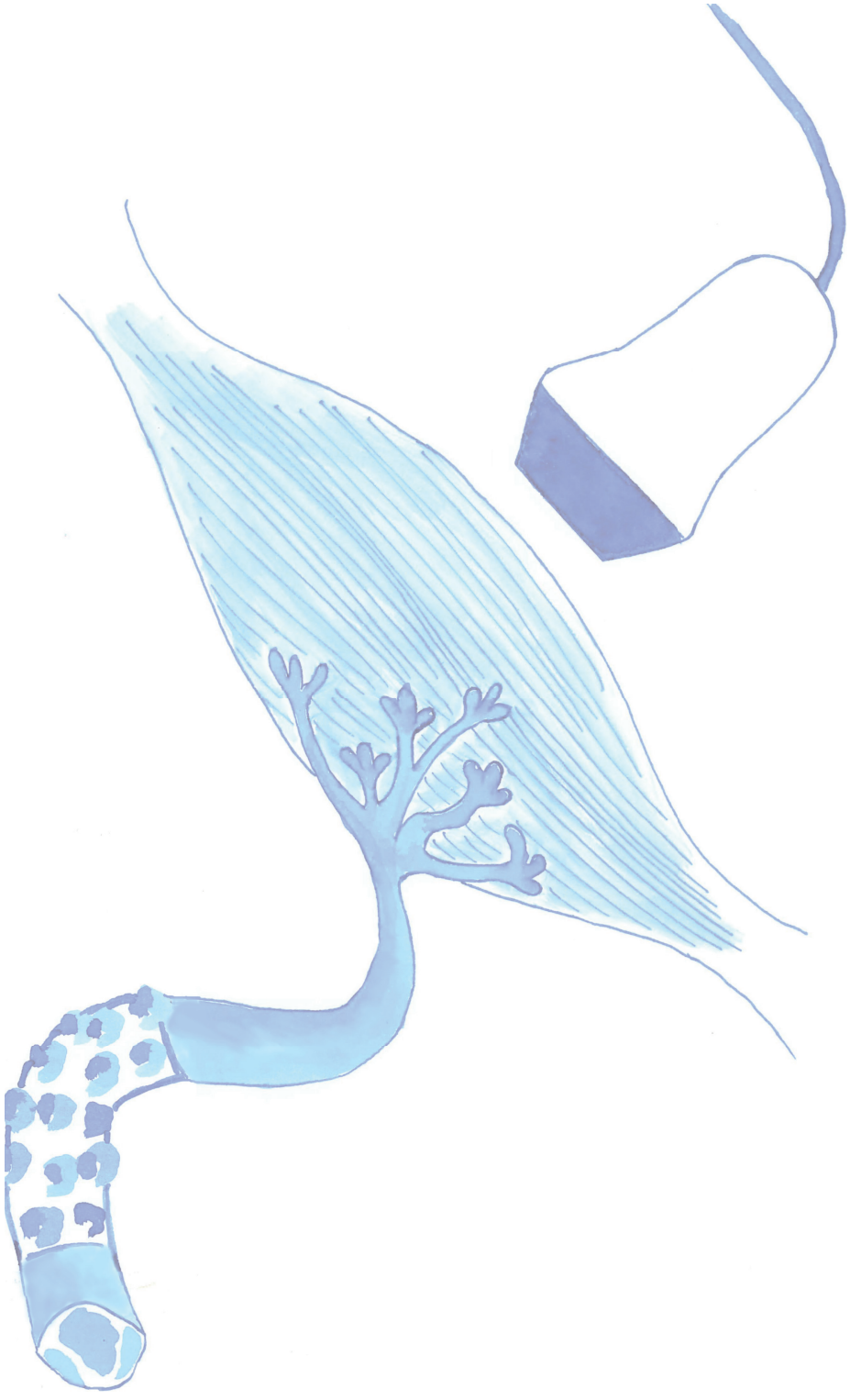
REFERENCES

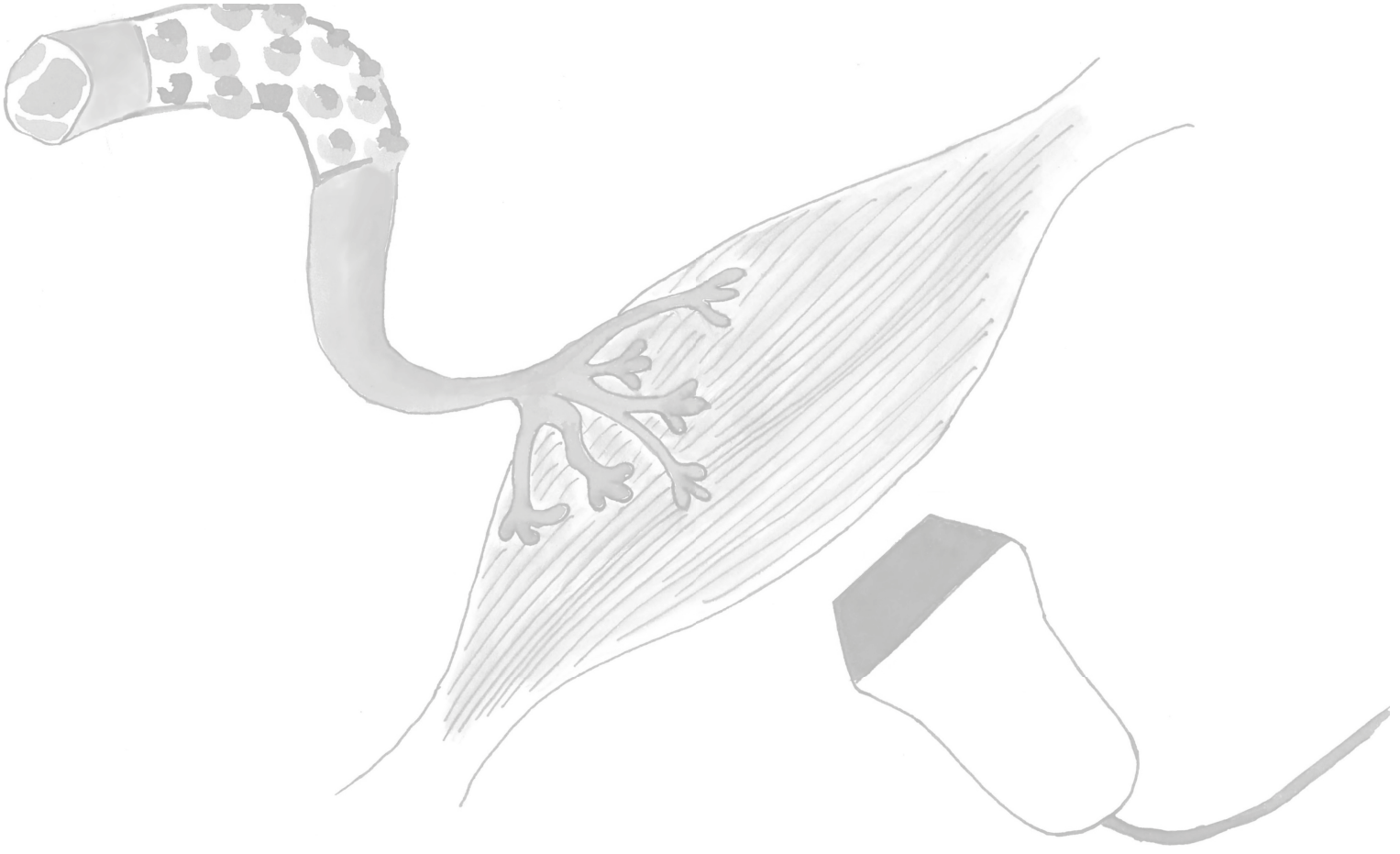
1. Boyd KU, Nimigan AS, Mackinnon SE. Nerve reconstruction in the hand and upper extremity. Clin Plast Surg. 2011;38(4):643e660.
2. Angius D, Wang H, Spinner RJ, Gutierrez-Cotto Y, Yaszemski MJ, Windebank AJ. A systematic review of animal models used to study nerve regeneration in tissue-engineered scaffolds. Biomaterials. 2012;33(32):8034e8039.
3. Calvert GT, Doolabh VB, Grand AG, Hunter DA, Mackinnon SE. Rat-strain differences in recovery following peripheral-nerve allotransplantation. J Reconstr Microsurg. 2001;17(3):185e191.
4. Giusti G, Kremer T, Willems WF, Friedrich PF, Bishop AT, Shin AY. Description and validation of isometric tetanic muscle force test in rabbits. Microsurgery. 2012;32(1):35e42.
5. Amillo S, Yanez R, Barrios RH. Nerve regeneration in different types of grafts: experimental study in rabbits. Microsurgery. 1995;16(9): 621e630.
6. Deumens R, Bozkurt A, Meek MF, et al. Repairing injured peripheral nerves: bridging the gap. Prog Neurobiol. 2010;92(3):245e276.
7. Nijhuis TH, de Boer SA, Wahegaonkar AL, et al. A new approach to assess the gastrocnemius muscle volume in rodents using ultrasound; comparison with the gastrocnemius muscle index. PLoS One. 2013;8(1):e54041.
8. Bain JR, Mackinnon SE, Hunter DA. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. Plast Reconstr Surg. 1989;83(1):129e138.
9. Hundepool CA, Nijhuis TH, Rbia N, Bulstra LF, Selles RW, Hovius SE. Noninvasive ultrasound of the tibial muscle for longitudinal analysis of nerve regeneration in rats. Plast Reconstr Surg. 2015;136(5):633ee639e.
10. Lee JY, Giusti G, Wang H, Friedrich PF, Bishop AT, Shin AY. Functional evaluation in the rat sciatic nerve defect model: a comparison of the sciatic functional index, ankle angles, and isometric tetanic force. Plast Reconstr Surg. 2013;132(5):1173e1180.
11. Mohseny B, Nijhuis TH, Hundepool CA, Janssen WG, Selles RW, Coert JH. Ultrasonographic quantification of intrinsic hand muscle cross-sectional area: reliability and validity for predicting muscle strength. Arch Phys Med Rehabil. 2015;96(5):845e853.
12. Members ATF, Kohn DF, Martin TE, et al. Public statement: guidelines for the assessment and management of pain in rodents and rabbits. J Am Assoc Lab Anim Sci. 2007;46(2):97e108.
13. Bouhemad B, Langeron O, Orliaguet G, Coriat P, Riou B. Effects of halothane and isoflurane on the contraction, relaxation and energetics of rat diaphragmatic muscle. Br J Anaesth. 2002;89(3):479e485.
14. Gross R, ed. The Physiology of Growth. New York, NY: Academic Press; 1978.
15. Sahakyants T, Lee JY, Friedrich PF, Bishop AT, Shin AY. Return of motor function after repair of a 3-cm gap in a rabbit peroneal nerve: a comparison of autograft, collagen conduit, and conduit filled with collagen-GAG matrix. J Bone Joint Surg Am. 2013;95(21):1952e1958.
16. Shrout PE, Fleiss JL. Intraclass correlations: uses in assessing rater reliability. Psychol Bull. 1979;86(2):420e428.
17. Shin RH, Vathana T, Giessler GA, Friedrich PF, Bishop AT, Shin AY. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery. 2008;28(6):452e457.
18. Yi TI, Han IS, Kim JS, Jin JR, Han JS. Reliability of the supraspinatus muscle thickness measurement by ultrasonography. Ann Rehabil Med. 2012;36(4):488e495.
19. Takai Y, Katsumata Y, Kawakami Y, Kanehisa H, Fukunaga T. Ultrasound method for estimating the cross-sectional area of the psoas major muscle. Med Sci Sports Exerc. 2011;43(10): 2000e2004.
20. Reeves ND, Maganaris CN, Narici MV. Ultrasonographic assessment of human skeletal muscle size. Eur J Appl Physiol. 2004;91(1): 116e118.
21. Mendis MD, Wilson SJ, Stanton W, Hides JA. Validity of real-time ultrasound imaging to measure anterior hip muscle size: a comparison with magnetic resonance imaging. J Orthop Sports Phys Ther. 2010;40(9):577e581.

22. Schmitz HC, Beer GM. The toe-spreading reflex of the rabbit revisitedefunctional evaluation of complete peroneal nerve lesions. Lab Anim. 2001;35(4):340e345.
23. Meffert RH, Frey SP, Jansen H, Ochman S, Raschke MJ, Langer M. Muscle strength quantification in small animals: a new transcutaneous technique in rabbits. J Orthop Res. 2008;26(11): 1526e1532.
24. Brodbelt DC, Blissitt KJ, Hammond RA, et al. The risk of death: the confidential enquiry into perioperative small animal fatalities. Vet Anaesth Analg. 2008;35(5):365e373.
25. Singh I. One-sided dominance in the limbs of rabbits and frogs, as evidenced by asymmetry in bone weight. J Anat. 1971;109(pt 2): 271e275.



PART III

Implementation and improvement



6

Comparable functional motor outcomes after repair of peripheral nerve injury with an elastase-processed allograft in a rat sciatic nerve model

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ABSTRACT

Background

A bridging nerve autograft is the gold standard for the repair of segmental nerve injury that cannot be repaired directly. However, limited availability and donor site morbidity remain major disadvantages of autografts. Here, a nerve allograft decellularized with elastase was compared with an autograft regarding functional motor outcome in a rat sciatic segmental nerve defect model. Furthermore, the effect of storage on this allograft was studied.

Methods

Sixty-six Lewis rats (250-300 g) underwent a 10-mm sciatic nerve reconstruction using either a cold- (n=22) or frozen-stored (n=22) decellularized nerve allograft or an autograft (n=22). Sprague-Dawley rats (300-350 g) served as full major histocompatibility complex-mismatched donors. Functional motor outcome was evaluated after 12 and 16 weeks. Ankle angle, compound muscle action potential (CMAP), isometric tetanic force, wet muscle weight and histomorphometry were tested bilaterally.

Results

For CMAP and isometric tetanic force, no significant differences were observed between groups. In contrast, for ankle angle, histomorphometry and muscle weight, the cold-stored allograft performed comparable to the autograft, while the frozen-stored allograft performed significantly inferior to the autograft. At week 16, ankle angle was 88.0±3.1% in the cold-stored group, 77.4±3.6% in the frozen-stored group and 74.1±3.1% in the autograft group ($P<.001$); At week 16, the muscle weight showed a recovery up to 71.1±4.8% in the autograft group, 67.0±6.6% in the cold-stored group and 64.7±3.7% in the frozen-stored group ($P<.05$).

Conclusions

A nerve allograft decellularized with elastase, if stored under the right conditions, results in comparable functional motor outcomes as the gold standard, the autograft.

INTRODUCTION

Traumatic injuries to peripheral nerves can cause considerable disability and economic burden[1]. Although highly prevalent in military conflicts, peacetime injuries commonly result from trauma secondary to motor vehicle accidents, penetrating trauma, industrial injuries and falls. Five percent of all patients admitted to Level I trauma centers have a peripheral nerve injury[2-4]. The majority of peripheral nerve injuries requires surgical reconstruction to restore sensation and function[5]. The gold standard for nerve injuries that cannot be repaired directly is the nerve autograft[6]. Typically, the sural nerve is harvested and sectioned into cables to fit the diameter and length of the defect[7]. However, a limitation of using autografts is their limited diameter and length, the associated donor site morbidity and their limited supply[8]. This has constrained the ability to optimally reconstruct nerves of patients with multiple or large segmental defects where the length of the nerve graft needed far exceeds the availability and necessitates prioritization of the nerves to be reconstructed. In the last decades, several alternatives have been proposed to replace the autograft, each with its own advantages and disadvantages[9-14]. Currently available reconstruction options are nerve grafts (auto- and allograft), biological conduits (*e.g.* arterial and venous) and synthetic conduits (collagen, polyglycolic acid and caprolactone)[14].

One of the proposed reconstruction alternatives is the use of a nerve allograft. Theoretically, a nerve allograft will more closely mimic the structure of the autograft than an empty conduit. The first allografts introduced required the use of immunosuppressants in order to prevent rejection[15]. Although progress has been made in the processing of nerve allografts in order to prevent rejection, further development is required before these can be used instead of an autograft, especially in longer nerve gaps. Clinical studies have demonstrated that processed allografts have a sensory nerve recovery similar to that of autografts but that motor recovery remains inferior to the autograft[16,17]. Previous research by our group has shown that, in a rat model, the allograft nerve processed by AxoGen (AxoGen, Alachua, Florida) showed comparable results to the autograft at 12 weeks. However, at 16 weeks, there was a statistically significant difference, where the allograft isometric motor strength degraded while the autograft continued to improve strength[9]. This inferior motor recovery stimulated the search for a better, optimized nerve allograft. The allograft process was improved by the addition of elastase, and demonstrated significant improvements in an *in vitro* study where different processing techniques and storage protocols were compared[18]. Based on histological outcome, the allograft process was improved and storage (frozen *vs*. cold) was found to have a significant influence on the structure of the grafts. In order to improve motor function after nerve reconstruction using processed nerve allografts, our lab recently improved the processing technique of the nerve allograft by using the enzyme elastase and comparing the effect of frozen and cold storage[18]. The purpose of this study was to study whether the use of an allograft nerve that was decellularized by means of elastase in a segmental sciatic nerve gap rat model resulted in improved motor nerve function. In addition, we investigated whether this effect was influenced by the storage conditions of the

decellularized allograft. The main and novel finding of this study is that this decellularized nerve allograft, if stored properly, results in comparable functional motor outcomes as the gold standard, the nerve autograft.

MATERIALS & METHODS

After approval by the Institutional Animal Care and Use Committee, 66 Lewis rats (weighing 250-300 grams and aged 10-12 weeks) were used for the experiments. Twenty-two Sprague-Dawley rats (weighing 300-350 grams and aged 10-12 week) served as full major histocompatibility complex (MHC)-mismatched nerve donors. Lewis rats were used as they are known for their reduced tendency for autotomy[19]. Animals were randomly divided into 3 groups, each treated for a 10 mm sciatic nerve gap. Group I (n=22) served as a control and was treated with a nerve autograft. Group II (n=22) had a nerve gap reconstructed with a processed nerve allograft that was cold stored ('allograft cold'). Group III (n=22) had a similar procedure except that the processed allograft was freeze-stored ('allograft frozen'). The rats were given food and water *ad libitum* and were individually housed with a 12-hour light-dark cycle.

Nerve processing

Twenty-two Sprague-Dawley rats weighing 300-350 grams, were sacrificed with an intraperitoneal injection of pentobarbital. The sciatic nerve on both sides was exposed with a mid-gluteal incision, the nerve was carefully dissected after which a 15 mm segment was harvested aseptically. The proximal cut was made in all animals at the same anatomical landmark, namely where the nerve exits the muscle. Nerve allografts were prepared as previously described[18,20]. Briefly, nerve segments were placed in RPMI medium, followed by subsequent steps using different detergents, two enzymatic steps and gamma irradiation[18]. In the end, the processed allografts were either stored in phosphate buffered saline at 4°C ('allograft cold') or in Ringers Lactate at -80°C ('allograft frozen') for 14 days.

Surgical procedure

Rats were anesthetized with an intraperitoneal mixture of ketamine (Ketaset®, 100 mg/ml, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Vettek™, 100 mg/ml, Bluesprings, MO) 10:1 mixture, administering a dose of 1 ml/kg body weight after initial induction with isoflurane. Anesthesia was maintained with additional doses of ketamine only. Ringers Lactate was administrated subcutaneously to prevent dehydration, and body temperature was maintained with a heating pad. The left sciatic nerve was exposed with a mid-gluteal incision. A 10 mm sciatic nerve segment was excised under an operating microscope (Zeiss OpMi6, Carl Zeiss Surgical, Oberkochen, Germany). In the control group (group I), the nerve segment was reversed and put back. In groups II and III, a 10 mm nerve allograft, cold or frozen, respectively, was used to bridge the 10 mm nerve gap using 10-0 nylon epineural sutures. For this we used 6 stitches. The muscle was approximated and the skin was closed with 5-0 Vicryl rapid sutures. Postoperatively trimethoprim/sulfadiazine 30 mg/kg (Tribrissen,

Five Star Compounding Pharmacy, Clive, IA) was administered to prevent infection and buprenorphine (Buprinex®, 0.1 ml/kg, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) served as an analgesic.

Evaluation of Motor Functional Outcome

For the sacrificial procedure, at 12 and 16 weeks, the animals were anesthetized as described above. The passive ankle angle, compound muscle action potential (CMAP), isometric tetanic force and wet muscle weight were tested bilaterally. Distal nerve segments were analyzed for histomorphometry.

Ankle Angle

The maximum passive plantar flexion angle of the ankle was measured bilaterally in all animals to determine the ankle contracture angle as described previously[8,21]. The angle between the anterior aspect of the tibia and the dorsal aspect of the foot with the ankle in maximal passive plantar flexion was measured.

Electrophysiology

The sciatic nerve was exposed as described previously. A miniature bipolar electrode (Harvard Apparatus, Holliston, MA) was attached proximal to the nerve graft. Recording electrodes were placed subcutaneously to the tibialis anterior muscle and a ground electrode was placed in the surrounding tissue. CMAP was measured using an EMG (VikingQuest, Nicolet Biomedical, Madison, WI). The maximal amplitude was recorded. In a similar fashion the contralateral side was measured.

Maximum Isometric Tetanic Force

For obtaining the maximum isometric tetanic force, the peroneal nerve, distal of the nerve graft was exposed. The force measurements were executed as previously described[22]. The tibial muscle was carefully freed from its insertion while preserving the neurovascular pedicle. The hind limb was stabilized with 2 Kirschner wires (DePuy Orthopedics) in the distal femur and ankle joint. The distal tendon of the tibial muscle was attached to a force transducer (MDB-0.5, Transducer Techniques, Temecula, CA) using a custom clamp with the tendon aligned in the anatomical position. The force transducer signal was processed and analyzed with a computer using LabView software (National Instruments). A miniature bipolar electrode (Harvard Apparatus) was attached to the peroneal nerve. The nerve was stimulated with a stimulator (Grass SD9, Grass Instrument Co., Quincy, MA). After establishing the optimal preload or muscle resting length, the stimulus intensity was increased until maximum tetanic muscle force was reached.

Wet Muscle Mass

After bilateral force testing, animals were sacrificed with an overdose of pentobarbital intraperitoneally. The tibial muscles of both hind limbs were carefully dissected and

weighed immediately to obtain muscle mass ratio.

Histomorphometry

Nerve segments of the peroneal nerve were excised and stored in Trumps solution (37% formaldehyde and 25% glutaraldehyde) and subsequently embedded in spur resin. 1 µm sections were cut and stained with 1% Toluidine Blue. Images were acquired with a digital camera and analysis was performed using Image ProPlus Software (Media Cybernetics Inc., Bethesda, MD) where nerve area, total myelin area, number of axons and total axon area were obtained in semi-automatic fashion. The N-ratio was calculated as the total myelinated fiber area divided by the total tissue cable area, which provides information regarding the number of sprouting events of the regenerating nerve.[30,31]

Statistical Analysis

Statistical analysis was performed by use GraphPad Prism 5 software (GraphPad Software, CA, USA). The groups were compared with respect to ankle contracture, electrophysiology, maximum isometric tetanic force, muscle weight and histomorphometry. Data are expressed as a percentage of the contralateral (healthy) side to diminish intra-animal differences. One-way analysis of variance (ANOVA) followed by a Bonferroni correction for multiple testing was used for statistical analysis. All results are presented as mean ± standard deviation (SD). A P-value of .05 was considered significant.

RESULTS

All animals survived the surgical procedure and no complications were observed. A summary of the experimental results is presented in Table 1.

Ankle Angle

The percentage of recovery of the ankle angle contracture of the experimental side compared to the contralateral side was 80.2 ± 3.1% in group I, 73.7 ± 3.9% in group II and 73.1 ± 4.2% in group III at 12 weeks. At 16 weeks the recovery was 88.0 ± 3.1% in group I, 77.4 ± 3.6% in group II and 74.1 ± 3.1% in group III. A significant difference was observed between groups I and III ($p<.001$) at both 12 and 16 weeks postoperatively (Figure 1).

Electrophysiology

Recovery of the CMAP at 12 weeks was 41.9 ± 14.4% in group I and 44.5 ± 15.1% and 40.8 ± 5.3% in group II and III, respectively. At 16 weeks the recovery increased to 44.0 ± 21.9% in group I and 56.2 ± 14.0% and 53.5 ± 12.7% in group II and III, respectively. Group comparison showed no statistically significant difference between all groups at both time points (Figure 1).

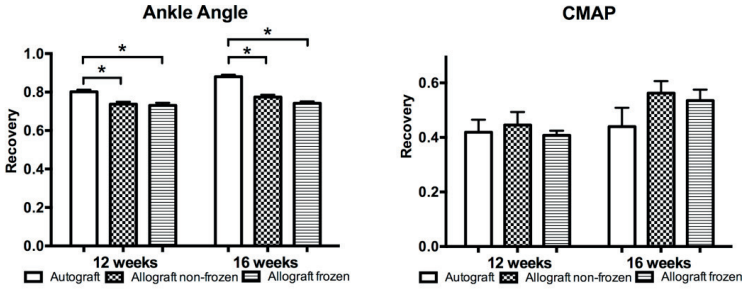


Figure 1. Group comparison for ankle angle contracture and CMAP (compound muscle action potential). Results are expressed as a percentage of the contralateral, normal side and are presented as mean ± standard deviation. *Indicates significance (P<.05).

Isometric Tetanic Force

In group I, the percentage of muscle force recovery at 12 weeks was found to be 42.3 ± 5.8%. Group II showed the highest recovery with 48.7 ± 7.6% and group III was 43.2 ± 10.1%. In the late follow-up time, at 16 weeks, the muscle force was recovered to 53.9 ± 12.0% in group I and 55.4 ± 12.7% and 50.0 ± 11.4% in group II and III, respectively. No statistically significant differences were found when the groups were compared at the early and late follow-up times (Figure 2).

Muscle Weight

The muscle mass ratio of the tibial muscle at the 12th follow-up week was 63.7 ± 4.9%, 60.2 ± 4.7%, 58.3 ± 4.1% in group I, II and III, respectively. At 16 weeks, the muscle weight showed a recovery up to 71.1 ± 4.8% in the autograft group, 67.0 ± 6.6% in the cold allograft group and 16 64.7 ± 3.7% in the frozen allograft group. At both time points, a statistically significant difference was observed between groups I and III (P<.05 both at week 12 and 16). The autograft performed better than the frozen allograft. No difference was found when comparing the autograft to the cold allograft (Figure 2).

Histomorphometry

Figure 3 shows the normalized results of the histomorphometry of the different groups 16 weeks postoperatively. Total nerve area, myelin area and axon area were statistically lower in the frozen allograft group when compared to the autograft (P<.05). The N-ratio showed no statistically significant differences between the three groups (P=.14). The cold stored allograft did not significantly differ from the nerve autograft (Figures 3 and 4).

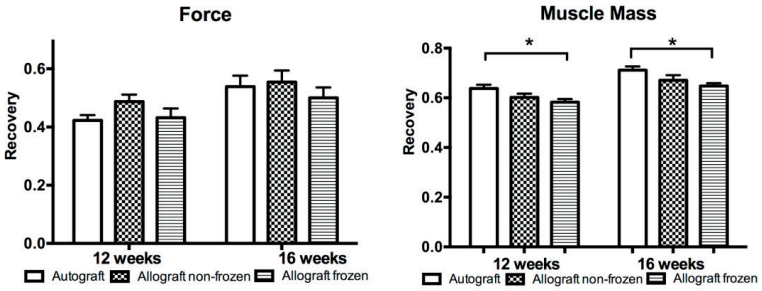


Figure 2. Group comparison for isometric tetanic force and muscle weight. Results are expressed as a percentage of the contralateral, normal side and are presented as mean ± standard deviation. *Indicates significance P<.05).

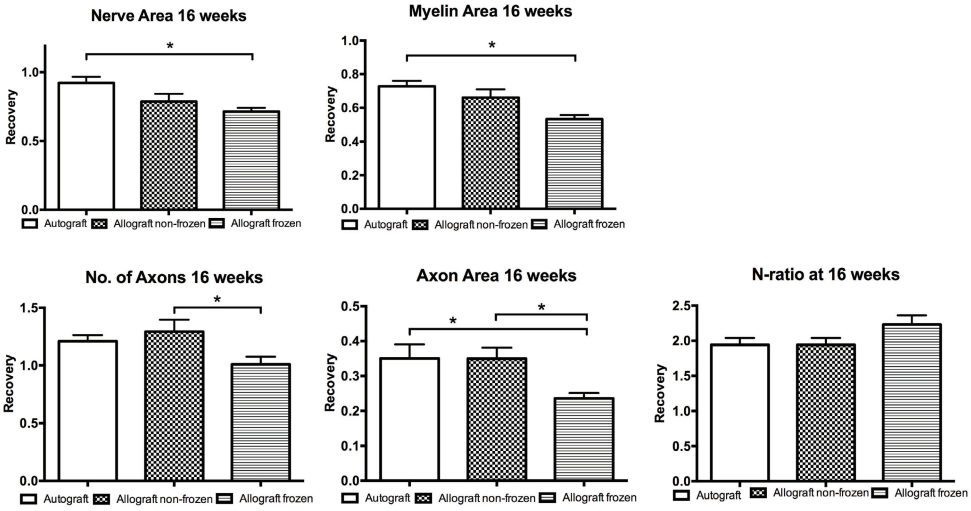


Figure 3. Group comparison of histomorphometry of nerve area, myelin area, number of axons, total axon area and N-ratio at 16 weeks postoperative. Results are expressed as a percentage of the contralateral, normal side and are presented as mean ± standard deviation. *Indicates significance (P<.05).

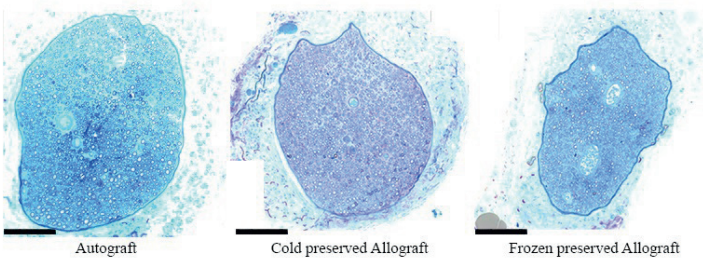


Figure 4. Histological transverse sections of the peroneal nerve at 20 x magnification. Nerve size was similar between the 3 groups at both time points. Scale bar represents 100 mm.

Table 1. Summary of results of test for all groups.							
		Group I		Group II		Group III	
Surgical intervention		*Autograft*		*Cold stored Allograft*		*Frozen stored Allograft*	
No. of animals tested		11	11	11	11	11	11
Sacrifice time (wk)		12	16	12	16	12	16
Maximum passive plantar flexion ankle angle (%)		80.2 ± 1.0	88.0 ± 0.9	73.7 ± 1.2	77.4 ± 1.1	73.1 ± 1.3	74.1 ± 0.1
CMAP (%)		41.9 ± 4.6	44.0 ± 6.9	44.6 ± 4.8	56.2 ± 4.4	40.8 ± 1.7	53.5 ± 4.0
Maximum isometric tetanic tension (%)		42.3 ± 1.8	53.9 ± 3.8	48.7 ± 2.4	55.4 ± 4.0	43.2 ± 3.2	50.0 ± 3.6
Tibialis anterior wet muscle weight (%)		63.7 ± 1.5	71.1 ± 1.5	60.2 ± 1.5	67.0 ± 2.1	58.3 ± 1.3	64.7 ± 1.2
Histomorphometry of peroneal nerve (%)	Nerve area	74.6 ± 4.9	92.2 ± 4.4	86.9 ± 5.9	78.7 ± 5.6	76.7 ± 3.6	71.4 ± 2.7
	Myelin area	55.5 ± 4.1	72.8 ± 3.3	75.7 ± 6.9	66.1 ± 4.9	57.3 ± 3.2	53.4 ± 2.3
	No. of axons	120.1 ± 11.5	130.0 ± 5.4	138.4 ± 9.5	130.3 ± 10.4	101.8 ± 4.7	101.0 ± 6.6
	Axon area	29.8 ± 3.7	35.0 ± 4.0	30.7 ± 3.0	35.0 ± 3.1	18.9 ± 11.3	23.6 ± 1.5

Results are depicted as a percentage compared to the contralateral, healthy side and expressed as mean ± standard deviation.

DISCUSSION

The goal of this study was first, to evaluate the functional outcome of the elastase processed nerve allograft in an animal segmental motor nerve defect and second, to compare the effect of frozen and cold storage of the nerve allograft on functional outcome. Developing a nerve allograft that is unlimited in supply, without the harmful donor side morbidity and with equivalent outcomes as the autograft would be a tremendous advance in peripheral nerve surgery. It is essential in the optimization of longitudinal nerve regeneration that this alternative has a 3-dimensional structure[23]. Additionally, this scaffold should cause no immunological reaction, avoiding the need for administering immunosuppressive agents.

Our results demonstrate that there is a superior regeneration potential of the cold-stored elastase processed nerve allograft compared to the frozen stored allograft. This graft also showed statistically equivalent results compared to the nerve autograft, with regards to the functional outcome. The novelty of this study lies in the fact that this elastase-processed allograft results in comparable *motor* and not just s*ensory* recovery as the autograft.

Previous studies were executed to evaluate the allograft's performance, both *in vitro* and *in vivo*. Hudson described one of the first processing protocols for the nerve allograft[24,25] which has been modified by several others. Moore *et al*. modified and tested the protocol but unfortunately, investigated only 4 animals per group and therefore lacked power to draw any

conclusions[26]. Whitlock *et al*. studied the effect of the first commercially processed allograft (AxoGen, Alachua, Florida) in 14- and 28 mm gaps in rats and evaluated walking track analysis, muscle mass and histomorphometry. They found comparable results between the allograft and a collagen conduit, but the allograft was not able to outperform the autograft 12 weeks post-operatively[12]. Interestingly, the study of Whitlock was the basis for the clinical study with the commercially available nerve allograft (*i.e.* the Ranger study) without the confirmation of comparable results at late recovery. Giusti *et al*. evaluated early and late recovery at 12 and 16 weeks, using AxoGen processed allograft rat nerves and compared these to autograft nerves. While both the allograft and the autograft had comparable results at the early recovery time of 12 weeks, 16 weeks post-surgery the autograft outperformed the AxoGen processed rat allograft nerve[9]. This could be explained by the fact that the allografts still showed intraluminal remaining debris. This could reduce or inhibit the nerve regenerative capacity since the debris results in a fibrotic intra-luminal occlusive blockade. This degradation of motor function could be secondary to the inflammatory reaction as a consequence of the immunogenicity. As such, the ultimate goal is to process a nerve allograft by removing all cells and cellular debris, which are targeted in an immune response, while preserving the extracellular matrix. To this end we aimed to optimize the processing technique of the nerve allograft in a previous study. In addition to developing a novel processing technique, the optimal storage technique was studied. Therefore we challenged different processing and storage protocols *in vitro* and described our elastase processed nerve allograft[18].

To this end, in this *in vivo* study the regenerative capacity of the elastase processed nerve allograft was evaluated. Motor function analysis is the most reliable and important way to evaluate the neuroregenerative capacity of different nerve grafts[22]. An interesting observation is the significantly superior result (*i.e.* 3 out of 5 assessments) of the cold stored graft compared to the frozen graft. This was observed in both early and late nerve regeneration. The cold preserved allograft showed no statistically significant difference -and thus comparable results- with the nerve autograft. The difference between the two storage techniques was in line with the previous *in vitro* study[18]. First, the results of this study confirm the superior effect of the cold preservation as found in our *in vitro* study. Second, when comparing the results of this study to the *in vivo* study with the AxoGen processed allograft (Giusti *et al*.), as described above, improved regeneration was observed at 16 weeks, favoring the elastase processed allograft, compared to the degradation in motor function which was observed at the 16 week time point in the previous *in vivo* study[9].

Ankle contracture, although minimal, was observed in all animals at both time points. Ankle contracture has shown to be correlated with muscle force and muscle weight[21,27]. In our study a difference was found in ankle contracture between the autograft group and the frozen allograft group at both time points. No difference was observed when comparing the cold allograft group to the autograft.

Isometric tetanic muscle force testing is a reliable method to test motor nerve recovery[22]. In this study, the differences between the groups were, with regard to muscle force, not statistically different. This suggests that the processed allograft can produce results similar to the autograft. The results of the electrophysiological testing were similar to the isometric tetanic force testing. Wet muscle mass showed, independent of recovery time, comparable results between the nerve autograft and the cold preserved allograft. On the other hand, significantly lower scores were found for the frozen allograft when compared to the autograft, suggesting an inferior regeneration potential. Histomorphometrical analysis of the peroneal nerve, distal to the nerve graft, showed statistically comparable results between the cold preserved allograft and the autograft for all parameters investigated. The frozen allograft showed inferior values to the autograft, which is in line with the functional testing.

Clinically, the use of commercially available decellularized human nerves (AxoGen, Alachua, Florida) for motor nerve reconstruction have been reported in three case reports and recently in a larger multicenter study[17,28,29]. The case reports show results that are not compared to any other treatment and therefore lack the ability to show equality or even superiority of the allograft to the autograft. In our opinion, the results of the multicenter study did not provide satisfying results despite the positive conclusions made[16,17]. Similar to the case reports, no control patients were included, and thus the possibility to put the results in perspective are unfortunately lacking. Another study with this allograft was used for the evaluation of reconstruction of sensory nerve defects but also did not compare the nerve allograft with the nerve autograft[10]. Although this has been a step forward, we believe that the introduction of a new allograft needs careful evaluation in both *in vitro* and *in vivo* studies to thoroughly investigate neuroregenerative capacity. Not before non-inferiority or superiority has been proven, clinical testing is justified.

We recognize the limitations of this study in particular the use of the rat model. The rat has a rapid neuroregenerative capacity, a limited gap size and its size prohibits serial immunological testing. Although the regeneration is argued to be too fast, significant differences between groups have been demonstrated in previous studies, as well as with this study. The limitation of the nerve gap length and the immunological testing could be overcome by using a larger animal.[33,34] Furthermore, some may argue that the current work has limited novelty as the concept of an acellular nerve allograft was proposed in the early 1990's and since then many have investigated the optimal way to prepare acellular nerve allografts. Recently, some researchers even used mesenchymal stem cells and cytokine therapy.[35-38] As discussed above, we feel that the present study does move the field forward because unlike in previous studies we demonstrate that with our elastase-processed allograft, motor recovery, in addition to sensory recovery is comparable to that which can be obtained by use of an autograft.
Finally, we did not perform walking track analysis in the present study. The reasons we chose not to incorporate this test in our set of experiments lies in the fact that previous studies have

shown that walking track analysis is poorly correlated with other measurements of functional outcome.[21,32]

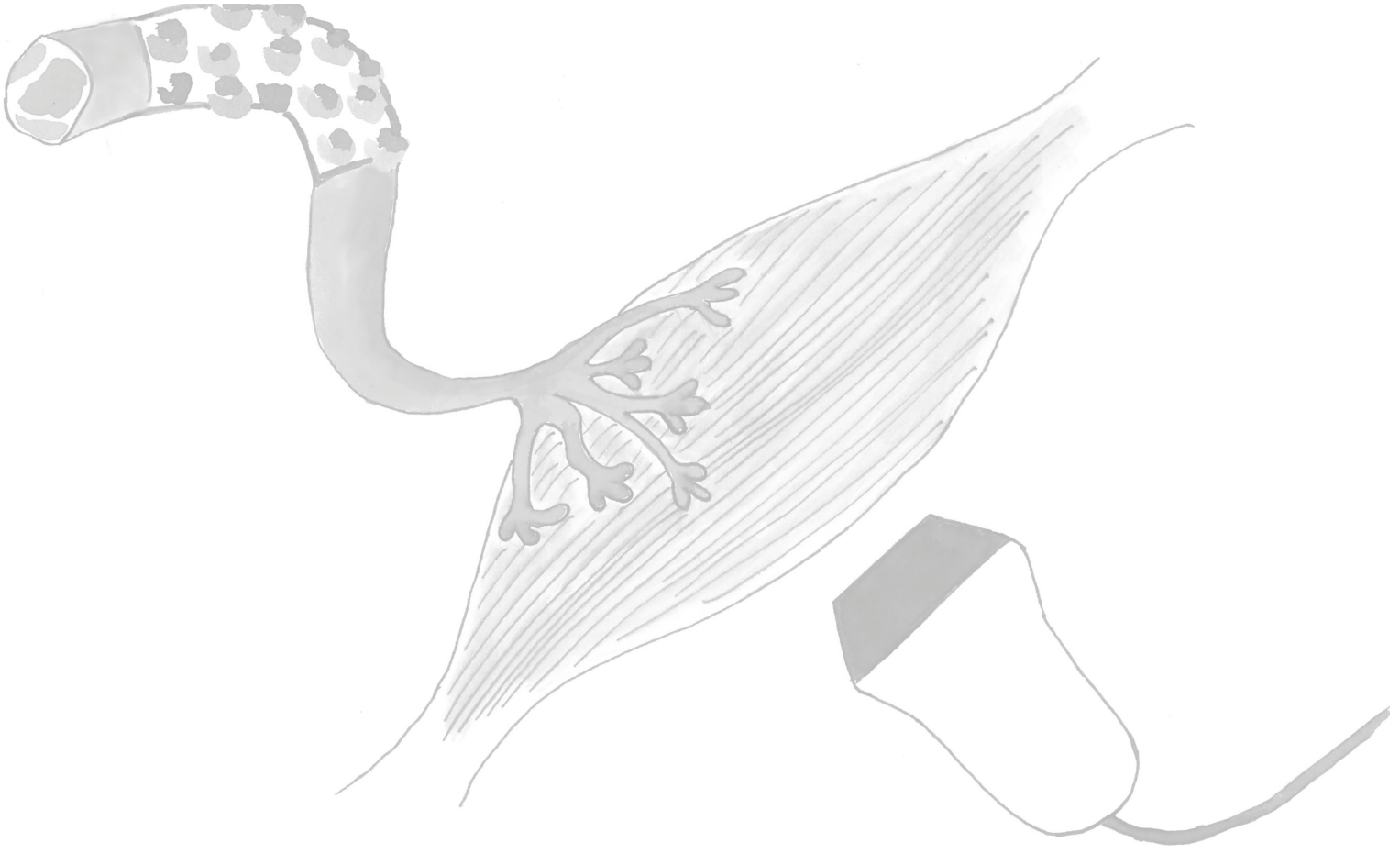
CONCLUSION

These limitations notwithstanding, we can conclude that the repair of a peripheral nerve injury with an elastase-processed allograft, if stored under the right conditions, results in comparable functional motor outcomes as the nerve autograft. These results are a step towards clinical application of allografts which may eventually overcome the limitations of autografts which to this day remain the gold standard.

REFERENCES

1. Jaquet JB, Luijsterburg AJ, Kalmijn S, Kuypers PD, Hofman A, Hovius SE. Median, ulnar, and combined median-ulnar nerve injuries: functional outcome and return to productivity. The Journal of trauma 2001;51(4):687-692.
2. Noble J, Munro CA, Prasad VS, Midha R. Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. The Journal of trauma 1998;45(1):116-122.
3. Taylor CA, Braza D, Rice JB, Dillingham T. The incidence of peripheral nerve injury in extremity trauma. American journal of physical medicine & rehabilitation / Association of Academic Physiatrists 2008;87(5):381-385.
4. Campbell BT, Saleheen H, Borrup K, McQuay J, Luk S, Hiscoe J, Lapidus G. Epidemiology of trauma at a level 1 trauma center. Connecticut medicine 2009;73(7):389-394.
5. Kovachevich R, Kircher MF, Wood CM, Spinner RJ, Bishop AT, Shin AY. Complications of intercostal nerve transfer for brachial plexus reconstruction. The Journal of hand surgery 2010;35(12):1995-2000.
6. Millesi H. Nerve grafting. CLIN PLAST SURG 1984;11(1):105-113.
7. Berger A, Millesi H. Nerve grafting. Clin Orthop Relat Res 1978(133):49-55.
8. FF IJ, Nicolai JP, Meek MF. Sural nerve donor-site morbidity: thirty-four years of follow-up. Annals of plastic surgery 2006;57(4):391-395.
9. Giusti G, Willems WF, Kremer T, Friedrich PF, Bishop AT, Shin AY. Return of motor function after segmental nerve loss in a rat model: comparison of autogenous nerve graft, collagen conduit, and processed allograft (AxoGen). J Bone Joint Surg Am 2012;94(5):410-417.
10. Karabekmez FE, Duymaz A, Moran SL. Early clinical outcomes with the use of decellularized nerve allograft for repair of sensory defects within the hand. Hand (N Y) 2009;4(3):245-249.
11. Li ST, Archibald SJ, Krarup C, Madison RD. Peripheral nerve repair with collagen conduits. Clinical materials 1992;9(3-4):195-200.
12. Whitlock EL, Tuffaha SH, Luciano JP, Yan Y, Hunter DA, Magill CK, Moore AM, Tong AY, Mackinnon SE, Borschel GH. Processed allografts and type I collagen conduits for repair of peripheral nerve gaps. MUSCLE NERVE 2009;39(6):787-799.
13. Shin RH, Friedrich PF, Crum BA, Bishop AT, Shin AY. Treatment of a segmental nerve defect in the rat with use of bioabsorbable synthetic nerve conduits: a comparison of commercially available conduits. The Journal of bone and joint surgery American volume 2009;91(9):2194-2204.
14. Griffin JW, Hogan MV, Chhabra AB, Deal DN. Peripheral nerve repair and reconstruction. J Bone Joint Surg Am 2013;95(23):2144-2151.
15. Mackinnon SE, Doolabh VB, Novak CB, Trulock EP. Clinical outcome following nerve allograft transplantation. Plast Reconstr Surg 2001;107(6):1419-1429.
16. Cho MS, Rinker BD, Weber RV, Chao JD, Ingari JV, Brooks D, Buncke GM. Functional outcome following nerve repair in the upper extremity using processed nerve allograft. The Journal of hand surgery 2012;37(11):2340-2349.
17. Brooks DN, Weber RV, Chao JD, Rinker BD, Zoldos J, Robichaux MR, Ruggeri SB, Anderson KA, Bonatz EE, Wisotsky SM, Cho MS, Wilson C, Cooper EO, Ingari JV, Safa B, Parrett BM, Buncke GM. Processed nerve allografts for peripheral nerve reconstruction: a multicenter study of utilization and outcomes in sensory, mixed, and motor nerve reconstructions. Microsurgery 2012;32(1):1-14.
18. Hundepool CA, Nijhuis TH, Kotsougiani D, Friedrich PF, Bishop AT, Shin AY. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. Neurosurg Focus 2017;42(3):E4.
19. Carr MM, Best TJ, Mackinnon SE, Evans PJ. Strain differences in autotomy in rats undergoing sciatic nerve transection or repair. Annals of plastic surgery 1992;28(6):538-544.
20. Giusti G, Lee J, Kremer T, Friedrich P, Bishop AT, Shin AY. The influence of vascularization of transplanted processed allograft nerve on return of motor function in rats. Microsurgery. 2016 Feb;36(2):134-43.

21. Lee JY, Giusti G, Wang H, Friedrich PF, Bishop AT, Shin AY. Functional evaluation in the rat sciatic nerve defect model: a comparison of the sciatic functional index, ankle angles, and isometric tetanic force. Plast Reconstr Surg 2013;132(5):1173-1180.
22. Shin RH, Vathana T, Giessler GA, Friedrich PF, Bishop AT, Shin AY. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery 2008;28(6):452-457.
23. Lee JY, Giusti G, Friedrich PF, Archibald SJ, Kemnitzer JE, Patel J, Desai N, Bishop AT, Shin AY. The effect of collagen nerve conduits filled with collagen-glycosaminoglycan matrix on peripheral motor nerve regeneration in a rat model. The Journal of bone and joint surgery American volume 2012;94(22):2084-2091.
24. Hudson TW, Zawko S, Deister C, Lundy S, Hu CY, Lee K, Schmidt CE. Optimized acellular nerve graft is immunologically tolerated and supports regeneration. Tissue Eng 2004;10(11-12):1641-1651.
25. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. Tissue Engineering 2004;10(9-10):1346-1358.
26. Moore AM, MacEwan M, Santosa KB, Chenard KE, Ray WZ, Hunter DA, Mackinnon SE, Johnson PJ. Acellular nerve allografts in peripheral nerve regeneration: A comparative study. MUSCLE NERVE 2011;44(2):221-234.
27. Lin FM, Pan YC, Hom C, Sabbahi M, Shenaq S. Ankle stance angle: a functional index for the evaluation of sciatic nerve recovery after complete transection. J Reconstr Microsurg 1996;12(3):173-177.
28. Guo Y, Chen G, Tian G, Tapia C. Sensory recovery following decellularized nerve allograft transplantation for digital nerve repair. J Plast Surg Hand Surg 2013;47(6):451-453.
29. Gunn S, Cosetti M, Roland JT, Jr. Processed allograft: novel use in facial nerve repair after resection of a rare racial nerve paraganglioma. Laryngoscope 2010;120 Suppl 4:S206.
30. Itoh S, Takakuda K, Ichinose S, Kikuchi M, Schinomiya K. A study of induction of nerve regeneration using bioabsorbable tubes. Journal of reconstructive microsurgery 2001;17(2):115-123.
31. Vleggeert-Lankamp C. The role of evaluation methods in the assessment of peripheral nerve regeneration through synthetic conduits: a systematic review. Laboratory investigation. J Neurosurg. 2007 Dec;107(6):1168-89.
32. Dellon AL, Mackinnon SE. Sciatic nerve regeneration in the rat. Validity of walking track assessment in the presence of chronic contractures. Microsurgery. 1989;10(3):220-5.
33. Sahakyants T, Lee JY, Friedrich PF, Bishop AT, Shin AY. Return of motor function after repair of a 3-cm gap in a rabbit peroneal nerve: a comparison of autograft, collagen conduit, and conduit filled with collagen-GAG matrix. J Bone Joint Surg Am. 2013 Nov 6;95(21):1952-8.
34. Giusti G, Kremer T, Willems WF, Friedrich PF, Bishop AT, Shin AY. Description and validation of isometric tetanic muscle force test in rabbits.Microsurgery. 2012 Jan;32(1):35-42.
35. Zhang Y, Zhang H, Zhang G, Ka K, Huang W. Combining acellular nerve allografts with brain-derived neurotrophic factor transfected bone marrow mesenchymal stem cells restores sciatic nerve injury better than either intervention alone. Neural Regen Res. 2014 Oct 15;9(20):1814-9.
36. Song Y, Wang Z, Wang Z, Zhang H, Li X, Chen B. Use of FK506 and bone marrow mesenchymal stem cells for rat hind limb allografts. Neural Regen Res. 2012 Dec 5;7(34):2681-8.
37. Wang Y, Zhao Z, Ren Z, Zhao B, Zhang L, Chen J, Xu W, Lu S, Zhao Q, Peng J. Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration. Neurosci Lett. 2012 Apr 11;514(1):96-101
38. Liu G, Cheng Y, Guo S, Feng Y, Li Q, Jia H, Wang Y, Tong L, Tong X. Transplantation of adipose-derived stem cells for peripheral nerve repair. Int J Mol Med. 2011 Oct;28(4):565-72.



7

Functional outcome after reconstruction of a long nerve gap in rabbits using optimized decellularized nerve allografts

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Submitted

ABSTRACT

Background

Processed nerve allografts are a promising alternative to nerve autografts providing an unlimited readily available supply while avoiding donor site morbidity and the need for immunosuppression. Currently, clinically available nerve allografts do not provide satisfactory results for motor reconstruction. The aim of this study was to evaluate functional recovery after reconstruction of a long nerve gap using an optimized nerve allograft. Furthermore, the influence of storage techniques was evaluated.

Methods

Nerve allografts were decellularized using elastase and detergents and stored at either 4 or -80°C. In 36 New Zealand White rabbits, a 3-cm peroneal nerve gap was repaired with either an autograft (group 1, control) or cold- (group 2) or frozen-stored (group 3) processed nerve allograft. Nerve recovery was evaluated using various measurements including longitudinal ultrasound measurements and electrophysiology (CMAP), isometric tetanic force (ITF), wet muscle weight (MW) and histomorphometry at 24 weeks follow-up.

Results

The cold preserved allograft provided earlier regeneration than the frozen-stored allograft as seen with longitudinal ultrasound measurements. Ultrasound furthermore showed significantly inferior recovery in group 3 than in both other groups ($p < 0.05$). MW and ITF both showed similar outcomes in the autograft and cold-stored allograft groups ($p = 0.096$ (MW) and $p = 0.286$ (ITF)). MW and ITF confirmed inferiority of the frozen-stored allograft to the autograft ($p < 0.01$ (MW) and $p = 0.02$ (ITF)).

Conclusions

Nerve reconstruction with the cold-stored optimized nerve allograft results in successful recovery of a long motor nerve defect in a rabbit model, statistically equivalent to the gold standard nerve autograft. Freeze-storage of the processed allograft impairs the functional outcome.

INTRODUCTION

The treatment of peripheral nerve injuries where direct tension free end-to-end suture is not possible still represents a clinical challenge.[1] The current gold standard, the autologous nerve graft, has important disadvantages including donor site morbidity, chance of painful neuromas and a limited amount of available tissue. Avoiding donor site morbidity would be a great advantage, especially for young children who require nerve grafting after, for example, birth related brachial plexus injury. The limited amount of available nerve graft is a problem particularly for large defects such as in brachial plexus trauma cases. A shortage of donor material may require the surgeon to prioritize reconstructive options, impeding optimal results. Furthermore, the additional procedure to harvest the sural nerve may lengthen operative time, especially in obese patients.[2]

Processed nerve allografts are a promising alternative, providing a readily available and unlimited supply while avoiding donor site morbidity and the need for immunosuppression. Although some successful recovery using decellularized nerve allografts in the clinical setting is reported for sensory nerve gaps, comparative studies that show satisfactory results for mixed and motor nerve defects are lacking.[3-5]

To improve the functional results of processed nerve allografts, elastase has been reported to optimized the decellularization technique.[6] This optimized elastase processed nerve allograft was subsequently implemented in a 1-cm gap in the rat model, yielding promising functional results and indicating that storage technique can influence outcome.[7] However, previous studies have shown that successful recovery in a short gap in the rat model does not guarantee success in larger gaps.[8,9] Longer gaps can be created in a large animal model, such as the rabbit model. The rabbit model not only allows for longer nerve gaps, but the rabbit's neuroregenerative and immunological properties also more closely mimic the human situation, providing an important step prior to research in a clinical setting. [10,11]

In this study, we aimed to evaluate the functional recovery after reconstruction of a 3-cm nerve gap using the optimized processed nerve allograft in the rabbit model. Furthermore, since little is known about the effect of storage temperature on functional recovery, the influence of storage technique was also evaluated.

MATERIALS AND METHODS

After obtaining approval from our institutional animal care and use committee (IACUC), 36 male New Zealand White rabbits (Harlan Laboratories, Inc, Indianapolis, IN), weighing 2.5–3 kg, were randomly divided into three experimental groups. All animals underwent a 3-cm peroneal nerve reconstruction. In group 1 a reversed autograft segment was used (control group), in group 2 the cold stored allograft was used and in group 3 the frozen stored allograft was used to bridge the gap. The surgical side was randomized to diminish the effect of side preference.[9,11,12] Animals were housed individually with a 12-hour light–dark cycle. Food and water were provided *ad libitum*.

At baseline and during follow-up multiple ultrasound measurements of the tibialis anterior muscle were obtained. Six months after reconstruction, animals underwent a non-survival procedure to evaluate functional motor recovery. Measurements included ankle angle, compound muscle action potentials (CMAP), isometric tetanic force measurements, wet muscle mass and nerve histomorphometry.

Allograft preparation

Dutch Belted rabbits served as donors for the allografts to obtain a Major Histocompatibility Complex (MHC) mismatch with the recipient New Zealand White rabbits.[13] Twelve donor animals were anesthetized with a combination of ketamine (35mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (5mg/kg, Vettek, Bluesprings, MO) IM and subsequently killed with an overdose of pentobarbital IV (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI). A 4-cm peroneal nerve segment was harvested bilaterally and processed as described previously.[6] Briefly, nerve segments were subsequently placed in different detergent based and enzymatic solutions. Segments were stored at 4°C (cold stored allograft) or -80°C (frozen stored allograft) until use.

Index surgical procedure

Anesthesia was induced with a combination of ketamine (35mg/kg) and xylazine (5mg/kg) IM. Animals were intubated and ventilated with 1-2% isoflurane using a Bain ventilation system (Harvard Apparatus, Holliston, MA) during the procedure. Intravenous lactated Ringer's solution was used to maintain hydration and animal temperature was maintained at 37°C with a heating pad.

With the animals in lateral position, a 10-cm longitudinal incision was made on the posterolateral aspect of the thigh. The biceps femoris was separated from the gluteus medius and vastus lateralis to expose the sciatic nerve and its peroneal branch. A 3-cm segment of the peroneal nerve was dissected at a level 6 cm distal to the border of external obituratory. The nerve segment was excised, reversed and used as an autograft (group 1) or replaced by a 3-cm cold stored (group 2) or frozen stored (group 3) processed allograft nerve. Grafts were sutured with aid of a surgical microscope (Zeiss OpMi 6; Carl Zeiss Surgical, Oberkochen, Germany) using 10-0 nylon epineural sutures (Ethicon, Inc., Somerville, NJ). Figure 1 shows an example of the nerve graft in situ. Wounds were closed in layers using bioabsorbable sutures (Ethicon, Inc., Somerville, NJ). The procedure was performed in a sterile fashion. To prevent postoperative infections, animals received 5 mg/kg enrofloxacin (Baytril; Bayer Animal Health, Shawnee Mission, KS) and 4 mg/kg carprofen (Rimadyl; Pfizer Animal Health, New York, NY) subcutaneously daily for 5 days postoperatively. Buprenorphine (0.18 mg/kg, Buprinex; Reckitt Benckiser Pharmaceuticals, Inc, Richmond, VA) was administered subcutaneous for pain control. An Elizabethan collar was placed on the rabbits until adequate healing of the wound.

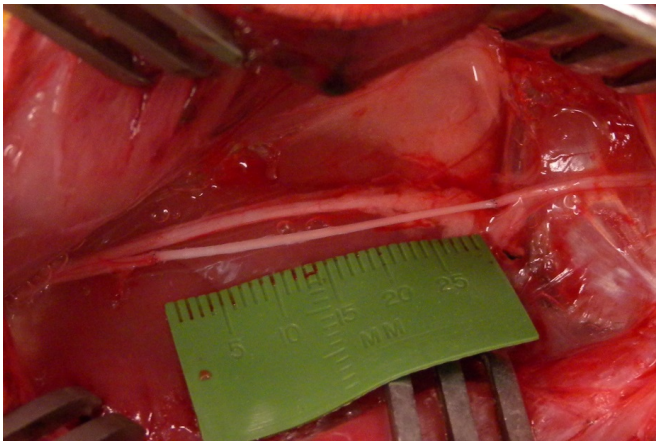


Figure 1. The cold preserved nerve allograft after index surgery.

Ultrasound measurements

Ultrasound measurements of the tibialis anterior muscle were obtained as previously described.[14] Anesthesia is not needed for this procedure. Measurements were obtained bilaterally at baseline and at 10, 16 and 24 weeks after surgery. All ultrasound measurements were obtained twice and the average value was used for further calculations. Images were analyzed using Adobe Photoshop CS5 Extended (Adobe Systems, Inc, San Jose, CA).

Non-survival procedure

After a follow-up time of 24 weeks, animals underwent a non-survival procedure including extensive functional testing. Anesthesia was induced similar to the index procedure and maintained using Propofol 0.2-0.6 mg/kg/min IV (Fresenius Kabi, Lake Zurich, IL). Infusion rates were managed based on corneal reflex, respiration and heart rate. Propofol was preferred over isoflurane because of the lathers influence on skeletal muscle contraction.[15] Ventilation was regulated using a Bain ventilation system after intubation. A 5% dextrose solution was administered through IV drip to prevent muscle fatigue and body temperature was maintained at 37° with a heating pad. At the end of the procedure, while under anesthesia, the rabbits were killed with an overdose of pentobarbital IV and samples of the peroneal nerves and tibial muscles harvested.

Ankle angle

The maximum passive plantar flexion angle of the ankle was measured bilaterally using a goniometer to evaluate the presence and severity of possible ankle contractures.

Electrophysiology (CMAP)

Next, the sciatic nerve and its peroneal branch were exposed through a posterolateral incision. CMAP amplitudes were measured bilaterally as previously described.[11,14]

Isometric tetanic force

Maximum isometric tetanic force was measured bilaterally as previously described.[11,14] Briefly, the exposed tibialis anterior tendon was connected to a force transducer (MDB-50; Transducer Techniques, Temecula, CA). The peroneal nerve was stimulated distal to the graft to first determining the optimal initial muscle tension, followed by measurement of the isometric tetanic muscle force at 100, 125, 150, 175 and 200 Hz.

Histomorphometry

After euthanizing the animals, the lower body was perfused with 10% formalin. 2.5-mm segments of the peroneal nerve distal from the graft were harvested and fixed in Trump fixative solution. In addition, segments from the contralateral side at a similar level were collected. Samples were embedded in spur resin, cut into 1-µm sections and toluidine blue (Fisher Scientific, Pittsburgh, PA) stained. Slides were digitalized and analyzed using Image-Pro software (Image-Pro Plus 7.0; Media Cybernetics, Bethesda, MD). From the images at 200× magnification, first the total tissue cable area was measured. Next, the myelinated fiber area, the number of axons and total axon area of representative sections of each slide were measured. Nerves were analyzed with regard to the total tissue cable area, the N ratio, axon ratio and nerve fiber density. The N ratio was calculated as the myelinated fiber area divided by the tissue cable area and the axon ratio was obtained by dividing the axon area by the tissue cable area. The number of axons was divided by the tissue cable area to obtain the nerve fiber density.[9,14]

Wet muscle mass

The tibialis anterior muscle of both sides was carefully dissected from the surrounding tissues and directly weighed in grams.

Statistical analysis

All outcome measurements were obtained bilaterally and normalized to correct for biological inter animal variation. Data was normalized by expressing the results as percentage recovery. This was calculated by dividing the value of the operated side by that of the contralateral (healthy) side.

One-way analysis of variance (ANOVA) followed by a Bonferroni correction for multiple testing was used for statistical analysis. All results are presented as mean ± standard deviation (SD) unless otherwise noted. A *p*-value of 0.05 or less was considered significant.

RESULTS

Three animals did not survive surgery. One animal was resuscitated during surgery, but excluded from further follow-up because neurological damage was suspected after prolonged low saturations during CPR. The remaining 32 animals (n=11 for the autograft group, n=10 for the cold stored graft group and n=11 for the frozen stored graft group) survived the 24-week follow-up period. Unfortunately, not all animals survived all outcome tests. The number of

animals we were able to obtain data from is indicated for each outcome measure below and in Figure 3.

Autotomy

Eight animals developed superficial licking wounds at the dorsum of the foot on the operated side (3 in group 1, 4 in group 2 and 1 in group 3). An Elizabethan collar was placed until the wounds healed. All wounds healed without complications.

Ultrasound measurements

Longitudinal ultrasound results are depicted in Figure 2. After 24 weeks follow-up, ultrasound showed recovery rates of 102.4 ± 6.9% in group 1, 88.9 ± 13% in group 2 and 72.3 ± 17.5% in group 3. When comparing the three groups, we found that the cold stored allograft performed similar to the autograft (p = 0.078). The frozen stored allograft showed significantly lower recovery rates in comparison to the cold stored allograft (p = 0.018) and autograft (p < 0.001). Results are depicted in Figure 3a.

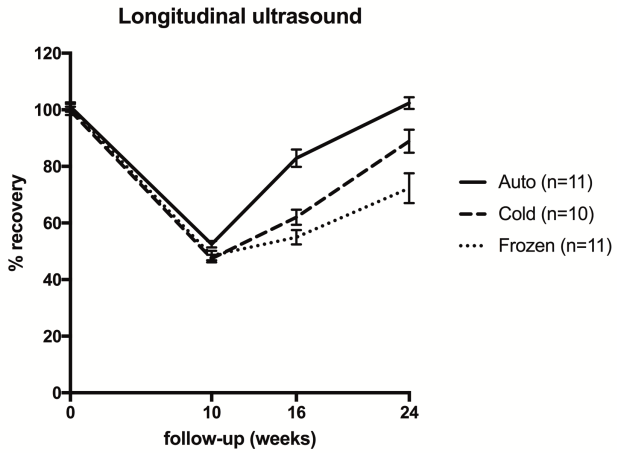


Figure 2. Longitudinal ultrasound results expressed as percentage recovery (cross sectional tibialis anterior muscle area of the operated side divided by that of the contralateral (healthy) side). For each time point the group mean ± SEM is depicted.

Ankle angle

Measured ankle angles relative to the control side were 98.5 ± 2.3% for group 1, 98.6 ± 2% for group 2 and 98.7 ± 6.3% for group 3. Differences between groups were not significant (p = 1.00). Measurements of the ankle angle could be obtained from all animals.

Electrophysiology (CMAP)

Electrophysiology results were highest in the autograft group with 86.3 ± 40.3%, followed by 50.5 ± 39.8 for group 3 and 46.7 ± 16.8% for group 2. Differences between the three

experimental groups were not significant ($p > 0.05$ for all comparisons). Results are depicted in Figure 3b.

Isometric tetanic force

In group 1, the percentage muscle force was 105.4 ± 14.2%. Group 2 and 3 showed percentages of 77 ± 20.5% and 62.1 ± 45.8% respectively. Differences between the cold stored allograft group and both other groups were not significant ($p = 0.286$ for auto vs. cold and $p = 1.00$ for cold vs. frozen). However, the frozen stored allograft performed statistically worse that the autograft ($p = 0.02$). The isometric tetanic force measurements could be completed in 9 animals in group 1, 7 animals in group 2 and 11 animals in group 3 (Figure 3c).

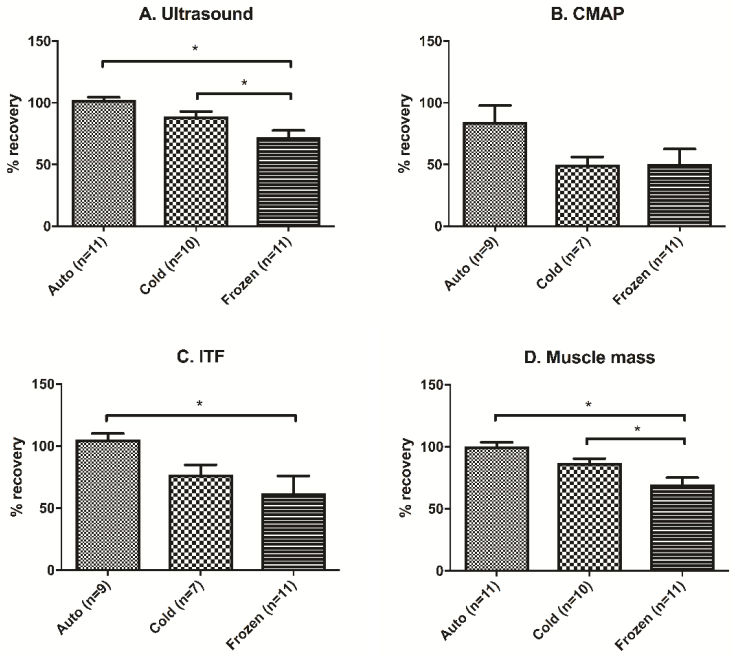


Figure 3. Assessment of recovery using Ultrasound (A), Electrophysiology (CMAP, B), Isometric Tetanic Force (ITF, C) and wet muscle mass (D). Results are expressed as percentage of the contralateral (healthy) side.
*: p < 0.05, **: p < 0.01, ***: p < 0.001. Error bars present the mean ± standard deviation (SD).

Histomorphometry

Relative to the contralateral (healthy) side, the total tissue cable area was 77.3 ± 32.3% in group 1, 65.8 ± 41% in group 2 and 55.5 ± 25.2% in group 3. For the nerve fiber density, results were 160.4 ± 65.4%, 200.4 ± 175.1% and 173.2 ± 150.9% for group 1, 2, and 3 respectively. Axon ratios were 109 ± 59.9% (group 1), 85 ± 76.7% (group 2) and 69.6 ± 68.2 (group 3). The N ratios were 108.7 ± 26.4%, 99.9 ± 23.1% and 91.2 ± 22.1% group 1, 2, and 3 respectively. None of the group comparisons were significantly different for any of the measured

parameters ($p > 0.05$). Representative examples of the toluidine blue slides are shown in Figure 4. Histomorphometry data could be obtained from all animals that survived surgery.

Wet muscle mass

Finally, the wet muscle mass expressed as percentage recovery was 100.5 ± 10.1% for group 1, 87.1 ± 10.6% for group 2 and 69.5% ± 18.3 for group 3. Results were significantly lower for the frozen stored allograft group compared to both the autograft group ($p < 0.001$) and cold stored allograft group ($p = 0.018$). Recovery rates of the autograft and cold stored allograft were similar ($p = 0.096$). This is depicted in Figure 3d.

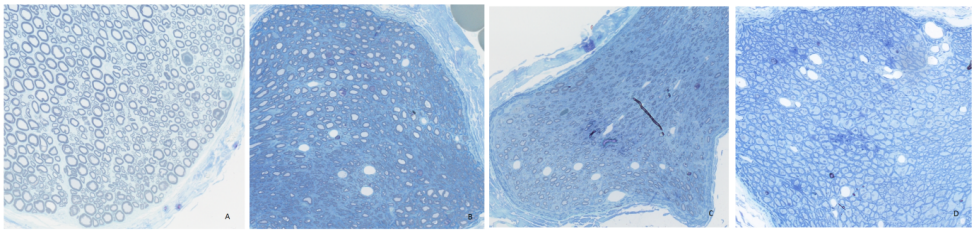


Figure 4. Representative histological sections of the toluidine blue stained nerve at 200x magnification. A: healthy control, B: autologous nerve graft (group 1), C: cold stored processed nerve allograft (group 2), D: frozen stored processed nerve allograft (group 3).

DISCUSSION

The aim of this study was to evaluate the functional recovery after reconstruction of a 3-cm nerve gap using our elastase processed (optimized) nerve allograft in the rabbit model. To also evaluate the effect of storage temperature, three groups were compared: the autograft, the cold stored processed allograft, and the frozen stored processed allograft.

Longitudinal ultrasound measurements showed that all three groups reached the smallest muscle cross sectional area at the 10-week time point. This can be explained by the occurrence of muscle atrophy after nerve transection and is in line with results of previous studies.[14,16] In all three groups, a subsequent increase in muscle cross sectional area was seen, indicating nerve regeneration and muscle reinnervation. A trend can be seen (Fig. 2) where nerve regeneration in the frozen stored allograft group seems to be slower than in the other two groups. This is confirmed by the results at 24 weeks, where the frozen-stored graft was significantly inferior to both other groups. Similar results were obtained with the muscle weight measurements (Fig. 3).

Unfortunately, not all animals survived the initial surgery and/or complete sacrifice procedure. Rabbits are known to be very sensitive to anesthesia and surgical procedures.[17] The sacrifice procedure is lengthy (approx. 4 hours per animal) and very invasive. As a result, in the cold stored allograft group, we could only obtain ITF measurements in 7 animals. Therefore, the calculations involving ITF data of the cold-stored group may be slightly

underpowered to detect significant differences. Nevertheless, it could be shown with adequate power that the frozen-stored allograft is significantly inferior to the autograft based on ITF data (Fig. 3). Both the electrophysiology and histology results did not show any significant differences between the three groups ($p > 0.05$ for all group comparisons).
In summary, the cold-stored allograft yielded similar results to the autograft group in all five outcome measurements. The inferiority of the frozen-stored allograft in comparison to the autograft group is shown by 3 out of 5 functional outcome measurements.

The lack of satisfactory results of processed allografts for mixed and motor nerve defects currently limit clinical use of processed nerve allografts to sensory nerve defects <3 cm.[3-5] To improve functional results of processed nerve allografts, our lab recently optimized the decellularization technique. It was shown in vitro that the addition of elastase significantly reduced the amount of axons and immunogenicity, while maintaining good ultrastructural properties. Furthermore, we found a significantly deteriorated ultrastructure in the frozen-stored group.[6] This was an important finding as the nerve allograft that is currently available for human use is stored at -80°C, but little is known about the effect of storage temperature on functional recovery. In a subsequent in vivo study, we compared both the cold-stored and frozen-stored elastase processed allografts to the autograft in a 1-cm sciatic nerve gap in the rat model. This study showed that the cold-stored nerve allograft reached results that are comparable to the autograft while the frozen-stored graft yielded inferior functional outcomes.[7]
As previous studies have shown that successful recovery in the rat model does not guarantee success in larger gaps in a larger animal model, the next step was to test this improved nerve allograft in a larger animal model.[8,9]

Only few studies have evaluated functional recovery after reconstruction of long mixed or motor nerve gaps with acellular nerve allografts. Yan et al.[18] reconstructed 6-cm sciatic nerve gaps in a rat model, using acellular nerve allografts with or without an interposition isograft. After 20 weeks follow-up, no measurable muscle force could be obtained in either groups. It is possible that there is a limit to the maximum length that can be bridged using acellular nerve grafts. Furthermore, based on Marquardt et al's.[19] study, it seems that their processing technique is less favorable in comparison to ours. Reconstruction of a 3-cm gap in rats using the same decellularized allografts results in significantly lower gastrocnemius muscle mass compared to autograft reconstruction.
Boriani et al.[20] used a similar study design as our study. They first optimized their decellularization techniques followed by a (pilot) in vivo study. Here they reconstructed a 1-cm tibial nerve gap in rabbits with either their optimized nerve allograft or an autograft. Only 3 animals per group were used and unfortunately motor recovery was not evaluated. Histomorphometry on toluidine blue dyed slides, at 12 weeks follow-up, showed no difference between their decellularized allograft and autograft. This is similar to our results. Jiang et al.[21] reconstructed 4-cm ulnar nerve defects in rhesus monkeys with either an

autograft, or decellularized allografts with and without added allogenic Schwann cells. Five months after surgery, CMAP and histological slides were evaluated. They conclude that their decellularized allograft (without added Schwann cells) is inferior to the nerve autograft. In our study we found similar results of the decellularized nerve allograft (both cold and frozen stored) in comparison to the autograft, measured by CMAP and histomorphometry. Unfortunately, there are no force measurements to compare to our results. Vasudevan et al.[22] compared two decellularization techniques to autograft and silicone tube reconstruction (3.5-cm gap in the rat). Twelve weeks after surgery, the detergent free decellularized allograft group has significantly lower gastrocnemic muscle mass in comparison to the autograft group. This might indicate that our decellularization technique is more successful. Furthermore, the detergent free process developed by Vasudevan et al.[22] is quite lengthy (3 weeks) in comparison to our 5 day decellularization protocol.[6]

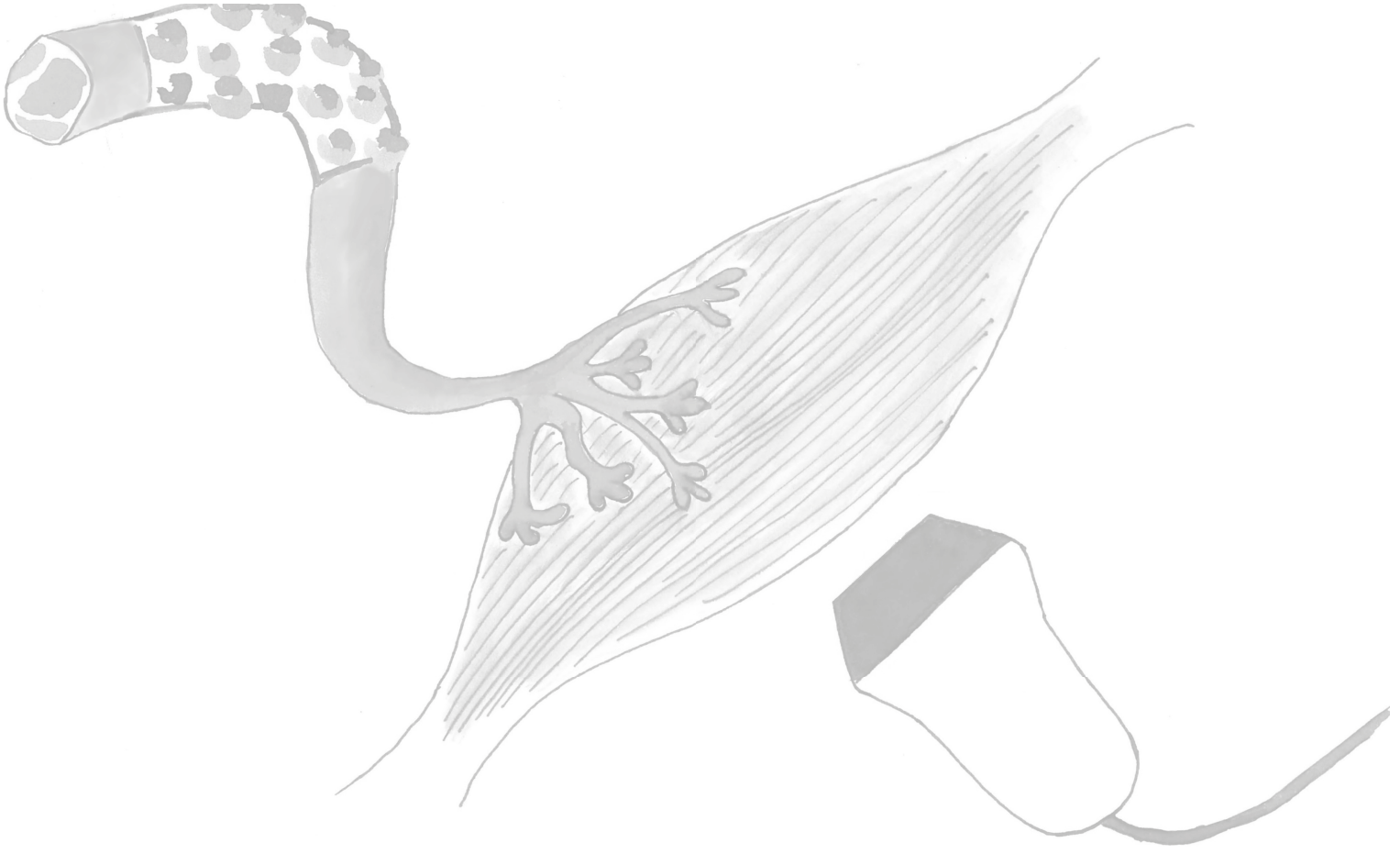
A limitation of this study is the relatively short animal survival time. Although the control group (autograft) reached recovery rates of approximately 100% for all outcome measurements, it is possible that the processed nerve allograft groups did not have sufficient time to reach their final outcome. A possible explanation is that in the processed nerve allografts all Schwann cells and other supportive cells are removed, possibly resulting in lower levels of important growth factors.[23] This could be addressed in future studies by, for example, the addition of growth factor producing stem cells. Another explanation could be the occurrence of a delayed immune response to the allograft.[24] However, this seems less likely considering the low levels of immunogenicity found in our previous in vitro work.[6]

In conclusion, this study shows that the cold-stored optimized nerve allograft yields functional recovery equivalent to the gold standard autograft in the reconstruction of a long motor nerve defect. In contrast, however, the frozen-stored nerve allograft yields impaired functional recovery.

REFERENCES

1. Bassilios Habre S, Bond G, Jing XL, Kostopoulos E, Wallace RD, Konofaos P. The Surgical Management of Nerve Gaps: Present and Future. Ann Plast Surg. 2017.
2. Ehretsman RL, Novak CB, Mackinnon SE. Subjective recovery of nerve graft donor site. Ann Plast Surg. 1999;43(6):606-612.
3. Brooks DN, Weber RV, Chao JD, et al. Processed nerve allografts for peripheral nerve reconstruction: a multicenter study of utilization and outcomes in sensory, mixed, and motor nerve reconstructions. Microsurgery. 2012;32(1):1-14.
4. Cho MS, Rinker BD, Weber RV, et al. Functional outcome following nerve repair in the upper extremity using processed nerve allograft. J Hand Surg Am. 2012;37(11):2340-2349.
5. Rinker B, Zoldos J, Weber RV, et al. Use of Processed Nerve Allografts to Repair Nerve Injuries Greater Than 25 mm in the Hand. Ann Plast Surg. 2017;78(6S Suppl 5):S292-S295.
6. Hundepool CA, Nijhuis TH, Kotsougiani D, Friedrich PF, Bishop AT, Shin AY. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. Neurosurg Focus. 2017;42(3):E4.
7. Hundepool CA, Bulstra LF, Kotsougiani D, et al. Optimizing decellularization techniques to create a novel nerve allograft: Return of motor function with decellularized nerve allografts using elastase in a rat sciatic nerve model. Manuscript submitted for publication. 2018.
8. Lee JY, Giusti G, Friedrich PF, et al. The effect of collagen nerve conduits filled with collagen-glycosaminoglycan matrix on peripheral motor nerve regeneration in a rat model. J Bone Joint Surg Am. 2012;94(22):2084-2091.
9. Sahakyants T, Lee JY, Friedrich PF, Bishop AT, Shin AY. Return of motor function after repair of a 3-cm gap in a rabbit peroneal nerve: a comparison of autograft, collagen conduit, and conduit filled with collagen-GAG matrix. J Bone Joint Surg Am. 2013;95(21):1952-1958.
10. Amillo S, Yanez R, Barrios RH. Nerve regeneration in different types of grafts: experimental study in rabbits. Microsurgery. 1995;16(9):621-630.
11. Giusti G, Kremer T, Willems WF, Friedrich PF, Bishop AT, Shin AY. Description and validation of isometric tetanic muscle force test in rabbits. Microsurgery. 2012;32(1):35-42.
12. Singh I. One-sided dominance in the limbs of rabbits and frogs, as evidenced by asymmetry in bone weight. J Anat. 1971;109(Pt 2):271-275.
13. Kniha H, Randzio J, Gold ME, et al. Growth of forelimb allografts in young rabbits immunosuppressed with cyclosporine. Ann Plast Surg. 1989;22(2):135-141.
14. Bulstra LF, Hundepool CA, Friedrich PF, Nijhuis TH, Bishop AT, Shin AY. Motor Nerve Recovery in a Rabbit Model: Description and Validation of a Noninvasive Ultrasound Technique. J Hand Surg Am. 2016;41(1):27-33.
15. Bouhemad B, Langeron O, Orliaguet G, Coriat P, Riou B. Effects of halothane and isoflurane on the contraction, relaxation and energetics of rat diaphragmatic muscle. Br J Anaesth. 2002;89(3):479-485.
16. Hundepool CA, Nijhuis TH, Rbia N, Bulstra LF, Selles RW, Hovius SE. Noninvasive Ultrasound of the Tibial Muscle for Longitudinal Analysis of Nerve Regeneration in Rats. Plast Reconstr Surg. 2015;136(5):633e-639e.
17. Brodbelt DC, Blissitt KJ, Hammond RA, et al. The risk of death: the confidential enquiry into perioperative small animal fatalities. Vet Anaesth Analg. 2008;35(5):365-373.
18. Yan Y, Hunter DA, Schellhardt L, et al. Nerve stepping stone has minimal impact in aiding regeneration across long acellular nerve allografts. Muscle Nerve. 2018;57(2):260-267.
19. Marquardt LM, Ee X, Iyer N, et al. Finely Tuned Temporal and Spatial Delivery of GDNF Promotes Enhanced Nerve Regeneration in a Long Nerve Defect Model. Tissue Eng Part A. 2015;21(23-24):2852-2864.
20. Boriani F, Fazio N, Fotia C, et al. A novel technique for decellularization of allogenic nerves and in vivo study of their use for peripheral nerve reconstruction. J Biomed Mater Res A. 2017;105(8):2228-2240.

21. Jiang CQ, Hu J, Xiang JP, Zhu JK, Liu XL, Luo P. Tissue-engineered rhesus monkey nerve grafts for the repair of long ulnar nerve defects: similar outcomes to autologous nerve grafts. Neural Regen Res. 2016;11(11):1845-1850.
22. Vasudevan S, Huang J, Botterman B, Matloub HS, Keefer E, Cheng J. Detergent-free Decellularized Nerve Grafts for Long-gap Peripheral Nerve Reconstruction. Plast Reconstr Surg Glob Open. 2014;2(8):e201.
23. Wang Y, Zhao Z, Ren Z, et al. Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration. Neurosci Lett. 2012;514(1):96-101.
24. Giusti G, Willems WF, Kremer T, Friedrich PF, Bishop AT, Shin AY. Return of motor function after segmental nerve loss in a rat model: comparison of autogenous nerve graft, collagen conduit, and processed allograft (AxoGen). J Bone Joint Surg Am. 2012;94(5):410-417.



8

A simple dynamic strategy to deliver stem cells to decellularized nerve allografts

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ABSTRACT

Background

The addition of adipose-derived Mesenchymal Stromal Cells (MSCs) to decellularized nerve allografts may improve outcomes of nerve reconstruction. Prior techniques used for cell seeding are traumatic to both the MSCs and nerve graft. An adequate, reliable and validated cell seeding technique is an essential step for evaluating the translational utility of MSC-enhanced decellularized nerve grafts. The purpose of this study was to develop a simple seeding strategy with an optimal seeding duration.

Methods

A dynamic bioreactor was used to seed rat and human MSCs separately onto rat and human decellularized nerve allografts. Cell viability was evaluated by MTS assays and cellular topology after seeding was determined by SEM microscopy. Cell density and distribution were determined by LIVE/DEAD assays and Hoechst staining at 4 different time points (6, 12, 24 and 72 hours). The validity and reliability of the seeding method were calculated.

Results

Cells remained viable at all time points, and MSCs exhibited exponential growth in the first 12 hours of seeding. Seeding efficiency increased significantly from 79.5% at 6 hours to 89.2% after 12 hours of seeding ($p = 0.004$). Both intra-rater ($r = 0.97$) and inter-rater reliability ($r = 0.92$) of the technique were high.

Conclusions

This study describes and validates a new method to effectively seed decellularized nerve allografts with MSCs. This method is reproducible, distributes cells homogenously over the graft and does not traumatize the intra-neural architecture of the allograft. Utilization of this validated seeding technique will permit critical comparison of graft outcomes.

INTRODUCTION

Segmental injury to peripheral nerves occurs in approximately 5% of all extremity traumas.[1] However, a nerve graft substitute that is biologically equal to autologous nerve tissue has yet to be developed.[2] One of the most promising commercially available alternatives is the decellularized nerve allograft.[3] The presence of an internal structure and extracellular matrix components (ECM) in processed allografts are critical for guiding cell migration and nerve fiber elongation.[4] However, outcomes to date suggest that allografts are not equivalent to autografts, especially for the reconstruction of long gaps or motor nerves.[5-7] Because Schwann cells and axons are eliminated after decellularization, the addition of supporting cells has been proposed to improve allografts. Perivascular MSCs that are derived from the stromal vascular fraction of adipose tissue have many clinical advantages, including easy accessibility, rapid proliferation and immunomodulatory effects.[8] Multiple studies have shown that MSCs have a beneficial effect on nerve regeneration and that MSCs can potentially provide the necessary support for nerve regeneration through local production of essential growth factors.[9,10,11]

Despite progress in our understanding of the beneficial roles of MSCs in peripheral nerve repair, differences in seeding strategies have resulted in varying outcomes and these differences have precluded comparisons of results between studies. Ikebe and colleagues demonstrated that protocols in clinical trials on MSCs varied widely and highlighted the importance of developing optimized and standardized methods.[12] An adequate cell seeding technique is essential for evaluating the potential of stem cells on nerve regeneration. Ideally, the optimal seeding technique should distribute cells homogeneously, have a high cell seeding efficiency and minimize cell/graft injury. In addition, such seeding techniques should be reproducible, easy to use and validated. For clinical use, the technique should also be rapid and user independent.[13]

Cell seeding on a solid support proceeds generally by either static or dynamic seeding. [14 15,16] In previous studies, dynamic cell seeding resulted in more efficient and homogeneous cell seeding compared to static seeding.[17]

The purpose of this study was to develop a simple seeding method to efficiently seed a decellularized nerve allograft with MSCs, which would be optimally suited for clinical strategies applications for nerve repair. We also investigated the optimal seeding duration and validated the consistency of our approach.

MATERIALS AND METHODS

General design

To determine if cell-seeding duration has an effect on cell attachment and seeding efficiency, cell viability, density and migration into the graft were evaluated at 4 different time points of

incubation (t= 6, 12, 24 and 72 hours). These time points were based on literature and potential human clinical translation (preoperative planning); the critical factor was to determine the shortest and most effective seeding time possible.[18] The seeding method, cell viability and effect of seeding duration were first evaluated on rat MSCs seeded onto rat nerve allografts. When the rat process was successful, the seeding method and cell viability were tested on human MSCs seeded onto human nerve allografts.

Allograft preparation

This study was approved by the IACUC institutional review committee and our Institutional Review Board. Previous work by our laboratory on decellularization techniques resulted in reduced immunogenicity, diminished cellular debris and better maintenance of the ultrastructure when compared to the commercially available processed nerve allograft.[19] Sciatic nerve segments of 2 cm were therefore harvested from a total of 36 donor male Sprague-Dawley rats weighing 250-350 grams (corresponding to 59-70 days old) (Harlan, Indianapolis, IN). After Isoflurane induction, rats were euthanized with an overdose of sodium pentobarbital. Sciatic nerves were aseptically excised, cleared of peripheral fat and connective tissue and decellularized.

The human motor nerves (thoracodorsal and long thoracic nerves) were obtained from one 61-year old fresh male human cadaver. Subjects with a history of a condition that affects the peripheral nervous system were excluded. All segments were decellularized using the protocol described by Hundepool et al.[19] Briefly, the samples were treated with different detergents including Triton X- 200, sulfobetaine-16, and sulfobetaine-10 and enzymatic solutions. Finally, the nerves were sterilized using γ-radiation and stored at 4°C for a maximum of 14 days before seeding. All chemicals were purchased from Sigma (St. Louis, MO, USA) and all solutions were autoclaved or filter-sterilized before use.

Mesenchymal stem cells

Rat MSCs were obtained from isogenic male Lewis rats weighing 250-350 grams (corresponding to 59-70 days old), as previously described by Kingham et al.[20] Sprague-Dawley donor nerves were seeded with Lewis rat MSCs to obtain a major histocompatibility complex mismatch, similar to the human allografts.[21] After euthanasia, the inguinal fat pad was carefully dissected and minced using a sterile razor blade. The tissue was enzymatically digested for 2h at 37 °C using 0.15% collagenase type 1 (Worthington, Biochemicals, Lakewood, NJ). The solution was passed through a 70-um filter to remove undissociated tissue, neutralized by adding modified Eagle's medium (α-MEM; Invitrogen, UK) containing 10% fetal bovine serum and centrifuged at 800 x g for 5 min. The stromal cell pellet was re-suspended in advanced Minimum Essential Medium (MEM) (Life Technologies, Grand Island, NY) containing 5% Platelet Lysate (PLTMax; Mill-CreekLifeSciences, Rochester, MN), 2 mmol/L Glutamax (Life Technologies, Grand Island, NY), 2 U/ml heparin and 1% penicillin/streptomycin solution (Cellgro, Coming, NY). The cultures were maintained at

subconfluent levels in a 37 °C incubator with 5% CO_2 and passaged with TrypLE (Invitrogen, UK). The multi-potency of the rat MSCs was demonstrated by differentiation into osteogenic and adipogenic lineages.

Human MSCs were isolated from abdominal lipo-aspirates from a representative 41-year-old male donor with written individual consent and institutional approval. Cell isolation and culture conditions have previously been described.[22-24] These cells have been routinely used at our institute for clinical trial applications and have been extensively tested for cell surface markers, RNA-seq transcriptome profiles, and multi-lineage potential.[8,22,25] All MSCs used in this study were of passage 5.

Seeding technique

The Lewis rat MSCs were then seeded onto the Sprague Dawley decellularized rat allografts at different durations of incubation (t=6, 12, 24 and 72 hours). The human MSCs were seeded onto the decellularized human allografts at one time point (t=12 hours). This technique involves a 9-step process as depicted in Figure 1 and described in detail in the appendix. An overview of the 9 steps is as follows:

1. Nerve allograft harvest
2. Nerve decellularization[19]
3. Cell culture and proliferation according to cell type.
4. Removal of harmful remnants of decelullarization process by placing nerve grafts in cell culture medium and soak for 2 hours.
5. Combine nerves with the cells in a 15 mL TubeSpin® Bioreactor tube containing 10 ml cell culture medium and 1 million cells/nerve, with a maximum of 4 nerve segments/tube.
6. Place tubes horizontally in a bioreactor rotator system (RevolverTM Labnet, Edison, USA) and secure the tubes. Balance the tubes on the rotator (maximum of 6 tubes per side) with a rotation axis of 30°.
7. Turn rotator on at fixed speed 18 rpm to remove the gravitational component and place the rotator in an incubator at 37 degree Celsius (98.6 Fahrenheit) and 5% CO2.
8. Incubate for 12 hours.
9. Place seeded nerves in a culture dish after 12 hours of bioreactor seeding.

Cell viability

MTS-assay

To determine the influence of the graft and possible remnants of the decellularization process and seeding strategy on the viability of the MSCs, MTS assays (Aqueous One Cell Proliferation Assay, Promega, Madison, USA) were performed on MSC seeded nerves according to the manufacturer's instructions. The wells with the nerve samples were pHEMA (Poly(2-hydroxyethyl methacrylate)) coated to prevent migration of cells to the well. Wells filled with medium and MTS reagent only served as negative control and for subtraction of the background signal. Wells incubated with MSCs served as positive control. MTS assays were

performed in triplicate for each group at multiple time points after incubation (t= 1, 2, 3 and 7 days). Results were analyzed using a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, USA) at an absorbance wavelength of 490 nm.

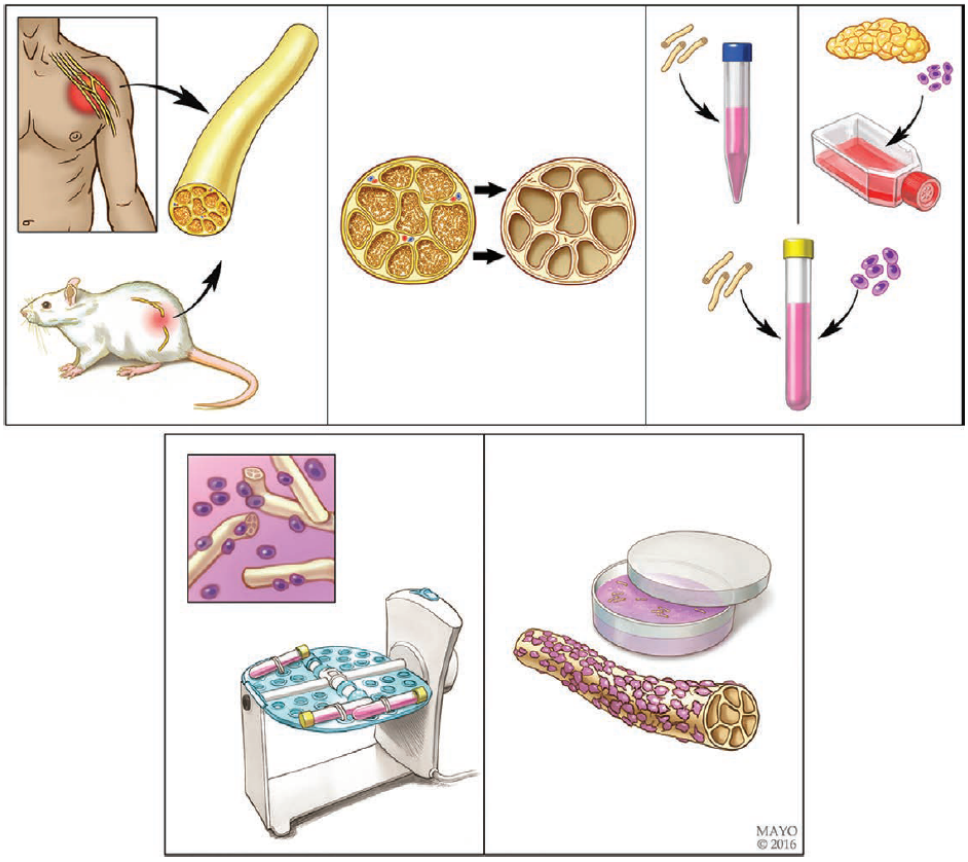


Figure 1. Seeding technique. This technique involves a 9-step process as depicted above and described in detail in appendix 1. (Above, left) Nerve graft harvest, (above, center) nerve decellularization, (above, right) cell culture and isolation, (below, left) bioreactor seeding, and (below, right) static storage before grafting. The seeding method, cell viability, and effect of seeding duration were first evaluated on rat mesenchymal stromal cells seeded onto rat nerve allografts. Then, the seeding method and cell viability were tested on human mesenchymal stromal cells seeded onto human nerve allografts. (Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved. Mayo © 2016.)

Seeding efficiency, cell density and distribution

Scanning Electron Microscopy (SEM)

Seeded samples (n=1 per time point) were fixed in 2% Trump's fixative solution (37% formaldehyde and 25% glutaraldehyde) and processed for SEM. Samples were rinsed, fixed and desiccated with (i) PBS (2×), (ii) water (2×), (iii) 10% ethanol, (iv) 30% ethanol, (v) 50% ethanol, (vi) 70% ethanol, (vii) 90% ethanol, (viii) 95% ethanol, and (ix) 100% ethanol (2×).

Samples were then placed in a critical point dryer while still in 100% ethanol and subsequently attached to an aluminum stub by double-sided conductive tape. The final preparation included sputter-coating for 90 seconds with gold–palladium. Images of the surface of the graft were obtained at 3 different magnifications (150x, 500x and 1800x)(Hitachi S-4700 field emission SEM). Seeding efficiency, determined as the percentage of graft coverage, and cell morphology were observed by one investigator. To obtain inter-rater and intra-rater reliability of the seeding efficiency, results were compared with pilot data from two independent investigators at two different points in time.

Live/Dead stain

After incubation at 37°C for 6, 12, 24 and 72 hours, seeded samples (n=3 per time point) were stained using a standard LIVE/DEAD® Cell Viability Assay (Invitrogen, Life Technologies, Grand Island, USA) following manufacturer's instructions. Live cells were identified by the incorporation of the membrane permeable Calcein AM stain within a cell, whereas dead cells were identified by the binding of Ethidium homodomer-1 to the nucleic acids of cells with damaged plasma membranes. The cells were visualized using confocal microscopy (Zeiss LSM 780; Zeiss).

Hoechst stain

Following the Live/Dead stain, seeded samples (n=2 per time point) were Hoechst 33342 stained (Invitrogen, Life Technologies, Grand Island, USA). The seeded grafts were longitudinal visualized using confocal microscopy (Zeiss LSM 780; Zeiss). Subsequently, to evaluate migration into the graft, samples (n=1 per time point) were suspended in OCT (Tissue-Tek, Sakura, Torrance, USA) and snap frozen. 15-µm cross sections of different levels of the nerve were evaluated for the presence of cell nuclei (Hoechst 33342 stained) to determine cell penetration into the graft.

Image analysis

All images were analyzed using Image J (NIH, Bethesda, USA). For SEM images, to determine the seeding efficiency, images were converted to 8-bit and the "MaxEntropy" automatic thresholding method was applied. "MaxEntropy" used the mean of grey levels as the threshold.[26] The fraction below the threshold (the part of the graft not covered by cells) was then calculated using the "Measure Area Fraction" function and was calculated by subtracting this fraction 100%. In the Live/Dead stained samples, the number of live and dead cells were determined per microscopic field. The number of positive Hoechst stained cells (nuclei) were manually counted. All Hoechst stained samples were also post-fixed with 10% Neutral Buffered Formalin (Thermo Fisher Scientific, Massachusetts), wrapped in foil and stored at 4°. After the final time-point all nerves were transferred to a 48 well plate and fluorescence was quantified using a microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, USA) at an absorbance wavelength of 340/460nm.

Statistical analysis

Cell counts were performed at least three times and results were expressed as the mean ± SD. The data was analyzed using the one-way analysis of variance (ANOVA) with Bonferoni's *post hoc* correction for multiple comparisons. For non-normally distributed data the Kruskal-Wallis test was used. To assess the validity and reliability of these measurements, the inter-rater and intra-rater reliability of this method was calculated using the intraclass correlation coefficient (ICC). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Cell viability

Results from MTS assays demonstrate that all cells have robust mitochondrial metabolic activity at all time points as is expected from actively proliferating cells. MTS absorbance increases between days 1 (average 0.65 ± 0.33) and 3 (1.27 ± 0.06), while decreasing between days 3 and 7 (0.82 ± 0.13), consistent with a more active proliferative phase during the first few days when MSCs settle on the decellularized construct.

Cell density, distribution and seeding efficiency

Scanning Electron Microscopy

All following results are based on rat-tissue. To evaluate cell morphology and coverage of cells seeded on the graft, SEM images were made. Manual quantification of the cell distribution was not possible, as the cells formed aggregates on the graft. MSCs attaching to the decellularized nerve allograft formed flat (layered) sheets. Figure 2 depicts the seeded nerve allografts at the four time points and at 3 different magnifications (300, 100 and 30 um). The cells appeared evenly distributed throughout the nerve grafts and the number of cells/percentage of coverage gradually increased. Cell morphology was consistent throughout the time course of the experiment. Objective fraction analysis of the grey levels (seeding efficiency) indicates that there is a significant ($p = 0.004$) mean increase of 79.5% at t=6h to, the longer seeding durations, 89.2% at t=12h, 88.1% at t=24h and 89.7% at t=72h (figure 3).

Live/Dead stain

Cells remained viable on the nerve allograft at all time points. Figure 4 shows Live/Dead staining of MSCs on nerve allografts at the 4 different time points. From 6 to 12 hours in culture, the live cell count showed a 2-fold increase in cell attachment from 200 to 395 cells per microscopic field, while from 12 to 24 hours cell counts only marginally increased to 453 cells per microscopic field. Time point 72h showed a decreased cell count of 225 cells per field. However, these differences in cell number were not significant ($p=0.620$ and $p=0.826$) (Figure 5). At 12h, the nerve allograft was completely covered indicating proliferation of cells on the graft, while only a few dead cells were seen.

Hoechst stain

Figure 6 depicts nuclei of cells stained with Hoechst dye and figure 7 presents the corresponding manual cell count. An increase in cell numbers was seen between time points 6 and 12 hours. A 12-hour seeding duration exhibited greater cell attachment and proliferation when compared to 6 hours. No striking differences were found between 12 and 24 hours of seeding and a decrease was noted between 24 and 72 hours. None of the differences were significant (p=0.198).

To control for manual counting errors, fluorescence intensity was also analyzed using a microplate reader after fixation of the cells (Figure 8). The fluorescence data show trends in cell proliferation during the first 24h that are similar to those observed for manual cell counts, but differences in cell counts over time were not significant (p=0.392). MSCs did not migrate on a large scale but some positive staining was seen in the center of the graft, indicating that MSCs are dynamically seeded with a bioreactor can penetrate into the graft (Figure 9).

Technique reliability

Analysis of seeding efficiency of 3 different SEM images that were seeded for 12 hours, resulted in 90.2%, 88.4% and 87.6% and demonstrated a high intra-rater reliability (r = 0.97). Inter-rater reliability between two investigators was also high (r = 0.92).

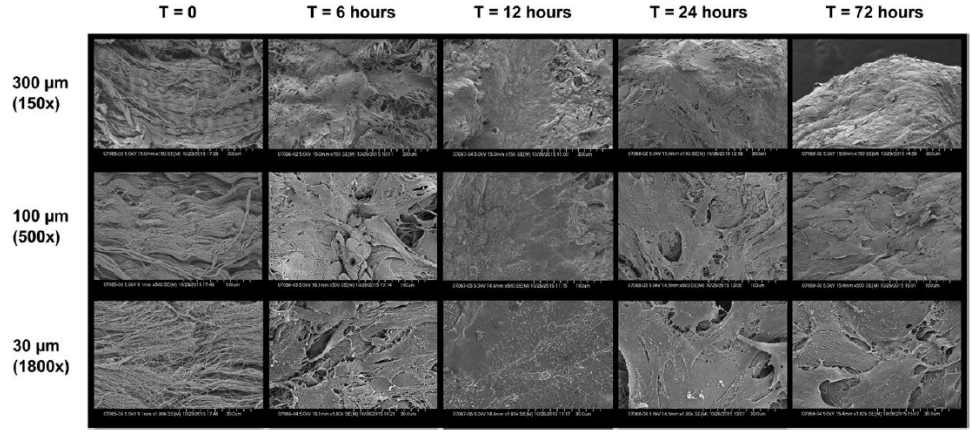


Figure 2. Scanning electron microscopic images of mesenchymal stromal cell–seeded nerve graft. Scale bars = 300 µm (original magnification, × 150), 100 µm (original magnification, × 500), and 30 µm (original magnification, × 1800). The mesenchymal stromal cell–seeded nerve graft is depicted here in three different magnifications. Illustrating the cell morphology and coverage of the unseeded decellularized graft (time point 0) and 6, 12, 24, and 72 hours after seeding. The unseeded nerve at time point 0 shows the longitudinal structure of the epineurium of the decellularized nerve. The later time points show the attached cells as flat (layered) sheets covering (and possibly penetrating) the epineurium. Cell morphology was consistent throughout the time course of the experiment; cells appeared distributed evenly throughout the nerve grafts and the number of cells gradually increased.

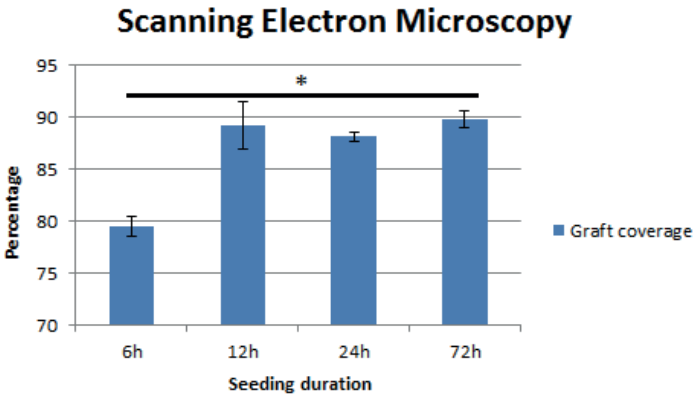


Figure 3. Seeding efficiency. Seeding efficiency was determined as the percentage of graft coverage imaged with scanning electron microscopy. Seeding efficiency increased significantly from 79.5 percent at 6 hours to 89.2 percent after 12 hours of seeding ($p = 0.004$). No significant differences were found among time points 12, 24, and 72 hours.

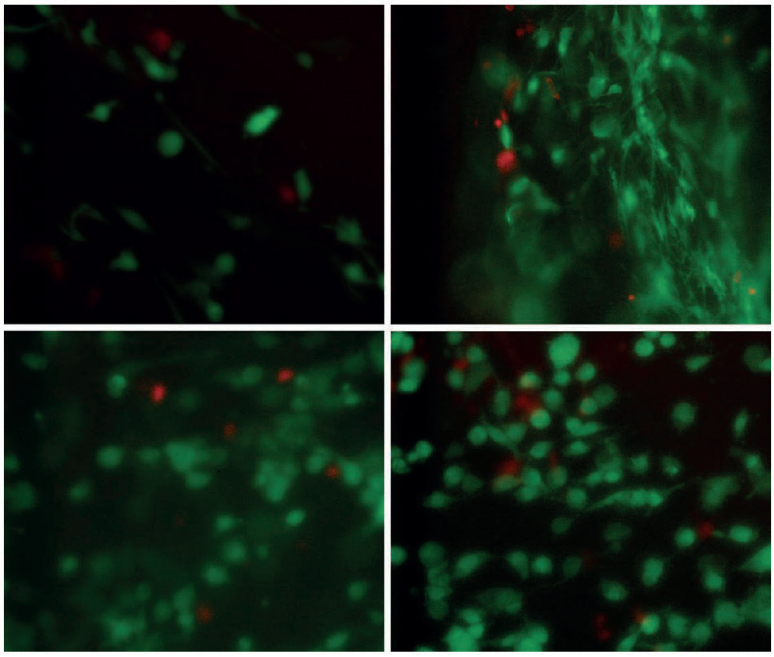


Figure 4. Live/Dead staining of MSC seeded allografts. Cellular viability and density is demonstrated after seeding the nerve allografts for A)6 hours, B)12 hours, C)24hours and D)72 hours. The cells were visualized using a confocal scanning microscopy at 40x magnification. Live cells were stained green and dead cells red.

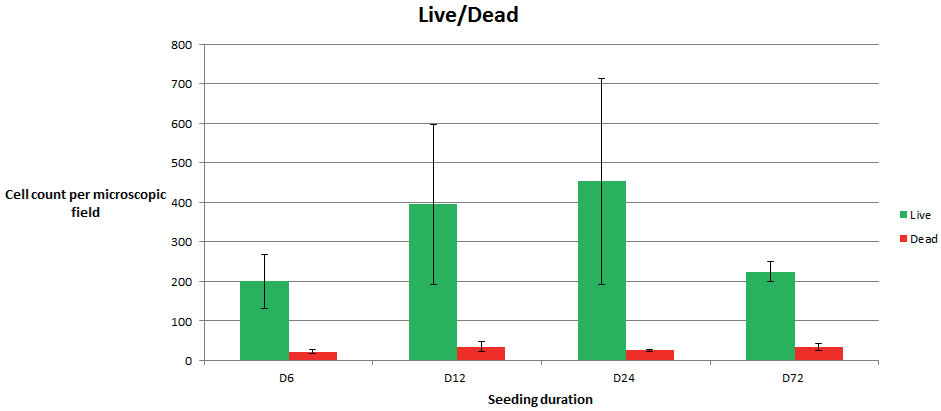


Figure 5. Live/Dead cell count. Average manual live (green) and dead (red) cell count per microscopic field per seeding duration. Error bars = SEM.

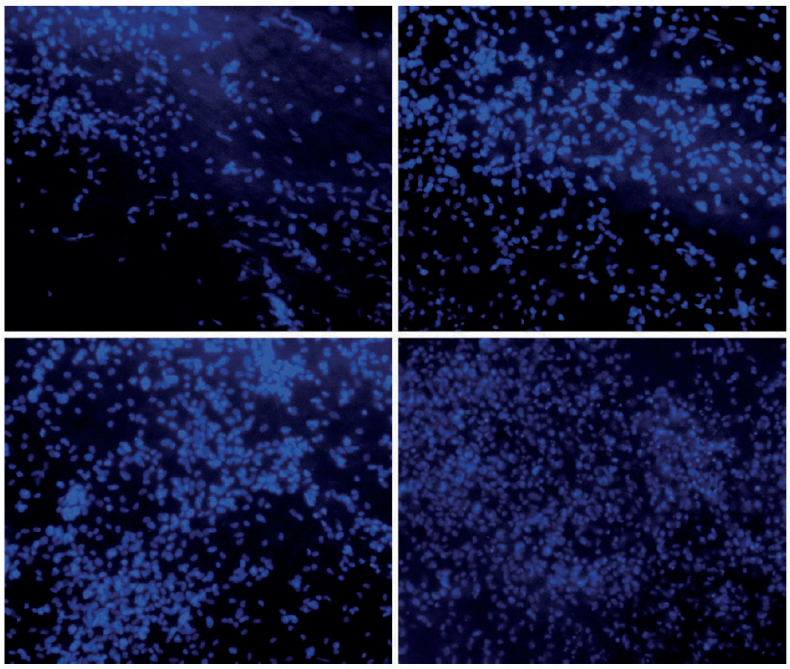


Figure 6. Hoechst longitudinal images. Cell nuclei were Hoechst stained after seeding the nerve allografts for (above, left) 6 hours, (above, right) 12 hours, (below, left) 24 hours, and (below, right) 72 hours. The cells were visualized using confocal scanning microscopy at 20× magnification.

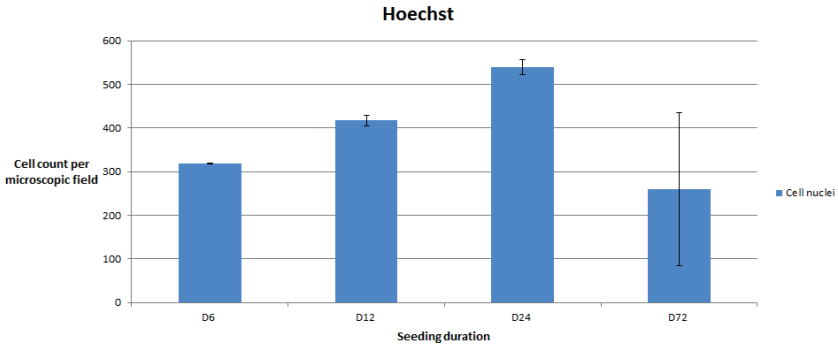


Figure 7. Hoechst cell count. The average manual Hoechst cell count per microscopic field per seeding duration is shown. Error bars = SD or SEM as indicated.

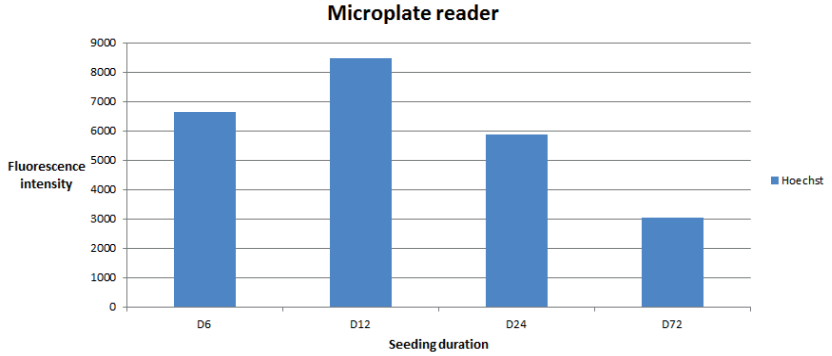


Figure 8. Hoechst fluorescence intensity. Demonstrates the differences in fluorescence intensity between different seeding durations as measured using a microplate reader.

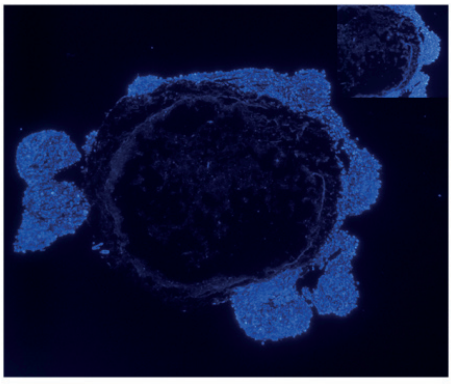


Figure 9. Hoechst cross-sectional images. A frozen cross-section is depicted after 12 hours of seeding and imaged at 5× magnification (inset = 10×). Samples of later time points contained freeze and sectioning artifacts and are therefore not depicted.

DISCUSSION

Prior techniques used for stem cell seeding in peripheral nerve research have primarily used static-seeding methods, such as the injection method (table 1).[4,27-34] Aside from the known disadvantages of static seeding, the process of (micro) injection can be traumatic to both stem cells and delicate intra-neural architecture and may lead to abnormal cell distribution. Additionally, possible aggregation of cells due to needle passage can result in clusters of cells in the vicinity of the injection point. Moreover, injecting cells into small acellular nerves can be difficult.[35,36] An alternative to the static injection technique is desirable, in particular for seeding of decellularized nerve allografts as they are delicate and abundant in extracellular matrix proteins. In this study, we describe a novel and technically straightforward seeding method that effectively and consistently seeds acellular nerve allografts in a dynamic bioreactor.

In this study, results from MTS assays indicate that the seeding does not affect MSC metabolic activity. SEM images showed that the rotating motion of the bioreactor enabled cells to distribute homogeneously over the graft and yielded a maximum seeding efficiency of 89.7%. This method also minimizes cell injury, as demonstrated by the Live/Dead stain. Furthermore, bioreactor based cell seeding is reproducible and not traumatic to intra-neural architecture.

Table 1. Stem cell delivery methods in peripheral nerve literature.

Author (year)	Seeding method	Application	Cell source	Concentration (cells/ml)
di Summa (2010)	Injected into	Fibrin nerve conduits	Schwann cells	$2x10^6$
Zhang (2010)	Microinjection	Xenogeneic acellular nerve matrix	AMSCs	$5x10^6$
Lui (2011)	Microinjection	Acellular nerve allografts	AMSCs	$2x10^6$
Jesuraj (2011)	Injection	Acellular nerve graft	Schwann cells	$1x10^6$
Nijhuis (2013)	Injection	Vein conduit	BMSC	$3x10^6$
Zhao (2014)	In fibrin glue (injected around nerve)	Acellular nerve	BMSC	$5x10^5$
Ozan (2016)	Injection	Autograft	AMSCs	$1x10^6$
Klein (2016)	Static onto	Type 1 collagen conduit	AMSCs	$2x10^6$
Sowa (2016)	Infused into lumen	Gelatin hydrogel	AMSCs	$1x10^4$

A secondary goal of this study was to determine the optimal seeding duration. After 12 hours the surfaces of the allografts were entirely covered with cells, and a significantly greater cell attachment and proliferation (seeding efficiency) was exhibited compared to shorter seeding durations. No statistical differences were found between 12, 24 and 72 hours of seeding,

which is consistent within all outcome measurements as well as previous observations showing that MSCs exhibit exponential growth in the first days of seeding.[25] Although not statistical significant, live cell counts showed a decrease at 72 hours of incubation. This is secondary to the death of cells that do not attach to the nerve graft; although the exact effect is unknown, these dead cells could potentially secrete harmful signals. Also, longer incubation times could theoretically increase cell damage from the bioreactors rotational forces. We therefore hypothesize no further decrease in cell counts after 72 hours of seeding when nerves are placed in a culture dish in the incubator after 12hours of seeding in the bioreactor and cell media is changed after 72-96 hours.

Infiltration was studied by Hoechst stains of cross-sections and MSCs did not migrate on a large scale. The presence of flat sheets of cells occluding the superficial pores may have limited the ability of MSCs to penetrate the inner aspects of the grafts. In contrast, it has been hypothesized that longer culture periods (weeks) may allow cells to proliferate and migrate toward the center of the graft.[37] While mechanisms underlying the neurotrophic potential of MSCs remain largely unknown, it is postulated that the local production of growth factors promote neuronal surival, and help guide axons during regeneration.[9,10] Although the transition from MSC to Schwann cell has been reported previously, other investigators have reported regeneration with few remaining MSCs and in the absence of Schwann cell-like differentiation, suggesting alternative mechanisms of support, such as enhancing host repair mechanisms.[11,38,39] Growthfactors could penetrate the graft by diffusion and therefore the role of MSC migrating into the nerve allograft may not be an important variable. Zhao et al. compared the effect of MSCs injected inside or outside an decellularized nerve graft and concluded that supplementing MSCs around nerve grafts was effective and did not destroy the graft while having the same effect on nerve regeneration as injecting MSCs inside the grafts.[31] Another concern with numerous cells in the lumen of the graft is that the cells would block the nerve growth cone during regeneration.

One limitation of the study is the lack of direct comparison to other seeding methods. Static seeding techniques yield efficiencies of approximately 10-25% while dynamic techniques result in efficiencies, ranging from 60% to 90%.[40] Drawbacks of dynamic seeding strategies in general include prolonged seeding time in low-speed rotational systems and potential cell damage in high-speed rotational systems.[17] Our proposed bioreactor strategy does take longer than the static injection method, but decreases the potential risk of damage to both the cells and the nerve graft while improving seeding efficiency. Furthermore, dynamic seeding protocols allow the clinician to evaluate the seeded graft prior to transplantation, and when used for research purposes, the cells can be labeled and tracked. Other limitations include our small sample size and manual cell counts. The 3D structure of the nerves impedes automated cell counts and evaluation of infiltration of the cells was complicated by freeze and sectioning artifacts of the samples. These limitations notwithstanding, all outcome measurements were consistent and reproducible.

Leferink et al. and Tan et al. demonstrated the importance of seeding parameters.[41,42] There is extensive literature, which have repeatedly found dynamic techniques to be superior to static techniques in terms of cell seeding homogeneity and cell density.[43,44] Most of the older studies seeded synthetic conduits that do not have an inner matrix structure. The nerve allograft, however, is more vulnerable and may be more difficult to seed adequately. Jesuraj et al. demonstrated this in their study, the insertion of a 24-gauge needle in cold-preserved acellular nerve grafts resulted in tearing of the epineurium and this would allow the cells to escape the nerve graft.[29] With the proposed dynamic bioreactor seeding method, there is no risk of damaging of the cells or the graft, which makes it a more reliable method.

CONCLUSION

This study describes and validates a convenient and technically feasible method for effective seeding of cells onto decellularized nerve allografts using a dynamic bioreactor. This method can also be applied to seed other grafts or tubular structures. Cells are homogenously distributed over the graft, this method also minimizes cell injury, is reproducible, is operator independent and is not traumatic to the graft or the stem cells. Twelve hours of seeding appears to be the optimal time to obtain a homogenous seeding. Utilization of a validated, reproducible and effective seeding technique permits consideration of rigorously testing of cell-enhanced allografts for a range of biological parameters during optimization for possible use of these hybrid grafts in clinical applications for nerve repair.

APPENDIX 1

Protocol: Rotator cell seeding

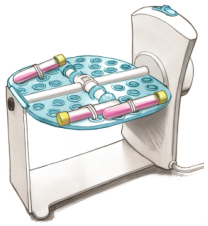
1. Harvest nerve segments under aseptic conditions and remove fatty and connective tissue from the epineurium. Place nerves immediately in Roswell Park Memorial Institute medium 1640 solution (RPMI) at 4°C until further processing.
2. Decellularize the nerves using the protocol as previously published.[19]

   All subsequent steps should be conducted in a laminar flow hood for sterility.

3. Grow cells in cell culture medium, according to standard protocol (check for each cell type the media and growing conditions). For AMSCs, Advanced Minimum Essential Media with the addition of 26.5 ml of Platelet Lysate, 1 ml of Heparin, 5 ml of Glutamax and 5 ml of Penicillin Streptomycin was used.

   Aim for approximately 1 million cells per sample that will be seeded.

4. Place samples in cell culture medium and soak for 2 hours to remove possible toxic residues.
5. Combine nerves with the AMSCs in a 15 ml TubeSpin Bioreactor tube containing 10 ml cell culture medium. Use 1 million AMSCs/sample, maximum of 4 samples/tube.
6. Place the tubes horizontally in a bioreactor rotator system (Revolver™ Labnet, Edison, USA) and secure the tubes with a rubber band. Balance the tubes on the rotator with a rotation axis of 30°.

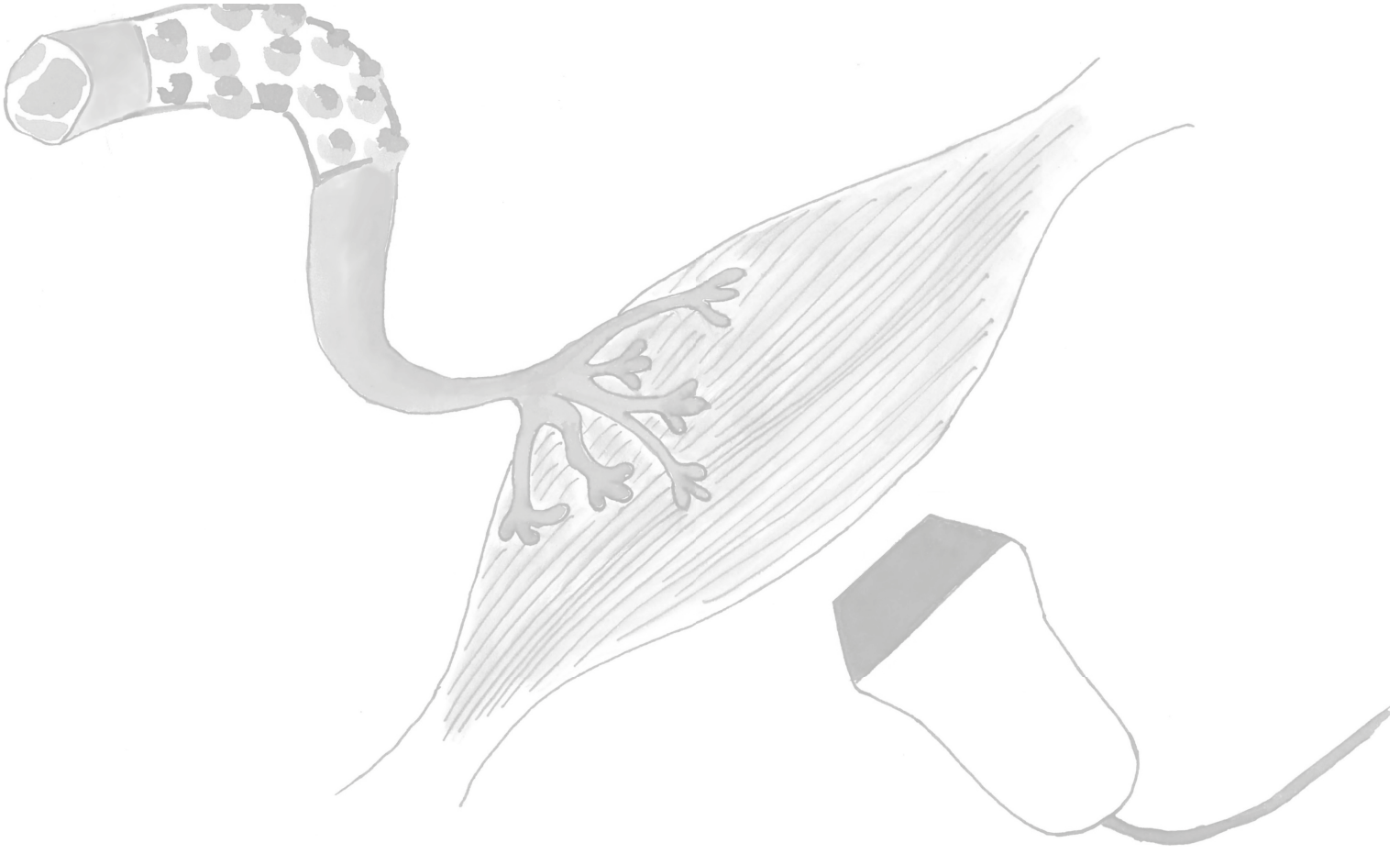


7. Turn rotator on at fixed speed of 18 rpm and place the rotator in an incubator at 37 degrees Celsius (98.6 Fahrenheit) and 5% CO2.
8. Incubate for 12 hours.
9. Place nerves in a culture dish after 12 hours of seeding.

REFERENCES

1. Robinson LR. Traumatic injury to peripheral nerves. Muscle Nerve. 2000;23(6):863-873.
2. Nectow AR, Marra KG, Kaplan DL. Biomaterials for the development of peripheral nerve guidance conduits. Tissue Eng Part B Rev. 2012;18(1):40-50.
3. Whitlock EL, Tuffaha SH, Luciano JP, et al. Processed allografts and type I collagen conduits for repair of peripheral nerve gaps. Muscle Nerve. 2009;39(6):787-799.
4. Zhang Y, Luo H, Zhang Z, et al. A nerve graft constructed with xenogeneic acellular nerve matrix and autologous adipose-derived mesenchymal stem cells. Biomaterials. 2010;31(20):5312-5324.
5. Cho MS, Rinker BD, Weber RV, et al. Functional outcome following nerve repair in the upper extremity using processed nerve allograft. J Hand Surg Am. 2012;37(11):2340-2349.
6. Brooks DN, Weber RV, Chao JD, et al. Processed nerve allografts for peripheral nerve reconstruction: A multicenter study of utilization and outcomes in sensory, mixed, and motor nerve reconstructions. MICROSURGERY. 2012;32(1):1-14.
7. Moore AM, MacEwan M, Santosa KB, et al. Acellular nerve allografts in peripheral nerve regeneration: a comparative study. Muscle Nerve. 2011;44(2):221-234.
8. Dudakovic A, Camilleri E, Riester SM, et al. High-resolution molecular validation of self-renewal and spontaneous differentiation in clinical-grade adipose-tissue derived human mesenchymal stem cells. J Cell Biochem. 2014;115(10):1816-1828.
9. Moore AM, Wood MD, Chenard K, et al. Controlled delivery of glial cell line-derived neurotrophic factor enhances motor nerve regeneration. J Hand Surg Am. 2010;35(12):2008-2017.
10. Pfister LA, Papaloizos M, Merkle HP, Gander B. Nerve conduits and growth factor delivery in peripheral nerve repair. J Peripher Nerv Syst. 2007;12(2):65-82.
11. Hundepool CA, Nijhuis TH, Mohseny B, Selles RW, Hovius SE. The effect of stem cells in bridging peripheral nerve defects: a meta-analysis. J Neurosurg. 2014.
12. Ikebe C, Suzuki K. Mesenchymal stem cells for regenerative therapy: optimization of cell preparation protocols. Biomed Res Int. 2014;2014:951512.
13. Buizer AT, Veldhuizen AG, Bulstra SK, Kuijer R. Static versus vacuum cell seeding on high and low porosity ceramic scaffolds. J Biomater Appl. 2014;29(1):3-13.
14. Solchaga LA, Tognana E, Penick K, et al. A rapid seeding technique for the assembly of large cell/scaffold composite constructs. Tissue Eng. 2006;12(7):1851-1863.
15. Mazzucotelli JP, Roudiere JL, Bernex F, Bertrand P, Leandri J, Loisance D. A new device for endothelial cell seeding of a small-caliber vascular prosthesis. Artif Organs. 1993;17(9):787-790.
16. Shirota T, He H, Yasui H, Matsuda T. Human endothelial progenitor cell-seeded hybrid graft: proliferative and antithrombogenic potentials in vitro and fabrication processing. Tissue Eng. 2003;9(1):127-136.
17. Villalona GA, Udelsman B, Duncan DR, et al. Cell-seeding techniques in vascular tissue engineering. Tissue Eng Part B Rev. 2010;16(3):341-350.
18. Thevenot P, Nair A, Dey J, Yang J, Tang L. Method to analyze three-dimensional cell distribution and infiltration in degradable scaffolds. Tissue Eng Part C Methods. 2008;14(4):319-331.
19. Hundepool CA, Nijhuis TH, Kotsougiani D, Friedrich PF, Bishop AT, Shin AY. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. Neurosurg Focus. 2017;42(3):E4.
20. Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G. Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. EXP NEUROL. 2007;207(2):267-274.
21. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. Tissue Engineering. 2004;10(9-10):1346-1358.
22. Crespo-Diaz R, Behfar A, Butler GW, et al. Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability. Cell Transplant. 2011;20(6):797-811.

23. Mader EK, Butler G, Dowdy SC, et al. Optimizing patient derived mesenchymal stem cells as virus carriers for a phase I clinical trial in ovarian cancer. J Transl Med. 2013;11:20.
24. Mahmoudifar N, Doran PM. Osteogenic differentiation and osteochondral tissue engineering using human adipose-derived stem cells. Biotechnol Prog. 2013;29(1):176-185.
25. Dudakovic A, Camilleri ET, Lewallen EA, et al. Histone deacetylase inhibition destabilizes the multi-potent state of uncommitted adipose-derived mesenchymal stromal cells. J Cell Physiol. 2015;230(1):52-62.
26. J.N. K. A new method for gray-level picture thresholding using the entropy of the histogram. Computer Vision, Graphics, and Image Processing. 1985;29(3):273-285.
27. di Summa PG, Kingham PJ, Raffoul W, Wiberg M, Terenghi G, Kalbermatten DF. Adipose-derived stem cells enhance peripheral nerve regeneration. J Plast Reconstr Aesthet Surg. 2010;63(9):1544-1552.
28. Liu GB, Cheng YX, Feng YK, et al. Adipose-derived stem cells promote peripheral nerve repair. Arch Med Sci. 2011;7(4):592-596.
29. Jesuraj NJ, Santosa KB, Newton P, et al. A systematic evaluation of Schwann cell injection into acellular cold-preserved nerve grafts. J Neurosci Methods. 2011;197(2):209-215.
30. Nijhuis TH, Bodar CW, van Neck JW, et al. Natural conduits for bridging a 15-mm nerve defect: comparison of the vein supported by muscle and bone marrow stromal cells with a nerve autograft. J Plast Reconstr Aesthet Surg. 2013;66(2):251-259.
31. Zhao Z, Wang Y, Peng J, et al. Improvement in nerve regeneration through a decellularized nerve graft by supplementation with bone marrow stromal cells in fibrin. Cell Transplant. 2014;23(1):97-110.
32. Abbas OL, Borman H, Uysal CA, et al. Adipose-Derived Stem Cells Enhance Axonal Regeneration through Cross-Facial Nerve Grafting in a Rat Model of Facial Paralysis. Plast Reconstr Surg. 2016;138(2):387-396.
33. Klein SM, Vykoukal J, Li DP, et al. Peripheral Motor and Sensory Nerve Conduction following Transplantation of Undifferentiated Autologous Adipose Tissue-Derived Stem Cells in a Biodegradable U.S. Food and Drug Administration-Approved Nerve Conduit. Plast Reconstr Surg. 2016;138(1):132-139.
34. Sowa Y, Kishida T, Imura T, et al. Adipose-Derived Stem Cells Promote Peripheral Nerve Regeneration In Vivo without Differentiation into Schwann-Like Lineage. Plast Reconstr Surg. 2016;137(2):318e-330e.
35. Jiang L, Jones S, Jia X. Stem Cell Transplantation for Peripheral Nerve Regeneration: Current Options and Opportunities. Int J Mol Sci. 2017;18(1).
36. Vitacolonna M, Belharazem D, Hohenberger P, Roessner ED. Effect of static seeding methods on the distribution of fibroblasts within human acellular dermis. Biomed Eng Online. 2013;12:55.
37. Griffon DJ, Abulencia JP, Ragetly GR, Fredericks LP, Chaieb S. A comparative study of seeding techniques and three-dimensional matrices for mesenchymal cell attachment. J Tissue Eng Regen Med. 2011;5(3):169-179.
38. Marcus AJ, Coyne TM, Black IB, Woodbury D. Fate of amnion-derived stem cells transplanted to the fetal rat brain: migration, survival and differentiation. J Cell Mol Med. 2008;12(4):1256-1264.
39. Prockop DJ. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). Clin Pharmacol Ther. 2007;82(3):241-243.
40. Orlando G. Regenerative Medicine Applications in Organ Transplantation. 1st Edtion ed. USA: Elsevier; 2013.
41. Leferink AM, Hendrikson WJ, Rouwkema J, Karperien M, van Blitterswijk CA, Moroni L. Increased cell seeding efficiency in bioplotted three-dimensional PEOT/PBT scaffolds. J Tissue Eng Regen Med. 2016;10(8):679-689.
42. Tan L, Ren Y, Kuijer R. A 1-min method for homogenous cell seeding in porous scaffolds. J Biomater Appl. 2012;26(7):877-889.
43. Hasegawa T, Miwa M, Sakai Y, et al. Efficient cell-seeding into scaffolds improves bone formation. J Dent Res. 2010;89(8):854-859.
44. Wendt D, Marsano A, Jakob M, Heberer M, Martin I. Oscillating perfusion of cell suspensions through three-dimensional scaffolds enhances cell seeding efficiency and uniformity. Biotechnol Bioeng. 2003;84(2):205-214.



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Seeding of decellularized nerve allografts with adipose-derived Mesenchymal Stromal Cells: An in-vitro analysis of the gene-expression and growth factors produced.

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* Both authors contributed equally to this work.

Submitted

SUMMARY

Mesenchymal stromal cells (MSCs) secrete many soluble growth factors and have previously been shown to stimulate nerve regeneration. MSC seeded processed nerve allografts could potentially be a promising method for large segmental motor nerve injuries. Further progress in our understanding of how the functions of MSCs can be leveraged for peripheral nerve repair is required before clinical translation can be made. The present study therefore investigated whether interactions of adipose-derived MSCs with decellularized nerve allografts can improve gene- and protein expression of growth factors that may support nerve regeneration.

Human nerve allografts (n=30) were decellularized and seeded with undifferentiated human adipose-derived MSCs. Subsequently, the MSCs and MSC seeded grafts were isolated at days 1, 3, 7, 14 and 21 days in culture for RNA expression analysis by qRT-PCR. Evaluated genes included NGF, BDNF, PTN, GAP43, MBP, PMP22, VEGF and CD31. Growth factor production was evaluated and quantified using Enzyme-Linked Immunosorbent Assay (ELISA).

Over 21 days, semi-quantitative RT-PCR analysis showed that adherence of MSCs to nerve allografts significantly enhances mRNA expression of neurotrophic, angiogenic, endothelial and myelination markers (e.g., BDNF, VEGF, CD31 and MBP). ELISA analysis revealed an upregulation of BDNF and reduction of both VEGF and NGF protein levels.

This study demonstrates that seeding of undifferentiated adipose-derived MSCs onto processed nerve allografts permits the secretion of neurotrophic and angiogenic factors that can stimulate nerve regeneration. These favorable molecular changes suggest that MSC supplementation of nerve allografts may have potential in improving nerve regeneration.

INTRODUCTION

Processed nerve allografts have shown promising results in peripheral nerve reconstruction. However, for large segmental motor nerve injuries results remain inferior to autograft reconstruction.[1,2,3-6] In autograft reconstruction, Schwann cells facilitate nerve regeneration by promoting myelination through growth factor production and interactions with the extracellular matrix (ECM).[7] The internal structure and ECM components of a nerve graft are critical for guiding cell migration and nerve fiber elongation. Therefore, in optimally decellularized allografts, the internal structure is preserved while cellular components, including Schwann cells, are removed to create a non-immunogenic graft.[8]

To improve the performance of allografts, an optimized environment of biological support must be created around the allograft. Many authors have studied the in vivo delivery of growth factors, mostly using delivery mechanisms such as micropumps and microspheres. This constant infusion has not shown to support nerve regeneration and in some cases even impaired nerve regeneration.[9-11]

In different stages of nerve regeneration there is a biological demand for different growth factors.[12] With this understanding, we addressed whether cell-based production of local growth factors mimics the biological requirements in the microenvironment of the allograft. Undifferentiated Mesenchymal stromal cells (MSCs), including those isolated from the stromal vascular fraction of adipose tissue, have trophic functions in tissue repair. They can secrete many soluble growth factors, including VEGF, NGF, BDNF and interleukins and have previously been shown to stimulate nerve regeneration.[13-15]

Although MSCs have proven to be beneficial for nerve regeneration, the actual mechanism is yet unclear. MSCs are thought to support peripheral nerve regeneration via local production of growth factors rather than as active participants in the regeneration process and it is postulated that the remaining nerve allograft extracellular matrix (ECM) still has biological activity that influences the MSCs and their differentiation.[16,17]

The combination of a patient's own adipose derived stem cells and the readily available processed nerve allograft is potentially a promising method for individualized peripheral nerve repair, providing results equal to the patient's own harvested nerves. However, further understanding of how the capacities of MSCs can be leveraged for peripheral nerve repair is required before clinical translation can be considered. The present study therefore investigated whether interactions of adipose-derived MSCs with decellularized nerve allografts can increase mRNA and protein expression of growth factors that may support nerve regeneration.

MATERIALS AND METHODS

General design

This study was approved by our Institutional Review Board (IRB 13-008081). To determine the interaction between the optimized decellularized nerve allograft and MSCs, a total of 30 human cadaver nerve segments were decellularized and seeded with clinical grade human MSCs. Subsequently, the MSCs and MSC seeded grafts were cultured for 21 days and sampled at various time points (1, 3, 7, 14 and 21 days). Changes in gene expression profiles (phenotype) of the MSCs were quantified and the production of growth factors was measured (Figure 1).

Allograft preparation

Human motor nerves (thoracodorsal and long thoracic nerves) were obtained from one fresh human cadaver within 12 hours post mortem (male, Caucasian, age 62). The subject had no known history of conditions that affect the peripheral nervous system. A total of 30 3-cm nerve segments were aseptically removed, cleared of peripheral fat and connective tissue, and were decellularized using the protocol described by Hundepool et al.[18] Briefly, the samples were treated with a series of detergents including Triton X- 200, sulfobetaine-16, sulfobetaine-10, chondroitinase and elastase. The nerves were sterilized using γ-radiation and stored at 4°C for 7 days prior to seeding. All chemicals were purchased from Sigma (St. Louis, MO, USA) and all solutions were autoclaved or filter-sterilized before use.

Isolation of mesenchymal stromal cells

Human MSCs were isolated from lipo-aspirates from a representative 41-year-old male donor with written individual consent and institutional approval. These cells have been routinely used at our institution for clinical trial applications and have been extensively tested for cell surface markers, RNA-sequence transcriptome profiles, and multi-lineage potential.[19,20] Cell isolation and culture conditions have previously been described.[19,21,22] Cell culture expansion for this experiment started with passage 4 cells and were passaged 2 additional times to obtain 35 million cells (1 million per nerve graft). All the MSCs used in the experiment were of passage 6 and all the experiments were performed at the same time in parallel.

Cell seeding

A total of 25 human nerve allografts were seeded with clinical grade human undifferentiated MSCs using a dynamic bioreactor as previously described.[23] Briefly, nerve allografts were combined with MSCs in a 15 mL TubeSpin® Bioreactor tube containing 10 ml cell culture medium and 1 million cells per nerve (max. 4 nerves per tube). Tubes were secured on a bioreactor rotator system (Revolver™ Labnet, Edison, USA) with a rotation axis of 30° at a fixed speed of 18 rpm. The rotating device was placed in an incubator for 12 hours at 37 degree Celsius and 5% CO_2. After incubation, seeded nerves were placed in a culture dish, containing advanced Minimum Essential Medium (MEM) (Life Technologies, Grand Island, NY)

containing 5% human platelet lysate, 2 mmol/L Glutamax, 2 U/ml heparin and 1% penicillin/streptomycin solution. Cell culture media was changed every 3-4 days.

A

Group	Description	Time points (days)	N	Outcome measurements
I	Seeded nerve graft (nerve graft + MSCs)	T= 1 T= 3 T= 7 T= 14 T= 21	n=5 per experimental group for each time point	**qPCR** analysis for: experimental genes: NGF, BDNF, GDNF, PTN, GAP43, VEGFA, CD31, MPZ, PMP22, MBP, COL1A1, COL3A1, LAMB2, FBLN1, CASP3, EGR1, CD96 reference genes: GAPDH, AKT1 **ELISA** for: NGF, BDNF, GDNF, VEGFA
II	Only MSCs (in static culture)			
III	Only nerve graft (negative control)	T= 0		

B

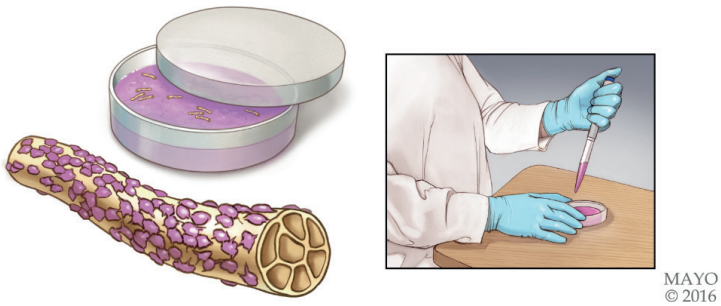

Figure 1. Experimental design. **A**. A total of 25 human nerve allografts were decellularized and in vitro seeded with clinical grade human MSCs. Subsequently, the MSC seeded grafts and MSCs cultured in media only were cultured for 21 days and at various time points (1, 3, 14 and 21 days), total RNA was extracted, reverse transcribed into cDNA and qRT-PCR was performed for genes essential for nerve regeneration and additional genes to map MSC characteristics including proliferation, apoptosis, myelination and ECM molecules. Growth factor production was evaluated and quantified using Enzyme-Linked Immunosorbent Assay (ELISA). **B**. Shows an image of MSCs seeded onto a decellularized nerve graft.

Quantitative Real Time Reverse-Transcriptase Polymerase Chain Reaction (qRT-PCR)

To quantify changes in relative gene expression profiles of MSCs in culture and MSCs seeded onto the decellularized nerve allografts, qRT-PCR was performedat day 1, 3, 7, 14 and 21. Total RNA was extracted from 5 samples using the miRNeasy Mini Kit (Qiagen, Valencia, CA) and RNA yield was evaluated using a Nanodrop 2000 (Thermo Scientific, Inc., Waltham, MA) followed by reverse transcription into cDNA by RT-PCR using SuperScript III (Invitrogen, Carlsbad, CA). The resulting cDNA libraries were analyzed by qRT-PCR (C1000 Touch Thermalk Cycler, BioRad, Hercules, CA) using SYBR Green detection and specific primers for the panel of genes represented in Table 1. All genes were chosen from the literature and have previously been shown to be important for nerve regeneration.[24-27] All samples were analyzed in triplicate and results were normalized to the reference gene AKT1 within each sample. AKT1 was selected as a housekeeping gene because it remains constant among a larger number of tissues, cells and conditions better than other commonly used normalizing genes (e.g., GAPDH and HPRT1; pers. obs.). Decellularized nerve allografts without MSCs (n=5) were

used as a negative control. The differences in gene expression levels were quantified using the comparative delta crossover threshold ($2^{-\Delta\Delta Ct}$) method.[20,26]

Table 1: mRNA primer sequences

Gene ID	Biology	Forward primer	Reverse primer
GAPDH	Reference gene	CCCGGTACACCACGTTCTTC	TGTGGTCATGAGTCCTTCCA
AKT1	Reference gene	ATGGCGCTGAGATTGTGTCA	CCCGGTACACCACGTTCTTC
NGF	Neurotrophic marker	ATACAGGCGGAACCACACTCAG	ATACAGGCGGAACCACACTCAG
BDNF	Neurotrophic marker	AGAGGCTTGACATCATTGGCTG	CAAAGGCACTTGACTACTGAGCATC
GDNF	Neurotrophic marker	CACCAGATAAACAAATGGCAGTGC	CACCAGATAAACAAATGGCAGTGC
PTN	Neurotrophic marker	ACTGGAAGTCTGAAGCGAGC	CTTCTTCTTAGATTCTGCTTGAGGT
GAP43	Neurotrophic marker	GTCCACTTTCCTCTCTATTTC	TGTTCATTCCATCACATTGA
VEGFA	Angiogenic marker	ATCTGCATGGTGATGTTGGA	GGGCAGAATCATCACGAAG
PECAM/ CD31	Angiogenic marker	AACAGTGTTGACATGAAGAGCC	AACAGTGTTGACATGAAGAGCC
MPZ	Myelination marker	GAGGAGGCTCAGTGCTATGG	GCCCGCTAACCGCTATTTCT
PMP22	Myelination marker	GTTAAAGGGAACGCCAGGA	AGTTTCTGCAGCCCAAAGGA
MBP	Myelination marker	GGCTGTGCAACATGTACAAGGA	GGACAGTCCTCTCCCCCTTTCC
COL1A1	ECM protein	GTAACAGCGGTGAACCTGG	CCTCGCTTTCCTTCCTCTCC
COL3A1	ECM protein	TTGAAGGAGGATGTTCCCATCT	ACAGACACATATTTGGCATGGTT
LAMB2	ECM protein	ACACGCAAGCGAGTGTATGA	AATCACAGGGCAGGCATTCA
FBLN1	ECM protein	AGAGCTGCGAGTACAGCCT	CGACATCCAAATCTCCGGTCT
CASP3	Apoptosis protein	GGACCTGTGGACCTGAAAAA	AGTTCGGCTTTCCAGTCAG
EGR1	Transcription factor	ACCCCTCTGTCTACTATTAAGGC	TGGGACTGGTAGCTGGTATTG
CD96	Immunoglobulin	AGATTGTGTGATGAAGGACATGG	AGATTGTGTGATGAAGGACATGG

Protein isolation and Enzyme-linked immunosorbent Assay (ELISA)

Production of growth factors by MSCs was evaluated by analysis of cell supernatants by Enzyme-Linked Immunosorbent Assay (ELISA). Cell supernatants were collected at 1, 3, 7, 14, and 21 days and centrifuged at 1,500 rpm for 10 min at 4°C. Aliquots were stored at -80°C. Since ELISA is a costly technique, four sandwich ELISA kits were selected for the most referenced factors and availability: NGF, GDNF, VEGF (catalog numbers: EHNGF, EHGDNF, KHG0111, Thermofisher, Waltham, MA) and BDNF (catalog number: MBS355324, MyBioSource, San Diego, CA). All reagents, samples and standards were prepared as per the manufacturer's instructions. Briefly, samples and standards were loaded in triplicates onto wells and incubated at room temperature for 2.5 hours. Plates were rinsed four times, and incubated with 100 µl Biotinylated Antibody for 1 hour. Plates were rinsed again four times and incubated with 100 µl Streptavidin HRP reagent, and subsequently rinsed four more times. The HRP reaction was initiated by addition of 100 µl TMB Substrate to each well and

incubated in the dark for 30 minutes. Reactions were terminated by addition of 0.2 M sulfuric acid and absorbance was measured at 450 nm on a SpectraMax190 microplate reader (Molecular Devices, Inc., Sunnyvale, CA). Samples were analyzed in triplicate and absorbance values of advanced MEM were used as negative controls. A standard curve was plotted to determine concentrations (pg/ml).

Statistical analysis

The Kolmogorov-Smirnoff test was applied to test for a normal distribution. To detect differences in gene expression between sampled groups, data were analyzed using the Mann-Whitney U test. A value of $p<0.05$ was considered statistically significant. All results are reported as mean ± standard deviation.

RESULTS

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Many soluble growth factors are secreted by MSCs and the interaction of MSCs with the allograft may alter expression of nerve-related growth factors or angiogenic proteins that are collectively conducive for allograft rejuvenation. Therefore, we examined whether seeding MSCs altered expression of select growth factors and cell adhesion molecules linked to neurogenesis or angiogenesis. The interaction between the regeneration-associated genes is likely to be a very dynamic process and therefore multiple time-points were studied.

As a negative control we first evaluated the gene expression of the decellularized nerve graft without cells. No RNA levels were detectable at time point 0 and therefore no comparisons were made for baseline expression (with the MSC group).

Figures 2a-d demonstrate the relative MSC gene expression levels clustered by genes that share a common function. NGF is important for the development and maintenance of the sympathetic and sensory nervous systems.[12] qPCR analysis revealed that NGF mRNA levels were down regulated when MSCs were seeded on a decellularized (motor) nerve graft, suggesting that upon adhesion to the substrate, MSCs become less involved in sensory nerve growth.

The BDNF-gene promotes the survival and differentiation of selected neuronal populations of the peripheral nervous system and participates in axonal growth.[28] qPCR analysis showed a significant up regulation of the BDNF maker upon seeding, suggesting that the interaction between MSCs and the nerve allograft triggers axonal growth.

We measured GDNF to assess the potential of MSCs in preventing motor neuron apoptosis (induced by axotomy), qPCR analysis revealed that GDNF mRNA levels significantly decreased

when MSCs were seeded, suggesting that the beneficial effect of seeded MSCs is not caused by the prevention of motor neuron apoptosis.[29]

The PTN-gene induces neurite outgrowth and has significant roles in cell growth and survival.[30] qPCR analysis showed that PTN mRNA levels were increased upon MSC seeding, suggesting that neurite outgrowth is supported in response to MSC adhesion.

GAP43 is expressed at high levels in neuronal growth cones during development and axonal regeneration and is considered a crucial component of an effective regenerative response[30]. qPCR analysis showed that GAP43 levels were up regulated upon seeding, suggesting that axonal regeneration is promoted upon adhesion to the graft.

The VEGFA growth factor plays a role in angiogenesis.[31] CD31 is involved in leukocyte migration, angiogenesis and integrin activation.[32] MPZ is required for the proper formation and maintenance of myelin. PMP22 is produced by Schwann cells, it plays a crucial role in the development and maintenance of myelin. MBP has a role in myelin formation and stabilization.[33] All of the above mentioned markers were highly expressed in the MSC seeded nerves but minimally in the MSCs only, suggesting that upon adhesion, the MSCs increase angiogenesis and myelination.

In vitro expansion of the MSCs resulted in high levels of ECM-related gene expression in both groups. COL1A1 and COL3A1 encode collagen type 1 and 3 that are found in most connective tissues. LAMB2, also an ECM marker, plays a role in cell adhesion, differentiation, migration and signaling as well as neurite outgrowth, it regulates all stages of Schwann cell development. Furthermore, FBLN also plays a role in cell adhesion and migration within the ECM.[34] This up regulation suggests that seeded MSCs retain their adhesion, migration and proliferation potential.

Of the other genes that were evaluated, CASP3 was measured to assess the potential of seeded MSCc to mediate cell apoptosis.[35] EGR1 functions as a transcriptional regulator, plays a role in the regulation of cell survival, proliferation and cell death.[36] CD96 is involved in the late phase of the immune response. qPCR analysis revealed a moderate up regulated expression of these markers over 21 days in the seeded MSCs (Figure 2d), suggesting that upon adhesion, the remaining nerve ECM can trigger cell apoptosis and cell survival while involving the late immune response.

In summary, for individual time-points there were significant differences for the following genes: NGF, BDNF, GDNF, PTN, GAP43, VEGF, CD31, PMP22, MBP, COL1A1, FBLN1, EGR1 and CD96. The majority of these differences occurred in the first week (D0-D7). No significant differences were found in MPZ, COL3A1, LAMB2, FBLN1 and CASP3 expression (Figure 2-d).

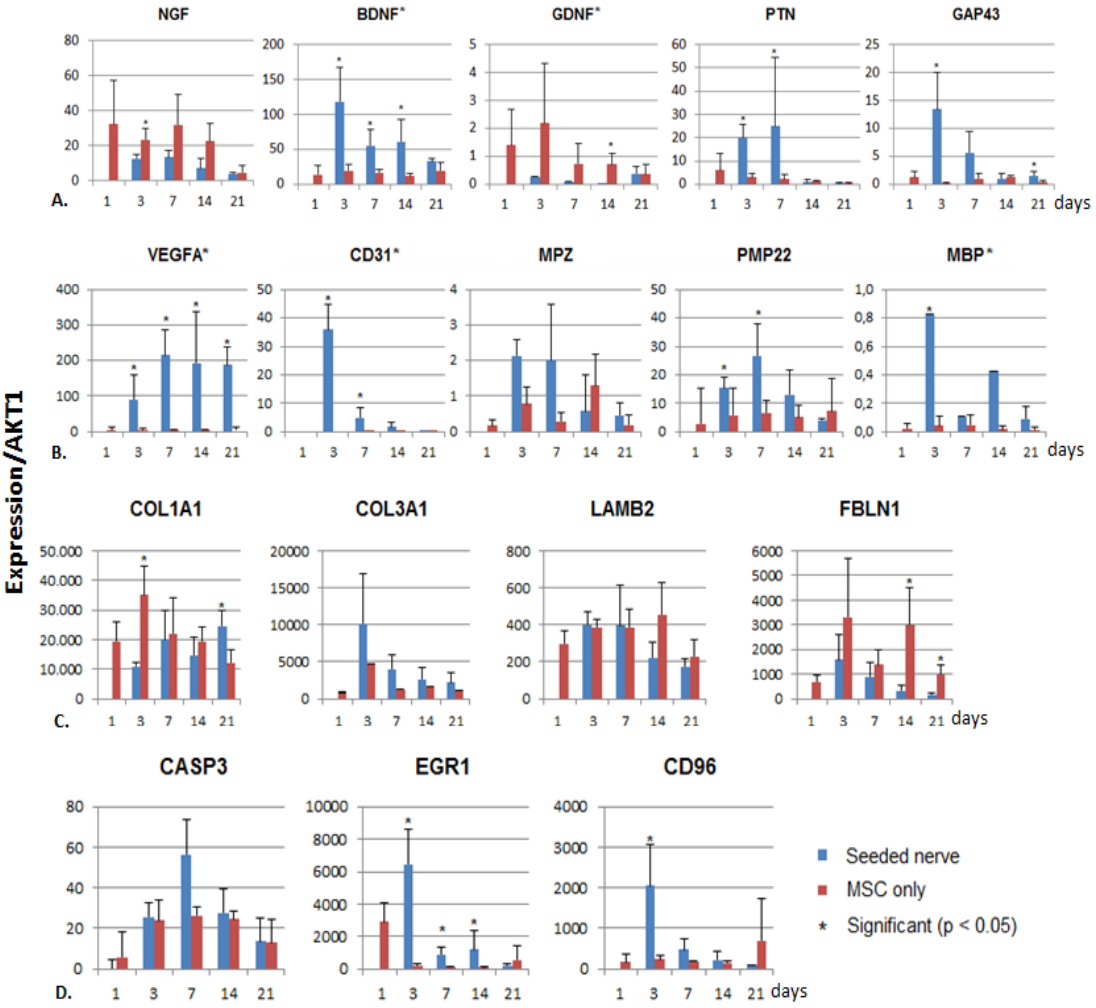

Figure 2. Gene expression clustered by genes that share a common function. **A.** Neurotrophic factors **B.** Angiogenic factors **C.** Extracellular matrix (ECM) factors **D.** Other factors: apoptosis protein (CASP3), transcription factor (EGR1) and immunoglobulin (CD96)

Those genes that showed significant changes in gene expression over 21 days were BDNF ($p = 0{,}021$), GDNF ($p = 0{,}043$), VEGF ($p = 0{,}021$), CD31 ($p = 0{,}021$) and MBP ($p = 0{,}021$). Neurotrophic, angiogenic and myelination related gene-expression (BDNF, VEGF, CD31 and MBP) was significantly upregulated in the seeded MSCs while in contrast the neurotrophic marker GDNF was significantly upregulated in the MSC only group (Figure 2-d).

Protein isolation and Enzyme-linked immunosorbent Assay (ELISA)

To assess whether changes in mRNA levels for secreted factors in MSCs is reflected by

corresponding changes in proteins secreted into the medium, we performed Enzyme-Linked Immunosorbent Assay (ELISA) assays. Figure 3 depicts the production of three factors analyzed by ELISA over 21 days.

Similar to the qPCR results, we found a decreasing trend for the NGF protein, suggesting that regulation at the protein level (e.g., translation, protein stability or secretion) is altered in MSCs attached to nerve allografts. The NGF protein production in the first 24 hours was comparable in both groups. At individual time points there were significant differences at time points 3 days ($p = 0.017$) and 7 days ($p = 0.003$).

ELISA assays revealed a significant ($p = 0.011$) upregulated BDNF protein production over the time course of 21 days by the seeded MSCs (393.48 pg/ml ± 64.52 at 24 hours compared to18.73 pg/ml ± 7.21 in the MSC only group), correlating with the significant upregulation of BDNF gene expression.
The significant upregulation of GDNF gene-expression in the MSC only group did not correlate with an increased GDNF-protein production. ELISA growth factor analysis showed no detectable protein production in either group.

Table 2. Summary of seeding induced changes of growth factor production.

Gene ID	Biology	mRNA	Protein
NGF	Neurotrophic marker	↓	↓
BDNF	Neurotrophic marker	↑↑*	↑↑*
GDNF	Neurotrophic marker	↓*	
PTN	Neurotrophic marker	↑	
GAP43	Neurotrophic marker	↑	
VEGFA	Angiogenic marker	↑↑*	↓↓*
PECAM/CD31	Angiogenic marker	↑↑*	
MPZ	Myelination marker	↑	
PMP22	Myelination marker	↑	
MBP	Myelination marker	↑*	
COL1A1	ECM protein	↓	
COL3A1	ECM protein	↑	
LAMB2	ECM protein	↓	
FBLN1	ECM protein	↓	
CASP3	Apoptosis protein	↑	
EGR1	Transcription factor	↑	
CD96	Immunoglobulin	↑	

Results are summarized and arrows indicate an increased or decreased expression in comparison to the unseeded MSCs. * While there were significant differences for individual time points, overall only the factors BDNF, GDNF, VEGFA, CD31 and MBP were significantly different between groups.

In contrast to qPCR gene expression of the seeded MSCs which revealed a significant upregulation in VEGFA mRNA levels, production of VEGFA protein in the seeded MSCs was very high in the first 24 hours (665.58 pg/ml ± 93.57) but decreased strikingly until day 21,

while the MSCs only produced stable significantly increasing (p = 0.028) levels of VEGFA protein over the 21 days (405.61 pg/ml ± 54.97 at day 1 to 692.78 pg/ml ± 72.23 at day 21). Collectively, the results (summarized in Table 2) show that interactions of MSCs with decellularized nerve allografts modulates gene expression in MSCs including the stimulation of mRNAs for key proteins that support nerve growth and/or homeostasis.

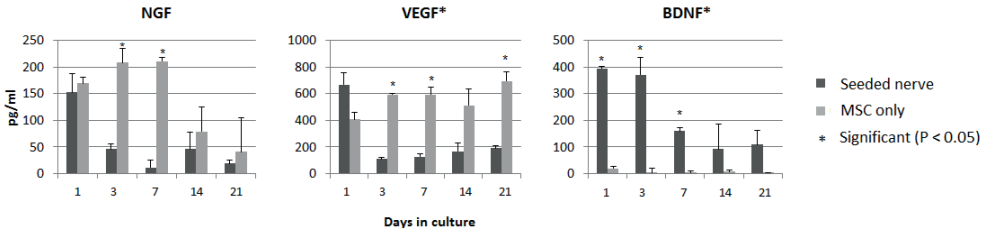

Figure 3. Growth factor production. ELISA analysis of the cell supernatants over 21 days is depicted.

DISCUSSION

This study investigated whether interactions of adipose-derived MSCs with decellularized nerve allografts can increase mRNA and protein expression of growth factors that may support nerve regeneration.

In the decellularized nerve grafts, no RNA levels were detectable, which was as expected. The purpose of the decellularization process is to render a scaffold that is devoid of cells and that does not elicit an immune response. Also, the decellularization process most likely caused degradation of the RNA of the cells that produced the ECM. In this study we did confirm presence of the residual ECM, but previous studies have repeatedly demonstrated preservation of the ECM after nerve decellularization.[8,18]

Three of the five neurotrophic markers and all myelination and angiogenic markers where increased in MSCs seeded onto a decellularized nerve graft when compared to MSCs alone (Table 2). NGF and GDNF showed a reduced expression in the seeded MSCs. This can be caused by multiple factors, for some growth factors, upregulation might not only be triggered by ques from the nerve allograft, but also by factors from the wound environment that were not included in our in vitro model.[37] Furthermore, we used motor nerve allografts and NGF has been previously found to be up regulated in sensory nerves but not in motor nerves.[24])

The high levels of ECM related gene expression in both groups demonstrate that the seeded MSCs retain cell-adhesion, collagen anabolic activity and cell-to-cell communication. This is in line with previous reports, influencing stem cell fate by interaction with a specific matrix, to create a so-called stem cell niche, is not a new concept; cell adhesion molecules have been shown to be capable of activating signaling pathways associated with promotion of self-renewal and stemness of MSCs.[38]

Discrepancies in protein and mRNA levels, such as we found for VEGF, could be due to translational control, as well as selective differences in protein stability or secretion. For GDNF, ELISA analysis showed no detectable protein production in either group. Therefore, we were unable to verify whether GNDF gene expression upregulation increased GDNF protein production. In both groups GDNF expression measured by qPCR was very low, which might have caused the undetectable protein levels. Another (technical) explanation could be that there were large amounts of cell supernatants (1-10 milliliters) stored frozen, while for the ELISA analysis only small amounts (micro liters) were used, this could have resulted in low concentrations despite adequate centrifugation. Also, there are many post-transcriptional mechanisms involved in turning mRNA into protein, and proteins may differ substantially in their half-lives.[39]

Strengths and limitations

Strenghts of this study include the use of a previously validated non-traumatic seeding technique.[23] Cells can also be injected into the graft using micro-injection, but this damages the carefully preserved ultrastructure and should therefore be avoided.[40] A major strength of this study, is that only human tissue was used; results are therefore translatable to clinical studies. Also, the MSCs and the nerve allograft were from different donors, which is comparable to the potential future situation where banked nerve allografts could be seeded with a patient's own MSCs.

A limitation of this study is the lack of biological replicates, which is due to technical limitations and the scarcity of eligible nerve allograft donors. We however do not expect to find any difference within allograft donors since all grafts are decellularized by the same protocol. Another limitation is that ELISA was not performed for all factors. Since ELISA is a costly technique, only four ELISA kits were selected on availability and references in the literature.

In this in vitro model we investigated the direct interaction between nerve allografts and MSCs. Other factors such as wound healing responses will likely influence MSC behavior;[37] therefore, future studies should focus on the specific role of MSCs seeded onto nerve allografts in an in vivo model of nerve regeneration. Long-term in-vivo studies will permit assessment of whether the seeded MSCs and their secreted factors also provide better functional outcomes.

CONCLUSION

This study demonstrates that seeding of undifferentiated adipose-derived MSCs onto processed nerve allografts permits the sustained secretion of neurotrophic and angiogenic factors that can stimulate nerve regeneration. Our results imply that for clinical translation, cells might not have to be pre-differentiated prior to surgery and that the trophic functions in tissue repair most likely occur in the first week after surgery. The combination of autologous MSCs and readily available processed nerve allografts is potentially a promising method for individualized peripheral nerve repair.

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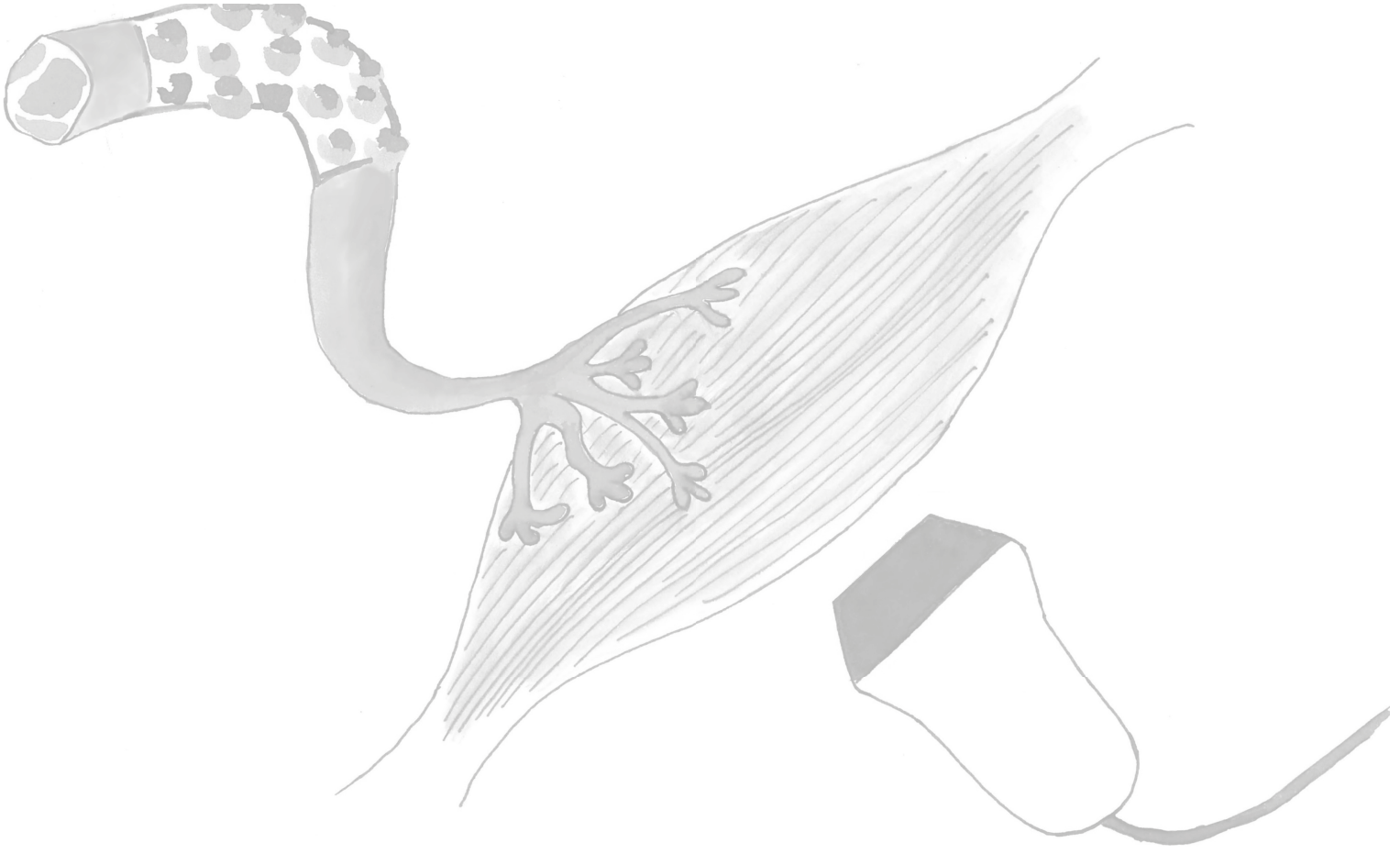
REFERENCES

1. Brooks DN, Weber RV, Chao JD, et al. Processed nerve allografts for peripheral nerve reconstruction: a multicenter study of utilization and outcomes in sensory, mixed, and motor nerve reconstructions. Microsurgery. 2012;32(1):1-14.
2. Cho MS, Rinker BD, Weber RV, et al. Functional outcome following nerve repair in the upper extremity using processed nerve allograft. J Hand Surg Am. 2012;37(11):2340-2349.
3. Szynkaruk M, Kemp SW, Wood MD, Gordon T, Borschel GH. Experimental and clinical evidence for use of decellularized nerve allografts in peripheral nerve gap reconstruction. Tissue Eng Part B Rev. 2013;19(1):83-96.
4. Giusti G, Willems WF, Kremer T, Friedrich PF, Bishop AT, Shin AY. Return of motor function after segmental nerve loss in a rat model: comparison of autogenous nerve graft, collagen conduit, and processed allograft (AxoGen). J Bone Joint Surg Am. 2012;94(5):410-417.
5. Moore AM, MacEwan M, Santosa KB, et al. Acellular nerve allografts in peripheral nerve regeneration: a comparative study. Muscle Nerve. 2011;44(2):221-234.
6. Rbia N, Shin AY. The Role of Nerve Graft Substitutes in Motor and Mixed Motor/Sensory Peripheral Nerve Injuries. J Hand Surg Am. 2017;42(5):367-377.
7. Chernousov MA, Carey DJ. Schwann cell extracellular matrix molecules and their receptors. Histol Histopathol. 2000;15(2):593-601.
8. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. Tissue Eng. 2004;10(9-10):1346-1358.
9. Hoyng SA, De Winter F, Gnavi S, et al. A comparative morphological, electrophysiological and functional analysis of axon regeneration through peripheral nerve autografts genetically modified to overexpress BDNF, CNTF, GDNF, NGF, NT3 or VEGF. Exp Neurol. 2014;261:578-593.
10. Lee JY, Giusti G, Friedrich PF, Bishop AT, Shin AY. Effect of Vascular Endothelial Growth Factor Administration on Nerve Regeneration after Autologous Nerve Grafting. J Reconstr Microsurg. 2016;32(3):183-188.
11. Piquilloud G, Christen T, Pfister LA, Gander B, Papaloizos MY. Variations in glial cell line-derived neurotrophic factor release from biodegradable nerve conduits modify the rate of functional motor recovery after rat primary nerve repairs. Eur J Neurosci. 2007;26(5):1109-1117.
12. Boyd JG, Gordon T. Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Mol Neurobiol. 2003;27(3):277-324.
13. Salgado AJ, Reis RL, Sousa NJ, Gimble JM. Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. Curr Stem Cell Res Ther. 2010;5(2):103-110.
14. Hundepool CA, Nijhuis TH, Mohseny B, Selles RW, Hovius SE. The effect of stem cells in bridging peripheral nerve defects: a meta-analysis. J Neurosurg. 2014;121(1):195-209.
15. Kingham PJ, Kolar MK, Novikova LN, Novikov LN, Wiberg M. Stimulating the neurotrophic and angiogenic properties of human adipose-derived stem cells enhances nerve repair. Stem Cells Dev. 2014;23(7):741-754.
16. Armstrong SJ, Wiberg M, Terenghi G, Kingham PJ. ECM molecules mediate both Schwann cell proliferation and activation to enhance neurite outgrowth. Tissue Eng. 2007;13(12):2863-2870.
17. di Summa PG, Kalbermatten DF, Raffoul W, Terenghi G, Kingham PJ. Extracellular matrix molecules enhance the neurotrophic effect of Schwann cell-like differentiated adipose-derived stem cells and increase cell survival under stress conditions. Tissue Eng Part A. 2013;19(3-4):368-379.
18. Hundepool CA, Nijhuis TH, Kotsougiani D, Friedrich PF, Bishop AT, Shin AY. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. Neurosurg Focus. 2017;42(3):E4.
19. Crespo-Diaz R, Behfar A, Butler GW, et al. Platelet lysate consisting of a natural repair proteome supports human mesenchymal stem cell proliferation and chromosomal stability. Cell Transplant. 2011;20(6):797-811.

20. Dudakovic A, Camilleri E, Riester SM, et al. High-resolution molecular validation of self-renewal and spontaneous differentiation in clinical-grade adipose-tissue derived human mesenchymal stem cells. J Cell Biochem. 2014;115(10):1816-1828.
21. Mader EK, Butler G, Dowdy SC, et al. Optimizing patient derived mesenchymal stem cells as virus carriers for a phase I clinical trial in ovarian cancer. J Transl Med. 2013;11:20.
22. Mahmoudifar N, Doran PM. Osteogenic differentiation and osteochondral tissue engineering using human adipose-derived stem cells. Biotechnol Prog. 2013;29(1):176-185.
23. Rbia N, Bulstra LF, Bishop AT, van Wijnen AJ, Shin AY. A simple dynamic strategy to deliver stem cells to decellularized nerve allografts. Accepted at Plast Reconstr Surg. 2018.
24. Hoke A, Redett R, Hameed H, et al. Schwann cells express motor and sensory phenotypes that regulate axon regeneration. J Neurosci. 2006;26(38):9646-9655.
25. Jesuraj NJ, Nguyen PK, Wood MD, et al. Differential gene expression in motor and sensory Schwann cells in the rat femoral nerve. J Neurosci Res. 2012;90(1):96-104.
26. Jesuraj NJ, Santosa KB, Macewan MR, et al. Schwann cells seeded in acellular nerve grafts improve functional recovery. Muscle Nerve. 2014;49(2):267-276.
27. Kingham PJ, Reid AJ, Wiberg M. Adipose-derived stem cells for nerve repair: hype or reality? Cells Tissues Organs. 2014;200(1):23-30.
28. Karpova NN. Role of BDNF epigenetics in activity-dependent neuronal plasticity. Neuropharmacology. 2014;76 Pt C:709-718.
29. Skaper SD. Neurotrophic Factors: An Overview. Methods Mol Biol. 2018;1727:1-17.
30. Blondet B, Carpentier G, Lafdil F, Courty J. Pleiotrophin cellular localization in nerve regeneration after peripheral nerve injury. J Histochem Cytochem. 2005;53(8):971-977.
31. Shibuya M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. Genes Cancer. 2011;2(12):1097-1105.
32. DeLisser HM, Christofidou-Solomidou M, Strieter RM, et al. Involvement of endothelial PECAM-1/CD31 in angiogenesis. Am J Pathol. 1997;151(3):671-677.
33. Li J, Parker B, Martyn C, Natarajan C, Guo J. The PMP22 gene and its related diseases. Mol Neurobiol. 2013;47(2):673-698.
34. Barros CS, Franco SJ, Muller U. Extracellular matrix: functions in the nervous system. Cold Spring Harb Perspect Biol. 2011;3(1):a005108.
35. Reid AJ, Sun M, Wiberg M, Downes S, Terenghi G, Kingham PJ. Nerve repair with adipose-derived stem cells protects dorsal root ganglia neurons from apoptosis. Neuroscience. 2011;199:515-522.
36. Calogero A, Arcella A, De Gregorio G, et al. The early growth response gene EGR-1 behaves as a suppressor gene that is down-regulated independent of ARF/Mdm2 but not p53 alterations in fresh human gliomas. Clin Cancer Res. 2001;7(9):2788-2796.
37. Clements MP, Byrne E, Camarillo Guerrero LF, et al. The Wound Microenvironment Reprograms Schwann Cells to Invasive Mesenchymal-like Cells to Drive Peripheral Nerve Regeneration. Neuron. 2017;96(1):98-114 e117.
38. Abdal Dayem A, Lee S, H YC, Cho SG. The Impact of Adhesion Molecules on the In Vitro Culture and Differentiation of Stem Cells. Biotechnol J. 2017.
39. Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol. 2003;4(9):117.
40. Jesuraj NJ, Santosa KB, Newton P, et al. A systematic evaluation of Schwann cell injection into acellular cold-preserved nerve grafts. J Neurosci Methods. 2011;197(2):209-215.



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General discussion and future research perspectives

The overarching aim of this thesis was to improve nerve reconstruction using an off the shelf peripheral nerve allograft that is unlimited in supply and can be individualized to each patient using stem cells, providing functional recovery comparable to autograft nerve. In this chapter the main findings of the studies in this thesis are discussed and suggestions for future research are given.

PART I: Current clinical use of nerve grafts

The first part of this thesis was focused on brachial plexus injury as this is currently one of the fields where nerve grafts are commonly used. In these often extensive injuries, long nerve gaps require large amounts of nerve graft. In those cases, the amount of available autologous tissue is not always sufficient so the availability of an off the shelf alternative would be very beneficial.[1]

In the treatment of brachial plexus injury, restoration of elbow flexion is considered the first priority to provide positioning of the hand in a useful position.[1] Reconstructive options for brachial plexus injury have continued to expand over the past few decades. Because available reconstructive options now allow for it, attention for reconstruction of other upper limb functions such as elbow extension is growing. Elbow extension is necessary for stabilization of the elbow without using the opposite arm and to lift the arm above the horizontal position.[2-5]

In **Chapter 2**, an overview of currently used techniques to restore elbow flexion and extension using nerve transfers is provided. When using the principal of "donor distal, recipient proximal", one will be able to obtain a tension free coaptation for most commonly used nerve transfers.[6] However, for a number of nerve transfers (including the medial pectoral nerve, spinal accessory nerve and sometimes intercostal nerves), nerve grafts are required to bridge the distance to the target nerve in a tension free fashion. Important disadvantages of using additional nerve grafts in nerve transfers include the additional reinnervation distance and the extra suture line that may form an additional burden for the growing axons. The actual clinical impact of an extra suture line is however still debatable.[7] In general, nerve transfers without interposition nerve grafts yield better results. A clear advantage of nerve grafts for additional length is that more nerve transfer sources become available for use. When using interposition nerve grafts, one should always aim to use the shortest length possible to obtain tension free coaptations.[8-14]

One thing that stood out is that described options for elbow flexion clearly outnumber the published options for triceps reinnervation. To address the paucity in reconstructive options for elbow extension, **Chapter 3** aimed to evaluate the quality and prevalence of restored elbow extension in brachial plexus injury patients that underwent a novel treatment. In these patients, part of the spinal accessory nerve (SAN) was transferred to the radial nerve branch of the triceps muscle, using long interposition nerve grafts to establish a tension free coaptation. After a median follow-up of 22.9 months, 52.4% of our cohort of 42 patients obtained meaningful recovery. The minimum follow-up for inclusion was 12 months, however

it is possible that a number of patients did not reach maximum recovery yet at their last follow-up. This was addressed in our subgroup analysis of patients with a minimum of 20 months of follow-up, showing that after a mean follow-up of 30 months, 69.5% reached meaningful recovery.
The long time to recovery that is necessary with this procedure may be explained by the long nerve grafts that were used (median 15 cm (range 10-25 cm)). Possibly due to the relatively small sample size, the influence of increased graft length in our cohort was not significant (p=0.058). However, in our study we could demonstrate an important negative effect of longer grafts with an Odds Ratio of 0.785 (95% Confidence Interval 0.611-1.008).
The presence of vascular injury seems to impair the chance of reaching meaningful recovery but contrary to previous studies, smoking and BMI did not show a significant effect, probably due to the distribution and the small number of patients who smoked (n=5) or were obese (n=8), causing a lack of power to detect any effects.[15] To allow for more advanced statistics to detect predictive factors for outcome, including more variables, one would ideally study a larger cohort.

Future research

- Currently, most studies on brachial plexus injury are (small) retrospective cohort studies. To generate volume it would be desirable to combine forces in prospective multicenter studies. This would allow for more advanced statistics to create predictive models to decide which patients should receive which operations for optimal functional outcome. Ideally a standard set of outcome measurements should be used including (objective) force measurement, validated questionnaires[16] and reports on physical therapy.
  Data on physical therapy is often unavailable in the current retrospective studies. However, the amount and kind of physical therapy patients receive after brachial plexus injury is a potentially important factor to improve functional outcome.[17]
  For functional outcome, ideally more objective measures should be used instead of the subjective modified MRC scale that is now frequently used.[18,19] Ideally both objective force and length of the "arm" (as moment = force x arm) should be taken into account. Also, the range of motion over which a patient has adequate power should be reported to get an accurate estimate of a patient's actual function instead of just the maximum force produced in a static position. Furthermore, values should always be compared to the healthy side as this is the best reference we have to what a patient's "normal strength" is.

PART II: Evaluation techniques

Animal models are frequently used to study alternative treatment strategies for peripheral nerve injury. The second part of this thesis was focused on the development of new evaluation techniques to measure nerve recovery in animal models. The use of ultrasound as a non-invasive tool to measure muscle recovery after nerve reconstruction was evaluated in both the rat and the rabbit model. The rationale was similar for both animal models. Currently

commonly used evaluation techniques, including muscle mass, force measurements, electrophysiology and histomorphometry, require animals to be euthanized. Non-invasive techniques would allow for serial measurements in the same animal, reducing the variability within studied groups and thereby reducing the required sample size and costs. Therefore we aimed to validate a non-invasive ultrasound technique to measure functional recovery in both the rat and the rabbit model.[20-22]

In the rat study **(Chapter 4)** we evaluated both the tibial and the gastrocnemic muscle, that are both innervated by different branches of the reconstructed sciatic nerve. Both intrarater (r=0.97) and interrater reliability (r=0.88) of ultrasound were high indicating excellent reliability and reproducibility in the rat model.

The rabbit model has some important advantages in comparison to the rat model, such as the possibility to reconstruct larger nerve gaps and the rabbit's neuroregenerative capacity more closely mimics the human situation. Therefore rabbits are commonly used as a next step after rat studies prior to research in a clinical setting.[20,23-26]

There are a couple of important differences between our two ultrasound validation studies. Often in the rabbit model, only the peroneal branch of the sciatic nerve, that innervates the tibialis anterior muscle, is reconstructed.[20,22] This is the model we used as well. One of the limitations of our study design in the rat model, was that we did not verify the intra- and inter-rater reliability within the same animal over time. We addressed this in the rabbit study **(Chapter 5)** by performing ultrasound measurement at different time points over time within the same animals. We showed that both inter-rater and intra-rater reliability were excellent (r=0.989 and 0.994 respectively). In line with our rat study and previous research, this indicates both high reliability and reproducibility of this method.[27] The very high correlation (r=0.97) between ultrasound and muscle mass may be explained by the fact that both share the same rationale for the measurement of motor nerve regeneration. Denervation will lead to atrophy in the target muscle, causing a decrease in muscle mass and muscle cross-sectional area. Both will increase when reinnervation occurs.[28]

Ultrasound was not compared with other imaging techniques such as MRI, as this has previously been reported.[29-32] We believe that ultrasound is preferred to more invasive and expensive techniques such as MRI. Besides the possibility to decrease numbers of animals needed for research, this non-invasive technique also has the major advantage of avoiding anesthesia risks, as animals can remain awake the whole procedure. Especially for rabbits this is very important as they are known to be vulnerable for anesthesia.[22,33]

Due to the high reliability and ease of use of ultrasound, the lab where all animal experiments were performed now includes ultrasound measurements in the standard test battery for nerve reconstruction studies in rabbits and rats. We hope the non-invasive ultrasound techniques will be implemented by other research groups studying nerve regeneration as well.

We showed that ultrasound is a reliable tool to measure early onset of muscle recovery. Currently, electrodiagnostic studies are the standard of care in clinical setting to evaluate for

nerve regeneration. Ultrasound may be preferred by patients over electrodiagnostic studies as they can be quite painful.[34]

Future research

- Ultrasound measurements can also potentially be used to evaluate functional recovery in subjects that are difficult to instruct so that measurement of maximum force is unreliable. An important group may be infants with neonatal brachial plexus injury.
- Furthermore, ultrasound may provide a non-invasive alternative to the often quite painful EMG studies. Ultrasound to follow up nerve recovery in clinical setting has already been validated for several muscles and we believe its use should be further stimulated.[35]

PART III: Implementation and improvement

Over the past decades, many alternatives for nerve autografts have been evaluated. All had the shared rationale of avoiding donor side morbidity and providing an unlimited supply. Processed nerve allografts proved to provide superior results to hollow conduits without the need for immunosuppression. Further development of processing techniques resulted in a commercially available product. Although clinical results are promising especially for sensory nerve defects, results for longer defects and motor nerve defects have not been satisfactory up to date.[36-45] Therefore, the allograft decellularization technique was modified with the addition of elastase, resulting in reduced cellular debris and better preservation of extracellular matrix (ECM) ultrastructure.[46]

The third part of this thesis was aimed at evaluating the functional outcome after reconstruction of nerve defects using the previously optimized nerve allografts followed by the development of strategies to further develop nerve allograft reconstruction.

Implementation of the optimized decellularized nerve allograft

Chapter 6: *Implementation in the rat model, 1-cm gap*

Following the promising in vitro results of the optimized processing technique, the next step was to implement this optimized decellularized nerve allograft in vivo in the rat model, to evaluate functional outcome. A 1-cm segmental nerve defect was reconstructed using either an autograft (control) or an elastase processed nerve allograft that was stored at either 4°C or -80°C. Storage at -80°C showed to significantly alter the ECM ultrastructure in vitro, however the effect on functional outcome was not known yet.[46] Functional results were evaluated at 12 and 16 weeks. Endpoints were chosen based on previous work from our lab.[42] In the rat model, we found that for isometric tetanic force (ITF) and compound muscle action potentials (CMAP), no significant differences were observed between the three groups. What stood out was that the cold stored graft yielded better results than the frozen stored graft in 3 out of 5 outcome measurements (ankle angle, histomorphometry and muscle weight). Between 12 and 16 weeks follow-up results continued to improve abating signs of a delayed immune response or other factors that can possibly impede nerve regeneration on the long

term.[42] Although these results were promising for the cold stored processed allograft, we acknowledged that the rat model is not suitable for direct clinical translation and further research was required.

Chapter 7: *Implementation in the rabbit model, 3-cm gap*

As explained previously, good results in a small gap in the rat model do not guarantee satisfactory results in a longer nerve gap in the rabbit model.[22,47] In the rabbit model, a 3-cm peroneal nerve defect was reconstructed with either an autograft (control) or cold- (4°C) or frozen-stored (-80°C) elastase processed nerve allograft and functional recovery was evaluated after 24 weeks. Looking at the longitudinal ultrasound measurements, a trend can be seen (Fig. 1) where nerve regeneration in the frozen stored allograft group seems to be slower and less successful than in the other groups. This is confirmed by the ultrasound results at 24 weeks, where the frozen-stored graft was significantly inferior to both other groups ($p<0.05$). Muscle weight and ITF yielded similar outcomes in the autograft and cold-stored allograft groups (muscle weight: $p=0.096$, ITF: $p=0.286$). Inferiority of the frozen-stored allograft was confirmed (muscle weight: $p<0.01$, ITF: $p=0.02$ for frozen-stored allograft vs. autograft).

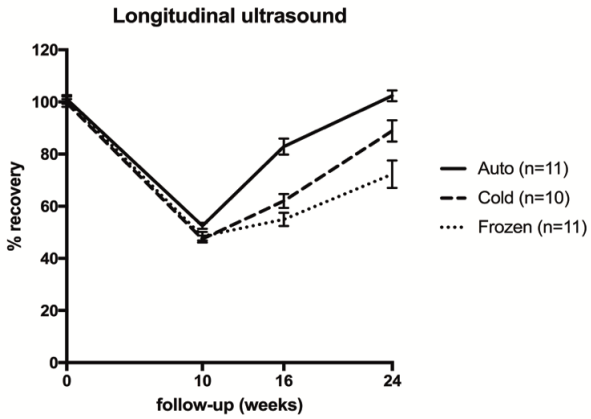


Figure 1. Longitudinal ultrasound results expressed as percentage recovery (cross sectional tibialis anterior muscle area of the operated side divided by that of the contralateral (healthy) side). For each time point the group mean±SEM is depicted.

Looking into recent literature, we found only few studies that evaluate functional recovery after reconstruction of long mixed or motor nerve gaps using acellular nerve allografts. Yan et al. [48] reconstructed 6-cm sciatic nerve gaps in a rat model, using acellular nerve allografts with or without an additional interposition isograft. After 20 weeks follow-up, no measurable muscle force could be obtained in either groups. Possibly, there is a limit to the length that can be bridged using acellular nerve grafts. A proposed theory is that regeneration over longer nerve gaps requests a greater proliferative demand on Schwann cells, resulting in increased

senescence of these Schwann cells eventually resulting in axonal growth arrest.[49,50] Furthermore, just like in any other wound healing process, scar formation (fibrosis) will occur during the nerve regeneration process. Especially fibrosis from the transected epineurium and within the transected segment may prohibit growth of regenerating axons.[51,52] However, this was not further evaluated in this thesis.

Vasudevan et al.[53] compared their detergent free decellularized nerve allograft to autograft reconstruction (3.5-cm gap in the rat). The detergent free decellularized allograft group had significantly lower gastrocnemic muscle mass in comparison to the autograft group at 12 weeks after reconstruction. This indicates that our decellularization technique seems to be more successful. Furthermore, the detergent free process developed by Vasudevan et al.[53] is quite lengthy (3 weeks) in comparison to our 5 day decellularization protocol.[46]

What is striking is that many studies do not use any true functional outcome measurements, but rely on histomorphometry and sometimes CMAP. Not even the easily obtainable wet muscle weight is used in many studies, making it nearly impossible to judge the true functional results and compare them to our outcomes. Histomorphometry measures are known to be prone to erroneous conclusions as the number of axons sprouting from the proximal nerve stump do not necessarily reflect the number of neurons emitting the axon sprouts. Hence, more axons does not always mean more function.[54]

In our ultrasound study, we found only a moderate correlation between CMAP and histomorphometry measurements and ultrasound outcomes. Meanwhile, correlations between ultrasound and force and muscle weight measurements were good. We believe that force measurements best represent actual functional outcome and that ultrasound provides an adequate alternative, considering the good correlation (0.83). To be able to compare results in the future, other groups are encouraged to use these measurements.

<u>Addition of stem cells</u>

Besides enthusiasm about the promising results of the elastase processed allograft for reconstruction of a long nerve defect, we acknowledge that there is room for further improvement. Our longitudinal ultrasound measurements after nerve reconstruction in the rabbit, showed that muscle reinnervation through the cold stored allograft is slower than through the autograft (Figure 1). We hypothesized that supportive cells are necessary, as the decellularized nerve allograft is devoid of cellular material while the autograft contains its native cells, such as Schwann cells. This may result in lower levels of important growth factors.[55] As addressed in the introduction of this thesis, the beneficial effect of adipose derived mesenchymal stem cells (MSCs) on nerve regeneration has been shown in multiple studies. MSCs are capable of producing growth factors (including NGF, BDNF, VEGF) and may be the key to boost nerve regeneration across acellular nerve allografts. However, the exact role and fate of MSCs in nerve regeneration have not been fully elucidated yet.[56-62]

First, we developed a technique to seed the decellularized nerve allograft with adipose derived MSCs **(Chapter 8)**. Using a custom bioreactor we developed a simple technique to

seed nerve allografts, that is easy to implement in other institutions. We choose to use a dynamic technique as opposed to a static strategy, as dynamic strategies have previously shown to increase cell seeding efficiency.[63-65]

There is currently no consensus on whether cells should be added only on the outside or (also) on the inside of the graft. Some previous work has used (micro)injection methods.[56,66-68] However, the injection process may damage both the MSCs as well as the, carefully preserved, allograft ultrastructure. Cell distribution may also be clustered in the injection tracts.[69,70] Therefore we choose to seed MSCs mainly on the outside of the nerve graft using a non-traumatic strategy. It is hypothesized that growth factors can penetrate the allograft by diffusion, rejecting the necessity for MSC presence inside the graft. This is supported by Zhao et al.[66] showing similar effect on nerve regeneration of MSCs injected inside the graft and MSCs placed around the graft.

Next, we evaluated the interaction of adipose derived MSCs and the ECM of the decellularized allograft by measurement of gene expression and growth factor production **(Chapter 9)**. We demonstrated that seeding of undifferentiated MSCs onto processed nerve allografts indeed permits secretion of angiogenic and neurotrophic factors that can potentially stimulate nerve regeneration.

Surprisingly, we found a reduced expression of NGF and GDNF for the MSC + allograft group. It is possible that specific ques are required to upregulate these growth factors that are normally provided by the wound microenvironment. We were not able to include these ques in our in vitro model.[71] Furthermore, we used motor nerve allografts for our experiments. Hoke et al.[72] previously found selective upregulation of NGF in reinnervated sensory nerves and not in motor nerves. The clinical relevance of the different characteristics of motor or sensory sources for nerve allografts is not well known yet.

There is an ongoing discussion whether to use differentiated (into Schwann cell-like cells) or undifferentiated MSCs. As Schwann cells play an important role in nerve regeneration, it has been hypothesized that differentiating MSCs towards Schwann cell-like cells would be beneficial.[56,73] However, while stimulation of the MSCs to express Schwann cell characteristics has shown to increase neurotrophic and angiogenetic growth factor production in vitro, this has not led to improved nerve regeneration and functional improvement in vivo when compared to undifferentiated MSCs.[59,61,74] MSCs do not seem to survive long-term, although the exact survival time is unknown, and there is little evidence that they execute Schwann cell functions such as creating new myelin sheets.[75] It is also unknown whether differentiated MSCs remain their differentiated state once transplanted. To avoid unnecessary delay, we have therefore chosen to work with undifferentiated MSCs in this thesis.

Future Research Perspectives

- There is a debate on the survival of transplanted MSCs and therefore the potential time frame of their effect. Furthermore, the eventual question is whether addition of MSCs does indeed improve functional outcome. In direct line with the work described in this thesis it would therefore be interesting to evaluate 1) survivability of the implanted cells

and 2) effect of added cells on functional outcome. In these studies, ideally more experimental groups using different cell types should be included. Not only should differentiated (into Schwann-cell like cells) MSCs be compared to undifferentiated MSC, but a combination of both should also be evaluated. Differentiated and undifferentiated MSCs may have different short and long-term effect that could potentially act synergistic.

- It is not well known if the rule “replace like with like” should be applied to the use of nerve allografts: i.e. should motor/sensory/mixed nerve gaps be reconstructed using allograft nerves from equal origin? More research is necessary to determine if graft source can significantly be of influence on functional recovery. This would be of high clinical importance. Current clinically available nerve allografts can be selected for size but not for donor source.
- If our research line is continued and our optimized nerve allograft gets FDA approval for clinical use, a future treatment algorithm may be as follows: the appropriate nerve allograft (size and origin) is selected off the shelf. Next it is seeded with either a patient’s own stem cells, or readily available stromal vascular fraction (more research needed).[76] Before this, some practical aspects still need to be addressed such as optimal storage conditions to guarantee a long shelf life and improvement of cell expansion techniques (this currently takes approximately 2 weeks).
- Up to date, allograft nerves have proven superior to artificially produced conduits. However technology will continue to improve so that in the future it may be possible to use 3D printing techniques to fabricate conduits with a proper microscopic ultrastructure. It may even be possible to print regeneration supporting drugs such as Tacrolimus (sustained release) into the material to locally support nerve regeneration.[77]

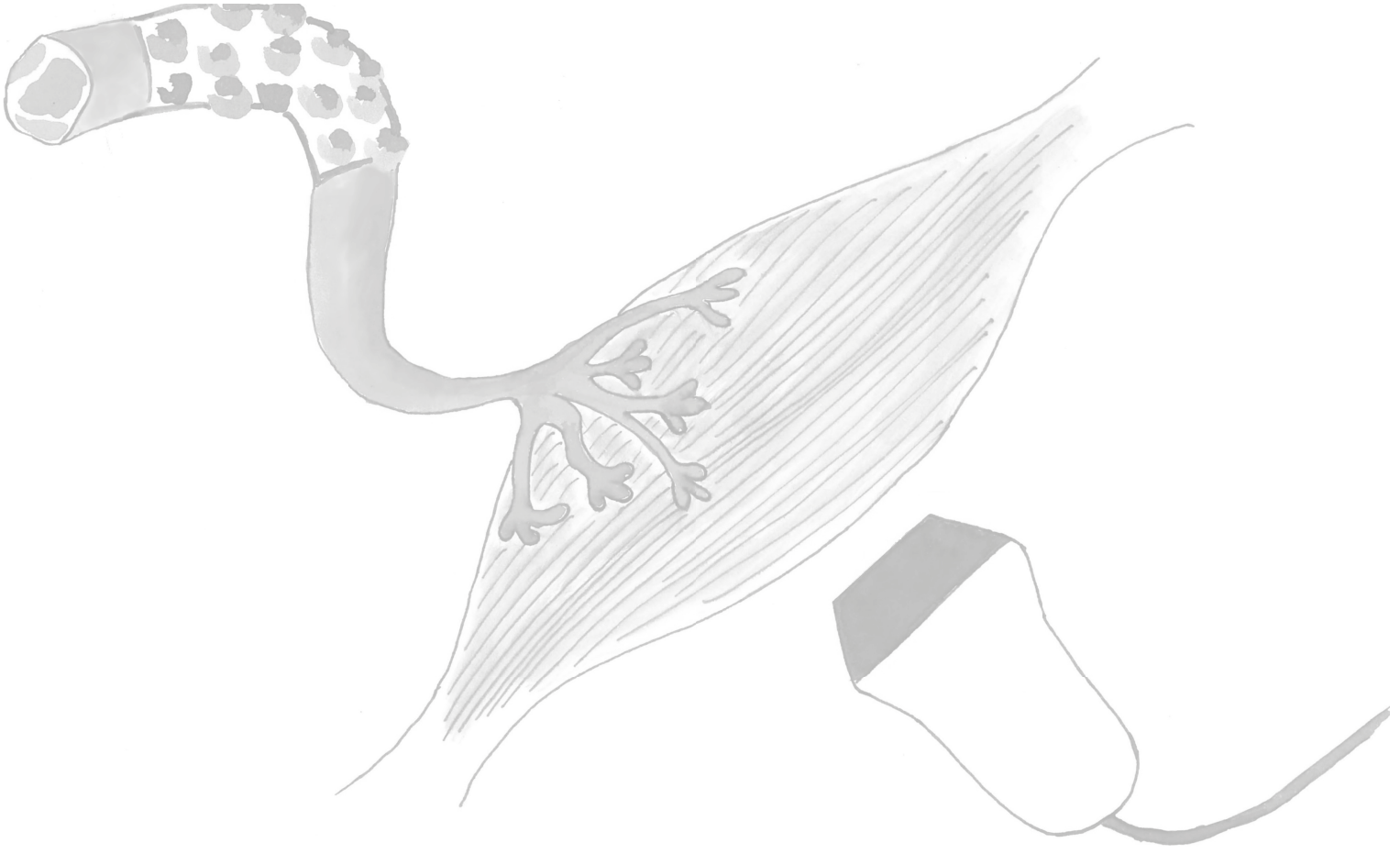
REFERENCES

1. Shin AY, Spinner RJ, Steinmann SP, Bishop AT. Adult traumatic brachial plexus injuries. J Am Acad Orthop Surg. 2005;13(6):382-396.
2. Bertelli JA. Lower trapezius muscle transfer for reconstruction of elbow extension in brachial plexus injuries. J Hand Surg Eur Vol. 2009;34(4):459-464.
3. Doi K, Sakai K, Kuwata N, Ihara K, Kawai S. Double free-muscle transfer to restore prehension following complete brachial plexus avulsion. J Hand Surg Am. 1995;20(3):408-414.
4. Doi K, Shigetomi M, Kaneko K, et al. Significance of elbow extension in reconstruction of prehension with reinnervated free-muscle transfer following complete brachial plexus avulsion. Plast Reconstr Surg. 1997;100(2):364-372; discussion 373-364.
5. Goubier JN, Teboul F. Transfer of the intercostal nerves to the nerve of the long head of the triceps to recover elbow extension in brachial plexus palsy. Tech Hand Up Extrem Surg. 2007;11(2):139-141.
6. Mackinnon SE. Donor Distal, Recipient Proximal and Other Personal Perspectives on Nerve Transfers. Hand Clin. 2016;32(2):141-151.
7. Yan Y, Wood MD, Hunter DA, Ee X, Mackinnon SE, Moore AM. The Effect of Short Nerve Grafts in Series on Axonal Regeneration Across Isografts or Acellular Nerve Allografts. J Hand Surg Am. 2016;41(6):e113-121.
8. Merrell GA, Barrie KA, Katz DL, Wolfe SW. Results of nerve transfer techniques for restoration of shoulder and elbow function in the context of a meta-analysis of the English literature. J Hand Surg Am. 2001;26(2):303-314.
9. Songcharoen P, Mahaisavariya B, Chotigavanich C. Spinal accessory neurotization for restoration of elbow flexion in avulsion injuries of the brachial plexus. J Hand Surg Am. 1996;21(3):387-390.
10. Tung TH, Novak CB, Mackinnon SE. Nerve transfers to the biceps and brachialis branches to improve elbow flexion strength after brachial plexus injuries. J Neurosurg. 2003;98(2):313-318.
11. Samardzic M, Rasulic L, Grujicic D, Milicic B. Results of nerve transfers to the musculocutaneous and axillary nerves. Neurosurgery. 2000;46(1):93-101; discussion 101-103.
12. Samardzic M, Grujicic D, Rasulic L, Bacetic D. Transfer of the medial pectoral nerve: myth or reality? Neurosurgery. 2002;50(6):1277-1282.
13. Malessy MJ, Thomeer RT. Evaluation of intercostal to musculocutaneous nerve transfer in reconstructive brachial plexus surgery. J Neurosurg. 1998;88(2):266-271.
14. Samii A, Carvalho GA, Samii M. Brachial plexus injury: factors affecting functional outcome in spinal accessory nerve transfer for the restoration of elbow flexion. J Neurosurg. 2003;98(2):307-312.
15. Lee JY, Kircher MF, Spinner RJ, Bishop AT, Shin AY. Factors affecting outcome of triceps motor branch transfer for isolated axillary nerve injury. J Hand Surg Am. 2012;37(11):2350-2356.
16. Mancuso CA, Lee SK, Saltzman EB, et al. Development of a Questionnaire to Measure Impact and Outcomes of Brachial Plexus Injury. J Bone Joint Surg Am. 2018;100(3):e14.
17. Milicin C, Sirbu E. A comparative study of rehabilitation therapy in traumatic upper limb peripheral nerve injuries. NeuroRehabilitation. 2018;42(1):113-119.
18. Mendell JR, Florence J. Manual muscle testing. Muscle Nerve. 1990;13 Suppl:S16-20.
19. Shahgholi L, Bengtson KA, Bishop AT, et al. A comparison of manual and quantitative elbow strength testing. Am J Phys Med Rehabil. 2012;91(10):856-862.
20. Giusti G, Kremer T, Willems WF, Friedrich PF, Bishop AT, Shin AY. Description and validation of isometric tetanic muscle force test in rabbits. Microsurgery. 2012;32(1):35-42.
21. Lee JY, Giusti G, Wang H, Friedrich PF, Bishop AT, Shin AY. Functional evaluation in the rat sciatic nerve defect model: a comparison of the sciatic functional index, ankle angles, and isometric tetanic force. Plast Reconstr Surg. 2013;132(5):1173-1180.
22. Sahakyants T, Lee JY, Friedrich PF, Bishop AT, Shin AY. Return of motor function after repair of a 3-cm gap in a rabbit peroneal nerve: a comparison of autograft, collagen conduit, and conduit filled with collagen-GAG matrix. J Bone Joint Surg Am. 2013;95(21):1952-1958.

23. Amillo S, Yanez R, Barrios RH. Nerve regeneration in different types of grafts: experimental study in rabbits. Microsurgery. 1995;16(9):621-630.
24. Young RC, Wiberg M, Terenghi G. Poly-3-hydroxybutyrate (PHB): a resorbable conduit for long-gap repair in peripheral nerves. Br J Plast Surg. 2002;55(3):235-240.
25. Hill PS, Apel PJ, Barnwell J, et al. Repair of peripheral nerve defects in rabbits using keratin hydrogel scaffolds. Tissue Eng Part A. 2011;17(11-12):1499-1505.
26. Hsu SH, Chan SH, Chiang CM, Chen CC, Jiang CF. Peripheral nerve regeneration using a microporous polylactic acid asymmetric conduit in a rabbit long-gap sciatic nerve transection model. Biomaterials. 2011;32(15):3764-3775.
27. Nijhuis TH, de Boer SA, Wahegaonkar AL, et al. A new approach to assess the gastrocnemius muscle volume in rodents using ultrasound; comparison with the gastrocnemius muscle index. PLoS One. 2013;8(1):e54041.
28. Shin RH, Vathana T, Giessler GA, Friedrich PF, Bishop AT, Shin AY. Isometric tetanic force measurement method of the tibialis anterior in the rat. Microsurgery. 2008;28(6):452-457.
29. Mendis MD, Wilson SJ, Stanton W, Hides JA. Validity of real-time ultrasound imaging to measure anterior hip muscle size: a comparison with magnetic resonance imaging. J Orthop Sports Phys Ther. 2010;40(9):577-581.
30. Reeves ND, Maganaris CN, Narici MV. Ultrasonographic assessment of human skeletal muscle size. Eur J Appl Physiol. 2004;91(1):116-118.
31. Takai Y, Katsumata Y, Kawakami Y, Kanehisa H, Fukunaga T. Ultrasound method for estimating the cross-sectional area of the psoas major muscle. Med Sci Sports Exerc. 2011;43(10):2000-2004.
32. Yi TI, Han IS, Kim JS, Jin JR, Han JS. Reliability of the supraspinatus muscle thickness measurement by ultrasonography. Ann Rehabil Med. 2012;36(4):488-495.
33. Brodbelt DC, Blissitt KJ, Hammond RA, et al. The risk of death: the confidential enquiry into perioperative small animal fatalities. Vet Anaesth Analg. 2008;35(5):365-373.
34. Strommen JA, Daube JR. Determinants of pain in needle electromyography. Clin Neurophysiol. 2001;112(8):1414-1418.
35. Mohseny B, Nijhuis TH, Hundepool CA, Janssen WG, Selles RW, Coert JH. Ultrasonographic quantification of intrinsic hand muscle cross-sectional area; reliability and validity for predicting muscle strength. Arch Phys Med Rehabil. 2015;96(5):845-853.
36. Means KR, Jr., Rinker BD, Higgins JP, Payne SH, Jr., Merrell GA, Wilgis EF. A Multicenter, Prospective, Randomized, Pilot Study of Outcomes for Digital Nerve Repair in the Hand Using Hollow Conduit Compared With Processed Allograft Nerve. Hand (N Y). 2016;11(2):144-151.
37. Deslivia MF, Lee HJ, Adikrishna A, Jeon IH. Decellularized Nerves for Upper Limb Nerve Reconstruction: A Systematic Review of Functional Outcomes. J Reconstr Microsurg. 2015;31(9):660-667.
38. Rinker B, Zoldos J, Weber RV, et al. Use of Processed Nerve Allografts to Repair Nerve Injuries Greater Than 25 mm in the Hand. Ann Plast Surg. 2017;78(6S Suppl 5):S292-S295.
39. Rinker BD, Ingari JV, Greenberg JA, Thayer WP, Safa B, Buncke GM. Outcomes of short-gap sensory nerve injuries reconstructed with processed nerve allografts from a multicenter registry study. J Reconstr Microsurg. 2015;31(5):384-390.
40. Taras JS, Amin N, Patel N, McCabe LA. Allograft reconstruction for digital nerve loss. J Hand Surg Am. 2013;38(10):1965-1971.
41. Cho MS, Rinker BD, Weber RV, et al. Functional outcome following nerve repair in the upper extremity using processed nerve allograft. J Hand Surg Am. 2012;37(11):2340-2349.
42. Giusti G, Willems WF, Kremer T, Friedrich PF, Bishop AT, Shin AY. Return of motor function after segmental nerve loss in a rat model: comparison of autogenous nerve graft, collagen conduit, and processed allograft (AxoGen). J Bone Joint Surg Am. 2012;94(5):410-417.

43. Brooks DN, Weber RV, Chao JD, et al. Processed nerve allografts for peripheral nerve reconstruction: a multicenter study of utilization and outcomes in sensory, mixed, and motor nerve reconstructions. Microsurgery. 2012;32(1):1-14.
44. Whitlock EL, Tuffaha SH, Luciano JP, et al. Processed allografts and type I collagen conduits for repair of peripheral nerve gaps. Muscle Nerve. 2009;39(6):787-799.
45. Rbia N, Shin AY. The Role of Nerve Graft Substitutes in Motor and Mixed Motor/Sensory Peripheral Nerve Injuries. J Hand Surg Am. 2017;42(5):367-377.
46. Hundepool CA, Nijhuis TH, Kotsougiani D, Friedrich PF, Bishop AT, Shin AY. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. Neurosurg Focus. 2017;42(3):E4.
47. Lee JY, Giusti G, Friedrich PF, et al. The effect of collagen nerve conduits filled with collagen-glycosaminoglycan matrix on peripheral motor nerve regeneration in a rat model. J Bone Joint Surg Am. 2012;94(22):2084-2091.
48. Yan Y, Hunter DA, Schellhardt L, et al. Nerve stepping stone has minimal impact in aiding regeneration across long acellular nerve allografts. Muscle Nerve. 2018;57(2):260-267.
49. Poppler LH, Ee X, Schellhardt L, et al. Axonal Growth Arrests After an Increased Accumulation of Schwann Cells Expressing Senescence Markers and Stromal Cells in Acellular Nerve Allografts. Tissue Eng Part A. 2016;22(13-14):949-961.
50. Saheb-Al-Zamani M, Yan Y, Farber SJ, et al. Limited regeneration in long acellular nerve allografts is associated with increased Schwann cell senescence. Exp Neurol. 2013;247:165-177.
51. Eather TF, Pollock M, Myers DB. Proximal and distal changes in collagen content of peripheral nerve that follow transection and crush lesions. Exp Neurol. 1986;92(2):299-310.
52. Ngeow WC. Scar less: a review of methods of scar reduction at sites of peripheral nerve repair. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010;109(3):357-366.
53. Vasudevan S, Huang J, Botterman B, Matloub HS, Keefer E, Cheng J. Detergent-free Decellularized Nerve Grafts for Long-gap Peripheral Nerve Reconstruction. Plast Reconstr Surg Glob Open. 2014;2(8):e201.
54. Wood MD, Kemp SW, Weber C, Borschel GH, Gordon T. Outcome measures of peripheral nerve regeneration. Ann Anat. 2011;193(4):321-333.
55. Wang Y, Zhao Z, Ren Z, et al. Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration. Neurosci Lett. 2012;514(1):96-101.
56. di Summa PG, Kingham PJ, Raffoul W, Wiberg M, Terenghi G, Kalbermatten DF. Adipose-derived stem cells enhance peripheral nerve regeneration. J Plast Reconstr Aesthet Surg. 2010;63(9):1544-1552.
57. Hundepool CA, Nijhuis TH, Mohseny B, Selles RW, Hovius SE. The effect of stem cells in bridging peripheral nerve defects: a meta-analysis. J Neurosurg. 2014;121(1):195-209.
58. Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G. Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Exp Neurol. 2007;207(2):267-274.
59. Kingham PJ, Kolar MK, Novikova LN, Novikov LN, Wiberg M. Stimulating the neurotrophic and angiogenic properties of human adipose-derived stem cells enhances nerve repair. Stem Cells Dev. 2014;23(7):741-754.
60. Nijhuis TH, Brzezicki G, Klimczak A, Siemionow M. Isogenic venous graft supported with bone marrow stromal cells as a natural conduit for bridging a 20 mm nerve gap. Microsurgery. 2010;30(8):639-645.
61. Orbay H, Uysal AC, Hyakusoku H, Mizuno H. Differentiated and undifferentiated adipose-derived stem cells improve function in rats with peripheral nerve gaps. J Plast Reconstr Aesthet Surg. 2012;65(5):657-664.
62. Tomita K, Madura T, Sakai Y, Yano K, Terenghi G, Hosokawa K. Glial differentiation of human adipose-derived stem cells: implications for cell-based transplantation therapy. Neuroscience. 2013;236:55-65.
63. Mazzucotelli JP, Roudiere JL, Bernex F, Bertrand P, Leandri J, Loisance D. A new device for endothelial cell seeding of a small-caliber vascular prosthesis. Artif Organs. 1993;17(9):787-790.

64. Shirota T, He H, Yasui H, Matsuda T. Human endothelial progenitor cell-seeded hybrid graft: proliferative and antithrombogenic potentials in vitro and fabrication processing. Tissue Eng. 2003;9(1):127-136.
65. Villalona GA, Udelsman B, Duncan DR, et al. Cell-seeding techniques in vascular tissue engineering. Tissue Eng Part B Rev. 2010;16(3):341-350.
66. Zhao Z, Wang Y, Peng J, et al. Improvement in nerve regeneration through a decellularized nerve graft by supplementation with bone marrow stromal cells in fibrin. Cell Transplant. 2014;23(1):97-110.
67. Jesuraj NJ, Santosa KB, Newton P, et al. A systematic evaluation of Schwann cell injection into acellular cold-preserved nerve grafts. J Neurosci Methods. 2011;197(2):209-215.
68. Liu GB, Cheng YX, Feng YK, et al. Adipose-derived stem cells promote peripheral nerve repair. Arch Med Sci. 2011;7(4):592-596.
69. Jiang L, Jones S, Jia X. Stem Cell Transplantation for Peripheral Nerve Regeneration: Current Options and Opportunities. Int J Mol Sci. 2017;18(1).
70. Vitacolonna M, Belharazem D, Hohenberger P, Roessner ED. Effect of static seeding methods on the distribution of fibroblasts within human acellular dermis. Biomed Eng Online. 2013;12:55.
71. Clements MP, Byrne E, Camarillo Guerrero LF, et al. The Wound Microenvironment Reprograms Schwann Cells to Invasive Mesenchymal-like Cells to Drive Peripheral Nerve Regeneration. Neuron. 2017;96(1):98-114 e117.
72. Hoke A, Redett R, Hameed H, et al. Schwann cells express motor and sensory phenotypes that regulate axon regeneration. J Neurosci. 2006;26(38):9646-9655.
73. Bunge RP. The role of the Schwann cell in trophic support and regeneration. J Neurol. 1994;242(1 Suppl 1):S19-21.
74. Caddick J, Kingham PJ, Gardiner NJ, Wiberg M, Terenghi G. Phenotypic and functional characteristics of mesenchymal stem cells differentiated along a Schwann cell lineage. Glia. 2006;54(8):840-849.
75. Faroni A, Terenghi G, Reid AJ. Adipose-derived stem cells and nerve regeneration: promises and pitfalls. Int Rev Neurobiol. 2013;108:121-136.
76. Mohammadi R, Mehrtash M, Mehrtash M, Sajjadi SS. Nonexpanded Adipose Stromal Vascular Fraction Local Therapy on Peripheral Nerve Regeneration Using Allografts. J Invest Surg. 2016;29(3):149-156.
77. Tulaci KG, Tuzuner A, Karadas Emir H, et al. The effect of tacrolimus on facial nerve injury: Histopathological findings in a rabbit model. Am J Otolaryngol. 2016;37(5):393-397.



11

Summary

Peripheral nerve injuries occur in up to 3% of patients suffering extremity trauma and can have devastating impact on patients' daily functions and quality of life. The overarching aim of this thesis is to improve nerve reconstruction using an off the shelf peripheral nerve allograft that is unlimited in supply and can be individualized to each patient using stem cells, providing functional motor recovery comparable to autograft nerve.

PART I: Current clinical use of nerve grafts

In the first part, studies on the current clinical use of nerve grafts are presented. The clinical focus of this part is on brachial plexus injury. The brachial plexus is a complex network of nerves that arises from the neck and innervates the muscles and skin of the upper extremities. An injury to the brachial plexus, commonly caused by motor vehicle or snow mobile accidents, can thus result in a severely impaired or even completely flail arm. Timely and adequate reconstruction of the nerve damage is the main priority. These injuries typically require large amounts of donor nerve material that is frequently not available, causing clinical challenges. The availability of an off the shelf alternative, with equal functional results as the gold standard nerve autograft, would be very beneficial in this field. In **Chapter 2**, an overview of different techniques that are currently used to reconstruct elbow function after brachial plexus injury is provided. In general, nerve transfers without interposition nerve grafts yield better results. However, a clear advantage of nerve grafts for additional length is that more nerve transfer sources become available for use that would otherwise not be able to reach the target nerve in a tension free fashion. One thing that stood out in our review is that described options for elbow flexion clearly outnumber the published options for reconstruction of elbow extension.

To address this paucity, **Chapter 3** evaluated the quality and prevalence of restored elbow extension in brachial plexus injury patients that underwent a novel treatment. In these patients, part of the spinal accessory nerve (SAN) was transferred to the radial nerve branch of the triceps muscle, using long interposition nerve grafts to establish a tension free coaptation. After a median follow-up of 22.9 months, 52.4% of our cohort of 42 patients obtained meaningful recovery. Furthermore, in our subgroup analysis of patients with a minimum of 20 months of follow-up, showing that after a mean follow-up of 30 months, 69.5% reached meaningful recovery. We believe this technique provides an adequate option to restore elbow extension.

PART II Evaluation techniques

Animal models are frequently used to study alternative treatment strategies for peripheral nerve injury. Before implementing new nerve reconstruction strategies in vivo in animal models, we recognized there was a need for non-invasive follow-up methods. The second part of this thesis was therefor focused on the development of new evaluation techniques to measure nerve recovery in animal models.

In **Chapter 4**, we used ultrasound to evaluate both the tibial and the gastrocnemic muscle after sciatic nerve reconstruction in the rat model. Both intra-rater (r=0.97) and inter-rater

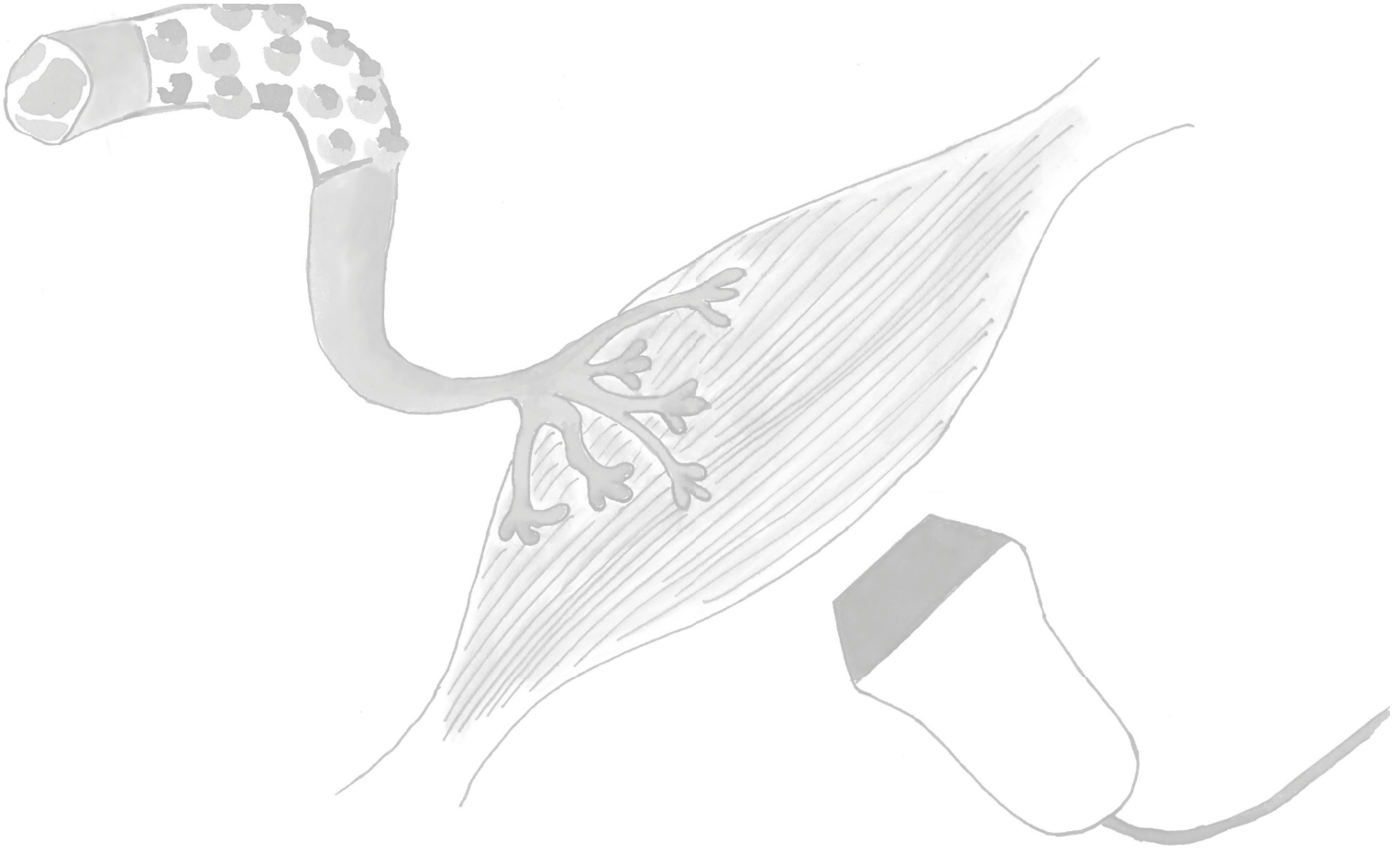
reliability (r=0.88) of ultrasound were high indicating excellent reliability and reproducibility in the rat model.

In **Chapter 5**, we developed and validated a similar technique for the rabbit model. The rabbit model is frequently used as a next step after the rat model. It allows for reconstruction of larger nerve gaps and the rabbit's neuroregenerative capacity more closely mimics the human situation. We showed that both inter-rater and intra-rater reliability were excellent (r=0.989 and 0.994 respectively). In line with our rat study and previous research, this indicates both high reliability and reproducibility of this method.
In conclusion, in both animal models ultrasound provides a reliable method to follow up muscle recovery after nerve reconstruction in a non-invasive fashion. Allowing for multiple measurements over time in the same animal, this could decrease inter-animal variation, diminish the number of test animals required in future studies and provide information on early nerve regeneration.

PART III: Implementation and improvement
The third part of this thesis was focused on the implementation and further improvement of the processed nerve allograft. In **Chapter 6**, we implemented our previously optimized nerve allograft (either stored at 4°C or -80°C) in a 1-cm gap in the rat model and compared outcomes to the autograft. We found that for all outcome measurements (ankle angle, electrophysiology, isometric tetanic force (ITF), muscle weight and histomorphometry), the elastase processed allograft that was cold stored yielded similar results compared to the autograft. Furthermore, the cold stored graft yielded better results than the frozen stored graft in 3 out of 5 outcome measurements.
In **Chapter 7**, a similar research design was implemented in the rabbit model, reconstructing a 3-cm peroneal nerve gap. After 24 weeks, ultrasound, muscle weight and ITF showed that the cold-stored allograft yielded similar outcomes in comparison to the autograft groups. The frozen stored group in contrast, yielded inferior functional outcome.
In conclusion, for both animal models, the cold stored optimized nerve allograft showed promising results. However, we acknowledges that there was room for further improvement. We hypothesized there was a need for a source of supportive and stimulating growth factors and that this may be provided by patient own adipose derived stem cells (MSCs) added to the nerve allograft. MSCs are capable of producing growth factors (including NGF, BDNF, VEGF) and may be the key to boost nerve regeneration across acellular nerve allografts. However, the exact role and fate of MSCs in nerve regeneration have not been fully elucidated yet.
In **Chapter 8,** we developed a simple technique to seed nerve allografts using a custom bioreactor, that is easy to implement in other institutions. This method was shown to be reproducible, distribute cells homogenously over the graft in an optimal seeding time of 12 hours, and does not traumatize the intra-neural architecture of the allograft.
In **chapter 9**, we aimed to clarify the potential role of the adipose derived MSCs in combination with our nerve allograft. Over 21 days, semi-quantitative RT-PCR analysis

showed that adherence of MSCs to nerve allografts significantly enhances mRNA expression of multiple neurotrophic, angiogenic, endothelial and myelination markers (BDNF, VEGF, CD31 and MBP), when compared to adipose derived MSCs that were expanded in absence of the nerve allograft. We thereby demonstrated that seeding of undifferentiated MSCs onto processed nerve allografts indeed permits secretion of growth factors that can potentially stimulate nerve regeneration.

In the final chapter of this thesis, **Chapter 10**, the main findings of the studies are discussed and suggestions for future research are given. We have taken a promising path by using allografts and stem cells on our mission to improve nerve reconstruction.



12

Nederlandse samenvatting

Perifeer zenuwletsel komt voor bij tot 3% van de patiënten met trauma van de extremiteiten. Letsel aan perifere zenuwen kan een zeer grote impact op het dagelijks functioneren en kwaliteit van leven hebben. Het overkoepelende doel van dit proefschrift is om de uitkomsten van zenuwreconstructies te verbeteren met behulp van allogene zenuwtransplantaten die vrijuit beschikbaar zijn en verder kunnen worden geïndividualiseerd voor iedere patiënt met stamcellen, zodat motorische uitkomsten het autologe transplantaat zullen kunnen evenaren.

DEEL I: Huidige klinische toepassing van zenuwtransplantaten

In het eerste deel worden studies gepresenteerd over het huidige klinische gebruik van zenuwtransplantaten. De klinische focus van dit deel is op plexus brachialis letsel. De plexus brachialis is een complex netwerk van zenuwen dat ontspringt vanuit de nek en de spieren en huid van de bovenste extremiteiten innerveert. Letsel van de plexus brachialis, vaak veroorzaakt door motor- en sneeuwscooterongevallen, kan leiden tot een zeer beperkte of zelfs geheel afwezige functie van de arm. Tijdige en adequate reconstructie van de zenuwbeschadiging is van hoogste prioriteit. Deze letsels vereisen vaak grote hoeveelheden zenuwtransplantaat, hetgeen niet altijd beschikbaar is vanuit de patiënt zelf en zo een klinische uitdaging vormt. Een vrij beschikbaar alternatief, dat even goed functioneel herstel geeft als autologe zenuwtransplantaten, zou dan ook van grote waarde zijn in dit veld.

In **hoofdstuk 2** wordt een overzicht gegeven van diverse technieken die momenteel gebruikt worden om de elleboog functie te herstellen na plexus brachialis letsel. Over het algemeen geven zenuw transfers zonder toegevoegde zenuwtransplantaten betere resultaten. Een voordeel van de toevoeging van zenuwtransplantaten is echter dat de extra lengte ervoor zorgt dat er meer zenuw transfer bronnen beschikbaar komen, die anders de distale zenuw niet zonder spanning zouden halen. Wat opviel in het overzichtsartikel is dat de beschreven opties voor herstel van elleboog flexie veel talrijker zijn dan de gepubliceerde opties voor elleboog extensie.

Hierop inspelend, is in **hoofdstuk 3** het herstel van elleboog extensie geëvalueerd in patiënten met plexus brachialis letsel die een nieuwe behandeling hebben ondergaan. In deze patiënten is een deel van de n. accessorius omgeleid naar de n. radialis tak die de triceps innerveert. Hierbij zijn lange autologe zenuwtransplantaten gebruikt om een spanningsloze coaptatie te bewerkstelligen. Na een mediane follow-up van 22.9 maanden, was functioneel nuttig herstel opgetreden bij 52.4% van onze 42 patiënten. Daarnaast vonden we dat in onze subgroep van patiënten met minimaal 20 maanden follow-up, zelfs 69.5% functioneel nuttig herstel bereikte na een follow-up van gemiddeld 30 maanden. Wij zijn van mening dat deze techniek een adequate optie biedt voor reconstructie van elleboog extensie.

DEEL II: Evaluatietechnieken

Diermodellen worden regelmatig gebruikt om alternatieve behandelopties voor perifeer zenuwletsel te bestuderen. Alvorens onze nieuwe reconstructieve technieken in vivo in diermodellen te testen, erkenden we dat er vraag was naar niet-invasieve meetmethoden.

Het tweede deel van dit proefschrift is daarom gericht op de ontwikkeling van nieuwe meetmethoden om zenuwherstel in diermodellen te evalueren.

In **hoofdstuk 4** is gebruik gemaakt van echografie om zowel de m. tibialis als de m. gastrocnemius te evalueren na reconstructie van de n. ischiadicus in de rat. De zogenaamde intra-rater (r=0.97) en inter-rater betrouwbaarheid (r=0.88) waren beiden uitstekend, hetgeen duidt op goede betrouwbaarheid en reproduceerbaarheid in het rat model.
In **hoofdstuk 5** hebben we een soortgelijke techniek ontwikkeld voor het konijnen model. Het konijn wordt vaak gebruikt als volgende stap na het rat model. Er kunnen langere zenuwdefecten worden gereconstrueerd en de zenuwregeneratie van het konijn is meer gelijk aan die van de mens. We vonden een uitstekende inter-rater en intra-rater betrouwbaarheid (r=0.989 and 0.994 respectievelijk). In lijn met de studie in het rat model en eerder onderzoek, indiceert dit dat de methode betrouwbaar en goed reproduceerbaar is.
Concluderend biedt echografie in beide diermodellen een betrouwbare techniek om spierherstel na zenuwreconstructie te evalueren op een niet-invasieve manier. Doordat er meerdere metingen in hetzelfde dier op verschillende tijdspunten mogelijk zijn, zal de variabiliteit afnemen waardoor het aantal benodigde proefdieren in de toekomst af zal nemen. Tevens is het mogelijk met deze methode in een vroeg stadium informatie te verkrijgen over zenuwherstel.

DEEL III: implementatie en verbetering
Het derde deel van dit proefschrift is gericht op de implementatie en verdere verbetering van het gedecellularizeerde allogene zenuwtransplantaat. In **hoofdstuk 6** is het eerder geoptimaliseerde allogene zenuwtransplantaat (bewaard op 4°C of -80°C) gebruikt om een 1-cm zenuwdefect in de rat te reconstrueren. Uitkomsten zijn vergeleken met autologe zenuwreconstructies. Alle uitkomstmaten (enkel hoek, elektrofysiologie, isometrische tetanische kracht, spiermassa en histomorfometrie), lieten zien dat het met elastase gedecellularizeerde allogene transplantaat dat koud bewaard was gelijke resultaten geeft als het autologe transplantaat. Daarnaast geeft het koud bewaarde transplantaat superieure resultaten ten opzichte van het bevroren bewaarde transplantaat in 3 van de 5 uitkomstmaten.
In **hoofdstuk 7** is een gelijke onderzoeksopzet toegepast in het konijnen model, waarbij een 3-cm n. peroneus defect is gereconstrueerd. Na 24 weken lieten echografie, spiermassa en isometrische tetanische krachtmetingen zien dat het koud bewaarde transplantaat vergelijkbare functie geeft als het autologe transplantaat. Het bevroren bewaarde allogene transplantaat daarentegen, gaf inferieure functionele resultaten.
Concluderend vonden we in beide diermodellen dat het koud bewaarde geoptimaliseerde allogene zenuwtransplantaat veelbelovende resultaten laat zien. Echter erkenden we wel dat er ruimte is voor verdere verbetering. Onze hypothese was dat er behoefte is aan een bron van ondersteunende en stimulerende groeifactoren en dat hierin zou kunnen worden voorzien door uit vet verkregen mesenchymale stamcellen (MSCs) van de patiënt zelf aan het

allogene transplantaat toe te voegen. MSCs kunnen diverse groeifactoren produceren (waaronder NGF, BDNF, VEGF) en kunnen potentieel een boost geven aan zenuwregeneratie over acellulaire transplantaten. Echter is de precieze rol van MSCs in zenuwregeneratie nog niet geheel helder.

In **hoofdstuk 8** wordt een nieuw ontwikkelde, eenvoudige techniek beschreven om de zenuwtransplantaten te bezaaien met mesenchymale stamcellen. De techniek is gemakkelijk te implementeren in andere instellingen. We hebben aangetoond dat de methode reproduceerbaar is en de stamcellen zich homogeen over het transplantaat verspreiden in een optimale incubatietijd van 12 uur. De structuur van het transplantaat wordt hierbij niet aangetast.

Het doel van het onderzoek als beschreven in **hoofdstuk 9** is om de potentiele rol van de uit vet verkregen mesenchymale stamcellen in combinatie met ons zenuwtransplantaat te verhelderen. Gedurende 21 dagen zagen we met behulp van semi-kwantitatieve RT-PCR analyse dat MSCs in combinatie met het allogene transplantaat significant hogere mRNA expressie lieten zien van diverse neurogene, angiogene, endotheliale en myelinisatie factoren (BDNF, VEGF, CD31 en MBP) in vergelijking met uit vet verkregen MSCs die zonder het transplantaat gekweekt waren. Hiermee toonden we aan dat ongedifferentieerde MSCs na contact met het geoptimaliseerde zenuwtransplantaat inderdaad in staat zijn om groeifactoren te produceren die zenuwregeneratie kunnen stimuleren.

In het laatste hoofdstuk van dit proefschrift, **hoofdstuk 10**, worden de belangrijkste bevindingen van de verschillende studies uiteengezet en bediscussieerd. Daarnaast worden suggesties voor toekomstig onderzoekgegeven. We zijn een veelbelovend pad ingeslagen door gebruik te maken van allogene zenuwtransplantaten en toevoeging van stamcellen om op deze wijze vorm te geven aan onze missie om de resultaten van zenuwreconstructies te verbeteren.

Appendices

List of publications

PhD portfolio

Curriculum Vitae

Dankwoord

List of Publications

1. **A simple dynamic strategy to deliver stem cells to decellularized nerve allografts.**
   Rbia N, Bulstra LF, Bishop AT, van Wijnen AJ, Shin AY. Plast Reconstr Surg. 2018 May 22
2. **Bone vascularized composite allotransplantation model in swine tibial defect: Evaluation of surgical angiogenesis and transplant viability.**
   Kotsougiani D, Hundepool CA, Bulstra LF, Friedrich PF, Shin AY, Bishop AT. Microsurgery. 2018 Mar 5.
3. **Spinal accessory nerve to triceps muscle transfer using long autologous nerve grafts for recovery of elbow extension in traumatic brachial plexus injuries.**
   Bulstra LF, Rbia N, Kircher MF, Spinner RJ, Bishop AT, Shin AY. J Neurosurg. 2017 Dec 8:1-7
4. **Evaluate muscle tension using intramuscular pressure device in rabbit tibialis anterior model for improved tendon transfer surgery.**
   Evertz L, Bulstra LF, Shin A, Kaufman KR. Physiol Meas. 2017 Jun 22;38(7):1301-1309.
5. **The learning rate in three dimensional high definition video assisted microvascular anastomosis in a rat model.**
   Kotsougiani D, Hundepool CA, Bulstra LF, Shin DM, Shin AY, Bishop AT. J Plast Reconstr Aesthet Surg. 2016 Nov;69(11):1528-1536.
6. **Recipient-derived angiogenesis with short term immunosuppression increases bone remodeling in bone vascularized composite allotransplantation: A pilot study in a swine tibial defect model.**
   Kotsougiani D, Hundepool CA, Bulstra LF, Friedrich PF, Shin AY, Bishop AT. J Orthop Res. 2016 Jul 29.
7. **Nerve Transfers to Restore Elbow Function.**
   Bulstra LF, Shin AY. Hand Clin. 2016 May;32(2):165-74.
8. **Motor Nerve Recovery in a Rabbit Model: Description and Validation of a Noninvasive Ultrasound Technique.**
   Bulstra LF, Hundepool CA, Friedrich PF, Nijhuis TH, Bishop AT, Shin AY. J Hand Surg Am. 2016 Jan;41(1):27-33.
9. **Noninvasive Ultrasound of the Tibial Muscle for Longitudinal Analysis of Nerve Regeneration in Rats.**
   Hundepool CA, Nijhuis TH, Rbia N, Bulstra LF, Selles RW, Hovius SE. Plast Reconstr Surg. 2015 Nov;136(5):633e-9e.

PhD Portfolio

Summary of PhD training and teaching

Name PhD student:	Liselotte Frederike Bulstra
Erasmus MC Department:	Plastic and Reconstructive Surgery and Hand Surgery
PhD period:	2014 – 2018
Promotor:	Prof. dr. S.E.R. Hovius
Copromotor:	Dr. T.H.J. Nijhuis

1. PhD training	**Year**	**Workload (ECTS / hours)**
General courses		
Human Subject Protection training program, Mayo Clinic	2015	0.7 ECTS
Research Integrity, Erasmus MC	2016	0.3 ECTS
English Biomedical Writing and Communications, Erasmus MC	2016	3 ECTS
Courses for the Quantitative Researcher, Erasmus MC	2016	1.4 ECTS
Veiligheidstraining MRI, Erasmus MC	2016	0.7 ECTS
Specific courses and training		
Microsurgery, Mayo Clinic Microvascular Surgery Training	2014	40 hours
NIHES Master Clinical Epidemiology	2015 - 2016	70 ECTS
Microsurgery, Erasmus MC Skillslab	2015 - 2016	152 hours
Seminars and workshops		
Kortjakje (5x)	2015 - 2017	25 hours
Workshop local transposition flaps, Erasmus MC Skillslab	2015	0.2 ECTS
Workshop funding and grant writing, Erasmus MC	2015	4 hours
Workshop Leading Through Conflict, Erasmus MC	2016	4 hours
(Inter)national conferences, oral presentations		
Description and validation of ultrasound in rabbits to measure motor recovery after peripheral nerve reconstruction.		
• Essercourse “On your nerves”, Rotterdam, NL	2014	10 hours
• Mayo Orthopedic Research Alumni Association (MORAA), Rochester, USA	2015	10 hours
• American Association for Surgery of the Hand (ASSH), annual meeting, Seattle, USA	2015	20 hours
• Dutch Society for Plastic Surgery (NVPC), scientific meeting (fall), Amsterdam, NL	2015	15 hours
• Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Leiden, NL	2015	10 hours

Optimizing decellularization techniques to create a novel nerve allograft: Return of motor function with decellularized nerve allografts using elastase in a rat sciatic nerve model

• Mayo Orthopedic Research Alumni Association (MORAA), Rochester, USA	2015	10 hours

Functional outcome after reconstruction of a long nerve gap in rabbits using decellularized nerve allografts

• Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Leiden, NL	2015	10 hours
• American Society for Peripheral Nerve (ASPN), Annual meeting, Scottsdale, USA	2016	20 hours
• Dutch Society for Plastic Surgery (NVPC), scientific meeting (fall), Rotterdam, NL	2016	15 hours
• International Federation of Societies for Surgery of the Hand (IFSSH), Triennial meeting, Buenos Aires, AR	2016	15 hours
• Traumaplatform 2018, Amsterdam, NL	2018	10 hours

Effectiveness and predictive factors of spinal accessory nerve to triceps transfer using autologous nerve grafts for recovery of elbow extension in traumatic brachial plexus injuries.

• Dutch Society for Plastic Surgery (NVPC), scientific meeting (fall), Rotterdam, NL	2016	15 hours

Description and validation of a simple histological nerve tissue scoring system.

• International Federation of Societies for Surgery of the Hand (IFSSH), Triennial meeting, Buenos Aires, AR	2016	15 hours

(Inter)national conferences, attendance only

American Society for Reconstructive Microsurgery (ASRM), American Society for Peripheral Nerve (ASPN), American Association for Hand Surgery (AAHS), combined annual meeting, Paradise Island, BS	2015	20 hours
American Society for Reconstructive Microsurgery (ASRM), American Association for Hand Surgery (AAHS), combined annual meeting, Scottsdale, USA	2016	20 hours
Federation of European Societies for Surgery of the Hand (FESSH), Annual meeting, Santander, ES	2016	15 hours
Hesperis Course, European Society for Organ Transplantation, Rome, Italy	2016	36 hours
Dutch Society for Plastic Surgery (NVPC), scientific meeting (fall), various locations. Spring 2016, fall 2017, spring 2018	2016 - 2018	15 hours

Grants and prizes

Van Beek Foundation, personal funding for Master Research, €750	2014
Trustfonds, personal funding for Research Fellowship, €2500	2014
Michael van Vloten Foundation, personal funding for Research Fellowship, €8000	2014
Esser Foundation, personal funding for Research Fellowship	2014 - 2016
AFSH Basic Science Award 2014, $20.000 *"Description and validation of ultrasound in rabbits to measure motor recovery after peripheral nerve reconstruction."*	2014
KNAW van Walree Foundation, Grant to present Master Research at the ASSH annual meeting 2015, €1000	2015
Mayo Clinic Center for Regenerative Medicine, $100.000	2015
AFSH Basic Science Award 2016, $20.000 *"Optimizing the regenerative capacities of the nerve allograft: addition of adipose derived stem cells"*	2016
Axogen Inc., $5.000	2016
NIH R01 2017, $1.250.000 *"Bridging the gap: Angiogenesis and stem cell seeding of processed nerve allograft"*	2017
Hippocrates Study Fund Prize 2017, €2000	2017
Grants received to visit conferences from Gerrit Jan Mulder Foundation and Stichting Erasmus Trustfonds.	2014 - 2017

2. Teaching	**Year**	**Workload (ECTS / hours)**
Lecturing		
Anatomy of the hand and arm (3rd year students)	2016	12 hours
Chronic diseases of the hand (2nd year students)	2016	8 hours
Skills		
Coach International Microsurgery course, Erasmus MC Skillslab	2015 - 2016	50 hours
Course Basics in Suture Techniques (1st year students)	2016	8 hours
Supervising students		
Summerstudents: A. Shin, D. Shin	2014 - 2015	80 hours
Minor students, review "Nerve transfers"	2015	15 hours
Minor students, review "Nerve allografts"	2015	15 hours
Minor students, review "Vascularization nerve grafts"	2016	15 hours

Curriculum Vitae

Liselotte Frederike Bulstra was born on January 14th 1991 in Utrecht, The Netherlands. After graduating from the Christelijk Gymnasium Utrecht in 2009 (cum laude), she started Medical School at the Erasmus University Rotterdam. In her first year of Medical School, she was selected for the Erasmus MC Honours Class, which she combined with her studies with great pleasure. Her special interest in plastic surgery was sparked during an elective rotation at the plastic surgery department of the Diakonessenhuis Utrecht. This interest was further fueled during several elective courses, including the Minor "Hoofdzaken van het hoofd-halsgebied".
During her third year, Liselotte obtained her first research experience at the department of Plastic and Reconstructive Surgery and Hand Surgery. In 2014, she commenced her PhD under supervision of Prof. dr. S.E.R. Hovius. She initiated this path with a 1.5 years international Research Fellowship at the Microvascular Research Laboratory, department of Orthopedic Surgery, division of Hand Surgery, Mayo Clinic, USA (Dr. A.Y. Shin and Dr. A.T. Bishop). Upon her return to Rotterdam, she enhanced her PhD with a Masters in Clinical Epidemiology at The Netherlands Institute for Health Sciences (NIHES).
Liselotte expects to finish her MD training in February 2019, after which she aims to fulfill her passion and become a plastic surgeon.

Dankwoord

De totstandkoming van dit proefschrift en deze bijzondere dag zou nooit geslaagd zijn zonder de geweldige hulp van velen. Wat een rijkdom om zoveel inspirerende, talentvolle, motiverende en vooral ook gezellige mensen om mij heen te mogen hebben.

Geachte prof. dr. Hovius, beste prof, dank voor de geweldige begeleiding tijdens de totstandkoming van dit proefschrift! De vele uren met u aan tafel (onder het genot van een kopje koffie of glaasje wijn) hebben dit boekje gemaakt tot wat het nu is. Het was erg leuk uw enthousiasme voor het onderwerp steeds weer te zien opvlammen. Uw kritisch houding met betrekking tot de interpretatie van de resultaten hebben de artikelen zeker naar een hoger niveau getild. Dank voor de vele inspirerende gesprekken en het vertrouwen dat u vanaf het begin in mij heeft gehad.

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