Stem cells in nerve reconstruction:

Hype, hope or reality?

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Stamcellen in zenuw reconstructie: Hype, hoop of realiteit?

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CHAPTER 1

General Introduction

BACKGROUND

Traumatic injuries to peripheral nerves are devastating life-altering injuries that occur in up to 3% of all patients admitted to level I trauma centers.[1,2] These injuries commonly result from motor vehicle accidents, penetrating trauma, falls or industrial accidents.[3] As every nerve has a specific function, symptoms depend on the type of nerve affected but might include numbness, tingling, hypersensivity, burning, pain, muscle weakness, lack of coordination or paralysis.[4] The nervous system is a complex collection of neurons that transmit signals between different parts of the body. In vertebrates it consists of two parts: the central nervous system and the peripheral nervous system. The central nervous system comprises the brain and the spinal cord, while the peripheral nervous system consists of all the nerves that lie outside the central nervous system. The peripheral nervous system sends information to and from the central nervous system, which allows the brain to react to external stimuli. The peripheral nervous system can be further divided into two parts: 1) the somatic nervous system that mediates voluntary movement and sensibility and 2) the autonomic nervous system that functions involuntary. In this thesis we will only focus on the somatic system, which consists of motor and sensory nerves. Motor nerves transmit signals from the central nervous system to the muscle and sensory nerves transmit information from the body (e.g. touch, sound, light) to the central nervous system. Mixed motor and sensory nerves have both functions.[5,6]

ANATOMY

A peripheral nerve is surrounded by a layer of connective tissue, which is called the epineurium. Within the epineurium, axons are bundled into fascicles that are surrounded by the perineurium. The individual axons are further surrounded by the endoneurium (figure 1). The blood supply to peripheral nerves derives from regional extrinsic vessels, lies on the outer surface and inner surface of the epineurium and courses through the epineurial capillary network.

At the cellular level, the nervous system consists of two main types of cells: neurons and glial cells. Neurons are specialized cells that consist of a cell body, one or more dendrites and an axon (figure 3). Neurons send signals to other cells through axons, which causes neurotransmitters to be released at the junctions. Glial cells are supportive cells that provide structural and metabolic support. Schwann cells are important types of glial

cells that generate myelin, a substance that surrounds the axon. This process is called myelination and ensures a rapid signal transmission.[7]

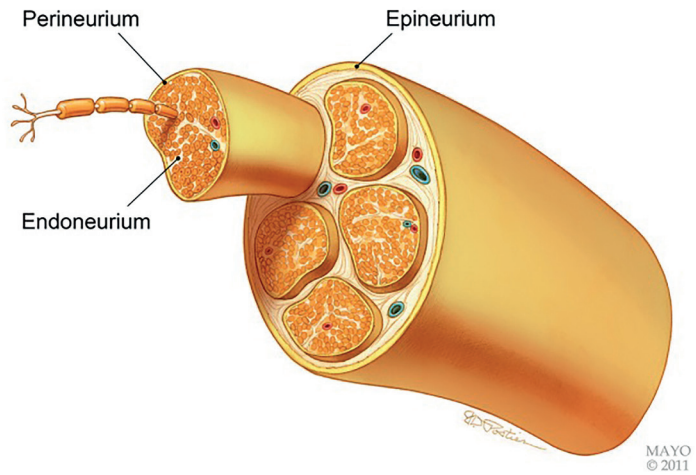

Figure 1. Schematic overview of the anatomy of peripheral nerves. (Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved.)

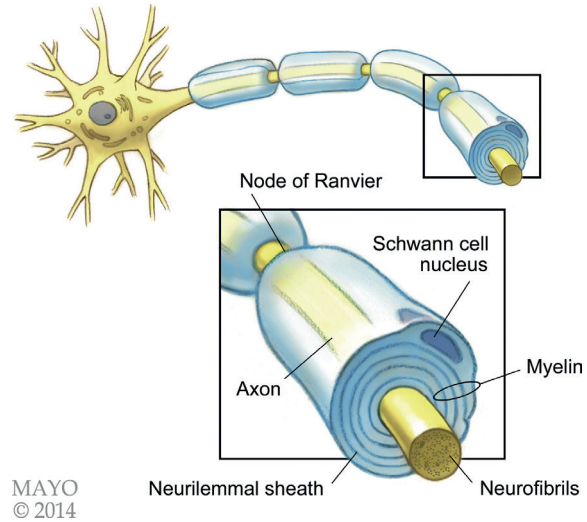

Figure 3. Myelinated axons are surrounded by Schwann cells to form a protective layer of myelin. (Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved.)

NERVE INJURY AND REGENERATION

Classification

There are several types of nerve injury and classification determines the prognosis and treatment strategy. Classification of nerve injury was described by Seddon in 1943 and by Sunderland in 1951. The classification of nerve injury described by Seddon comprised neurapraxia, axonotmesis and neurotmesis. In neurapraxia, the epineurium and axons are still intact. In axonotmesis, the epineurium is intact but the axons are disrupted and in neurotmesis, the nerve is completely transected.[8,9] Sunderland expanded this classification system to 5 degrees of nerve injury as depicted in figure 4. After neurapraxia or axotmesis, nerves can recover without surgical intervention. Neurotmesis requires surgical reconstruction.

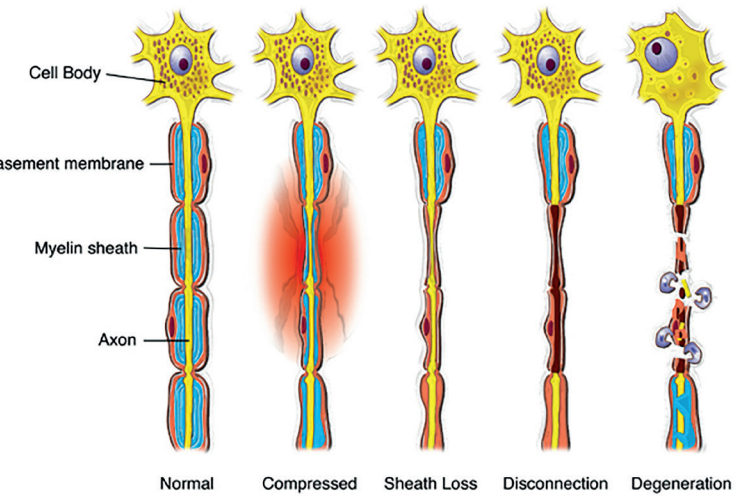

Figure 4. Classification of nerve injury according to Sunderland. (Permission requested form World J Stem Cells.)[10]

Pathophysiology

Following transection, changes occur at the site of injury and to components proximal and distal to it. First, chromatolysis (release of the Nissl granule in the cell body of a neuron) and swelling of the nucleus takes place.[11] Within forty-eight hours, Wallerian degeneration occurs, with breakdown of the axon distal to the level of transection (figure 5). Schwann cells and macrophages infiltrate to break down myelin and remove debris. Schwann cells

then start to proliferate by aligning longitudinally and forming the bands of Büngner, providing a scaffold for the axon to regenerate.[6] Following, numerous trophic and growth-inducing molecules, including neurotrophic and transcription factors, are secreted. [12-14] The concomitant interaction of Schwann cells and the surrounding environment promotes axonal sprouting at a rate of approximately 1 mm per day.[15] Evidence suggests that successful axonal regeneration and functional recovery depend on a delicate balance between both positive and negative growth signals.[16] Successful functional recovery also requires target organ reinnervation, which is influenced by the level and extent of the injury and by local biological factors. Most authors agree that poor functional recovery is expected if the growth cone fails to reinnervate the motor end plate by 12 months.[17] This sequence of nerve regeneration describes the ideal situation, however a complete nerve transection requires operative intervention.[18] When the perineurium is damaged, the regenerative nerve fascicles can escape their normal regenerative process. This leads to a proliferation of fibroblasts and Schwann cells in a disorganized fashion, leading to a neuroma formation.[15,19]

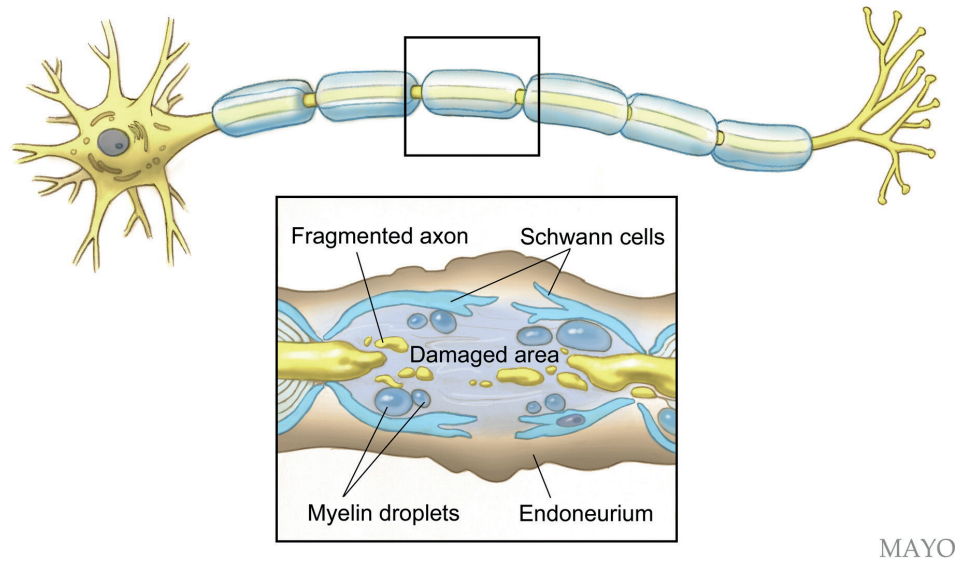

Figure 5. Wallerian degeneration. (Used with permission of Mayo Foundation for Medical Education and Research. All rights reserved.)

NERVE RECONSTRUCTION

Autografts and conduits

Despite numerous experimental studies, current clinical treatment of peripheral nerve defects rely heavily on the autograft: a healthy nerve sacrificed from elsewhere in the body.[20] The concept of nerve repair dates back to the seventh century, where Paulus Aegineta, a Greek physician, approximated cut nerve ends before wound closing.[21] However, it was not until 1861 during the American Civil War, that knowledge of peripheral nerve injury became available because nerve injuries were studied.[22] In 1872, the concept of primary repair was introduced by Huenter, suturing the epineurium of both nerve ends.[23] Later, it became evident that tension on the nerve repair carried the risk of decreased blood flow and proliferation of scar tissue. This led to the use of autologous nerve grafts to bridge a nerve gap up to 3 cm and results in recovery in up to 69% of the cases.[24,25] The harvest of an autograft nerve is however associated with several disadvantages such as sensory loss or neuroma formation at the donor site and at the repair site, size mismatch, scarring and fibrosis may occur, leading to poor regeneration.[20] To overcome these drawbacks, researchers have been searching for alternatives. In 1909 the use of biological conduits such as arterial and vein grafts was introduced.[17] Natural conduits are rich in extracellular matrix (ECM) components and contain viable cells and have therefore been considered as one of the first alternative grafting materials. However, empty veins have the tendency to collapse and they are also associated with donor site morbidity and therefore considered not ideal.[26] Subsequently, to avoid this donor site morbidity, synthetic conduits were introduced. Silicone conduits were the first synthetic conduits and another advantage included their readily availability in different sizes.[27,28] Despite this, the non-resorbable nature of silicone caused permanent fibrotic encapsulation of the implant leading to inflammation and potentially nerve compression that required a secondary surgical intervention.[20] These limitations have led to the development of 3 types of resorbable synthetic conduits: polyglycolic acid (PGA), collagen type 1, and caprolactone; many clinical studies followed.[15] Following, it was hypothesized that providing a synthetic mimic of the Schwann cell basal lamina in the form of a collagen-glycosaminoglycan (GAG) matrix would improve the bridging of the nerve gap and thus functional recovery. Again, initial early results were promising but when tested in a larger nerve gap it was outperformed by the autograft.[29,30] Currently, the use of synthetic nerve conduits is limited to gaps 3 cm or smaller in small-caliber sensory digital nerves. [31]

Nerve allografts

Nerve allografting, an alternative to autografts and synthetic nerve conduits, was first described in 1885 but reported poor clinical results.[32] Later, studies found that this poor result was caused by an immune rejection. Therefore, the first successful nerve allograft reconstructions required an 18-month-long course of immunosuppression.[33] Efforts to eliminate the need of immunosuppression have led to the development of decellularized nerve allografts.[17] Decellularized allografts prevent a host reaction to the implant by removing the immunogenic constituents of the graft. Even though these grafts are decellularized and in the process lose important growth promoting elements such as Schwann cells and growth factors, they retain the internal scaffold, laminin, and other structural extracellular components that are important for regeneration and therefore retain the beneficial characteristics of a nerve graft.[34,35] Since 2007, this decellularized nerve allograft is commercially available and major advantages of these grafts compared with autografts are that they are readily available for implantation and donor site morbidity and operative time are decreased.[15] However, despite the growing clinical popularity, studies suggest that allografts may still be inferior to fresh autografts in the ability to support nerve regeneration in large nerve gaps.[36-39] Long gap motor nerve injuries pose the biggest challenge to reconstruction and tend to have the poorest clinical outcome.[39-41]

Growth factors

Even with advanced surgical techniques, only around 50% of the patients achieve complete recovery of function.[42] To establish a more favorable environment for regenerating axons, researchers have introduced neurotrophic factors and ECM components to improve the effectiveness of nerve graft substitutes.[15] Neurotrophic and angiogenetic growth factors have been shown to have a beneficial effect on nerve regeneration in many studies.[43,44] But, results vary significantly when comparing different delivery mechanisms and concentrations, in some cases even interfering with regeneration.[45] Until now, a suitable mechanism to deliver the growth factors has not been established. There are two main limitations in the attempts that have been made so far: first, many groups use hollow conduits even though it has been shown that hollow conduits yield inferior results when compared to grafts that have an inner architecture.[43,46] The second problem is the growth factor delivery system. Delivery mechanisms such as micropumps and microspheres releasing a constant amount of a specific combination of growth factors over time have been used. This constant infusion has not shown much success and in some cases even impaired nerve regeneration.[47] In different stages of nerve regeneration there is a biological demand for different growth factors and the healthy body has a natural feedback mechanism to

provide the right factors at the right time in the right concentration. As it has been shown that a constant delivery of growth factors does not support nerve regeneration, it seems that the ability to adjust the production of local growth factors to the biological needs is key.[45] With this understanding, cell-based therapy is potentially ideal since it can respond to the demands placed on them by the local environment.

Mesenchymal stromal cells

The obvious choice for cell-based therapy to improve nerve graft substitutes would be Schwann cells, since they play an integral role in multiple facets of nerve regeneration. However, obtaining Schwann cells is limited by the invasive nature of harvesting and donor site morbidity.[46,48] For this reason, attention has been drawn to the use of mesenchymal stromal cells (MSCs). MSCs are undifferentiated precursors that can divide into daughter cells or that can differentiate along a variety of cell lineages.[46] MSCs have been shown to stimulate and support nerve regeneration. A number of different types of stem cells have been implemented in animal experiments, showing their beneficial effect on nerve regeneration. Frequently used cell types are MSCs derived from bone marrow and adipose tissue, among many other possible sources.[48] The use of adipose derived MSCs, compared to other cell types has many clinical advantages, such as easy accessibility, higher mesenchymal stem cell yields, rapid proliferation in culture and successful integration into host tissue with immunological tolerance.[48] Over the years, there has been an explosion of interest in MSCs that have the potential to repair tissue. Despite progress in the extensive studies on the role of MSCs in peripheral nerve repair, the actual mechanism of this effect is yet unclear and many questions remain before clinical translation can be considered.[49]

HISTORICAL BACKGROUND OF THIS THESIS

This thesis builds on the results of previous research conducted by two research groups to improve the effectiveness of nerve graft substitutes, with the emphasis on allografts seeded with stem cells.

First, a comparative study of bioabsorbable synthetic hollow nerve conduits, commercially available decellularized nerve allografts and a collagen conduit filled with a collagen/glycosaminoglycan (GAG) matrix was performed because lack of any internal structure was thought to be a possible explanation for the poor results in mixed nerve reconstruction.[29] Initial results were promising, however in a 3cm rabbit nerve defect, premature degradation

of the matrix-filled conduits and a failure to reinnervate the muscles were found. Autograft controls remained statistically superior to both empty and filled collagen conduits.[30]

To overcome the drawbacks of empty vein grafts that have the tendency to collapse, in the same period a study was performed where empty vein grafts were compared to saline filled vein grafts and to isogenic vein grafts pre-seeded with isogenic bone marrow stromal cells (BMSCs). The BMSC pre-seeded vein grafts were associated with better functional outcomes.[26] Subsequently, a vein muscle graft (vein graft filled with muscle to prevent collapse and support axon regeneration) was compared to a vein muscle graft injected with BMSCs and to an autograft. Twelve weeks after reconstruction, a strong indication was found for a beneficial effect of the BMSCs; however, both vein-muscle grafts were outperformed by the autograft.[26]

Next, decellularized nerve allograft was studied in the rat model, prepared identically to a commercially-marketed product (Axogen®). Functional outcome was found to be similar to autograft at 12 weeks, but failed to further improve at 16 weeks.[50] Histologic study of the supplied allograft demonstrated considerable cellular debris, suggesting that the degradation of functional outcome was secondary to either physical or immunologic mechanisms. Therefore, in a comparison of different processing and storage techniques of human sural nerves *in vitro*; and subsequent in vivo experiments, it was sought to understand how processing techniques and storage temperature effect functional outcomes of nerve grafting. Different processing protocols were evaluated based on the modified Hudson protocol.[34] The main difference between the different groups was the addition of elastase, and variable storage temperatures (-80°C or +4°C). Elastase is an enzyme that has been successfully used in the decellularization of various tissues such as heart valves and cartilage.[51] [52] The addition of elastase significantly reduced the amount of axonal debris and immunogenicity, while maintaining the ultrastructural properties. Freezing at -80°C damaged the ultrastructure, while its morphology in cold storage (4°C) remained similar to unprocessed controls.[53] After determining the optimal processing technique for decellularization of nerve allografts i*n vitro*, two *in vivo* studies were executed. A 1cm sciatic nerve defect was reconstructed in rats using either autografts, a cold (4°) stored decellularized allograft, or a frozen (-80°C) decellularized allograft. At both 12 and 16 weeks the processed cold stored allograft scored significantly better than the frozen allograft with regard to motor outcome. The elastase processed cold preserved allograft showed statistically similar results to the nerve autograft.[54] The elastase processed cold stored nerve allograft was then tested in a 3-cm peroneal nerve defect in the rabbit. When

autografts however were compared to the decellularized allograft they were found to re-innervate more rapidly and outperformed the decellularized allograft.[55] Subsequently, a meta-analysis was performed to summarize animal experimental studies on the effect of mesenchymal stromal cells as a luminal additive for nerve grafts in the reconstruction of peripheral nerve defects and results showed a beneficial effect of the MSCs in all studies.[56]

This thesis proposes to advance our line of work towards improvement of functional outcome after peripheral nerve injury by improving the effectiveness of decellularized nerve allografts. While commercially available decellularized nerve allografts are already a reality for small nerve defects, a solution for large mixed nerve defects still remains a big challenge. MSC's have been a hype over the years; can MSC seeded decellularized nerve allografts improve functional outcome of peripheral nerve reconstruction by turning hype and hope to reality?

AIM AND OUTLINE OF THIS THESIS

The overarching goal of this thesis is to further improve outcomes after nerve reconstruction by individualizing nerve allograft repair with the addition of adipose-derived MSCs. The aim of the first part was to investigate the clinical problem. In **chapter 2**, an evidence-based overview of the effectiveness of nerve conduits and allografts in motor and mixed sensory/motor nerve reconstruction is provided. In **chapter 3**, the outcomes of digital nerve gap reconstruction with the NeuraGen type 1 collagen nerve conduit and the Avance Nerve Graft are reported in a retrospective observational study.

The second part of this thesis focuses on the addition of adipose derived MSCs to decellularized nerve allografts and the in-vitro characteristics on human tissue, as well as the in-vivo characteristics in a rat-model. An adequate, reliable and validated cell seeding technique is an essential step for evaluating the translational utility of MSC-enhanced decellularized nerve grafts. Therefore in **chapter 4**, a new method to effectively seed decellularized nerve allografts with MSCs is described and validated. To understand how the functions of MSCs can be leveraged for peripheral nerve repair, in **chapter 5**, we investigated whether interactions of MSCs with decellularized nerve allografts can improve mRNA and protein expression of growth factors that may support nerve regeneration. After in-vitro testing, the MSC seeded nerve allograft was implemented in a rat model. As there is a paucity of information regarding the ultimate survivorship of implanted MSCs or if these cells remain where they are placed, in **chapter 6**, the in-vivo distribution and survival

of MSCs seeded on a decellularized nerve allograft was tracked using luciferase based bioluminescent imaging (BLI). In **chapter 7**, the molecular mechanisms underlying nerve repair by a decelullarized nerve allograft preseeded with autologous, undifferentiated, adipose derived MSCs are studied and compared to the unseeded allograft and autograft nerve.

In the general discussion (**chapter 8**) the results of this thesis are put into a broader perspective and compared to other recent publications. Furthermore, the implications of this research for future perspectives are discussed. In **chapter 9**, a summary in English and Dutch is provided.

REFERENCES

1. 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.
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. Robinson LR. Traumatic injury to peripheral nerves. *Muscle Nerve.* 2000;23(6):863-873.
4. Watson JC, Dyck PJ. Peripheral Neuropathy: A Practical Approach to Diagnosis and Symptom Management. *Mayo Clin Proc.* 2015;90(7):940-951.
5. Horner PJ, Gage FH. Regenerating the damaged central nervous system. *Nature.* 2000;407(6807):963-970.
6. Houdek MT, Shin AY. Management and complications of traumatic peripheral nerve injuries. *Hand Clin.* 2015;31(2):151-163.
7. Hall S. The response to injury in the peripheral nervous system. *J Bone Joint Surg Br.* 2005;87(10):1309-1319.
8. Seddon HJ, Medawar PB, Smith H. Rate of regeneration of peripheral nerves in man. *J Physiol.* 1943;102(2):191-215.
9. Sunderland S. A classification of peripheral nerve injuries producing loss of function. *Brain.* 1951;74(4):491-516.
10. Zack-Williams SD, Butler PE, Kalaskar DM. Current progress in use of adipose derived stem cells in peripheral nerve regeneration. *World J Stem Cells.* 2015;7(1):51-64.
11. Deumens R, Bozkurt A, Meek MF, et al. Repairing injured peripheral nerves: Bridging the gap. *Prog Neurobiol.* 2010;92(3):245-276.
12. Evans GR. Peripheral nerve injury: a review and approach to tissue engineered constructs. *Anat Rec.* 2001;263(4):396-404.
13. Walsh S, Midha R. Practical considerations concerning the use of stem cells for peripheral nerve repair. *Neurosurg Focus.* 2009;26(2):E2.
14. Dong MM, Yi TH. Stem cell and peripheral nerve injury and repair. *Facial Plast Surg.* 2010;26(5):421-427.
15. Rivlin M, Sheikh E, Isaac R, Beredjiklian PK. The role of nerve allografts and conduits for nerve injuries. *Hand Clin.* 2010;26(3):435-446, viii.
16. 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.
17. Lin MY, Manzano G, Gupta R. Nerve allografts and conduits in peripheral nerve repair. *Hand Clin.* 2013;29(3):331-348.
18. Rinker B, Vyas KS. Clinical applications of autografts, conduits, and allografts in repair of nerve defects in the hand: current guidelines. *Clin Plast Surg.* 2014;41(3):533-550.
19. Yuksel F, Kislaoglu E, Durak N, Ucar C, Karacaoglu E. Prevention of painful neuromas by epineural ligatures, flaps and grafts. *Br J Plast Surg.* 1997;50(3):182-185.
20. Busuttil F, Rahim AA, Phillips JB. Combining Gene and Stem Cell Therapy for Peripheral Nerve Tissue Engineering. *Stem Cells Dev.* 2017;26(4):231-238.
21. Konofaos P, Ver Halen JP. Nerve repair by means of tubulization: past, present, future. *J Reconstr Microsurg.* 2013;29(3):149-164.
22. Koehler PJ, Lanska DJ. Mitchell's influence on European studies of peripheral nerve injuries during World War I. *J Hist Neurosci.* 2004;13(4):326-335.
23. Millesi H. Microsurgery of peripheral nerves. *Hand.* 1973;5(2):157-160.
24. Millesi H. Bridging defects: autologous nerve grafts. *Acta Neurochir Suppl.* 2007;100:37-38.
25. Siemionow M, Brzezicki G. Chapter 8: Current techniques and concepts in peripheral nerve repair. *Int Rev Neurobiol.* 2009;87:141-172.
26. 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.
27. Lundborg G, Dahlin LB, Danielsen NP, Hansson HA, Larsson K. Reorganization and orientation of regenerating nerve fibres, perineurium, and epineurium in preformed mesothelial tubes - an experimental study on the sciatic nerve of rats. *J Neurosci Res.* 1981;6(3):265-281.
28. Lundborg G, Dahlin L, Dohi D, Kanje M, Terada N. A new type of "bioartificial" nerve graft for bridging extended defects in nerves. *J Hand Surg Br.* 1997;22(3):299-303.
29. 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.
30. 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.
31. Isaacs J, Browne T. Overcoming short gaps in peripheral nerve repair: conduits and human acellular nerve allograft. *Hand (N Y).* 2014;9(2):131-137.
32. E. A. Einige operationen an ner-ven. *Wien Med Presse.* 1885;26:1285.
33. Mackinnon SE, Doolabh VB, Novak CB, Trulock EP. Clinical outcome following nerve allograft transplantation. *Plast Reconstr Surg.* 2001;107(6):1419-1429.
34. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. *Tissue Engineering.* 2004;10(9-10):1346-1358.

35. Hudson TW, Zawko S, Deister C, et al. Optimized acellular nerve graft is immunologically tolerated and supports regeneration. *Tissue Eng.* 2004;10(11-12):1641-1651.
36. Griffin JW, Hogan MV, Chhabra AB, Deal DN. Peripheral nerve repair and reconstruction. *J Bone Joint Surg Am.* 2013;95(23):2144-2151.
37. 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.
38. Safa B, Shores JT, Ingari JV, et al. Recovery of Motor Function after Mixed and Motor Nerve Repair with Processed Nerve Allograft. *Plast Reconstr Surg Glob Open.* 2019;7(3):e2163.
39. 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.
40. 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.
41. 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.
42. Pfister BJ, Gordon T, Loverde JR, Kochar AS, Mackinnon SE, Cullen DK. Biomedical engineering strategies for peripheral nerve repair: surgical applications, state of the art, and future challenges. *Crit Rev Biomed Eng.* 2011;39(2):81-124.
43. 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.
44. 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.
45. 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.
46. Faroni A, Terenghi G, Reid AJ. Adipose-derived stem cells and nerve regeneration: promises and pitfalls. *International review of neurobiology.* 2013;108:121-136.
47. 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.
48. Jiang L, Jones S, Jia X. Stem Cell Transplantation for Peripheral Nerve Regeneration: Current Options and Opportunities. *Int J Mol Sci.* 2017;18(1).
49. 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.

50. 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.
51. Tedder ME, Simionescu A, Chen J, Liao J, Simionescu DT. Assembly and testing of stem cell-seeded layered collagen constructs for heart valve tissue engineering. *Tissue Eng Part A.* 2011;17(1-2):25-36.
52. Utomo L, Pleumeekers MM, Nimeskern L, et al. Preparation and characterization of a decellularized cartilage scaffold for ear cartilage reconstruction. *Biomed Mater.* 2015;10(1):015010.
53. 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.
54. Hundepool CA, Bulstra LF, Kotsougiani D, et al. Comparable functional motor outcomes after repair of peripheral nerve injury with an elastase-processed allograft in a rat sciatic nerve model. *Microsurgery.* 2018;38(7):772-779.
55. 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.
56. 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.

PART I

Clinical use of nerve graft substitutes

CHAPTER 2

The role of nerve graft substitutes in motor and mixed motor/sensory peripheral nerve injuries

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ABSTRACT

Alternatives to nerve autograft have been invented and approved for clinical use. The reported outcomes of these alternatives in mixed motor nerve repair in humans are scarce and marked by wide variabilities. The purpose of our Current Concepts review is to provide an evidence-based overview of the effectiveness of nerve conduits and allografts in motor and mixed sensory/ motor nerve reconstruction. Nerve graft substitutes have good outcomes in mixed/motor nerves in gaps less than 6 mm and internal diameters between 3 and 7 mm. There is insufficient evidence for their use in larger-gap and -diameter nerves; the evidence remains that major segmental motor or mixed nerve injury is optimally treated with a cabled nerve autograft.

INTRODUCTION

The gold standard treatment for reconstruction of a motor or mixed sensory/motor peripheral segmental nerve defect is autologous sensory cable nerve grafting.[1] Apart from the well-known drawbacks of this technique, such as donor site morbidity and a limited availability of donor nerves, the functional outcome has not been consistently successful, especially in terms of mixed sensorimotor nerve function.[2]

The repair of motor and mixed nerves presents additional challenges secondary to the need for precise identification of the fascicles. As Brushart's experiments[3-5] have demonstrated, motor pathways differ from sensory pathways and there is fundamental evidence that a pure sensory nerve graft is more effective in promoting sensory rather than motor axon regeneration.

Occasionally, a patient objects to autologous nerve grafting or there are no available donor nerves to be used; in these instances, nerve conduits could provide a readily available and relatively unlimited supply, offering an alternative to the nerve autograft. Nerve conduits made from various materials have been used since the late 1980s. They provide a protective environment that serves as a physical barrier to isolate the injured nerve from the surrounding tissues and also provide an enclosed chamber for the diffusion of neurotrophic factors released by the nerve ends.[6]

The first type 1 collagen nerve conduit approved by the U.S. Federal Drug Administration (FDA) in 2001 was NeuraGen (Integra Life Sciences, Plainsboro, NJ).[7] Two additional FDA-approved synthetic conduits: polyglycolic acid (PGA) and polylactide-caprolactone, subsequently became commercially available for nerve repair.[8] The first human nerve allograft transplantation was reported in 1878. However, rejection has been one of the major adverse effects in these early reports and immunosuppressive medications were required.[9] To overcome the disadvantage of immunosuppression, several authors have evaluated processing techniques including radiation, freeze-drying, and chemical techniques. Hudson et al[10] and Sondell et al[11] improved a chemical decellularization treatment in the late 1990s that removes myelin and Schwann cells while leaving the basal lamina tubes intact. Their research resulted in the development of the only commercially available decellularized human nerve allograft (Avance Nerve Graft; Axogen, Inc., Alachua, FL), which was approved for clinical use in 2007.

Bioabsorbable nerve conduits and decellularized allografts have been extensively studied for sensory nerve repairs and have demonstrated improved sensory recovery compared with direct nerve repair or nerve graft in several studies.[12-17] The data on the use of conduits/ allografts for mixed sensory/motor or pure motor nerve repair in humans, however, are scarce, consisting mainly of case reports and are marked by wide discrepancies and bias. Nerve conduits have been shown to be effective for mixed motor-sensory nerves in rat and monkey models of nerve repair,[18-20] but the translation from rat to human has been under debate because of the strong regenerative potential of the rat, which is in sharp contrast to the human patients, who often have major comorbidities or concomitant injuries.[21] In recent years, clinical reports have described both successful and failed motor reinnervation with bioabsorbable nerve conduits in the upper limb[22-24]; These inconsistent results have limited the current application to noncritical small-diameter sensory nerve defects of less than 3 cm.[25]

MATERIALS AND METHODS

Data collection

At present, there are no evidence-based guidelines that are applicable regarding the use of conduits/ allografts versus cabled autograft for the reconstruction of major motor or mixed peripheral nerve gaps. The question surgeons have is should an autologous nerve graft be harvested or should a nerve conduit or decellularized allograft nerve be used in these cases? We provide an evidence-based overview of the effectiveness of nerve

conduits and allografts in motor or mixed sensorimotor nerve reconstruction and define their role in current practice to assemble this Current Concepts article.

To guide this review, the authors applied the PRISMA (Preferred Reporting Items for Systematic reviews and Meta-analysis) Statement as a methodology.[26] All study types were considered and we took note of nerve and conduit type, defect length, and follow-up period. Participants of all ages were included and any type of FDA-approved synthetic conduit or allograft was included. Biological conduits such as autologous vein reconstructions were excluded. A MEDLINE literature search was performed for results of nerve grafting in the treatment of peripheral nerve injuries from 1978 to 2016. A restriction to the English language was applied and only clinical human studies were searched.

Reviewers selected the studies for inclusion, assessed methodological quality and validity, and extracted data. Titles and abstracts were screened to select potentially relevant studies. Full-text articles of the remaining studies were assessed for eligibility. Furthermore, studies were stratified according to whether the nerves reported were sensory, motor, or mixed nerves; only reports on motor or mixed nerves were included in the analysis. The quality of the evidence was assessed by the Oxford Centre for Evidence-Based Medicine Levels of Evidence.[27] Summary tables with descriptive comparisons are presented with primary study results. A quantitative analysis of the studies, however, was not possible because of diverse outcome measures and differences in study design.

The primary outcome was improvement in motor function; secondary outcome measurements were sensory recovery and complication rate. Given variations in reporting sensory and motor function, results as presented in the reviewed articles are reported as well as a standardized meaningful recovery when available (S3-4 or M3-5) on the Medical Research Council (MRC) scale.

RESULTS

Search results

The initial search in the databases identified a total of 1201 studies related to nerve grafting: 505 were potentially relevant based on the title and abstract; 47 full text reports were obtained from this subset for further examination and most studies were excluded because they did not focus on mixed/motor nerves. After full-text reading, only 21 clinical studies in peer-reviewed journals assessed the results of nerve conduits/allografts in

mixed or motor peripheral nerve injuries. Of the 15 clinical reports describing motor nerve reconstruction using synthetic conduits, 3 were case reports, 10 retrospective case series, and 2 prospective randomized controlled trials (Table 1). Of the 6 clinical reports using nerve allografts, 3 were case reports and 3 retrospective case series (Table 2).

Nonbioabsorbable synthetic nerve conduits

Stanec and Stanec[28] were one of the first to report their results on polytetrafluoroethylene tubes in the repair of median and ulnar nerves: 78.6% meaningful recovery was demonstrated in injuries with gaps from 1.5 to 4 cm, but for the larger gap lengths only 13.3% resulted in useful reinnervation. Braga-Silva[29] reconstructed 26 median and ulnar nerve injuries with a silicone tube. The technique was effective in the repair of gaps up to 3 cm, with better results in the ulnar nerves than in the median nerves. In 2004, excellent results were obtained by Lundborg et al[30] with the use of silicone tubes for median and ulnar nerve gaps (3e5 mm). In a prospective randomized study, comprising 30 patients, long-term outcome from silicone tube nerve repair was compared with routine microsurgical repair. No significant difference between the 2 techniques was found. In 8 of the 30 cases, a secondary surgical procedure was necessary to remove the tube owing to local discomfort; therefore, it was not recommended in favor of conventional reconstruction.[30]

Bioabsorbable synthetic nerve conduits

One year later, Ducic et al[31] reconstructed 2 cases of spinal accessory nerve injury, 1 with an autograft and 1 with a bioabsorbable conduit, the Neurotube (PGA). The conduit reconstruction reached M5 trapezius function by 3 months whereas the autograft reached M4 function by 6 months after reconstruction and had persistent donor site morbidity.[31] Ashley et al[32] performed a retrospective analysis of 5 patients who underwent Neurogen placement for birth-related brachial plexus palsy with a mean gap of 2 cm. Three patients exhibited a good recovery after 2 years of follow-up, 1 patient showed remarkable functional improvement, and 1 other patient did not recover at all. Donoghoe et al[33] reconstructed 2 median nerve gaps of 3-cm long with 4 separate 2.3-mm diameter Neurotubes (PGA). Each patient recovered 2 point discrimination by 2 years and both patients recovered abductor pollicis brevis motor function measured on electrodiagnostic study and/or pinch strength. Rosson et al[22] reviewed in 2009 the results of motor nerve reconstruction with PGA conduits. Average gap length was 2.8 cm and all patients had some return of motor function rated as M3. Two of the 6 patients, however, had tendon transfers to improve grip and pinch.

Up to the year 2008, reports have been mainly positive. However, in 2009, Moore et al[24] reported on 4 failed cases that underwent repair of large-diameter nerves with collagen and PGA-conduits. No functional motor recovery was noted and all patients experienced complications as neuroma pain.

In 2010, Wangensteen and Kallianen[8] published a case series of 126 patients receiving NeuraGen conduits. The series consisted mainly of sensory digital nerves, but included 21 large-caliber nerves. Overall, repair of these nerve defects led to recovery of nerve function in 43% on quantitative or qualitative evaluation. Gu et al[34] described a successful case in which a 30-mm-long median nerve defect was reconstructed with a PGA-nerve conduit. Motor function was recovered to M4 and sensory function had recovered to S3+ measured by static 2-point discrimination. Kuffler et al[35] enriched a collagen tube with autologous platelet-rich fibrin to repair a 12-cm ulnar nerve gap 3.25 years after injury. M4-5 motor function and appropriate vibration sensitivity was reached. On the contrary, Chiriac et al[36] reported less-positive results on the polylactide-caprolactone tube (Neurolac) in a series of 28 nerves. With an average gap length of 11 mm, 17 of the 28 cases had no recovery of sensation (61%) and 3 cases showed no progression of Tinel sign. Eight complications were observed, of which the most serious consisted of fistulizations and neuromas. Based on these results, the Neurolac was not recommended in hand nerve defect reconstructions.[36] A prospective clinical study on ulnar and median nerve repair by Boeckstyns et al[37] found no differences between collagen conduit repair and conventional microsurgical techniques in gaps of 6 mm or less. Dienstknecht et al[23] repaired traumatic median nerve lacerations of 1 to 2 cm in 9 patients with collagen conduits. Motor function recovered to M3 in 2 patients, M4 in 4, and M5 in 2 patients, measured by the Manual Muscle Strength Testing system. Recently, Liodaki et al[38] described 4 failed cases of upper extremity nerves, with gaps between 1 and 3 cm reconstructed by the NeuraGen tube. The authors did not seek to explain the clinical outcome but focused on the histological findings; histology showed characteristics of a scar neuroma without any signs of foreign body reaction in 3 cases and minimal foreign body in 1 case.

Nerve allografts

Mackinnon and Hudson[39] reported in 1992 on one of the first large mixed nerve allograft reconstructions. A 23-cm proximal complete sciatic nerve injury was repaired by a 10-cable nerve allograft procedure followed by immunosuppression. Protective sensibility was present after 18 months but there was no evidence of motor recovery. In 1996, Mackinnon[40] reported the successful recovery of (abnormal) sensibility across 8 donor nerve allografts,

bridging a 20-cm posterior tibial nerve defect. In 2001, the same author and colleagues[41] presented their results in 7 cases with a mean total defect length of 22 cm. One patient had rejection of the allograft and 3 patients regained motor nerve function.

Decellularized nerve allografts

As the decellularized nerve allograft became commercially available in 2007, the Multicenter Retrospective Study of Avance Nerve Graft Utilization, Evaluations and Outcomes in Peripheral Nerve Injury Repair (RANGER study) was initiated to analyze functional outcomes for upper extremity nerve repairs. The registry, sponsored by Axogen, Inc., included 12 study centers and most clinical data on nerve allografts have been published by these participating centers. Between 2001 and 2012, the majority of articles have been published about the use of nerve allografts for sensory nerve reconstruction.[15] In 2012, Brooks et al[42] reported on the safety and functional outcomes for 76 repairs (49 sensory, 18 mixed, and 9 motor nerves). Even though not all subjects had adequate follow-up, subgroup analysis was performed to determine the relationship to factors known to influence outcomes of nerve repair. A 100% meaningful recovery (S3-M3) was found in the 12 nerves of the short-gap group (5-14 mm). Meaningful recovery was seen in 77% of the 13 mixed nerves and 86% of the 7 motor nerves. Specifically, reinnervation based on electromyography alone was seen in 3 motor nerves with gaps of 12, 15, and 40 mm. Squintani et al[43] assessed the outcome of cryopreserved allografts in brachial plexus stretch injuries and compared direct neurotization with graft repair with nerve transfers with interposition of allografts. All patients had regained motor function equal to or greater than M3 at 2-year follow-up. Not all patients, however, presented with a complete brachial plexus lesion. Berrocal et al[44] bridged a 1.7-cm ulnar defect with an Avance nerve graft where 8 months after repair, no clinical or electromyography (EMG) evidence of reinnervation was present.

Table 1. Nerve conduits

Author	Conduit type	Nerve type	Mean age (range) (years)	Mean defect length (range) (cm)	Mean follow-up (months)	Movement scale	Sensory function (reported)	Motor function (reported)	Standardized meaningful recovery *	Complications	Level of evidence
Stanec [28]	ePTFE	Median + ulnar	30 (9-56)	1.5–6	30.4	- SMF - 2PD - Grip and pinch - MRC	-	-	78.6% of group 1, 13.3% in group 2	1 revision due to discomfort	Case-series
Braga-Silva [29]	Silicone	Median + ulnar	23 (18-26)	3 (2-5)	30	- Chanson et al. classification	-	-	Effective in gaps of up to 3 cm, better results in ulnar than median nerves	7 tubes removed due to discomfort	Case-series
Lundborg [30]	Silicone	Median + ulnar	33 (12-72)	0.3-0.5	60	- SMF - Grip - MRC	6 conventional, 9 tubular repairs reached >S3	-	No statistical significant differences	8 tubes removed due to discomfort	Randomised prospective study
Ducic [31]	Neurotube	SAN	52 (40-63)	1.5 and 2.2	9	- EMG		Autograft M4, Neurotube M5	Autograft recovered upper trapezius and partial lower trapezius. Tube recovered full shoulder abduction	-	Case-report
Ashley [32]	Neurogen	C5, C6	0.7	2	25	- MSC	-	1 of the 5 Good recovery MSC> 0.6, 3, excellent recovery (MSC >0.75) and 1 no recovery	80% good functional recovery	-	Case-series
Donoghoe [33]	Neurotube	Median	43 and 61	3	60	- 2PD - EMG - Physical testing	Both recovered 2PD	-Recovered abductor pollicis brevis function	-	-	Case-report
Dellon [22]	Neurotube	SAN, median, ulnar	47 (9-61)	2.8 (1.5-4)	39	- Strength - EMG	-	All some return of motor function, M3 or greater	100%	-	Case-series
Mackinnon [34]	NeuraGen, Neurotube	Median, ulnar, C5-6 roots	16 (0.25-43)	2.5	26	-2PD -EMG	Absent	Absent	Failed outcome	Neuroma , early conduit degradation	Case-series
Wangensteen [8]	NeuraGen	Multiple (digital + larger diameter nerves)	33 (7-79)	1.3 (0.3-2)	11	- 2PD - SWF - EMG - Subjective	35-45% sensory recovery	33% recovery on EMG	43% overall postoperative improvement	13% revisions	Case-series
Gu [35]	PGA	Median	55	0.3	36	- 2PD - MRC - CMAP	SMF 4.56, 2PD14, 9 and 9mm in thumb, index and middle fingers	APB recovered to M4. Grip and pinch strength 93.3% and 83.% of contralateral side	Sensory recovery to S3+ and motor recovery M4	-	Case-report

Table 1. Nerve conduits (continued)

Author	Conduit type	Nerve type	Mean age (range) (years)	Mean defect length (range) (cm)	Mean follow-up (months)	Movement scale	Sensory function (reported)	Motor function (reported)	Standardized meaningful recovery *	Complications	Level of evidence
Kuffler [36]	Collagen tube + platelet rich fibrin	Ulnar	48	12	36	- Vibrations - MRC scale - Subjective	Vibration sensitivity in small and ring finger	MRC 5, extrinsic muscles M4. Intrinsic muscles M0	Good motor function (M4-5) and vibration sensitivity	-	Case-report
Chiriac [37]	Neurolac	Multiple (digital + larger diameter nerves)	39	11 (0.2-2.5)	21.9	- DASH - Strength - 2PD - SWF	46.92 on SWF difference between two sides	Grip strength 64.62% of contralateral side	17 of 28 cases clinical failure	8 complications (fistula, neuroma)	Case-series
Boeckstyns [38]	NeuraGen	Median + ulnar	36 (21-66)	0.6 or less	24	- CMAP - Rosen scoring system	CMAP recovered	CMAP recovered to 50% of control side	No differences after 24 months.	7 of 17 conduits removed due to local discomfort	Randomized prospective study
Dienstknecht [39]	NeuroGen	Median nerve	25 (10–41)	1-2	21	- 2PD - Electrophysology - DASH	S2PD <3 in 3 patients, 6-10 in 4 patients and > 10 mm in 2 patients	M4 or higher in 6 patients	M4 or higher in 6 patients, 8 out of 9 satisfied	-	Case-series
Liodaki [40]	NeuraGen	Digital, median, radial, ulnar	14–50	1-3	6–17	-	Persisting pain and loss of sensation	No motor recovery	Failed outcome		Case-series

SWF = Semmes Weinstein Filament testing
2PD = 2 Point Discrimination
MRC = Medical Research Council Classification scale for muscle strength
APB = Abductor Pollicis Brevis muscle

* = Meaningful recovery = S3-S4 or M3-M5 on MRC scale

Table 2. Nerve allografts

Author	Graft type	Nerve type	Mean age (range) (years)	Mean defect length (cm)	Mean follow-up (months)	Movement scale	Sensory function (reported)	Motor function (reported)	Standardized meaningful recovery *	Complications	Level of evidence
Mackinnon [41]	Donor nerve	Sciatic	8	23	45	- Vibration - SWF - 2PD	SWF abnormal, no 2PD, protective sensibility	No motor recovery	-	-	Case-report
Mackinnon [42]	Donor nerve	Tibial	12	20	24	- Vibration - SWF - 2PD	SWF + 2PD present but abnormal	Intact motor branch	-	-	Case-report
Mackinnon [43]	Donor nerve	Sciatic, tibial, median. Ulnar, radial	3-24	21	29	- Vibration - SWF	6 out of 7 patients recovered light-touch sensation and protective sensation	3 of 7 patients regained motor function	-	1 allograft rejected	Case-series
Brooks [44]	AxoGen	Multiple (digital + larger diameter nerves)	41 ± 17 (18-86)	27 ± 14 (0.5-5)	264 ± 152	- 2PD - SWF - ROM - MRC	Static 2PD had an average score of 8 mm (4-15mm). Moving 2PD was 8mm (4-15mm). SWF with return to diminished light touch or better reported in 13 or 17 nerve repairs.	Return of meaningful motor function was observed at the level of M4-M5 in 9 of the cases and M3 in 6 cases.	Meaningful recovery sensory 88.6%, Mixed 77.0% and motor 85.7%.	No graft related adverse experiences	Case-series
Squintani [45]	Donor nerve	Brachial plexus	18-58	6.2 (4-10)	24	- Mackinnon Modified MRC	-	100% >M3	All of the patient regained motor function M3 or greater	-	Case-series
Berrocal [46]	AxoGen	Ulnar	20	1.7	8	- Grip strength - EMG	Absent sensation hypothenar and medial aspect of ring and small finger, tingling and numbness	Grip weakness, progressive clawing of the left hand	Absence of evidence of reinnervation	-	Case-report

SWF = Semmes Weinstein Filament testing
2PD = 2 Point Discrimination
MRC = Medical Research Council scale for muscle strength

* = Meaningful recovery = S3-S4 or M3-M5 on MRCC scale

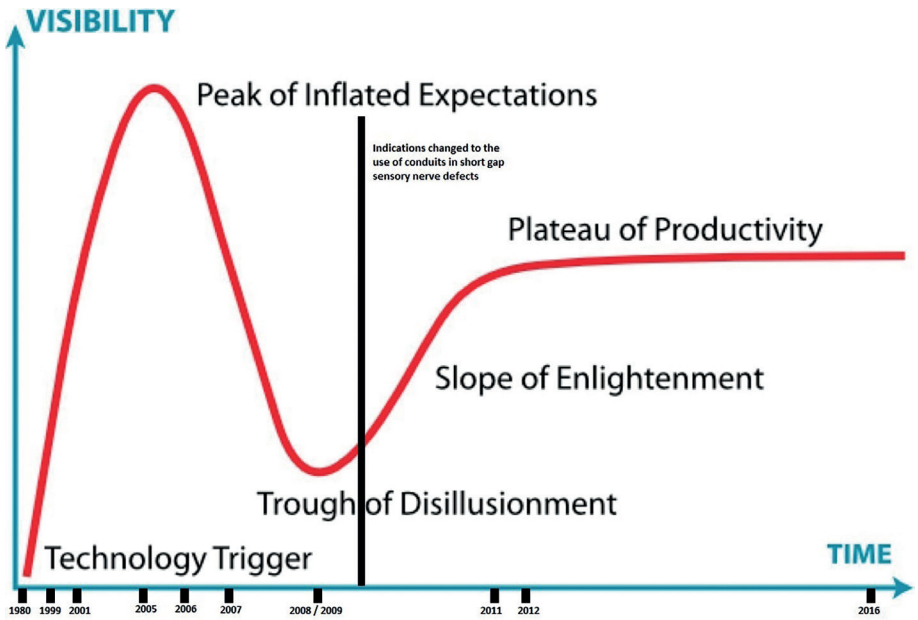

Figure 1. Gartner Hype Cycle interprets technology hype. (Reproduced with permission from Gartner Inc. Hype Cycle Research Methodology. Available at: www.gartner.com. Accessed August 17, 2016.[45]).

DISCUSSION

The evolution of nerve conduits has been the subject of experimental and clinical research over the past 2 decades. As with many technologies in medicine, the initial introduction of the conduit was promoted intensively. This evolution can be best described by the Gartner Hype Cycle (Fig. 1) which interprets technology hype.[45] Early rapid adoption of a new treatment started with a technology trigger in the 1980s, after which, early publicity produced a number of success stories but, in this peak of inflated expectations, the product did not really deliver what it promised. As more clinical failures were being recognized, the trough of disillusionment phase began in 2008, and evaluation of failed cases forced the refinement of indications for use of nerve graft substitutes. Finally, a plateau of productivity was reached when indications had been changed to the use of conduits in short-gap sensory nerve defects.

In reviewing the literature of conduits, it is difficult to make evidence-based conclusions secondary to the large number of variables. This is further confounded by the type of nerve reconstructed. The ability to compare studies is limited and it remains difficult to interpret the role of nerve conduits for mixed/motor nerves.

Summarizing the key findings, the best evidence for nerve conduits currently comes from the only 2 prospective randomized controlled trials that evaluated nerve gaps less than 6 mm long with diameters between 3.0 and 7.0mm, both of which reported excellent results for median and ulnar nerves.[30,37] However, Rosson et al[22] would consider the reconstruction of a defect size smaller than 5mm a conduit-assisted coaptation and did not include these patients in their analysis. The key takehome point is that there is insufficient high-quality evidence for the use of nerve conduits in larger-gap motor or mixed sensory/motor nerves.

When evaluating processed nerve allografts, a complicating factor is the variations of the clinically available nerve allograft studied by many authors. The best evidence for the only commercially available decellularized human nerve allograft is the industry sponsored RANGER study.[46] In this study, 18 mixed and 9 motor nerves were reconstructed with a mean gap of 29 mm. Meaningful recovery was seen in 77% of the mixed nerves and 86% of the motor nerves. The major weaknesses in this study include the small (mixed/motor nerve) sample size, its retrospective design, the differences in postoperative care, lack of quantitative measurements in 28% of the sample, and absence of a control group. In addition, they reported several MRC grade V outcomes after nerve reconstruction, which has demonstrated to be very difficult if not impossible to obtain.[46] No conclusions could be made regarding the correlation between outcome and gap length, patient age, or nerve type in the RANGER study, despite other studies that have highlighted the importance of these factors with respect to outcome. These discrepancies highlight the need for a prospective randomized study to evaluate the role of processed nerve allografts versus autograft in motor or mixed nerves.

In reviewing the literature, the limitations of the studies become evident. Outcome measurements are highly inconsistent and there is no objective measurement of motor strength. The use of the MRC grading, although highly used, is severely flawed. Modifications of the MRC grading system are widespread and differ from institution to institution. Grade IV encompasses nearly 80% of the spectrum and is often not compared with the contralateral side.[47] In a study evaluating the MRC scale to objective torque testing of the reconstructed musculocutaneous nerves, Shahgohli et al[46] demonstrated

the inconsistency of senior surgeons to grade biceps motor recovery. In fact, grade V outcomes were never achieved by torque test comparisons with the normal side, despite grade V outcomes by senior surgeon evaluation. The other glaring inconsistency is the critical misunderstanding of reinnervation times. Several studies report proximal nerve reconstruction at follow-up periods inconsistent with reinnervation physiology. In addition, there are no presurgery examinations that evaluate for anomalous innervations (such as Martin-Gruber anastomosis). Finally, EMG evidence of reinnervation is often reported. However, it is important to understand that EMG evidence of reinnervation does not imply successful motor outcomes.[48] It is important that EMG evidence of reinnervation be carefully followed up with clinical motor examination at the appropriate time from surgery.

In conclusion, both the nerve conduit and the allograft have demonstrated good outcomes in mixed/motor nerves with gap lengths of less than 6 mm and diameters between 3 and 7 mm. The evidence remains that major segmental motor or mixed motor/sensory nerve injury is optimally treated with a cabled nerve autograft. There is insufficient evidence for the use of conduits or allograft nerves in larger-gap and –diameter nerves and there are no clinical studies that directly compare nerve allografts and biological conduits with cabled nerve autograft. If we rely on basic science literature; the autograft remains statistically superior in all studies, but animal data do seem to show evidence for a superiority of allografts to collagen nerve conduits.[49,50] Future prospective randomized studies to compare allografts with nerve conduits would further define the exact value of these bridging tools in segmental motor and mixed nerve injuries.

REFERENCES

1. Siemionow M, Brzezicki G. Chapter 8: Current techniques and concepts in peripheral nerve repair. Int Rev Neurobiol 2009;87:141-72.
2. Seidel JA, Koenig R, Antoniadis G, Richter HP, Kretschmer T. Surgical treatment of traumatic peroneal nerve lesions. Neurosurgery 2008;62-3:664-73; discussion -73.
3. Brushart TM. Preferential reinnervation of motor nerves by regenerating motor axons. J Neurosci 1988;8-3:1026-31.
4. Brushart TM. Motor axons preferentially reinnervate motor pathways. J Neurosci 1993;13-6:2730-8.
5. Brushart TM, Seiler WAt. Selective reinnervation of distal motor stumps by peripheral motor axons. Exp Neurol 1987;97-2:289-300.
6. Belkas JS, Shoichet MS, Midha R. Peripheral nerve regeneration through guidance tubes. Neurological Research 2004;26-2:151-60.
7. Dellon AL, Mackinnon SE. An alternative to the classical nerve graft for the management of the short nerve gap. Plast Reconstr Surg 1988;82-5:849-56.
8. Wangensteen KJ, Kalliainen LK. Collagen tube conduits in peripheral nerve repair: a retrospective analysis. Hand 2010;5-3:273-7.
9. Albert E. Berichte des Naturwissenschaftlich-medizins in Innsbruck. 1878;Vol. 9, 1878 87. .
10. 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;10-11-12:1641-51.
11. Sondell M, Lundborg G, Kanje M. Regeneration of the rat sciatic nerve into allografts made acellular through chemical extraction. Brain Res 1998;795-1-2:44-54.
12. Weber RA, Breidenbach WC, Brown RE, Jabaley ME, Mass DP. A randomized prospective study of polyglycolic acid conduits for digital nerve reconstruction in humans. Plastic & Reconstructive Surgery 2000;106-5:1036-45; discussion 46-8.
13. Bertleff MJ, Meek MF, Nicolai JP. A prospective clinical evaluation of biodegradable neurolac nerve guides for sensory nerve repair in the hand. J Hand Surg Am 2005;30-3:513-8.
14. Taras JS, Amin N, Patel N, McCabe LA. Allograft nerve reconstruction for digital nerve loss. Journal of Hand Surgery 2013;1):e27.
15. 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 2009;4-3:245-9.
16. Lohmeyer JA, Siemers F, Machens HG, Mailander P. The clinical use of artificial nerve conduits for digital nerve repair: a prospective cohort study and literature review. J Reconstr Microsurg 2009;25-1:55-61.

17. Haug A, Bartels A, Kotas J, Kunesch E. Sensory recovery 1 year after bridging digital nerve defects with collagen tubes. Journal of Hand Surgery - American Volume 2013;38-1:90-7.
18. 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. J Bone Joint Surg Am 2009;91-9:2194-204.
19. Mackinnon SE, Dellon AL. A study of nerve regeneration across synthetic (Maxon) and biologic (collagen) nerve conduits for nerve gaps up to 5 cm in the primate. J Reconstr Microsurg 1990;6-2:117-21.
20. Archibald SJ, Shefner J, Krarup C, Madison RD. Monkey median nerve repaired by nerve graft or collagen nerve guide tube. J Neurosci 1995;15-5 Pt 2:4109-23.
21. Kaplan HM, Mishra P, Kohn J. The overwhelming use of rat models in nerve regeneration research may compromise designs of nerve guidance conduits for humans. Journal of Materials Science-Materials in Medicine 2015;26-8:226.
22. Rosson GD, Williams EH, Dellon AL. Motor nerve regeneration across a conduit. Microsurgery 2009;29-2:107-14.
23. Dienstknecht T, Klein S, Vykoukal J, Gehmert S, Koller M, Gosau M, Prantl L. Type I collagen nerve conduits for median nerve repairs in the forearm. J Hand Surg Am 2013;38-6:1119-24.
24. Moore AM, Kasukurthi R, Magill CK, Farhadi HF, Borschel GH, Mackinnon SE. Limitations of conduits in peripheral nerve repairs. Hand (N Y) 2009;4-2:180-6.
25. Ray WZ, Mackinnon SE. Management of nerve gaps: autografts, allografts, nerve transfers, and end-to-side neurorrhaphy. Exp Neurol 2010;223-1:77-85.
26. Moher D, Liberati A, Tetzlaff J, Altman DG, Group P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Int J Surg 2010;8-5:336-41.
27. Howick J C, et al. . Introduction to the 2011 Oxford Centre for Evidence-Based Medicine (OCEBM) Table of Evidence. Oxford, Oxford Centre for Evidence-Based Medicine. 2011.
28. Stanec S, Stanec Z. Reconstruction of upper-extremity peripheral-nerve injuries with ePTFE conduits. Journal of Reconstructive Microsurgery 1998;14-4:227-32.
29. Braga-Silva J. The use of silicone tubing in the late repair of the median and ulnar nerves in the forearm. Journal of Hand Surgery 1999;24 B-6:703-6.
30. Lundborg G, Rosen B, Dahlin L, Holmberg J, Rosen I. Tubular repair of the median or ulnar nerve in the human forearm: a 5-year follow-up. Journal of Hand Surgery - British Volume 2004;29-2:100-7.
31. Ducic I, Maloney CT, Jr., Dellon AL. Reconstruction of the spinal accessory nerve with autograft or neurotube? Two case reports. Journal of Reconstructive Microsurgery 2005;21-1:29-33; discussion 4.

32. Ashley WW, Jr., Weatherly T, Park TS. Collagen nerve guides for surgical repair of brachial plexus birth injury. Journal of Neurosurgery 2006;105-6 Suppl:452-6.
33. Donoghoe N, Rosson GD, Dellon AL. Reconstruction of the human median nerve in the forearm with the NeurotubeTM. Microsurgery 2007;27-7:595-600.
34. Gu J, Hu W, Deng A, Zhao Q, Lu S, Gu X. Surgical repair of a 30 mm long human median nerve defect in the distal forearm by implantation of a chitosan-PGA nerve guidance conduit. Journal Of Tissue Engineering & Regenerative Medicine 2012;6-2:163-8.
35. Kuffler DP, Reyes O, Sosa IJ, Santiago-Figueroa J. Neurological recovery across a 12-cm-long ulnar nerve gap repaired 3.25 years post trauma: Case report. Neurosurgery 2011;69-6:E1321-E6.
36. Chiriac S, Facca S, Diaconu M, Gouzou S, Liverneaux P. Experience of using the bioresorbable copolyester poly(DL-lactide-epsilon-caprolactone) nerve conduit guide NeurolacTM for nerve repair in peripheral nerve defects: report on a series of 28 lesions. Journal of Hand Surgery: European Volume 2012;37-4:342-9.
37. Boeckstyns ME, Sorensen AI, Vineta JF, Rosen B, Navarro X, Archibald SJ, Valss-Sole J, Moldovan M, Krarup C. Collagen conduit versus microsurgical neurorrhaphy: 2-year follow-up of a prospective, blinded clinical and electrophysiological multicenter randomized, controlled trial. J Hand Surg Am 2013;38-12:2405-11.
38. Liodaki E, Bos I, Lohmeyer JA, Senyaman O, Mauss KL, Siemers F, Mailaender P, Stang F. Removal of collagen nerve conduits (NeuraGen) after unsuccessful implantation: focus on histological findings. Journal of Reconstructive Microsurgery 2013;29-8:517-22.
39. Mackinnon SE, Hudson AR. Clinical application of peripheral nerve transplantation. Plastic & Reconstructive Surgery 1992;90-4:695-9.
40. Mackinnon SE. Nerve allotransplantation following severe tibial nerve injury. Case report. J Neurosurg 1996;84-4:671-6.
41. Mackinnon SE, Doolabh VB, Novak CB, Trulock EP. Clinical outcome following nerve allograft transplantation. Plastic & Reconstructive Surgery 2001;107-6:1419-29.
42. 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.
43. Squintani G, Bonetti B, Paolin A, Vici D, Cogliati E, Murer B, Stevanato G. Nerve regeneration across cryopreserved allografts from cadaveric donors: a novel approach for peripheral nerve reconstruction. Journal of Neurosurgery 2013;119-4:907-13.

44. Berrocal YA, Almeida VW, Levi AD. Limitations of nerve repair of segmental defects using acellular conduits. Journal of Neurosurgery 2013;119-3:733-8.
45. Gartner Inc. Gartner Hype Cycle. Available at: http://www.gartner.com/technology/research/methodologies/hype-cycle.jsp. Accessed August 17, 2016.
46. 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):856e862.
47. MacAvoy MC, Green DP. Critical reappraisal of Medical Research Council muscle testing for elbow flexion. J Hand Surg Am. 2007;32(2):149e153.
48. Sammer DM, Kircher MF, Bishop AT, Spinner RJ, Shin AY. Hemicontralateral C7 transfer in traumatic brachial plexus injuries: outcomes and complications. J Bone Joint Surg Am. 2012;94(2):131e137.
49. 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;39-6:787-99.
50. 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;94-5:410-7.
51. Chanson L, Michon J, Merle M, Delagoutte JP. Étude des résultats de la réparation de 85 nerfs dont 49 gros nerfs [in French]. Rev Chir Orthop. 1977;63(supp II):153e160.

CHAPTER 3

Collagen nerve conduits and processed nerve allografts for the reconstruction of digital nerve gaps: a single institution case series and review of the literature

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ABSTRACT

Background

A single institution case series and review of the literature on the outcomes of digital nerve gap reconstruction with the NeuraGen type 1 collagen nerve conduit (Integra Life Sciences, Plainsboro NJ) and the Avance Nerve Graft (Axogen, Inc, Alachua, FL).

Methods

37 patients were included with a minimal follow-up of 12 months. Primary outcome was postoperative sensory recovery measured by static two-point discrimination (S2PD) test or the Semmes-Weinstein monofilament test (SWM). Secondary outcome measurements were peri- or postoperative complications. Final outcome data was stratified to grade results as excellent, good or poor.

Results

The mean nerve gap length was 14 ± 4.9 mm for the collagen conduits versus 18.4 ± 9.3 for nerve allografts. After 12 months, outcomes were graded as excellent sensory recovery in 48% of the collagen conduit repairs and 39% of the nerve allografts (P = 0.608), good in 26% of the conduits and 55% of the allografts (P = 0.074) and poor in 26% of the conduits versus 6% of the allografts (P = 0.091). There was one neuroma and one infection reported. Graft rejection or extrusion was not observed.

Conclusions

Nerve conduits and processed nerve allografts offer convenient off-the-shelf options for digital nerve gap repair. Both techniques offer effective means of reconstructing a digital nerve gap <2.5 cm at a minimum of 12 months of follow-up. Future prospective randomized large sample size studies comparing nerve conduits with allografts are needed to perform subgroup analyses and to define their exact role in digital nerve injuries.

INTRODUCTION

Over the past 20 years, the reconstructive options for peripheral injuries that cannot be approximated by direct tensionless coaptation have expanded. In proximal injuries, nerve transfers are increasingly being performed.[1] However, in distal nerve injuries, interposition nerve grafting remains the most commonly used method.[2] Ten percent of all surgically treated hand injuries are accompanied by nerve injuries of which the digital nerves are most frequently injured.[3,4] Of these, about 18% require nerve grafting.[5] Autologous nerve grafting has historically been the most reliable method (the gold standard), but the procedure requires an additional operation and there is significant associated donor site morbidity.[6]

Current commercially available alternatives are biodegradable nerve conduits or processed/decellularized human nerve allografts. Conduits provide a protective semipermeable (absorbable) hollow environment to isolate the nerve from the surrounding tissue allowing for the collection of fibrin to serve as a scaffold for axonal regeneration toward the distal stump.[7] Processed nerve allografts consist of decellularized human nerves, maintaining the internal microstructure and extracellular matrix of native nerve tissue.[8]

Both techniques have been extensively studied for purely sensory nerve repairs; several studies have demonstrated high rates of recovery and very few complications or adverse events.[9,10] The majority of the literature supports the use of nerve graft substitutes for noncritical small-diameter sensory nerve defects of less than 3 cm.[11]

More specifically, data from animal studies have been relatively consistent; collagen nerve conduits have been found to be equivalent to direct suture for 5-mm defects, and inferior to autografts for 20-mm mixed motor sensory nerve deficits.[12,13] Processed nerve allografts have been repeatedly found to be similar or inferior to the nerve autograft, but superior to collagen conduits.[8,14-16] One study found that conduits demonstrated an unorganized pattern of mid-graft nerve fibers whereas allografts demonstrated evenly distributed nerve fiber regeneration.[17]

Clinical data, however, does not clearly report on the direct comparison of these nerve graft substitutes and debate exists on the preferred technique to repair digital nerve

defects. To this date, only one single comparative pilot study has concluded that nerve allografts had significantly improved sensory outcomes compared with hollow conduits.[18]

The aim of this study was to report a single institution case series and a review of the literature on the clinical outcomes of digital nerve gap reconstruction with the NeuraGen type 1 collagen nerve conduit (Integra Life Sciences, Plainsboro, NJ) and the Avance Nerve Graft (Axogen, Inc, Alachua, FL) to evaluate for potential differences in recovery outcomes and to define the exact role of both nerve graft substitutes in short gap digital nerve reconstruction.

MATERIALS AND METHODS

Study design and population

This study was designed as a retrospective review of cases of digital nerve injury that underwent nerve reconstruction with either a NeuraGen nerve conduit made from bovine type I collagen (Integra Lifesciences, Plainsboro, NJ) or processed nerve allograft (Avance Nerve Graft, AxoGen Inc, Alachua, FL) at a single tertiary referral trauma center between January 2005 and December 2015. The study was approved by the institutional review board (IRB number 16-002017). Patients were included if they were 18 years or older and had sustained injuries requiring reconstruction of at least one digital nerve that resulted in a nerve gap after resection to healthy nerve. Patients were excluded if the nerve transection was incomplete; the follow-up period was less than 12 months, if there was missing data on the sensory outcome, if repairs were delayed greater than three weeks or chronic or if patients had comorbidities known to affect the growth of neural structures.

Data collection

Data was extracted from the medical records, including age at surgery, body mass index (BMI), gender, the interval between injury and surgery and length and diameter of the used conduit or allograft. Remaining data such as injury-related factors including mechanism of injury, gap length, co-morbidities, smoking status, and concomitant injuries were obtained from the surgical and clinical notes.

Institutional practice

Indications for an acute repair of a digital nerve were a loss of 2PD sensation with or without concomitant injury to other structures. The selection for one technique versus the other was not pre-defined and was at the discretion of the attending hand surgeon.

Surgical technique

In patients with concomitant injury, the vascular, bone or tendon was first repaired. In the allograft group, the injured nerve was resected until healthy fascicles were visualized. The diameter of the injured nerve and the distance between nerve ends was measured by a paper surgical ruler with fingers extended in mm and a nerve allograft was chosen and prepared according to the manufacturer's guidelines. The nerve allograft was thawed in warm sterile saline and cut to bridge the gap. An average of 3 sutures was used for coaptation using 9-0 nylon sutures under an operative microscope. In the conduit group, after resection of proximal and distal nerve endings, the diameter and the distance between nerve ends was measured by a paper surgical ruler with fingers extended and a conduit with a minimally larger diameter and length was chosen to bridge the gap. The conduit was then hydrated in saline for 10 minutes and the nerve ends were placed into the conduit. To prevent blood clots in the conduit, the lumen was rinsed regularly with saline. One coaptation was then sutured to the conduit with a 9-0 nylon suture with a horizontal mattress stitch through the conduit and epineurium. The lumen was rinsed with saline and both nerve ends were secured into the conduit. Postoperatively, patients were immobilized for a minimum of three weeks if only nerve/vessel injury were present. If other tissues (tendon, bone) were repaired, immobilization was at a minimum three weeks and motion was commenced depending on the status of the other tissues injuries (i.e. bone union, tendon injury, etc.).

Outcome measurements

All patients were clinically evaluated by the operating hand surgeon or a certified hand therapist. The primary outcome was postoperative sensory recovery measured by the static two-point discrimination (S2PD) test or the Semmes-Weinstein monofilament test (SWM) (North Coast Medical, Inc, Morgan Hill, CA) [19,20] Secondary outcome measurements were peri or postoperative complications such as infection, extrusion, or graft rejection. The minimal follow-up was 12 months. Final outcome data was categorized according to the Mackinnon and Dellon classification to grade results as excellent, good or poor.[21] Not all patient records reported both a SWM and S2PD score. As it has been previously shown that the results of the Semmes-Weinstein test demonstrated a good correlation with the static 2PD test, these outcomes were both categorized in order to include as many patients as possible.[22,23] An excellent functional result was noted if S2PD was less than or equal to 6 mm and/or SWM showed normal light touch (1.65-2.83). A good functional result was noted if S2PD was between 7 and 15 mm and/or SWM showed diminished light touch (3.22-3.61).

A poor result was noted if S2PD was more than 15 mm and/or SWM diminished protective sensation and loss of protective sensation (3.84-6.65).

Statistical analysis

Descriptive statistics were used to present patient characteristics. Quantitative data were expressed as the mean ± standard deviation. Categorical data were expressed as frequencies and percentages. The Kolmogorov-Smirnoff test was applied to test for a normal distribution. To detect categorical differences between the outcomes of nerve conduit and processed nerve allograft reconstruction after 12 months, the data was analyzed using the Chi-Squared test. Numerical data was analyzed by the Mann-Whitney U test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

A total of 63 eligible nerve allografts and 79 nerve conduits were identified from 2007-2015 and met the inclusion criteria. Of these, a total of 37 patients (19 patients who received a nerve conduit repair and 18 patients who received decellularized nerve allografts) completed the 12-month follow-up and reported quantitative outcome data that could be analyzed. The baseline characteristics of these patients are outlined in table 1-3. There were no significant differences between groups for gender, follow-up, age, BMI, smoking habit, gap length, graft diameter, time to repair and mechanism of injury or concomitant injury. The gap size for the conduits was based on the perceived gap within the conduit. All repairs were performed within three weeks from the original injury. All patients had a complete nerve transection that required bridging of the nerve gap. The mean nerve gap length after debridement was 14 ± 4.9 mm for conduit repairs and 18.4 ± 9.3 mm for nerve allograft repairs. The diameter was 2.0 ± 0.0 mm for conduits and 2.3 ± 0.6 mm for nerve allografts. The mean follow-up for conduits was 477 ± 406 days and for allografts 432 ± 215 days. The mechanism of injury was stratified into two groups: lacerations and complex (amputation, avulsion, blast, compression, crush and gunshot injuries). The most common mechanism of injury with 63-67% in both groups was a laceration. In both groups, concomitant injuries such as vascular, tendon or fractures were common (95% versus 78%).

Table 1. Baseline characteristics for all patients enrolled in the study.

Characteristics	Collagen conduit Mean ± SD or no. (%) of patients	Processed allograft Mean ± SD or no. (%) of patients	P-value
Total	19	18	
Gender Male Female	 19 (100) 0	 15 (83) 3 (17)	0.105
Follow-up days	477 ± 406	432 ± 215	0.420
Age	38 ± 13	41 ± 18	0.271
BMI	27 ± 5	28 ± 7	0.500
Smoking	5 (26)	5 (28)	0.909
Time to repair Acute	 19 (100)	 18 (100)	0.900
Gap length in mm	14 ± 4.9	18.4 ± 9.3	0.208
Graft diameter in mm	2 ± 0.0	2.3 ± 0.6	0.055
Mechanism of injury Laceration Complex	 12 (63) 7 (37)	 12 (67) 6 (33)	0.823
Concomitant injury	18 (95)	14 (78)	0.132

Baseline characteristics of the study population are given and numbers are expressed as percentages of the total population.

* Variables with a P-value of ≤ of 0.05 were considered to be statistically significant.

Table 2. Allograft patient data.

No.	Age	Sex	Etiology	Standardized meaningful recovery *	Adverse events	Concomitant injury
1	56	M	laceration	excellent	none	none
2	46	F	laceration	excellent	none	vascular
3	60	F	laceration	good	none	none
4	19	M	laceration	excellent	none	vascular
5	9	M	complex	good	none	none
6	45	M	complex	excellent	none	compartment syndrome
7	49	M	complex	good	none	bone
8	21	F	laceration	excellent	none	none
9	72	M	laceration	good	none	vascular, tendon
10	35	M	complex	good	none	bone
11	29	M	complex	good	neuroma	vascular, tendon, bone
12	55	M	laceration	good	none	vascular, tendon, bone

Table 2. Allograft patient data. (continued)

No.	Age	Sex	Etiology	Standardized meaningful recovery *	Adverse events	Concomitant injury
13	18	M	laceration	excellent	none	vascular, tendon, bone
14	37	M	laceration	poor	none	vascular, tendon, bone
15	40	M	complex	good	none	vascular, tendon, bone
16	61	M	laceration	good	none	vascular, tendon
17	60	M	laceration	good	none	vascular, tendon, bone
18	28	M	laceration	excellent	none	vascular, tendon

Raw data of the allograft population is depicted.
* Mackinnon classification

Table 3. Conduit patient data.

No.	Age	Sex	Etiology	Standardized meaningful recovery *	Adverse events	Concomitant injury
1	26	M	laceration	excellent	none	vascular, tendon
2	55	M	complex	poor	none	vascular, tendon, bone
3	20	M	complex	poor	none	vascular, tendon
4	31	M	laceration	excellent	none	vascular, tendon, bone
5	50	M	complex	excellent	none	vascular, tendon, bone
6	38	M	laceration	excellent	none	vascular, tendon
7	29	M	laceration	good	none	tendon
8	28	M	complex	poor	infection	vascular, tendon, bone
9	39	M	laceration	excellent	none	vascular, tendon
10	62	M	laceration	good	none	vascular, tendon, bone
11	26	M	complex	excellent	none	vascular, bone
12	50	M	laceration	poor	none	vascular, tendon, bone
13	12	M	laceration	excellent	none	none
14	33	M	complex	poor	none	vascular
15	38	M	complex	excellent	none	vascular, tendon, bone
16	51	M	laceration	excellent	none	vascular, tendon, bone
17	47	M	laceration	good	none	vascular, tendon, bone
18	47	M	laceration	good	none	vascular, tendon, bone
19	33	M	laceration	good	none	vascular, tendon

Raw data of the conduit population is depicted.
* Mackinnon classification

Sensory outcome after 12 months of follow-up

A total of 19 nerve conduit repairs and 18 processed nerve allograft reconstructions completed a follow-up of at least 12 months after surgery. The mean S2PD at 12 months for collagen conduits was 9.8 ± 3.8 mm and 8.5 ± 3.7 mm for the processed allografts. Excellent sensory recovery was reached by 48% of the collagen conduit repairs and 39% of the processed nerve allografts (P = 0.608), good sensory recovery was achieved in 26% of the conduits and 55% of the allografts (P = 0.074) and poor outcomes occurred in 26% of the conduits versus 6% of the nerve allografts (P = 0.091). The difference between both groups was not statistically significant with regard to sensory outcome. Results are depicted in table 4.

Table 4. Sensory outcome after a minimum of 12 months of follow-up.

	Collagen conduit no. (%) of patients	**Processed allograft** no. (%) of patients	**P-value**
Total	19	18	
Excellent	9 (48)	7 (39)	0.608
Good	5 (26)	10 (55)	0.074
Poor	5 (26)	1 (6)	0.091

Final sensory outcome data were categorized according to the Mackinnon and Dellon classification to grade results as excellent, good, or poor.

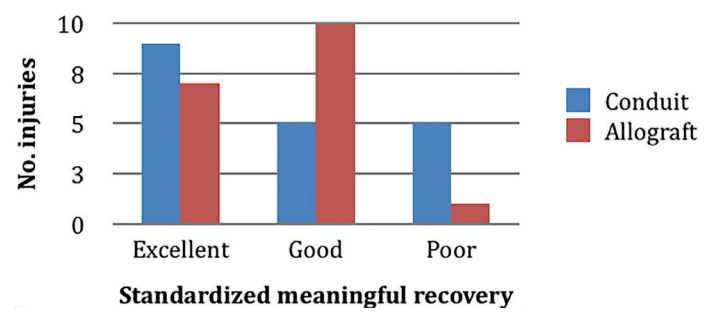

Figure 1. Sensory outcome.

Secondary outcome measurements after 12 months of follow-up

After 12 months there were no signs of extrusion or graft rejection. However, one neuroma arose after a processed allograft repair and one patient developed allodynia and complex regional pain syndrome 5 months after allograft repair. This was treated by a ganglion block and the patient recovered light touch sensation. A single infection was reported after collagen conduit reconstruction (P = 0.378).

DISCUSSION

The purpose of this study was to review a single institution's clinical experience with the NeuraGen collagen conduits and Avance processed human nerve allografts in digital nerve reconstruction, to define the exact role of both nerve graft substitutes. In this study, no statistical differences were found in sensory recovery or complications after 12 months of follow-up. This could suggest that both techniques offer effective means of reconstructing a digital nerve gap <2.5 cm and that the decision to choose either the conduit or the processed allograft should be made based on other parameters.

Previously, other researchers also noticed a lack of consensus regarding the most optimal management of digital nerve repair. Mermans et al. analyzed whether there was evidence for the superiority of one technique above the other in a meta-analysis. They included studies on end-to-end neurorrhaphy, nerve grafts, conduits or end-to-side neurorrhaphy and found no statistically significant difference in outcome between operation techniques.[24]

Kim et al. found that the average S2PD measurements for end-to-end primary neurorrhaphy, nerve grafts, synthetic conduits, vein conduits and muscle/muscle in vein reconstructions were 8.21 mm, 10.21 mm, 7.49 mm, 9.09 mm and 8.57 mm, respectively. According to the Mackinnon classification, all reported surgical repair techniques achieved good results (6-15 mm) and the study did not find any significant difference in recovery of sensibility among different techniques of digital nerve repair.[25]

Paprottka et al. summarized and compared the outcomes of digital nerve repair with the following methods: end-to-end and end-to-side coaptations, nerve grafts, artificial conduit, vein-muscle, and muscle-in-vein reconstructions. There was practically no particular technique superior to another and no clear treatment recommendations could be made. The authors conclude that a decision should be based on personal preference and clinical experience and recommend performing a vein graft or artificial conduit for gap lengths shorter than 10 mm; for gaps ranging from 10 to 30 mm an artificial conduit or posterior interosseous nerve graft and for gaps longer than 30 mm a sural nerve graft, medial antebrachial cutaneous nerve graft or end to side coaptation.[26]

Important to note, is that none of these three review articles included data on processed nerve allografts due to the lack of comparative useful literature at the time of publication.

Braga Silva et al. performed a systematic literature review that included data on processed nerve allografts and concluded that both allografts and collagen tubes were the most studied and best options.[27] Another review elaborated further on the question which commercially available conduit is better than the other and focused on nerve diameter and gap size. Data is shown where the quality of recovery decreased with longer gaps and the authors, therefore, conclude that nerve conduits are effective for 1 to 2-cm gaps in small diameter nerves. The authors also conclude that a direct comparison of the clinical results of acellular allograft repairs and nerve conduits was not possible based on the available literature.[11]

An overview of the literature, on collagen conduits and processed nerve allografts used for digital nerve reconstruction, is provided in table 5. Clinical studies often present different scoring systems that render direct comparison difficult, therefore we stratified all the results to the criteria as modified by Mackinnon and Dellon.[21] The recovery rates vary, but overall all studies report high numbers of meaningful recovery. For collagen conduits excellent to good recovery ranges from 50% to 89% and for nerve allografts it ranges from 83% to 100%. The results in this study compare favorably to these rates with a good or excellent recovery in 78% of the conduits and 94% of the nerve allografts. The poor recovery rate in the allograft literature ranges from 0% - 16% versus 0% - 38% for conduits, this is in accordance with the rates found in this study, 6% of nerve allografts had a poor outcome versus 26% of the nerve conduits.

Recently, Means et al. published a pilot study on the direct comparison of processed nerve allografts and hollow-conduits for digital nerve reconstructions in the hand. The authors found a statistically significant greater recovery (S2PD) at 12 months, for patients in the processed allograft treatment group. Moving 2PD, DASH (disabilities of the arm, shoulder, and hand) questionnaire, thermal discretion and pain assessment scores were not significantly different.[18] This study also compared results of participants with a minimum of 6-month follow-up and showed higher recovery rates for the allograft repairs at this time point, but these results were not statistically significant. Any difference in the rate of recovery of sensation between the two groups would be very relevant, however in the literature, there is evidence demonstrating that nerve regeneration after tubulization is not terminated after 12 months.[28]

Overall very few complications or adverse events after the use of collagen conduits or nerve allografts have been described in the literature.[10,29] This is consistent with our results.

Wangensteen et al. reported on two minor post-operative nerve conduit complications: erythema around the wound and partial wound dehiscence.[30] Haug et al. reported two wound infections after nerve conduit reconstruction.[31] Means et al. reported one adverse event in the processed allograft group resulting from a severe skin infection at the injury site that was determined to be unrelated to the treatment. The conduit group, however, had two possible product related events: 1. Persistent pain at the repair site and 2. Tube extrusion, osteomyelitis and fungal infection of the hand.[18]

It is notable that wound infections are considered product related in some studies, while in others skin infections were considered to be unrelated to the treatment. Also, tube extrusions have been described as a serious adverse event of polyglycolic conduits but this problem has not been previously encountered with collagen conduits.[32]

Based on these results and the current literature, both techniques seem to provide effective means of reconstructing sensation of a digital nerve gap < 2.5 cm. An advantage of allografts compared with nerve conduits is the similarity with autografts in structure and handling. An advantage of conduits compared with allografts is the storage condition; if the defect size changes during surgery, a different size conduit can be used without wasting the product. This is in contrast to the nerve allograft that is stored frozen, requires additional space for the appropriate storage freezer, needs time to defrost and cannot be frozen again if unused. This study was underpowered to detect any differences between the two groups, however there seems to be a trend for improved outcomes in nerve allograft reconstruction for larger gaps which corresponds with the literature.[18] Prognostic factors that have previously been associated with improved outcomes are young age, a short gap length (<1.3 cm), repair within 15 days after injury and sharp lacerations.[24-26]

Decision-making should be based upon surgical experience, gap length, wound condition, the extent of injury, operation time and additional costs and storage factors. Based on the results of this study, either collagen conduits or processed nerve allografts can be successfully used to restore sensation in digital nerve gap reconstructions < 2.5 cm. Future prospective studies should look for differences in the quality of sensation and rate of recovery between the two groups and define the exact role of nerve graft substitutes in digital nerve injuries.

Benefits of this study model include its multi-surgeon approach, its literature review and its diverse population with both lacerations and complex injuries. This study is limited by

the small sample size, high number of loss to follow-up, multiple variations among groups, the surgeon's unblinded assessment of the outcome and its retrospective nature. Were the patients that continued to follow-up doing so poorly that they kept coming back or were the patients doing so good that formal sensory testing was performed to document their excellent recovery? Observational study designs are associated with loss of patients to follow-up, no treatment randomization and inconsistent evaluation of results. This study is therefore at risk for selection bias, however, no significant differences were found among the study populations. Due to the small sample size, this study was not sufficiently powered to detect small differences and to perform subgroup analyses. Furthermore, quantitative measurements applied in this study were S2PD and SWM. Both measurements have some methodical limitations. The 2PD is the most frequently used test for the assessment of sensory outcome after nerve repair but the lack of standardization and extremely variable outcome make the method questionable. The SWM method is limited by the low inter-observer reliability.[22,33,34] Subsequently, most referenced studies were of small sample size and are of a low level of evidence.

CONCLUSION

In conclusion, nerve conduits and processed nerve allografts offer convenient off-the-shelf options for digital nerve gap repair. Based on this study, both techniques offer effective means of reconstructing a digital nerve gap < 2.5 cm after 12-months of follow-up. Future prospective randomized large sample size studies comparing nerve conduits with allografts are needed to perform subgroup analyses and to define their exact role in digital nerve injuries.

Table 5. Overview of the literature.
An overview of the literature on conduits and allografts in digital nerve injuries is provided.

Author	Conduit type	No. injuries	Mean age (range) (years)	Mean defect length (range) (mm)	Mechanism of injury	Time to repair (days)	Mean follow-up (months)	Sensory outcome and movement scale	Standardized outcome as reported in the studies	Results stratified to the Mackinnon and Dellon classification	Adverse events	Level of evidence
Rinker 2015	Allograft	24	43 ± 15 (23-81)	11 ± 3 (5-15)	Saw-related lacerations, sharp-type lacerations, amputations/avulsions, crush-type injuries, gunshot injuries	13 ± 41 (0-215)	16	-Static 2PD: 7.1 ± 2.9 (2-15mm) -Moving 2PD: 6.7 ± 3.3 (2-15mm) -SWM: 29 of 33 repairs with light touch and normal sensation returning in 16 and 7 of those repairs	* S3 or greater in 92% with 84% reporting recovery at the S3+ or S4 level	Excellent or good: 84% Poor: 16%	None	Retrospective
Guo 2013	Allograft	5	28.6 (18-39)	23 (18-28)	Table saw or gunshot injury	≤ 8	13.2	-2PD: 6 mm -SWM: 4.31	-	-	None	Retrospective
Karabekmez 2009	Allograft	10	44 (23-65)	22.3 (5-30)	Traumatic transection, neuroma resection	-	9 (5-12)	-Static 2PD: 5.5 mm -Moving 2PD: 4.4 mm	*** Excellent: 5 of 10 (50%) Good: 5 of 10 (50%) Poor: none	Excellent: 50% Good: 50% Poor: -	None	Retrospective
Taras 2013	Allograft	18	39 (18-76)	11 (5-30)	Sharp lacerations, crush type injuries	29 (2-262)	15 (12-20)	-Static 2PD: 7.1 mm (5-8 mm) -Moving 2PD: 5.4 mm (2-8 mm) -SWM: 0.08 to 279 g.	**** Excellent: 7 of 18 (39%) Good: 8 of 18 (44%) Fair: 3 of 18 (17%) Poor: none	Excellent: 39% Good: 61% Poor: -	None	Prospective

Table 5. Overview of the literature. (continued)
An overview of the literature on conduits and allografts in digital nerve injuries is provided.

Author	Conduit type	No. injuries	Mean age (range) (years)	Mean defect length (range) (mm)	Mechanism of injury	Time to repair (days)	Mean follow-up (months)	Sensory outcome and movement scale	Standardized outcome as reported in the studies	Results stratified to the Mackinnon and Dellon classification	Adverse events	Level of evidence
Wangensteen 2010	Collagen conduits	26	33 (7-79)	11.7 (2.5-20)	Lacerations, crush, cat bite, gunshot wound, blast and drill injury	1 (0-7300)	9		35% reporting improvement and 31% going on to a revision operation	-	Erythema, wound dehiscence	Retrospective
Schmauss et al. 2014	Collagen conduit	20	40 (20-75)	8.8 (6-15)	Cut injuries and crush injuries	-	58.1 (29-84)	-2PD: 6.8 mm (3-15)	***** Excellent: 15% Good: 50% Fair: 20% Poor: 15%	Excellent: 15% Good: 70% Poor: 15%	-	Prospective
Taras 2011	Collagen conduits	22	44 (22-72)	12 (5-17)	Sharp or semi sharp	6 (1-19)	20 (12-59)	-Static 2PD: 5 mm (6 patients did not recover S2PD) -Moving 5PD: 5 mm	** Excellent: 13 of 22 (59%) Good: 3 of 22 (14%) Fair: 6 of 22 (27%) Poor: none	Excellent: 59% Good: 41% Poor: -	None	Prospective

Abbreviations: Semmes Weinstein Monofilament (SWM), 2 Point Discrimination (2PD).
* = Meaningful recovery = S3-S4 on MRCC scale
** = Modified Weber scale = Excellent (M2PD ≤ 4mm or S2PD ≤ 6), Good (M2PD 5- 7mm or S2PD 7-8mm), Fair (M2PD ≤ 8mm or S2PD > 8), Poor (M2PD > 8mm or S2PD = 8)
*** = Mackinnon classification = Excellent (M2PD ≤ 3mm or S2PD ≤ 6), Good (M2PD 4-7mm or S2PD 7-15mm), Poor (absence of S2PD or M2PD)
**** = Taras outcome criteria = Excellent (M2PD ≤ 4mm or S2PD ≤ 6), Good (M2PD 5-7mm or S2PD 7mm), Fair (M2PD = 8mm or S2PD = 8), Poor (M2PD > 8mm or S2PD > 8)
***** = Modified guidelines of the ASSH = Excellent (2PD ≤ 6), Good (2PD 6-10mm), Fair (2PD = 11-15mm), Poor (2PD > 15mm)

REFERENCES

1. Moore AM. Nerve Transfers to Restore upper Extremity Function: A Paradigm Shift. *Front Neurol.* 2014;5:40.
2. 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.
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. Renner A, Cserkuti F, Hankiss J. Late results after nerve transplantation on the upper extremities. *Handchir Mikrochir Plast Chir.* 2004;36(1):13-18.
5. de Medinaceli L, Prayon M, Merle M. Percentage of nerve injuries in which primary repair can be achieved by end-to-end approximation: review of 2,181 nerve lesions. *Microsurgery.* 1993;14(4):244-246.
6. Stang F, Stollwerck P, Prommersberger KJ, van Schoonhoven J. Posterior interosseus nerve vs. medial cutaneous nerve of the forearm: differences in digital nerve reconstruction. *Arch Orthop Trauma Surg.* 2013;133(6):875-880.
7. Li ST, Archibald SJ, Krarup C, Madison RD. Peripheral nerve repair with collagen conduits. *Clin Mater.* 1992;9(3-4):195-200.
8. 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.
9. Taras JS, Jacoby SM, Lincoski CJ. Reconstruction of digital nerves with collagen conduits. *Journal of Hand Surgery.* 2011;36(9):1441-1446.
10. 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. *Journal of Reconstructive Microsurgery.* 2015;31(5):384-390.
11. Isaacs J, Browne T. Overcoming short gaps in peripheral nerve repair: conduits and human acellular nerve allograft. *Hand (N Y).* 2014;9(2):131-137.
12. Krarup C, Archibald SJ, Madison RD. Factors that influence peripheral nerve regeneration: an electrophysiological study of the monkey median nerve. *Ann Neurol.* 2002;51(1):69-81.
13. Waitayawinyu T, Parisi DM, Miller B, et al. A comparison of polyglycolic acid versus type 1 collagen bioabsorbable nerve conduits in a rat model: an alternative to autografting. *J Hand Surg Am.* 2007;32(10):1521-1529.
14. 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.
15. 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.
16. 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.
17. Johnson PJ, Newton P, Hunter DA, Mackinnon SE. Nerve endoneurial microstructure facilitates uniform distribution of regenerative fibers: a post hoc comparison of midgraft nerve fiber densities. *J Reconstr Microsurg.* 2011;27(2):83-90.
18. 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.
19. Dellon AL, Mackinnon SE, Crosby PM. Reliability of two-point discrimination measurements. *J Hand Surg Am.* 1987;12(5 Pt 1):693-696.
20. Weinstein SJ. Somatosensory changes after penetrating brain wounds in man. *Harvard University Press, Cambridge.* 1960(4-11):60,62.
21. Mackinnon SE, Dellon AL. Clinical nerve reconstruction with a bioabsorbable polyglycolic acid tube. *Plastic and Reconstructive Surgery.* 1990;85(3):419-424.
22. Hirasawa Y, Katsumi Y, Tokioka T. Evaluation of sensibility after sensory reconstruction of the thumb. *J Bone Joint Surg Br.* 1985;67(5):814-819.
23. Rebecca Saunders RA, Susan Burke, James Higgins, Michael McClinton. *Hand and Upper Extremity Rehabilitation.* 2015.
24. Mermans JF, Franssen BB, Serroyen J, Van der Hulst RR. Digital nerve injuries: a review of predictors of sensory recovery after microsurgical digital nerve repair. *Hand (N Y).* 2012;7(3):233-241.
25. Kim JS, Bonsu NY, Leland HA, Carey JN, Patel KM, Seruya M. A Systematic Review of Prognostic Factors for Sensory Recovery After Digital Nerve Reconstruction. *Ann Plast Surg.* 2018;80(5S Suppl 5):S311-S316.
26. Paprottka FJ, Wolf P, Harder Y, et al. Sensory recovery outcome after digital nerve repair in relation to different reconstructive techniques: meta-analysis and systematic review. *Plast Surg Int.* 2013;2013:704589.
27. Braga Silva J, Marchese GM, Cauduro CG, Debiasi M. Nerve conduits for treating peripheral nerve injuries: A systematic literature review. *Hand Surg Rehabil.* 2017;36(2):71-85.
28. Schmauss D, Finck T, Liodaki E, et al. Is nerve regeneration after reconstruction with collagen nerve conduits terminated after 12 months? The long-term follow-up of two prospective clinical studies. *Journal of Reconstructive Microsurgery.* 2014;30(8):561-568.

29. Taras JS, Amin N, Patel N, McCabe LA. Allograft reconstruction for digital nerve loss. *Journal of Hand Surgery.* 2013;38(10):1965-1971.
30. Wangensteen KJ, Kalliainen LK. Collagen tube conduits in peripheral nerve repair: a retrospective analysis. *Hand (N Y).* 2010;5(3):273-277.
31. Haug A, Bartels A, Kotas J, Kunesch E. Sensory recovery 1 year after bridging digital nerve defects with collagen tubes. *J Hand Surg Am.* 2013;38(1):90-97.
32. Duncan SFM, Kakinoki R, Rizzo M, Kang W. Extrusion of a neurotube: A case report. *Ochsner Journal.* 2015;15(2):191-192.
33. Lundborg G, Rosén B. The two-point discrimination test - Time for a re-appraisal? *Journal of Hand Surgery.* 2004;29(5):418-422.
34. Huber JL, Maier C, Mainka T, Mannil L, Vollert J, Homann HH. Recovery of mechanical detection thresholds after direct digital nerve repair versus conduit implantation. *J Hand Surg Eur Vol.* 2017;42(7):720-730.

PART II

Mesenchymal stromal cells in nerve regeneration

CHAPTER 4

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% CO2 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 timepoint (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 (Revolver™ 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.

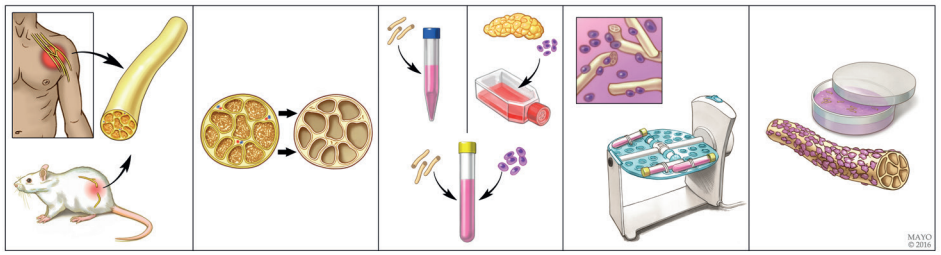


Figure 1. Seeding technique. This technique involves a 9-step process as depicted above and described in detail in the appendix. A) Nerve graft harvest, B) Nerve decellularization, C) Cell culture and isolation, D) Bioreactor seeding, E) Static storage before grafting. The seeding method, cell viability and effect of seeding duration were first evaluated on rat MSCs seeded onto rat nerve allografts. Secondly, the seeding method and cell viability were tested on human MSCs seeded onto human nerve allografts.

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.

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 340/460 nm.

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 µg). 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).

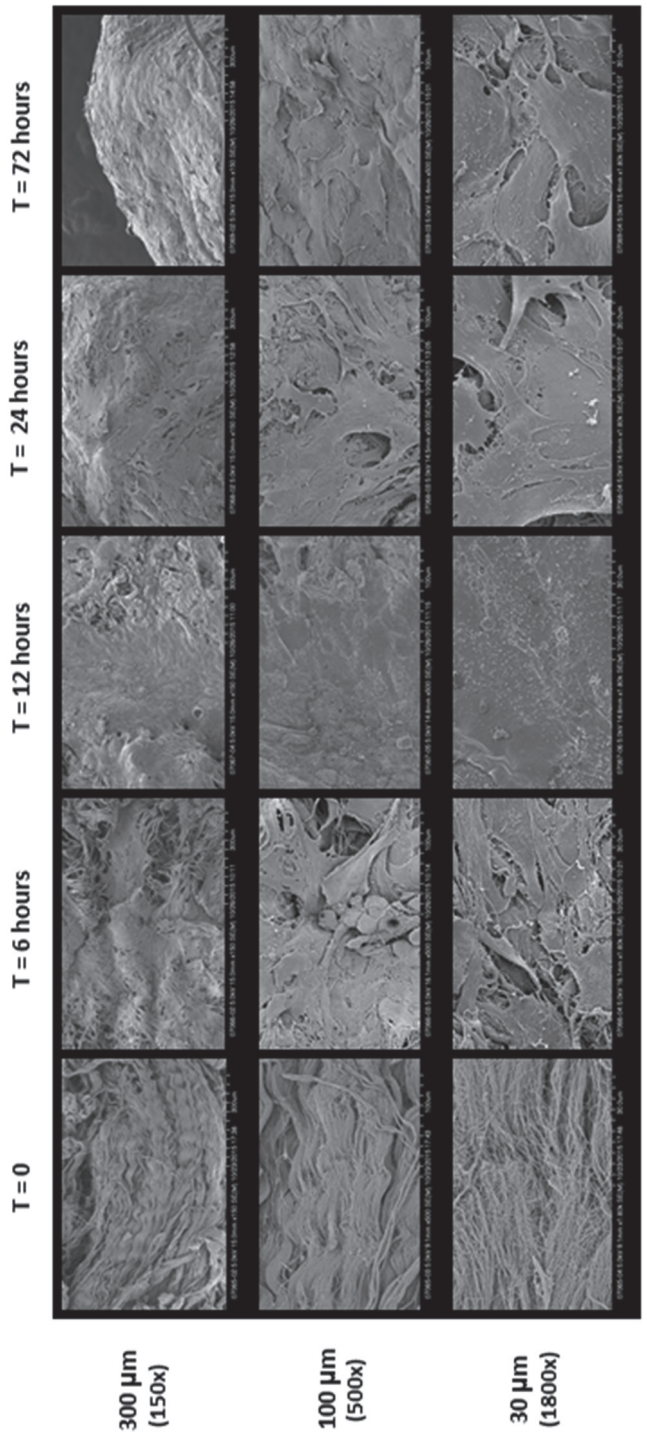


Figure 2. Scanning electron microscopy images of MSC seeded nerve graft. Scale bars = 300µm (150x), 100µm (500x) and 30µm (1800x). The MSC seeded nerve graft is depicted here in 3 different magnifications. Illustrating the cell morphology and coverage of the unseeded decellularized graft (t=0) and after 6, 12, 24 and 72 hours of 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 evenly distributed throughout the nerve grafts and the number of cells gradually increased.

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).

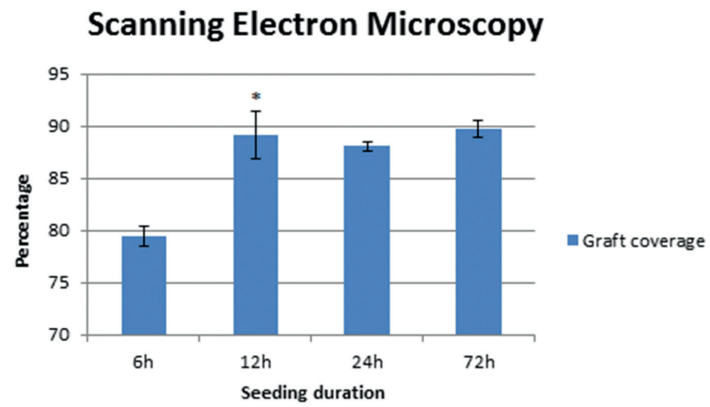


Figure 3. Seeding efficiency. Seeding efficiency was determined as the percentage of graft coverage imaged with scanning electron microscopy. Seeding efficiency increased significantly from 79.5% at 6 hours to 89.2% after 12 hours of seeding (p = 0.004). No significant differences were found between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

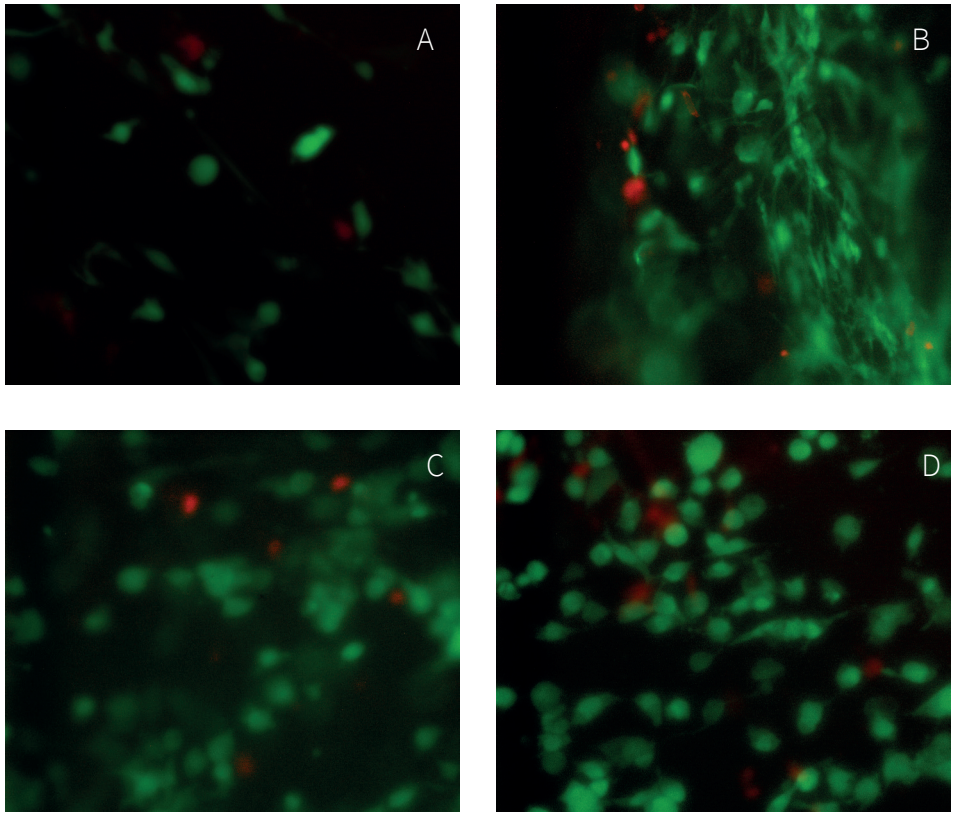


Figure 4. Live/Dead staining of MSC seeded allografts. Cellular viability and density is demonstrated after seeding the nerve allografts for A)6 hours, B)12 hours, C)24hours and D)72 hours. The cells were visualized using a confocal scanning microscopy at 40x magnification. Live cells were stained green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

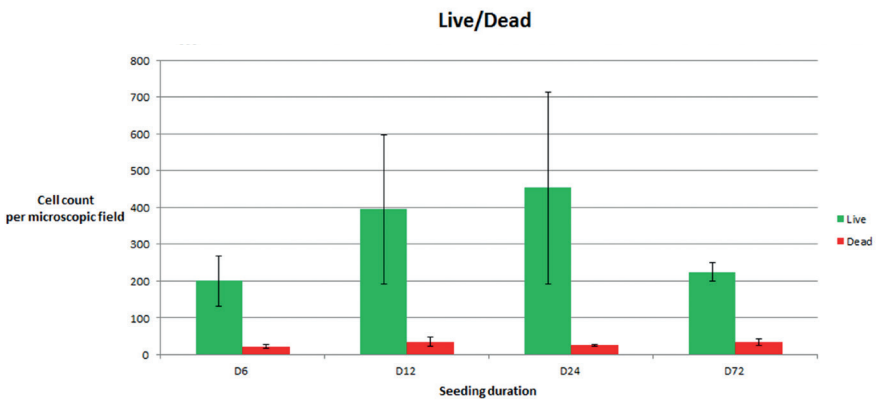


Figure 5. Live/Dead cell count. Average manual live and dead cell count per microscopic field per seeding duration. Error bars represent the standard error of the mean.

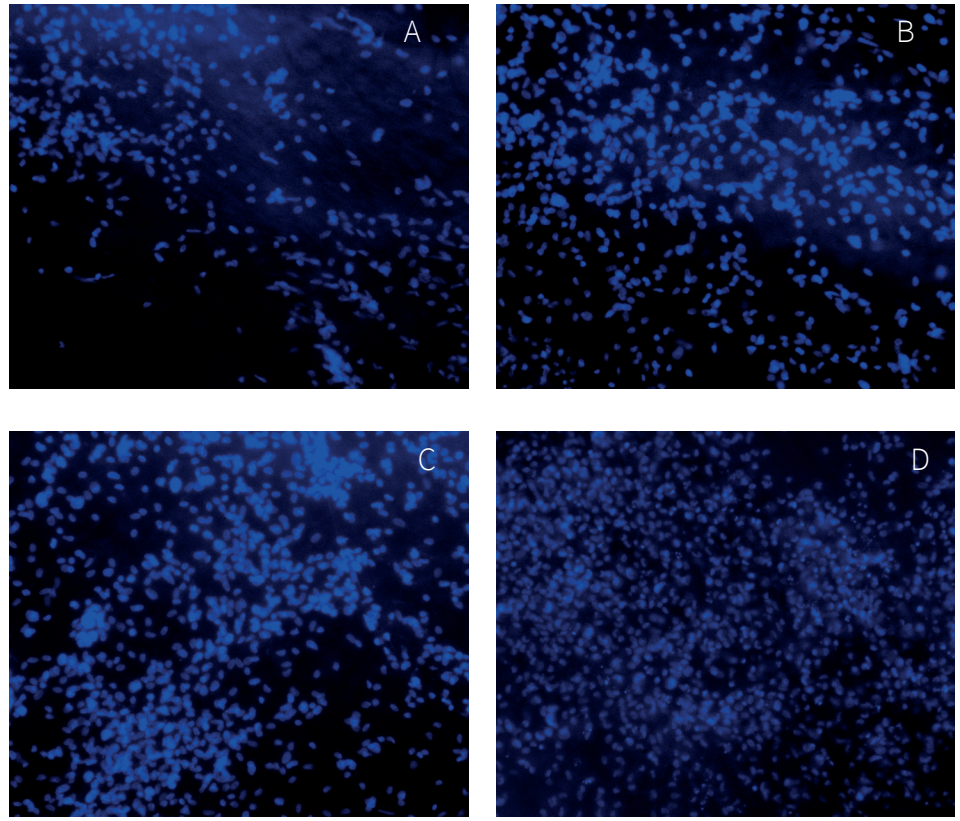


Figure 6. Hoechst longitudinal images. Cell nuclei were Hoechst stained after seeding the nerve allografts for A)6 hours, B)12 hours, C)24hours and C)72 hours. The cells were visualized using a confocal scanning microscopy at 20x magnification.

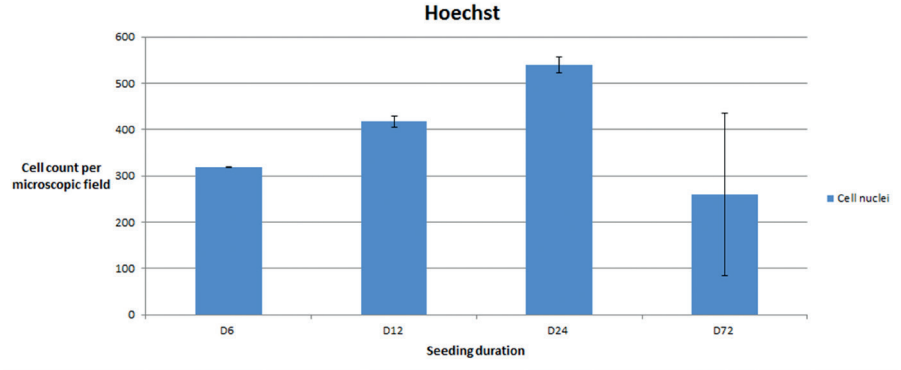


Figure 7. Hoechst cell count. Shows the average manual Hoechst cell count per microscopic field per seeding duration. Error bars represent the standard deviation or standard error of the mean as indicated.

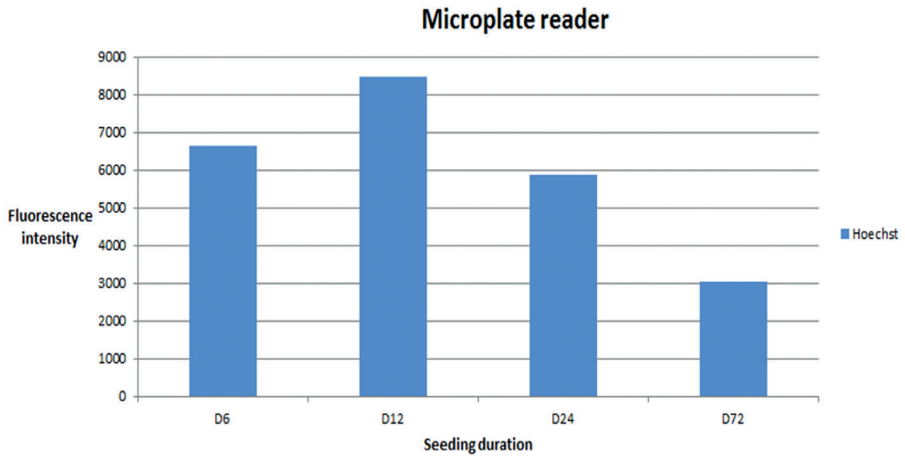


Figure 8. Hoechst fluorescence intensity. Demonstrates the differences in fluorescence intensity between different seeding durations as measured using a micr oplate reader.

Figure 9. Hoechst cross-sectional images. A frozen cross-section is depicted after 12 hours of seeding and imaged at 5x magnification (inset = 10x). 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)	Micro-injection	Xenogeneic acellular nerve matrix	AMSCs	$5x10^6$
Lui (2011)	Micro-Injection	Acellular nerve al-lografts	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 12 hours 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 a 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

Protocol: Rotator cell seeding

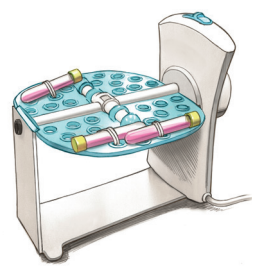
1. Harvest nerve segments under aseptic conditions and remove fatty and connective tissue from the epineurium. Place nerves immediately in Roswell Park Memorial Institute medium 1640 solution (RPMI) at 4°C until further processing.
2. Decellularize the nerves using the protocol as previously published.[19]

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 (RevolverTM 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.

CHAPTER 5

Seeding 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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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 repairing 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 3, 7, 14 and 21 days in culture for RNA expression analysis by RT-qPCR. 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, quantitative RT-qPCR 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 nerve reconstructions.[1-6] In autograft reconstruction, Schwann cells facilitate nerve regeneration by promoting myelination through growth factor production and interaction 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 that have previously been shown to stimulate nerve regeneration.[13-15] MSCs are preferred to embryonic stem cells for ethical concerns and reported teratoma formation. MSCs are also preferred over neural stem cells due to difficulties in their harvest and potential for neuroblastoma formation.[16, 17] MSCs can be obtained from multiple sources including adipose tissue, bone marrow, skin, dental pulp and hair follicles.[14] Adipose derived MSCs have some important advantages including the ease of accessibility with low donor morbidity, rapid proliferation with high yield and capability to produce favorable growth factors locally.[13, 18, 19]

Although MSCs have proven to be beneficial for nerve regeneration, the actual mechanism is currently 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.[20, 21]

The combination of a patient's own adipose derived stem cells and the high availability of processed nerve allograft is an attractive 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 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 surgical-grade decellularized nerve allografts 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 multiple time points (3, 7, 14 and 21 days). Changes in gene expression profiles (phenotype) of the MSCs were quantified and 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 decellularized using the protocol described by Hundepool et al.[22] 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.

A

Group	Description	Time points (days)	N	Outcome measurements
I	Seeded nerve graft (nerve graft + MSCs)	T= 0 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

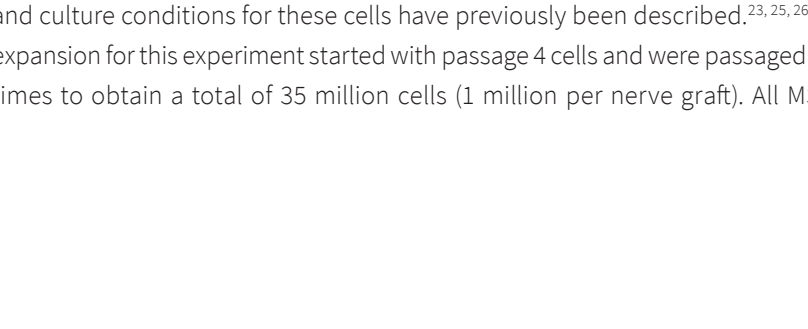

Figure 1. Experimental design.
A) A total of 25 human nerve allografts were decellularized and in vitro seeded with clinical grade human MSCs. Subsequently, the MSC seeded grafts and MSCs cultured in media only were cultured for 21 days and at various time points (3, 7, 14 and 21 days), total RNA was 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.

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 the following cell surface markers: CD73, CD90, CD105, CD44, CD14 and CD45, RNA-sequence transcriptome profiles, and multi-lineage potential.[23, 24] Cell isolation and culture conditions for these cells have previously been described.[23, 25, 26] Cell culture expansion for this experiment started with passage 4 cells and were passaged 2 additional times to obtain a total of 35 million cells (1 million per nerve graft). All MSCs used in

experiments were of passage 6 and all experiments were performed concurrently from the same source population of cells.

Cell seeding

A total of 25 human nerve allografts were seeded with clinical-grade human MSCs using a dynamic bioreactor as previously described.[27] 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 a solution of 5% human platelet lysate, 2 mmol/L Glutamax, 2 U/ml heparin and 1% penicillin/streptomycin. Cell culture media was changed every 3-4 days. The impact of seeding on MSC survival was previously determined to not affect the cell viability over a 7 day study period.[27]

For the control group, 250.000 cells/well were suspended in 6-well plates (Thermofisher, Waltham, MA) containing 2.5ml cell culture medium. The plates were placed in an incubator at 37 degree Celsius and 5% CO_2 for up to 21 days. Media was changed every 3-4 days.

Quantitative Real Time Reverse-Transcriptase Polymerase Chain Reaction (RT-qPCR)

To quantify changes in relative gene expression profiles of MSCs in culture and MSCs seeded onto the decellularized nerve allografts, RT-qPCR was performed at days 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) under the following conditions: 3 minutes at 65C, 1.5 hour at 37C and 5 minutes at 95C. The resulting cDNA libraries were analyzed by RT-qPCR (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 because of their importance in nerve regeneration.[14, 28-39] All MSC only samples were analyzed in triplicate and all MSC seeded nerves were analyzed in groups of five. Results were normalized to the reference gene *AKT1* within each sample and to the value of MSCs only at day 0. *AKT1* was selected as a normalization gene because it remains

constant among a larger number of tissues, cells and conditions than other commonly used normalizing genes (e.g., *GAPDH* and *HPRT1*; pers. obs.). In this study, the suitability of both *GAPDH* and *AKT1* as normalization markers were determined and validated by a comprehensive online tool based on four different algorithms (geNorm, NormFinder, BestKeeper and Delta Ct).[40-42] *AKT1* was identified as the most stable reference gene (Fig. 2). 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.[24, 43, 44]

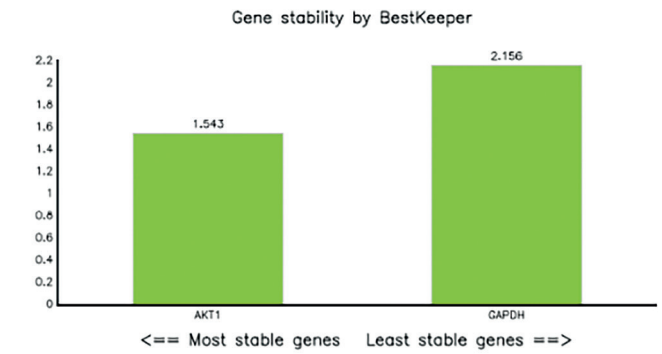

Figure 2. Ranking of candidate reference genes based on their expression stability calculated by comprehensive ranking. Comprehensive gene stability of reference genes AKT1 and GAPDH in human tissue. The y-axis represents the geometric mean of results obtained from geNorm, NormFinder, BestKeeper and Delta Ct. The x-axis represents ranking from least to most stable gene expression, lower values indicate more stable gene expression.

Table 1. mRNA primer sequences

Gene ID	Biology	Forward primer	Reverse primer	Literature reference
GAPDH	Reference gene	CCCGGTACACCAC-GTTCTTC	TGTGGTCATGAGTCCTTC-CA	Dudakovic A (2018)[30]
AKT1	Reference gene	ATGGCGCT-GAGATTGTGTCA	CCCGGTACACCAC-GTTCTTC	Staal SP 1987[36]
NGF	Neurotrophic marker	ATACAGGCGGAACCA-CACTCAG	ATACAGGCGGAACCA-CACTCAG	Kingham (2014)[14]
BDNF	Neurotrophic marker	AGAGGCTTGACAT-CATTGGCTG	CAAAGGCACTTGAC-TACTGAGCATC	Kingham (2014)[14]
GDNF	Neurotrophic marker	CACCAGATAAA-CAAATGGCAGTGC	CACCAGATAAACAAATG-GCAGTGC	Kingham (2014)[14]
PTN	Neurotrophic marker	ACTGGAAGTCTGAAG-CGAGC	CTTCTTCTTAGATTCT-GCTTGAGGT	Tezuka (1990)[37]
GAP43	Neurotrophic marker	GTCCACTTTCCTCTC-TATTTC	TGTTCATTCCATCA-CATTGA	Kingham (2014)[14]
VEGFA	Angiogenic marker	ATCTGCATGGTGAT-GTTGGA	GGGCAGAATCATCAC-GAAG	Kingham (2014)[14]
PECAM/CD31	Angiogenic marker	AACAGTGTTGACAT-GAAGAGCC	AACAGTGTTGACATGAA-GAGCC	Albeda (1991)[28]
MPZ	Myelination marker	GAGGAGGCTCAGT-GCTATGG	GCCCGCTAACCGC-TATTTCT	Shy (2006)[35]
PMP22	Myelination marker	GTTAAAGGGAACGC-CAGGA	AGTTTCTGCAGC-CCAAAGGA	Li (2017)[32]
MBP	Myelination marker	GGCTGTGCAACATG-TACAAGGA	GGACAGTCCTCTC-CCCCTTTCC	Zhou (2017)[39]
COL1A1	ECM protein	GTAACAGCGGT-GAACCTGG	CCTC-GCTTTCCTTCCTCTCC	Malfait (1993)[33]
COL3A1	ECM protein	TTGAAGGAGGAT-GTTCCCATCT	ACAGACACATATTTGG-CATGGTT	Chiarelli (2018)[29]
LAMB2	ECM protein	ACACGCAAGCGAGT-GTATGA	AATCACAGGGCAGG-CATTCA	Naba (2017)[34]
FBLN1	ECM protein	AGAGCTGCGAGTA-CAGCCT	CGACATCCAAATCTCCG-GTCT	Naba (2017)[34]
CASP3	Apoptosis protein	GGACCTGTGGACCT-GAAAAA	AGTTCGGCTTTCCAGT-CAG	Kingham (2014)[14]
EGR1	Transcription factor	ACCCCTCTGTCTAC-TATTAAGGC	TGGGACTGGTAGCTGG-TATTG	Wang (2018)[38]
CD96	Immunoglob-ulin	AGATTGTGTGAT-GAAGGACATGG	AGATTGTGTGATGAAGG-ACATGG	Islam (2018)[31]

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 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 based on relevance 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 triplicate 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 final 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 (RT-qPCR)

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 3a-d demonstrate the relative MSC gene expression levels clustered by genes that share a common function. 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 among groups for *MPZ*, *COL3A1*, *LAMB2* and *CASP3* expression (Figure 3d).

Genes that showed significant changes in 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 (*BDNF*, *VEGF*, *CD31* and *MBP*) gene expression values were significantly upregulated in the seeded MSCs, while the neurotrophic marker *GDNF* was significantly upregulated in the MSC group only (Figure 3d).

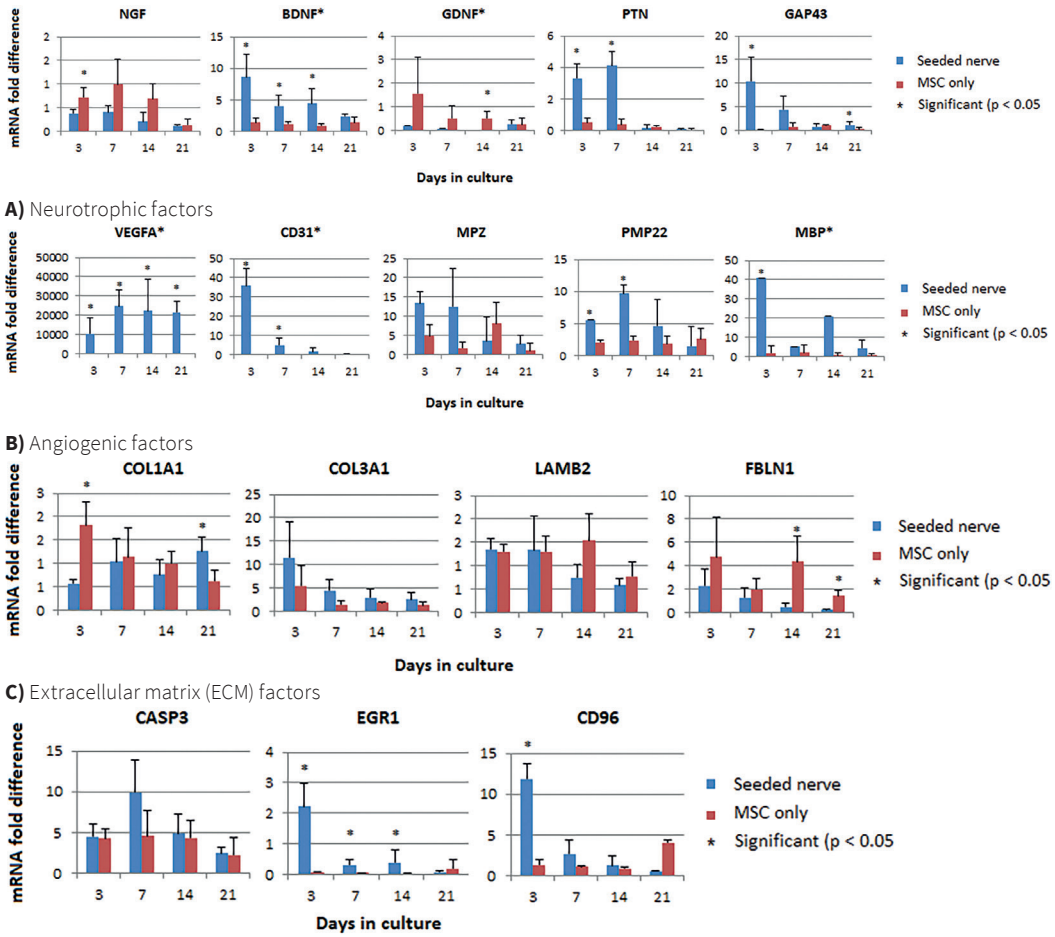

Figure 3. Gene expression clustered by genes that share a common function. The gene expression of each time point was normalized to AKT1 and the fold difference versus MSCs day 0 was calculated. The error bars represent the standard deviation (n=5).
* denotes p < 0.05 when compared to the gene expression in seeded nerves to MSCs only at that time point.

Protein isolation and Enzyme-linked immunosorbent Assay (ELISA)
Figure 4 depicts the production of three factors analyzed by ELISA over 21 days.

The NGF protein production in the MSCs only was significantly increased at individual time points 3 days with 207,99 pg/ml ± 28,47 versus 45,02 pg/ml ± 12,73 (p =0,017) and 7 days 210,78 pg/ml ± 6,69 versus 11,42 pg/ml ± 12,73 (p = 0,003).

Production of VEGFA protein in the seeded MSCs was low but increasing per time point from 108,95 pg/ml ± 8,43 at day 3 to 190,10 pg/ml ± 19,61 at day 21. While the MSCs only produced significantly increasing (p = 0.028) levels of VEGFA protein over the 21 days from 590,84 pg/ml ± 7,57 at day 3 to 692,78 pg/ml ± 72,23 at day 21.

ELISA assays revealed a significant (p = 0.011) upregulated BDNF protein production over the time course of 21 days by the seeded MSCs (370.16 pg/ml ± 13.37 at 72 hours compared to 18.73 pg/ml ± 7.21 in the MSC only group), correlating with the significant upregulation of BDNF gene expression. ELISA growth factor analysis showed no detectable GDNF-protein production in either sample group.

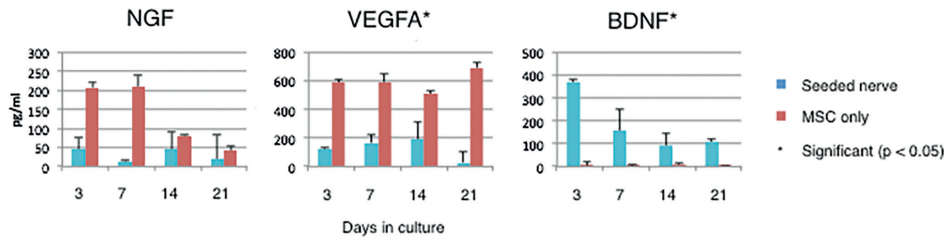

Figure 4. Growth factor production. ELISA analysis of the cell supernatants over 21 days is depicted.

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.

qRT-PCR and ELISA results

In the decellularized nerve grafts, no RNA levels were detectable, which was expected. The purpose of the decellularization process is to render a scaffold devoid of cells that does not elicit an immune response. Also, the decellularization process likely caused degradation of RNA in cells that produced the ECM. Although we did not confirm presence of the residual

ECM, previous studies have repeatedly demonstrated preservation of the ECM after nerve decellularization.[8, 22]

Many soluble growth factors are secreted by MSCs and the interaction of MSCs with an 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 (Figures 3a-d). 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).

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.

NGF is important for the development and maintenance of the sympathetic and sensory nervous systems.[12] 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.

We measured GDNF to assess the potential of MSCs to prevent 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.[45]

The reduced expression in the seeded MSCs for NGF and GDNF can be caused by multiple factors. For some growth factors, up regulation might not only be triggered by the nerve allograft structure, but also by factors from the wound environment that were not included in our in vitro model.[46] Furthermore, we used motor nerve allografts and NGF has been previously found to be up regulated in sensory nerves but not in motor nerves.[47]

The BDNF-gene promotes the survival and differentiation of selected neuronal populations of the peripheral nervous system and participates in axonal growth.[48] qPCR analysis showed a significant up regulation of the BDNF maker upon seeding, suggesting that the interaction between MSCs and the nerve allograft could stimulate axonal growth.

The PTN-gene induces neurite outgrowth and has significant roles in cell growth and survival.[49] qPCR analysis showed that PTN mRNA levels were increased in allografts seeded with MSCs.

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.[49] qPCR analysis showed that GAP43 levels were upregulated in cells after seeding. These results suggest that axonal regeneration and neurite outgrowth are promoted upon adhesion to the graft. However, the effect of genes upregulated in MSCs on axonal growth should be further evaluated, for example in neuronal co-culture models including the seeded nerve allograft.

The VEGFA growth factor plays an important role in angiogenesis.[50] CD31 is involved in leukocyte migration, angiogenesis and integrin activation.[51] MPZ is required for the proper formation and maintenance of myelin. PMP22 is produced by Schwann cells and

helps develop and maintain myelin. MBP is an integral component of myelin formation and stabilization.[52] All of the above-mentioned markers were highly expressed in the MSC seeded nerves but were scarcely detected in the MSCs alone, suggesting that upon adhesion, MSCs increase their capacity for angiogenesis and myelination.

In vitro expansion of 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, is typically upregulated in cells during adhesion, differentiation, migration and signaling as well as neurite outgrowth and regulation of Schwann cell development. Furthermore, FBLN is known to promote cell adhesion and migration within the ECM.[53] Observed upregulation of these markers suggests that seeded MSCs retain their adhesion, migration and proliferation potential. High levels of ECM related gene expression in both groups demonstrate that seeded MSCs retain cell-adhesion, collagen anabolic activity and cell-to-cell communication abilities. Influencing stem cell fate by interaction with a specific matrix, to create a so-called stem cell niche, is not a new concept. Indeed, cell adhesion molecules are capable of activating signaling pathways associated with the promotion of MSC self-renewal and retention of 'stemness'.[54]

Of the other genes that were evaluated, CASP3 was measured to assess the potential of seeded MSCs to mediate cell apoptosis.[55] EGR1 functions as a transcriptional regulator, plays a role in the regulation of cell survival, proliferation and cell death.[56] CD96 is involved in late phase immune responses. qPCR analysis revealed a moderate up regulated expression of these markers over 21 days in the seeded MSCs (Figure 3d), suggesting that upon adhesion, the remaining nerve ECM can trigger cell apoptosis and cell survival while eliciting a late immune response.

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 4).

Similar to qPCR results, we observed 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 significant upregulation of GDNF gene-expression in the MSC only group did not correlate with an increased GDNF-protein production.

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.[57]

Collectively, our results (summarized in Table 2) demonstrate that interactions of MSCs with decellularized nerve allografts modulate gene expression in MSCs including the stimulation of mRNAs for key proteins that support nerve growth and/or homeostasis.

Strengths and limitations

The seeding of decellularized allografts with MSCs was attempted by Hu et al.[58], who used decellularized allografts injected with bone-marrow derived MSCs to repair 40-mm ulnar nerve gaps in rhesus monkeys with good electrophysiological and immunohistochemistry results. However, other measures of functional outcome (muscle force and mass) were not evaluated. It remains unclear whether cell seeding strategies will enhance the in vivo performance of nerve allografts, and the source of seeded cells are the topic of considerable debate (both topics that need resolution before clinical translation of these strategies can be accomplished). For example, Wang et al.[59] and Fan et al.[60] tried seeding MSCs that were differentiated into schwann-cell like cells onto allografts. Wang et al. found that in comparison to Schwann cells, results of differentiated MSCs seem promising. Fan et al.[60] compared differentiated MSCs to undifferentiated MSCs and found that differentiated cells yielded better outcomes after nerve reconstruction. Kingham et al.[14] showed that pre-differentiation of MSC's resulted in increased secretion of neurotrophic and angiogenic factors. Most levels of these factors measured in the undifferentiated seeded cells in this study were also enhanced, suggesting that cells might not have to be pre-differentiated prior to surgery. Other researchers hypothesized that once stimulation factors are removed, the cells tend to revert back to their original phenotype.[61] A strength of this study is the use of a previously validated non-traumatic seeding technique.[27] Another major strength of this study, is that only human tissues were 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 the scarcity of eligible nerve allograft donations available for research purposes. We however do not expect to find any difference within allograft donors since all grafts are decellularized by the same protocol. Secondly, ELISA was not performed for all known growth factors. Since ELISA is a costly technique, only four ELISA kits were selected based on priority from the literature. Also, ELISA data did not incorporate the potential for differences in cell biology (e.g., growth, proliferation) among the seeded and control cell groups. Another limitation of this study is that no evidence of cell survival at later time points is demonstrated (in vitro), this would theoretically help to reassure the robustness of the useful induction of growth factors seen by seeding undifferentiated MSCs on decellularized nerves. In contrast, cells cultured for more than 21 days become extremely unpredictable, because of the number of times they have divided to cover the substrate. Furthermore, we focused on the clinical translatability. Cells that have been cultured for weeks would not be used in the clinical setting. In addition, we recently published an in vivo rat study demonstrating that implanted MSCs, seeded onto a decellularized nerve allograft, survived up to 29 days. Gradually diminishing signals were observed in the first week following implantation, concluding that MSCs have a finite survival after implantation.[62]

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.[46] Therefore, future studies should focus on the need of pre- differentiation and on the specific role of MSCs seeded onto nerve allografts in an in vivo model of nerve regeneration. Long-term in-vivo studies will allow direct assessment of whether seeded MSCs and their secreted growth factors also provide better functional nerve repair 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 suggest that for clinical translation, cells may not need differentiation prior to surgery and that the

trophic functions in tissue repair occur in the first week after surgery. The pre-operative combination of autologous MSCs and readily available processed nerve allografts is an exciting avenue for additional research aimed at customization of peripheral nerve repair in individualized medicine.

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: 1-14.
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: 2340-9.
3. Giusti G, Willems WF, Kremer T, et al. 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: 410-7.
4. Moore AM, MacEwan M, Santosa KB, et al. Acellular nerve allografts in peripheral nerve regeneration: a comparative study. Muscle Nerve 2011: 44: 221-34.
5. 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: 367-77.
6. 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: 83-96.
7. Chernousov MA, Carey DJ. Schwann cell extracellular matrix molecules and their receptors. Histol Histopathol 2000: 15: 593-601.
8. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. Tissue Eng 2004: 10: 1346-58.
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: 578-93.
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: 183-8.
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: 1109-17.
12. Boyd JG, Gordon T. Neurotrophic factors and their receptors in axonal regeneration and functional recovery after peripheral nerve injury. Mol Neurobiol 2003: 27: 277-324.
13. 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: 195-209.
14. 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: 741-54.
15. 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: 103-10.
16. Ladak A, Olson J, Tredget EE, Gordon T. Differentiation of mesenchymal stem cells to support peripheral nerve regeneration in a rat model. Exp Neurol 2011: 228: 242-52.
17. Safford KM, Hicok KC, Safford SD, et al. Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 2002: 294: 371-9.
18. 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: 2008-17.
19. Pfister LA, Papaloizos M, Merkle HP, Gander B. Nerve conduits and growth factor delivery in peripheral nerve repair. J Peripher Nerv Syst 2007: 12: 65-82.
20. 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: 2863-70.
21. 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: 368-79.
22. Hundepool CA, Nijhuis TH, Kotsougiani D, et al. Optimizing decellularization techniques to create a new nerve allograft: an in vitro study using rodent nerve segments. Neurosurg Focus 2017: 42: E4.
23. 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: 797-811.
24. 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: 1816-28.
25. 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.
26. Mahmoudifar N, Doran PM. Osteogenic differentiation and osteochondral tissue engineering using human adipose-derived stem cells. Biotechnol Prog 2013: 29: 176-85.
27. Rbia N, Bulstra LF, Bishop AT, van Wijnen AJ, Shin AY. A Simple Dynamic Strategy to Deliver Stem Cells to Decellularized Nerve Allografts. Plast Reconstr Surg 2018: 142: 402-13.

28. Albelda SM, Muller WA, Buck CA, Newman PJ. Molecular and cellular properties of PECAM-1 (endoCAM/CD31): a novel vascular cell-cell adhesion molecule. J Cell Biol 1991: 114: 1059-68.
29. Chiarelli N, Carini G, Zoppi N, Ritelli M, Colombi M. Transcriptome analysis of skin fibroblasts with dominant negative COL3A1 mutations provides molecular insights into the etiopathology of vascular Ehlers-Danlos syndrome. PLoS One 2018: 13: e0191220.
30. Dudakovic A, Gluscevic M, Paradise CR, et al. Profiling of human epigenetic regulators using a semi-automated real-time qPCR platform validated by next generation sequencing. Gene 2017: 609: 28-37.
31. Islam Z, Jahan I, Ahammad RU, et al. FAS promoter polymorphisms and serum sFas level are associated with increased risk of nerve damage in Bangladeshi patients with Guillain-Barre syndrome. PLoS One 2018: 13: e0192703.
32. Li LX, Dong HL, Xiao BG, Wu ZY. A Novel Missense Mutation in Peripheral Myelin Protein-22 Causes Charcot-Marie-Tooth Disease. Chin Med J (Engl) 2017: 130: 1779-84.
33. Malfait F, Wenstrup R, De Paepe A. Classic Ehlers-Danlos Syndrome1993.
34. Naba A, Pearce OMT, Del Rosario A, et al. Characterization of the Extracellular Matrix of Normal and Diseased Tissues Using Proteomics. J Proteome Res 2017: 16: 3083-91.
35. Shy ME. Peripheral neuropathies caused by mutations in the myelin protein zero. J Neurol Sci 2006: 242: 55-66.
36. Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A 1987: 84: 5034-7.
37. Tezuka K, Takeshita S, Hakeda Y, et al. Isolation of mouse and human cDNA clones encoding a protein expressed specifically in osteoblasts and brain tissues. Biochem Biophys Res Commun 1990: 173: 246-51.
38. Wang LF, Liu YS, Yang B, et al. The extracellular matrix protein mindin attenuates colon cancer progression by blocking angiogenesis via Egr-1-mediated regulation. Oncogene 2018: 37: 601-15.
39. Zhou Y, Simpson S, Jr., Charlesworth JC, et al. Variation within MBP gene predicts disease course in multiple sclerosis. Brain Behav 2017: 7: e00670.
40. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett 2004: 26: 509-15.
41. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002: 3: RESEARCH0034.

42. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 2004: 64: 5245-50.
43. Jesuraj NJ, Santosa KB, Macewan MR, et al. Schwann cells seeded in acellular nerve grafts improve functional recovery. Muscle Nerve 2014: 49: 267-76.
44. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001: 25: 402-8.
45. Skaper SD. Neurotrophic Factors: An Overview. Methods Mol Biol 2018: 1727: 1-17.
46. 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: 98-114 e7.
47. Hoke A, Redett R, Hameed H, et al. Schwann cells express motor and sensory phenotypes that regulate axon regeneration. J Neurosci 2006: 26: 9646-55.
48. Karpova NN. Role of BDNF epigenetics in activity-dependent neuronal plasticity. Neuropharmacology 2014: 76 Pt C: 709-18.
49. Blondet B, Carpentier G, Lafdil F, Courty J. Pleiotrophin cellular localization in nerve regeneration after peripheral nerve injury. J Histochem Cytochem 2005: 53: 971-7.
50. 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: 1097-105.
51. DeLisser HM, Christofidou-Solomidou M, Strieter RM, et al. Involvement of endothelial PECAM-1/CD31 in angiogenesis. Am J Pathol 1997: 151: 671-7.
52. Li J, Parker B, Martyn C, Natarajan C, Guo J. The PMP22 gene and its related diseases. Mol Neurobiol 2013: 47: 673-98.
53. Barros CS, Franco SJ, Muller U. Extracellular matrix: functions in the nervous system. Cold Spring Harb Perspect Biol 2011: 3: a005108.
54. 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.
55. Reid AJ, Sun M, Wiberg M, et al. Nerve repair with adipose-derived stem cells protects dorsal root ganglia neurons from apoptosis. Neuroscience 2011: 199: 515-22.
56. 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: 2788-96.
57. Greenbaum D, Colangelo C, Williams K, Gerstein M. Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 2003: 4: 117.

58. Hu J, Zhu QT, Liu XL, Xu YB, Zhu JK. Repair of extended peripheral nerve lesions in rhesus monkeys using acellular allogenic nerve grafts implanted with autologous mesenchymal stem cells. Exp Neurol 2007: 204: 658-66.
59. Wang Y, Zhao Z, Ren Z, et al. Recellularized nerve allografts with differentiated mesenchymal stem cells promote peripheral nerve regeneration. Neurosci Lett 2012: 514: 96-101.
60. Fan L, Yu Z, Li J, Dang X, Wang K. Schwann-like cells seeded in acellular nerve grafts improve nerve regeneration. BMC Musculoskelet Disord 2014: 15: 165.
61. Keilhoff G, Goihl A, Langnase K, Fansa H, Wolf G. Transdifferentiation of mesenchymal stem cells into Schwann cell-like myelinating cells. Eur J Cell Biol 2006: 85: 11-24.
62. Rbia N, Bulstra LF, Thaler R, et al. In Vivo Survival of Mesenchymal Stromal Cell-Enhanced Decellularized Nerve Grafts for Segmental Peripheral Nerve Reconstruction. J Hand Surg Am 2018.

CHAPTER 6

In-vivo survival of mesenchymal stromal cell-enhanced decellularized nerve grafts for segmental peripheral nerve reconstruction.

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ABSTRACT

Purpose

Adipose-derived Mesenchymal Stromal Cells (MSCs) have emerged as promising tools for peripheral nerve reconstruction. There is a paucity of information regarding the ultimate survivorship of implanted MSCs or if these cells remain where they are placed. The aim of the present study was to track the in-vivo distribution and survival of MSCs seeded on a decellularized nerve allograft reconstruction of a peripheral nerve defect using luciferase based bioluminescent imaging (BLI).

Methods

To determine the in-vivo survivability of MSCs, autologous Lewis rat-MSCs were stably labeled with luciferase by lentiviral particles. Labeled cells were dynamically seeded onto a Sprague Dawley decellularized rat nerve allograft and used to bridge a 10 mm sciatic nerve defect. MSC survival was determined by performing in-vivo bioluminescence imaging to detect living cells. Twelve animals were examined at 24 hours after implantation, 3, 7, 9, 11, 14 days and at daily intervals thereafter if signal were still present.

Results

Results demonstrated that labeled MSCs could be detected for up to 29 days. Gradually diminishing BLI signals were observed within the first week following implantation. Implanted MSCs were not detected anywhere other than the site of surgery.

Conclusions

This study provides experimental evidence that MSCs seeded on decellularized nerve allografts can survive in-vivo, but have finite survival after implantation. There was no evidence of migration of MSCs to surrounding tissues. This understanding of the viability and distribution of implanted MSCs can be considered for future strategies to optimize nerve graft related therapies.

INTRODUCTION

Outcomes following peripheral motor nerve injury remain poor.[1] It has been shown experimentally that even in the best case scenario, only half of the neurons regenerate their axons into the distal stump.[2] Where sensory recovery can be obtained with some of the current reconstructive options (synthetic conduits and processed acellular allografts), obtaining satisfactory motor nerve recovery still remains a challenge.[3] Acellular nerve allografts, in contrast to synthetic conduits, contain the natural nerve structure and the essential nerve extracellular matrix (ECM), but lack Schwann cells and axons. Supplementing acellular nerve grafts with cells may improve outcomes of peripheral nerve reconstruction.[4,5]

Adipose-derived Mesenchymal Stromal Cells (MSCs) have emerged as promising tools for regenerative therapies and have been shown to have positive effects in a large number of experimental peripheral nerve injury studies.[4-6] Particularly, MSC implantation decreases muscular atrophy, while facilitating sorting of axons and myelination and reducing inflammation. [7] MSCs may promote tissue regeneration through different mechanisms, including cell differentiation, cell fusion and paracrine effects via the secretion of various growth factors.[8] However, the long-term fate and effects of implanted MSCs, their definitive mechanism of action and survivability are not well understood.[9] [10]

One obstacle in the routine use of MSCs is the reported low survivorship of implanted MSCs. In general, fewer than 5% of implanted cells persist at the site of implantation.[11,12] The major causes of poor in vivo survival of MSCs include ischemia at the site of implantation, extracellular matrix degradation, oxidative stress, inflammation and immune rejection.[13] Within these environments, the majority of MSCs undergo apoptosis prior to participating in lineage differentiation and cellular integration.[14] It therefore appears that the therapeutic effect of cell-based therapies does not fundamentally depend on the persistence of cells at the site of tissue injury, but a clear understanding of the survivorship, localization and identity of administered cells over time remains of great interest.[15]

Luciferase-based non-invasive bioluminescence imaging (BLI) allows real-time in vivo monitoring of location and proliferation of luciferase-expressing MSCs.[16] In this study we used BLI to investigate the survival, viability and distribution of autologous MSCs seeded onto an acellular rat nerve allograft used to reconstruct a sciatic nerve defect.

MATERIALS AND METHODS

To determine the survivability of MSCs seeded on a decellularized nerve allograft in vivo, autologous Lewis rat-MSC were stably labeled with luciferase using lentiviral particles. Cells were dynamically seeded onto a Sprague Dawley decellularized rat nerve allograft and used to bridge a 10 mm sciatic nerve defect. MSC survival was determined by performing in-vivo bioluminescence imaging to detect living MSCs. Twelve animals were examined at 24 hours after implantation, 3 days, 7 days, 9 days, 11 days, 14 days, and at daily intervals thereafter if signal were still present. The presence of luciferase was demonstrated by immunohistochemical data. This study was approved by our IACUC institutional review committee.

Isolation and characterization of rat MSCs

Rat MSCs were obtained from isogenic Lewis rats, as previously described by Kingham et al.[17] 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 (aMEM) (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).

Lentiviral transduction

The MSCs were lentivirally transduced to express the firefly luciferase. This method has previously been shown to display minor cytotoxicity with no differences in viability and cell proliferation between labeled and unlabeled cells.[18,19] Fifty to sixty percent confluent MSCs passage 2 were labeled luciferase positive using a replication incompetent lentiviral vector (PLVX-puro). CMV based lentivirus particles were prepared by transfection of 4 plasmids (pMDLg/PRRE, Prsv-REV and pMD2.G and PLVX-puro) (Addgene, Cambridge, Massachusetts) into human HEK293 cells using a high-efficiency transfection reagent (Promega, Wisconsin). Cell media containing the viral particles was collected and the media was used to infect the rat MSCs for three times 8 hours. Infected cells were split after 24-hours and drug-selected with Puromycin (InVivoGen, San Diego, CA) for 48-hours to guarantee a luciferase positive cell population. Subsequently cells were cultured in aMEM containing 5% Platelet Lysate, 2 mmol/L Glutamax, 2 U/ml heparin and 1% penicillin/streptomycin solution. Cell cultures were maintained in a 37 °C incubator with 5% CO2 until 90% confluent.

In-vitro viability assay

To determine the luciferase activity in-vitro, a luciferase assay system (Steady-Glo® Luciferase Assay System, Promega, Madison, USA) was performed following manufacturer's instructions on the transfected MSCs. Different quantities (1.250, 2.500, 5.000, 10.000) of transfected MSCs were plated onto 96-well plates in 100 µl medium. A row of wells was plated with unlabeled cells as controls. To every well, 15 µl of luciferase assay reagent was added and light produced was measured by a microplate reader (Tecan, Maennedorf, Switzerland)). The correlation between luminescence intensity and the number of MSCs was graphed and analyzed by a regression plot.

Preparation of allografts

A total of 15 Rat sciatic nerve segments of 1.5 cm were harvested from Sprague-Dawley rats weighing 250-350 grams (Harlan, Indianapolis, IN) after isoflurane induction and euthanasia with an overdose of sodium pentobarbital. To closely mimic the human nerve allografting procedure, Sprague-Dawley rats were chosen as donors as there is a major histocompatibility mismatch to the Lewis rat (host animal).[20] Sciatic nerves were aseptically excised, cleared of peripheral fat/connective tissue and decellularized using the protocol utilizing elastase as previously described.[21] 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.

Surgical procedure and seeding method

Lewis rats (N=15) were anesthetized with an intraperitoneal cocktail 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. Ringer's lactate solution 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 GmbH, Oberkochen, Germany). Decellularized Sprague-Dawley donor nerves (N=12) were seeded with 1 million (clinical grade) Lewis rat MSCs passage 5.[22] A dynamic bioreactor was used to seed the MSCs, using the protocol as previously described and validated by Rbia et al.[23] Briefly, the nerve allografts were soaked for 2 hours in cell culture medium to remove harmful remnants of the decellularization process, nerves were combined with the cells in a 15 ml Bioreactor tube containing 10 ml cell culture medium and 1 million cells/nerve, with a maximum of 4 nerve segments/tube. Following, the tubes were placed and secured horizontally in a bioreactor rotator system (Revolver™ Labnet, Edison, USA), tubes were balanced on the rotator with a rotation axis of 30°, the rotator was turned on at fixed speed 18 rpm to remove the gravitational component and was place in an incubator at 37 degree Celsius (98.6 Fahrenheit) and 5% CO2. The nerves and cells were incubated for 12 hours and subsequently the MSC seeded allograft was used to bridge the 10 mm nerve gap using 10-0 nylon epineural sutures. In the control group (N=3), a processed nerve allograft without MSCs was used to bridge the gap. The muscle was approximated with 5-0 Vicryl rapid sutures and the skin was closed with the same 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.

Bioluminescence imaging

Luciferase activity was assessed at different time points using an in-vivo imaging system (IVIS ; 100; Xenogen, Alameda, California, now Caliper Life Sciences, Hopkinton, Massachusetts). After induction of anesthesia with Isoflurane, 2 ml D-Luciferin solution (VivoGlo™Luciferin, In Vivo Grade, Promega, Madison, USA) in 0.9% NaCl (15 mg/ml) was injected intraperitoneally prior to imaging. To reduce background signals, the skin was shaved locally. Twenty minutes after injection, the rats were positioned in the IVIS chamber and peak luminescence was measured by taking sequential images with an integration time of 5 minutes and a delay time of 1 minute using Living Image Software for IVIS.

Immunohistochemistry

Immunohistochemical staining was obtained to confirm that the Luciferase labeled MSCs were successfully seeded onto the nerve allograft and to study whether the nerve repair was successful. If the BLI signal was faded, anesthesia was induced and all animals were sacrificed with an intraperitoneal overdose of Fatal-Plus (Vortech Pharmaceuticals,

Dearborn, MI). The nerve allografts were explanted over a length from 5mm proximal to the graft to 5mm distal to the graft. Samples were fixed in 4% Paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), suspended in Tissue-Tek OCT Compound (optimal cutting temperature) (Sakura Finetek, Torrance, CA, USA), and snap frozen in liquid nitrogen-cooled Isopentane (Sigma-Aldrich, St. Louis, MO, USA). Transverse sections (5 µm thick) were cut on a cryostat (Leica CM1850) at different levels within the repair site. Next, immunohistochemical staining was obtained for Luciferase and Schwann-cell marker S100. The immunohistochemical staining procedure was performed using the Leica Bond RX Stainer (Leica Microsystems, Buffalo Grove, IL, USA). Frozen sections slides were stored at -80C and dried for 2 hrs. at room temperature prior to staining. The slides were manually postfixed in 4% paraformaldehyde and retrieved online using Epitope Retrieval 1 (Leica Microsystems, Buffalo Grove, IL, USA) for 5 minutes. Sections were incubated for 60 minutes at 24˚C in the following primary antibodies: polyclonal S100 anti–rabbit (Dako Z0311, Agilent Technologies, Santa Clara, CA, USA) was used at 1:5000 and polyclonal anti-rabbit Firefly Luciferase (Abcam ab21176) was used at 1:2000. Sections were then washed with PBS and incubated with secondary antibodies (Alexa Fluor 568 or 488 Goat-anti-Rabbit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) at 24˚C for 60 minutes. The Research Detection System (Leica DS9455, Leica Microsystems, Buffalo Grove, IL, USA) was used and included Rodent Block R (Biocare RBR962, Biocare Medical, Pacheco, CA, USA). Cell nuclei were stained with Hoechst 33342 (Invitrogen H1399, Thermo Fisher Scientific, Waltham, MA, USA). Once completed, slides were removed from the stainer and rinsed for 5 minutes in distilled water. Slides were coverslipped using ProLong Gold antifade media (Invitrogen) and were directly observed by a confocal microscope (LSM 780, Zeiss, Oberkochen, Germany).

Image analysis

The optical fluorescent image data was displayed in units of photons and quantification of the luminescence signal was performed by the Living Image Software as units of total flux (photon/sec) in an area of interest subtracted from the background signal. Quantitative values were expressed as the mean ± standard error of the mean (SEM). Regression plots were used to describe the association between bioluminescence and days after implantation.

RESULTS

This study examined the survival, viability and distribution of autologous MSCs seeded onto an acellular rat nerve allograft used to reconstruct a sciatic nerve defect. The multi-lineage potential of rat MSCs after expanses in PLTmax was demonstrated upon differentiation in osteogenic or adipogenic media, which revealed Oil Red and Alizarin Red positive cells (Figure 1).

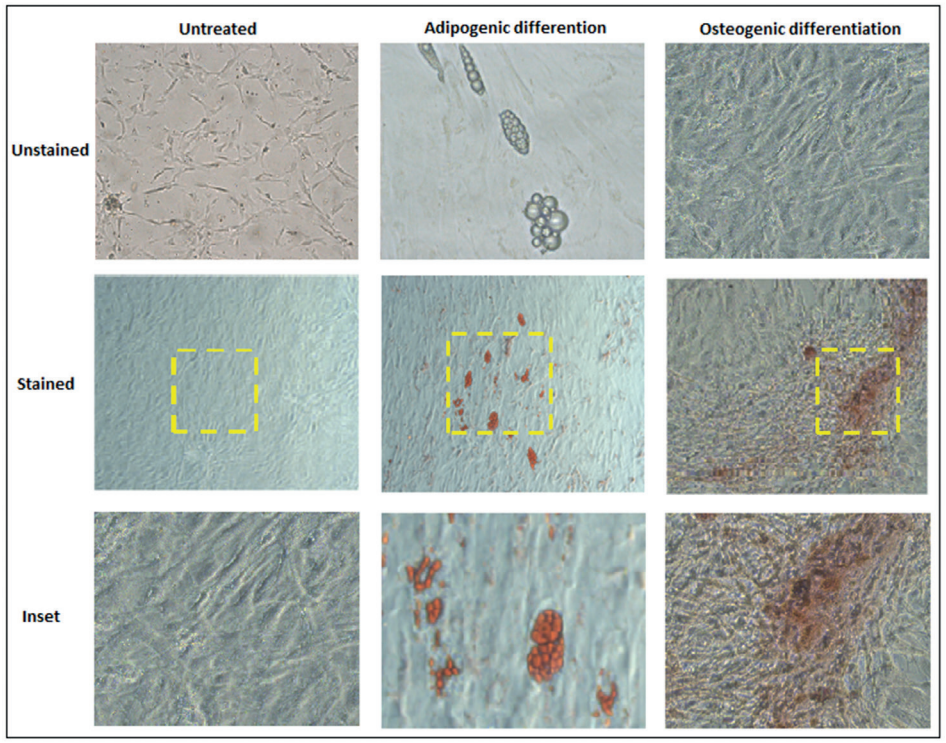

Figure 1. Characterization of rat MSCs. The multi-potency of the rat MSCs was confirmed by differentiation into osteogenic and adipogenic lineages. Osteogenic differentiation was performed by Alizarin Red staining and adipogenic differentiation by Oil Red staining. Inset, 10x magnification. The control group (untreated cells) did not stain positive.

In-vitro viability

Light production capacity of the lentivirally transduced MSCs was measured in lysates from predetermined numbers of cells (i.e., 1250, 2500, 5000 and 10,000). The minimum cell number needed for light production was not determined, as the in-vivo cell dose was predetermined at 1 million cells.[22] Luciferase was expressed steadily in-vitro and the

unlabeled control group did not produce bioluminescence. Figure 2 depicts the linear regression plot of light production versus cell number. The number of MSCs was linearly correlated with light production, indicating that the cells could be used to quantitatively track the luciferase signal in an in-vivo model.

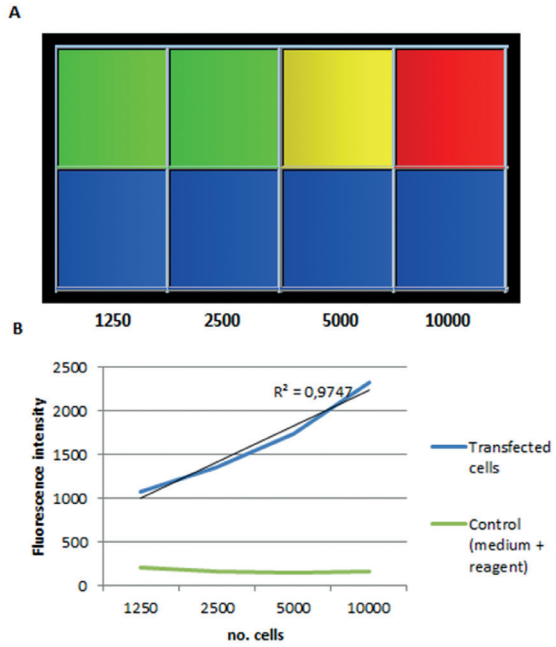

Figure 2. In-vitro viability assay. In-vitro bioluminescence imaging of serial dilution of lentivirally transduced MSCs (1.250, 2.500, 5.000, 10.000 cells). Luciferase was expressed steadily in-vitro and the unlabeled control group did not produce bioluminescence. (A) The colors indicate the light production level: blue, lowest intensity; red, highest intensity. (B) Plots of light production extracted from the images versus the number of MSCs. Quantitative analysis showed a linear relationship.

Bioluminescence imaging

All animals survived the surgical procedure and no complications were observed. The control group showed no signal and the BLI signal was not detected anywhere other than the site of surgery. After surgery, noninvasive imaging was performed at day 1, 3, 7, 9, 11, 14 days, and at daily intervals thereafter if signal were still present. After 24 hours, the bioluminescence signals were clearly observed. In one animal, no signal could be detected 24 hours after implantation. This animal was therefore removed from all data analyses. Figure 3 depicts serial bioluminescent imaging of the rats, demonstrating a gradually diminishing MSC survival over 29 days. Dramatic decreases of BLI signals, from

6.28 x 10^4 ± 1.29 x 10^4 to 3.73 x 10^4 ± 7.95 x 10^3 (photons/sec/cm2/steradian), were observed within the first week following implantation (figure 4.) At day 30, no signals were detected.

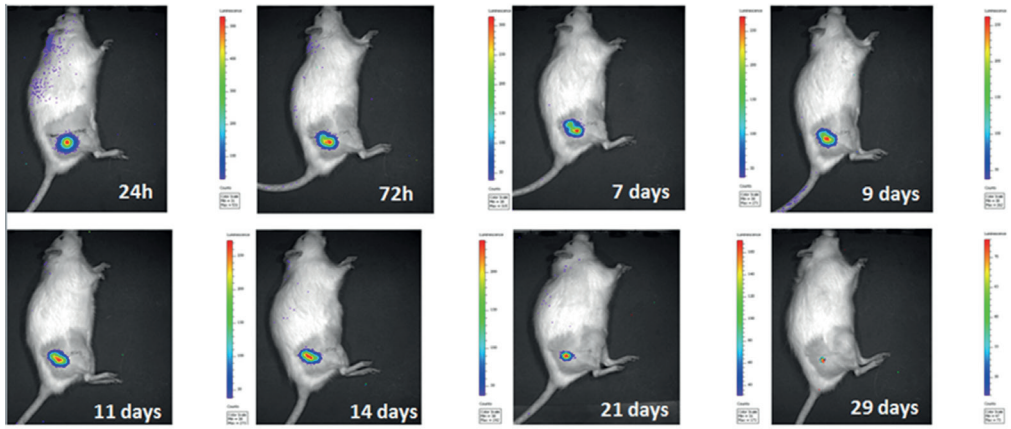

Figure 3. In-vivo bioluminescence. Serial bioluminescent imaging of the rats demonstrates a gradually diminishing MSC survival over 29 days. At day 30, no signals were detected. No visible signal was observed in the control group. The color bar indicates the bioluminescence intensity in photons per seconds cm^2 per steradian, from the lowest (blue) to the highest (red).

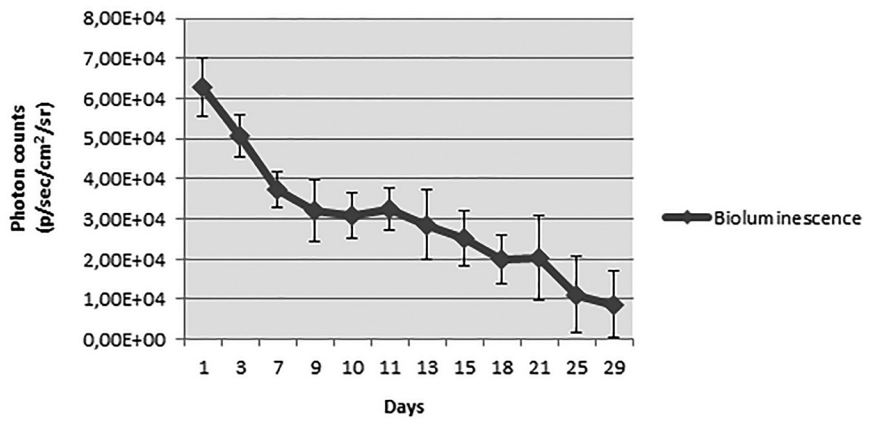

Figure 4. Quantification of bioluminescence. BLI signals were extracted from the analysis software Living Image, and plotted versus the elapsed time post- implantation. Dramatic decreases of BLI signals (photons/sec/cm2/steradian), were observed within the first week following implantation. Bars represent mean ± SEM (N=11).

Immunohistochemistry

Immunohistochemical staining was obtained to confirm that the Luciferase labeled MSCs were successfully seeded onto the nerve allograft and to study whether the nerve repair was successful. Luciferase-labeled MSCs were observed in the peripheral areas of the nerve graft (Figure 5). In contrast, no Luciferase-labeled cells were observed in the negative control group (Figure 5).

Analysis of the mid portion of the nerve grafts by Immunohistochemical staining for Schwann-cell marker S100 revealed that nerve regeneration occured in all groups (Figure 6).

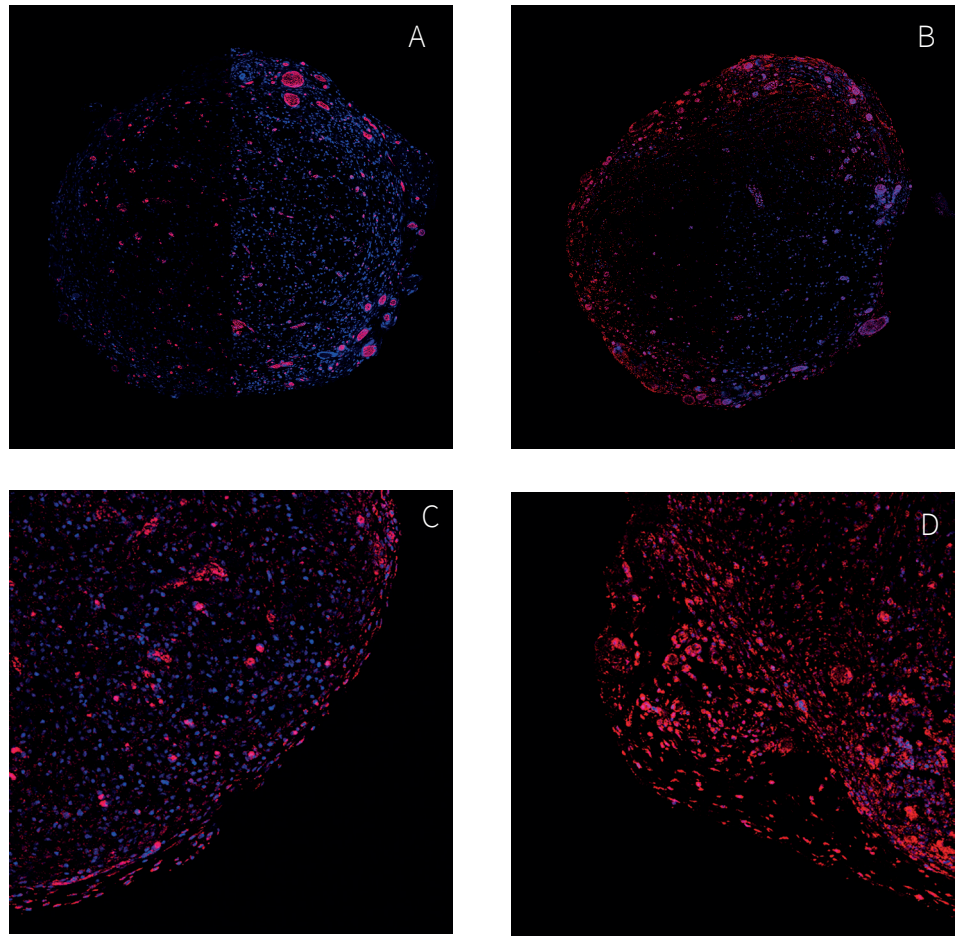

Figure 5. Immunohistochemical stain for Luciferase-positive cells. A) Negative control (Luciferase stain, but no Luciferase in the MSCs), B) Luciferase labeled cells are visible in the peripheral aras of the nerve graft, C and D) Close-up.

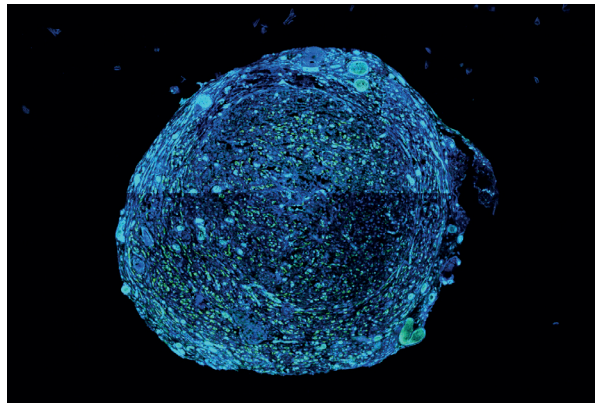

Figure 6. Immunohistochemical stain for S100. Analysis of the mid portion of the nerve grafts revealed that nerve regeneration occurred in all groups.

DISCUSSION

In this study, we successfully monitored the in-vivo survival and distribution of MSCs seeded on decellularized allografts used to reconstruct a rat sciatic defect by cell tracking using luciferase labeling and BLI. This method is a noninvasive, longitudinal approach for in vivo monitoring of implanted cells. The results demonstrated that the labeled MSCs could be detected for up to 29 days in vivo. The BLI signal declined rapidly within the first week following implantation; suggesting early cell death, and gradually diminishing BLI signals were observed until the end of the experiment. Furthermore, we showed that the cells on the allograft remained at the implantation site and there was no evidence of migration from the sciatic nerve to any other location. In one animal, no signal could be detected 24 hours after implantation. Because immune responses, labelling errors or detection limits are unlikely, and because bioluminescence can only be detected if the cells are viable[24], it is possible that cells may have undergone apoptosis or vacated the graft during surgery.

Survival of MSCs (delivered to nerve injury sites) is rarely reported in studies of stem cell therapy for nerve reconstruction with decellularized allografts.[25-27] There is a notable discrepancy in the finding of these studies; different survival patterns have been described in the literature, such as a gradual decrease, stabilized signals or gradually increased signals in the first days after implantation. In a study by Ma and colleagues the fate of Schwann cells seeded on nerve guides was examined. The authors observed a gradual decrease of the in-vivo signal intensity over time, as detected with IVIS, and transcutaneous

signal could be detected up to 7 days after implantation.[28] A recent study by Strohschein used luciferase-based BLI to investigate the survival of autologous MSCs implanted into a severely crushed soleus muscle of the rats and could track the MSCs until day 7 irrespective of local or intra-arterial administration.[24] Results from studies by Wolbank and others indicated that BLI allowed for real time tracking of luciferase labelled MSCs for at least 20 days after implantation within fibrin matrix subcutaneously in nude mice. It was also found that the reporter gene expression did not interfere with cell phenotypes and stem cell properties.[29]

Other studies reported that 5-bromo-2-deoxyurudine (BrdU) labeled MSCS in 3-D collagen scaffolds were identifiable for 30 days in a porcine model.[30] Table 1 provides a summary of previous studies reporting on in-vivo stem cell survival in relation to peripheral nerve grafts.

Table 1. Stem cell survival in peripheral nerve literature.

Author (year)	Seeding method (cells/ml)	Cell labeling method	Cell source	Survival
Erba (2010)[1]	Transplanted in an artificial nerve conduit	GFP-labelling	ADSCs	< 14 days
Murakami et al. (2003)[2]	Embedden in collagen gel	BrdU-labelling	Amniotic fluid/ MSCs	Up to 10 days, none at 4 weeks
Dezawa et al. (2001)[3]	Transplanted into the cut ends of sciatic nerves	GFP-labelling	BMSCs	More than 3 weeks
Shimizu et al. (2007)[4]	Filled into a transpermeable tube	GFP- labelling	BMSCs	More than 3 weeks
Strohschein (2015)[5]	Transplanted into a crushed soleus muscle	Luciferase-labelling	BMSCs	Until day 7
Ming-san ma (2009)[6]	Seeded in nerve guides	Luciferase-labelling	Scwann cells	Up to 7 days
Suganuma (2013)[7]	Seeded in a nerve tube	PKH-26-labelling	AMSCs	14 days
Tennstaedt (2015)[8]	Implanted into striatum of mouse brain	Luciferase	hNSCs	14 days
Do Won Hwang (2014)[9]	Embedded within a scaffold	Luciferase	hNSCs	8-14 days

Table 1. Stem cell survival in peripheral nerve literature. (continued)

Author (year)	Seeding method (cells/ml)	Cell labeling method	Cell source	Survival
Nakabayashi (2013)[10]	Into an injured tibialis anterior muscle	Luciferase	MSCs	Up to 7 days
Min (2006)[11]	Into myocardium	Luciferase	MSCs	Up to 8 days
Zhuo (2013)[12]	Intravenous administration	Luciferase	MSCs	Up to 7 days
Lassailly (2010)[13]	Intravenous injection	Lipophilic dyes	Human HL60 cells	14 days
Lequeux (2011)[14]	Subcutaneous implantation of cells seeded in collagen scaffold	BrdU labeling	ADSCs	4 weeks
Wolbank(2007)[15]	Subcutaenous within fibrin matrix	Luciferase	ADSCs	20 days

A summary is provided of previous studies reporting on in-vivo stem cell survival (in peripheral nerve literature). Abbreviations: green fluorescent protein (GFP), bromodeoxyuridine (BrdU), adipose-derived stem cells (ADSCs), mesenchymal stromal cells (MSC), bone marrow stromal cells (BMSCs), red fluorochrome (PKH-26), human neural stem cells (hNSCs).

Interpretation of the literature is further complicated by the different methods to track MSCs. To date, a variety of fluorescent dyes have been used that can reversibly or irreversibly bind to the cell nucleus (5-bromo-2'-deoxyuridine (BrdU)) or the cell membrane (PKH26). The membrane labeling process is simple but the usefulness is limited to the short term (10-11 days) and the dye may be cytotoxic to cells. Fluorescent dyes also fade with serial passages and rapidly contaminate neighboring cells by macrophage-mediated phagocytosis or other mechanisms, resulting in false positive contamination of host cells. Nucleus labeling has no effects on the MSCs differentiation/proliferation potential and is not cytotoxic, but is not suitable for non-invasive methods of detection. Another concern is that dyes tend to be relatively resistant to degradation and therefore may not lose their fluorescence once the cells die, which could lead to a non-cell-associated signal.[31]

Genetic labeling with a GFP or luciferase reporter gene in combination with bioluminescence imaging has been used extensively and does not alter the proliferation or differentiation capacity of the cells.[18,19,32] It does not require sacrifice of the animal and is a well-established method for longitudinal cell viability monitoring.[24,29,33,34] Genetic labeling with GFP or luciferase reporter gene with BLI is not deleterious to transduced cells and it appears to be a relatively long-lasting method. The lentiviral vector transduces both

replicating and quiescent cells, which is an unique feature as other Retroviruses can infect only quiescent cells. Genetic labeling is stable and does not degrade until cell death and provides information of the cells' viability and proliferation. The non-invasiveness is a marked advantage compared to conventional fluorescent cell viability assays.[16]

Limitations of genetic labeling include the vector and the protein could theoretically be transmitted to host cells resulting in contamination. This technique is not suitable for use with fresh uncultured cells and it should be noted that some host tissues may have endogenous fluorescence, which would result in a false positive signal.[29] Although BLI provides an unique methodology, quantitative analysis must be approached with caution, and validation for each specific application is necessary. [35] In this study, the minimal number of detected cells in vivo was not pre-determined and no histology was performed on surrounding tissues, therefore it might be possible that a very small fraction of cells did migrate to surrounding tissues and that these were not detected by the BLI method. Although, it has been previously demonstration, in transplant patients, that MSCs do not migrate between tissues.[36] The BLI method has also been previously used in many studies to monitor MSC survival in-vivo and migration.[24] Wu et al. performed in vivo imaging of MSCs and harvested the surrounding tissue and detected no migration to other organs.[19] Similarly, Eckhard et al. and Wolbank et al. demonstrated no BLI signals in other tissues or organs by immunofluorescence staining.[29,37]

Bioluminescence signals vary per cell source, recipient species, grafting material, concentration and cell delivery method.[24,38] The low cell viability is commonly attributed to: 1) anchorage-related programmed cell death (anoikis) that occurs due to the loss of ECM attachment. 2) Nutrient and oxygen deprivation in the injured environment. 3) Inflammatory cells such as neutrophils, monocytes and macrophages, which are recruited by chemokines or cytokines, inducing apoptosis.[14,39]

Previously, investigators have placed great emphasis on the retention and differentiation of implanted MSCs.[36,38] To improve the survival of the implanted MSCs, strategies have been developed, such as pretreatment with cytokines, growth factors, genetic modifications and hypoxic preconditioning.[39] It appeared that differentiated MSCs have a greater propensity for survival compared with undifferentiated MSCs.[40] In contrast, multiple studies have supported the hypothesis that MSCs contribute to an improved functional outcome through a secretory effect rather than in-vivo differentiation. Supporters of in-vivo differentiation must clearly reconcile the fact that differentiation takes longer than

the average reported survival time of the MSCs. In-vitro differentiation (with induction agents) of MSCs to Schwann-cell like cells occurs after approximately 21 days.[41] Looking at the lifespan of the MSCs in this study, it is possible that a fraction of the cells have differentiated. As differentiated cells would still be viable and carry the luciferase gene, they are still detectable by BLI. Some investigators have noted therapeutic effects with few remaining cells and in the absence of differentiation, suggesting alternative mechanisms of support, such as enhancing host repair mechanisms.[24,38] Alternatively, it could be that MSCs are able to rapidly pass on their effect to other cells that subsequently mediate tissue repair or that the clean-up process of the MSCs may be a trigger for the therapeutic effects.[36]

Another popular method to increase the viability of MSCs by the use of scaffolds. Both naturally and synthetically derived scaffolds provide structural support and can provide physical and chemical cues to guide growth and integration into the host tissue.[14] The findings of this study have clearly demonstrated that MSCs seeded on decellularized nerve allografts can survive in vivo for a limited period of time and there was no evidence of migration of MSCs from the decellularized nerve allograft. Understanding the viability and distribution of the implanted MSCs in-vivo is a prerequisite for better understanding the role of MSCs in the therapeutic process. Future studies should further focus on the fate of implanted MSCs to ascertain the role of MSC in aiding peripheral nerve regeneration.

REFERENCES

1. Fairbairn NG, Meppelink AM, Ng-Glazier J, Randolph MA, Winograd JM. Augmenting peripheral nerve regeneration using stem cells: A review of current opinion. *World J Stem Cells.* 2015;7(1):11-26.
2. Welin D, Novikova LN, Wiberg M, Kellerth JO, Novikov LN. Survival and regeneration of cutaneous and muscular afferent neurons after peripheral nerve injury in adult rats. *Exp Brain Res.* 2008;186(2):315-323.
3. 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.
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. 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.
6. Zhang YR, Ka K, Zhang GC, et al. Repair of peripheral nerve defects with chemically extracted acellular nerve allografts loaded with neurotrophic factors-transfected bone marrow mesenchymal stem cells. *Neural Regen Res.* 2015;10(9):1498-1506.
7. Jiang L, Jones S, Jia X. Stem Cell Transplantation for Peripheral Nerve Regeneration: Current Options and Opportunities. *Int J Mol Sci.* 2017;18(1).
8. Baldari S, Di Rocco G, Trivisonno A, Samengo D, Pani G, Toietta G. Promotion of Survival and Engraftment of Transplanted Adipose Tissue-Derived Stromal and Vascular Cells by Overexpression of Manganese Superoxide Dismutase. *Int J Mol Sci.* 2016;17(7).
9. Kingham PJ, Reid AJ, Wiberg M. Adipose-derived stem cells for nerve repair: hype or reality? *Cells Tissues Organs.* 2014;200(1):23-30.
10. Erba P, Mantovani C, Kalbermatten DF, Pierer G, Terenghi G, Kingham PJ. Regeneration potential and survival of transplanted undifferentiated adipose tissue-derived stem cells in peripheral nerve conduits. *J Plast Reconstr Aesthet Surg.* 2010;63(12):e811-817.
11. Burdick JA, Mauck RL, Gerecht S. To Serve and Protect: Hydrogels to Improve Stem Cell-Based Therapies. *Cell Stem Cell.* 2016;18(1):13-15.
12. Sart S, Ma T, Li Y. Preconditioning stem cells for in vivo delivery. *Biores Open Access.* 2014;3(4):137-149.
13. Hwang DW, Jin Y, Lee DH, et al. In vivo bioluminescence imaging for prolonged survival of transplanted human neural stem cells using 3D biocompatible scaffold in corticectomized rat model. *PLoS One.* 2014;9(9):e105129.

14. Hyun JS, Tran MC, Wong VW, et al. Enhancing stem cell survival in vivo for tissue repair. *Biotechnol Adv.* 2013;31(5):736-743.
15. Agrawal H, Shang H, Sattah AP, Yang N, Peirce SM, Katz AJ. Human adipose-derived stromal/stem cells demonstrate short-lived persistence after implantation in both an immunocompetent and an immunocompromised murine model. *Stem Cell Res Ther.* 2014;5(6):142.
16. Peeters M, van Rijn S, Vergroesen PP, et al. Bioluminescence-mediated longitudinal monitoring of adipose-derived stem cells in a large mammal ex vivo organ culture. *Sci Rep.* 2015;5:13960.
17. 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.
18. De Vocht N, Bergwerf I, Vanhoutte G, et al. Labeling of Luciferase/eGFP-expressing bone marrow-derived stromal cells with fluorescent micron-sized iron oxide particles improves quantitative and qualitative multimodal imaging of cellular grafts in vivo. *Mol Imaging Biol.* 2011;13(6):1133-1145.
19. Dai M, Xu P, Hou M, Teng Y, Wu Q. In vivo imaging of adipose-derived mesenchymal stem cells in female nude mice after simulated childbirth injury. *Exp Ther Med.* 2015;9(2):372-376.
20. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. *Tissue Eng.* 2004;10(9-10):1346-1358.
21. 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.
22. Chen BK, Staff NP, Knight AM, et al. A safety study on intrathecal delivery of autologous mesenchymal stromal cells in rabbits directly supporting Phase I human trials. *Transfusion.* 2014.
23. Rbia NB, L.F.; Bishop, A.T.; van Wijnen, A.J.; Shin, A.Y. A simple dynamic strategy to deliver stem cells to decellularized nerve allografts. *Accepted at Plast Reconstr Surg.* 2018.
24. K S, P R, T W, G ND, C P, vR P. In Vivo Bioluminescence Imaging - A Suitable Method to Track Mesenchymal Stromal Cells in a Skeletal Muscle Trauma. *Open Orthop J.* 2015;9:262-269.
25. Wang D, Liu XL, Zhu JK, et al. Bridging small-gap peripheral nerve defects using acellular nerve allograft implanted with autologous bone marrow stromal cells in primates. *Brain Res.* 2008;1188:44-53.
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. Zhao Z, Wang Y, Peng J, et al. Repair of nerve defect with acellular nerve graft supplemented by bone marrow stromal cells in mice. *Microsurgery.* 2011;31(5):388-394.

28. Ma MS, Van Dam G, Meek M, Boddeke E, Copray S. In vivo bioluminescent imaging of Schwann cells in a poly(DL-lactide-epsilon-caprolactone) nerve guide. *Muscle Nerve.* 2009;40(5):867-871.
29. Wolbank S, Peterbauer A, Wassermann E, et al. Labelling of human adipose-derived stem cells for non-invasive in vivo cell tracking. *Cell Tissue Bank.* 2007;8(3):163-177.
30. Lequeux C, Oni G, Mojallal A, Damour O, Brown SA. Adipose derived stem cells: efficiency, toxicity, stability of BrdU labeling and effects on self-renewal and adipose differentiation. *Mol Cell Biochem.* 2011;351(1-2):65-75.
31. Progatzky F, Dallman MJ, Lo Celso C. From seeing to believing: labelling strategies for in vivo cell-tracking experiments. *Interface Focus.* 2013;3(3):20130001.
32. Schomann T, Mezzanotte L, Lourens IM, de Groot JC, Frijns JH, Huisman MA. Lentiviral transduction and subsequent loading with nanoparticles do not affect cell viability and proliferation in hair-follicle-bulge-derived stem cells in vitro. *Contrast Media Mol Imaging.* 2016;11(6):550-560.
33. Lucignani G, Rodriguez-Porcel M. In vivo imaging for stem cell therapy: new developments and future challenges. *Eur J Nucl Med Mol Imaging.* 2011;38(2):400-405.
34. Wang J, Jokerst JV. Stem Cell Imaging: Tools to Improve Cell Delivery and Viability. *Stem Cells Int.* 2016;2016:9240652.
35. Sadikot RT, Blackwell TS. Bioluminescence imaging. *Proc Am Thorac Soc.* 2005;2(6):537-540, 511-532.
36. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol.* 2014;5:148.
37. Bai X, Yan Y, Coleman M, et al. Tracking long-term survival of intramyocardially delivered human adipose tissue-derived stem cells using bioluminescence imaging. *Mol Imaging Biol.* 2011;13(4):633-645.
38. Walsh SK, Kumar R, Grochmal JK, Kemp SW, Forden J, Midha R. Fate of stem cell transplants in peripheral nerves. *Stem Cell Res.* 2012;8(2):226-238.
39. Lee S, Choi E, Cha MJ, Hwang KC. Cell adhesion and long-term survival of transplanted mesenchymal stem cells: a prerequisite for cell therapy. *Oxid Med Cell Longev.* 2015;2015:632902.
40. 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.
41. Han I, Kwon BS, Park HK, Kim KS. Differentiation Potential of Mesenchymal Stem Cells Is Related to Their Intrinsic Mechanical Properties. *Int Neurourol J.* 2017;21(Suppl 1):S24-31.

CHAPTER 7

Gene expression and growth factor analysis in early nerve regeneration following segmental nerve defect reconstruction with a mesenchymal stromal cell-enhanced decellularized nerve allograft

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ABSTRACT

Purpose

The purpose of this study was to evaluate the molecular mechanisms underlying nerve repair by a decelullarized nerve allograft seeded with adipose-derived mesenchymal stromal cells (MSCs) and compare it to the unseeded allograft and autograft nerve.

Methods

Undifferentiated MSCs were seeded onto decellularized nerve allografts and used to reconstruct a 10 mm gap in a rat sciatic nerve model. Gene expression profiles of genes essential for nerve regeneration and immunohistochemical staining (IHC) for PGP9.5, NGF, RECA-1 and S100 were obtained 2 weeks postoperatively.

Results

Semi-quantitative RT-PCR analysis showed that the angiogenic molecule *VEGFA* was significantly increased in seeded allografts and transcription factor *SOX2* was down regulated in seeded allografts. Seeded grafts showed a significant increase in immunohistochemical markers NGF and RECA-1, when compared to unseeded allografts.

Conclusions

MSCs contributed to the secretion of trophic factors. A beneficial effect of the MSCs on angiogenesis was found when compared to the unseeded nerve allograft, but implanted MSCs did not show evidence of differentiation into SC-like cells.

INTRODUCTION

Nerve graft substitutes remain inferior to autografts for the repair of motor and mixed peripheral nerve injuries.[1] Nerve regeneration is a complicated process highlighted by Wallerian degeneration, axonal sprouting, and myelination.[2] In response to injury, Schwann cells (SCs) produce high levels of neurotrophic growth factors.[3] Successful crossing of a nerve gap depends on the formation of a new extracellular matrix (ECM) scaffold, over which blood vessels, fibroblasts and SCs can migrate and regenerate towards the distal nerve stump.[4] Acellular nerve grafts are rich in ECM components, however they lack viable SCs.[5] Supplementing acellular nerve grafts with supporting cells may improve outcomes.[6]

Previously, researchers have successfully supplemented acellular nerve grafts with cultured SCs.[7,8] However, efficiently obtaining autologous SCs for clinical use is difficult as it requires harvest of a donor nerve and time to culture and proliferate. [6] Adipose-derived mesenchymal stromal cells (MSCs) are easily accessible, rapidly expandable, capable of survival/integration within the host tissue and can be guided into non-mesenchymal lineages, such as neurons, astrocytes and SC-like cells to support nerve regeneration.[2,9] *In vitro* differentiation is a time consuming process and limits clinical applicability. Thus application of undifferentiated MSCs, which have been recently shown to improve nerve conduction velocity[10], is an attractive alternative.

Evaluations of nerve regeneration have relied heavily on functional evaluations. Although these approaches have the potential to be clinically relevant, they do not provide insight into the mechanisms underlying the neurotrophic potential of MSCs.[11] Despite the popular theory that release of growth factors is a potential mechanism of the cells' restorative capacity (figure 1), quantitative analysis of neurotrophic factor release from implanted MSCs is rarely reported.[12]

Questions regarding cell fate and differentiation remain unanswered. The purpose of this study was to evaluate the molecular mechanisms underlying nerve repair with a decelullarized nerve allograft seeded with isogenic, undifferentiated, adipose-derived MSCs.

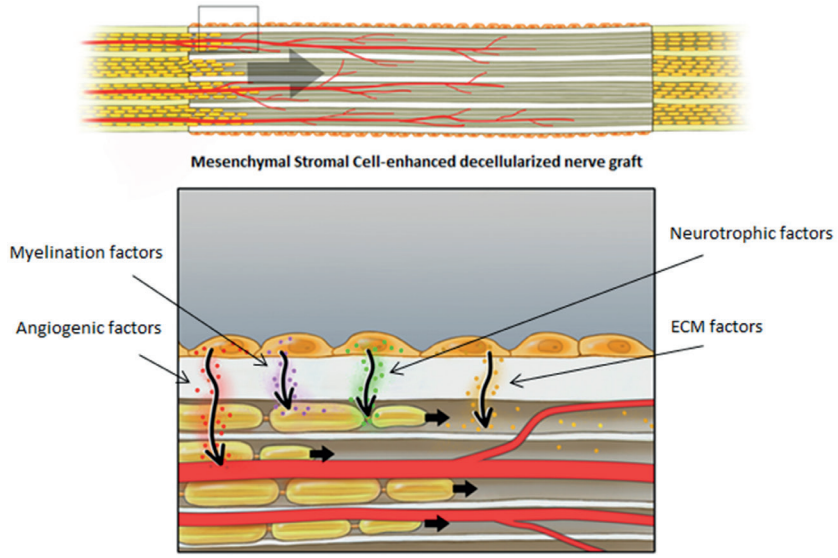


Figure 1. Cell-based therapy. The proposed mechanism of how cell-based therapy can create a more favorable environment for peripheral nerve regeneration is depicted.

MATERIALS AND METHODS

After IACUC institutional review committee approval, isogenic Lewis rat MSCs were dynamically seeded onto Sprague Dawley rat decellularized nerve allografts (N=9) and used to bridge a 10 mm sciatic nerve defect in Lewis rats. Sprague-Dawley rats were chosen as nerve donors, as there is a major histocompatibility mismatch to the recipient Lewis rat.[13] This mismatch would mimic the human clinical situation. The fate of implanted MSCs was determined by evaluating gene expression profiles 2 weeks postoperatively. IHC staining was obtained for neurite outgrowth, angiogenesis and SCs to determine whether up or down regulation of growth factor levels had functional consequences for early nerve regeneration. Outcomes were compared to unseeded allografts (N=9) and autograft controls (N=9).

Isolation of rat MSCs

MSCs were obtained from the inguinal fat pad of inbred Lewis rats, as previously described by Kingham et al.[14] Cultures were maintained at subconfluent levels in a 37°C incubator with 5% CO_2 and passaged with TrypLE (Invitrogen). MSCs were then lentivirally tranduced to express the firefly luciferase, as previously described.[15] This method has been shown to display no differences in viability and cell proliferation between labeled/unlabeled cells and luciferase was expressed steadily in vitro. All MSCs used in the experiment were of passage 5 and have previously been shown to be capable of multilineage differentiation.[16] Undifferentiated MSCs were used as they have been shown to respond to the demands placed on them by the local environment.

Preparation of allografts

A total of 18 rat sciatic nerve segments of 1.5 cm were harvested from Sprague-Dawley rats, weighing 250-350 grams (Harlan, Indianapolis, IN). Sciatic nerves were aseptically excised, cleared of peripheral fat tissue and decellularized using the protocol utilizing elastase as previously described.[17] Prior to surgery, 9 decellularized allografts were dynamically seeded with passage 5 Lewis rat MSCs as previously described.[18] 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. After 12 hours of incubation, seeded nerves were used for surgery.

Surgical procedure

Lewis rats (N=27) weighing 250-350 grams (Harlan) were anesthesized and surgical procedures were performed under standard aseptic conditions, as previously described by Hundepool et al.[19] All allografts were cut to 10mm. In group I, seeded allografts (N=9) were used to bridge the 10 mm nerve gap using 10-0 nylon epineural sutures (Ethicon, Inc., Somerville, NJ). In group II, acellular allografts (N=9) were used. In the control group III, autologous nerve segments were reversed and sutured back.

Outcome measurements

Two weeks postoperatively, anesthesia was induced using isoflurane and all animals (N=27) were sacrificed with an intraperitoneal overdose of Fatal-Plus (Vortech Pharmaceuticals, Dearborn, MI).

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

Changes in relative gene expression profiles of MSCs seeded allografts were evaluated by qRT-PCR and compared to unseeded allografts and autograft controls (N=5 per group). Nerve segments were harvested, frozen in QIAzol Lysis Reagent (Qiagen, Valencia, CA) and stored at -80°C. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen) 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). Resulting cDNA libraries were analyzed by qRT-PCR (C1000 Touch Thermalk Cycler, BioRad, Hercules, CA) using SYBR Green detection with specific primers, chosen from the literature, for a panel of genes essential for nerve regeneration (table 1). Results were analyzed to map MSC characteristics including proliferation, apoptosis, myelination and ECM-production.[8,20-22] Samples were analyzed in triplicate and results were normalized to the reference housekeeping gene GAPDH within each sample and then normalized to the unseeded allograft group. Differences in gene expression levels were quantified using the comparative delta crossover threshold (2^ (−ΔΔ Ct)) method.[23]

Table 1. mRNA primer sequences.

Gene ID	Biology	Forward primer	Reverse primer
GAPDH	Reference gene	TACCAGGGCTGCCTTCTCTTG	GGATCTCGCTCCTGGAAGATG
NGF	Neurotrophic marker	CACTCTGAGGTGCATAGCGT	CTATTGGTTCAGCAGGGGCA
GDNF	Neurotrophic marker	CGCTGACCAGTGACTCCAATA	GCGACCTTTCCCTCTGGAAT
PTN	Neurotrophic marker	GCCGAGTGCAAACAAACCAT	TGATTCCGCTTGAGGCTTGG
GAP43	Cytoplasmic protein	GATAACTCGCCGTCCTCCAA	CTACAGCTTCTTTCTCCTCCTCA
VEGFA	Angiogenic marker	CAGAAAGCCCATGAAGTGGTG	CTTCATCATTGCAGCAGCCC
PECAM1/ CD31	Angiogenic marker	TTGTGACCAGTCTCCGAAGC	TGGCTGTTGGTTTCCACACT
MPZ	Myelination marker	AGGCCGAGATGCCATTTCAA	CCCATACCTAGTGGGCACTTTT
PMP22	Myelination marker	GTCTGGTCTGCTGTGAGCAT	GCCATTGGCTGACGATGGTG
MBP	Myelination marker	TCTGGCAAGGACTCACACAC	AAATCTGCTGAGGGACAGGC
COL1A1	ECM protein	AAGTCTCAAGATGGTGGCCG	TCGATCCAGTACTCTCCGCT
COL3A1	ECM protein	CCCGGCAACAATGGTAATCC	GACCTCGTGCTCCAGTTAGC
LAMB2	ECM protein	AGTACCCACACGGATGGAGTG	CTCGAGAACAGCCAGGTACA

Table 1. continued

Gene ID	Biology	Forward primer	Reverse primer
FBLN1	ECM protein	GCAGACACCTTTCGCCAAGA	CGTGACAGCCCTCAGAAAGA
CASP3	Apoptosis protein	GGAGCTTGGAACGCGAAGAA	ACACAAGCCCATTTCAGGGT
EGR1	Transcription factor	CACCTGACCACAGAGTCCTTTT	GTTGGAGGGTTGGTCATGCT
SOX2	Transcription factor	AGTGGTACGTTAGGCGCTTC	CCCAGCAAGAACCCTTTCCT
CCNB2	Protein coding gene	ACCAGTGCAGATGGAGACAC	GACTGCAAAGCCTCAAGCTG
FABP4	Protein coding gene	TGAAAGAAGTGGGAGTTGGCTT	TGGTCGACTTTCCATCCCAC

Sequences for primers used in qPCR reactions (ECM = extracellular matrix). The following genes were analyzed: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), nerve growth factor (*NGF*), glial cell line-derived neurotrophic factor (*GDNF*), pleiotrophin (*PTN*), growth associated protein 43 (*GAP43*), vascular endothelial cell growth factor alpha (*VEGFA*), platelet endothelial cell adhesion molecule 1 (*PECAM1/CD31*), myelin protein zero (*MPZ*), peripheral myelin protein 22 (*PMP22*), myelin basic protein (*MBP*), collagen type 1 (*COL1A1*) and 3 (*COL3A1*), laminin subunit beta 2 (*LAMB2*), fibulin 1 (*FBLN1*), caspase 3 (*CASP3*), early growth response protein (*EGR1*), sex determining region y-box 2 (*SOX2*), cyclin B2 (*CCNB2*) and fatty acid binding protein 4 (*FABP4*).

Immunohistochemical staining

Sciatic nerves (N=4) of the operated sites were explanted over a length from 5mm proximal and distal to the graft, fixed in 4% Paraformaldehyde (Sigma), suspended in Tissue-Tek OCT Compound, and snap frozen. Transverse sections (5 µm thick) were cut on a cryostat at different levels within the middle of the repair site (figure 2). IHC staining was obtained for PGP9.5, NGF, RECA-1 and S100. Luciferase labeled MSCs were also double stained for Luciferase and S100 to study MSC differentiation to SC like cells. Slides were stored at -80˚C and prior to staining dried for 2 hours. Slides were manually post fixed and retrieved online using Epitope Retrieval 1 (Leica Microsystems). Sections were incubated for 60 minutes at 24˚C in the following primary antibodies: polyclonal S100 anti–rabbit (Dako Z0311) was used at 1:5000; polyclonal PGP9.5 anti–rabbit (Dako Z5116) was used at 1:500; monoclonal RECA-1 anti–mouse (Abcam ab9774) was used at 1:200; monoclonal NGF anti–rabbit (Abcam ab52918) was used at 1:150 and polyclonal anti-rabbit Firefly Luciferase (Abcam ab21176) was used at 1:2000. Sections were then washed with PBS and incubated with secondary antibodies (Alexa Fluor 568 or 488 Goat-anti-Rabbit (Invitrogen) at 24˚C for 60 minutes. The Research Detection System (Leica DS9455) was used and included Rodent Block R (Biocare RBR962). Cell nuclei were stained with Hoechst 33342 (Invitrogen H1399). Once completed, slides were rinsed in distilled water, coverslipped and observed by a confocal microscope (LSM 780, Zeiss). Nerve areas were captured at 10x (tile-scan) and 20x magnification.

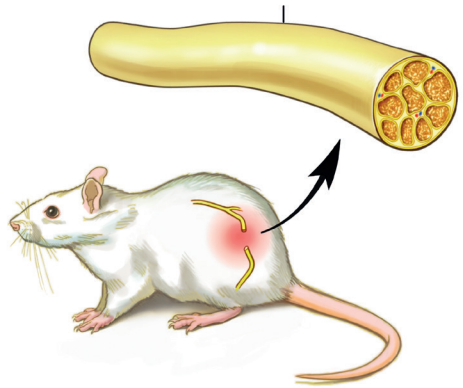


Figure 2. Section levels. Transverse sections were cut on a cryostat at different levels within the middle of the nerve.

Image analysis

Fluorescence intensity in the nerve cross-sections was measured using ImageJ (NIH, Bethesda) to quantify differences between the grafts. All images were obtained with the same settings and analysis was performed on the antibody monolayer, without Hoechst. A representative area was selected and the integrated density was measured. To determine and correct for the background signal, three areas of the image that had no fluorescence were selected and mean gray value was measured. Results were used to calculate the corrected total cell fluorescence (CTCF).[24]

Statistical analysis

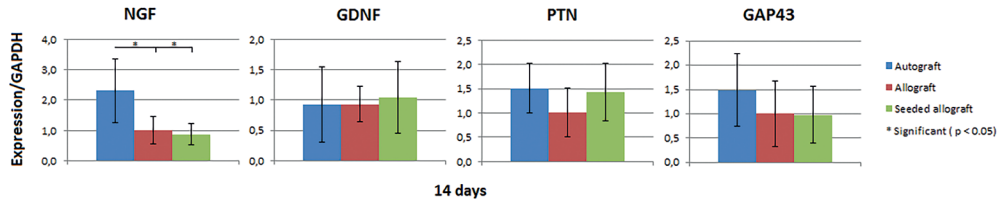
The Kolmogorov-Smirnoff test was applied to test for a normal distribution. To detect differences between groups, data were analyzed using the Kruskal-Wallis test followed by the Dunn-Bonferroni post hoc test. Statistically significance was set at $p<0.05$. All results are reported as mean ± standard deviation (SD).

RESULTS

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

All animals survived and there were no surgical complications. Figures 3a-d demonstrate the expression levels clustered by genes sharing a common function. *NGF*, *GDNF*, *PTN* and *GAP43* (figure 3a) were chosen as representative neurotrophic factors as they have been shown to promote neuronal survival and axonal regeneration after peripheral

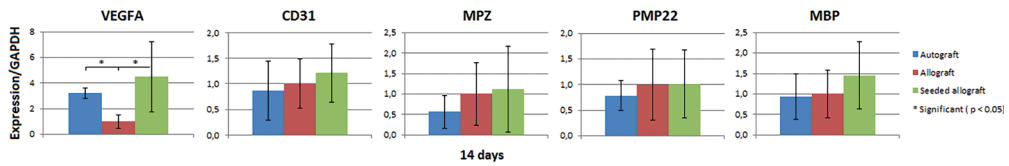
nerve injury.[11] Analysis showed significantly higher *NGF* mRNA levels after autograft reconstruction when compared to the unseeded allograft (2.3 fold increase, p=0.047) or the seeded nerve allograft (2,6 fold increase p = 0.038). There was no significant difference between the allograft groups (p = 0.863). For the expression of *GDNF*, *PTN* and *GAP43*, no significant differences were found between the groups (p = 0.849, 0.344, 0.557).



A) *NGF, GDNF, PTN* and *GAP43* mRNA expression levels were measured in the autograft, allograft and MSC seeded nerve allograft ($n = 5$). Relative expression levels are shown with regard to the allograft (value = 1). * $p < 0.05$.

Gene expression for the angiogenic molecule *VEGFA*, endothelial marker *CD31* and myelination factors *MPZ*, *PMP22* and *MBP* is depicted in figure 3b. Analysis showed a significant 4.5 fold increase of VEGFA expression in seeded nerve allografts (p = 0.009) and a significant 3.2 fold increase in autografts (p = 0.014) when compared to the unseeded nerve allografts. For *CD31*, an increasing trend (1.2 fold) was observed as well in the seeded allograft group, however differences were not significant (p = 0.616).

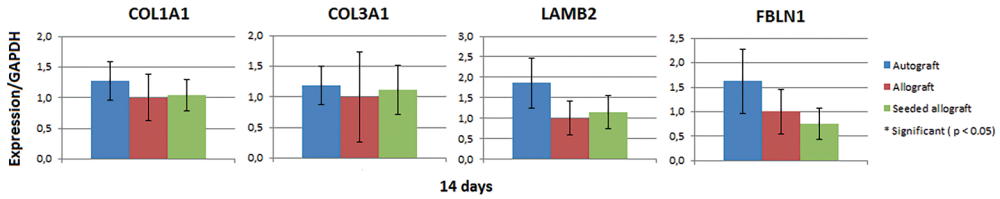
Myelination markers *MPZ*, *PMP22* and *MBP*, required for formation and maintenance of myelin, were all equally expressed.



B) *VEGFA, CD31, MPZ, PMP22* and *MBP* mRNA expression levels were measured. Relative expression levels are shown with regard to the allograft (value = 1). * $p < 0.05$.

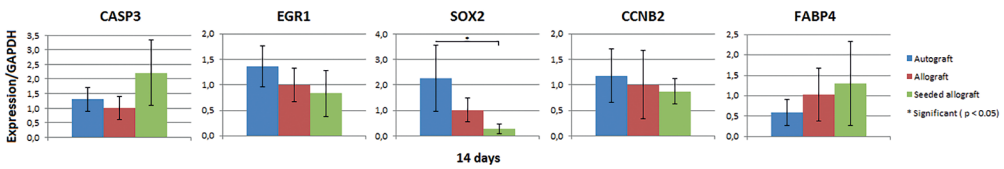
ECM-related markers *COL1A1* and *COL3A1* were highly expressed in all groups and no significant differences were found.

The *LAMB2* and *FBLN1* genes play a role in cell adhesion, differentiation and migration. Expression was moderate in all groups and differences were non-significant (figure 3c).[25]



C) *COL1A1, COL3A1, LAMB2* and *FBLN1* mRNA expression levels were measured. Relative expression levels are shown with regard to the allograft (value = 1).

Additional genes (figure 3d) were evaluated to map MSC characteristics including proliferation and apoptosis. A non-significant increase (2.2 fold) in *CASP3* in MSC seeded allografts when compared to the autograft and unseeded allograft was found. For *EGR1*, no significant differences were found. The *SOX2* gene mRNA level was significantly down regulated (0.3 fold, p= 0.006) in MSC seeded allografts. No significant differences were found for *CCNB2* and *FABP4*.



D) *CASP3, EGR1, SOX2, CCNB2* and *FABP4* mRNA expression levels were measured. Relative expression levels are shown with regard to the allograft (value = 1).

Figure 3. Relative gene expression 14 days postoperatively.

Immunohistochemical staining

PGP9.5 was assessed to quantify the amount of newly formed (sensory) axons. The marker was present in all three groups and representative images are depicted in figure 4a. The CTCF score in the nerve autograft (95.2 ± 30.9) was increased when compared to the unseeded allograft (33.4 ± 6.8) and MSC seeded allograft (60.6 ± 36.9) respectively. The MSC seeded graft showed an increased expression but not signficant when compared to the unseeded allograft (figure 4b).

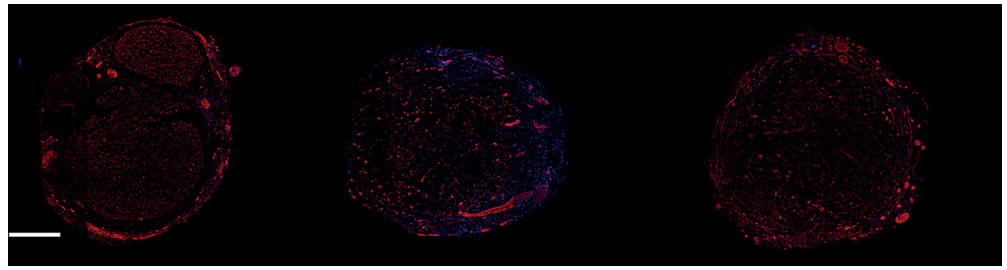


Figure 4a. PGP9.5 expression. Sections at the mid-graft stained with the (sensory) axonal marker PGP9.5 showed axons in the autograft, allograft and MSC seeded nerve allograft. Representative images are depicted with the double layer (DAPI). Magnification x10. Scale bar reflects 5 mm. *n* = 4 animals per group.

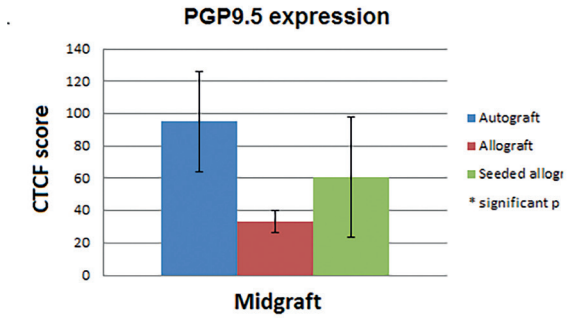


Figure 4b. PGP9.5 staining intensity quantification. Quantitative analysis of PGP9.5 staining by calculating and comparing the average CTCF-score among groups. Images were analyzed without DAPI. No significant differences were found. *n* = 4 animals per group.

NGF is critical in regeneration, survival and maintenance of neurons and is expressed during early neural development.[26] NGF was relatively highly expressed in all three groups (figure 5a). Both the CTCF score of the nerve autograft (245.2 ± 79.8) and the MSC seeded allograft (175.9 ± 16.1) were significantly increased (p= 0.007 and p= 0.018) when compared to the unseeded allograft (122.6 ± 40.1). There were no significant differences between the autograft and seeded allograft (figure 5b).

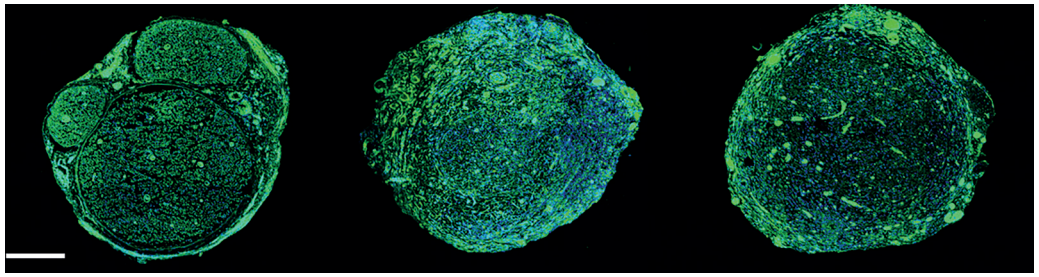


Figure 5a. NGF expression. Sections at the mid-graft stained for NGF showed presence of the marker in the autograft, allograft and MSC seeded nerve allograft. Representative images are depicted with the double layer (DAPI). Magnification x10. Scale bar reflects 5 mm. n = 4 animals per group.

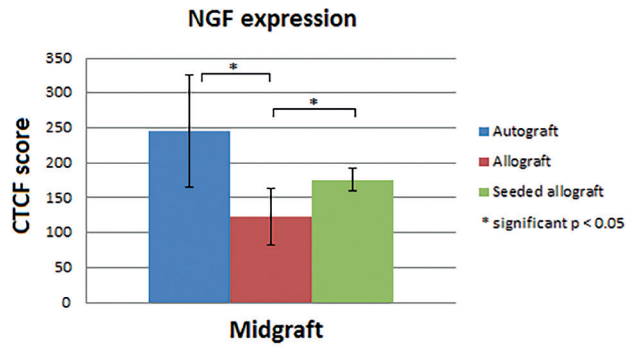


Figure 5b. NGF staining intensity quantification. Quantitative analysis of NGF staining by calculating and comparing the average CTCF-score among groups. Images were analyzed without DAPI. A significant increase of NGF expression in the autograft and MSC seeded allograft was found when compared to the unseeded nerve allograft. *n* = 4 animals per group. * $p < 0.05$.

RECA-1 is expressed in rat endothelial cells and was used to study angiogenesis. [26] RECA-1 was highly expressed in the autograft and MSC seeded allograft group (figure 6a). Both the CTCF score of the nerve autograft (282.6 ± 91.9) and the MSC seeded allograft (187.5 ± 26.5) were significantly increased (p= 0.007 and p= 0.018) when compared to the unseeded allograft (48.4 ± 36.5). There were no significant differences between the autograft and seeded allograft (figure 6b).

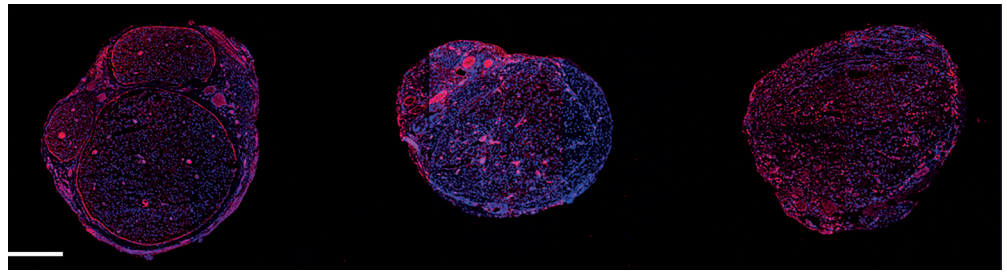


Figure 6a. RECA-1 expression. Sections at the mid-graft stained for endothelial marker RECA-1 showed presence of the marker in the autograft, allograft and MSC seeded nerve allograft. Representative images are depicted with the double layer (DAPI). Magnification x10. Scale bar reflects 5 mm. *n* = 4 animals per group.

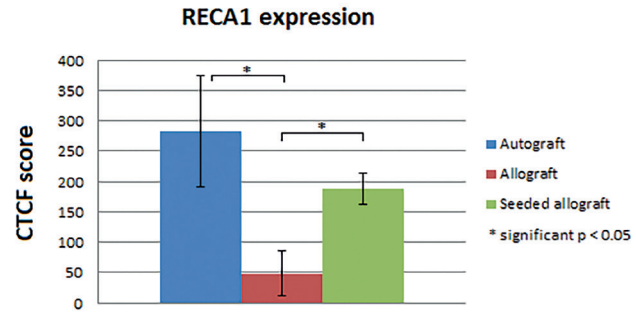


Figure 6b. RECA-1 staining intensity quantification. Quantitative analysis of RECA-1 staining by calculating and comparing the average CTCF-score among groups. Images were analyzed without DAPI. A significant increase of RECA-1 expression in the autograft and MSC seeded allograft was found when compared to the unseeded nerve allograft. *n* = 4 animals per group. * $p < 0.05$.

S100 was measured to assess the amount of SC-like cells. The marker was highly expressed in all groups and representative images are depicted in figure 7a. Quantification of the expression showed no significant differences between groups in CTCF score for the nerve autograft (342.8 ± 82.6), unseeded allograft (245.9 ± 71.6) and MSC seeded allograft (263.7 ± 93.3) respectively (figure 7b).

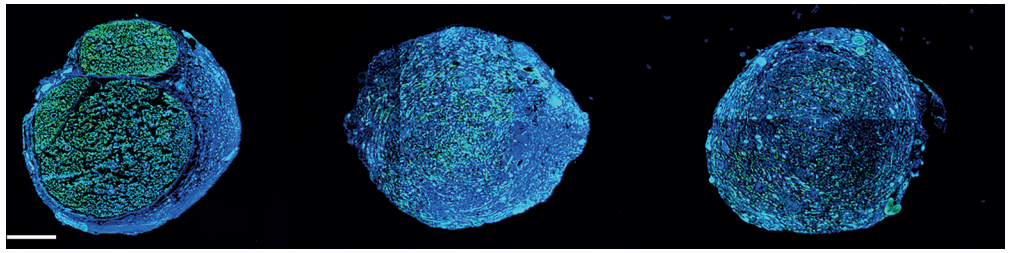


Figure 7a. S100 expression. Sections at the mid-graft stained for SC marker S100 showed presence of the marker in the autograft, allograft and MSC seeded nerve allograft. Representative images are depicted with the double layer (DAPI). Magnification x10. Scale bar reflects 5 mm. *n* = 4 animals per group.

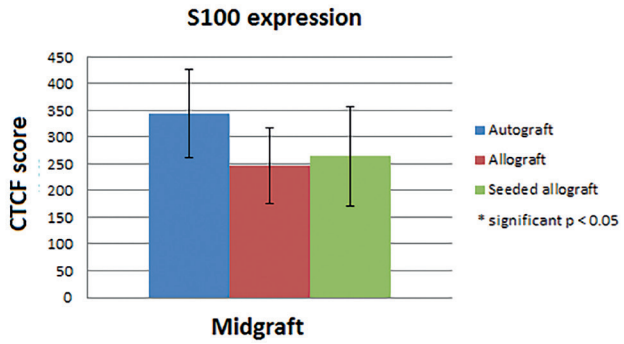


Figure 7b. S100 staining intensity quantification. Quantitative analysis of S100 staining by calculating and comparing the average CTCF-score among groups. Images were analyzed without DAPI. No significant differences were found. *n* = 4 animals per group.

Luciferase labeled MSCs were double stained for Luciferase and S100 to study MSC differentiation to SC-like cells. Luciferase positive cells were detected in high abundance in the peripheral areas of the nerve graft, but only a few showed co-staining with S100. Many luciferase positive cells were negative for S100, indicating that the implanted cells did not differentiate in large numbers into SC-like cells (figure 8).

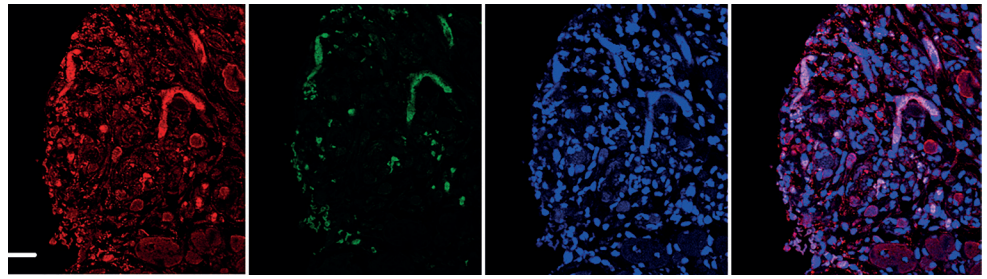


Figure 8. Luciferase and S100 double stain. Sections at the mid-graft of MSC seeded nerve allografts have been stained for anti-Luciferase (red) and SC marker S100 (green). Cell nuclei were stained by DAPI (blue). Overlapping images are shown in the right column. Many Luciferase positive cells are negative for S100, indicating that the implanted cells did not differentiate into a SC phenotype. Magnification x20. Scale bar reflects 100 µm. *n* = 4 animals.

DISCUSSION

We report on early regenerative parameters following nerve repair by a decellularized nerve allograft pre-seeded with adipose-derived MSCs and compare it to the unseeded allograft and autograft nerve.

NGF is important for the development and maintenance of the sympathetic and sensory nervous systems.[11] *NGF* gene-expression was significantly increased in autografts, which correlates with the increasing trend in NGF staining intensity. However, in seeded allografts NGF staining intensity was significantly increased when compared to unseeded allografts, suggesting that MSC seeded nerve allografts support the regeneration of sensory nerves to a greater extent than unseeded nerve allografts. NGF is not expressed by motor neurons, is barely detectable in healthy sciatic nerves, and following nerve injury it shows a biphasic up regulation in the distal nerve stump during the first week of regeneration.[11] *NGF* mRNA decrease can be explained by either the motor origin of the nerve allografts, or the solitary 2-week time point. It is possible that in both allograft groups, levels were increased in the first week and were already normalized at week 2.

Both the *VEGFA*-gene and *CD31* are involved in angiogenesis and endothelial cell growth. *VEGFA* plays a role in wound healing, can promote neovascularization and has a neurotrophic effect in enhancing the survival of SCs and protecting neurons from ischemic injury.[27] *VEGFA* molecule was significantly increased in seeded allografts and *CD31* showed the same increasing trend. This correlates with the significantly increased RECA-1 intensity in seeded nerve grafts, however the intensity was also significantly increased in

autografts. Previous studies have reported beneficial effects of VEGFA on vascularization, resulting in improved regeneration in acellular grafts.[28] Kingham et al. showed that in addition to acting directly on the nervous system, (differentiated) MSCs were able to boost vascularization at the area of nerve injury.[29] Similar to our results, the authors showed an increased VEGFA secretion and RECA-1 staining intensity. Other studies have confirmed the correlation between increased vascularization and enhanced nerve regeneration within acellular conduits.[30,31]

CASP3 was measured to assess the potential of seeded MSCs to mediate cell apoptosis. We hypothesized that MSCs would reduce apoptosis, but the increase in seeded allografts suggests that a portion of the cells go into apoptosis. We previously showed that implanted undifferentiated MSCs do not survive longer than 4 weeks and that the number of cells gradually diminishes over time.[15] Kingham et al. showed that CASP3 levels were significantly reduced when nerve conduits were filled with stimulated cells, while unstimulated MSCs had no significant effect. [29] This could suggest that differentiated cells survive longer than undifferentiated cells.

The *SOX2* gene is a key transcription factor in the regulation of pluripotency and neural differentiation. Cells expressing *SOX2* are capable of proliferating and producing differentiated neural cell types.[32] Transcription factor *SOX2* was significantly down-regulated in seeded allografts, suggesting that after 2 weeks, seeded MSCs no longer proliferate and may have reached a differentiated state. MSCs may not have differentiated into SC-like cells as there was no increase in myelination markers and only a moderate increase in S100 staining intensity. This is in line with previous reports that found no histological evidence of MSC transdifferentiation into SC-like cells within 14-days follow-up.[33,34] Others concluded that the therapeutic effect was maintained for several weeks after there were no significant quantities of viable cells and concluded that the regenerative effect of MSCs was mediated by an initial boost of released growth factors or by an indirect effect on endogenous SC activity.[2,35] In line with these results, Wang et al. detected only few S100 positive cells after implanting MSCs in an acellular nerve allograft.[36]

The anti-Luciferase and S100 double stain confirmed that implanted cells did not differentiate in large numbers into a SC phenotype (figure 8). This is consistent with previous reports, concluding that adipose-derived MSCs do not differentiate into SCs but probably secrete some type of humoral factor or VEGFA that promotes the proliferation or migration of SCs.[37] Sowa et al. also concluded that undifferentiated implanted adipose-

derived MSCs did not differentiate into SC but do promote peripheral nerve regeneration at the injured site.[38]

In vitro it has been shown that the cues of the ECM increased neurotrophic gene expression.[25] *In vivo*, undifferentiated cells may undergo differentiation in response to local stimuli. The hypoxic milieu of the wound might have triggered the MSCs to produce angiogenic molecules; which could be the underlying mechanism of MSCs restorative capacity. Wang et al. concluded that *in vivo* differentiation was safer, site-dependent and entirely under the control of signals from the endogenous microenvironment.[39]

Undifferentiated MSCs can potentially differentiate into unwanted cell types or form teratomas, however this risk is very low. Because undifferentiated MSCs only develop into cells of mesodermal lineages, while the composition of teratoma requires all three germ cell layers.[40] Klein et al. reported there is controversy regarding whether this risk of malignant transformation is evident for adipose-derived MSCs and reported no tumor formations in their study.[10] Nonetheless, for future clinical implementation this potential risk has to be investigated.

Strengths of this study include the use of a non-damaging cell seeding technique and a design that mimics the human situation with major histocompatibility complex mismatched allograft donors and recipients.

We recognize the limitations of this study. Successful axonal regeneration depends on a dynamic balance between growth factors and analysis of multiple time points would provide more insight.

No functional measurements were performed because 2 weeks is too early for any motor reinnervation to occur. Future studies should focus on the growth factor expression at multiple time-points and should determine if the initially improved regenerative response of MSCs enhanced decellularized nerve allografts results in enhanced functional recovery.

CONCLUSION

We aimed to evaluate the molecular mechanisms underlying nerve repair by a decelullarized nerve allograft seeded with undifferentiated MSCs. We confirmed that MSCs contribute to the secretion of trophic factors, resulting in a beneficial effect of the MSCs on angiogenesis. Implanted MSCs did not show evidence of differentiation into SC-like cells.

REFERENCES

1. 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.
2. Jiang L, Jones S, Jia X. Stem Cell Transplantation for Peripheral Nerve Regeneration: Current Options and Opportunities. *Int J Mol Sci.* 2017;18(1).
3. di Summa PG, Kalbermatten DF, Pralong E, Raffoul W, Kingham PJ, Terenghi G. Long-term in vivo regeneration of peripheral nerves through bioengineered nerve grafts. *Neuroscience.* 2011;181:278-291.
4. Rodriguez FJ, Verdu E, Ceballos D, Navarro X. Nerve guides seeded with autologous schwann cells improve nerve regeneration. *Exp Neurol.* 2000;161(2):571-584.
5. Walsh S, Midha R. Practical considerations concerning the use of stem cells for peripheral nerve repair. *Neurosurg Focus.* 2009;26(2):E2.
6. 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.
7. Sun XH, Che YQ, Tong XJ, et al. Improving nerve regeneration of acellular nerve allografts seeded with SCs bridging the sciatic nerve defects of rat. *Cell Mol Neurobiol.* 2009;29(3):347-353.
8. 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.
9. Georgiou M, Golding JP, Loughlin AJ, Kingham PJ, Phillips JB. Engineered neural tissue with aligned, differentiated adipose-derived stem cells promotes peripheral nerve regeneration across a critical sized defect in rat sciatic nerve. *Biomaterials.* 2015;37:242-251.
10. 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.
11. 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.
12. 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.
13. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. *Tissue Eng.* 2004;10(9-10):1346-1358.

14. 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.
15. Rbia N, Bulstra LF, Thaler R, Hovius SER, van Wijnen AJ, Shin AY. In Vivo Survival of Mesenchymal Stromal Cell-Enhanced Decellularized Nerve Grafts for Segmental Peripheral Nerve Reconstruction. *J Hand Surg Am.* 2018.
16. Rbia N, Bulstra LF, Thaler R, Hovius SER, van Wijnen AJ, Shin AY. In-vivo survival of mesenchymal stromal cell-enhanced decellularized nerve grafts for segmental peripheral nerve reconstruction. *Submitted.*
17. 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.
18. 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.
19. Hundepool CA, Bulstra LF, Kotsougiani D, et al. Comparable functional motor outcomes after repair of peripheral nerve injury with an elastase-processed allograft in a rat sciatic nerve model. *Microsurgery.* 2018;38(7):772-779.
20. 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.
21. 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.
22. Kingham PJ, Reid AJ, Wiberg M. Adipose-derived stem cells for nerve repair: hype or reality? *Cells Tissues Organs.* 2014;200(1):23-30.
23. 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.
24. Jensen EC. Quantitative analysis of histological staining and fluorescence using ImageJ. *Anat Rec (Hoboken).* 2013;296(3):378-381.
25. 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.
26. Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol.* 2000;10(3):381-391.
27. Fan L, Yu Z, Li J, Dang X, Wang K. Schwann-like cells seeded in acellular nerve grafts improve nerve regeneration. *BMC Musculoskelet Disord.* 2014;15:165.

28. 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.
29. 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.
30. Hobson MI, Green CJ, Terenghi G. VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy. *J Anat.* 2000;197 Pt 4:591-605.
31. Sowa Y, Imura T, Numajiri T, Nishino K, Fushiki S. Adipose-derived stem cells produce factors enhancing peripheral nerve regeneration: influence of age and anatomic site of origin. *Stem Cells Dev.* 2012;21(11):1852-1862.
32. Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH. In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. *Cell Stem Cell.* 2007;1(5):515-528.
33. Shen J, Duan XH, Cheng LN, et al. In vivo MR imaging tracking of transplanted mesenchymal stem cells in a rabbit model of acute peripheral nerve traction injury. *J Magn Reson Imaging.* 2010;32(5):1076-1085.
34. Santiago LY, Clavijo-Alvarez J, Brayfield C, Rubin JP, Marra KG. Delivery of adipose-derived precursor cells for peripheral nerve repair. *Cell Transplant.* 2009;18(2):145-158.
35. Kappos EA, Engels PE, Tremp M, et al. Peripheral Nerve Repair: Multimodal Comparison of the Long-Term Regenerative Potential of Adipose Tissue-Derived Cells in a Biodegradable Conduit. *Stem Cells Dev.* 2015;24(18):2127-2141.
36. Wang Y, Jia H, Li WY, Tong XJ, Liu GB, Kang SW. Synergistic effects of bone mesenchymal stem cells and chondroitinase ABC on nerve regeneration after acellular nerve allograft in rats. *Cell Mol Neurobiol.* 2012;32(3):361-371.
37. Suganuma S, Tada K, Hayashi K, et al. Uncultured adipose-derived regenerative cells promote peripheral nerve regeneration. *J Orthop Sci.* 2013;18(1):145-151.
38. 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.
39. Wang D, Liu XL, Zhu JK, et al. Bridging small-gap peripheral nerve defects using acellular nerve allograft implanted with autologous bone marrow stromal cells in primates. *Brain Res.* 2008;1188:44-53.
40. Zhang R, Rosen JM. The role of undifferentiated adipose-derived stem cells in peripheral nerve repair. *Neural Regen Res.* 2018;13(5):757-763.

CHAPTER 8

General discussion

GENERAL DISCUSSION

The overarching aim of this thesis was to improve functional outcome after peripheral nerve reconstruction by improving the effectiveness of decellularized nerve allografts with the addition of adipose-derived MSCs. Can MSC seeded decellularized nerve allografts improve outcome of peripheral nerve reconstruction by turning hype and hope to reality? In this chapter, the results of this thesis are put into a broader perspective and compared to other recent publications. Furthermore, the implications of this research for future perspectives are discussed.

PART I: CLINICAL USE OF NERVE GRAFT SUBSTITUTES

The aim of the first part of this thesis was to investigate the clinical problem and to define the exact role of current nerve graft substitutes in peripheral nerve reconstruction. Although, the nerve autograft is still the golden standard for segmental peripheral nerve reconstruction, there are many situations where there is a need for clear surgical guidelines. As the amount of autografts, that can be harvested is limited and in some cases is undesirable or even impossible. When should a surgeon decide to use nerve graft substitutes and which ones should be used in which situation? In some cases with multiple large nerve injuries, nerve graft substitutes might be necessary in addition to nerve autografts. To date, there are no formal guidelines that are applicable regarding the use of graft substitutes for the reconstruction of motor, mixed and sensory peripheral nerve gaps.

In **Chapter 2**, an evidence-based overview of the effectiveness of nerve conduits and allografts in motor and mixed sensory/motor nerve reconstruction is provided. We reviewed the literature specifically on evidence in motor and mixed nerves and found 21 clinical studies of which 2 prospective randomized controlled trials and 19 case reports or case series. The best evidence comes from the randomized controlled trials that conclude that nerve graft substitutes have good outcomes in mixed/motor nerves in gaps less than 6 mm and internal diameters between 3 and 7 mm. There were case reports describing successful functional recovery after the repair of larger defects, but there were also case reports reporting complete failures. Therefore, there was insufficient evidence for their use in larger-gap and -diameter nerves and it remains that major segmental motor or mixed nerve injury is optimally treated with a cabled nerve autograft. Some authors would consider the reconstruction of a defect size smaller than 5 mm a conduit-assisted

coaptation and one could argue what the additional value of the nerve graft substitute is in these cases. This study affirms that despite many years of studies, the field of peripheral nerve research still lacks high quality prospective studies on the reconstructive options of motor and mixed nerve injuries and confirms that improvement of functional outcome after segmental peripheral motor nerve injury is desirable. Since this study was published research has further advanced and small sample size studies have been published reporting that functional outcomes of processed nerve allografts in mixed and motor nerve repairs compare favorably to historical controls for nerve autograft and exceed those for hollow tube conduit.[1,2] However, direct comparisons with control groups are still lacking.

On the contrary; in an isolated digital nerve injury when tension free primary repair is not possible, the harvest of an autograft is undesirable and nerve graft substitutes are used more frequently. In **Chapter 3**, the goal was to retrospectively compare the outcomes of digital nerve gap reconstruction with the NeuraGen type 1 collagen nerve conduit to the Avance Nerve Graft. Since both substitutes have been commercially available for several years, the aim was to evaluate for potential differences in outcomes and to define the exact role of both alternatives in digital nerve reconstruction. We identified all eligible nerve allografts and conduits and soon found out that only a small minority of the patients completed the 12-month follow-up and reported quantitative outcome data that could be analyzed. The sample size was too low to perform subgroup analyses and the study would suffer from selection bias. Therefore the data was used to present a single institution case series and a review of the literature was added. Based on these results together, we concluded that no differences were found in sensory recovery and post-operative complications after 12 months of follow-up. We suggest that the decision to choose either the conduit or processed allograft should be based on other parameters such as surgical experience, gap length, wound condition, the extent of injury, operation time and additional costs and storage factors. To define the exact role of both nerve graft substitutes prospective large sample size studies with subgroup analyses are needed comparing nerve conduits with nerve allografts.

FUTURE PERSPECTIVES

- In the field of peripheral nerve research there is a need of high quality prospective studies in particular on motor/mixed nerve repair but also on sensory nerve reconstruction. These

studies would allow subgroup analyses to define what patient would benefit from what type of treatment.

- Another option would be to actively collect a large amount of data with outcome measurements and a follow-up of at least 12 months in order to perform observational studies that allow analyses by propensity score matching.

- Motor outcome measures currently are highly inconsistent, future research should focus on an universal objective measurement of motor strength to make results comparable.

- The same holds for sensory scoring systems, up to date, many authors use different scoring systems or make their own modification. Future research should use one scoring system to make comparisons among studies.

PART II: MESENCHYMAL STROMAL CELLS IN NERVE REGENERATION

In 1963, the authors Till and McCulloch published one of the first high impact papers on regenerative stem cells within mice bone marrow.[3] In 2001, researchers discovered that enzymatic digestion of adipose tissues followed by plating of the resulting stromal vascular fraction generated a homogenous population of adipocyte progenitors with stem cell-like characteristics and a year later, the multi potency of these cells was confirmed.[4-6] Since then, the adipose-derived MSCs are considered the most popular cell type due to the ease of harvest and abundant number of cells; to date a PubMed search yields over 5700 entries. In 2004, the first clinical studies published their results in the field of bone grafting and Crohn's disease.[7] For peripheral nerve repair however, despite convincing in-vitro and in-vivo studies, there is still no clear evidence for clinical implementation.[8] This is mainly because the beneficial effect has not outperformed the nerve autograft and because questions (and concerns) remain on the fate of implanted cells as the mechanism behind the proposed beneficial effect of MSCs is still not fully elucidated. In the **second part** of this thesis we decided to improve the most promising nerve graft substitute, the optimally decellularized nerve allograft, by addition of the most popular type of MSC, the adipose-derived mesenchymal stromal cell. Also, we studied the mechanism behind the proposed beneficial effect of cell therapy.

In **chapter 4**, the purpose was to develop a simple and reliable cell seeding strategy. Methods of MSC delivery have not been standardized, but the injection technique has been previously used in many studies.[9-11] This technique has been reliable for the delivery of MSCs to empty synthetic nerve conduits, however the nerve allograft contains an intra-neural structure and it has been previously emphasized that the micro-injection of cells into nerve ends or grafts can be traumatic to the delicate intra-neural architecture and can result in unpredictable cell distribution.[12] We thought that an adequate, reliable and validated cell seeding technique was an essential step for evaluating the translational utility of MSC-enhanced decellularized nerve grafts. It has been a point of discussion whether the MSCs were needed on the outside of the graft or on the inside of the graft. Many researchers believe the supportive cells are needed inside the graft to help guide axons during regeneration. This method however seeds the MSCs on the outside of the nerves in order to not traumatize the intra-neural architecture of the allograft and also because it had been previously hypothesized that necessary growth factors can penetrate the graft by diffusion.[13,14] This method was used in the subsequent studies of this thesis.

In **chapter 5**, we took the next step and studied the interaction of mesenchymal stromal cells and decellularized nerve allografts at a cellular level. MSCs may theoretically promote tissue regeneration through different proposed mechanisms including differentiation into Schwann-like cells, via the secretion of various growth factors, neuroprotective abilities, immunoregulation, the promotion of myelination of regenerating axons or neoangiogenesis.[15,16,17] MSCs originally attracted attention because of their multipotency, but many authors have not found evidence of engraftment of differentiation. [6,12] Furthermore MSCs may not survive in the long term, and there is little evidence that they execute Schwann cell functions like the formation of new myelin sheaths. And also it is postulated that the remaining nerve allograft ECM still has biological activity that influences the MSCs.[18,19] Therefore, in this chapter we hypothesize that MSCs support peripheral nerve regeneration via local production of growth factors rather than as active participants in the regeneration process and investigated whether the interaction of mesenchymal stromal cells and decellularized nerve allografts can improve gene- and protein expression of growth factors that may support nerve regeneration. Based on previous research we expected to see an increase in all neurotrophic factors gene expression that correlates with protein expression and a decrease in apoptosis marker.[20,21] Surprisingly, we found both increased neurotrophic factors and decreased factors. Also, not all variables correlated with the protein expression, the apoptosis marker was increased and angiogenic markers were highly increased. Obviously, there are many factors that can influence the results

of this study such as the use of motor origin nerve allografts, the donor MSCs origin and passage of the cells, the different time points and the qRT-PCR procedure. MSCs can be transplanted in their undifferentiated state or can undergo a period of in vitro differentiation into SC-like cells. In this study undifferentiated MSCs were used as there is some evidence that suggests that these cells can respond to the demands placed on them by the local environment.[22,23,24] Superiority of differentiated cells has not been shown and some investigators claim that in-vitro differentiation incurs an unnecessary delay, limiting clinical applicability.[12] This study confirms that MSCs respond to the mechanical properties of the extracellular matrix, which suggests a possible synergistic interaction between these cells and the extracellular milieu. Furthermore, in this chapter we conclude that cells may not need pre-differentiation prior to surgery and that the trophic functions in tissue repair occur in the first week after surgery.

In the literature, MSCs have also been shown to possess immunogenic properties with immunosuppressive potential.[25] In this study immunological markers where not included and therefore we are unable to make a statement on the immunoregulation by MSCs. As there was a paucity of information regarding the ultimate survivorship of implanted mesenchymal stromal cells or if these cells remain where they are placed, the aim of **chapter 6** was to track the in-vivo distribution and survival of mesenchymal stromal cells seeded on a decellularized nerve allograft reconstruction of a peripheral nerve defect, using luciferase based bioluminescent imaging.

MSCs were lentivirally transduced to express the firefly luciferase and Luciferin was injected intraperitoneally prior to imaging. Luciferase activity (production of a yellow fluorescent light) was assessed at different time point using an in-vivo imaging system. The bioluminescence signal declined rapidly within the first week following implantation; suggesting early cell death. Gradually diminishing BLI signals were observed until the end of the experiment. This corresponds to the growth factor production described in chapter 5, showing a decline in the first weeks after seeding. We found no evidence of migration of cells to surrounding tissues. Which is an import finding concerning the safety of MSC implantation. It can be discussed whether the BLI method is sensitive enough to state this strongly, but it confirms the finding of other studies that harvested the surrounding tissue and detected no migration to other organs.[26-28] This chapter demonstrates that implanted MSCs have a finite survival and therefore strengthens the hypothesis that MSCs contribute to an improved functional outcome through an initial secretory effect rather than in-vivo Schwann cell like differentiation.

In **chapter 7**, we aimed to evaluate the molecular mechanisms underlying in vivo nerve repair by a decellularized nerve allograft preseeded with autologous, undifferentiated, adipose-derived mesenchymal stromal cells. Growth factor production in vivo was measured 2 weeks postoperatively and immunohistochemistry staining was obtained for neurite outgrowth, angiogenesis and SCs to determine whether up or down regulation of growth factor levels had functional consequences for early nerve regeneration. The 2 week time point was specifically chosen to be able to measure differences between groups in regeneration distance or velocity, as we expected already full recovery at later time points. The main findings of this study were a significant increase in gene-expression of the angiogenic molecule VEGFA in seeded nerve allografts, which correlated with a significant increase of the endothelial marker RECA-1. The in-vivo increase in angiogenic molecules correlates with the in-vitro increase of angiogenic markers, found in chapter 5. For the factors PGP9.5 and S100 we found no significant differences between autografts, allografts and seeded allografts after 2 weeks of follow-up, this could suggest that at this time point there aren't a lot of newly formed axons or SC-(like) cells yet. In contrast, NGF is expressed during early neural development and showed no significant differences in gene expression but a significant increase in both the autograft and the seeded allograft at the protein level when compared to the unseeded allograft. These differences between gene expression and protein production were also seen in chapter 5 and can have technical causes but there are also many post-transcriptional mechanisms involved in turning mRNA into protein. It has also been shown that qRT-PCR displays a broader dynamic range and higher sensitivity than immunohistochemistry.[29] In addition, PGP9.5 and NGF mark sensory axons exclusively, while the donor nerve allograft has a motor origin. The effect of motor versus sensory nerve grafting seems not clear yet. Some studies report that the motor nerve architecture plays an important role in nerve regeneration in a mixed nerve gap model, while others report similar outcomes for motor versus sensory grafts.[30,31]

Luciferase labeled MSCs were double stained for Luciferase and S100 to study MSC differentiation to SC-like cells and confirmed that implanted cells did not differentiate in large numbers into a SC phenotype. The in-vitro SC-like differentiation process takes three weeks; therefore when looking back, it would have been valuable to include this time point. The results in this chapter are consistent with previous reports, concluding that adipose-derived MSCs do not differentiate into SCs but probably secrete some type of humoral factor or VEGFA that promotes the proliferation or migration of SCs.[32] These results are supported by the literature, where it has been shown that MSCs secrete cytokines, growth factors and bioactive molecules with trophic, paracrine effects at variable concentrations

in response to local microenvironmental cues.[25] The increase in angiogenic molecules can thus be explained by hypoxia in the woundbed. Fairbarn et al. confirmed that hypoxia has been found to potentiate the angiogenic and neurogenic influence of some MSCs, leading to improved blood vessel and nerve fiber formation.[12] We therefore hypothesize that the ultimate fate of implanted mesenchymal stromal cells could depend on the exogenous microenvironment and that site-dependent in-vivo differentiation could be the underlying mechanism of MSCs restorative capacity.

FUTURE PERSPECTIVES

This thesis proposed to advance the line of work towards improvement of functional outcome after peripheral nerve injury by improving the effectiveness of decellularized nerve allografts. We evaluated if MSC seeded decellularized nerve allografts can improve functional outcome of peripheral nerve reconstruction by turning the hype of MSCs and hope for a solution for large mixed nerve defects, to a reality. We developed a simple and reliable cell seeding strategy, took the next step and studied the interaction of MSCs and decellularized nerve allografts at a cellular level, tracked the in-vivo distribution and survival of MSCs seeded on a decellularized nerve allograft reconstruction and aimed to evaluate the molecular mechanisms underlying in vivo nerve repair. Although this thesis proves that adipose-derived MSCs are a promising tool for nerve allograft repair and reveals some of the mechanism behind the proposed beneficial effect of cell therapy, still some questions remain.

- First, after two weeks, the MSC enhanced nerve allograft did not outperform the nerve autograft. Di summa et al. already showed no short-term benefits after 2 weeks of reconstructing a rat sciatic nerve gap with a MSC seeded nerve conduit, which suggests that a longer follow-up time is needed.[33] Before definitive conclusions can be drawn, functional testing of the used MSC seeded decellularized nerve allograft should be performed at later time points.

- Secondly, MSCs can be implanted in an undifferentiated state or can undergo in-vivo differentiation into Schwann-like cells. Benefits of differentiation include superior in-vivo viability, enhanced neurotrophic factor secretion and a decreased risk of differentiation into aberrant lineages.[12,34] Although, differentiation of MSCs improves their neurotrophic potential, it has been suggested that cells dedifferentiate once removed from the differentiation medium.[6] The positive effects of undifferentiated MSCs have also been

reported and results from this thesis suggest that undifferentiated cells may undergo in-vivo differentiation in response to local stimuli. Also, undifferentiated cells are better clinical applicable as there is no unnecessary delay of in-vitro differentiation. Up to date, there is no consensus on whether to use differentiated or undifferentiated cells and therefore future studies should focus on direct comparisons.

- Additionally, co-cultures of MSCs with autologous Schwann cells could improve the MSC phenotypic stability and may also be further studied.[35]

- Finally, clinical trials are necessary. The scarce clinical publications are case series and there is a need for higher quality studies.[8] In order to implement MSCs in clinical practice, a standardized protocol to isolate MSCs for clinical application is necessary. Due to current governmental regulations, cells should not be manipulated (in vitro) and should be implanted during the same operative session.

We believe this thesis contributes to understanding the role of MSCs in nerve regeneration, MSC seeded nerve allografts aren't a reality yet but we trust that in the future it will be possible to make MSCs therapy a possibility in peripheral nerve reconstruction.

MAIN CONCLUSIONS OF THIS THESIS

- Commercially available nerve graft substitutes have good outcomes in mixed/motor nerves in gaps less than 6 mm and internal diameters between 3 and 7 mm.

-There is insufficient evidence for the use of commercially available nerve graft substitutes in large-gap and -diameter nerves; the evidence remains that major segmental motor or mixed nerve injury is optimally treated with a cabled nerve autograft.

- Nerve conduits and processed nerve allografts offer both convenient off-the-shelf options for digital nerve gaps <2.5 cm.

- A dynamic seeding method distributes cells homogenously over the graft and does not traumatize the intra-neural architecture of the allograft.

- Seeding of undifferentiated adipose-derived mesenchymal stromal cells onto processed nerve allografts permits the secretion of neurotrophic and angiogenic factors in-vitro.

- Mesenchymal stromal cells seeded onto decellularized nerve allografts survive for up to 29 days in vivo.

- Mesenchymal stromal cells seeded onto decellularized nerve allografts do not differentiate into Schwann cell like cells.

- Mesenchymal stromal cells seeded onto decellularized nerve allografts contribute to angiogenesis in-vivo.

- The ultimate fate of implanted mesenchymal stromal cells might depend on the exogenous microenvironment.

- Site-dependent in-vivo differentiation could be the underlying mechanism of stromal cell's restorative capacity.

- A beneficial effect of mesenchymal stromal cells on the early regeneration was found when compared to the unseeded nerve allograft.

- The early regeneration of decellularized nerve allografts pre-seeded with mesenchymal stromal cells is not superior to the nerve autograft.

REFERENCES

1. Safa B, Shores JT, Ingari JV, et al. Recovery of Motor Function after Mixed and Motor Nerve Repair with Processed Nerve Allograft. *Plast Reconstr Surg Glob Open.* 2019;7(3):e2163.
2. Isaacs J, Safa B. A Preliminary Assessment of the Utility of Large-Caliber Processed Nerve Allografts for the Repair of Upper Extremity Nerve Injuries. *Hand (N Y).* 2017;12(1):55-59.
3. Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature.* 1963;197:452-454.
4. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* 2001;7(2):211-228.
5. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* 2002;13(12):4279-4295.
6. Armaiz Flores A, Wang H. The Use and Delivery of Stem Cells in Nerve Regeneration: Preclinical Evidence and Regulatory Considerations. *Ann Plast Surg.* 2018;80(4):448-456.
7. Zuk PA. The adipose-derived stem cell: looking back and looking ahead. *Mol Biol Cell.* 2010;21(11):1783-1787.
8. Toyserkani NM, Jorgensen MG, Tabatabaeifar S, Jensen CH, Sheikh SP, Sorensen JA. Concise Review: A Safety Assessment of Adipose-Derived Cell Therapy in Clinical Trials: A Systematic Review of Reported Adverse Events. *Stem Cells Transl Med.* 2017;6(9):1786-1794.
9. 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.
10. Liu GB, Cheng YX, Feng YK, et al. Adipose-derived stem cells promote peripheral nerve repair. *Arch Med Sci.* 2011;7(4):592-596.
11. 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.
12. Fairbairn NG, Meppelink AM, Ng-Glazier J, Randolph MA, Winograd JM. Augmenting peripheral nerve regeneration using stem cells: A review of current opinion. *World J Stem Cells.* 2015;7(1):11-26.
13. 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.
14. 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.
15. Baldari S, Di Rocco G, Trivisonno A, Samengo D, Pani G, Toietta G. Promotion of Survival and Engraftment of Transplanted Adipose Tissue-Derived Stromal and Vascular Cells by Overexpression of Manganese Superoxide Dismutase. *Int J Mol Sci.* 2016;17(7).
16. Kingham PJ, Reid AJ, Wiberg M. Adipose-derived stem cells for nerve repair: hype or reality? *Cells Tissues Organs.* 2014;200(1):23-30.
17. Erba P, Mantovani C, Kalbermatten DF, Pierer G, Terenghi G, Kingham PJ. Regeneration potential and survival of transplanted undifferentiated adipose tissue-derived stem cells in peripheral nerve conduits. *J Plast Reconstr Aesthet Surg.* 2010;63(12):e811-817.
18. 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.
19. 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.
20. 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.
21. 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.
22. Tohill M, Mantovani C, Wiberg M, Terenghi G. Rat bone marrow mesenchymal stem cells express glial markers and stimulate nerve regeneration. *Neurosci Lett.* 2004;362(3):200-203.
23. Chen X, Wang XD, Chen G, Lin WW, Yao J, Gu XS. Study of in vivo differentiation of rat bone marrow stromal cells into schwann cell-like cells. *Microsurgery.* 2006;26(2):111-115.
24. Rando TA. Stem cells, ageing and the quest for immortality. *Nature.* 2006;441(7097):1080-1086.
25. Baer PC. Adipose-derived mesenchymal stromal/stem cells: An update on their phenotype in vivo and in vitro. *World J Stem Cells.* 2014;6(3):256-265.
26. Bai X, Yan Y, Coleman M, et al. Tracking long-term survival of intramyocardially delivered human adipose tissue-derived stem cells using bioluminescence imaging. *Mol Imaging Biol.* 2011;13(4):633-645.
27. Wolbank S, Peterbauer A, Wassermann E, et al. Labelling of human adipose-derived stem cells for non-invasive in vivo cell tracking. *Cell Tissue Bank.* 2007;8(3):163-177.
28. Dai M, Xu P, Hou M, Teng Y, Wu Q. In vivo imaging of adipose-derived mesenchymal stem cells in female nude mice after simulated childbirth injury. *Exp Ther Med.* 2015;9(2):372-376.

29. Sinn HP, Schneeweiss A, Keller M, et al. Comparison of immunohistochemistry with PCR for assessment of ER, PR, and Ki-67 and prediction of pathological complete response in breast cancer. *BMC Cancer.* 2017;17(1):124.
30. Moradzadeh A, Borschel GH, Luciano JP, et al. The impact of motor and sensory nerve architecture on nerve regeneration. *EXP NEUROL.* 2008;212(2):370-376.
31. Ali SA, Rosko AJ, Hanks JE, et al. Effect of Motor versus Sensory Nerve Autografts on Regeneration and Functional Outcomes of Rat Facial Nerve Reconstruction. *Sci Rep.* 2019;9(1):8353.
32. Suganuma S, Tada K, Hayashi K, et al. Uncultured adipose-derived regenerative cells promote peripheral nerve regeneration. *J Orthop Sci.* 2013;18(1):145-151.
33. di Summa PG, Kingham PJ, Campisi CC, Raffoul W, Kalbermatten DF. Collagen (NeuraGen(R)) nerve conduits and stem cells for peripheral nerve gap repair. *Neurosci Lett.* 2014;572:26-31.
34. Faroni A, Smith RJ, Reid AJ. Adipose derived stem cells and nerve regeneration. *Neural Regen Res.* 2014;9(14):1341-1346.
35. Khalifian S, Sarhane KA, Tammia M, et al. Stem cell-based approaches to improve nerve regeneration: potential implications for reconstructive transplantation? *Arch Immunol Ther Exp (Warsz).* 2015;63(1):15-30.

CHAPTER 9

Summary

Nederlandse samenvatting

SUMMARY

In **chapter 1**, a general introduction on peripheral nerve injury and reconstruction and an overview of the aims of this thesis called “stem cells in nerve reconstruction: hype, hope or reality?” is provided. This thesis builds on the results of previous research conducted to improve the effectiveness of nerve graft substitutes. The overarching goal of this thesis is to further improve outcomes after nerve reconstruction by individualizing nerve allograft repair with the addition of adipose-derived MSCs.

In the **first part** of this thesis the clinical outcome after nerve reconstruction is investigated.

In **chapter 2**, an evidence-based overview of the effectiveness of nerve conduits and allografts in motor and mixed sensory/motor nerve reconstruction is provided. A MEDLINE literature search was performed from 1978 to 2016 and the main finding was that nerve graft alternatives have good outcomes in mixed/motor nerves in gaps less than 6 mm and internal diameters between 3 and 7 mm. There was insufficient evidence for the use in larger-gap and-diameter nerves; the evidence remained that major segmental motor or mixed nerve injury is optimally treated with a cable nerve autograft.

In **chapter 3**, we performed a single institution case series and review of the literature on the outcomes of digital nerve gap reconstruction with the NeuraGen type 1 collagen nerve conduit and the Avance Nerve Graft. 37 patients were included with a minimal follow-up of 12 months and primary outcome was postoperative sensory recovery measured by static two-point discrimination (S2PD) test or the Semmes-Weinstein monofilament test (SWM). After 12 months, outcomes were graded as excellent sensory recovery in 48% of the collagen conduit repairs and 39% of the nerve allografts, good in 26% of the conduits and 55% of the allografts and poor in 26% of the conduits versus 6% of the allografts. We conclude that nerve conduits and processed nerve allografts offer convenient off-the-shelf options for digital nerve gap repair. Both techniques offer effective means of reconstructing a digital nerve gap <2.5 cm at a minimum of 12 months of follow-up and future prospective randomized large sample size studies comparing nerve conduits with allografts are needed to perform subgroup analyses and to define their exact role in digital nerve injuries.

Taken together, chapter 2 and 3 depict the current clinical guidelines for the use of nerve graft substitutes. In small sensory nerve gaps, both conduits and nerve allografts have good outcomes. In long motor and mixed nerve gaps, the nerve autograft remains the golden standard and alternatives are desirable.

In the **second part** of this thesis we focused on the addition of adipose derived MSCs to decellularized nerve allografts and investigated the mechanism behind the proposed beneficial effect of cell therapy by evaluating the in-vitro characteristics on human tissue, as well as the in-vivo characteristics in a rat-model.

In **chapter 4**, the purpose was to develop and validate a simple cell seeding strategy with an optimal seeding duration. Prior techniques used for cell seeding are traumatic to both the mesenchymal stromal cells and the nerve graft. A dynamic bioreactor was used to seed both rat and human cells onto rat and human decellularized nerve allografts and the cells remained viable at all time points. Seeding efficiency was 89.2% after 12 hours of seeding and both intra-rater (r = 0.97) and inter-rater reliability (r = 0.92) were high. The method was reproducible, distributed cells homogenously over the graft and did not traumatize the intra-neural architecture of the allograft.

In **chapter 5**, we took the next step and investigated the interaction of mesenchymal stromal cells and decellularized nerve allografts. The method described in chapter 4, was used to seed human mesenchymal stromal cells onto human decellularized nerve allografts and qRT-PCR and ELISA was performed to examine the gene expression and growth factor production. Over 21 days, gene expression analysis showed that adherence of mesenchymal stromal cells to nerve allografts significantly enhanced the expression of neurotrophic, angiogenic and myelination markers. ELISA analysis showed a correlating up regulation of the BDNF growth factor, but a reduction of both VEGF and NGF protein levels. This study demonstrates that the seeding of undifferentiated adipose-derived mesenchymal stromal cells onto processed nerve allografts permits the secretion of neurotrophic and angiogenic factors; these favorable molecular changes suggest that cell supplementation of nerve allografts may have potential in improving nerve regeneration.

In **chapter 6**, we aimed to track the in-vivo distribution and survival of mesenchymal stromal cells seeded on a decellularized nerve allograft reconstruction of a peripheral nerve defect, by using luciferase based bioluminescent imaging. Autologous Lewis rat-MSCs were stably labeled with luciferase by lentiviral particles. Labeled cells were

dynamically seeded onto a Sprague Dawley decellularized rat nerve allograft and used to bridge a 10 mm sciatic nerve defect. Results demonstrated that labeled mesenchymal stromal cells could be detected for up to 29 days and gradually diminishing signals were observed within the first week following implantation. There was no evidence of migration of cells to surrounding tissues.

In **chapter 7**, we aimed to evaluate the molecular mechanisms underlying nerve repair by a decellularized nerve allograft preseeded with autologous, undifferentiated, adipose-derived mesenchymal stromal cells and comparing it to the unseeded allograft and autograft nerve. Undifferentiated MSCs were seeded onto decellularized nerve allografts and used to reconstruct a 10 mm gap in a rat sciatic nerve model. Gene expression profiles of genes essential for nerve regeneration and immunohistochemical staining (IHC) for PGP9.5, NGF, RECA-1 and S100 were obtained 2 weeks postoperatively to determine the early regeneration. The main finding of this study was a significant increase in the angiogenic molecule VEGFA in the seeded nerve allografts, which correlated with a significant increase of the endothelial marker RECA-1. The seeded cells did not show evidence of differentiation into Schwann cells. The in-vivo increase in angiogenic molecules correlates with the in-vitro increase of angiogenic markers, found in chapter 5. We conclude that the ultimate fate of implanted mesenchymal stromal cells could depend on the exogenous microenvironment and that site-dependent in-vivo differentiation could be the underlying mechanism of stromal cell's restorative capacity. At 2 weeks postoperatively, a beneficial effect of the cells was found when compared to the unseeded nerve allograft. However, at the protein level, there was no improvement when compared to the nerve autograft.

In **Chapter 8** the results of this thesis are put into a broader perspective. In the field of peripheral nerve research there is still a need of high quality prospective studies in particular on motor/mixed nerve repair but also on sensory nerve reconstruction. We suggest that the decision to choose either a nerve conduit or processed allograft should be based on parameters such as surgical experience, gap length, wound condition, the extent of injury, operation time and additional costs and storage factors. We confirmed that seeded MSCs respond to the mechanical properties of the extracellular matrix of the nerve allograft, which suggests a possible synergistic interaction between these cells and the extracellular milieu. Furthermore, MSCs may not need pre-differentiation prior to surgery and most trophic functions in tissue repair occur in the first week after surgery. We demonstrated that implanted MSCs have a finite survival, which strengthens

the hypothesis that MSCs contribute to an improved functional outcome through an initial secretory effect rather than in-vivo Schwann cell like differentiation. A double stain for Luciferase labeled MSCs and S100 further confirmed that implanted cells did not differentiate in large numbers into a SC phenotype. Other main findings were a significant increase in gene-expression of the angiogenic molecule VEGFA in seeded nerve allografts, which correlated with a significant increase of the endothelial marker RECA-1 and with the in-vitro increase of angiogenic markers, found in chapter 5. This thesis proves that adipose-derived MSCs are a promising tool for nerve allograft repair and reveals some of the mechanism behind the proposed beneficial effect of cell therapy, but still questions remain before MSC seeded nerve allografts can become a reality. Future research should focus on longer follow-up time, functional testing of the MSC seeded decellularized nerve allograft and clinical trials are necessary before implementing the optimized MSC seeded nerve allograft in clinical practice.

NEDERLANDSE SAMENVATTING

In **hoofdstuk 1** wordt een algemene inleiding gegeven over perifere zenuwbeschadiging en reconstructie alsook een overzicht van de doelstellingen van dit proefschrift getiteld “stamcellen in zenuwreconstructie: hype, hoop of realiteit?” Dit proefschrift is gebaseerd op de resultaten van eerder onderzoek naar verbetering van de effectiviteit van zenuw conduits en allografts. Het overkoepelende doel van dit proefschrift is om de resultaten na de zenuwreconstructie verder te verbeteren door het herstel met de zenuw allograft te individualiseren met de toevoeging van uit vetcellen verkregen stamcellen.

In het **eerste deel** van dit proefschrift wordt de klinische uitkomst na zenuwreconstructie onderzocht.

In **hoofdstuk 2** wordt een evidence-based overzicht gegeven van de effectiviteit van zenuw conduits en allografts in de reconstructie van motorische en gemengde zenuwen. Een MEDLINE literatuuronderzoek werd uitgevoerd voor studies gepubliceerd tussen 1978 en 2016 en de belangrijkste bevinding was dat zowel conduits als allografts goede resultaten hebben in motorische zenuw defecten van minder dan 6 mm lang en diameters tussen de 3 en 7 mm. Er was onvoldoende bewijs voor het gebruik in langere defecten en voor de reconstructie van zenuwen met grotere diameters; het bewijs blijft dat tot op heden segmentale motorische en gemengde zenuw letsels tot optimaal behandeld worden met een bekabelde zenuw autograft.

In **hoofdstuk 3** hebben we de resultaten van sensorische digitale zenuwdefecten bestudeerd in een retrospectieve observationele studie en presenteren we een overzicht van de literatuur. Resultaten van de NeuraGen type 1 colageen zenuw conduit werden vergeleken met de resultaten van de Avance Nerve allograft. 37 patiënten werden geïncludeerd met een minimale follow-up van 12 maanden en de primaire uitkomstmaat was postoperatief sensorisch herstel gemeten met statische twee punts discriminatie test (S2PD) of de Semmes-Weinstein monofilamenttest (SWM). Na 12 maanden werden de uitkomsten geclassificeerd als excellent in 48% van de reparaties van de conduit groep en 39% van de allograft groep, de uitkomst was geclassificieerd als goed in 26% van de conduits en 55% van de allografts en slecht in 26% van de conduits versus 6 % van de allograft. We concluderen dat zenuw conduits en gedecellulariseerde zenuw allografts beide handige beschikbare opties zijn voor sensorisch zenuw herstel. Beide technieken herstellen na 12 maanden het gevoel in de vingers in het geval van een zenuwdefect < 2,5 cm. Toekomstige prospectieve gerandomiseerde grote studies die zenuw conduits vergelijken met zenuw allografts zijn nodig om subgroep analyses uit te voeren en om hun exacte rol in digitale zenuwletsels te definiëren.

Deze twee hoofdstukken samen geven een overzicht van de huidige klinische richtlijnen voor het gebruik van conduits en allografts in perifere zenuwletsels. In kleine sensorische letsels hebben zowel conduits als allografts goede resultaten. In segmentale motorische en gemengde zenuwletels blijft de zenuw autograft de gouden standard en zijn alternatieven wenselijk.

In het **tweede deel** van dit proefschrift hebben we ons gericht op de toevoeging van uit vet weefsel verkregen stamcellen aan de gedecellularizeerde zenuw allograft. Ook hebben we het mechanisme achter het voorgestelde gunstige effect van de cel therapie bestudeerd door de in-vitro kenmerken op humaan weefsel, evenals de in vivo kenmerken in een rat-model te evalueren.

In **hoofdstuk 4** was het de bedoeling om een eenvoudige techniek te ontwikkelen en te valideren om stamcellen op de zenuw allograft te brengen in een optimale tijdsduur. Eerder beschreven technieken zijn traumatisch voor zowel de stamcellen als het zenuw transplantaat. Een dynamische bioreactor werd gebruikt om zowel humane als rat stam cellen op gedecellularizeerde allografts aan te brengen en de cellen bleven op alle tijdspunten in leven. De efficiëntie van het aanbrengen was 89,2% na 12 uur en zowel de intra-rater (r=0,97) als de inter-rater beoordelaarsbetrouwbaarheid (r=0,92) waren hoog. De methode was reproduceerbaar, de cellen werden homogeen over het transplantaat verdeeld en de intra-neurale architectuur van de allograft werd niet beschadigd.

In **hoofdstuk 5** hebben we de volgende stap genomen en onderzochten we de interactie tussen stamcellen en de gedecellulariseerde zenuw allograft. De methode beschreven in hoofdstuk 4 werd gebruikt om de stamcellen op de allograft aan te brengen en qRT-PCR en ELISA werden gebruikt om de genexpressie en productie van groeifactoren te onderzoeken. Over een periode van 21 dagen toonde de genexpressie analyse aan dat de interactie tussen de stamcellen en de allograft zorgt voor een significant toegenoemen expressie van neurotrofe, angiogene en myeline markers. ELISA-analyse toonde een correlerende opregulatie van de BDNF-groeifactor, maar een vermindering van zowel VEGF als NGF groeifactoren. Deze studie toont aan dat het samenvoegen van ongedifferentieerde uit vetweefsel verkregen stamcellen met gedecellulariseerde zenuw

allografts de uitscheiding van neurotrofe en angiogene factoren mogelijk maakt; deze gunstige moleculaire veranderingen suggereren dat celsuppletie van zenuw allografts potentie heeft voor de verbetering van zenuwregeneratie.

In **hoofdstuk 6** hadden wij als doel om de in-vivo distributie en overleving van met stamcel bezaaide zenuw allografts te volgen door middel van Luciferase-gemedieerde bioluminescentie. Autologe Lewis rat-stamcellen werden in-vitro gemarkeerd met Luciferase en deze gemarkeerde cellen werden dynamisch samengevoegd met Sprague Dawley gedecellularizeerde rat-zenuw allografts en gebruikt om een 10mm lang ischiadicus zenuw defect te reconstrueren. Met behulp van bioluminescentie werden de stamcellen in beeld gebracht. De resultaten toonden dat de gemarkeerde stamcellen tot 29 dagen postoperatief werden gedetecteerd en geleidelijk aan afnemende signalen werden waargenomen binnen de eerste week na implantatie. Er was geen bewijs van migratie van cellen naar omliggend weefsel.

In **hoofdstuk 7** hebben we ons gericht op het evalueren van de moleculaire mechanismen die ten grondslag liggen aan zenuwherstel met een met autologe ongedifferentieerde uit vet weefsel verkregen stamcellen bezaaide zenuw allograft en deze te vergelijken met allografts zonder stamcellen en autograft reconstructies. Ongedifferentieerde stam cellen werden dynamisch toegevoegd aan gedecellulariseerde allografts en gebruikt om een 10mm ischiadicus rat-zenuw defect te reconstrueren. Genexpressieprofielen van genen die essentieel zijn voor zenuwregeneratie en immunohistochemische kleuring (IHC) voor axonale maker PGP9.5 en NGF, voor de endotheel marker RECA-1 en Schwan cel marker S100 werden 2 weken na de operatie verkregen. De belangrijkste bevindingen van deze studie waren een significante toename van de angiogene factor VEGFA in de met stamcel bezaaide allografts, dit correleerde met een significante toename van de endotheel marker RECA-1. De geimplanteerde stamcellen vertoonden geen tekenen van Schwan cel differentiatie. De in-vivo toename van angiogene factoren correleert met de in-vitro gevonden toename van angiogene factoren in hoofdstuk 5. We concluderen dat het uiteindelijke lot van geimplanteerde stamcellen zou kunnen afhangen van de exogene micro-omgeving en dat omgevingsafhankelijke differentiatie het onderliggende mechanisme van de herstellende capaciteit van stamcellen is. Twee weken postoperatief werd een gunstig effect van de stamcellen gezien ten opzichte van de allograft zonder stamcellen. Echter, op het eiwit niveau, werd geen verbetering gevonden ten opzichte van de zenuw autograft.

In **hoofdstuk 8** worden de resultaten van dit proefschrift in een breder perspectief geplaatst. Op het gebied van perifeer zenuwonderzoek is er nog steeds behoefte aan prospectieve studies van hoge kwaliteit, met name over motororisch / gemengd zenuwherstel, maar ook over sensorische zenuwreconstructies. We suggereren dat de beslissing om een zenuwconduit of een gedecellulariseerde allograft te kiezen gebaseerd moet zijn op parameters zoals chirurgische ervaring, defect lengte, wond conditie, de mate van het letsel, operatietijd en extra kosten en opslag factoren. We bevestigden dat geïmplanteerde stamcellen de mechanische eigenschappen van de extracellulaire matrix van het zenuwallograft detecteren en erop reageren, wat een mogelijke synergetische interactie tussen deze cellen en het extracellulaire milieu suggereert. Bovendien hoeven stamcellen voorafgaand aan de operatie geen pre-differentiatie te ondergaan en de meeste trofische functies vinden plaats in de eerste week na de operatie. We hebben aangetoond dat geïmplanteerde stamcellen een eindige overleving hebben, wat de hypothese versterkt dat stamcellen bijdragen aan een verbeterd functioneel resultaat door een initieel secretoir effect in plaats van in-vivo Schwann-celachtige differentiatie. Een dubbele kleuring voor met luciferase gemerkte stamcellen en S100 bevestigde verder dat geïmplanteerde cellen niet in grote aantallen in een Schwann-cel fenotype differentiëren. Andere belangrijke bevindingen waren een significante toename in genexpressie van het angiogene molecuul VEGFA in met stamcel bezaaide zenuw allografts, wat correleerde met een significante toename van de endotheel marker RECA-1 en met de in vitro toename van angiogene markers, te vinden in hoofdstuk 5. Dit proefschrift bewijst dat van vet-weefsel afgeleide stamcellen een veelbelovende tool zijn voor zenuwallograft herstel en onthult een deel van het mechanisme achter het voorgestelde gunstige effect van celtherapie, maar er blijven vragen bestaan voordat met stam cel bezaaide zenuwallografts een realiteit kunnen worden. Toekomstig onderzoek zou zich moeten richten op een langere follow-up-tijd, functionele testen van de met stamcel bezaaide zenuw allograft en klinische trials zijn noodzakelijk voordat de geoptimaliseerde zenuw allograft in de klinische praktijk wordt geïmplementeerd.

APPENDICES

List of publications

PhD Portfolio

Curriculum Vitae

Dankwoord

Erasmus MC
Universitair Medisch Centrum Rotterdam

LIST OF PUBLICATIONS

1. 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.

2. The Role of Nerve Graft Substitutes in Motor and Mixed Motor/Sensory Peripheral Nerve Injuries. **Rbia N**, Shin AY. J Hand Surg Am. 2017 May;42(5):367-377.

3. High Prevalence of Chronic Pain With Neuropathic Characteristics After Open Reduction and Internal Fixation of Ankle Fractures. **Rbia N**, van der Vlies CH, Cleffken BI, Selles RW, Hovius SER, Nijhuis THJ. Foot Ankle Int. 2017 Sep;38(9):987-996.

4. 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.

5. Ultrasound assessment of the sural nerve in patients with neuropathic pain after ankle surgery. **Rbia N**, Nijhuis THJ, Roukema GR, Selles RW, van der Vlies CH, Hovius SER. Muscle Nerve. 2018 Mar;57(3):407-413.

6. 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.

7. Meisje met zwellingen aan de vingers. **N. Rbia**, J. Damman, E.J. Mendels. Accepted at Nederlands Tijdschrift voor Dermatologie en Venereologie

8. In-vivo survival of mesenchymal stromal cell-enhanced decellularized nerve grafts for segmental peripheral nerve reconstruction. **N. Rbia**, L.B. Bulstra, R. Thaler, S.E.R. Hovius, A.J. van Wijnen, A.Y. J Hand Surg Am. 2019 Jun;44(6):514.e1-514.e11. doi: 10.1016/j.jhsa.2018.07.010. Epub 2018 Oct 6.

9. Collagen nerve conduits and processed nerve allografts for the reconstruction of digital nerve gaps: a single-institution case series and review of the literature. **N. Rbia**, L.B. Bulstra, T.M. Saffari, S.E.R. Hovius, A.Y. Shin. World Neurosurg. 2019 Jul;127:e1176-e1184. doi: 10.1016/j.wneu.2019.04.087. Epub 2019 Apr 16.

10. Seeding decellularized nerve allografts with adipose-derived mesenchymal stromal cells: an in vitro analysis of the gene expression and growth factors produced. **N. Rbia**, L.B. Bulstra, E.A. Lewallen, S.E.R. Hovius, A.J. van Wijnen, A.Y. Shin. J Plast Reconstr Aesthet Surg. 2019 Aug;72(8):1316-1325. doi: 10.1016/j.bjps.2019.04.014. Epub 2019 May 9.

11. Adhesion, distribution, and migration of differentiated and undifferentiated mesenchymal stem cells (MSCs) seeded on nerve allografts. F. Mathot, **N. Rbia**, A.T. Bishop, S.E.R. Hovius, A.J. van Wijnen, A.Y. Shin. J Plast Reconstr Aesthet Surg. 2019 May 22. pii: S1748-6815(19)30231-1. doi: 10.1016/j.bjps.2019.05.030. [Epub ahead of print]

12. Gene expression and growth factor analysis in early nerve regeneration following segmental nerve defect reconstruction with a mesenchymal stromal cell-enhanced decellularized nerve allograft. **N. Rbia**, L.B. Bulstra, P.F. Friedrich, A.T. Bishop, T.H.J. Nijhuis, A.Y. Shin. Accepted in PRS Global Open.

13. Gene expression profiles of differentiated and undifferentiated adipose derived mesenchymal stem cells dynamically seeded onto a processed nerve allograft. Femke Mathot; **Nadia Rbia**; Roman Thaler; Allen T Bishop; Andre van Wijnen, Ph.D.; Alexander Y Shin. Accepted in Gene.

PHD PORTFOLIO

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD student: Nadia Rbia Erasmus MC Department: Plastic and Reconstructive Surgery and Hand Surgery Research School: NIHES	PhD period: 2015-2019 Promotor(s): Prof. dr. S.E.R. Hovius Co-promotor: Dr. T.H.J. Nijhuis

1. PhD training	Year	Workload (Hours/ECTS)
General academic skills		
NIHES Master of Clinical Epidemiology	2013-2014	70 ECTS
General courses		
Biomedical English Writing and Communication	2016-2017	3 ECTS
Research Integrity	2017	0.3 ECTS
EndNote, Pubmed and other databases, Medical Library, Erasmus MC Rotterdam	2013	30 hours
Specific courses		
Laboratory animal science and Institutional Review Board (IRB), Mayo Clinic College of Medicine, Rochester, MN, USA	2015	24 hours
Microsurgery; Mevr. J.M Hekking, Skillslab-Plastic and reconstructive surgery	2014-2017	164 hours
Microsurgery; Mayo Microvascular Surgery Training Center, Mayo Clinic College of Medicine, Rochester, MN, USA	2015-2016	60 hours
Seminars and workshops		
Advanced Musculoskeletal Utrasound Course, LUMC Leiden	2014	16 hours

1. PhD training	Year	Workload (Hours/ECTS)
Presentations, (inter)national conferences		
Annual meeting American Society for Peripheral Nerve (ASPN), Hawaï, USA	2017	20 hours
International Federation of Societies for Surgery of the Hand (IFSSH), Buenos Aires, Argentina – *best paper session*	2016	20 hours
Annual meeting American Society for Peripheral Nerve (ASPN), Arizona, USA	2016	20 hours
Congress of the European Society for Trauma and Emergency Surgery (ECTES), Amsterdam	2015	20 hours
International Symposium on Peripheral Nerve Regeneration (ISPNR), Turin, Italy – *travel grant winner*	2014	20 hours
Symposium Experimenteel Onderzoek Heelkundige Specialismen (SEOHS), Maastricht	2013	20 hours
Other		
Organizing the 19th Esser Course: CMC1 osteoarthritis	2013	24 hours
Organizing the 20th Esser Course: masterclass neuropathic pain	2013	50 hours

2. Teaching	Year	Workload (Hours/ECTS)
Supervising practicals and excursions, Tutoring		
Supervising minor students	2015 + 2016	48 hours
Research collaborator Mayo Clinic	2016-2017	60 hours
Grants		
Contributed together with team members and PI dr. A.Y. Shin to NIH R01 grant application	2017	$1.250.000
American Foundation for Surgery of the Hand	2016	$20.000
Catharina van Tussenbroek fonds	2016	€4.000
Center for Regenerative Medicine	2015	$50.000
Esser Foundation	2015	€40.000
Michael van Vloten fonds	2015	€10.000

CURRICULUM VITAE

Nadia Rbia was born on april 5th 1989 in s'-Gravenhage, the Netherlands. After graduating from the gymnasium in 2007, she started medical school at the Erasmus University in Rotterdam. Her first research project at the department of Plastic and Reconstructive Surgery and Hand surgery was part of her master's program in 2013. Later that year, she enrolled in a second master's program in clinical epidemiology at the Netherlands Institute for Health Sciences (NIHES) which led to two first author publications. In 2014 she was qualified for a PhD project at the department of Plastic and Reconstructive Surgery and Hand Surgery at the Erasmus MC (Prof. S.E.R. Hovius). First, she worked for six months as a resident not in training in Rotterdam and in May 2015 she did a research fellowship at the Microvascular research laboratory at the department of Orthopedic Surgery, division of Hand Surgery at the Mayo Clinic, Rochester, Minnesota, USA under the supervision of dr. A.Y. Shin and dr. A.T. Bishop. From April 2016 to April 2017 she was a full time PhD student and during this period she assisted surgical procedures at the Mohs Klinieken in Amsterdam. In April 2017 she started as a resident not in training in Dermatology at the Admiraal de Ruyter Hospital in Goes while finishing this PhD thesis. In October 2017 the research projects in this thesis were part of a successful NIH grant application of A.Y. Shin, making it possible to continue the line of research. In May 2018 she started working at the department of Dermatology at the Erasmus MC and in July 2018 she was accepted as a trainee. In January 2019 she started her residency (Dr. C. van Montfrans) and she will continue to improve her clinical and scientific skills.