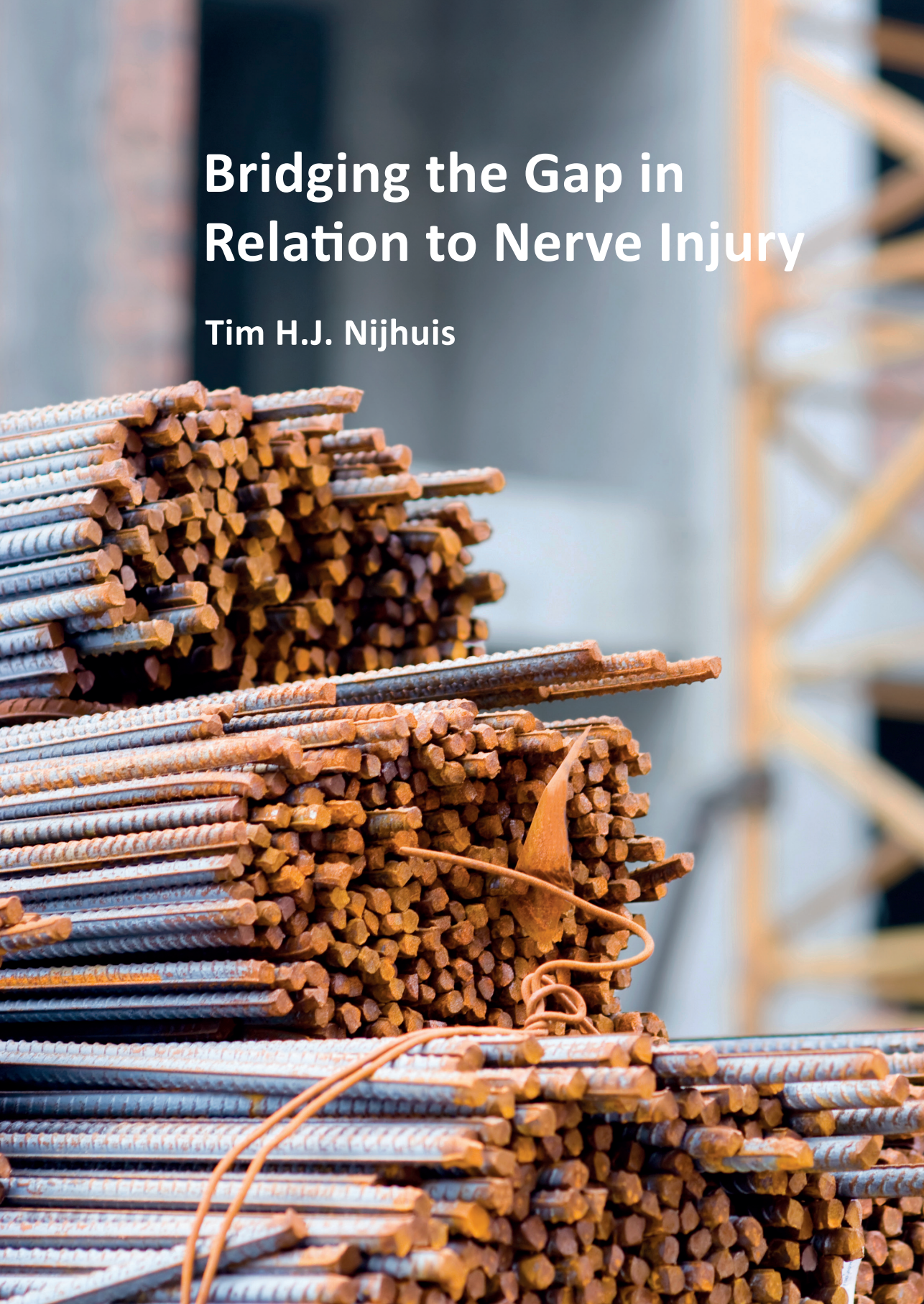
Bridging the Gap in Relation to Nerve Injury

Tim H.J. Nijhuis



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Het overbruggen van een defect en de relatie met zenuwschade

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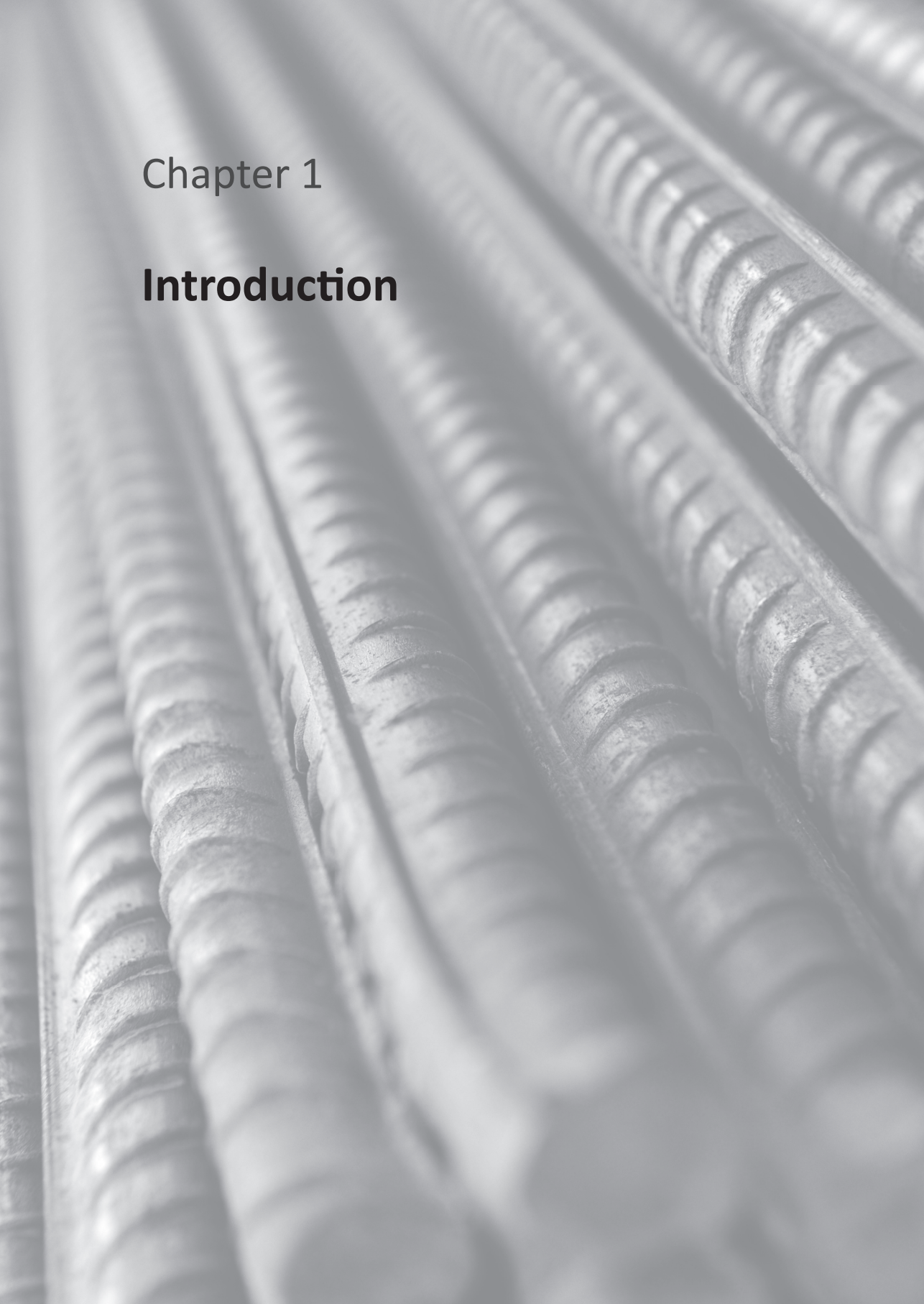

Chapter 1

Introduction

1.1 Bridging the Gap in Relation to Nerve Injury

The title of this thesis has been chosen after careful reflection. The different meanings reflect the research performed in this thesis:

- Nerve regeneration after bridging a large nerve defect is an important subject of this thesis.
- The second interpretation of this title should be sought in the translational aspect of this thesis. We tried to implement the use of Bone Marrow Stromal Cells (BMSCs) in nerve reconstruction in a conduit often used in clinical setting. A bench to bedside approach!
- The third interpretation refers to the bridge between experimental and human research. In this thesis both types of studies have been conducted to answer research questions related to nerve injury and one aspect of clinical follow-up, i.e. cold intolerance!

1.2 Clinical Cases: 2 Different Nerve Trauma

To appreciate the complexity of nerve trauma and the different approaches of treatment strategies, one does not need to look further than any outpatient clinic or emergency room (ER). The following two cases illustrate two typical injuries with different treatment strategies and outcomes, and varying degrees of success.

Case 1

Steve Johnson is renovating his house; he is a schoolteacher and has little experience in working with tools necessary to cut wood and steel. It is a Saturday morning and Steve Johnson is cutting floor parts using his circular saw. In a split second the timber floor part cuts loose from the circular saw, and his thumb is amputated from the hand.

In the ER the surgeon decides to reconnect the thumb to the hand. After a long surgery session, in which blood flow is restored, the bone repositioned, and the nerve stumps directly connected using sutures, a long period of recovery and training is required to restore feeling and function of the lacerated thumb.

Sixteen months after surgery Steve Johnson still often suffers from small burnings because his diminished feeling doesn't make him realize when an object is hot. In addition, he is complaining of intolerance to cold, specifically in the area of the hand that is innervated by the nerve that was lacerated.

Case 2

James Duggan is a healthy 16 year old boy. He is brought to the ER because he fell through a glass window while running in the living room. His left arm is severely injured, including a complete laceration of the ulnar nerve, leaving a gap of 4 cm. In the operating theater the surgeon decides to bridge the nerve defect using an autologous donor nerve from the left foot of the patient.

The patient recovers as expected, but the rehabilitation progresses slowly. 24 months after surgery the patient still complains of suboptimal function and, most importantly, he doesn't have full sensation. This results in bruises and burnings, and he can't walk outside because of aching pain in the palm of his left hand and arm when exposed to cold. Additionally, at the donor site of his foot, the patient is complaining of loss of sensation and sometimes irritating pain, symptomatic for neuroma formation at the proximal stump.

Although both patients were treated according to the best treatment protocols at the time of their injuries, they still suffer from serious and irreversible problems. The following work was undertaken to progress our understanding of nerve regeneration after nerve reconstruction and contribute to the improved outcome for patients like Steve and James.

1.3 Introduction

Although today's treatment of nerve injuries in the upper extremity is often satisfactory, the clinical outcome after a peripheral nerve injury still regularly shows loss of sensory and motor function, which is frequently accompanied by pain and discomfort. Although the incidence of peripheral nerve injury is estimated at a relatively low 1.6 – 2.8%, the need for research into optimal treatment strategies is necessary and ongoing for these complications that are frustrating and often debilitating for the patients.[57,75]

Over the last thirty years, both clinical and experimental research have increased the knowledge of understanding the pathophysiology of nerve injuries, reconstruction techniques and the healing processes. However, we are still learning how to improve recovery of function after treatment of nerve injury.[42]

The reason for the failure of clinical improvement despite these insights should be sought in a number of factors. Alongside some fundamental problems described in Chapter 1.4 is a summary of the previously-known predicting factors of age, cooperation, motivation and cognitive capacity of the patient. Additionally, recent studies suggest the involvement of the plasticity of the brain; here, sensory re-education and hand therapy are predicting factors.[42]

Hence, "improvement" in its broadest sense plays a central role in this thesis. Not only is our research focused on improvement of nerve reconstruction, but also on the improvement of evaluation techniques used for testing nerve function. We seek to find a new, minimally invasive evaluation technique, as well as a novel reconstruction technique combining vein, muscle and stem cells to bridge the defect. We demonstrate this via a proposed new approach to evaluate the presence of neural fibers in the footpaths of rodents.

To achieve optimal nerve reconstruction and thus to conquer problems related to the nerve regeneration process, it is important to understand the physiology of nerve degeneration and regeneration first. The basic anatomy of the peripheral nerve is therefore the second part of this introduction. Consequently, the reconstruction after a peripheral nerve trauma is discussed. The fourth part of this chapter will elucidate the problems related with and/or after nerve reconstruction. The final part will discuss the role of cellular therapy in nerve reconstruction and will introduce the bone marrow stromal cells in particular.

1.4 Basic Anatomy of the Peripheral Nerve

The peripheral nerve (PN) is the combination of connective tissue and neural components and is defined as the conducting or functional unit of the nerve cell. In other words, the nerve connects the brain to the motor and sensory endplates. A difference should be made between unmyelinated and myelinated fibers. Unmyelinated fibers are composed of several axons,

enveloped as a group by a single Schwann cell. Myelinated fibers, on the other hand, consist of a single axon, enveloped individually by a single Schwann cell. A multilaminated myelin sheath is formed by Schwann cells' membrane wrapping around the myelinated nerve fibers.[35]

The connective tissue structures of the PN consist of three distinct sheaths; from outermost to innermost these are the epineurium, perineurium and endoneurium, respectively. The epineurium surrounds the entire nerve trunk and blends with the connective tissue of nearby parts. The perineurium is the middle-level, and is made up of compact, concentrically arranged cellular layers. The endoneurium encloses all individual fascicles of longitudinally running nerve fibers and, thus, is the innermost sheath surrounding the Schwann cells and the nerve fibers within (Figure 1).[23]

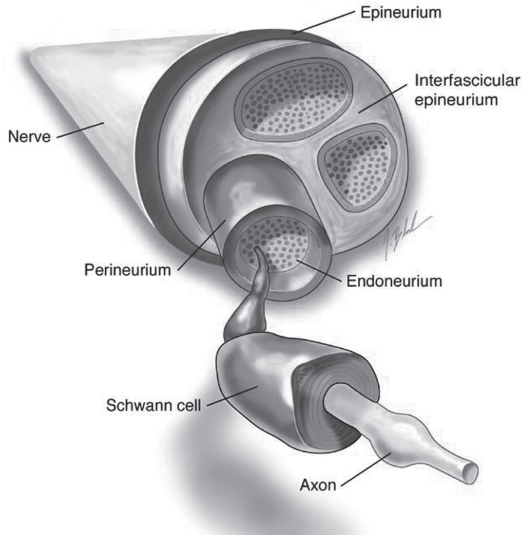

Figure 1 | Schematic composition of a peripheral nerve

Morphological Changes after Nerve Damage and Regeneration

After a peripheral nerve injury, difficult pathophysiologic changes including morphologic and metabolic changes occur at the site of injury, but also to the proximal and distal nerve segments. Both segments have to be separated to completely understand the main changes.

Proximal Segment

Transection of a peripheral nerve (i.e. axon) results in an exposure of the intracellular compartment of the axon to the extracellular environment and thus leads to neural death.[54,56,85] This process initiates a cascade of events to restore the damaged connections with a delay of 3-42 days.[16,26,70] The most important phenomenon is the degeneration of axons in a proximal direction from the nerve transection, leaving empty endoneurial tubes (the basal laminae of the Schwann cells) behind, which elaborates the regeneration by guiding the sprouting unmyelinated axons.[69]

Distal Segment

Immediately after the injury, the distal segment undergoes a process known as the Wallerian degeneration. Schwann cells and macrophages are recruited, and over a period of 3 – 6 weeks, phagocytize all the myelin and cellular debris (i.e. the result of disintegration of the axons).[26] The upregulation of Schwann cells causes the synthesis of several types of neurotrophic factors such as Neural Growth Factor (NGF).[29] Eventually, after a few months of regeneration, the invasion of sprouting axons results in a compartmentation of numerous miniature fascicles, which connect the CNS with the different motor and sensory end-plates.

1.5 Reconstruction of the Peripheral Nerve

It may be redundant to mention that the primary goal of nerve reconstruction is to allow reinnervation of the target organs by guiding regenerating sensory, motor and autonomic axons into the environment of the distal nerve without losing a significant number of axons at the site of injury.[7] In 1795 Cruikshank was the first to report successful nerve regeneration after surgical reconstruction, and, in the years following, extensive experimental and clinical studies investigating the peripheral nerve anatomy and physiology led to the introduction of microsurgery for the reconstruction of the peripheral nerve in the 20th century.[52,67] Despite the development and improvement of techniques and different repair methods in the last century, the rate of successful recovery from peripheral nerve lesions is far from optimal.[33,43,82] To reconstruct both small and large nerve gaps, a number of techniques is available to the surgeon. A clear distinction can be made between the direct and indirect (i.e. placement of graft or conduit) repair.[67] However, for both direct and indirect repair, optimal regeneration starts with precisely aligning the nerve stumps, creating minimal tension, and connecting the stumps atraumatically with minimal tissue damage and a minimal number of sutures.[17,21,28,44,45,67,68,74,77]

The primary surgical intervention of peripheral nerve laceration is immediate: direct end-to-end repair of the distal and proximal stump. This technique is recommended if the gap is small and if the two ends can be approximated with minimal tension.[20,44,73] The most common used end-to-end repair is the epineural repair, which requires aligning of the nerve ends and

placing 4-8 sutures through the epineurium alone to connect the stumps without any protrusion of fascicles.[49,82] A recent modification of this technique is the epineural sleeve technique, which reduces the compression and tension at the site of injury, and creates a biological chamber to preserve loss of axoplasmic fluid leakage.[41,68,77]

Indirect repair is necessary when the two nerve stumps cannot be approximated and coaptated without tension. Ever since its first clinical introduction in 1885 by Albert, the autologous nerve graft is considered to be the gold standard for bridging a discontinuity in a peripheral nerve.[67] The autologous nonvascularized nerve graft meets the criteria for an ideal nerve conduit, since it is a permissive and stimulating scaffold, contains the Schwann cell basal laminae, neurotrophic factors, and adhesion molecules.[1] Limiting factors for this type of reconstruction are the diameter of the donor nerve and the vascularization of the surrounding tissue, since the graft is nonvascularized and is completely dependent of the diffusion from the surrounding tissues and capillary in-growth from the nerve ends.[1,53] An obvious disadvantage to this treatment is the need for a second incision, donor site morbidity and prolonged surgery duration. The donor side morbidity is the combination of symptoms such as numbness, neuropathic pain and/or cold intolerance. This stimulates the search for new conduit/grafts to bridge the nerve gap. Such a conduit can either be biological or artificial, but should provide an environment for outgrowing axons, the growth of Schwann cells and neurotrophic stimulation of the distal stump.[51]

Biological Conduits

Arteries, veins, muscle and epineural sheath are all considered to be biological conduits. The vein is the most studied biological conduit, and will therefore be discussed here. The possibility to use a vein as a nerve conduit was first described by Chiu et al in 1980, and supported by Suematsu et al. in 1988.[11,71] The first successful clinical application was performed in 1990, with the reconstruction of digital nerves.[12] Research continued to focus on the vein as a conduit, and one observed drawback was the fact that the vein has the tendency to collapse.[84] A solution was proposed by Brunelli et al., which was to combine the vein with muscle, which not only prevents the vein from collapsing but also provides adhesion for the growing nerve fibers.[3,6] This technique is already applied in multiple clinical cases with good results, but remains inferior to the autologous nerve graft.[4,5]

Artificial Conduits

Artificial conduits are another alternative, and have evolved in the last 25 years to be clinically acceptable.[19] Five products are FDA approved for clinical use.[51,68] These approved conduits can be divided in resorbable (either polyglycolic acid, Poldy-DL-lactide-ε-caprolactone or collagen I) and nonresorbable (polyvinyl alcohol hydrogel).[51,67] Currently, no engineered graft conduit is as

effective as an autologous nerve graft, and, therefore, the biological conduits are favored over the artificial grafts by most surgeons.[33]

1.6 Problems Related to Nerve Repair

Neural Adhesion

Local tissue damage as a consequence of the injury will initiate migration and proliferation of fibroblasts and other cells that play a roll in the primary healing process of the tissue. In addition, the damage will stimulate extraneural collagen deposition. Collagen fibers, enveloping the nerve, can contract/shorten, increasing the risk of dysfunction and pain.[31,72]

Speed of Axon Regeneration

In humans, axonal disruption axons grow by 1 – 6 mm / day. After total loss of continuity of the nerve, growth slows to 1 – 2mm / day. The speed of this regeneration is dependent on multiple factors age, severity and level of injury, co-morbidity and species. Slower axon regeneration will induce formation of scar tissue at the suture site and distal of the lesion.[24,66,72,79]

Quantity of Axon Regeneration

When the complete regeneration over the injured site fails, the result will be loss of functional recovery. One of the causes for this incomplete regeneration could be the already mentioned intraneural scarring, which will prevent axons to reach their target organ.

Donor Site Morbidity

One of the problems related to the autologous nerve graft is donor side morbidity. The donor nerves used for nerve grafting are commonly expendable sensory nerves whose harvest results in no significant complications. A few examples are the sural nerve, the lateral antebrachial cutaneous nerve (LACN), anterior division of the medial antebrachial cutaneous nerve (MACN), doral cutaneous branch of the ulnar nerve, and uperficial sensory branch of the radial nerve (SSR).[30,49]

Cold Intolerance

Cold intolerance is defined as abnormal pain after exposure to mild or severe cold, with or without discoloration, numbness, weakness, or stiffness of the hand and fingers.[8,22,36,39,40] Symptoms of cold intolerance develop in the first months after injury, and generally do not decrease over time.[13,15,32,50,55,60,64] It has been reported that in the majority of peripheral nerve-injured patients, cold intolerance is the most bothersome, prolonged and disabling symptom, affecting both work and leisure activities.[2,25,40,55,60] Ruijs et al. studied the prevalence of cold intolerance in patients

with ulnar or median nerve injuries, and found that 56% of the patients with a single nerve injury and 70% with a combined nerve injury suffered pathological cold intolerance according to the CISS questionnaire.[62] Consequently, Ruijs et al. performed a study investigating the re-warming patterns, and described a strong relation between the sensory recovery and the presence of active re-warming in nerve injury patients. However, they could not relate cold intolerance to the re-warming patterns.[63]

Neuropathic Pain

Another problem, which often occurs after peripheral nerve injury, is neuropathic pain. The pathophysiology is explained by the manifestation of maladaptive plasticity in the nervous system.[14] The risk of developing a persistent neuropathic pain is ≈5% in small nerve traumas, whereas injury to larger nerves, for example the sciatic nerve, creates a risk of developing neuropathic pain up to 30% – 60%.[34,38,46] Although treatment targeted at the primary pathology is obviously essential, understanding the mechanisms responsible for the maladaptive plasticity offers specific therapeutic opportunities to prevent the development of neuropathic hypersensitivity and normalize function in established neuropathic pain.[14] Experimental research should therefore be conducted to increase the knowledge of neuropathic pain in order to deliver adequate therapy and treatment of neuropathic pain.

1.7 Cellular Therapy

Despite the progress that has been made in the last 2 decades, nerve conduits have remained inferior to the autologous nerve graft. As a result, cellular therapy has been introduced. The concept behind this approach is based on the fact that nerve transection induces the secretion of fluids from the both nerve ends and represents one of the first steps in the regeneration cascade. This fluid contains structural components such as neurotrophic factors but also macrophagic cells to break down myelin and neurons.[18,66] It is reasoned that if the luminal additive contains similar substances, it could enhance or fasten the nerve regeneration. Additives can be separated into cellular, structural and neurotrophic components.[83] Since in this thesis the supportive effect of bone marrow stromal cells in nerve regeneration is one of the major aims, only the cellular components of luminal additive will be discussed:

Schwann Cells

Schwann cells are considered the main support cells of the peripheral nervous system and are responsible for supporting axons through forming the myelin sheath to increase conduction speed and removal of debris.[48] The Schwann cells contribute to nerve regeneration by producing and secreting neurotrophic factors, and by organizing themselves into a series of cylinders to

help guide the regenerating axons and therefore could make Schwann cells an attractive choice as a supportive cellular therapy inside the conduit. A recent review by Terenghi described the problems related to the use of autologous Schwann cells, since after harvesting the cells they have to be cultured for up to 10 weeks before having grown enough cells for transplantation. This unavoidable delay could impair the nerve regeneration, as a fast reconstruction is advised in nerve repair.[76] Allogenic Schwann cells, however, could be a solution to this problem, since the cells have already been harvested and grown in culture. These cells can therefore be used whenever the surgeon needs to bridge the nerve gap with a conduit and a luminal supportive additive is required. Unfortunately, the support of Schwann cells did lead to regeneration, but mostly not comparable to the regeneration potential of a nerve autograft.[61] Despite this drawback, a review by Yan et al.[83] showed that significant research has been carried out on this topic, and the beneficial effect of Schwann cells has been described in different types of conduit including collagen,[59] synthetic[27] and vein[84].

Bone Marrow Stromal Cells (BMSCs)

These cells are found in the bone marrow and are therefore found in larger amounts and easier to obtain.[83] Similar to Schwann cells the BMSCs must be cultured before injection, but only 5 – 8 passages are sufficient to grow sufficient undifferentiated cells. BMSCs have the capacity to migrate extensively, and, because they can transdifferentiate into their surrounding tissue of bone, cartilage and fat, they can also differentiate into neural tissue.[65,78] Kielhoff et al. confirmed that the BMSCs have the potential to differentiate into SC-like cells and can therefore support nerve regeneration,[37] and, as a consequence of this differentiation, the mesenchymal stem cells will synthesize and secrete neurotrophins.[10,47] There are also studies that have used undifferentiated mesenchymal stem cells in a nerve conduit and confirmed a stimulation of the axonal regrowth and motor function recovery.[9,58,81] Additionally, Walsch and Midha stated in their literature review on the augmentation of stem cells that the use of BMSCs as a luminal additive increases electrophysiological and/or behaviroal recovery compared to an empty conduit.[80] The evidence of the beneficial effect of the BMSCs is promising, and, for that reason, of particular interest in this thesis. We will investigate the effect of BMSCs when a vein is used as a carrier in large neural gaps.

1.8 General Aim and Outline of the Thesis

The aim of this thesis first part was to investigate the regenerative effect of BMSCs when used in the vein as a carrier using up-to-date evaluation techniques. The working hypothesis is that BMSCs will enhance nerve regeneration when used as a cellular therapy in vein graft reconstruction model.

The vein is accepted for bridging large neural gaps and has been part of the surgeon's arsenal of different nerve conduits for many years. The main investigative model employed was the rat sciatic nerve model, which is often used in peripheral nerve research, both for the assessment of functional and histological regeneration parameters.

Chapter 2 is a study conducted in collaboration with the Cleveland Clinic microsurgery laboratory, and the main objective was to compare the regenerative effect of the vein filled with Bone Marrow Stromal Cells (BMSCs) with the vein left empty.

Chapter 3 investigates the possibility of a new minimally invasive ultrasound technique for the placement of a needle near the sciatic nerve. This needle needs to be placed for accurate stimulation of the nerve to be able to retrieve Compound Muscle Action Potentials (CMAPs). The introduction of an evaluation technique will create the possibility to measure the CMAPs in the same animal over time, which will reduce animal use and increase the accuracy of the nerve regeneration model in rodents.

Chapter 4 was designed after the interpretation of the results of Chapter 3. We modified the vein graft supported with BMSCs to a vein-muscle graft supported with BMSCs. This modification originated in Italy, and prevented the vein from collapsing. The major aim was to elucidate the regeneration potential of this particular graft. Another goal was to track our injected mesenchymal stem cells in-vivo by means of a fluorescent label. This label could possibly shed light on the in-vivo differentiation in our conduit and help explain the regeneration process in our conduit.

In **chapter 5** skin biopsies from the rat hind footpath were taken to evaluate the regeneration of all sensory skin fibers 12 weeks after reconstruction of a 15mm nerve defect using an autograft. Visualization of the skin epidermal sensory nerve fibers is useful to show the regeneration in the most distal terminals of the injured nerve. The peptidergic (visualized with CGRP and Substance P) and non-peptidergic fibers (visualized with P2X3) are responsible for signalling noxious and termed nociception. The Aδ-fibers are visualized using NF200 staining.

In **chapter 6** the same evaluation technique as in chapter 5 is used and allowed us to visualize the re-innervation of the sensory skin fibers after reconstructing the nerve using a vein-muscle graft with and without additional BMSCs as a luminal additive.

Part II of this thesis comprises two clinical studies investigating cold intolerance in patients with a hand fracture. **Chapter 7** is a retrospective study in which we analysed the presence of cold intolerance in patients who had suffered a hand fracture. The prevalence of cold intolerance was determined using the CISS questionnaire.

In **chapter 8** infrared videothermography was used to continuously monitor re-warming patterns of the hand and fingers after short term cooling in both healthy volunteers and hand fracture patients. In this study we determined whether patients with and without cold intolerance after a hand fracture or healthy controls have a diminished re-warming after a cold stimulus.

In **Chapter 9** the implications of this research for future research and clinical practice are discussed.

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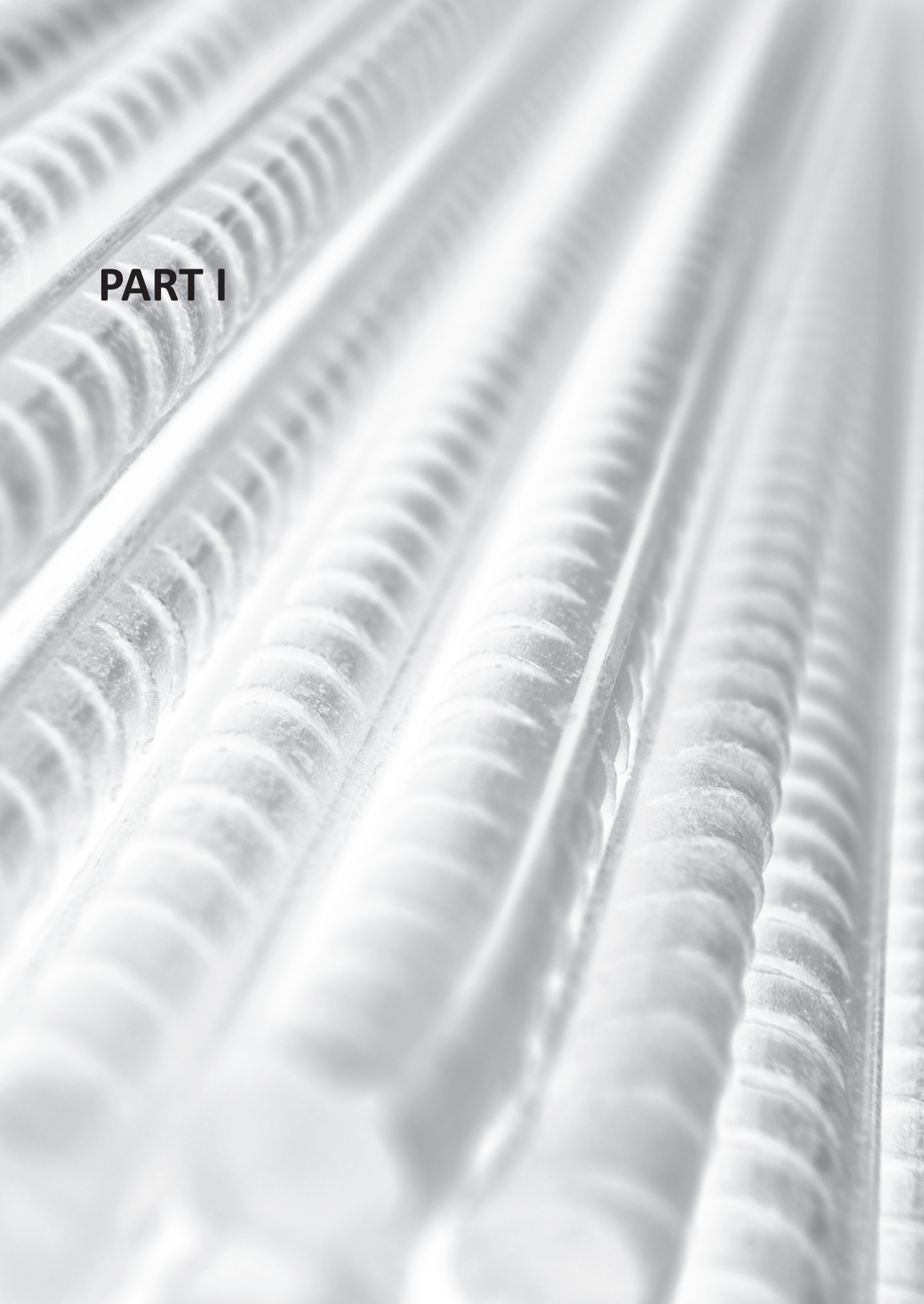

PART I

Chapter 2

Isogenic Venous Graft Supported with Bone Marrow Stromal Cells as a Natural Conduit for Bridging a 20mm Nerve Gap

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Abstract

Introduction

In this study we introduce a technique for bridging large neural gaps, using an isogenic vein graft supported with isogenic bone marrow stromal cells (BMSCs).

Methods

In three groups a nerve defect of 20mm was bridged with a vein graft. Our first experimental group comprised an empty venous graft, in group II the venous nerve graft was filled with saline where as in group III the venous nerve graft was filled with isogenic BMSCs. The animals were tested for functional recovery up to 3 months post repair.

Results

Our results show that the BMSC filled venous graft resulted in significantly better regeneration of the nerve defect compared to controls, as confirmed by the functional recovery measured by somatosensory evoked potentials, toe spread, pin prick and gastrocnemius muscle index.

Conclusion

The results in this study confirm the hypothesis that the vein graft supported with BMSC is associated with better functional nerve regeneration as compared to the saline filled vein graft and the empty vein graft and therefore can be considered a promising method for the reconstruction of large nerve defects.

Introduction

Five percent of all open wounds in the extremities are complicated by peripheral nerve trauma.[14] The primary surgical intervention of peripheral nerve laceration is immediate end-to-end repair of the distal and proximal stump. This technique is recommended if the gap is small and the two ends can be approximated with minimal tension.[20,27]

When the nerve gap is too large for primary repair, an alternative approach is required to establish a tension-free coaptation.

Nerve grafts such as the autologous non-vascularised peripheral nerve grafts are widely used.[26] The use of a nerve graft remains the gold standard, since the graft is the source of neurotrophic factors and will minimize the foreign body reaction. However, despite the fact that the results are satisfactory, a limited amount of donor nerves can be considered and significant donor site morbidity such as scarring, neuroma formation, and loss of sensation have been described.[15]

The morbidity of sacrificing normal functioning nerves as nerve grafts has resulted in a search for alternatives.

Over the last decades multiple studies using experimental models have been performed with nerve gaps of different materials and there are several tubes approved by the US Food and Drug Administration (FDA) for clinical applications.[21,30] Although these developments are encouraging, the risk of inflammation, high costs, and mixed results have opened a new search for natural conduits.

Vein grafts are more abundantly available in a wide variety of sizes and lengths, have less donor morbidity than autografts, and provide a metabolically supportive environment for the regenerating axons. Furthermore the wall of the vein acts as a barrier against scar ingrowth.[33] Other experimental studies have clinically, electrophysiologically and histologically demonstrated that the vein graft is capable of axon regeneration.[2,5,16,24] In addition, Levine et al. revealed an expression of nerve growth factor in the femoral vein of a rodent, which could benefit the nerve regeneration.[17] On the other hand, it has been reported that contact between the endothelial cells of the vein graft and the regenerating axons would result in fibrosis, which causes nerve constriction and impairment of axon regeneration.[13]

In recent studies bone marrow stromal cells (BMSCs) have been used as a cellular therapy to promote nerve regeneration. BMSCs are multipotential cells that contribute to the regeneration of different type of tissues such as bone, cartilage, fat, and muscle. Moreover, multipotency of BMSCs is augmented by the expression of many cytokines and neurotrophic factors.[4] In addition, the BMSCs are capable of supporting nerve regeneration and promote axonal regeneration.[4,7,11,18,22,28,31,32] Cuevas et al. showed that undifferentiated BMSCs exert a beneficial effect against peripheral nerve injury.[9,10] Consequently a recent literature review by Walsh et al. concluded that cell-based strategies in clinical setting are promising, however the

optimization of cell delivery and the combination of grafting material with the cells should be investigated.[29]

Therefore, in the search for a graft capable of bridging short nerve defects with adequate regeneration and minimal side effects (i.e. neural scarring and / or neuroma formation), previous studies combined a vein graft supported with BMSCs as a natural conduit in a 8mm and 16mm sciatic nerve defect in rodents.[7,11]

In this study we describe the application of the BMSC supported vein graft to bridge a 20mm nerve defect. Our hypothesis is that the BMSC supported vein graft is more suitable for peripheral nerve reconstruction than a saline filled vein graft or an empty vein graft.

Materials and Methods

Animals

Seventy-eight Lewis rats, weighing 240 to 280 grams and cared for under the guidelines for the Care and Use of Laboratory animals published by the National Institutes of Health and the guidelines of the Cleveland Clinic Animal Research Committee, were used.

Animals were pair-housed in hooded cages at room temperature on a 12-hour light/dark schedule, and were given food and water ad libitum. Surgical procedures and electrophysiological evaluations were performed under subcutaneous ketamine cocktail anaesthesia (ketamine, xylazine, acepromazine, 30, 6, and 1 mg per kilogram, respectively). The surgical procedure was performed by one surgeon using standard aseptic microsurgical techniques under the operating microscope (Zeiss OP-MI 6-SD; Carl Zeiss, Goettingen, Germany) on sciatic nerve of the left hind limb, and the right limb sciatic nerve served as control.

Bone Marrow Stromal Cell Preparation

Fresh bone marrow cells were harvested aseptically from tibias and femurs of 8 adult isogenic rats. Both ends of the bones were cut and the marrow was flushed with 10ml of alpha-MEM medium. After centrifugation, cell suspension was lysed with 0.85% NH_4Cl for 5 min. The suspension was then filtered through 70-mm nylon mesh, and re-suspended with culture alpha-MEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 25 ng/ml amphotericin B. The cells were placed in a 75 cm^2 flask and incubated at 37°C in 5% CO_2 for 3 days. The non-adherent cells were removed by replacing the medium three times a week. After 5 to 8 passages, when the BMSC culture reached confluence, BMSCs were lifted by 0.25% trypsin and 1 mM% EDTA in PBS for 5 min, washed in alpha-MEM medium and prepared for transplantation.

Surgical Procedure

Twenty-four donor rats were used to harvest two isogenic vein grafts. A longitudinal mid-line incision of 35mm was created in the neck of the donor rat. The right and left external jugular veins were dissected, ligating all side branches and ligating both proximal and distal ends of the graft with 10 – 0 nylon sutures (Bear Microsurgery, Arosurgical instruments, Newport Beach), leaving a 20mm graft. The harvested veins were kept on a straight irrigator in a 0.9% saline filled sterile container, while preparing the recipients.

The recipient rats were anesthetized according to the previous described protocol and an oblique skin incision was made from the posterior thigh to the knee of the left hind limb. The sciatic nerve was exposed from the sciatic notch to the popliteal bifurcation. A 20 mm gap was created in the exposed sciatic nerve and bridged with the venous graft.

The graft was coaptated proximally to the sciatic nerve and distally to the posterior tibial nerve using 6 10/0 nylon sutures (Bear Microsurgery, Arosurigcal instruments, Newport Beach, USA) creating a 'pull-over sleeve', which was first described by Siemionow et al.[19,25] The muscle was closed using 4 5/0 Vicryl sutures (Ethicon Inc, Johnson & Johnson Somerville USA), followed by closing the skin using 5/0 Vicryl sutures (Ethicon Inc, Johnson & Johnson Somerville USA).

Experimental Groups

After coaptation, the rats were randomly divided into three experimental groups of 16 rats each. In group I the vein graft remained empty after coaptation, in group II the vein graft was filled with 0.08ml 0.9% saline after coaptation and in group III the vein graft was filled with $3.5 – 4 \times 10^6$ BMSCs suspended in 0.08 ml AlphaMem medium. Eight animals from each group were evaluated and euthanized after 6 weeks while the remaining eight animals were euthanized after 12 weeks.

Functional Assessment

The nerve regeneration in the 3 different groups was evaluated at 1, 3, 6 and 12 weeks by pinprick and the toe-spread test.

At 6 and 12 weeks somatosensory evoked potentials (SSEP) were recorded and the gatrocnemius muscle index (GMI) was calculated.

The pinprick test was used to assess the recovery of sensory function. A pinching stimulus was applied with forceps to the skin of the hind limb starting distal at the toes ascending up towards the knee. The withdrawal reaction was graded on a 0-3 point scale. Presence of withdrawal during stimulus above the ankle, the metatarsal area, and at the level of the toes was graded 1, 2 and 3 respectively. An absent withdrawal during skin stimulation was marked with '0'.

The toe-spread test is a commonly used test to assess the motor function recovery. Toe spread was evaluated by raising the animal by the tail and observing voluntary reaction of the toes. When full toe extension and abduction was observed a normal reaction was assigned. No reaction was given 0 points. Any sign of toe movement was awarded 1 point, abduction of the toes graded with 2 points and extension and abduction 3 points.

Somatosensory evoked potentials were measured using a Nihon Kohden Neuromaster MEB-2200 (Tokyo, Japan).

To retrieve the SSEPs, stimulating electrodes were placed subcutaneously (near the Achilles tendon and at the dorsum of the foot). Two holes were drilled in parietal bones of the cranium and recording electrodes were placed over the area of the somatosensory cortex. An increasing electrical stimulus (0-10 mA/200μs) was applied to the stimulating electrodes until active cortical responses were seen. At least 250 average responses were averaged to produce one waveform at motor threshold intensity. At least 3 waveforms were recorded for each animal for both operated and contralateral side.

The typical, triphasic waveform was registered with characteristic latencies and a series of negative and positive potentials. The N1 latency was marked as the time point when the first upward deflection (negativity) was seen in the cortical channel. This is typically followed by a prominent downward deflection called the P1 response (a typical example of this waveform is depicted in Figure 1). The later upward deflection following the P1 potential was labeled as the N2 potential. The amplitude was defined as a difference between the amplitude of the P1 and N2 potential. Control values were obtained from unoperated side. We analyzed the latencies and amplitudes at each time point within the experimental groups by comparing them with the control values. We evaluated the difference between experimental groups at each time point by calculating the ratios between the operated and unoperated latencies and amplitude to avoid the anesthesia influence.

Gastrocnemius muscle index was measured to assess the denervation atrophy at 6 and 12 weeks follow-up. The muscle was excised form the lower leg and wet muscle weight was measured immediately using a digital scale (Ohaus Precision Standard, Germany). The contralateral gascrocnemius was also harvested and the muscle weight index was calculated by dividing the muscle weight from the operated side by that from the contralateral side.

Statistical Analysis

Pearsons correlation and Anova-analysis were used to determine statistical significance. P values of less then 0.05 were considered as significant.

Results

All operations went uneventful and in all animals it was possible to evaluate the results of our intervention.

Pinprick Test

As shown in Figure 2, a significant difference in withdrawal between group I and III was found at 1 week post grafting ($p = 0.0146$). At 3 weeks the withdrawal reaction of group I and II was significantly worse ($p = 0.0146$) compared to group III. At 12 weeks after surgery all animals reached the maximum value of 3 points.

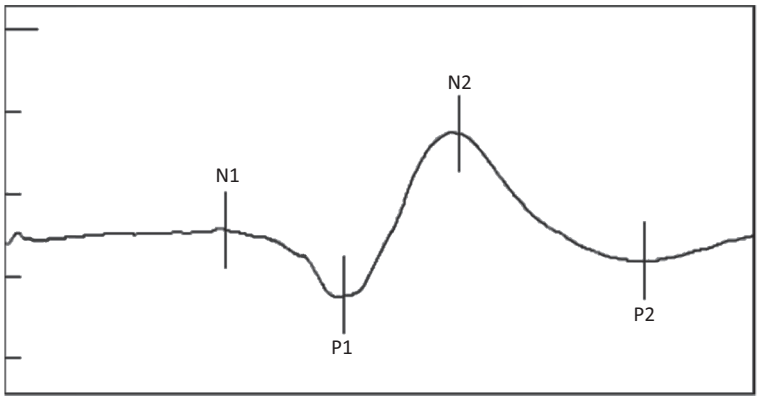

Figure 1 | Normal somatosensory evoked potential recorded from an unoperated side. The N1 latency was marked as the time point when the first upward deflection (negativity) was seen in the cortical channel. This is typically followed by a prominent downward deflection called the P1 response. The later upward deflection following the P1 potential was labeled as the N2 potential. The amplitude was defined as a difference between the amplitude of the P1 and N2 potential.

Toe-spread Test

Figure 3 shows the results between the three different groups at different time points. For all three groups significant improvement of toe-spread over time was found ($p < 0.0001$). The BMSC filled vein graft was found to have a significantly better toe-spread compared to the saline filled and the empty vein graft at 6 weeks ($p = 0.0342$ and $p = 0.0016$, respectively) and at 12 weeks ($p = 0.0373$ and $p = 0.0206$, respectively). No significant differences between the saline filled vein graft and the empty graft were found at 6 and 12 weeks.

Somatosensory Evoked Potentials

Mean values of P1 and N2 latencies, as well as amplitude were shown in Table 1. The operated/unoperated side ratios for latencies and amplitude were calculated and depicted in Table 2.

There was a significant elongation of P1 and N2 latencies in group I at 6 and 12 weeks compared to the contralateral side ($p = 0.0005$ / $p = 0.0330$ and $p = 0.0001$ / $p = 0.0001$).

A significant elongation of the N2 latency in the BMSC and saline filled grafts at 12 weeks was found compared to the control N2 latency ($p = 0.0015$ and $p = 0.0030$, respectively).

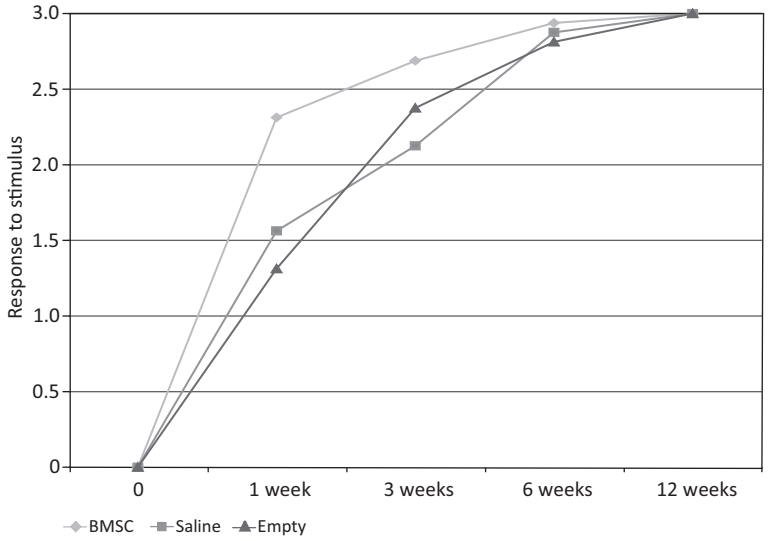

Figure 2 | Results of Pinprick test at day 7, day 21, 3 weeks, 6 weeks and 12 weeks after grafting groups: empty vein graft (Empty), saline filled vein graft (Saline) and BMSC filled vein graft (BMSC).

Table 1 | Mean SSEP latencies [ms] and amplitudes [μV] of the operated side at 6 and 12 weeks. Control values – unoperated side (mean ± SD).

	6 weeks			12 weeks		
	P1Lat	**N2Lat**	**Amplitude**	**P1Lat**	**N2Lat**	**Amplitude**
Group I	20,99 ± 3,98	28,77 ± 5,26	5,48 ± 4,1	19,10 ± 1,13	26,32 ± 1,16	6,73 ± 1,42
Group II	18,54 ± 2,65	25,53 ± 2,58	5,25 ± 3,0	18,59 ± 1,79	24,73 ± 1,84	7,23 ± 4,45
Group III	17,51 ± 2,62	25,07 ± 3,45	6,56 ± 4,2	17,27 ± 1,92	24,84 ± 1,89	8,36 ± 3,27
Control	16,82 ± 1,45	24,00 ± 2,91	19,18 ± 13,37	16,82 ± 1,45	24,00 ± 2.91	19,18 ± 13,37

Table 2 | Mean SSEP latencies and amplitude ratios (operated side / unoperated side) at 6 and 12 weeks (mean ± SD).

	6 weeks			12 weeks		
	P1Lat	**N2Lat**	**Amplitude**	**P1Lat**	**N2Lat**	**Amplitude**
Group I	1,22 ± 0,22	1,15 ± 0,21	0,56 ± 0,40	1,18 ± 0,12	1,15 ± 0,07	0,44 ± 0,27
Group II	1,14 ± 0,12	1,06 ± 0,28	0,48 ± 0,39	1,13 ± 0,12	1,09 ± 0,10	0,31 ± 0,17
Group III	1,09 ± 0,08	1,11 ± 0,12	0,51 ± 0,28	1,01 ± 0,08	1,08 ± 0,07	0,32 ± 0,2

Comparing the latencies between the three groups at 6 weeks group I was significantly more elongated compared to group III. A significant difference was found at 12 weeks comparing the P1 latency between group I and group III ($p = 0.0356$) and between group II and group III

(p = 0.047). The N2 latency was significantly more elongated in group I when compared to group III (p = 0.048). No significantly differences in amplitude were found when comparing the three groups. Detailed results are depicted in Figures 4, 5 and 6.

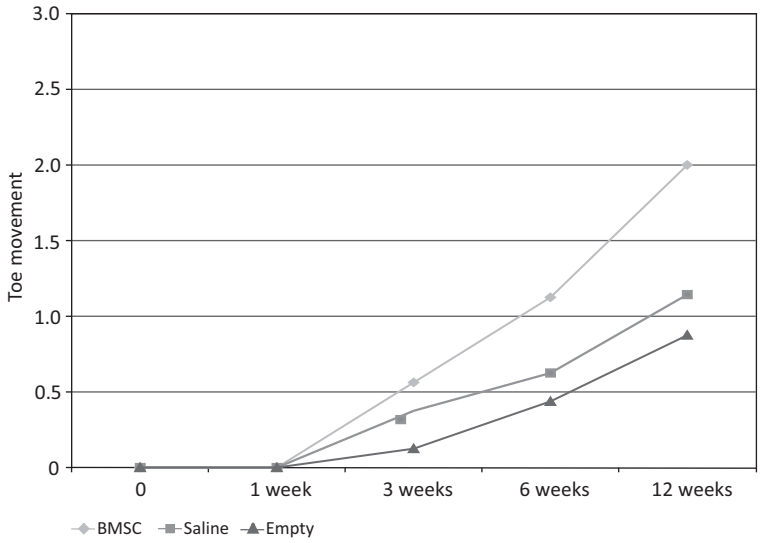

Figure 3 | Results of Toe-spread test at day 7, day 21, 3 weeks, 6 weeks and 12 weeks after grafting groups: empty vein graft (Empty), saline filled vein graft (Saline) and BMSC filled vein graft (BMSC).

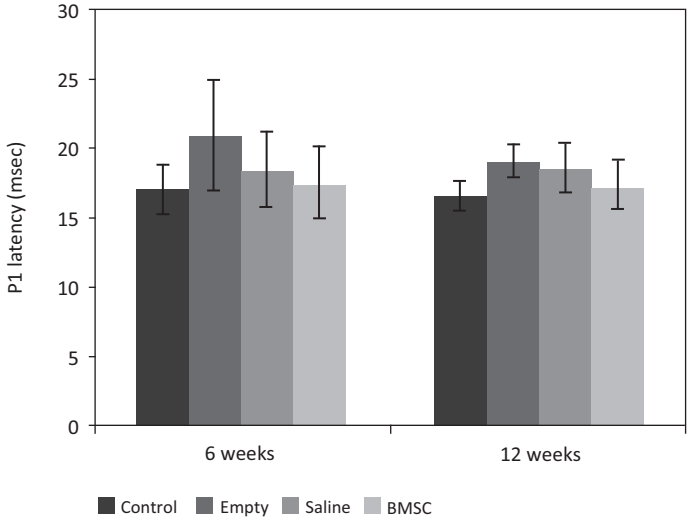

Figure 4 | Results of somatosensory evoked potentials at 6 and 12 weeks after grafting: empty vein graft (Empty), saline filled vein graft (Saline), BMSC filled vein graft (BMSC) and the control latency (Control).

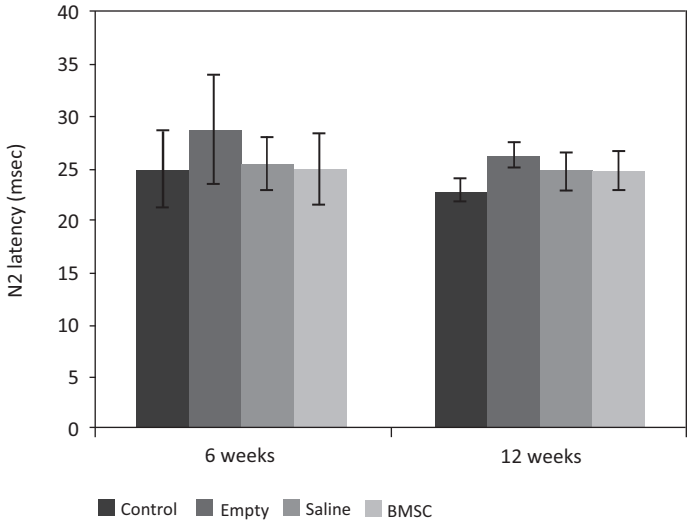

Figure 5 | Results of somatosensory evoked potentials at 6 and 12 weeks after grafting: empty vein graft (Empty), saline filled vein graft (Saline), BMSC filled vein graft (BMSC) and the control latency (Control).

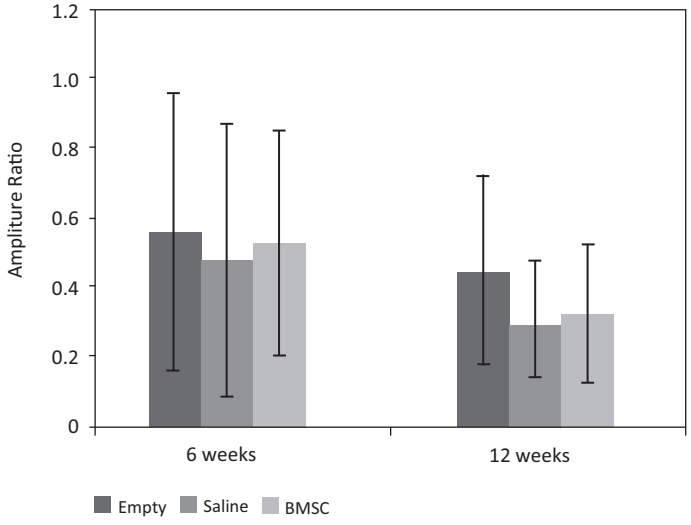

Figure 6 | Results of somatosensory evoked potentials at 6 and 12 weeks after grafting: empty vein graft (Empty), saline filled vein graft (Saline), BMSC filled vein graft (BMSC) and the control latency (Control).

Gastrocnemius Muscle Index

GMI at 6 weeks for groups I, II and III was 27.23 ± 4.36%, 29.70 ± 4.18% and 33.05 ± 5.96%, respectively. At 12 weeks post grafting GMI for groups I, II, III was 17.18 ± 3.65%, 17.22 ± 2.32% and 21.68 ± 1.89%, respectively.

GMI was higher for the BMSC filled vein graft compared to the saline filled vein graft and the empty vein graft at 6 weeks ($p = 0.2147$ and $p = 0.0428$, respectively) and at 12 weeks ($p = 0.0012$ and $p = 0.0079$). For all three groups a significant decrease of muscle weight between 6 and 12 weeks was found ($p < 0.0005$). A graphic illustration is depicted in Figure 7.

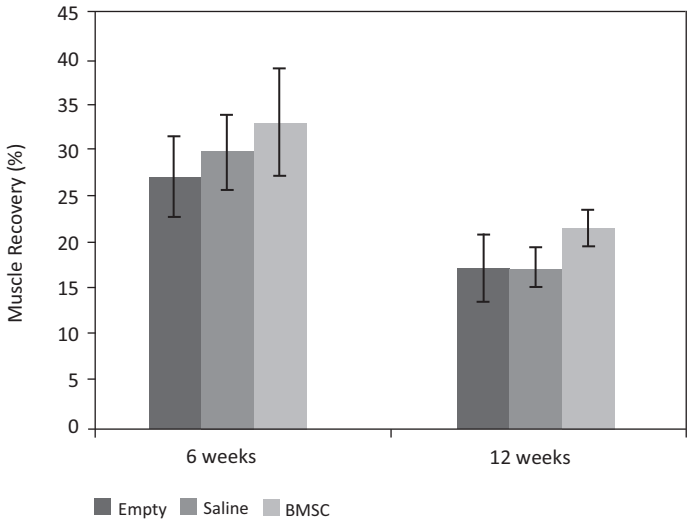

Figure 7 | Results of gastrocnemius muscle index at 6 and 12 weeks after grafting: empty vein graft (Empty), saline filled vein graft (Saline) and BMSC filled vein graft (BMSC).

Discussion

The results in this study show a minimal withdrawal after pin prick stimulus, one week after grafting. Consequently, a maximum withdrawal reaction was measured in all groups at 12 weeks.

Toe-spread test revealed a strong correlation between the improvement of the toe spreading with progression of time in all three groups, with significant differences between the BMSC filled vein graft and the other two vein grafts at 6 and 12 week post grafting.

The SSEPs used to evaluate neurophysiologic recovery showed that at 12 weeks post grafting only the BMSC vein graft had comparable P1 latencies to the contralateral side and for

that reason can be considered to have normal potentials. In contrast, the N2 latency remained significantly different in all three groups compared to the contralateral side at 12 weeks post grafting. These results demonstrate that the vein graft alone does not have the capability to reach a somatosensory potential as the normal sciatic nerve. This finding is confirmed by earlier performed studies concerning the vein graft.[1,15,33] Despite the fact that these results indicate a significant decrease in the GMI between 6 and 12 weeks, the GMI of the BMSC filled vein graft is significantly higher than the saline filled and empty vein graft and therefore support the results yielded from the SSEPs. The increase in muscle atrophy in the operated limb between 6 and 12 weeks in all groups reveals that muscle re-innervation has not yet been established. Despite the fact that the motor end plates have not yet been reconnected through axons with the peripheral nerves, the BMSC filled vein graft showed significant less atrophy compared to the empty vein graft.

In current literature two explanations for the contribution of BMSCs to nerve regeneration exist. The first explanation is that BMSCs have the capability to differentiate into neural lineages including neurons, astrocytes, oligodendrocytes, microglia and most important Schwann Cells.[4,18,22,28,31,32]

The second explanation of the contributing role of BMSCs is that they may produce many different cytokines and growth factors and positively impact neural cell survival.[3,8,23,33]
The supporting role of BMSC in combination with the vein graft has been previously described,[5,11] however both studies used a smaller gap to evaluate the regeneration potential and functional evaluation was not performed.

Even though the results in this study indicate that the vein graft filled with BMSCs is a reliable conduit for reconstruction of 20 mm nerve defects, future studies should focus on the histological evaluation of this graft. A limitation of our study could be the used pin prick evaluation technique, since a minimal withdrawal in all three groups was observed already one week after grafting and all groups showed a maximal withdrawal reaction at 12 weeks. These findings seem to indicate a full and successful regeneration in all groups, which is not supported with our other results.

In summary this study applied the vein graft over a larger (20 mm) defect than previously described in literature. The introduction of this technique in long neural gaps is of importance for clinical application, since autologous nerve grafting is currently the procedure of choice, but when insufficient length is available an alternative is needed. Hence, studies investigating regeneration in large neural gaps are of particular interest for the surgeon. Furthermore, the sleeve technique for graft coaptation used as described by Siemionow et al.,[25] prevents the fascicles from sprouting and will minimize the leakage when injecting the BMSCs into the graft.
These results prove that injecting the BMSCs into the vein graft not only improves nerve regeneration but also prevents the graft from collapsing and therefore limit the nerve regeneration process as described by Chiu et al.[6,12] Moreover, the relative ease of isolation of substantial cell

numbers via bone marrow aspiration could make BMSC attractive for additional cellular therapy in clinical setting. To this end we should focus on nerve regeneration after injecting a vein with aspirated bone marrow. The results presented in this study confirm the hypothesis that the vein graft supported with BMSC is associated with better functional nerve regeneration as compared to the saline filled vein graft and the empty vein graft and therefore can be considered a promising method for the reconstruction of large nerve defects.

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Chapter 3

Ultrasound-guided Needle Positioning Near the Sciatic Nerve to Elicit Compound Muscle Action Potentials from the Gastrocnemius Muscle of the Rat

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Abstract

Introduction

The use of ultrasound-guided electrode positioning in near-nerve myography was investigated. This is a minimally invasive technique that allows repeated measurements to increase accuracy and hence decreases animal numbers.

Methods

Ultrasound imaging of the sciatic nerve was performed in nine rats using a 55 MHz high-end transducer. Once visualised, a monopolar needle electrode was placed through the skin near this nerve. Upon stimulation, two surface electrodes, placed over the gastrocnemius muscle, recorded compound muscle action potentials (CMAPs). Reproducibility was tested having two teams of investigators perform the recordings consecutively. Reliability of the procedure was determined by comparing the ultrasound method to the conventional technique, which requires an incision through muscle and skin to expose the sciatic nerve.

Results

In all animals the sciatic nerve was visible on ultrasound images. Both methods showed CMAP latencies. Reproducibility was excellent, resulting in a coefficient of variation for duration of 2.1% and for amplitude 6.7%. Interclass correlation coefficient was 0,828 for duration. Comparing the three different measurements no significant differences were found and our new method can therefore be considered reliable and comparable to the conventional method.

Conclusion

Ultrasound-guided near-nerve needle positioning is a reproducible and reliable minimally invasive method for selectively eliciting CMAPs, which allows repeated CMAP measurements for studying nerve regeneration in rats.

Introduction

Peripheral neuropathies often result in a reduction of the number of muscle fibers (and, hence, of force) that can be recruited voluntarily or through electrical stimulation of the affected nerves. In neurophysiological testing, this reduction is reflected in a low compound muscle action potential (CMAP) amplitude.[7] Conversely, CMAP amplitude is sometimes used to evaluate the nerve regeneration process in neuropathy in both animal studies and a clinical setting.[2,10] A recent review study has evaluated various methods for the assessment of peripheral nerve regeneration in rodents, including the CMAP.[9] Because the majority of the reviewed studies demonstrated a significant difference in the CMAP amplitude recorded from regenerating nerves over time, using the CMAP for evaluation of nerve regeneration was highly recommended in future animal experiments.

The conventional method to retrieve CMAPs in rats requires surgical exploration of the sciatic nerve before placing the needles necessary to stimulate the nerve selectively. This invasive procedure can result in sacrificing the animal, because of the trauma created by making the incision and splitting the muscle to expose the sciatic nerve. Consequently, multiple animals are needed to track nerve regeneration over time. Being able to study the affected nerve in the same animal at different time points would not only reduce the number of animals required for such research, but also minimize unwanted variability from differences between animals interfering with the effects of the regeneration process.

A recent animal study by Kuffler demonstrates that sonography can work as a diagnostic tool for nerve damage while eliminating the need for invasive exploratory surgery.[5] Furthermore, earlier studies by our department have shown that ultrasound-guided positioning of stimulus electrodes near the sciatic nerve of rabbits allowed the retrieval of compound nerve action potentials (CNAP) and compound muscle action potentials (CMAP). We concluded that ultrasound could be helpful for evaluating the progress of nerve regeneration by means of minimally invasive (and, hence, repeatable) tests.[3,4]

The present study was designed to evaluate the feasibility of high-end ultrasound imaging of the rat sciatic nerve, and to explore the possibility of using ultrasound guidance for the placement of needles to elicit CMAPs.

Material and Methods

Animals and Anaesthesia

Nine Wistar rats, weighing 250 – 450 g, were studied under general anaesthesia (Isoflurane, 1 – 2% in O2). Animal status was monitored by visual inspection (i.e. breathing) and the animal's temperature was controlled with external heating by means of a heating pad. Upon completion

of the experiments, animals were sacrificed by means of surgically applied bleeding in the thorax. The experimental protocol was approved by the local Animal Experiments Committee according to the National Experiments on Animals Act and conducted according to this law that serves the implementation of Directive 86/609/EC of the Council of Europe.

Study Procedures

The study was designed to allow both an assessment of reproducibility/interoperator variability and a comparison with the gold standard invasive technique. Two different teams were involved during the measurements. First, Team 1 executed the near-nerve myography technique, Team 2 followed after a short break in the same animal. The animal remained anaesthetized and in the same position, but stimulus and recording electodes and the ultrasound probe were removed. Finally, either Team 1 or Team 2 continued with the conventional technique, by making a skin incision and splitting the gluteal septum in order to accurately place the needle.

The sciatic nerves of the rats were visualized using a Vevo 770 system (VisualSonics Inc., Toronto, Canada), which is equipped with broadband mechanical scanning single element transducers. The Vevo 770 is a commercially available high-resolution ultrasound system designed for small animal research. It offers spatial resolution down to 30 microns. The system was equipped with RMV (real-time micro visualisation) scan head no. 708, which has a frequency of 27.5 – 82.5 MHz and a maximum field of view of 11 mm.

After visual confirmation of the nerve's location, the transducer was mounted in a custom-made frame to fixate its position. Following visual re-confirmation and minor adjustments of the position of the transducer, a monopolar needle electrode was placed near the sciatic nerve using the ultrasound image as a guide (see Figure 1). This needle was subsequently used as cathode for near-nerve stimulation, together with a subdermal needle as anode. CMAPs were recorded by means of two round surface recording electrodes that were connected to the skin with a conductive EEG paste. The active electrode was placed over the lateral side of the gastrocnemius muscle, the passive electrode on the medial side (inside of the thigh). Because the electrodes did not easily stick to the rodent skin and, if not properly secured, disconnected during stimulation, the hair of the leg was shaved and waxed before placing the electrodes and the electrodes were additionally taped after placement.

The CMAPs were elicited and registered using a standard clinical electromyography (EMG) system (Viking IV; Nicolet Biomedical, Madison, Wisconsin). Batches of 10 signals were averaged and CMAP peak-peak amplitude and duration (onset latency to positive peak) were stored. Finally, a longitudinal skin incision was made and the sciatic nerve was exposed. Two needle electrodes were then placed near the nerve and used to elicit 10 CMAPs (conventional invasive myography) on the same surface electrodes as used for the minimally invasive procedure.

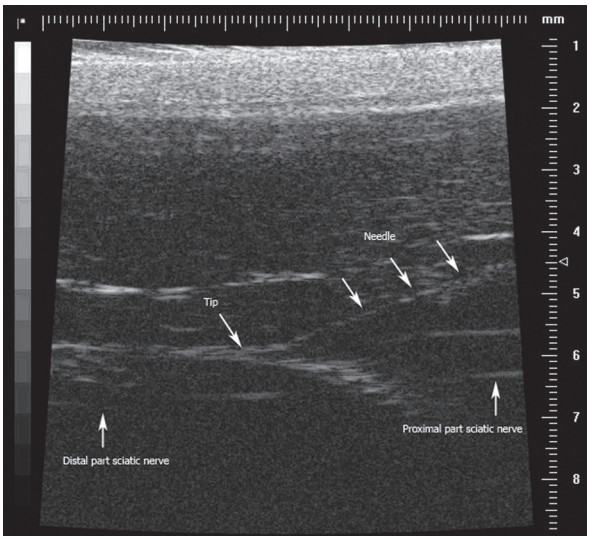

Figure 1 | Placement of needle near the sciatic nerve.

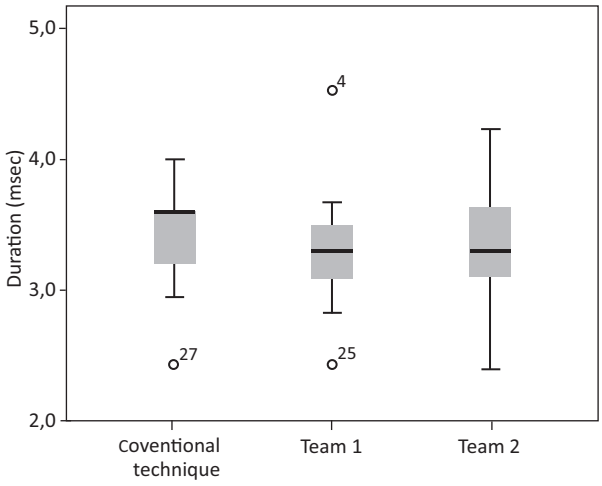

Figure 2 | Boxplot of CMAP durations for the three teams.

Statistical Analysis

ChiSquare analysis was used to compare the results of the two teams and the two approaches. To assess the reproducibility of our method, the coefficient of variation and the intraclass

correlation coefficient were determined. The intraclass correlation coefficient is the ratio of variance of interest (between-subject variance) over variance of interest and error variance (between-subject plus within-subject variance).[6] The magnitude of the intraclass correlation coefficient indicates the resemblance of the units in the same group.

Statistical analysis was performed using SPSS (ChiSquare and ICC) and Excel (CoV).

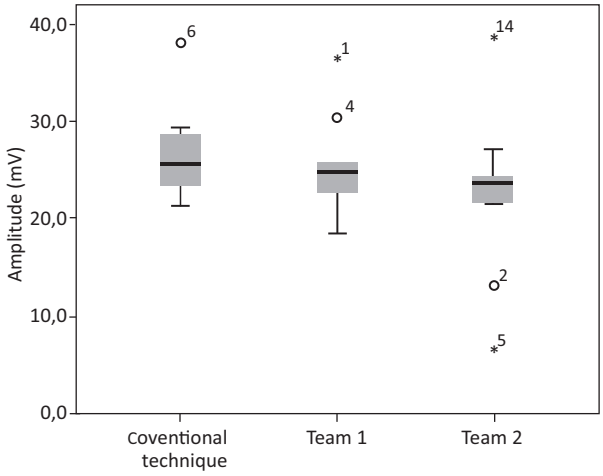

Figure 3 | Boxplot of CMAP amplitudes for the three teams.

Results

Ultrasonic appearance

The sciatic nerve was scanned in longitudinal direction, starting at the trifurcation level and moving in proximal direction. In all cases, the nerves could easily be traced and ultrasound-guided near-nerve needle placement proved to be well feasible. The nerve consisted of a hypoechoic interior, surrounded by a hyperechoic rim. Anatomically, the nerve is more superficial in the distal part of the leg, where the trifurcation of the sciatic nerve into the peroneal, tibial, and sural nerve could be clearly identified.

Compound muscle action potentials

In all animals, both ultrasound-guided and intra-operative near-nerve needle placement resulted in consistent signals. The resulting CMAPs mostly appeared as biphasic potentials. Their amplitude and duration are provided in Table 1, per animal and per recording.

Table 1 | Compound Muscle Action Potential values for all animals.

	Duration			Amplitude		
Animal	T_1 (ms)	T_2 (ms)	CI (ms)*	T_1 (mV)	T_2 (mV)	CI (mV)*
1	3,5	2,8	3,3	36,4	13,1	2,9
2	4,5	4,2	3,0	30,4	6,59	38,0
3	2,8	3,1	4,0	18,4	27,1	29,4
4	3,3	3,7	3,6	24,8	24,4	22,2
5	3,3	3,4	3,6	25,8	38,6	28,2
6	3,1	3,1	3,6	25,7	23,3	21,3
7	3,4	3,3	3,2	22,8	23,7	23,5
8	3,7	3,6	4,0	23,4	24,2	25,7
9	2,4	2,4	2,4	22,8	21,5	23,5
Mean	3,3 (SD0,5)	3,3 (SD0,5)	3,4 (SD0,5)	25,6 (SD9,4)	22,5 (SD8,8)	23,8 (SD9,3)

* CI stands for Conventionally invasive.

The mean CMAP latency was 3,3ms for teams 1 and 2; team 3 retrieved a latency of 3,4ms. The mean CMAP amplitude for team 1, 2 and 3 was 25,6mV, 22,5mV and 23,8mV respectively. ChiSquare analysis showed no statistically significant difference between the conventional invasive and new minimally invasive methods for both duration ($p = 0,292$) and amplitude ($p = 0,398$). The median coefficient of variation for the repeated recordings of Teams 1 and 2 was 2.1% for duration and 6.7% for amplitude. The intraclass correlation coefficient for these recordings was 0,828 for duration and 0,508 for amplitude.

Discussion

In all studied animals, the sciatic nerve could be visualised using our ultrasound equipment despite its restricted field of focus. Furthermore, this equipment enabled precise needle placement near the nerve, which allowed reproducible registration of CMAPs that were indistinguishable from those registered with the conventional invasive procedure. We therefore conclude that our technique appears to be a reliable and minimally invasive alternative for the measurement of CMAPs. The use of ultrasound can improve various aspects of the study of nerve function in rats. Specifically, we have demonstrated that this technique allows repeated CMAP measurements in the same animal, effectively eliminating an important source of unwanted variability in studies that aim to evaluate a process such as nerve regeneration over time. Furthermore, for this type of research fewer animals suffice than for conventional studies that allowed only a singe recording per animal.

A point of concern regarding our measurements is the learning curve. The first three measurements were responsible for significant variance in our results. After excluding these measurements for our statistical analysis, we found a median CoV of 1.5% and 3.4% for duration and amplitude, respectively. We would recommend to practice this new technique using at least 3 animals prior to the actual measurements.

In addition to Kuffler conclusions, we agree that the ultrasound equipment used is not sufficient to determine the nerve regeneration (i.e. repopulation).[5] In this study, however, visualising the repopulation was not our primary concern. The ultrasound equipment was only used to assist in placing the needle near the sciatic nerve to elicit the CMAPs. The ultrasound equipment proved to be helpful as a assisting tool not only for this specific research topic, but also in today's outpatient clinic ultrasound is gaining application as an accurate and reliable instrument for visualising peripheral nerves.[1,8]

The obvious next step is to introduce this technique in studies that assess peripheral nerve regeneration in rats and other animals, perhaps even in humans. However, our approach requires highly specialized equipment. The resolution of the current clinical ultrasound equipment, with frequencies ranging between 5 and 20 MHz, does not allow easy visualisation of smaller or deeper nerves. However, there is a clear trend toward ever higher insonation frequencies with high-resolution imaging transducers; hence we expect this limitation will automatically resolve over time.

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Chapter 4

Natural Conduits for Bridging a 15-mm Nerve Defect: Comparison with the Vein Supported by Muscle and Bone Marrow Stromal Cells with a Nerve Autograft

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Abstract

Introduction

The nerve autograft for the reconstruction of a nerve defect results in donor site morbidity. This detrimental consequence drives the search for alternatives. We used a vein-muscle graft and injected this conduit with Bone Marrow Stromal Cells (BMSCs).

Methods

In 60 Lewis rats a 15 mm sciatic nerve defect was bridged with either a nerve autograft, a vein filled with muscle, or a vein filled with muscle and BMSCs. Functional recovery up to 3 months post reconstruction was evaluated Extensive histology was performed to confirm the presence of the BMSCs and to evaluate regeneration staining neural tissue sections for Schwann cells and presence of Neural Growth Factor.

Results

Twelve weeks after grafting all animals responded with a toe-spread and pinprick reaction, significant differences were found between the autograft and the vein graft at 12 weeks. Six weeks post grafting no difference was found when comparing the GMI between the three groups. Group I had a significant increase in GMI at twelve weeks compared to group II and group III. The CMAP measurements showed comparable results at 6 weeks post grafting. Twelve weeks after reconstruction the autograft outperformed the vein-muscle grafts. The addition of BMSCs did result in better CMAPs. Histological analysis showed improved early regeneration in group III.

Conclusion

This study demonstrated the beneficial effect of BMSCs as a luminal additive in a conduit. However, our data do not demonstrate sufficient benefit to warrant clinical implementation at this stage, since our conduit is not on par comparing regeneration to that of the nerve autograft.

Introduction

Throughout the years, many materials have been used for grafting nerve defects. Autologous nerve grafts are widely used and the autologous non-vascularised perineural nerve graft can be considered the golden standard of treatment in the clinical setting.[18] However, harvesting a donor nerve requires an extra operating site and the removal of a sensory nerve (usually the sural nerve) thus resulting in a sensory deficit at the donor site. Furthermore, scarring, neuroma formation, neuropathic pain and other symptoms have all been correlated to donor site morbidity.[17,22]

Veins form a frequently described alternative for nerve grafting.[2,4,9,19,29,36] They can be harvested with limited donor site morbidity and provide a metabolically supportive environment for the regenerating axons. Schwann cells from the proximal part of the lacerated nerve invade the vein and start proliferating, resulting in a similar regeneration process as in the nerve graft.[15,16] However, the tendency of an empty vein to collapse when used for bridging gaps hinders the recovery process.[4,9,19,29] This problem may be solved by placing a piece of muscle in the graft, which prevents its collapse and has been shown to yield promising results.[2,3,5] Furthermore, the muscle appears to provide an adequate matrix for the nerve fibers growing inside the vein.[1]

Despite the array of grafts available, a clear need exists for new techniques that can improve the outcome of nerve reconstruction. Cellular therapy and the use of stem cells in particular have gained much interest over the last decade. We hypothesized that the ability of these cells to differentiate into other cell types may have therapeutic potential when used in combination with a graft. Indeed, previous work by Cui et al. has demonstrated enhanced nerve regeneration using embryonic stem cells and revealed successful regeneration when transplanted in an epineural tube.[13]

The present study was designed to compare a new grafting technique (the muscle supported vein graft and additional cellular therapy from the BMSCs) to the autologous nerve graft. We combine functional and histological evaluation techniques in order to make this comparison and to investigate the beneficial effect of the BMSCs.

Methods

The experimental protocol was approved by the Animal Experiments Committee according to the National Experiments on Animals Act and conducted according to this law that serves the implementations of Directive 86/609/EC of the Council of Europe. Eighty isogenic adult female Lewis rats, weighing 180 – 200 grams, were used. Surgical procedures and electrophysiological evaluations were performed under general anaesthesia (Isoflurane, 1 – 2% in a mixture of O_2 / $N_2$0). Sixty animals were randomly allocated to one of two groups. The first group, consisting of 30 animals, was evaluated 6 weeks post-surgery, the other group of 30 animals after 12 weeks.

These two groups were subdivided into three groups of 10 rats each. Group I was treated with a donor autograft. Group II was treated with a vein-muscle graft and Group III with the vein-muscle graft and additional injection of BMSCs. The remaining 20 animals served as autograft donors of the animals in group I and for harvesting bone marrow to isolate BMSCs.

Bone Marrow Stromal Cell Preparation

Fresh bone marrow cells were harvested aseptically from tibias and femurs of 20 adult rats. Both ends of the bones were cut and the marrow was flushed with 10 ml of D-MEM medium. After centrifugation, the cell suspension was lysed with 0.85% NH_4Cl for 5 min. The suspension was then filtered through a 40-mm nylon mesh, and re-suspended in culture D-MEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were placed in a 75-cm^2 flask and incubated at 37°C in 5% CO_2 for three days. Non-adherent cells were removed by replacing the medium three times a week. After five to eight passages, when the BMSC culture reached confluence, BMSCs were lifted by 0.25% trypsin and 1 mM% EDTA in PBS for 5 min and washed in alpha-MEM medium. BMSCs were labelled with red membrane dye PKH-26 (Sigma-Aldrich, UK) in order to evaluate their capacity to differentiate into neuronal and other tissue types as well as to track their migration into lymphoid organs and the contralateral sciatic nerve. PKH-26 staining was performed in accordance with the manufacturer's instructions. The stromal cells were incubated with PKH-26 dye in Diluent C buffer solution at room temperature for 5 min. Labelling was stopped by incubation with 1% BSA in PBS for 1 min and complete D-MEM medium. Following a final wash in complete D-MEM medium, labelled stromal cells were prepared to a final concentration of 3×10^6 cells in 0.05 ml. This solution was kept in a 1 ml syringe and the solution was then injected in the two ends of the vein using a small syringe (27G), injecting 0.025 ml in each side.

Surgical Technique

The surgical procedure was performed by a single surgeon and assistant using standard aseptic microsurgical techniques under the operating microscope (Zeiss OP-MI 6-SD; Carl Zeiss, Goettingen, Germany) on the sciatic nerve of the left hind limb. The right limb sciatic nerve served -if possible- as pairwise control. In the donor animals an oblique skin incision was made in the left gluteal region, exposing the sciatic nerve through a gluteal muscle-splitting incision and externally dissected to excise a 15 mm segment of the nerve for use as a nerve graft in Group I. The same procedure was performed in the recipient animals, carefully excising the 15 mm nerve defect to the proximal anatomical landmark (i.e. the sciatic notch). For Group II and Group III the jugular vein was used as a graft, which was harvested through a longitudinal mid-line incision of 35 mm in the neck. The left external jugular vein was dissected and both the proximal and the distal end of the vein were ligated. A small muscle fragment of 1½ x 1½ mm^2, was cut out of the gluteal muscle and placed inside the lumen of the vein using a straight irrigator. Next, the graft

was connected to the nerve stumps using 6 10/0 Ethilon sutures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands) at each coaptation side (Figure 1). In all three groups the muscle was closed using 2 6/0 Vicryl Rapide sutures, followed by closing the skin using 6/0 Vicryl Rapide Sutures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands).

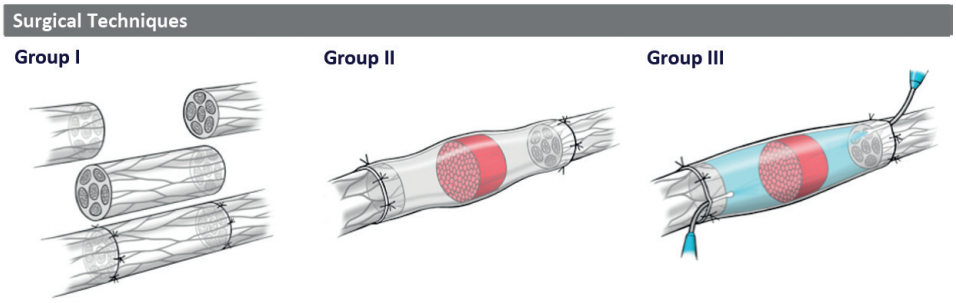

Figure 1 | Surgical techniques used in this study. Group I comprises an autologous nerve graft, Group II an empty vein-muscle graft and Group III a vein-muscle graft with BMSCs.

Functional Assessment

Sensory recovery was evaluated at 1, 3, 6, and 12 weeks regeneration time, by means of stimulating three different points along the lateral side of the foot (pinprick test).[14,30] Reflexes were considered positive when the paw was withdrawn. The toe-spread test is a commonly used test to assess motor function recovery.[25,27] Toe-spread was evaluated in the same sessions as the pinprick test, by raising the animal and observing voluntary reaction of the toes, which was graded with up to 3 points.

Electrophysiology

To evaluate nerve conduction and the translation of the electrical impulse into muscle contraction, we recorded the compound muscle action potential (CMAP) as evoked response from the gastrocnemius muscle upon electrical stimulation of the sciatic nerve proximally from the lesion site. The CMAP is a validated way of assessing nerve regeneration.[33] We used a minimally invasive technique to evoke the CMAPs, as described in more detail elsewhere.[26] Briefly, we employed an 10 – 18 MHz probe (LA435) and Esaote MyLabFive ultrasound system (Esaote Europe, The Netherlands) to visualize the nerve and position the stimulus electrodes. The CMAPs that were subsequently elicited were recorded in batches of 25 responses and then averaged. These averages were quantified by means of signal onset latency, amplitude, and duration.

Gastrocnemius Muscle Index (GMI)

The GMI was measured to assess denervation atrophy at 6 and 12 weeks regeneration time. Following the electrophysiological evaluation, the muscle was excised from the lower leg and wet muscle weight was measured immediately using a digital scale. The contralateral gastrocnemius was also harvested and the GMI was calculated by dividing the muscle weight from the operated side by that from the contralateral side.

Immunofluorescence Staining

Expression of growth factors involved in nerve regeneration was evaluated with monoclonal antibodies and immunofluorescence staining. The freshly dissected nerve graft was first divided into proximal, medial and distal parts. Differences between these sites are likely linked to (and can thus provide information on) different stages of the regeneration process over time. A nerve section from the contralateral nerve was taken as control. All parts were snap-frozen in liquid nitrogen. Tissue sections were subsequently cut for 4 mm slides and fixed for 10 min in acetone. Next, the slides were incubated with rabbit anti-rat NGF (Sigma) and S-100 (Sigma) monoclonal antibody for 30 min. The binding of primary antibodies was detected using a fluorescent goat anti-rabbit immunoglobulin (FITC, Sigma) in accordance with the manufacturer's instructions. Slides were mounted in Vectashield mounting medium with DAPI and were analyzed using a camera (DFC 350 FX R2 v1.9.0; Leica, Wetzlar, Germany) connected to a microscope (DM5500B; Leica, Wetzlar, Germany), using imaging software Leica Application Suite Advanced Flouresence (LASAF v2.0.0 build 1929, Leica, Wetzlar, Germany). The nerve areas where captured in different magnification (10x, 20x, 40x). Before analysis, all the images were filtered exactly the same way with the imaging program (Cell^D v3.1 build 1276, Olympus, Tokyo, Japan) to reduce noise and sharpen them. Expression of the Neural Growth Factor (NGF) and Schwann cell (S-100) marker immunoreactivity were assessed for staining intensity by three independent observers that were blinded to the treatment. Intensity of staining was scored as absent (0), weak (1); moderate (2); strong (3) and powerful (4) expression. The inter rater reliablity of the observers was evaluated prior to the scoring, which gave a Cronbach's Alpha of 0.973. Therefore the scores of the four observers were averaged and used for statistical analysis. Double staining of PKH (red) with neurotrophic factors was used to assess co-localization of BMSC expression for Neural Growth Factor (NGF) and Schwann cells (S-100) (both green), respectively.

Histology

To evaluate the presence of muscle in the vein graft, we examined our frozen cross-sectional samples by hematoxilin and eosin (H&E) staining.

Statistical Analysis

Unpaired student T-tests were used to determine statistical significance. P values of less then 0.05 were considered significant. Spearman's rho was calculated to assess the correlation between the CMAP and the wet muscle weight.

Results

No early or late postoperative complications were detected in any of the animals. However, we lost two animals during surgery due to anesthesia complications.

Pinprick Test

Six weeks after surgery, there was little reaction to the pinprick test and no sign of toe movement in most animals (Figure 2). There were no significant differences between the three groups. Twelve weeks post-surgery, pinprick reaction had improved. Group I (nerve graft) had a significantly better reaction than Group II & III (vein grafts) ($p \leq 0.004$). Furthermore, Group III (with BMSCs) tended to have better reactions than Group II ($p = 0.081$).

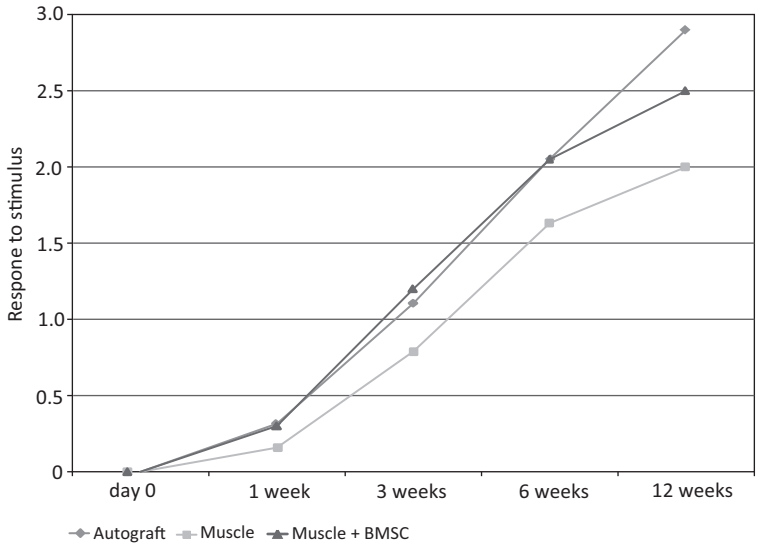

Figure 2| Mean results of the Pinprick test at week 1, 3, 6, and 12 after grafting. (Left) Presence of withdrawal during stimulus above the ankle, the metatarsal area, and at the level of the toes was graded 1, 2 and 3, respectively. No withdrawal during skin stimulation was marked with '0'.

Toe-spread Test

For all three groups significant improvement of toe-spread over time was found ($p < 0.001$; Figure 3). The autograft resulted in a significantly better toe-spread than the vein grafts at 12 weeks ($p \leq 0.004$). Group III performed worse than Group I ($p = 0.054$), but better than Group II ($p = 0.049$).

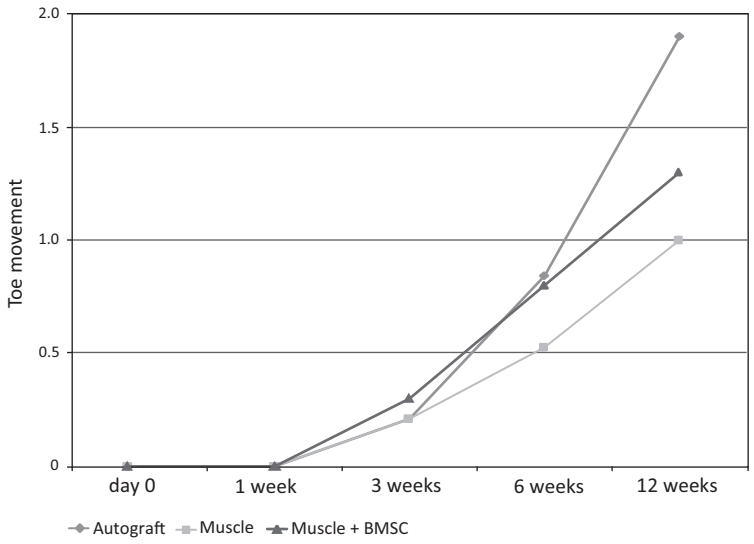

Figure 3 | Mean results of the Toe-spread test at week 1, 3, 6, and 12 after grafting. No reaction: 0 points. Any sign of toe movement: 1 point. Abduction of the toes: 2 points. Extension and abduction: 3 points.

Electrophysiology

After six weeks of recovery, the onset latency of the CMAP in Group I was significantly longer than that in Groups II & III ($p = 0.014$ and $p = 0.029$, respectively) (Table 1). Furthermore, in all three groups this latency was significantly different compared to the control value ($p = 0.001$, $p = 0.014$ and $p = 0.007$, respectively). There were no differences in duration between groups or between the groups and their controls. However, amplitude was significantly decreased in all three groups compared to the control values ($p < 0.001$) and was significantly lower in Group II than in Group I ($p = 0.008$).

Twelve weeks after surgery onset latency had normalized, but amplitude remained significantly decreased in all three groups compared to control values ($p < 0.001$). Amplitude was significantly higher in Group I than in Groups II and III ($p < 0.05$). Both peak latency and duration in Group I were significantly longer than in Group II and Group III and when compared to the control values ($p < 0.008$). Between Groups II and III, no differences were found.

Table 1 | Mean CMAP latencies [ms], amplitudes [mV] and duration [ms] of the operated side at 6 and 12 weeks. Control values – unoperated side (mean ± SD).

	6 weeks				12 weeks			
	Onset Latency	**Peak Latency**	**Duration**	**Amplitude**	**Onset Latency**	**Peak Latency**	**Duration**	**Amplitude**
Group I	1,89 ± 0,92	5,2 ± 0,6	3,3 ± 0,8	9,8 ± 6,4	1,04 ± 0,11	4,8 ± 1,0	3,7 ± 0,9	13,3 ± 2,8
Group II	1,06 ± 0,11	4,8 ± 0,7	3,7 ± 0,6	9,7 ± 2,6	1,13 ± 0,24	3,9 ± 0,6	2,9 ± 0,6	10,0 ± 2,8
Group III	1,04 ± 0,08	4,6 ± 0,3	3,6 ± 0,3	12,4 ± 1,3	1,00 ± 0,29	4,0 ± 0,5	3,1 ± 0,5	9,5 ± 2,3
Control	1,23 ± 0,19	4,7 ± 0,8	3,5 ± 0,8	36,8 ± 10,9	1,08 ± 0,13	4,2 ± 0,3	3,2 ± 0,4	40,0 ± 9,6

Gastrocnemius Muscle Index (GMI)

In Group I, GMI increased between six and twelve weeks ($p < 0.001$) whereas it decreased in Groups II and III ($p < 0.001$). At twelve weeks, the GMI was significantly higher for Group I than for Groups II and III ($p < 0.001$) (Figure 4). Moreover, there was a high correlation between CMAP amplitude and wet muscle weight ($R = 0.774$, $p < 0.001$).

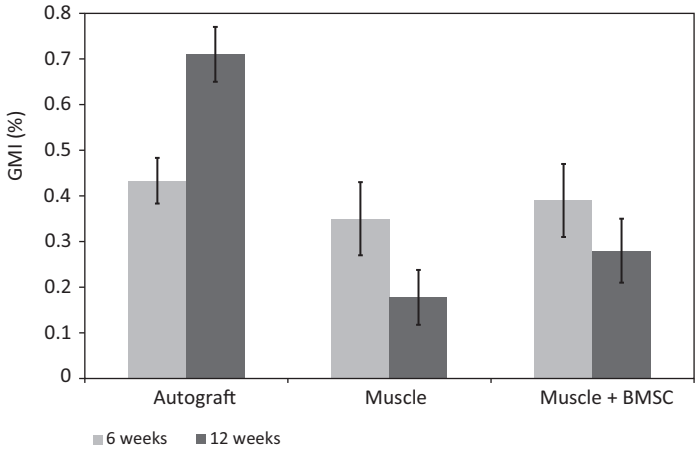

Figure 4 | Results of the Gastrocnemius Muscle Index at 6 and 12 weeks after grafting groups: Group I (Autograft), Group II (Muscle) and Group III (Muscle + BMSC).

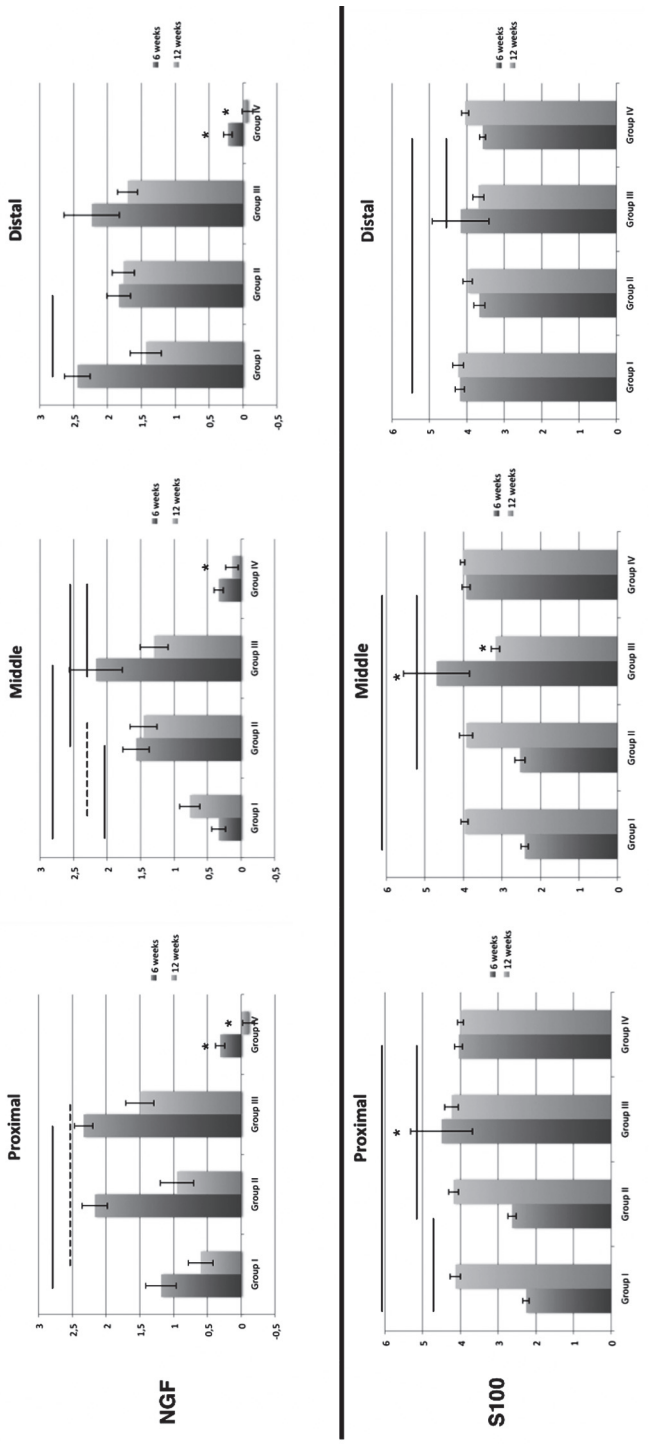

Figure 5 | Results of the histological analysis evaluating NGF (top) and S-100 (bottom) expression in the Proximal, Middle and Distal nerve sections at 6 and 12 weeks after grafting. Group I: (Autograft, Group II: Muscle, Group III: Muscle + BMSC, Group IV: contralateral side served as control. Statistically significant differences are indicated with an asterisk if $p < 0.05$ when the marked group is compared to the three other groups at the same time point. The continuous line indicates the significant differences between the 6 weeks groups, the interrupted line illustrates differences in between groups 12 weeks after grafting.

Immunofluorescence Staining

NGF

Typical NGF staining examples are depicted in Figure 6. Compared to the healthy control nerve (Group IV) all segments, except the middle section of Group I, showed significantly increased staining intensity of NGF ($p < 0.010$) (Figure 5, top). Twelve weeks after grafting, the NGF staining intensity was decreased in all Groups compared to the six weeks sections (again with the exception of the middle section of Group I). However, in all operated Groups the staining was still significantly more than in Group IV ($p < 0.050$).

S-100

Figure 7 provides images of the sections taken with the immunofluorescence microscope. At six weeks, the S-100 expression was significantly decreased in all segments except the distal segment in Group II, compared to the healthy control nerve (Group IV) ($p < 0.050$). Significant differences were found between groups in the proximal and middle sections, but not in the distal segments. Twelve weeks post grafting, expression of S-100 in the proximal and distal sections was similar in all groups and all sections, with relatively low values only in the middle section in Group III (see Figure 5, bottom).

Bone Marrow Stem Cells (BMSCs) were clearly visible at both six and twelve weeks (Figure 8). More importantly, evaluation of the S-100 staining revealed double staining in all sections in Group III.

Histology

H&E stained sections did not show any remaining muscle tissue at 6 and 12 weeks post-operatively. However, interestingly, the vein did not collapse at both time points, indicating presence of extra cellular matrix from the muscle preventing the vein from collapsing.

Discussion

This study suggests that recovery following our new grafting technique (using a vein filled with a small fragment of muscle and injected with BMSCs) appears to have a beneficial effect when compared to the vein model with muscle support only (Group II); However both conduits are not on par with the recovery after grafting the defect using an autologous nerve graft (Group I).

Results from sensory testing, motor function assessments, the electrophysiological evaluation, the gastrocnemius muscle index (GMI), and histological evaluation all converge in these respects.

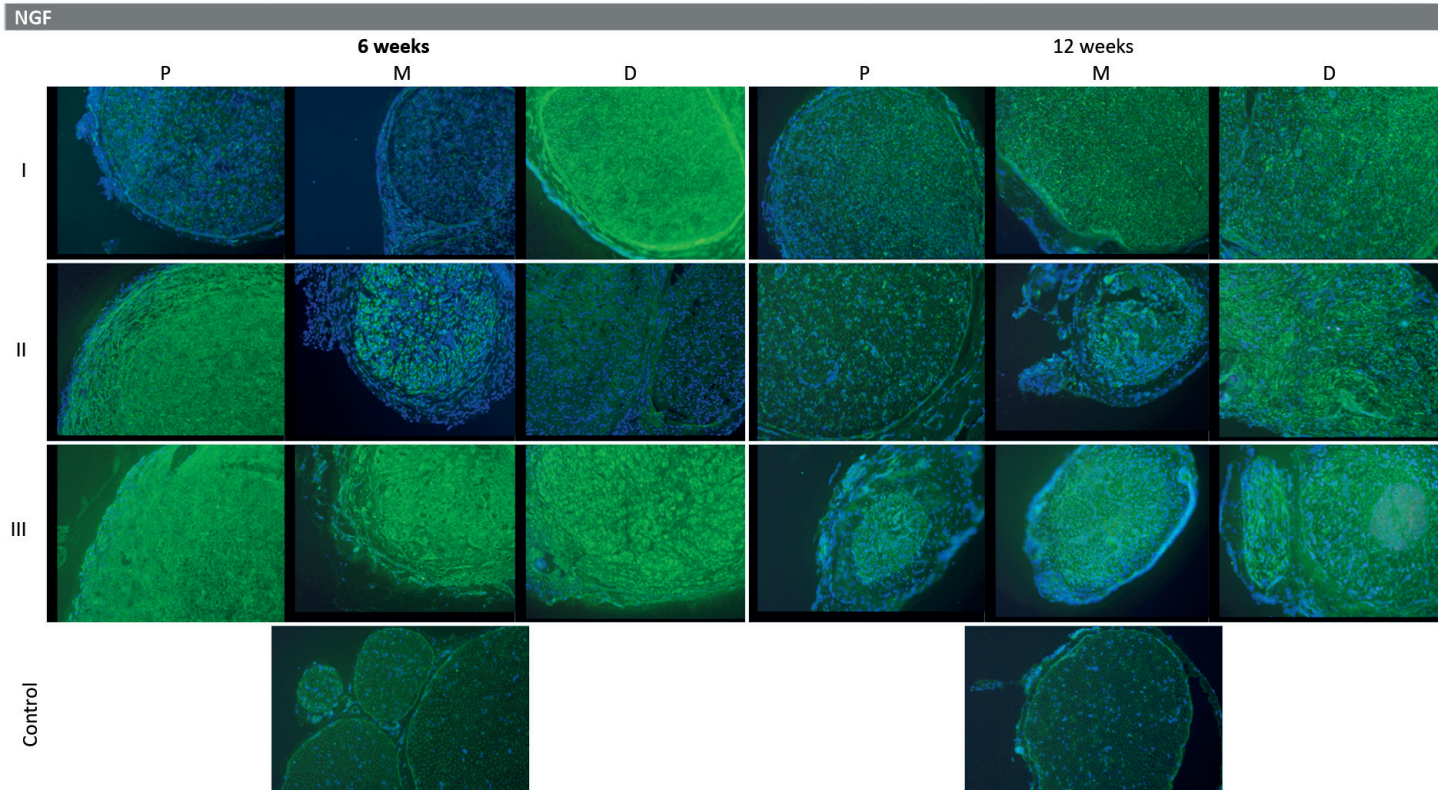

Figure 6 | An overview of the NGF expression in the different sections (P = proximal, M = middle, D = distal) at 6 and 12 weeks after grafting groups: Group I (Autograft), Group II (Muscle), Group III (Muscle + BMSC), Control (healthy control nerve). After staining the tissue with NGF and Fit C (green staining), all the slides were mounted with vectamount medium with Dapi (blue staining) for visualizing the epineurium and vessel wall. Pictures are taken with a 10x magnification.

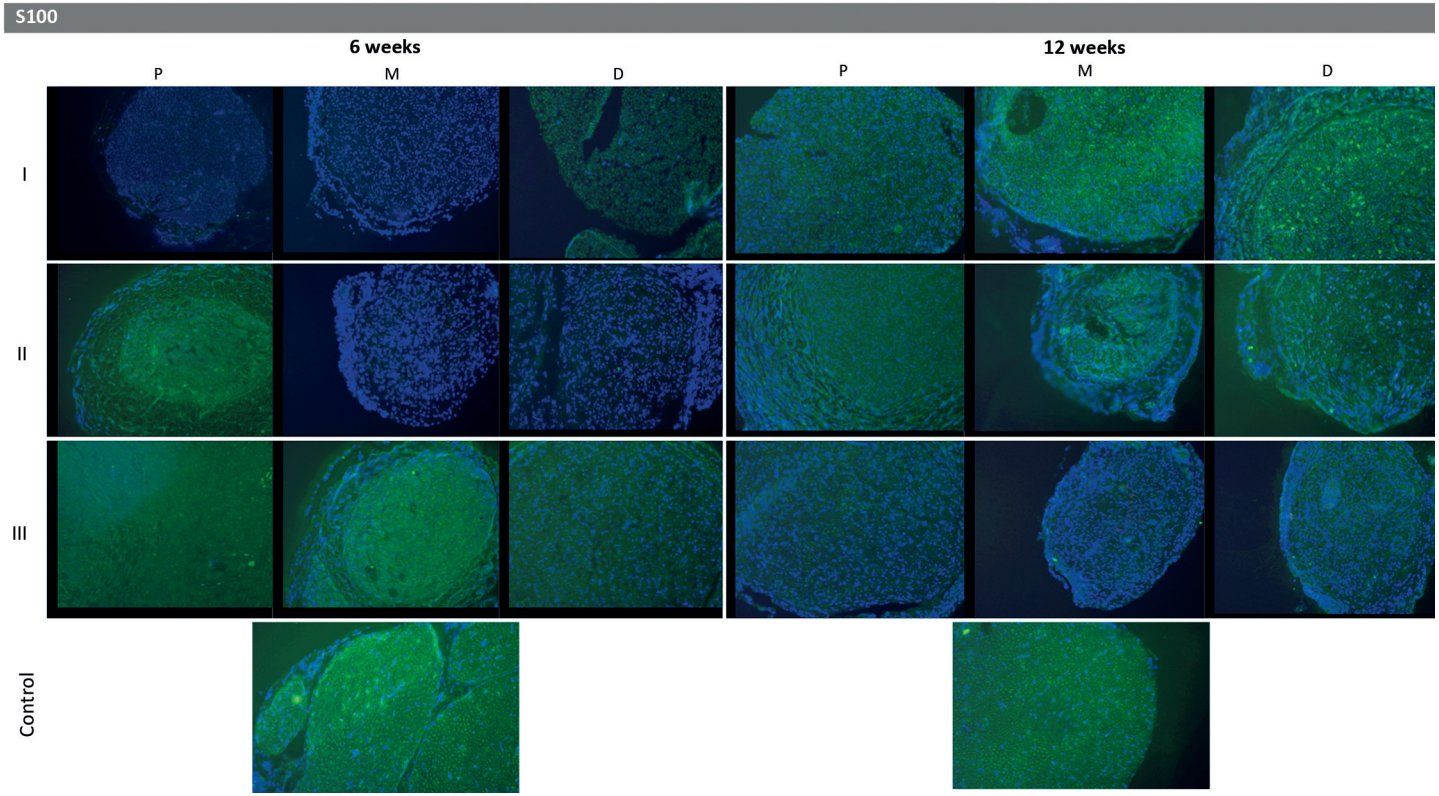

Figure 7 | An overview of the S-100 expression in the different sections (P = proximal, M = middle, D = distal) at 6 and 12 weeks after grafting groups: Group I (Autograft), Group II (Muscle), Group III (Muscle + BMSC), Control (healthy control nerve). After staining the tissue with S-100 and Fit C (green staining), all the slides were mounted with vectamount medium with Dapi (blue staining) for visualizing the epineurium and vessel wall. Pictures are taken with a 10x magnification.

Differences in the recovery process between the groups appear most pronounced in the GMI. Whereas the autograft resulted in a significant increase in muscle weight over time, both vein-muscle grafts led to more atrophy between six and twelve weeks post-surgery. This suggests that the nerve reconstructed with the nerve autograft had already reinnervated the muscle through newly formed axons, whereas in the other two groups muscle reinnervation had not yet been established.

The staining for neural growth factor (NGF) and S-100 (a marker for Schwann cells) in three sections of the reconstructed nerve at two time points allowed us to evaluate the regeneration progress histologically. Our analysis demonstrated a beneficial effect of the BMSCs especially in early regeneration. Not only could we still find the labeled BMSCs inside the vein, but – more importantly – we also found a stronger staining intensity of NGF and S-100 in Group III compared to Group II, six weeks post-operatively. Twelve weeks post-operatively, Group I revealed the least NGF expression. Possibly, the newly formed axonal sprouts in the autologous donor nerve had already progressed beyond the evaluated sections. S-100 expression, predominantly present in differentiated Schwann cells was similar to the healthy control nerve in the proximal and distal section. The middle section, however, continued to show less expression, which might be an indication of continued regeneration of the Schwann cell population at this site in the graft.

In the literature, two explanations for the contribution of BMSCs to nerve regeneration are mentioned. The first is that BMSCs may produce cytokines and growth factors that positively impact neural cell survival.[7,8,10,20,23,24,31,34-36] The second explanation is that BMSCs can differentiate into neural lineages including neurons, astrocytes, oligodendrocytes, microglia and, most important in this context, Schwann cells.[8,11,12] In line with the work of Tohill et al.,[32-34] our results provide support for the latter possibility, because the reaction of the labeled BMSCs to the S-100 staining shows that they can have the capability to transdifferentiate into cells with a Schwann cell-like phenotype. A similar finding has been published by Tohill et al., who found mesenchymal cells differentiating into a Schwann cell-like phenotype[32] and later demonstrated that differentiated mesenchymal stem cells can have the same characteristics as Schwann cells in rats.[6,21]

Our hypothesis regarding the muscle segment was that it could prevent collapse of the vein in the early post-operative phase. As the muscle fragment was avascularised, we expected it to become atrophic over time. Indeed, the H&E staining on our histology samples did not reveal any muscle tissue 6 and 12 weeks post-operatively. As previous work has already shown that any remaining muscle tissue does not obstruct the regenerating nerve,[28] we can safely assume that incorporation of muscle in a vein graft will not influence nerve regeneration other than by preventing the vein from collapsing. Therefore, it may well be used in further experiments employing vein grafts.

The continuous search for new and better grafting techniques has always been stimulated by the limitations of the nerve autograft. However, taking the results of this study into consideration, we can conclude that the nerve autograft clearly outperforms the vein-muscle graft in our animal model and that injecting the vein-muscle graft with BMSCs did have a tendency to increase regeneration.

For the future, the most important question is if clinicians will be able to accept donor-site morbidity as a side effect of good nerve regeneration or will consider use of new natural conduits (e.g. the proposed vein-muscle-BMSC graft) and accept less favourable results. However, since we did elucidate a tendency of the BMSCs to improve nerve regeneration, at this stage, further research is needed to optimize vein-muscle-BMSC grafts in large animal models to enhance clinical outcomes.

Acknowledgements

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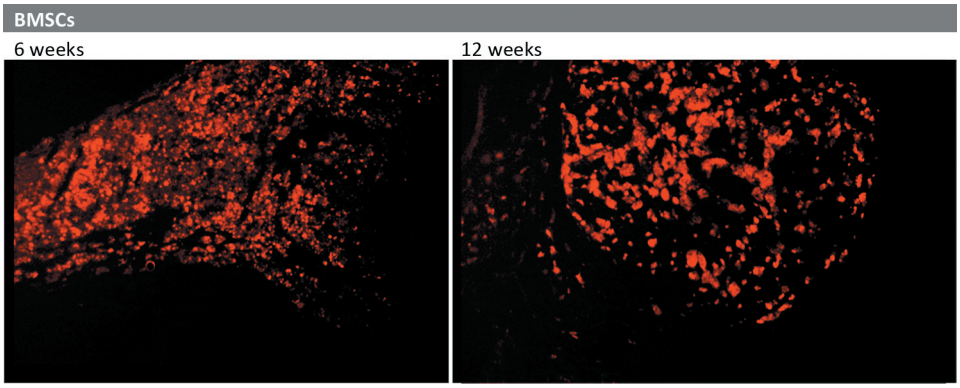

Figure 8 | Typical example of the strong PKH-26 dye staining at 6 and 12 weeks post grafting in group III. Pictures are taken with a 20x10 magnification

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Chapter 5

Re-innervation of Subgroups of Specific Sensory Nerve Fibers of the Skin following Nerve Autograft Reconstruction in a Rat Model

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Submitted

Abstract

Introduction

For large peripheral nerve injuries the nerve autograft is still considered the best strategy for reconstruction. Visualization of the skin epidermal sensory nerve fibers is useful to show the regeneration in the most distal terminals of the injured nerve. The peptidergic (visualized with CGRP and Substance P) and non-peptidergic fibers (visualized with P2X3) are responsible for signalling noxious and termed nociception. The Aδ-fibers are visualized using NF200 staining.

This study investigates the re-innervation of these different skin neural fibers in the rat foot sole, 12 weeks after reconstructing a nerve defect with an autograft.

Methods

In 5 animals, a 15 mm sciatic nerve defect was reconstructed using an autograft. Five healthy animals served as control.

Twelve weeks after grafting, the sole of the foot of the operated hindpaw was excised and stained according to the necessary protocols for CGRP, Substance P, P2X3 and NF200. Control staining was performed with PGP 9.5 (a pan-neuronal marker).

Von Frey hairs were used to assess the mechanical threshold and sensibility after reconstruction.

Results

PgP 9.5 staining demonstrated a 70% re-innervation of the skin sensory nerve fibers. The peptidergic fibers re-innervated the skin for 70%, and the non-peptidergic fibers regenerated up to 35%. The myelinated fibers had a 80% re-innervation 12 weeks after reconstruction of the nerve defect. The withdrawal response of the operated paw was significantly delayed as compared to the healthy control response.

Conclusion

The present study demonstrated that 84 days post operative the foot sole is not fully re-innervated with skin nerve fibers and the peptidergic fibers had a stronger regeneration capacity than the non-peptidergic fibers.

Introduction

Peripheral nerve injuries are still a frequent problem after trauma to the extremities. In lengthy nerve gaps therapeutic repair strategies preclude direct end-to-end coaptation of the nerve ends, since traction on coaptation can result in ischemia of the nerve and therefore negatively influence nerve regeneration. The clinical gold standard for large nerve defects is the use of a nerve autograft.[3,4,17,29] Typically sensory nerve autografts, such as the sural nerve or the medial / lateral antebrachial cutaneous nerve, are used for reconstruction.[2,7] Considering the significant risk of donor site morbidity and complication after reconstruction, such as neuroma formation and / or loss of sensibility, the urge for autograft replacement is still present.[15,23] However to do so, it is of pivotal importance to understand the regeneration process distally from the nerve autograft, especially in the terminals of the regenerated nerve, where the external stimuli are conveyed into sensory information.

Previous studies investigating the regeneration of large peripheral nerve defects, when bridging the gap with other grafts than an autograft, incorporate an extensive functional and histological evaluation of the nerve itself.[16,18,24] Interestingly there are no reports examining the re-innervation of the terminal sensory fibers of a nerve (i.e. the nerve fibers in a rat foot sole) that is reconstructed by a nerve autograft.

Sensory fibers innervating the skin can be subdivided in two major groups; the unmyelinated C fibers and myelinated Aϑ-fibers. The unmyelinated C fibers can be distinguished into two groups, namely the peptidergic and non-peptidergic fibers. The peptidergic fibers contain the neuropeptides calcitonin gene-related peptide (CGRP) and substance P and the non-peptidergic fibers express P2X purinoceptor 3 (P2X3). The Aδ-fibers are solely peptidergic and more specifically purely CGRP positive and can be visualized with a Neurofilament 200 (NF200) antibody. Peptidergic fibers are responsive to the neurotrophic factor Nerve Growth Factor (NGF), and non-peptidergic fibers are responsive to Glial cell line-derived nerve factor (GDNF). These neurotrophins are thought to be secreted in substantial quantities after peripheral nerve injury and ultimately result in regeneration of the peptidergic and non-peptidergic fibers after peripheral nerve injury.[20,28]

In the clinical situation, Figure 1 illustrates the relation of the different fibers and the skin biopsies stained with PGP 9.5, a pan neuronal marker, are correlated with pathological neural diseases. Regarding this and the possibility to discriminate between the different kinds of sensory skin fibers, it may be a valuable tool to examine the regeneration of nerve terminals after a reconstruction and correlate this to behavioural response.[11,12]

The last two decades of research investigating peripheral nerve regeneration has resulted in no other conclusion than the superiority of the autograft in the arsenal of nerve grafts available.[8] Understanding in the re-innervation rate of the four different subgroups of nerve sensory skin fibers can help us understand the physiology of peripheral nerve regeneration at large.

The goal of the present study is to investigate the distribution of skin neural fibers in the rat foot sole, 12 weeks after reconstructing a 15mm nerve defect with an autograft. These data are also correlated with the behavioural responses to mechanical stimuli.

Methods

Animals

The experimental protocol was approved by the Animal Experiments Committee according to the National Experiments on Animals Act and conducted according to this law that serves the implementations of Directive 86/609/EC of the Council of Europe. Ten isogenic adult female Lewis rats, weighing 180 – 200 grams, were used. Animals were pair-housed in hooded cages at room temperature on a 12-hour light/dark schedule, and were given food and water ad libitum. Surgical procedures and electrophysiological evaluations were performed under general anaesthesia (Isoflurane, 1 – 2% in a mixture of O_2/N_20).

Surgical Technique

The surgical procedure was performed by a single surgeon and one assistant using standard aseptic microsurgical techniques under the operating microscope (Zeiss OP-MI 6-SD; Carl Zeiss, Goettingen, Germany) on the sciatic nerve of the left hind limb. In five animals the sciatic nerve defect was reconstructed using an autograft. Five healthy animals served as a control. After an oblique skin incision was made in the left gluteal region of the donor the sciatic nerve was exposed through a gluteal muscle-splitting incision and externally dissected to isolate a 15 mm segment of the nerve. The nerve was transected proximally and distally to obtain the 15 mm segment. The autograft was retrieved from an isogenic donor animal and connected to the nerve stumps using 6 10/0 Ethilon sutures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands) at each coaptation side. The muscle was closed using 2 6/0 Vicryl Rapide sutures, followed by closing the skin using 6/0 Vicryl Rapide Sutures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands).

Evaluation of Mechanical Hypersensitivity

Twelve weeks post operative, the mechanical threshold of the hind paws was measured using Von Frey hairs. Each Von Frey hair was applied for 2 s at 5 s intervals. The threshold was set at 3 paw lifts. For testing the rats were placed in a plastic box (perforated floor) and able to move freely. The medial and lateral side where stimulated at the transition point from glabrous skin to hairy skin. The center foot sole was stimulated at the very center point of the foot sole. The withdrawal response was noted and used for analysis.

Immunohistochemistry

Tissue Preparation

After sacrificing the animal, the complete foot sole of the left and right hind paws were surgically resected (Figure 1). The foot soles were then placed in a 10 per cent sucrose solution for 24hrs, washed and rinsed with glycerol solution and placed in freezer.

Immunohistochemical Staining

The 40 µm sections were pre-incubated (90 min, room temperature (RT)) in a mixture containing bovine serum albumin (BSA 2%) phosphate buffered saline (PBS, pH 7,4). (Fraction V, Roche) and 0.5% Triton X-100. Thereafter, the sections were rinsed in PBS and incubated for 48 hours in a cocktail of 2% BSA containing antibody; goat anti-CGRP antibody (Abcam, United States) (1/30.000), NF-200 (1/35.000), Substance P (1/500), P2X3 (1/25.000), PGP 9.5 (1/15.000). Subsequently, sections where incubated with a secondary antibody rabbit anti-goat for 90 min room temperature. Sections were further processed using a Vectastain Elite ABC kit (Vector, Burlingame, CA) (90 min at RT). Finally, 3,-3' diaminobenzidine (DAB) enhanced by the glucose oxidase-nickel-DAB method (Kuhlmann and Peschke, 1986) was used to reveal antigenic sites. The sections were mounted on gelatinized slides, air dried overnight, dehydrated using absolute ethanol (< 0.01% methanol), transferred to xylene and coverslipped with Permount (Fisher, Hampton, NH). Immunoreactivity for CGRP was completely abolished when primary antibodies were omitted.

Analysis

A total of 8 series of sections of the complete footpath were used for analysis, making selection in a lateral, center and medial side per section and dividing the footpath in a lateral and distal segment. The sections where put in serial order from proximal to distal by using the brain as an anatomical marker. The sections were analysed systematically to visualize the location of the labelled fibers in the dermis, between the dermis and epidermis (crossing fibers) as well as the number of (apparently) terminal fibers within the epidermis using an Olympus BH microscope equipped with a Lucivid miniature monitor and Neurolucida™ software (MicroBrightField, Inc., Colchester, VT). The fibers were quantified using a 20 x 10 objective. The fibers in all three sections (i.e. dermis, crossing and terminal) of the most medial, of the center and of the lateral side were counted for analysis of all sections. Per group, the results were averaged and compared with the average results in the control group. Errors in the variations were assessed as the standard error of the mean (SEM). An unpaired t-test was performed for all evaluations. An illustration of our study model is depicted in Figure 1.

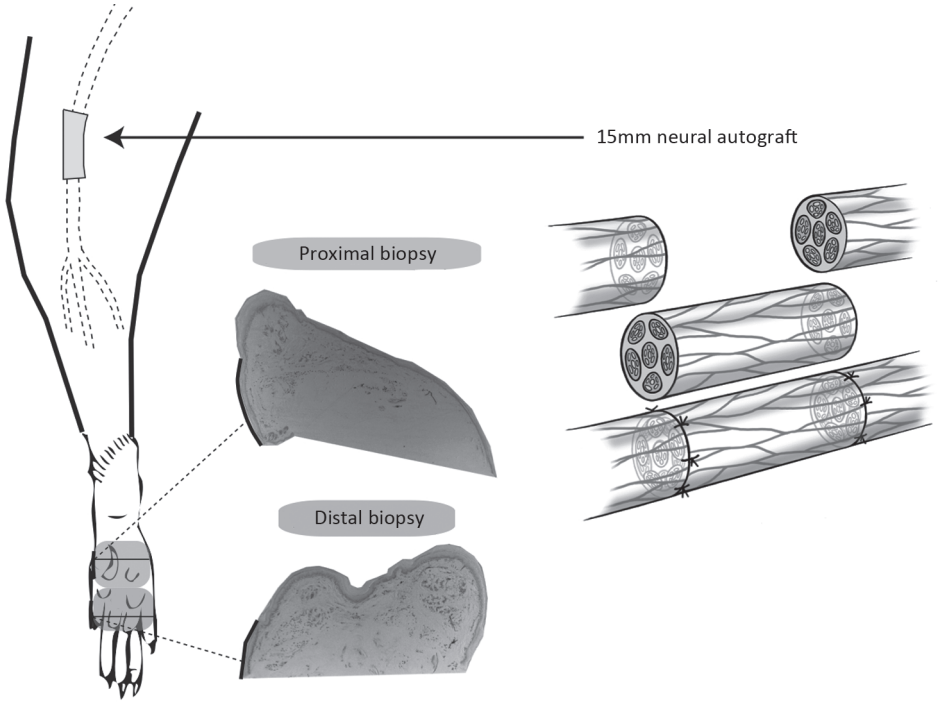

Figure 1 | Detailed image illustration of our model. Depicted are the 15 mm nerve autograft reconstruction and the biopsies taken at 12 weeks for both proximal and distal segments in the rat foot sole.

Results

Evaluation of Mechanical Hypersensitivity

The mechanical withdrawal of both the operated paw was determined. The operated leg showed a significant increase in the withdrawal threshold, compared to the healthy control ($p = 0.019$). The mean threshold for the animals treated with the autograft was 10.7 gram (SD ± 3.09) in contrast to the threshold for healthy animals, which showed a withdrawal reaction at 8.5 gram (SD ± 2.23) (Figure 2).

Immunohistochemistry

Normal innervation of the sensory nerve skin fibers is plotted in Figure 3. Figure 4 depicts an example of our staining in the different groups for the markers used. Figure 5 illustrates the regrowth of fibers in all three layers (i.e. the upper dermis, dermal-epidermal junction -crossing-

and epidermis). Regeneration percentage was only given for the epidermal areas, since this area is the most important for sensory transmission. For the myelinated fibers we used the crossing as the target area to measure regeneration percentage (Table 1).

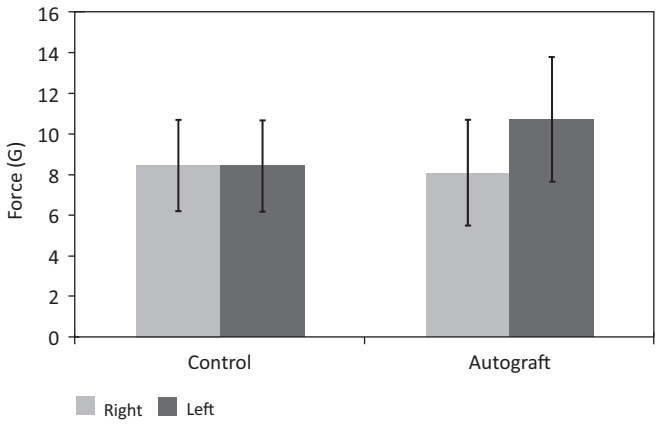

Figure 2 | This figure illustrates the results from the Von Frey Test. The operated leg showed a significant increase in the withdrawal threshold, compared to the healthy control ($p = 0.019$). The SD is visualised with the error bars.

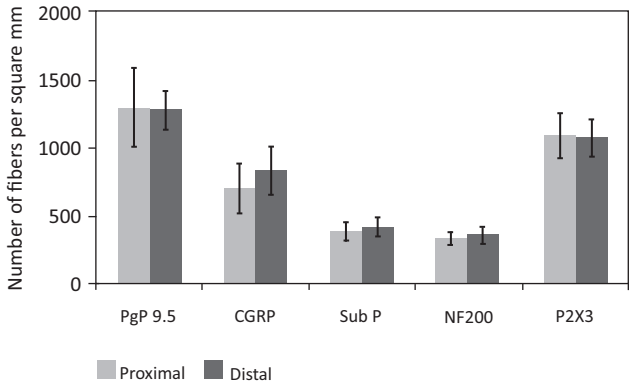

Figure 3 | Illustration of the normal distribution of the different sensory nerve fibers in the rat foot sole for the proximal and distal segment. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers. SEM is visualised with the error bars.

Table 1 | Regeneration percentage of the sensory nerve fibre subgroups, as reflected by their AB staining pattern, 12 weeks post operative and compared to the respective control. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers.

	Proximal (%)			Distal (%)		
	Lateral	**Central**	**Medial**	**Lateral**	**Central**	**Medial**
Pgp 9.5	68	77	71	69	61	75
CGRP	73	56	88	65	59	67
Subst P	68	46	65	56	74	64
NF200	69	47	72	57	55	76
P2X3	36	32	50	25	22	44

Pgp 9.5

Epidermis

Our healthy control had an average number of fibers in the lateral, central and medial side of the proximal segment of the foot sole of 1516, 1698, 1481 fibers per mm^2, respectively. Distal we found a total number of 1453, 1670, 1451 fibers per mm^2, respectively. The group treated with the nerve autograft had a total number of fibers per mm^2 for the lateral, central and medial side in the proximal side of 1025, 1307, 1051, 12 weeks post operative, respectively. Distal we found 998, 1023, 1095 fibers per mm^2 respectively.

Regeneration percentage for the lateral side of the foot sole for the proximal and distal segment was 68% and 69%, respectively. For the central side a percentage of 77% and 61% was found for the proximal and distal side. The medial side progressed up to 71% and 75% regeneration for the proximal and distal segments. A statistical trend was found comparing the autograft to the healthy controls ($p < 0.09$), which indicates that the autograft still had less fibers compared to the healthy control.

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 1197, 1461 and 1234 fibers per mm^2 in the proximal segment of the footpath. Distally 1208, 1427 and 1205 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 907, 1107 and 943 per mm^2 in the lateral, central and medial sections, respectively. Distal we found 889, 933 and 959 fibers per mm^2 respectively.

Fibre regeneration was significantly lower in all segments when compared to healthy controls ($p < 0.037$), except for the proximal central footpad area.

Upper Dermis

Innervating fibers in the lateral, central and medial side with 1088, 1279 and 1131 fibers per mm^2 in the proximal segment of the footpath. Distally 1033, 1273 and 1032 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 883, 1056 and 903 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 884, 892 and 883 fibers per mm^2 respectively. For both the lateral and proximal lateral side no significant difference between the 2 groups were found ($p > 0.129$). In the distal segment, the central area was still significantly different ($p < 0.001$) in contrast to the proximal side. The medial area for the proximal and distal segments was significantly different comparing both groups ($p < 0.048$).

CGRP

Epidermis

These peptidergic nerve fibers are innervating the lateral, central and medial side with 932, 1369 and 949 fibers per mm^2 in the proximal segment of the footpath. Distally 1136, 1624, 1174 fibers per mm^2 in the three sections were found. Group I had a total number of regenerated fibers of 1136, 1624, 1174 per mm^2 in the lateral, central and medial sections, respectively. The percentage of recovery was proximal for the three sections 73%, 56% and 88%, distal a recovery of 65%, 59% and 67% for the lateral, central and medial sections. Except for the proximal medial footpad area, all segments were significant different from the healthy controls ($p < 0.023$).

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 667, 913 and 527 fibers per mm^2 in the proximal segment of the footpath. Distally 738, 1026 and 742 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 441, 569 and 512 per mm^2 in the lateral, central and medial sections, respectively. Distal we found 468, 679 and 528 fibers per mm^2 respectively. Comparing the two groups, for both proximal and distal segment the number of fibers in lateral area was still significantly reduced ($p < 0.014$). The central area showed no significant difference, the medial area in the autograft treated group was distally still significant less ($p = 0.010$), proximally no significant difference was found.

Upper Dermis

Innervating fibers in the lateral, central and medial side with 497, 606 and 408 fibers per mm^2 in the proximal segment of the footpath. Distally 534, 815 and 557 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 304, 302 and 318 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 295, 375 and 332 fibers per mm^2 respectively. The fibers in all area's in both segments, except for the proximal medial area, were still significant less compared to the control group ($p < 0.062$).

Substance P

Epidermis

This subclass peptidergic fibre -in our control group- had a total of 459, 645, 556 fibers per mm^2 proximal and distal 564, 644, 620 in the lateral, central and medial sections. Three months post operative a total number of fibers of 314, 293, and 358 per mm^2 in the lateral, central and medial sections regenerated proximally. In the distal segment regenerated a total of 315, 474, 398 per mm^2. Looking at the regenerative percentage, the autograft did suffice proximal 68%, 46%, 65% and distally 56%, 74%, 64% regeneration.

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 667, 913 and 527 fibers per mm^2 in the proximal segment of the footpath. Distally 738, 1026 and 742 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 441, 569 and 512 per mm^2 in the lateral, central and medial sections, respectively. Distal we found 468, 679 and 528 fibers per mm^2 respectively. In the lateral area the proximal segment had significant less fibers in the autograft group ($p = 0.031$). The central area in both segments had less fibers compared to the control group ($p < 0.010$). The medial area was significant different in the distal area ($p = 0.003$).

Upper Dermis

Innervating fibers in the lateral, central and medial side with 497, 606 and 408 fibers per mm^2 in the proximal segment of the footpath. Distally 534, 815 and 557 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 304, 302 and 318 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 295, 375 and 332 fibers per mm^2 respectively. The lateral area was comparable in both groups for both segments. The central area in the autograft group was not comparable to the control group for both segments ($p < 0.018$). The medial area had significant less fibers in the autograft group in the distal segment ($p = 0.013$).

NF-200

Epidermis

The total number of myelinated fibers in our control group was proximally 272, 433 and 316 per mm^2 for the lateral, central and medial segments. Distally we found 377, 395 and 354 fibers per mm^2 for the lateral, central and medial side. Our operated animals had an average of 187, 205 and 226 fibers per mm^2 for the lateral, central and medial segments proximally. Distally an average of 194, 216 and 268 fibers per mm^2 for the lateral, central and medial segments was quantified. Taking these results in consideration, we can report a regeneration of 69%, 47% and 72% for the lateral, central and medial areas respectively. This is reflected statistically since all

comparisons between the autograft reconstruction and the healthy control were not significant different ($p > 0.231$), except for the proximal footpad comparing the central area between the two groups ($p = 0.036$).

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 272, 433 and 316 fibers per mm^2 in the proximal segment of the footpath. Distally 337, 395 and 354 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 187, 205 and 226 per mm^2 in the lateral, central and medial sections, respectively. Distal we found 194, 216 and 268 fibers per mm^2 respectively.

Comparing both groups in the proximal segment significant differences were found in the central and medial area ($p < 0.025$). Distally, except for the medial area, the lateral and central areas were significantly less in the operated group ($p < 0.012$).

Upper Dermis

Innervating fibers in the lateral, central and medial side with 528, 677 and 523 fibers per mm^2 in the proximal segment of the footpath. Distally 571, 717 and 626 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 452, 492 and 518 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 503, 480 and 560 fibers per mm^2 respectively. In the dermis the central area still showed significant less fibers in the autograft group in both the proximal and distal segment ($p < 0.009$). All the other segments were not significantly different.

P2X3

Epidermis

These non-peptidergic fibers, proximally, had 1036, 1402, 1030 fibers per mm^2 in the three specific sections. Distally 1034, 1386, 977 fibers per mm^2 were present. On the operated side, proximal 369, 447 and 518 fibers per mm^2 were found and in the distal segment we encountered 262, 298 and 425 fibers per mm^2 in the lateral, central and medial sections. The regeneration percentage for the proximal segment was 36%, 32% and 50%, distal a percentage of 25%, 22% and 44% was found.

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 981, 1322 and 975 fibers per mm^2 in the proximal segment of the footpath. Distally 984, 1297 and 942 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 375, 419 and 485 per mm^2 in the lateral, central and medial sections, respectively. Distal we

found 280, 318 and 415 fibers per mm^2 respectively. All area's in all segments had still significant less fibers in the autograft group compared to the healthy control group ($p < 0.001$).

Upper dermis

Innervating fibers in the lateral, central and medial side with 1083, 1425 and 1060 fibers per mm^2 in the proximal segment of the footpath. Distally 1126, 1383 and 1019 fibers per mm^2 in the three sections were found. The autograft treated group had a total number of regenerated fibers of 388, 457 and 503 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 313, 368 and 449 fibers per mm^2 respectively. All areas in all segments had still significant fewer fibers in the autograft group compared to the healthy control group ($p < 0.020$).

Discussion

This study examined the re-innervation of sensory nerve fibers of the glabrous skin in a rat hind paw at 12 weeks postoperatively, after reconstructing a 15 mm gap using a neural autograft. The main findings were: 1) that there was 70% re-innervation of PGP 9.5-IR skin fibers; 2) that peptidergic fibers (CGRP, Substance P) had a re-innervation percentage of 70% in the skin; 3) that non-peptidergic fibers had a re-innervation rate of 35%; 4) that myelinated fibers had a re-innervation percentage of 80% at 12 weeks postoperatively.

The foot sole of a rat can be divided into foot pads (i.e. touch cushions) and intermediate thin skin. For quantification of the skin nerve fibers we used the intermediate thin area, because this was also the location where the Von Frey mechanical test was performed. Regarding this area we could make, therefore, a clear correlation between the behavioural signs and the histological findings.

In a healthy animal all sensory fibers, except the myelinated fibers, end in the epidermis and are thought to play an essential role in transducing pain and innocuous stimulation.[25,31] To calculate a regeneration percentage of the fibers in the four subgroups, the fibers in the epidermis were quantified. To evaluate the regeneration of the myelinated fibers (i.e. Aδ fibers) the dermal-epidermal junction was used as the quantification area, since these fibers loose their myelin sheet in this dermal-epidermal crossing.[10]

The present study used different immunohistochemical markers to distinguish between specific kinds of fibre populations. While these markers are well characterized in previous studies, we cannot exclude the possibility that these markers in the fibers may fall below detection level, although the fibers may still be present. To minimize the risk of missing fibers, we used the PGP 9.5 antibody, which is a pan-neuronal marker and stains all nerve fibers to verify if the peptidergic, non-peptidergic and myelinated fibers are not an underestimation.

PGP 9.5

In the current study we found at 12 weeks postoperatively 70% of epidermal PGP-IR fibers re-innervated the foot sole compared to control group. Previous studies have shown that after a crush injury to the sciatic nerve, there is a complete re-innervation of PGP 9.5-IR fibers in the upper dermis at 5 weeks and epidermal fibers at 12 weeks.[19,22,27] This discrepancy with the present study may have a few reasons. 1) axons, which regenerate after complete transection of the mixed sciatic nerve, rarely return to the original Schwann cell string. The incidence of axonal misdirection is likely to be very high 2) axons have to regenerate over a 12 mm gap 3) previous studies used Sprangue Dawley male rats, however the present study uses Lewis female rats. Testosterone is known to accelerate nerve regeneration and therefore may explain the better regeneration rate in the previous reports that used male rats instead of females.[9]

In the present study we found that the regenerated PGP 9.5 fibers were thinner and penetrated the epidermis in lesser extension compared to the control group, which was in line with previous reports. Majority of the fibers in the epidermis are unmyelintaed C-fibers that terminate as free nerve endings. However linear Bands of Büngner do not form when unmyelinated C-fibers degenerate, which are important for guiding the regrowing axon. Therefore, the regenerative C-fibers may be mislead which in turn may lead to incomplete skin regeneration. Other studies that used the chronic constructive injury (CCI)[1], which consists of four-loosely catgut ligations made around the sciatic nerve, showed a hyper re-innervation of PGP-IR skin fibers compared to control levels at 8 and 12 weeks.[20,30] However this is a neuropathic pain model where the excessive sprouting of fibers could be explained by substantial secretion of neurotrophic factors induced by partial nerve injury and inflammation.

CGRP

An average regeneration percentage in all six segments of 70 % was found for the CGRP fibers. Local axon synthesis of CGRP is critical for the Schwann cell proliferation during regeneration in adult peripheral nerve regrowth.[14,26] Previous reports showed that CGRP expression increased substantially in regenerative growth cones after nerve injury.[14,26] Therefore, it is not surprising that the CGRP fibers have a substantial regenerative percentage compared to the non-peptidergic P2X3 fibers. These fibers are the peptidergic group of fibers that react to the neurotrophic Nerve Growth Factor (NGF), which is known to be secreted during peripheral nerve injury by Schwann cells, keratinocytes and other non-neuronal cells. NGF acts as a guidance cue for embryonic mammalian dorsal root ganglions and in the adult as a target-derived maintenance factor. However, after peripheral nerve injury the NGF that is secreted from the target tissue, for example the skin, may lead to incorrect target cell re-innervation. This may explain the incomplete re-innervation pattern that is seen in a regeneration model.

Substance P

Substance P is a peptidergic neuropeptide that is thought to have a role in sensation as a transducer of pain in small unmyelinated C nerve fibers. This peptidergic neuropeptide is co-localized with CGRP, however CGRP is not entirely co-localized with Substance P.[21] In the present study we found an overall regeneration percentage in the autograft group of approximately of 60% of Substance P-IR fibers compared to the control group. This suggest that Substance P has a slower re-innervation pattern compared to CGRP fibers. Previous reports showed that CGRP and sympathic fibers achieve higher levels of reinnervation after a crush injury compared to Substance P.[19] A possible explanation could be that these NGF responsive fibers compete for NGF after peripheral nerve injury as described earlier. In addition a small portion of Substance P fibers are NGF-insensitive and, therefore, may be more likely to sprout in lesser extent.

Previous reports showed that hyper-reinnervation of peptidergic fibers, following peripheral nerve injury, often was accompanied with neuropathic pain.[6,20,25,30] However the incomplete CGRP and Substance P re-innervation is in support with our behavioural data that shows that this autograft group does not develop hypersensitivity to mechanical stimuli.

NF-200

The present study showed a re-innervation percentage of 75% for the NF-200-IR skin fibers compared to control group. This is an interesting finding, because previous research that looked at re-innervation pattern in skin a sciatic nerve, after a CCI of the sciatic nerve, showed a permanent loss of myelinated fibers 18 months post operatively.[20] However this model displays a neuropathic pain model, where it was hypothesised that the unmyelinated fibers play a substantial role in inducing and maintaining neuropathic pain. In our autograft model the myelinated fibers showed the quickest regeneration rate. An explanation could be sought that primary survival of these animals needs the Aδ fibers. These peptidergic fibers are responsible for the nerve signalling associated with acute pain and constitute the afferent portion for the reaction of noxious stimuli and/or abnormal temperature sensations. This explanation is further strengthened by our NF-200 regeneration with an average percentage of 75% for the proximal and distal foot sole.

P2X3

Few studies have considered the re-innervation pattern of non-peptidergic fibers[5] whereas these fibers account for more than 50% of unmyelinated C-fibers. Non-peptidergic fibers are not responsive to NGF, but are responsive to glial cell-line-derived neurotrophic factor (GDNF). In the present study we found a re-innervation pattern of 35% of skin P2X3-IR fibers in the autograft group. This re-innervation has not progressed as far as that of the peptidergic fibers. In vitro studies show that DRG neurons with lesioned sciatic nerve secrete much lower levels of GDNF than NGF in culture.[13] This could be a possible explanation for the slow re-innervation of non-peptidergic fibers, which is supported by study conducted by Riberio et. al.[20]

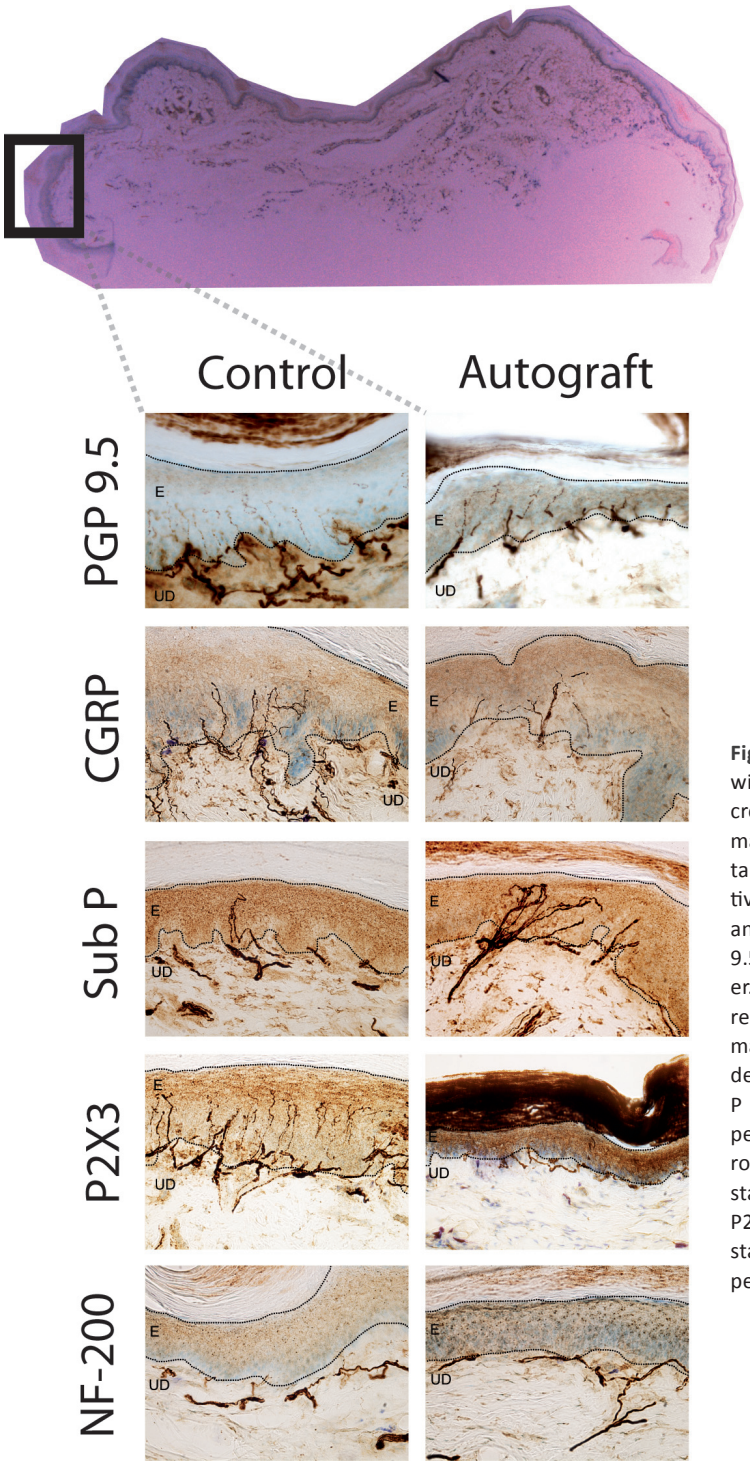

Figure 4 | An overview with example of the micrographs of the different markers used. Pictures are taken with a 20x10 objective for both the proximal and distal segment. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers.

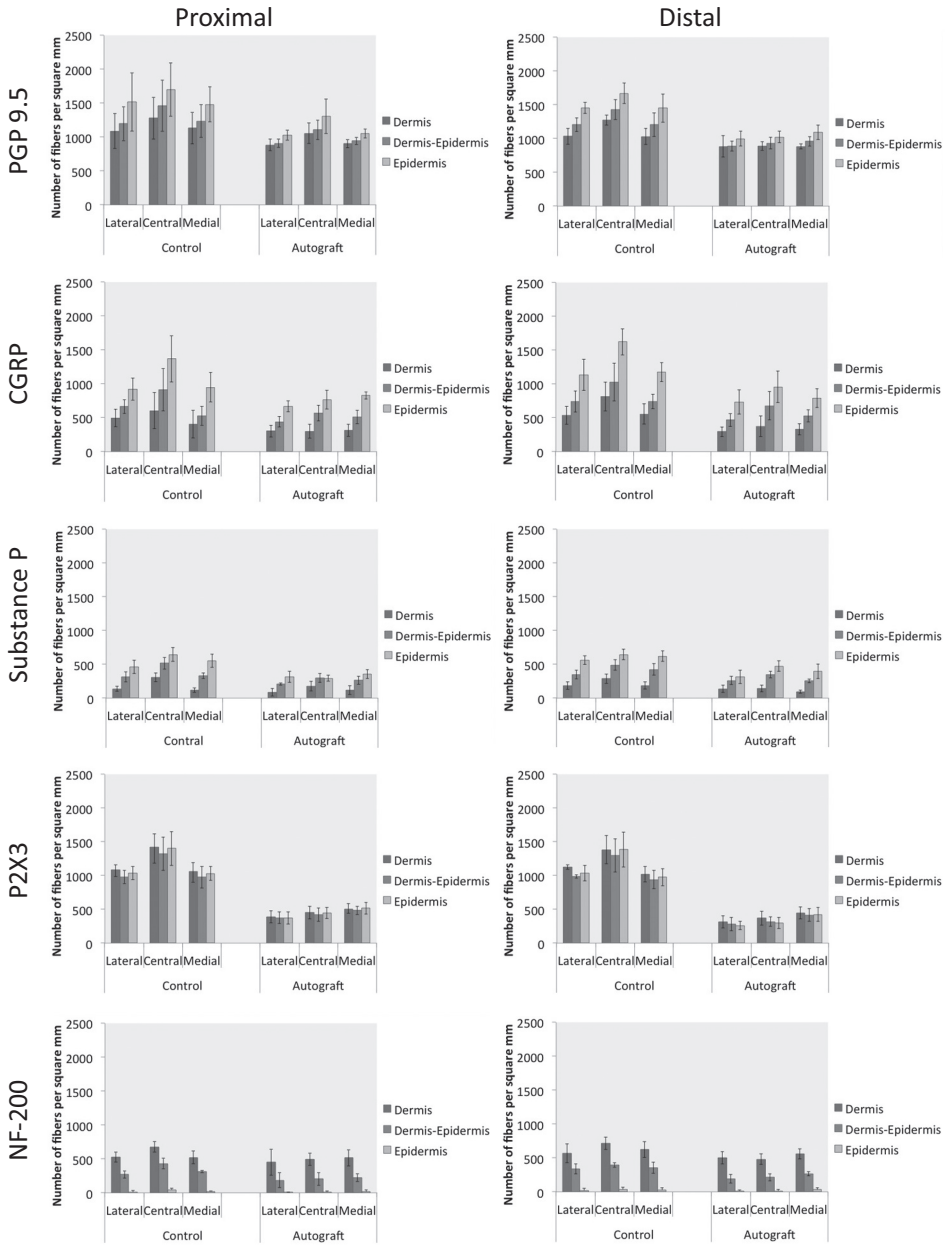

Figure 5 | The re-innervation of the skin nerve fibers, 12 week post operatively. SEM is visualised with the error bars. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers. SEM is visualised with the error bars.

The present study found that 84 days post operative the foot sole is not fully re-innervated with skin nerve fibers, even though the nerve defect was limited to 15 mm and the complete distance to cover was no more than 50 mm. This incomplete regeneration was further strengthened by our Von Frey test, which showed a higher mechanical withdrawal threshold in the autograft reconstruction group compared to the control group. In addition, earlier studies conducted by our group supported by means of functional and histological data supported this finding.

These findings are helpful in the understanding of neural regeneration after grafting a nerve defect with the autologous nerve graft. Especially the particular findings that illustrate a stronger regeneration of the peptidergic fibers compared to the non-peptidergic fibers 12 weeks post operative (Figures 6 and 7). This could be explained by the fact that peptidergic fibers are responsible for transferring noxious stimuli and temperature sensations to the central nervous system (CNS). Further projects should investigate the re-innervation of skin nerve fibers in the foot sole after grafting nerve defects with other conduits than the nerve graft.

In this study, however, we only focused on the re-innervation of the foot sole after autologous nerve grafting, since in literature such a meticulous analysis has not been made before and it contributes to the knowledge available in the field of peripheral nerve reconstruction. This study provides a baseline for the number of fibers after reconstructing a large defect using an autograft.

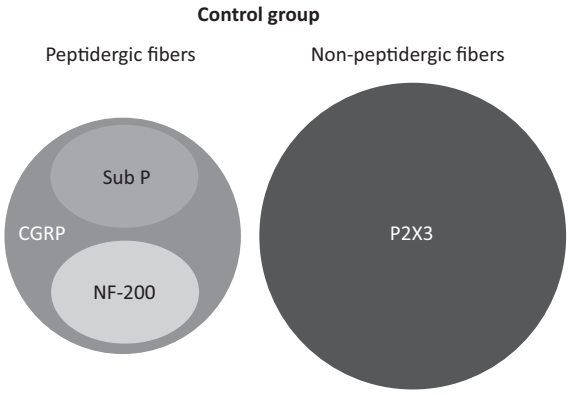

Figure 6 | Distribution of the five different subgroups of sensory nerve fibers in the healthy rat foot sole. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers. SEM is visualised with the error bars.

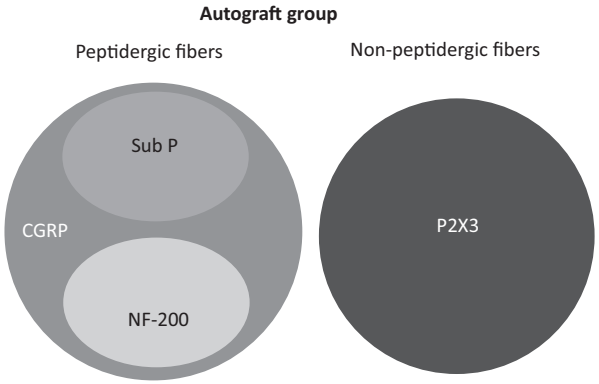

Figure 7 | Distribution of the five different subgroups of sensory nerve fibers in the rat foot sole 12 weeks after reconstructing the 15 mm nerve defect using an autograft. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers. SEM is visualised with the error bars.

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Chapter 6

Re-innervation Pattern of Subgroups of Specific Sensory Nerve Fibers of the Skin after a Vein-muscle Graft Reconstruction supported with BMSCs in a Rat Model

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Submitted

Abstract

Introduction

An alternative to the autograft in experimental nerve reconstruction is the vein-muscle graft supported with BMSCs.

Visualizing the skin epidermal sensory nerve fibers can illustrate the regeneration in the most distal terminal of the injured nerve. The different peptidergic (visualized with CGRP and Substance P) and non-peptidergic fibers (visualized with P2X3) are responsible for signalling noxious and termed nociception. The Aδ-fibers are visualized using NF200.

This study investigates the re-innervation of these skin neural fibers in the rat foot sole, 12 weeks after reconstructing a nerve defect with a vein-muscle graft and a vein-muscle graft with BMSCs.

Methods

In 5 animals a 15 mm sciatic nerve defect was reconstructed using a vein-muscle graft. Five animals were reconstructed with a vein-muscle graft supported with BMSCs.

Twelve weeks after grafting, the sole of the foot of the operated hindpaw was excised and stained according to the necessary protocols for CGRP, Substance P, P2X3 and NF200. Control staining was performed with PGP 9.5 (a pan-neuronal marker).

Results

The vein-muscle graft with BMSCs had approximately 50% re-innervation of the sensory skin fibers (stained with PgP 9.5). The myelinated fibers had an increased re-innervation of approximately 190% favouring the vein-muscle graft with BMSCs. The vein-muscle graft with the BMSCs had a stronger regeneration compared to the vein-muscle graft for the non-peptidergic fibers (18% vs 9.5%).

Conclusion

Twelve weeks after reconstructing a nerve defect using a vein-muscle graft with/without BMSCs, we found strong indications for the beneficial effect of the supportive cellular therapy (i.e. the BMSCs). Compared to the autograft both conduits were outperformed.

Introduction

Peripheral nerve injury is a common problem in trauma. Reconstruction methods are often disappointing regarding the poor functional outcome and therefore prompts researchers to investigate alternative reconstruction techniques. Especially larger nerve defects that cannot be reconstructed with a conventional method, such as a primary end-to-end coaptation, are in need for an appropriate technique to restore innervation of the end organs.[8,17,32]

The clinical gold standard for bridging large nerve defects is the use of an autologous nerve graft. Disadvantages of this technique are the inevitable donor site morbidity and limited length of available graft material. In addition some of the morbidity of the donor site are scarring, neuroma formation and loss of sensation.[2,7,13,17] An alternative introduced by our group is the vein-muscle graft supported with BMSCs. We showed that a vein-muscle graft filled with BMSCs outperformed the conventional vein-muscle graft, which resulted in functional and histological improvement.

Previously, our group demonstrated the re-innervation of the different sensory subgroups of fibers in the skin in after an autograft reconstruction.[22] However there are no reports about the re-innervation pattern of sensory nerve fibers in the skin following reconstruction using a conventional vein-muscle graft or the vein-muscle graft filled with BMSCs. Consequently, no studies have been reported about the beneficial effect of BMSCs on the regeneration of the skin fibers after vein-muscle graft reconstruction.

The different sensory nerve subgroups in the foot sole were visualized three months post operative using different stainings. Pgp 9.5 is a pan neural marker that stains all sensory nerve fibers and thus visualizing the total regrowth of the fibers. Two major classes of sensory fibers in the skin are peptidergic and non-peptidergic fibers. The peptidergic fibers were stained with cGRP and SubstanceP, visualising the small unmyelinated C nerve fibers, that are responsible for the fast transduction of pain and temperature. NF200 visualises all the peptidergic myelinated fibers in the skin, the fast pain and temperature sensations. These fibers lose their myelination in the dermal/epidermal junction. P2X3 visualises all non-peptidergic C nerve fibers for the non-acute sensations.

We hypothesise that the BMSCs enhance neural re-innervation in the rat foot sole and that the injection of these cells in the lumen of the graft results in better regeneration as can be expected in conventional neural reconstruction using a vein-muscle graft alone.

Methods

Animals

The experimental protocol was approved by the Animal Experiments Committee according to the National Experiments on Animals Act and conducted according to this law that serves the implementations of Directive 86/609/EC of the Council of Europe. Ten isogenic adult female Lewis rats, weighing 180 – 200 grams, were used. Animals were pair-housed in hooded cages at room temperature on a 12-hour light/dark schedule, and were given food and water ad libitum. Surgical procedures and electrophysiological evaluations were performed under general anaesthesia (Isoflurane, 1 – 2% in a mixture of O_2/N_20).

Surgical Technique

The surgical procedure was performed by a single surgeon and assistant using standard aseptic microsurgical techniques under the operating microscope (Zeiss OP-MI 6-SD; Carl Zeiss, Goettingen, Germany) on the sciatic nerve of the left hind limb. After an oblique skin incision was made in the left gluteal region of the donor the sciatic nerve was exposed through a gluteal muscle-splitting incision and externally dissected to isolate a 15 mm segment of the nerve. The nerve was transected proximally and distally to obtain the 15 mm segment. The jugular vein served as the conduit, which was harvested through a longitudinal mid-line incision of 35 mm in the neck. The left external jugular vein was dissected and both the proximal and the distal end of the vein were ligated. A small muscle fragment of 1½ x 1½ mm^2, was cut out of the gluteal muscle and placed inside the lumen of the vein using a straight irrigator. Next, the graft in group I (i.e. the vein-muscle graft) was connected to the nerve stumps using 6 10/0 Ethilon sutures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands) at each coaptation side. The muscle was closed using 2 6/0 Vicryl Rapide sutures, followed by closing the skin using 6/0 Vicryl Rapide Sututures (Ethicon, Johnson & Johnson, Amersfoort, the Netherlands). In group II (the vein-muscle graft with BMSCs) the cells were injected after connecting the graft to both the proximal and distal nerve stumps. A detailed illustration is depicted in Figure 1.

Bone Marrow Stromal Cell Preparation

Fresh bone marrow cells were harvested aseptically from tibias and femurs of 20 adult rats. Both ends of the bones were cut and the marrow was flushed with 10 ml of D-MEM medium. After centrifugation, the cell suspension was lysed with 0.85% NH_4Cl for 5 min. The suspension was then filtered through a 40-mm nylon mesh, and re-suspended in culture D-MEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were placed in a 75-cm^2 flask and incubated at 37°C in 5% CO_2 for three days. Non-adherent cells were removed by replacing the medium three times a week. After five to eight passages, when the BMSC culture reached confluence, BMSCs were lifted by 0.25% trypsin and 1 mM% EDTA in PBS for 5 min and washed in alpha-MEM medium. BMSCs

were labelled with red membrane dye PKH-26 (Sigma-Aldrich, UK) in order to evaluate their capacity to differentiate into neuronal and other tissue types as well as to track their migration into lymphoid organs and the contralateral sciatic nerve. PKH-26 staining was performed in accordance with the manufacturer's instructions. The stromal cells were incubated with PKH-26 dye in Diluent C buffer solution at room temperature for 5 min. Labelling was stopped by incubation with 1% BSA in PBS for 1 min and complete D-MEM medium. Following a final wash in complete D-MEM medium, labelled stromal cells were prepared to a final concentration of 3×10^6 cells in 0.05 ml. This solution was kept in a 1 ml syringe and the solution was then injected in the two ends of the vein using a small syringe (27G), injecting 0.025 ml in each side.

Evaluation of Mechanical Hypersensitivity

Twelve weeks post operative, the mechanical threshold of the hind paws was measured using the Von Frey hairs. Each Von Frey hair was applied for 2 s at 5 s intervals, with a maximum of 5 repeated measures. The threshold was set at 3 paw lifts. For testing the rats were placed in a plastic box (perforated floor) and able to move freely. The medial and lateral side where stimulated at the transition point from glabrous skin to hairy skin. The center foot sole was stimulated at the very center point of the foot sole. The withdrawal response was noted and used for analysis.

Immunohistochemistry

Preparation of Tissue

After sacrificing the animal, the complete foot sole of the left and right hind paws were surgically resected. The footsoles were then placed in a 10 percent sucrose solution for 24hrs, washed and rinsed with glycerol solution and placed in freezer. This was performed according to the protocol described by Duraku et al.[10,22]

Immunohistochemical Staining

The sections were pre-incubated (90 min at room temperature (RT)) in a mixture containing bovine serum albumin (BSA 2%) phosphate buffered saline (PBS, pH 7,4). (Fraction V, Roche) and 0.5% Triton X-100. Thereafter, the sections were rinsed in PBS and incubated for 48 hours in a cocktail of 2% BSA containing antibody; goat anti-CGRP antibody (Abcam, United States) (1/30.000), NF-200 (1/35.000), Substance P (1/500), P2X3 (1/25.000), PGP 9.5 (1/15.000). Subsequently, sections where incubated with a secondary antibody rabbit anti-goat for 90 min room temperature. Sections were further processed using a Vectastain Elite ABC kit (Vector, Burlingame, CA) (90 min at RT). Finally, 3,-3' diaminobenzidine (DAB) enhanced by the glucose oxidase-nickel-DAB method (Kuhlmann and Peschke, 1986) was used to reveal antigenic sites. The sections were mounted on gelatinized slides, air dried overnight, dehydrated using absolute ethanol (< 0.01% methanol), transferred to xylene and coverslipped with Permount (Fisher, Hampton, NH). Immunoreactivity for CGRP was completely abolished when primary antibodies were omitted.

Analysis

A total of 8 series of sections of the complete footpath was used for analysis, making selection in a lateral, center and medial side per section and dividing the footpath in a lateral and distal segment. The sections where put in serial order from proximal to distal by using the brain as an anatomical marker as described by Duraku et al.[10] The sections were analyzed systematically to visualize the location of the labeled fibers in the dermis, between the dermis and epidermis (crossing fibers) as well as the number of (apparently) terminal fibers within the epidermis using an Olympus BH microscope equipped with a Lucivid miniature monitor and Neurolucida™ software (MicroBrightField, Inc., Colchester, VT). The fibers were quantified using the 20 x 10 objective. The fibers in all three sections (i.e. dermis, crossing and terminal) of the most medial, of the center and of the lateral side were counted for analysis of all sections. Per group, the results were averaged and compared with the average results in the control group. Errors in the variations were assessed as the standard error of the mean (SEM). An unpaired t-test was performed for all evaluations. An illustration of our study model is depicted in Figure 1.

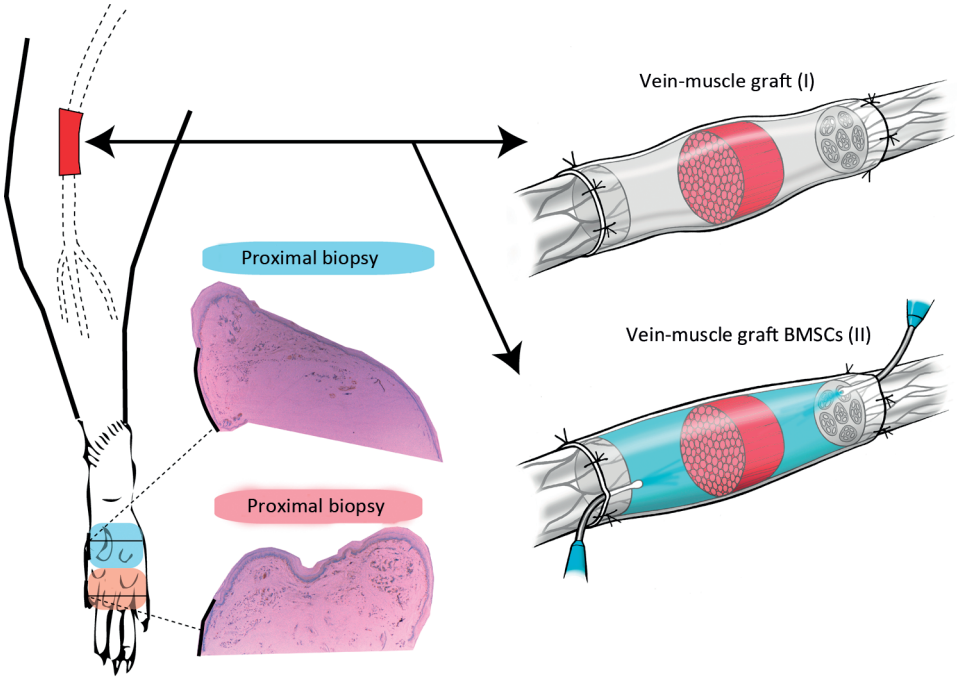

Figure 1 | Detailed illustration of the surgical model of the vein-muscle grafts used and the location of the skin biopsies.

Results

Evaluation of Mechanical Hypersensitivity

The mechanical withdrawal of both the operated and the non-operated paws were determined. The mean threshold for the animals treated with the vein-muscle graft (group I) was 10.7 gram (SD ± 3.09) and the animals with the vein-muscle graft with BMSCs (group II) showed a withdrawal reaction at 8.5 gram (SD ± 2.23) (Figure 2).

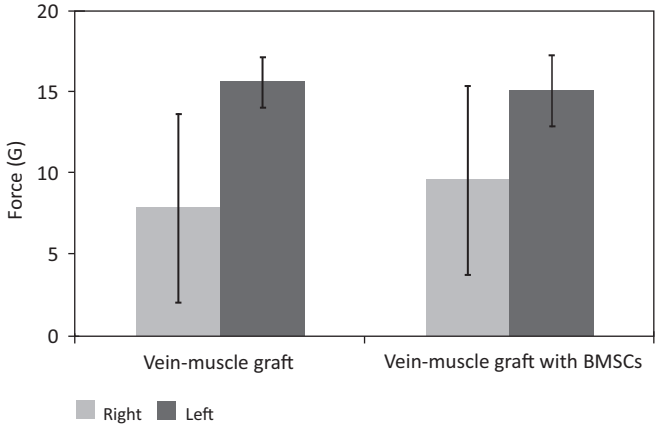

Figure 2 | The Von Frey test illustrating the mechanical withdrawal of the animals in both groups. The SD is visualised with the error bars.

Immunohistochemistry

The results section is subdivided in five individual stainings, starting with Pgp 9.5 than discussing peptidergic and non-peptidergic fibers. Figure 3 illustrates our staining intensity in the different groups, showing images using our 20 x 10 microscope. Figure 4 illustrates the regrowth of fibers in all three layers (i.e. the upper dermis, crossing and epidermis). Regeneration percentage was only given for the epidermal areas, since this area is the most important for sensory transmission. For the myelinated fibers we used the crossing as the target area to measure regeneration percentage (Table 1).

Table 1 | Regeneration percentage of the sensory nerve fiber subgroups, as reflected by their AB staining pattern, 12 weeks post operative and compared to the respective control. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers.

	Proximal (%)		Distal (%)	
	Group I	**Group II**	**Group I**	**Group II**
Pgp 9.5	30	44	28	37
CGRP	70	70	61	59
Subst P	53	67	42	48
NF200	36	57	26	59
P2X3	14	22	5	14

Pgp 9.5

Epidermis

The vein muscle graft (group I) had an average of fibers in the lateral, central and medial side of the proximal segment of the footsole of 437, 501 and 453 fibers per mm^2, respectively. Distal we found a total number of 411, 450 and 431 fibers per mm^2, respectively. The group treated with the vein-muscle graft filled with BMSCs (group II) had a total number of fibers per mm^2 for the lateral, central and medial side in the proximal side of 638, 786 and 645, 12 weeks post operative, respectively. Distal we found 571, 565 and 543 fibers per mm^2 respectively. No significant differences were found between the two groups for both the proximal and distal sections ($0{,}162 < p < 0{,}420$).

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 369, 405 and 386 fibers per mm^2 in the proximal segment of the footpath. Distally 374, 377 and 364 fibers per mm^2 in the three sections were found in the vein-muscle graft treated group. The group treated with the vein-muscle graft had a total number of 534, 627 and 593 regenerated fibers of per mm^2 in the lateral, central and medial sections, respectively. Distal we found 508, 529 and 497 fibers per mm^2 respectively.

Comparing the proximal sections for both groups we found a trend for the BMSC treated group to outperform the vein-muscle graft alone ($0.096 < p < 0.131$). The distal sections were found comparable ($0{,}183 < p < 0{,}470$).

Upper Dermis

Innervating fibers in the lateral, central and medial side with 395, 417 and 386 fibers per mm^2 in the proximal segment of the footpath. Distally 371, 372 and 366 fibers per mm^2 in the three sections were found for the vein-muscle graft group. The vein-muscle-BMSC treated group had a total number of regenerated fibers of 498, 613 and 573 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 492, 536 and 483 fibers per mm^2 respectively. Proximal the vein-muscle grafts injected with the BMSCs showed a trend to outperform the vein-muscle graft alone, in the central segment ($p = 0,086$) and the medial segment ($p = 0,049$). Distally both groups were not significant different ($p > 0,139$).

CGRP

Epidermis

These peptidergic nerve fibers are innervating the lateral, central and medial side with 624, 738 and 901 fibers per mm^2 in the proximal segment of the footpath in group I. Distally 628, 934 and 848 fibers per mm^2 in the three sections were found. Group II had a total number of regenerated fibers of 777, 745 and 756 per mm^2 in the lateral, central and medial proximal segment, respectively. Distally we found 644, 890 and 779 fibers per mm^2 in the lateral, central and medial segment, respectively. In the proximal section comparable results were found ($p > 0,264$). Both groups showed comparable results ($0,719 < p < 0,921$) in the distal segment.

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 416, 489 and 503 fibers per mm^2 in the proximal segment of the footpath in group I. Distally 386, 573 and 477 fibers per mm^2 in the three sections were found. Group II had a total number of regenerated fibers of 400, 463 and 476 per mm^2 in the lateral, central and medial sections, respectively. Distal we found 359, 546 and 448 fibers per mm^2 respectively. Both groups had no significant difference in regenerated fibers for all segment ($p > 0,723$).

Upper Dermis

The vein-muscle graft had in the lateral, central and medial side 218, 233 and 272 fibers per mm^2 in the proximal segment of the footpath. Distally 204, 284 and 263 fibers per mm^2 in the three sections were found. Group II had a total number of regenerated fibers of 258, 278 and 308 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 223, 321 and 278 fibers per mm^2 respectively. For all sections the number of fibers were comparable between group I and group II ($p > 0,732$).

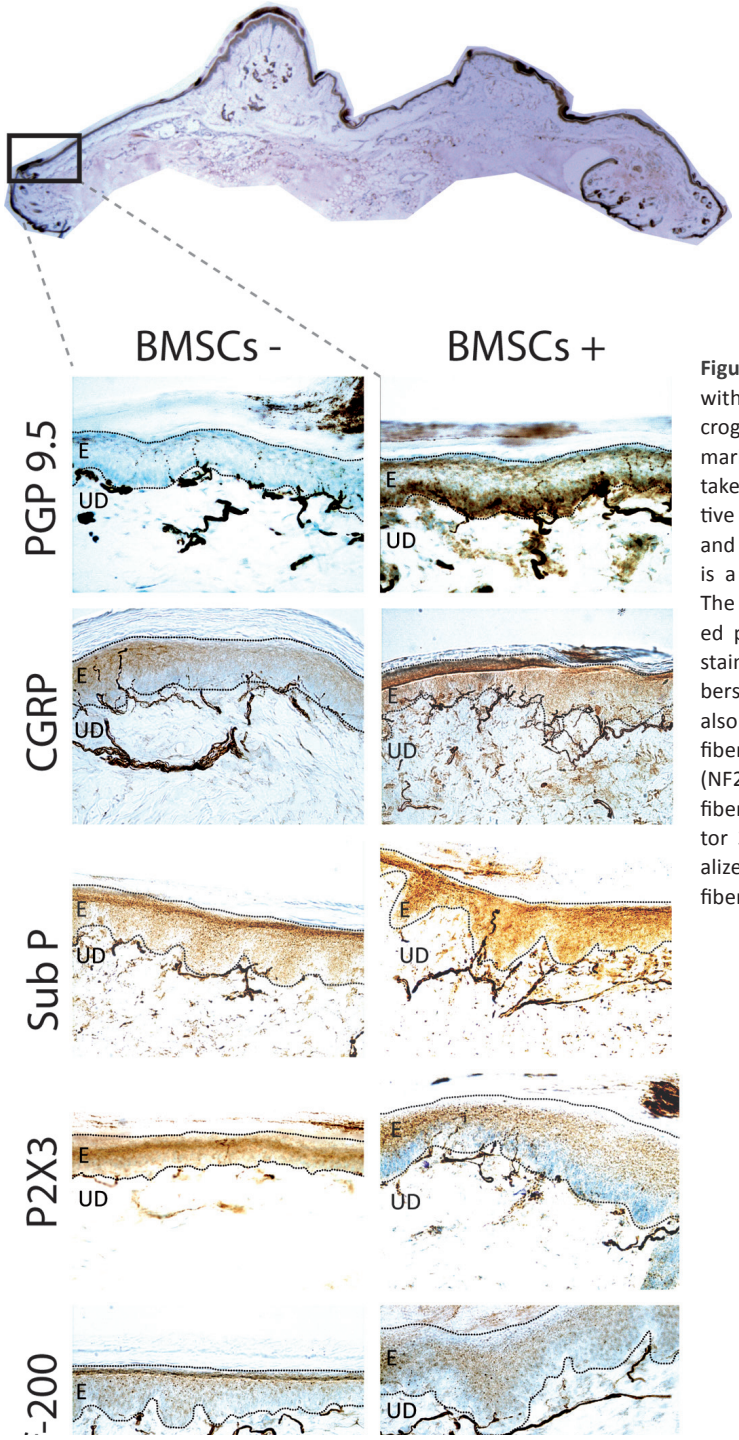

Figure 3 | An overview with example of the micrographs of the different markers used. Pictures are taken with a 20x10 objective for both the proximal and distal segment. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers.

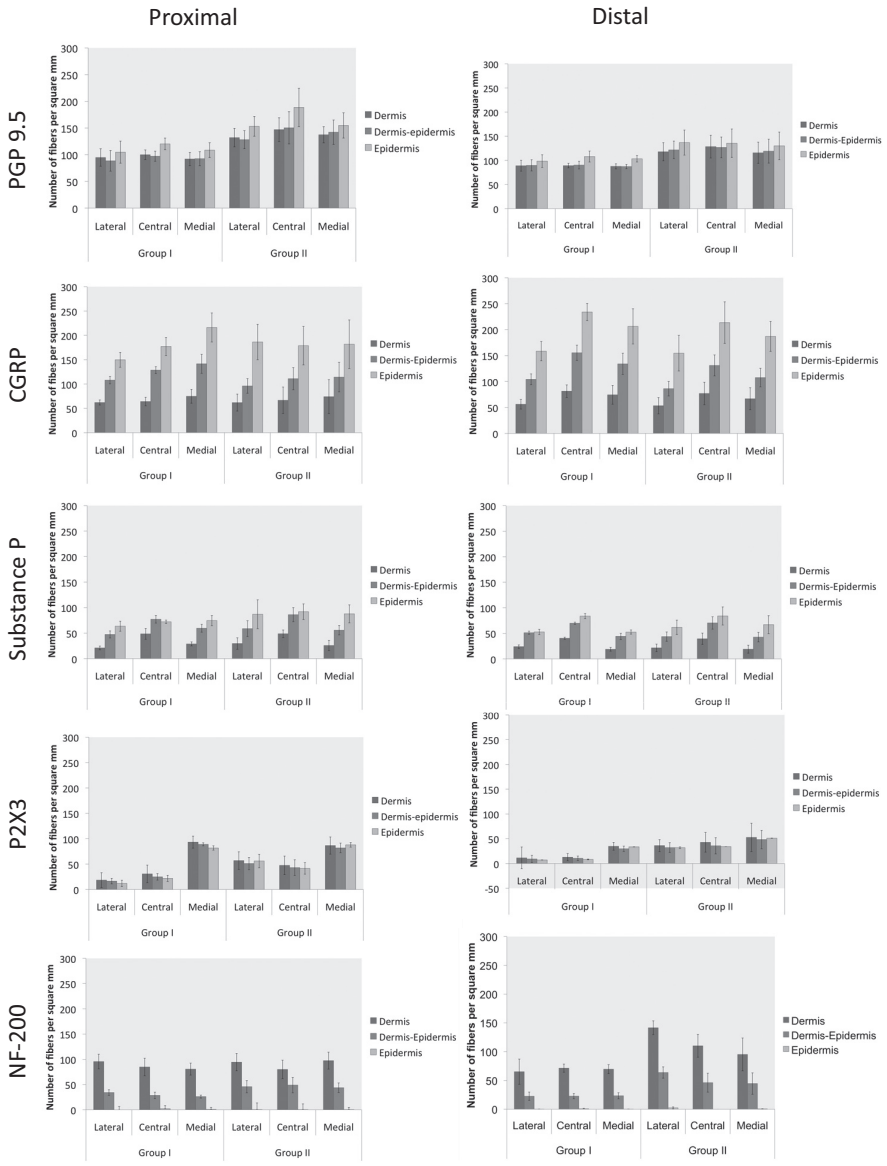

Figure 4 | The re-innervation of the skin nerve fibers, 12 week post operatively. SEM is visualised with the error bars. PgP 9.5 is a pan neuronal marker. The calcitonin gene-related peptide (CGRP) marker stains the peptidergic fibers. Substance P (Sub P) also stains the peptidergic fibers. Neurofilament 200 (NF200) stains the Aδ-fibers. The P2X purinoceptor 3 (P2X3) staining visualizes all non-peptidergic fibers. SEM is visualised with the error bars.

Substance P

Epidermis

This subclass peptidergic fiber -in group I- had a total of 265, 301 and 311 fibers per mm^2 proximal and distal 224, 343 and 208 in the lateral, central and medial sections. Three months post operative a total number of fibers of 363, 383 and 366 per mm^2 in the lateral, central and medial sections regenerated proximally in group II. In the distal segment regenerated a total of 258, 350 and 279 fibers per mm^2. Both groups had a comparable number of fibers in both the proximal and distal sections for all segments ($p > 0,324$).

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 186, 309 and 238 fibers per mm^2 in the proximal segment of the footpath. Distally 200, 289 and 183 fibers per mm^2 in the three sections were found. The vein-muscle grafts treated with BMSCs had a mean total number of regenerated fibers of 245, 359 and 233 per mm^2 in the lateral, central and medial sections, respectively. Distal we found 182, 293 and 178 fibers per mm^2 respectively. Both groups had a comparable number of fibers in both the proximal and distal sections for all segments ($p > 0,427$).

Upper Dermis

Innervating fibers in the lateral, central and medial side with 68, 175 and 95 fibers per mm^2 in the proximal segment of the footpath. Distally 87, 128 and 61 fibers per mm^2 in the three sections were found. Group III had a mean total number of regenerated fibers of 123, 203 and 108 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 90, 164 and 79 fibers per mm^2 respectively. Both groups had a comparable number of fibers in both the proximal and distal sections for all segments ($p > 0,288$).

NF-200

Epidermis

The total number of myelinated fibers in group I was proximally 0, 9 and 3 per mm^2 for the lateral, central and medial segments. Distally we found 1, 3 and 1 fibers per mm^2 for the lateral, central and medial side. Groups II had an average of 2 fibers per mm^2 for the lateral, central and medial segments proximally. Distally an average of 10, 0 and 3 fibers per mm^2 for the lateral, central and medial segments was quantified. For all segments in both the proximal and distal sections the number of fibers was not significantly different between the two groups ($p > 0.141$).

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 143, 119 and 109 fibers per mm^2 in the proximal segment of the footpath. Distally 94, 95 and 98 fibers per mm^2 in the three sections were found. Group II had a total number of regenerated fibers of 191, 204 and 183 per

mm^2 in the lateral, central and medial sections, respectively. Distal we found 266, 193 and 186 fibers per mm^2 respectively. In the lateral segment of the distal section group II outperformed group I ($p = 0.010$). The other segments were comparable between the two groups ($p > 0.113$).

Upper Dermis

Innervating fibers in the lateral, central and medial side 398, 353 and 336 fibers per mm^2 in the proximal segment of the footpath. Distally 272, 298 and 290 fibers per mm^2 in the three sections were found. Group II had a total number of regenerated fibers of 393, 333 and 405 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 590, 459 and 397 fibers per mm^2 respectively. In the lateral segment of the distal section group II outperformed group I ($p = 0.016$). The other segments were comparable between the two groups ($p > 0.104$).

P2X3

Epidermis

These non-peptidergic fibers, proximally, had 50, 92 and 341 fibers per mm^2 in the three specific sections in group I. Distally 25, 32 and 118 fibers per mm^2 were present. Group II, proximal 234, 173 and 366 fibers per mm^2 were found and in the distal segment we encountered 133, 143 and 213 fibers per mm^2 in the lateral, central and medial sections. In the lateral segment of the proximal section group II outperformed group I ($p = 0.038$). The other segments were comparable between the two groups ($p > 0.262$).

Dermal-epidermal Junction

These fibers are innervating the lateral, central and medial side with 69, 111 and 373 fibers per mm^2 in the proximal segment of the footpath. Distally 38, 43 and 124 fibers per mm^2 in the three sections were found. Group II had a total number of regenerated fibers of 213, 180 and 342 per mm^2 in the lateral, central and medial sections, respectively. Distal we found 134, 149 and 202 fibers per mm^2 respectively. In the lateral segment of the proximal section group II outperformed group I ($p = 0.054$). The other segments were comparable between the two groups ($p > 0.306$).

Upper Dermis

Innervating fibers in the lateral, central and medial side with 78, 128 and 398 fibers per mm^2 in the proximal segment of the footpath. Distally 49, 56 and 152 fibers per mm^2 in the three sections were found. Group III had a total number of regenerated fibers of 238, 198 and 361 fibers per mm^2 in the lateral, central and medial sections, respectively. Distal we found 151, 178 and 220 fibers per mm^2 respectively. In the lateral segment of the proximal section group II outperformed group I ($p = 0.057$). The other segments were comparable between the two groups ($p > 0.302$).

Discussion

This study examined the re-innervation of sensory nerve fibres in a rat hind paw glabrous skin at 12 weeks postoperatively, after reconstructing a 15 mm gap using a vein-muscle graft and a vein-muscle graft supported with BMSCs. The main findings were: 1) the BMSCs treated vein grafts showed approximately 50% increased re-innervation of PGP 9.5-IR skin; 2) that myelinated fibers had an increased re-innervation of approximately 190% at 12 weeks postoperatively favouring the vein-muscle graft with BMSCs. 3) the vein-muscle graft filled with BMSCs outperformed the vein-muscle graft in regeneration of most of the sensory fibers.

The present study used different immunohistochemical markers (PGP 9.5, CGRP, Substance P, P2X3, NF-200) to distinguish between specific kinds of fibre populations. While these markers are well characterized in previous studies, we cannot exclude the possibility that these markers in the fibres may fall below detection level, although the fibres may still be present. Therefore, we used the PGP 9.5 antibody, which is a pan-neuronal marker and stains all nerve fibres to verify if the peptidergic, non-peptidergic and myelinated fibres are not underestimated.

PGP 9.5

In the current study we found that the vein-muscle graft with BMSCs 3 months post operatively had an average regeneration of 41% of the epidermal PGP-IR fibers compared to control group, defined in our previous study. The vein-muscle graft without the cellular therapy had an average regeneration of 29 % compared to the control group.

Both conduits did allow regeneration, but the autograft as a conduit resulted in a much higher PGP-IR level (i.e. up to 70%). This finding is confirmed by our functional results comparing these conduits.[21] A possible explanation could be the environment within the nerve, which is rich of fascicles and nerve sustainable factors and cytokines.[1] The vein provides a metabolically supportive environment for the regenerating axons. Schwann cells from the proximal part of the lacerated nerve invade the vein and start proliferating, resulting in a comparable regeneration process as in the nerve graft.[11,12] However, clearly, the autograft outperformed our vein-muscle graft with and without BMSCs. The BMSCs did gave a profound stimulation in regeneration, since we found evidence for a regeneration improvement of 12% compared to the vein-muscle graft.

CGRP

Both conduits had a comparable regeneration percentage 12 weeks post reconstruction. CGRP is one of the most abundant fibers produced in both peripheral and central neurons[25] and derived mainly from the cell bodies of motor neurons. In the case of nerve injury, CGRP is synthesized in the anterior horn of the spinal cord and contribute to a successful regeneration. Conversely, CGRP is derived from posterior root ganglion when synthesized in the posterior horn of the spinal cord and may be linked to the transmission of pain.[4]

Interestingly, the amount of regenerated peptidergic sensory skin fibers was similar in the autograft group, as described in our previous study, and in the vein-muscle graft group regardless of the BMSCs filling. This is in somewhat contrast to the functional data (Toe spread, Pinprick, CMAP, GMI), which proved that the autograft group was superior to the vein-muscle graft group.[22] In addition, the total amount of skin nerve fibers (PGP 9.5) showed a different regeneration pattern, favouring the autograft group, which is in line with the functional data presented previously. This suggests that the regenerative rate of sensory CGRP fibers is independent of the conduit used. The addition of BMSCs did not seem to influence the regeneration of the CGRP fibers in this matter. Intra-axonal translation of CGRP is of critical importance for the regeneration of injured nerve fibers, since it regulates Schwann cell proliferation.[15,28]

Substance P

The sensory function of Substance P is of pivotal importance in pain regulation, since it joins the excitatory neurotransmitter glutamate in the communication of painful stimulation to the CNS.[9] Clinicians treating chronic pain often use Capsaicin to reduce the level of Substance P. The pathogenesis of this specific agonist, TRPV1, is explained either by reducing the number of C-fibers or making these fibers more tolerant. In relation to this treatment option, a study from Park et al. showed that naked mole rats that lack cutaneous C fibers reactive to Substance P are insensitive to pain. Administering painful stimulation to the skin provoked no reaction.[23] In this study, group I did not have as much regeneration as compared to group II, the vein-muscle graft supported with BMSCs outperformed the vein-muscle graft by 10%. Based on these findings, non-vital sensory regeneration in group II already reached a higher level compared to group I 3 months operative.

The regeneration of Substance P fibers in the autograft was similar to the number of regenerated CGRP fibers. However, in the present study, we found the amount of regenerated Substance P skin fibers in the vein-muscle graft group -with / without BMSCs lower that the number of CGRP fibers. For that reason we could assume that presence of Schwann cells is of critical importance for the regeneration of Substance P fibers. Which in turn explains the delayed re-innervation compared to the CGRP fibers, since CGRP upregulates Schwann cell proliferation. Schwann cells upregulate p75 receptor and secrete NGF in substantial quantity. These differentiated Schwann cells are essential for the guidance and ultimately for the direction of regenerated axons. However in the vein-muscle graft these guidance Schwann cells are absent, in contrast the autograft group, therefore may be an explanation for the slower regeneration rate of sensory Substance P fibers. The vein-muscle graft supported with BMSCs outperformed the vein-muscle graft without the cellular additive. This beneficial effect can be explained by the theory that BMSCs have the capability to transdifferentiate to Schwann-like cells and therefore may enhance the regeneration of sensory Substance P skin fibers.[3,5,6,16,18-20,26,27,30,31]

This explains the difference in Substance P regeneration comparing the autograft to the vein-muscle grafts.

NF-200

This marker stains the myelinated fibers. As discussed in our previous study these fibers are responsible for signalling stimuli referring acute pain and / or abnormal temperature sensations.[22] The regeneration in group II outperformed the regeneration of the fibers in group I (58 vs 31 percent).

Another finding were the comparable regenerated NF-200 fibers between the autograft and the vein-muscle graft supported with BMSCs. In addition we found a significant lower number of NF-200 fibers in the empty vein-muscle graft. A possible explanation for this difference is the presence of the Schwann cells in the autograft group and the transdifferentiated BMSCs in the vein-muscle graft, which support the regeneration rate. These Schwann cells are of pivotal importance for the myelinisation of the axons. Therefore it is uncertain if the lower number of NF-200 fibers are due to an incomplete myelinisation or to a lower number of myelinated fibers.

P2X3

The P2X3 receptor subunit, a member of the purinergic receptors, is localized in the sensory neurons. A study by Valchanova investigated the role of P2X3 in relation to the information transferred by the specific neurons. The found that in normal rats 40% of the neurons expressed P2X3 and 70 percent of these fibers was reduced during Capsaicin treatment.[29] Another group elucidated that the non-peptidergic fibers are not responsive to NGF, but are responsive to glial cell-line-derived neurotrophic factor (GDNF).[14,24]

In this study the fibers had the lowest regeneration percentage as compared to the other sensory fibers. The vein-muscle graft supported with the BMSCs outperformed the empty vein-muscle graft, resulting in a significant higher regeneration percentage (18 vs 9.5%). This suggests again that the Schwann cells are of critical importance for the regeneration of non peptidergic fibers. However it remains elusive why non-peptidergic have a dramatic slower regeneration rate compared to peptidergic fibers. An explanation might be that secretion of GDNF occurs at much lower concentration than of the peptidergic specific NGF after a peripheral nerve injury.

Taking the findings of these specific markers in consideration we can conclude that peptidergic fibers proliferate more dominant compared to the non-peptidergic fibers. Another finding is the more profound proliferation of the NF-200 stained fibers in the vein-muscle conduit filled with BMSCs.

However, a limitation to this study is the lack of statistical power, resulting in the lack of statistically significant differences between most of the comparisons between the two groups. The small number of animals in this study could explain this. The encouraging results do stimulate larger animal groups for investigating to confirm the results statistically in all stainings.

An important observation is that, in addition to our previous report, the sensory fibers transferring the vital pain and abnormal temperature sensation have the most profound regenerated fibers 12 week after nerve reconstruction.[22] This strengthens our theory that in nerve regeneration these fibers have priority, since they are of essential importance for survival. A next step following this interpretation should be the start of a human study to investigate the re-innervation of neural sensory fibers after trauma.

The beneficial effect of the Bone Marrow Stromal Cells is already investigated in other studies specifically interested in nerve regeneration. And the basic concept of the beneficial effect can be explained by the transdifferentation of the BMSCs into Schwann cell-like phenotypes and (thus) the influence on the production of neural cytokines.[3,5,6,16,18-20,26,27,30,31] The results of this study strengthen the findings as described above, since we found a higher number of fibers in the group that was reconstructed with the vein-muscle graft supported with BMSC. Thus, we found an indication that the BMSCs help regenerate the traumatized nerve better.

This study elucidated the re-innervation of the neural sensory fibers after reconstructing a nerve defect using the vein-muscle graft with and without BMSCs. We found strong indications for the beneficial effect of the supportive cellular therapy (i.e. the BMSCs).

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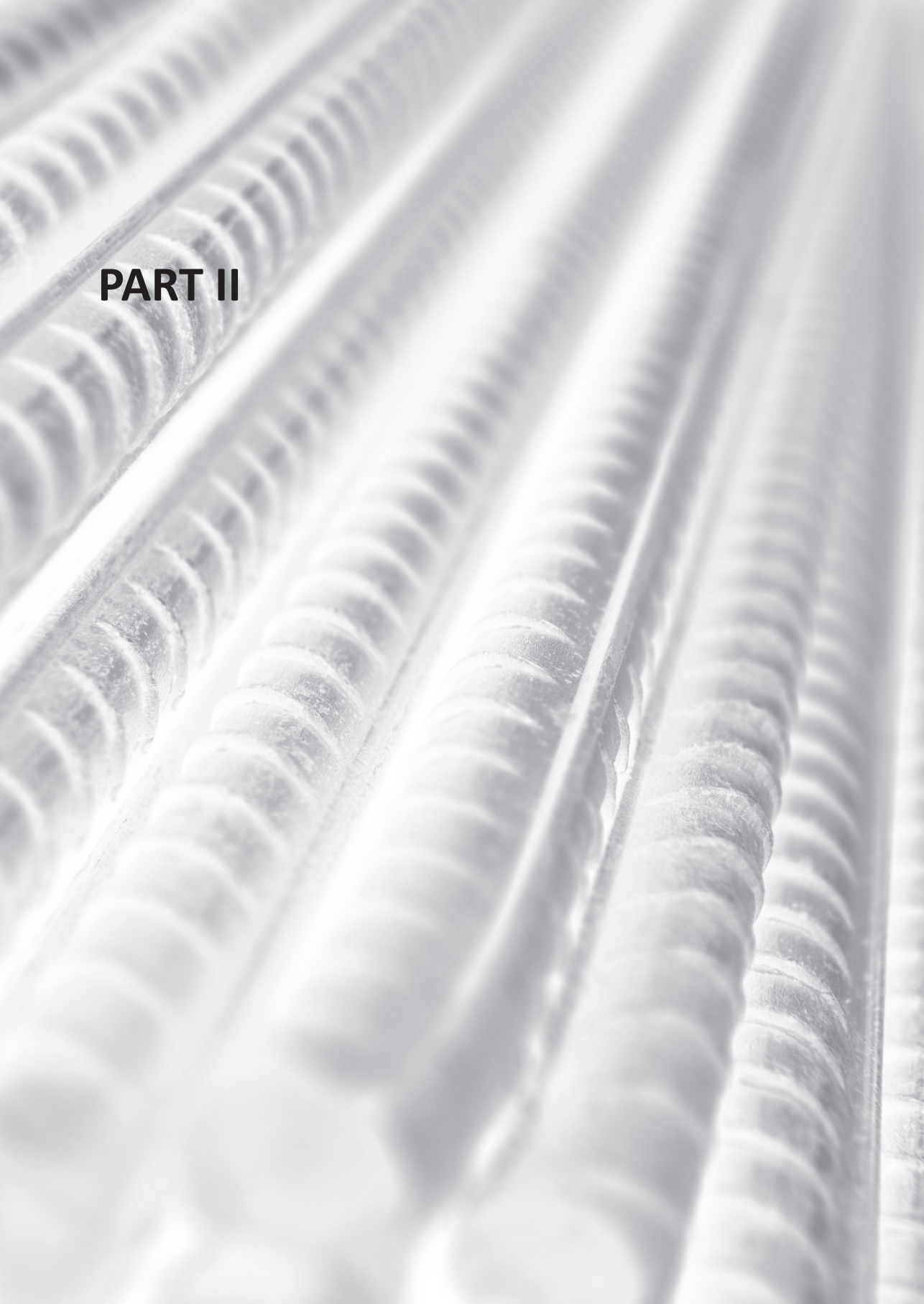

PART II

Chapter 7

Prevalence and Severity of Cold Intolerance in Patients with a Hand Fracture

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Abstract

Introduction

Cold intolerance is a well-known phenomenon that develops in the first months after hand injury and generally does not decrease over time. A high prevalence of cold intolerance after nerve injury and replantation is described in literature. In this study, we evaluate the prevalence and severity of cold intolerance in patients with a hand fracture.

Methods

To evaluate the prevalence and severity of cold intolerance, 129 patients treated for a hand fracture completed the Cold Intolerance Symptom Severity (CISS) questionnaire. This questionnaire assesses the severity of the cold intolerance using 6 questions. The CISS test score ranges between a minimum of 0 and a maximum of 100. The cut-off point for the diagnosis cold intolerance is ≥ 30. Patients with nerve and/or vascular injuries were excluded. The response rate was 59%. The mean CISS score of the patients was 23. Thirty-eight percent (95 confidence interval: 24 – 53%) was diagnosed with pathological cold intolerance.

Conclusion

Cold intolerance is increasingly accepted as a serious problem for patients with trauma in their extremities. We found that 38% of our patients with hand fractures had pathological cold intolerance.

Introduction

Cold intolerance after fracture of the hand is not well documented. Cold intolerance is defined as abnormal pain after exposure to mild or severe cold, with or without discoloration, numbness, weakness, or stiffness of the hand and fingers.[3,7,13,16,18] Symptoms of cold intolerance develop in the first months after injury and generally do not decrease over time.[4,5,10,20,23,25,27] While current treatment includes the use of pharmacological agents, operations of the autonomic nervous system and biofeedback techniques, behavioral change such as a change of occupation is still considered the most effective strategy.

Although cold intolerance is a frequent sequel of upper limb trauma, the prevalence is particularly high after nerve injury. It has been reported that in the majority of peripheral nerve-injured patients, cold intolerance is the most bothersome, prolonged and disabling symptom, affecting both work and leisure activities.[1,8,18,23,25] Ruijs et al. (2007) studied the prevalence of cold intolerance in patients with ulnar or median nerve injuries and found that 56% of the patients with a single nerve injury and 70% with a combined nerve injury suffered pathological cold intolerance according to the CISS questionnaire. In addition, cold intolerance has been documented in other hand disorders such as Raynaud's disease[12,19,21,22], upper extremity trauma,[15] digital replantations[1,2,8,9,11,17,24,25,28] and after raising a radial forearm flap.[29,30]

In most studies, cold intolerance has been evaluated using single 'yes' or 'no' questions.[3-5,16,18] More recent questionnaires such as the Cold Intolerance Severity Scale,[20] Blond McIndoe[10] and the Cold Intolerance Symptom Severity (CISS) questionnaire[10,26,27] contain multiple questions on the impact of cold intolerance in the patients daily life and calculate a sum score, expressing the severity of cold intolerance in more detail than a single yes or no question. These questionnaires do not appear to have been used to study cold intolerance after hand fractures. Therefore, we used the CISS questionnaire to examine the prevalence and severity of cold intolerance in patients with a hand fracture.

Methods

One hundred and twenty-nine patients treated for one or more hand fractures between 2005 and 2006 were asked to participate in this study. Patients had between one and seven fractures of the metacarpals or phalanges. All patients received treatment at the Erasmus MC in Rotterdam, The Netherlands. Exclusion criteria were a nerve or vascular injury, vascular and systemic diseases (such as Raynaud's disease, diabetes, and rheumatoid arthritis), age lower than 18 years, use of vasodilatating medicines, previous hand fractures, nerve lesions, and previous hand surgery. All these criteria were evaluated using a questionnaire with, for their greater part, closed questions and an open question on additional diseases to exclude systemic and vascular diseases. The

fractures types were categorized by their anatomic description. The Medical Ethics Committee of the Erasmus MC approved this study and all participants signed informed consent.

Patients completed the Cold Intolerance Symptom Severity (CISS) questionnaire which was developed by Irwin et al. (1997)and was modified by Ruijs et al. (Table 1).[26,27] The CISS test score ranges between a minimum of 0 and a maximum of 100. The cut-off point for the diagnosis cold intolerance is ≥ 30, based on the 95% confidence interval in normal subjects.[26] We also recorded age, profession, use of medication, hand dominance, side of injury, number of fractures, type of fractures, number of hospital visits, and costs of treatment.

Patients were contacted by a letter that included the CISS questionnaire, a form with the additional questions and consent form. If there was no response, the patient was contacted by telephone and the questionnaire was scored by telephone.

Table 1 | CISS Questionnaire.

Question	Score*
1. Which of the following symptoms of cold intolerance do you experience in your injured limb on exposure to cold?	
Pain, numbness, stiffness, weakness, aching, skin colour change (white/bluish white/ blue)	Not scored
2. How often do you experience these symptoms? (Please tick)	
Continuously / all the time	10
Several times a day	8
Once a day	6
Once a week	4
Once a month or less	2
Never†	0
3. When you develop cold induced symptoms, on your return to a warm environment are the symptoms relieved (please tick):	
Not applicable†	0
Within a few minutes	2
Within 30 minutes	6
After more than 30 minutes	10
4. What do you do to ease or prevent your symptoms occurring? (please tick)	
Take no special action	0
Keep hand in pocket	2
Wear gloves in cold weather	4
Wear gloves all the time	6
Avoid cold weather / stay indoors	8
Other (please specify)	10

Continued Table 1 | CISS Questionnaire

Question	Score*
5. How much does cold bother your injured hand in the following situations (please score 0-10)	
Holding a glass of ice water‡	10
Holding a frozen package from the freezer‡	10
Washing in cold water‡	10
When you get out of a hot bath/shower with air room temperature‡	10
During cold wintry weather	10
6. Please state how each of the following activities have been affected as a consequence of cold induced symptoms in your injured hand and score each (please score 0-4)	
Domestic chores	4
Hobbies and interests	4
Dressing and undressing	4
Tying your shoe laces	4
Your job	4

*In question one, patients are asked to score their symptoms as follows: "Please give each symptom a score between 0 and 10, where 0 = no symptoms at all and 10 = the most severe symptoms you can possibly imagine." However, the scores given in this question do not count towards the final CISS test score.
†These are two questions developed by Ruijs et al. to improve the scoring
‡These are the questions of the McCabe cold sensitivity test.

Data analysis

Descriptive values such as mean, median and standard deviation were calculated with the SPSS 16.0. Independent t-tests were used to test for significant differences between the responders and non-responders. One way analysis of variance (ANOVA) was used to test for effects of fracture type, fracture location and type of work on the CISS score. Pearson correlation was used to test for relations between the CISS score and a number of variables.

Results

The characteristics of responders and non-responders are presented in Table 2. Seventy-six (58.9%) of the 129 patients completed our questionnaire. The average age of responders at the time of injury was 39 years (range 15 – 75); 50 were men. Statistical differences between the responders and non-responders were found for age and gender (Table 2) but not for the other patient or fracture characteristics.

The main cause of the injury was falling (62%), followed by punching (13%), accidents with a machine (10%), entrapment (8%) and other (e.g. sports and unknown, 7%). The metacarpals

were injured in 42% of cases. The mean follow-up time of the responders, defined as the number of days between the injury and completion of the questionnaire, was 669 days (median follow-up time was 674 days).

Table 2 | Characteristics of responders and non-responders.

Characteristic	Responders	Non-responders	p-value
Age Mean (SD)	39 (SD 17)	32 (SD 13)	0.003
Male : female	50 : 26	40 : 13	0.016
Duration since injury (in days) (Median)	674	732	0.346
Fracture Geometry			
Transversal	20	21	
Comminuted	18	9	
Avulsion	12	10	
Oblique	11	6	
Spiral	8	6	0.874
Longitudinal	1	0	
Unknown	6	1	
Fracture Location			
Metacarpal	32	25	
Distal phalanx	20	14	
Proximal phalanx	14	8	0.910
Middle phalanx	5	5	
Unknown	5	1	
Dominant hand affected			
Yes : no : unknown	35 : 35 : 6	26 : 21 : 6	0.685

P-values indicate the differences between the responders and the non-responders.

Mean CISS score for all patients was 27 (range 0 – 75). Twenty-nine (38%; 95% confidence interval 24 – 52%) of the patients had a CISS score larger than 30, indicating pathological cold intolerance. In Figure 1, the distribution of CISS scores can be seen, showing a large group of patients with a low CISS score (< 10), while the other scores between 10 and 80 are more or less equally distributed. Figure 2 shows the CISS scores plotted against age, indicating no relation between the age of the patient and the CISS score.

No correlation was found between fracture type or location and the CISS score. In addition, no correlation was found between time since injury and CISS score, and no significant differences were found between the CISS scores and white collar workers, blue collar workers, and

unemployed. However, significant correlations were found between CISS score and the number of fractures, the number of hospital visits and the number of rehabilitation visits (see Table 3), indicating that more severe or complex injuries lead to higher CISS scores.

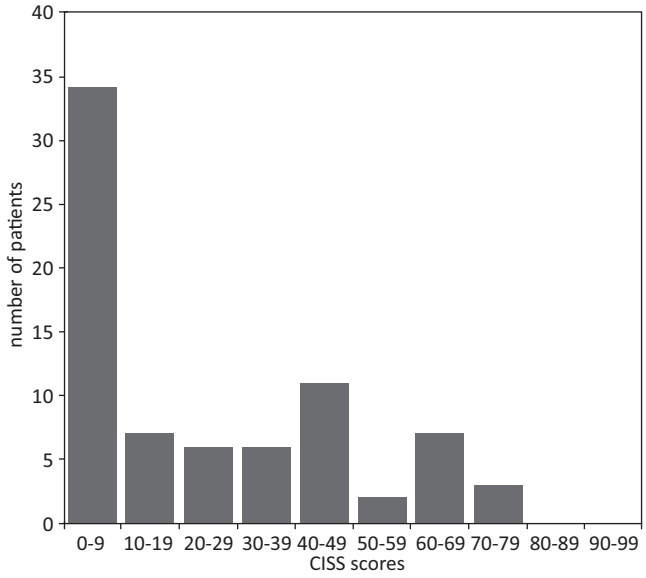

Figure 1 | Histogram indicating the number of patients with scores in the different CISS-score ranges.

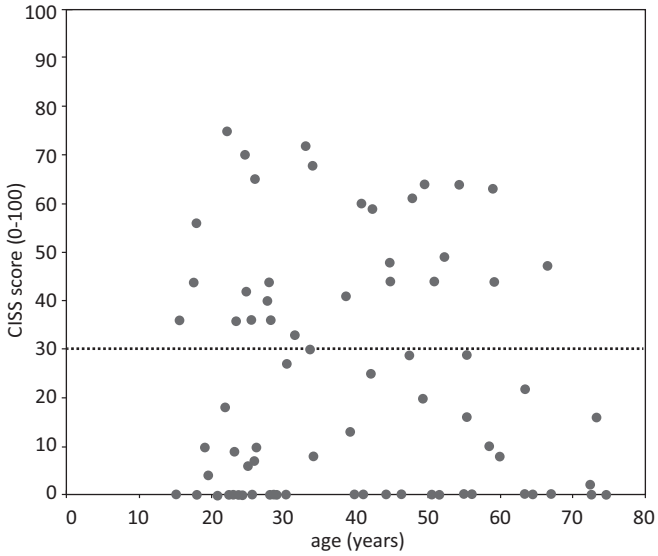

Figure 2 | Scatter plot of age versus CISS score for all patients. The dotted line indicates the cut-off value for pathological cold intolerance.

Table 3 | Pearson correlations between CISS score and variables indicating the severity of the fracture (number of fractures in the same hand, number of visits and absence of work) and CISS scores for the different fracture types, fracture locations and work type.

Variables	Correlation with CISS	P-value
Number of fractures	0.237	0.039
Number of hospital visits	0.250	0.036
Absence at work due rehabilitation	0.201	0.175
Number of rehabilitation visits	0.263	0.027
	CISS mean (SD)	
Fracture type		
Avulsion (N=12)	32.7 (29.6)	
Comminuted (18)	22.8 (25.1)	
Longitudinal (1)	0	
Oblique (11)	19.6 (25.4)	0.706
Spiral (8)	16.1 (17.0)	
Transverse (20)	24.8 (23.0)	
Unknown (6)	19.5 (24.2)	
Fracture location		
Metacarpal (32)	20.5 (22.6)	
Proximal phalanx (14)	26.4 (22.1)	
Middle phalanx (5)	6.6 (14.8)	0.699
Distal phalanx (20)	26.0 (28.2)	
Unknown (5)	19.0 (27.0)	
Work type		
Blue collar (24)	27.3 (26.7)	
White collar (18)	24.2 (23.0)	0.309
Unemployed (26)	16.8 (23. 4)	

The p-values indicate the significance of the correlation coefficients or the significance of the overall effects of fracture type, fracture location and work type on the CISS score.

Discussion

Cold intolerance is increasingly accepted as a serious problem for patients with trauma in their extremities. The prevalence and severity of cold intolerance is unknown. We found that 38% of our patients with hand fractures had pathological cold intolerance.

There are a number of limitations of this study. Firstly, it should be noted that the CISS questionnaire consists of questions that describe only the symptoms of cold intolerance (see

Table 1). Since the etiology of cold intolerance is unknown, it is not clear whether the score relates to disease severity or progression. However, we believe that the questionnaire indicates the severity of the complaints of cold intolerance (the higher the score, the more severe the complaints). A second limitation is the low response rate of 59%. A low response rate is commonly seen in these populations.[6] In the unlikely case that none of the non-responders suffered cold intolerance, our study would indicate a minimum rate of 22%. It should also be noted that the follow-up since injury is relatively long. Although most studies related to other injuries in the upper limb indicate that cold intolerance develops in the first few months after trauma and generally does not decrease significantly over time.[4,5,10,20,23,25,27]

It should be noted that we do not have data on the presence of cold intolerance before injury. However, the cut-off value of 30 for the CISS questionnaire is based on an evaluation of the range of values found in healthy subjects (95% confidence interval). Therefore, on statistical grounds, it could be expected that approximately 5% of the subjects had a CISS score larger than 30 before the trauma.

The prevalence in our study on patients with hand fractures (38%) is lower than those found after single nerve injury (56%) and combined nerve injury (70%) (Ruijs et al. 2007). It is also lower than the 83% prevalence in replantation patients.[14] These higher rates may be explained by the more extensive nerve injury in those pathologies as well as the vascular damage in replantation cases. However, at present, the relation between cold intolerance and nerve damage or vascular damage remains unclear.

The pathophysiology and predictors of cold intolerance remain controversial. Although the symptoms have been associated with reduction of blood flow in response to cold, the reason for the decreased flow is unclear. For example, the assumption that cold intolerance after finger replantation is caused by macrovascular problems or capillary microcirculatory failure seems incorrect based on the results of Klein-Weigel et al., (2007) who demonstrated that diminished skin vessel density reduced thermal modulation capacities in the fingertips of cold-sensitive replanted digits.

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Chapter 8

Re-warming Patterns in Hand Fracture Patients with and without Cold Intolerance

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Abstract

Introduction

It is often assumed that cold intolerance is associated with abnormalities in the skin temperature due to changes in the blood flow of the hands. In this prognostic study, we determined whether patients with and without cold intolerance after a hand fracture or healthy controls have a diminished re-warming after a cold stimulus.

Methods

The severity of cold intolerance was evaluated using the Cold Intolerance Symptom Severity (CISS) questionnaire. To determine if abnormal re-warming plays a major role in the underlying pathophysiology of cold intolerance, a cold-stress test was applied 30 months after the patients recovered from a hand fracture. Temperature during the re-warming phase was measured using videothermography.

Results

Thirteen control subjects and 18 patients participated. Control subjects did not report any symptoms of cold intolerance (CISS score 0) and no loss of sensibility was measured. Mean CISS score of all patients was 27.8; nine patients scored above the cut-off value for normal cold intolerance.

No significant differences were found in the re-warming patterns between 1) the affected and non-affected hand of the patients, 2) the dominant and non-dominant hand of the control subjects, and 3) the patients and controls.

Conclusions

The results of this study revealed no relation between the severity of cold intolerance and re-warming patterns after cold stress testing. This may suggest that temperature regulation of the hands in this patient group may not be responsible for the symptoms of cold intolerance.

Introduction

Cold intolerance is a disabling symptom that is defined as abnormal pain following exposure to mild cold that often occurs after trauma.[4,10,16,18,21,26,31,33] In a study on patients with a hand fracture, the prevalence of cold intolerance was found to be 38%. In addition, cold intolerance is also commonly reported by patients with Raynaud's disease,[12,22,24,25] upper extremity trauma, digital replantations,[1,2,6,7,11,15,20,28,29,35] the hand and arm vibration syndrome and after a radial forearm flap.[36,3]. In many studies, the presence of cold intolerance is determined using a single yes/no question. Alternatively, the severity of cold intolerance can be determined in more detail using the Cold Intolerance Symptom Severity (CISS) questionnaire, developed by Irwin et al.[10] and adjusted by Ruijs et al.[32]

The pathophysiology of cold intolerance is still unclear.[4] Nevertheless, it is assumed that cold intolerance is related to vascular dysfunction.[11] In a healthy person, after extensive cooling of the extremity, the thermoregulatory system increases the blood flow to the extremity to counteract the decrease in hand temperature and prevent pain and/or frostbite.[17] One of the substances involved in this thermoregulatory process is Nitric Oxide (NO), having a direct effect on the smooth muscle activity.[13] In this process sympathetic fibers play a key role, providing control of adrenergic neurotransmitters by peripheral nerves and vasculature. As described by Ruch et al., the vascular flow can be separated in nutritional flow and thermoregulatory flow, from which only thermoregulatory flow determines the digital temperatures.[30] Thermoregulation of the skin in part relies on input from temperature sensors and nerves located in the peripheral location. There are also alternative hypotheses on the pathophysiology of cold intolerance. For example, experimental studies are performed on Transit Receptor Potential (TRP) channels, which are poly model receptors that respond to temperature, pain and pressure. Recent studies suggest that an up-regulation of TRP channels after peripheral nerve trauma may also explain cold intolerance.[3,14]

When vascular dysfunction exists, abnormal re-warming will be one of the results. One way to elucidate abnormal re-warming is to perform a cold-stress test.[19] In this test, both hands are cooled in water and the re-warming pattern is recorded. Measuring temperature during re-warming after a cold stress test in cold intolerant patients has not been extensively studied. In cold intolerant patients with measureable peripheral nerve damage, a diminished thermoregulation was observed.[34] Until now, it is still unclear if, and to what extent, cold intolerance is indeed related to abnormal re-warming and not, for example, to neurological changes or interaction between both.[5,6]

The aim of this study was to investigate the correlation between cold intolerance and abnormal re-warming after a cold stress test. In order to elucidate this relation we analyzed re-warming patterns for possible thermoregulatory dysfunction in control subjects and in hand fracture patients with and without cold intolerance. Additionally, we wanted to study the correlation between the score of the CISS questionnaire and the re-warming pattern.

Methods

Subjects

We invited patients form an earlier study on the incidence and severity of cold intolerance after a hand fracture to participate.[27] All subjects completed the cold intolerance symptom severity (CISS) questionnaire,[10,23,32] which has a minimum score of 0 and a maximum score of 100. Based on the range of normative values, a patient with a score of 30 or higher has abnormal cold tolerance'.[32] Thirteen control subjects (five men and eight women) participated in this study. Average age was 38 year (range from 22 to 58 years), one subject was left hand dominant, twelve right hand dominant (see Table 1). Three subjects smoked 2 – 10 cigarettes a day (average 6/ day), but were asked to refrain from smoking starting the evening before testing. In addition, eighteen hand fracture patients (nine women and nine men) participated in this study. The mean time after fracture was 135 weeks with a range of nineteen weeks. All patients had one or more fractures in their metacarpal and/or phalangeal bone(s). Average age at injury was 45 years (range from 21 to 75 years) and 17 patients were right hand dominant. Five subjects smoked between 3 – 40 cigarettes a day, these patients were asked to refrain from smoking starting the evening before testing. This study was approved by the Medical Ethical Committee of our hospital and all patients and control subjects gave their written informed consent (MEC 2008-088). No external funding was used to fulfill this study.

Measurements

During the patient's and control subjects acclimatization for 15 minutes in a room with a mean temperature of 23°C (range: 22.5 to 23.5) and a relative humidity of 50% (range: 45 to 55), the CISS question list was completed. Hereafter the possible loss of sensibility in the patients hands was measured using Semmes Weinstein monofilaments (North Coast Medical Inc, Morgan Hill, CA) and outcome was classified according to Imai et al.[9] On each hand, 5 filaments ranging from 2.83 till 6.65 gram were used. Filaments were placed for 1 to 2 seconds on the fingers and we asked the patient to indicate without looking which finger was touched.

Cooling and Temperature Recording

For cooling, both hands were immersed up to the ulnar styloids for 90seconds in a water bath with a constant temperature of 13°C. 'Traynor and McDermid showed that immersion of hands in 13 degrees C water for 90 seconds is sufficient to initiate active rewarming [38].

After removing the hands from the water, the examiner carefully and quickly blotted the hands dry using a cotton towel. Temperature measurements of the affected and unaffected hand were made with the hands placed on a clean towel. The delay between the removal of the hands from the water and the start of the thermographic recording was less than 10 seconds.

Skin temperature from the dorsal side of both hands was registered with a computer-assisted videothermography (ThermaCAM SC2000, Flir Systems, Berchem, Belgium) which calibrated automatically. The measurement protocol was previously used by Ruijs et al.[34] The thermal sensitivity was 0.05°C at 30°C, the spectral range 7.5 to 13.0 µm, and the built-in digital video has 320x240 pixels (total 76.800 pixels). Data were obtained through a high-speed (1Hz) data acquisition system (ThermaCAM Researcher 2001 HS, Berchem, Belgium) connected to a desktop-PC. The distance between the camera and the hands was 70cm; the pixel size of the temperature recordings was 0.8 x 0.8mm. Temperature was measured until both hands had re-warmed to a stable temperature of 34°C with a minimum duration of ten minutes and a maximum of thirty minutes.

Table 1 | Characteristics of study population.

Characteristic	**Control group N=13 (Sd)**	**Patient Group N=18 (Sd)**
Gender (M: F)	8: 5	9: 9
Age (years)	38 (22 – 58)	45 (21 – 75)
BMI (kg/m²)	25.0 (21 – 28)	24.0 (18 – 35)
CISS-Score	0	27.8 (0 – 75)
Smokers (cigarettes a day)	3 (2 – 10)	6 (2-40)
Fracture Geometry	**(%)**	**N (%)**
Transverse		7 (39)
Spiral		4 (22)
Avulsion		2 (11)
Comminuted		2 (11)
Oblique		2 (11)
Longitudinal		1 (6)
Semmes Weinstein		
A (Normal) (2.83)	13 (100)	15 (83)
B (Residual texture) (3.61)		1 (6)
C (Residual protective sensation) (4.31)		2 (11)
D (Loss off protective sensation) (4.56)		0
E (Residual deep pressure) (6.65)		0

Statistical Methods

Temperature data were exported to text files using FLIR Thermacam Researcher Pro (version 2001-HS). For each digit, temperature was measured in the middle of the nail bed placing a circle with an average diameter of 8 mm (see Figure 1). Figure 2 shows a thermographic image of an example of normal active re-warming after applying the cold stress test. The active re-warming starts distal in the fingertips and proceeds in proximal direction. Data was plotted in a time-temperature curve for further analysis of the re-warming (Figures 3 and 4).

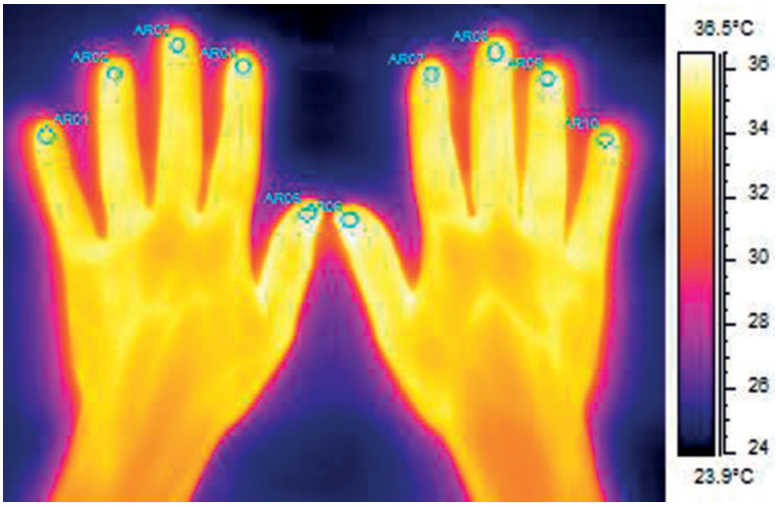

Figure 1 | Videothermographic image of both hands.

The temperature readings of each digit, stored in a text file, were imported to Matlab® (version 7.1) to be analyzed. In Matlab® the calibration points of the camera were removed and a second order low pass Butterworth filter with a cut-off frequency of 0.015Hz was applied. An algorithm was developed in Matlab® to automatically determine the start and stop events of the active re-warming in each curve (Figures 3 and 4). We defined the active re-warming as the time between the maximum increase and decrease of the second temperature derivative[31]. Since the automatically selected points were not always accurate because of, for example, irregularities due to calibrations of the camera or movement of the fingers, all selected events were visually verified and, when needed, manually corrected.

To quantify the re-warming, the duration of re-warming (between the start and stop events) and the area under the curve (Q) between these events was calculated (Figures 3 and 4). This Q-value was divided by time to calculate the average re-warming per second (Q/s), indicating the re-warming velocity of the digit.

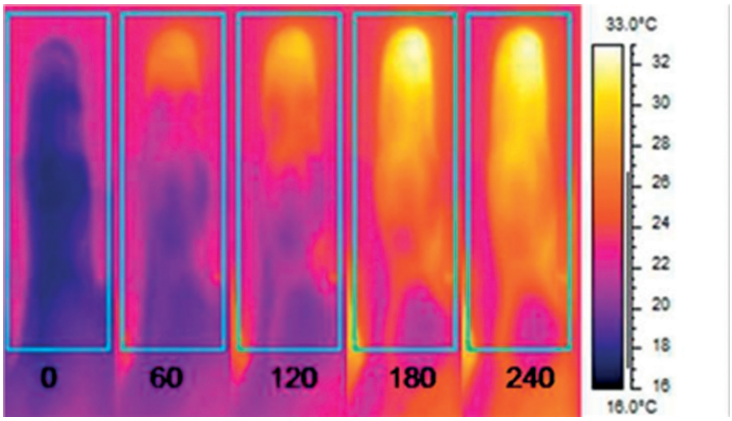

Figure 2 | Consecutive thermographs during the active re-warming phase of a control subject. Active re-warming starts in the fingertips and proceeds in proximally.

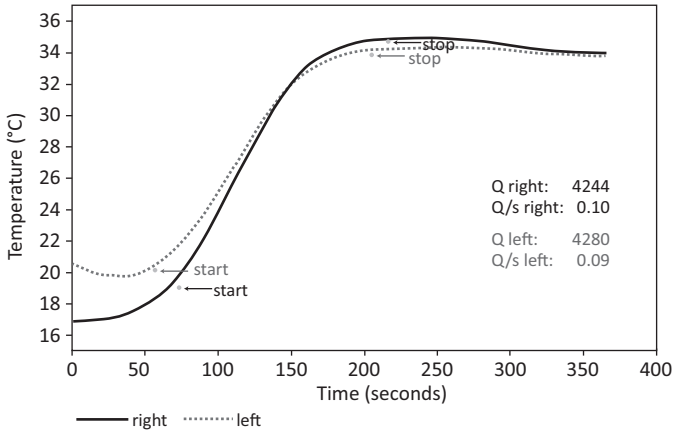

Figure 3 | Re-warming curve single digit of a control subject. Q value as indication of efficiency of heating in a digit, Q/s average re-warming per second. The x-axis indicates the time in seconds, the y-axis the temperature in degrees Celsius.

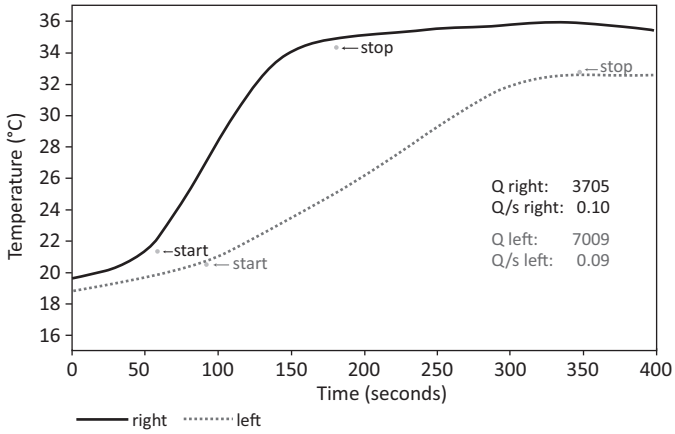

Figure 4 | Re-warming curve of a single digit in a patient with a fracture in the left hand and no injury in the right hand. Q value as indication of efficiency of heating in a digit, Q/s average re-warming per second. The x-axis indicates the time in seconds, the y-axis the temperature in degrees Celsius.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0. The Q and Q/s value of the affected hands were compared with the opposite digits, and the Q-value between patients and control subjects where compared.

The Pearson correlation was used to describe the relation between the re-warming time, indication of energy added (Q), re-warming velocity (Q/s) of the digits and the CISS score.

Results

Mean CISS score of the control subjects was 0, indicating that all of the control subjects had normal cold tolerance. The mean CISS score of the patients was 27.8 (range: 0 – 75). Nine of the 18 patients had abnormal cold intolerance based on the CISS Score.

The Semmes Weinstein test did not show abnormal sensitivity in any of the control subjects. Three of the eighteen patients had a decreased sensibility in the fractured finger as assessed by using the Semmes Weinstein test (Table 1).

Cooling the hands in water of 13°C was not experienced as unbearable pain. Figure 3 and 4 show typical examples of a normal and an abnormal re-warming curve. The normal active re-

warming of the control subject in Figure 3 starts approximately 50 – 80 seconds after removing the hands from the cold water. This active re-warming continues until the hands reach an average temperature of 34 degrees Celsius. Figure 4 illustrates an abnormal re-warming pattern in a patient with a hand fracture and with a high level of cold intolerance (CISS = 61), affected hand (left) and unaffected contralateral (right) are plotted. The re-warming curve illustrates a noteworthy reduction in the rate of active re-warming in the affected hand compared to the non affected hand.

Although re-warming was abnormal in a number of hand fracture patients, we did not find systematic and meaningful differences between groups, when comparing the duration of re-warming, Q and Q/s. Additionally, no differences between the affected and unaffected hand of the patients, between the dominant and non dominant hand of the controls, and between the affected hand of patients and the hands of the control subjects were found for the determined variables. Consequently, no significant correlations between the variables describing the re-warming patterns (duration, Q and Q/s) for the patients and the CISS scores were found. We also compared only the patients with cold intolerance to the healthy subjects and again found no significantly different re-warming between both groups (for all determined variables $p \geq 0.48$).

Discussion

The aim of this study was to determine if abnormal re-warming after a cold stress test could be related to cold intolerance. In this study, we found no difference in re-warming patterns between the fractured hand and the non-affected hand of patients with and without cold intolerance or between patients and controls. Furthermore, no correlations for CISS score and the re-warming variables (duration, Q and Q/s) were found, indicating that the severity of cold intolerance in hand fracture patients is not related to the re-warming pattern. These findings indicate that thermoregulatory dysfunction may not be cause of the experienced cold intolerance in these patients.

Disturbed thermoregulation as a result of pathology within the extremities has been reported in a number of studies. For example, the study of Jayanetti et al. revealed abnormal re-warming curves in children with Raynaud's phenomenon.[12] In addition, Suominen et al. reported that a radial forearm flap affects the thermoregulatory system of the donor hand resulting in abnormal re-warming.[36] Greenstein et al. reported that subjects with Raynaud's phenomenon have an impaired thermoregulation which he suggests to explain the cold sensitivity in this patient group.[8]

In this study we found abnormal re-warming in both healthy subjects and patients. Secondly, we found no relation between re-warming patterns after cold stress testing in patients with cold intolerance recovering from a hand fracture and subjective cold intolerance.

This finding may seem in contrast with the findings from Ruijs et al.,[34] who reported abnormal re-warming in peripheral nerve injury patients with cold intolerance. However, an important difference between both studies was that Ruijs et al. studied nerve injury patients after trauma whereas in the present study patients with nerve injuries were excluded, although damage to the more distal and smaller nerve branches as a result of the trauma cannot be excluded. Our finding of an absent relation between re-warming after cold stress testing and subjective scoring cold intolerance is in line with a study by Traynor and MacDermid on healthy subjects.[38] In addition, the study of Suominen et al., also concluded that abnormal re-warming in patients with a radial forearm flap did not correlate with subjective cold intolerance.[36]

The lack of relation between re-warming and cold intolerance may have important implications for understanding cold intolerance, since it may indicate that disrupted re-warming may not be responsible for the symptoms of cold intolerance. It also put the findings of Ruijs et al. of a relation between abnormal re-warming and subjective cold intolerance in nerve injury patients in a different perspective, indicating that these factors may both be symptoms of nerve injury but without cold intolerance being the direct result of disrupted re-warming. In addition, it can be concluded that direct vascular pathophysiology in, for example Raynaud phenomenon, could cause cold intolerance.

Although this study indicates that cold intolerance is not related to abnormal re-warming and that other mechanisms have to be present, quantifying re-warming may still be important since patients may have more complaints if they have cold intolerance in combination with disrupted re-warming. Future studies are needed to investigate these relations in more detail.

The present study has a number of limitations. A first limitation is that we found our algorithm to determine the start and stop of the active re-warming not working successfully in a number of recordings. Therefore, in the cases where the algorithm failed, we manually selected start and stop of the active re-warming. Although two researchers independently selected both points, we especially encountered difficulties when defining these events in the situation of abnormal re-warming. A second limitation was that the number of patients in this study was relatively small (n = 18), leading to a relatively small statistical power. However, in addition to finding no significant differences, we also found no trends of differences between patients and controls and between involved and uninvolved hand, as well as no trends of significance in the correlation between CISS scores and re-warming data, indicating that clinical relevant effects are not present. A third limitation could be the absence of a second cold stress test evaluation to increase the reliability of the results. On the other hand, Traynor and MacDermid concluded that the results between two cold stress test evaluations in the same group were comparable.[38] Another study of Ruijs et al. with a almost identical measurement design concluded that the test retest reliability for the Q value, maximum slope (Q/s) and the cold stress testing was good.[34] Hence, an additional evaluation in both patient and control groups seems unnecessary.

This study revealed that there is no direct relation between cold intolerance in patients with a hand fracture and abnormal re-warming after cold stress testing. As a result, alternative hypotheses are needed to understand cold intolerance, such as mechanisms based on changes in the properties of the temperature-sensory system or other neurological mechanisms. Future studies should include a longitudinal follow-up on a larger and similar patient group starting earlier after injury, this may provide more detailed information about the developments and the courses of the cold intolerance.

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Chapter 9

Discussion

General Discussion and Future Perspectives

In the treatment of peripheral nerve injuries it is recommended to think T3: Time, Tension and Total coaptation.

Delayed nerve reconstruction often results in less optimal regeneration and to surgically restore the nerve should therefore be executed as soon as possible. Dvali and Mackinnon recommended to perform this primary surgery within 72 hours up to 7 days post trauma.[27]

Secondly, it is important to reconstruct the nerve without tension. Nerve tension can result in ischemia as a consequence of a decreased vascular flow. To achieve a suitable restoration of nerve anatomy and therefore a functional vascular system, a tensionless connection should be sought. Therefore, at the trauma site a graft should be placed between the proximal and distal stump to reconnect the nerve when both stumps can't be connected without tension. This might result in extra scar formation since a second coaptation is necessary, however this limitation is less traumatizing than tension on the coaptated nerve.[25,66]

Another key-issue in nerve reconstruction is to strive for total coaptation. If the proximal and distal stumps are nicely positioned and the properties of the nerve are respected, the axons can traverse the surgical coaptation without restriction.[21,27,64]

Unsuccessful nerve regeneration or problems related to nerve injury can be invalidating, since sensory and/or motor loss, scarring, neuroma formation, cold intolerance and neuropathic pain all fit within the scope of these related problems.

Part I of this thesis focuses on developing a new way of nerve reconstruction, combining cellular therapy, Bone Marrow Stromal Cells (BMSCs), with respected grafting techniques (i.e. the vein graft and the vein muscle graft). Also, two new evaluation techniques are introduced. The second part of this thesis describes a vast occurring problem in nerve injury: cold intolerance. We investigated the possible relation between cold intolerance and abnormal re-warming patterns in patients with a hand fracture to explore if the pathophysiology of cold intolerance is either vascular or neurogenic derived.

Part I

Vein Graft and Bone Marrow Stromal Cells

Despite decades of clinical experience and numerous experimental studies, still, the autologous nerve graft is considered the golden standard. However, donor site morbidity inevitably occurs when using an autologous nerve graft and another problem related to the nerve graft is the limited length available. Therefore, the search for alternative grafting materials has been ongoing and both natural and artificial materials have been used.[11,63] In the early nineties the possibility of a vein as suitable grafting material was introduced. Vein grafts are abundantly

available in a wide variety of sizes and lengths, have less donor morbidity than autografts, and provide a metabolically supportive environment for the regenerating axons. Furthermore the wall of the vein acts as a barrier against scar ingrowth.[69] Other experimental studies have clinically, electrophysiologically and histologically demonstrated that the vein graft is capable of axon regeneration.[5,12,41,55] Despite the less successful regenerative potential compared to the autograft, the vein is still often used in clinical practice. In summary, the use of vein grafts is supported because of the availability in a wide variety of sizes and lengths; they have less donor site morbidity than autografts, and provide a metabolically supportive environment for the regenerating axons.[1,3,5,6,12-14,34,35,39,41,45,49,55,69]

For that reason we thought the vein would be the ideal conduit for testing the beneficial effect of the BMSCs. The use of BMSCs as a luminal additive have already been published in several articles and the beneficial effect is explained by their capability to trans-differentiate and/or their capability to produce cytokines and growth factors.[10,14,15,19,20,47,50,53,67,68]

We were interested to test the effect of BMSCs on nerve regeneration after grafting a nerve defect using a vein and found that injecting the veins with these BMSCs result in a significant improvement in nerve regeneration.

Prior to our study, two studies investigated the nerve regeneration after grafting a nerve defect using a 8mm and 16mm vein graft filled with BMSCs and also found a beneficial effect in regeneration after filling the vein with BMSCs.[14,30]

Our study not only confirmed these results, but also showed that these effects were present after grafting a longer defect (i.e. 20mm). We evaluated regeneration on the basis of functional tests without additional histological analysis.

The obvious next step was to investigate the combination of both the functional and histological evaluation of nerve regeneration. In a new experiment this was performed and compared to an autologous nerve graft. Battiston et al emphasized that the vein graft has a tendency to collapse and a piece of muscle should be placed inside the vein to create a sustainable nerve conduit.[3,4,33] However they did not use BMSCs. Based on the results of Battiston et al, we used a vein graft filled with muscle and subsequently added BMSCs.

The findings of our study showed that the autologous nerve graft had significantly better regeneration compared to the vein-muscle graft and the vein-muscle graft filled with BMSCs by both functional and histological parameters. The addition, however, of BMSCs to the vein-muscle graft had a beneficial effect when compared with the vein and muscle graft only.

Taking the findings of these two studies into consideration, we conclude that the BMSCs have indeed a beneficial effect on nerve regeneration and that the addition of a small piece of muscle, as filler of the vein graft to prevent collapsing of the graft, does not lead to a better outcome. Another finding during these studies is the importance of recording either CMAPs or another electrophysiological test. These tests are an objective trustworthy parameter to have an indication for regeneration. A concern regarding these tests is that the invasive procedure

necessary to record these values requires sacrificing the animal afterwards. It is impossible to follow peripheral nerve regeneration in the same animal longitudinally in time. A solution could be found in the development of a minimally invasive method.

A Minimally Invasive Method for Measuring CMAPs

Parallel to the studies afore mentioned, we investigated a new approach to measure the compound muscle action potentials using a minimally invasive technique. The standard technique of retrieving these potentials is to position two needle electrodes near the sciatic nerve. However, an invasive procedure is necessary to visualize the nerve and place the electrodes.[22,23,43] Since the CMAPs are a reliable method of evaluating nerve regeneration, we wanted to modify this technique to be able to measure regeneration over time in the same animal without the need of exposing the animal to an invasive procedure. We used ultrasound equipment to visualize the sciatic nerve and tested if live ultrasound images allowed accurate placement of the needle electrode near the nerve. We found this modified technique highly accurate and reliable and resulted in comparable results with the standard technique. Not only yielded this study good results, but we have also experienced that the ultrasound technique is easy to learn as confirmed by high intraobserver and interobserver correlations. Moreover, this technique requires only the use of a clinically available ultrasound machine with a 20 MHz probe in addition to the equipment necessary for the conventional method. Another major advantage with the use of this technique is that regeneration can be measured longitudinally in one and the same animal and the number of animals needed for these types of experiments can be reduced.

Sensory Nerve Fibers in the Rat Foot Sole after Reconstruction

Next we studied the regeneration of the different subgroups of sensory nerve fibers in the footpath of the rat hind limb 12 weeks after reconstructing a nerve defect with an autologous nerve graft, a vein-muscle graft and a vein-muscle graft filled with BMSCs. The different sensory fibers innervating the skin can be subdivided in two major classes: peptidergic and non-peptidergic fibers. The peptidergic fibers contain the neuropeptides calcitonin gene-related peptide (CGRP), substance P and myelinated fibers. These fibers, especially the myelinated fibers, are associated with signalling acute pain and the afferent portion for the reaction of noxious stimuli and/or abnormal temperature sensations. The non-peptidergic fibers contain P2X purinoceptor 3 (P2X3) and are associated with the C-fibers as the afferent portion for chronic pain.

Our results show re-innervation 12 weeks postoperative in approximately 70% of the peptidergic fibers in the sole of the foot in the autograft reconstruction group. The non-peptidergic fibers only re-innervated the skin up to 35%. Incomplete regeneration is further confirmed by the Von Frey test, which demonstrated a higher mechanical withdrawal threshold in the autograft reconstruction group compared to the control group. This finding illustrates the difference in regeneration progress between the peptidergic and the non-peptidergic fibers.

We could therefore conclude that in nerve regeneration the fibers responsible for acute pain regenerate faster compared to the other fibers.

A possible explanation for this faster regeneration could be that the peptidergic fibers have a better chemotactic signal to NGF. NGF, together with the neurotrophins BDNF, NT-3, NT-4/5 and NT-6, is produced by Schwann Cells. The Schwann cells proliferate as soon as their contact with the axons has been disrupted and this proliferation results into the formation of the bands of Bunger. Hence a high intensity of NGF can be found distal to the nerve lesion and thus stimulate the peptidergic fibers.[32,36]

This explanation can be translated to the next project. The re-innervation of the subgroups of sensory nerve skin fibers after reconstructing a nerve defect with a vein-muscle graft and a vein-muscle graft supported with BMSCs was visualized. Although the results were not on par with the re-innervation of the autograft treated group, we did find a similar regeneration pattern. Faster regeneration of the peptidergic fibers as compared to the non-peptidergic fibers was a profound similarity. This finding is supported by the functional and histological findings in our previous study (i.e. chapter 4).

Three months postoperative both groups did not suffice enough re-innervation to compete with the autograft reconstruction. The group reconstructed with the vein-muscle graft supported with BMSCs, however, outperformed the vein-muscle graft treated group. The beneficial effect of the BMSCs could be explained by their capability to transdifferentiate into Schwann cells or producing cytokines such as NGF. As a result the peptidergic fibers will be stimulated to re-innervate faster.

Visualizing the peptidergic and non-peptidergic fibers has only been used in experiments investigating neuropathic pain.[17,24,26,29,44,52] We report the analysis of these fibers in the footpath of the rat hind limb following nerve reconstruction. To have an understanding of the regeneration of the sensory subgroups in rodent skin can result in a better understanding of the neural regeneration after trauma in humans. The functional limitations, the neuropathic pain and cold intolerance are possibly all complications as a result of an unsuccessful regeneration.
In the second part of this thesis we have focused on one aspect of unsuccessful regeneration (i.e. cold intolerance).

Part II

Cold Intolerance

Cold intolerance is defined as abnormal pain after exposure to mild or severe cold, with or without discoloration, numbness, weakness, or stiffness of the hand and fingers.[7,28,40,42,46] Symptoms of cold intolerance develop in the first months after injury and generally do not decrease over time.[16,18,37,48,51,54,62] Current treatment options include the use of pharmacological agents, surgery

of the autonomic nervous system and biofeedback techniques. Unfortunately behavioral change such as a change of occupation is still considered the most effective strategy.

Although no objective test is available, different questionnaires have been developed to evaluate presence of cold intolerance accurately.[8,9]

Cold intolerance is often seen after reconstruction of upper limb trauma, the prevalence is particularly high after nerve injury. Literature reports that in the majority of peripheral nerve-injured patients, cold intolerance is the most bothersome, prolonged and disabling symptom, affecting both work and leisure activities.[2,31,46,51,54] Ruijs et al. (2007) studied the prevalence of cold intolerance in patients with ulnar or median nerve injuries and found that 56% of the patients with a single nerve injury and 70% with a combined nerve injury suffered pathological cold intolerance according to the Cold Intolerance Symptom Severity (CISS) questionnaire.[58,60] This questionnaire includes 6 questions about the impact of the cold intolerance in daily life. The CISS test score ranges between a minimum of 0 and a maximum of 100. The cut-off point for the diagnosis cold intolerance is ≥ 30.[59]

We decided to investigate the prevalence in patients who suffered from a hand fracture using the CISS questionnaire. Our results showed a prevalence of 38%. The high prevalence encouraged us to initiate a study investigating if cold intolerance could be explained by an abnormal re-warming pattern or that another explanation should be sought. We measured re-warming patterns after cooling of the hands to elucidate if thermoregulatory dysfunction plays a major role in the underlying pathophysiology of cold intolerance in patients with a hand fracture. Vascular flow can be subdivided in a nutritional flow (20%) and a thermoregulatory flow (80%).[56] One of the substances involved in the regulation of the thermoregulatory flow is Nitric Oxide (NO).[38] Sympathetic fibers play a key role in regulating this flow, providing control of adrenergic neurotransmitters by peripheral nerves and vasculature. Nerve damage and thus the denervation of all sensory fibers in the skin, could result in a dysfunctional thermoregulatory flow. This relation is confirmed by the findings of Ruijs et al.[57,61] In our study re-warming patterns after cooling were measured in both healthy subjects and patients who suffered a hand fracture. We found both abnormal and normal re-warming patterns in both groups.[65] Cold intolerance could -thus- not be explained by an abnormal re-warming. Hence a dysfunctional regulation for the thermoregulatory flow is not assumable and the sympathetic fibers are likely to have a normal function. If the nerve function is considered normal, the cause for cold intolerance after a hand fracture should be sought elsewhere.

In contrast to the studies performed by Ruijs et al., a pilot study (performed in our department) investigated the re-warming in rats with a nerve transection and described no abnormal re-warming as compared to the sham treated and the naïve animals.

In perspective, one should be careful to explain the cold intolerance using a specific dysfunctional system (i.e. vascular or neurogenic). However, it is assumable that after nerve transection, the peptidergic (or vital) skin fibers cannot communicate with the CNS. In return the

CNS cannot adapt the thermoregulatory flow after temperature changes. A consequence of this malfunction could be the occurrence of cold intolerance.

When patients with a hand fracture are seen in the outpatient clinic suffering from cold intolerance, the cause is not likely to be explained by an abnormal re-warming.

It is therefore inevitable to explain the cold intolerance using alternative hypotheses.

Main Conclusions of this Thesis

From this thesis the following conclusions can be drawn:

- The vein graft was applied in a larger neural defect than previously described in literature and gave satisfactory results.
- Bone marrow stromal cells (BMSCs) are a useful luminal additive, because of the easiness to isolate these cells and the large amount of cells in the bone marrow.
- Bone marrow stromal cells enhance nerve regeneration when injected into the vein graft.
- Ultrasound is helpful as an assisting tool for visualizing the sciatic nerve in rats and particularly when placing needles near the nerve accurately.
- Our new minimally invasive technique allows recording CMAPs in the same animal over time and thus evaluating nerve regeneration on an individual basis.
- The vein graft supported with muscle allows nerve regeneration and can be considered a suitable grafting method.
- The vein graft supported with muscle and BMSC results in superior nerve regeneration when compared to the vein-muscle graft only. The BMSC can therefore be considered as a contributive luminal additive in this rodent model.
- The vein-muscle graft supported with BMSC results in less optimal regeneration when compared to the autologous nerve graft.
- The autologous nerve graft, 12 weeks post operative, proved to have superior nerve regeneration when staining and comparing all the neural fibers in the footpath to the vein-muscle graft only and the vein-muscle graft supported with BMSC.
- In a reconstruction model, the nerve regeneration suggests a faster proliferation of the axons transferring noxious stimuli and temperature sensations (i.e. the acute pain fibers). Therefore we could possibly assume that restoring vital sensory function has priority in these animals.
- Cold intolerance has a prevalence of 38% in patients who suffered from a hand fracture with a mean CISS score of 56 / 100.
- Abnormal re-warming after cooling was not related to the cold intolerance complaints of patients with a hand fracture.

Future Perspectives for Further Research

On the basis of the findings in this thesis, I feel that future research in the field of experiments related to the field of nerve reconstruction should concentrate on the following perspectives:

- Although most studies show that conduits available cannot replace today's golden standard, the nerve autograft, we should not underestimate the effect of cellular therapy in the field of nerve reconstruction. Since others and we have elucidated the beneficial effect of BMSCs, future projects should look into nerve regeneration, combining the nerve autograft with the BMSCs. This might result in faster regeneration and therefore reduce the postoperative revalidation time and reduce the high costs involved in unsuccessful nerve regeneration.
- The role of ultrasound proved to be helpful in developing a minimally invasive technique to record the compound muscle action potentials (CMAPs). Therefore, in collaboration with the Mayo Clinic, we have initiated a new project. This project focuses on the evaluation of muscle atrophy, which ideally could replace the conventional method. Future projects could then measure CMAPs and muscle atrophy minimally invasive in the same animal over time. This will lead to the use of less animals and a stronger statistical analysis, since regeneration longitudinally in the same animal can be measured.
- Human studies should be initiated to measure muscle atrophy in the muscles of the upper extremity after nerve injury and reconstruction. Using ultrasound we could measure muscle diameters and correlate this to the progress of nerve regeneration. Combining muscle diameter and measuring CMAPs minimally invasive could assist in finding a conduit other than the nerve autograft in clinical setting. This bed to bench side approach is a next step in the research regarding peripheral nerve reconstruction.
- Cold intolerance is and probably will continue to have high impact on the patients suffering from both nerve and/or bone trauma. Future projects should try to understand the specific pathophysiology of this problem and consequently develop a therapeutic strategy, since today's treatment available is often dissatisfactory. Consequently pharmaceutical therapies to treat neuropathic pain should be tested in patients with a peripheral nerve injury.

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Chapter 10

Summary and Samenvatting

Bridging the Gap in Relation to Nerve Injury

In the case of nerve injury it is recommended to think of the T approach: Time, Tension and Total coaptation.

Delayed nerve reconstruction often results in less optimal regeneration and the surgical intervention to restore the nerve should therefore be executed as soon as possible.

Secondly, it is important to reconstruct the nerve without tension. Tension can result in ischemia of the nerve, since the vascular flow will decrease or even arrest. To achieve a suitable restoration of nerve anatomy and therefore a functional vascular system, a tensionless connection should be sought. Therefore, at the trauma site a graft can be placed between the proximal and distal stump to reconnect the nerve.

Another key-issue in nerve reconstruction is to strive for total coaptation. If the proximal and distal stumps are nicely positioned and the properties of the nerve are respected, the axons can traverse the surgical coaptation without restriction.

Unsuccessful nerve regeneration or problems related to nerve injury can be invalidating, since sensory and/or motor loss, scarring, neuroma formation, cold intolerance and neuropathic pain all fit within the scope of these related problems.

Part of this thesis focused on developing a new way of nerve reconstruction, combining cellular therapy with a respected grafting technique.

Two new techniques were introduced and the regeneration was evaluated using both functional and histological evaluation techniques. The second part of this thesis describes a vast occurring problem in nerve injury, cold intolerance. We investigated this problem in patients with a hand fracture. Consequently, a possible relation between this cold intolerance and abnormal re-warming patterns is sought to find out if unknown neural problems could be the cause in this group of patients other than a disrupted vascular flow due to the fracture.

In **Chapter 1** a complete overview of nerve anatomy and physiology is presented. Secondly, different reconstruction techniques are described, and the different problems related to nerve injury are introduced. The last topic that will be discussed is the role of cellular therapy in nerve reconstruction.

The study in **chapter 2** was designed to investigate the regenerative effect of a vein graft filled with Bone Marrow Stromal Cells (BMSCs) for bridging a 20 mm nerve defect. Despite decades of clinical experience and numerous experimental studies, the autologous nerve graft still is considered the golden standard. When choosing an autologous nerve graft the problem of donor side morbidity arises and then there is a limited length available. For that reason it is a challenge to find alternatives that have similar regeneration compared to the autologous nerve graft, but can bridge large nerve defects. As a consequence of that in the early nineties the possibility of a vein as suitable grafting material was introduced and despite the regenerative potential is less compared to the autograft, the vein is still often used in clinical practice. For that

reason we used the vein graft as a graft that could bridge a large nerve defect and in addition to that is empty, so could easily be filled with BMSCs. BMSCs as a luminal additive has already been published in several articles and the beneficial effect is explained by the capability to trans-differentiate and/or the production of cytokines and growth factors. We used a large nerve defect and proved that the use of BMSCs resulted in significant better regeneration when compared to the empty vein graft. However we did encourage more research to prove this beneficial effect not only with functional evaluation parameters, but also include a histological analysis.

Chapter 3 introduces a new approach of retrieving Compound Muscle Action Potentials (CMAPs) in rats. The standard technique of retrieving these potentials was to position two needle electrodes near the sciatic nerve. However, an invasive procedure was necessary to visualize the nerve and place the electrodes. Since the CMAPs are a reliable method of evaluating nerve regeneration, we wanted to modify this technique to be able to measure regeneration over time in the same animal without the need of exposing the animal to an invasive procedure. We used ultrasound equipment to visualize the sciatic nerve and tested if live ultrasound images allowed accurate placement of the needle electrode near the nerve. We found this modified technique highly accurate and reliable and resulted in comparable results with the standard technique. Therefore this new technique can be helpful in future experiments, since it allows multiple measurements in the same animal, without the need of surgery.

The aim of **chapter 4** was to modify the vein graft used in chapter 2 and we placed a small piece of muscle in the middle of the vein graft. This piece of muscle should reduce the veins tendency to collapse and therefore risk a sub-optimal regeneration. Also, the BMSCs were introduced to elucidate the beneficial effect in this graft. In addition to chapter 2 we now compared our modified grafting technique with the autologous nerve graft and not only evaluated functional but also histological parameters. The findings of our study showed that the autologous nerve graft had significant better regeneration compared to the vein-muscle graft and the vein-muscle graft filled with BMSCs by both functional and histological parameters. In this study, we found that the BMSCs also had a beneficial effect on regeneration, when comparing the vein-muscle graft and the vein-muscle graft with BMSCs.

Chapter 5 introduces a new approach for the evaluation of nerve regeneration. Although extensive knowledge is available regarding the regeneration when an autologous nerve graft is used for reconstructing large nerve defects, little is known about the regeneration of the sensory fibers of the skin. The skin sensory fibers can be divided in peptidergic fibers (containing CGRP and Substance P), which are responsible for the transmission of vital pain (temperature changes and acute pinch pain), and non-peptidergic fibers (containing P2X3) which transmit non-vital pain. Another group of fibers are the A-Delta fibers responsible for the quickest pain response transmission. In this chapter the re-innervation of the epidermal fibers after a nerve reconstruction using a nerve autograft is described. Twelve weeks post surgery the sensory fibers have been re-innervated for almost 70%. Another key finding is that the fibers responsible

for transmitting vital pain re-innervate faster compared to the fibers responsible for non-vital pain transmission. The myelinated fibers in the rat footpath showed to have the maximum re-innervation compared to the other fibers.

Chapter 6 uses the same evaluation technique as used in chapter 5, only this time the beneficial effect of BMSCs in a vein-muscle graft for nerve reconstruction was tested. The results of this study showed that the non-peptidergic fibers had comparable re-innervation in both groups, but that the regeneration of the peptidergic and myelinated fibers in the vein-muscle graft injected with BMSCs was more progressed.

Part II

The study in **chapter 7** is a retrospective study investigating the prevalence of cold intolerance in patients who suffered from a hand fracture. Since cold intolerance was such an invalidating problem in patients with nerve injury, with a prevalence described up to 56%, a relevant question was if such a high prevalence is found in patients with a hand fracture. To determine whether or not patients have cold intolerance, patients were asked to complete the CISS questionnaire. Seventy-six patients who suffered a hand fracture between 2005 and 2006 responded and we found a prevalence of 38%. The high prevalence encouraged us to initiate a study investigating if the cold intolerance could be explained by an abnormal re-warming pattern or that another explanation should be sought.

Chapter 8 describes the study in which we wanted to investigate re-warming patterns in patients who suffered from a hand fracture with and without cold intolerance. The aim of this study was to determine if thermoregulatory dysfunction plays a major role in the underlying pathophysiology of cold intolerance in patients with a hand fracture by studying re-warming patterns after cooling of the hands. We investigated 18 hand fracture patients and 13 control subjects. In both groups we found abnormal re-warming patterns and for that reasons no correlation was found between the cold intolerance complaints and an abnormal re-warming. For that reason we have to explain the cold intolerance using alternative hypotheses, such as mechanisms based on changes in the properties of the temperature-sensory system or other neurological mechanisms.

Samenvatting

Het overbruggen van zenuwdefecten in relatie tot zenuwletsel

Wanneer zenuwletsel optreedt en chirurgisch herstel noodzakelijk is, moet men proberen de volgende 3 aspecten in acht te nemen: tijd (1), spanning (2) en totale coaptatie (3).
Een vertraging in het herstellen van zenuwletsel resulteert vaak in minder goed herstel dan wanneer de ingreep binnen 24 uur plaatsvindt.

Ten tweede is het belangrijk te zorgen dat de zenuw zonder spanning gereconstrueerd wordt. Spanning op de beschadigde zenuw kan resulteren in ischemie van de betreffende zenuw, omdat de vascularisatie niet in stand kan worden gehouden. Het is daarom van belang te zorgen dat, wanneer de zenuw niet zonder spanning geapproximeerd en gehecht kan worden, een interponaat beschikbaar is. Een interponaat zorgt ervoor dat de zenuw zonder spanning kan genezen en bewerkstelligt daarmee wellicht een snellere regeneratie.

Ten derde moet gestreefd worden naar een zodanig chirurgisch herstel dat de proximale en distale stomp in die mate worden geapproximeerd dat de uitgroeiende axonen de coaptatie makkelijk kunnen overbruggen.

Niet succesvolle regeneratie of problemen als gevolg van het letsel kunnen invaliderend zijn. Men moet denken aan sensibel en/of motorisch verlies, littekenvorming, kans op neuromen, koude intolerantie en neuropathische pijn.

In het eerste gedeelte van dit proefschrift is gekeken naar nieuwe manieren voor het overbruggen van zenuwdefecten, waarbij een combinatie werd gezocht van het gebruik van stamcellen en een geaccepteerd interponaat.

Twee nieuwe technieken werden geïntroduceerd en de regeneratie werd gemeten met zowel histologische als functionele testen. Het tweede gedeelte van dit proefschrift beschrijft het klinisch onderzoek naar de prevalentie van koude intolerantie bij mensen met een hand fractuur. Daarna hebben we gekeken of het optreden van koude intolerantie verklaard kon worden door een afwijking in de vascularisatie en/of een neurogene oorzaak. Dit hebben we gedaan door te kijken naar een mogelijke afwijking in de opwarming.

In **hoofdstuk 1** wordt een compleet overzicht van de anatomie en fysiologie van de zenuw gegeven. Ook worden verschillende hersteltechnieken beschreven en de mogelijke problemen die kunnen optreden bij zenuwletsel en de reconstructie daarvan. Het laatste onderdeel dat in dit hoofdstuk aan bod komt is de rol van cellulaire therapie ter bevordering van de zenuwregeneratie en met name toegespitst op de rol van stromale beenmerg stamcellen.

De studie in **hoofdstuk 2** is zo opgezet dat in een dier-experimenteel model gekeken kon worden naar de regeneratie van de zenuw nadat een 20 mm defect hersteld is met een vene gevuld met de stromale beenmerg stamcellen (BMSC's). Na tientallen jaren klinische ervaring en veel experimentele studies, geldt de autologe donor zenuw nog steeds als het meest gebruikte en beste interponaat. Dit interponaat heeft als nadeel dat er sensible uitval optreedt in het betreffende donorgebied en dat de lengte van de donorzenuw te beperkt kan zijn. Verder onderzoek naar het vinden van een alternatieve manier van overbruggen van zenuwdefecten met vergelijkbare resultaten is daarom al jaren bezig en zowel autologe als artificieel geproduceerde materialen zijn gebruikt. In de jaren 90 is het gebruik van een vene als mogelijke overbrugging geïntroduceerd. Een voordeel van het gebruik van een vene is dat deze in verschillende lengte en grootte beschikbaar is, de schade aan de donorzijde niet tot zeer beperkt is en dat de binnenwand een milieu heeft waarin de regenererende axonen goed kunnen doorgroeien. Daarnaast voorkomt de vene wand fibrosering van de regenererende zenuw. Deze eigenschappen hebben er voor gezorgd dat dit tot vandaag een vaak gebruikt alternatief is in klinische reconstructie. Om die reden hebben we er voor gekozen om de vene te gebruiken voor het overbruggen van een zenuwdefect in een rattenmodel. Vervolgens werd de lege vene gevuld met de BMSC's. De BMSC's als aanvulling bij de reconstructie van zenuwdefecten was al eerder beschreven. Het stimulerende effect werd toegeschreven aan de mogelijkheid van deze cellen om te trans-differentiëren en/of zouden ze een rol spelen bij de productie van cyotokines en groeifactoren.

Wij hebben gekozen voor een grotere overbrugging dan daarvoor gedaan en hebben aangetoond dat de BMSC's hebben gezorgd voor een significant betere zenuwregeneratie. Wel hebben wij in dit project alleen gekeken naar functioneel herstel en niet gekeken naar de histologische analyse.

Hoofdstuk 3 introduceert een nieuwe manier voor het meten van de zogeheten "Compound Muscle Action Potentials (CMAP's)". Deze potentialen zeggen iets over de hoeveelheid spiermassa en hoe goed de geleiding van de zenuw naar de spier is. De conventionele manier om de CMAP's in ratten te meten was het plaatsen van 1 electrode dicht bij de ischiadicus zenuw. Echter, voor het plaatsen van deze electrode is een invasieve benadering nodig om de zenuw te visualiseren en de electrode goed neer te kunnen leggen. Aangezien de CMAP's een betrouwbare maat zijn om de zenuwregeneratie te evalueren is het interessant om deze in het verloop van de tijd te meten. Wij hebben ervoor gekozen om onder begeleiding van echo- beelden de naald dicht bij de zenuw te leggen en zodoende een invasieve procedure te kunnen vermijden. Deze nieuwe techniek bleek zeer betrouwbaar en nauwkeurig vergeleken met de conventionele methode. In toekomstige projecten is deze nieuwe methode goed toepasbaar, omdat hiermee in hetzelfde dier de CMAP's meerdere malen over tijd gemeten kunnen worden.

Het doel van **hoofdstuk 4** was om de regeneratie tot stand gekomen met de vene gevuld met BMSC's gebruikt in hoofdstuk 2 te verbeteren door het plaatsen van een stukje spier in het midden van de vene. Dit stukje spier voorkomt het dichtvallen van de vene en daarmee een sub-optimale regeneratie. Ook in dit project hebben we weer gekeken naar de regeneratie wanneer BMSC's worden geïntroduceerd in de vene, maar nu hebben we ook de vergelijking gemaakt met de autologe donor- zenuw. Daarnaast hebben we nu ook een uitgebreide histologische analyse toegepast. De bevindingen in deze studie laten zien dat de autologe donorzenuw significant betere regeneratie heeft vergeleken met de vene-spier interponaat en de vene-spier interponaat gevuld met de BMSC's. Wel zagen we dat de additie van BMSC's heeft geresulteerd in een betere regeneratie dan wanneer de vene-spier interponaat niet werd gevuld.

Hoofdstuk 5 introduceert een nieuwe benadering voor het evalueren van zenuw- regeneratie. Alhoewel er veel onderzoek is gedaan naar de regeneratie na autologe zenuwtransplantatie is de re-innervatie van de huid, en met name de epidermale vezels, in dit proces nog relatief onbekend. De sensorische vezels bestaan uit: peptiderge vezels (CGRP en Substance P bevattend) die zorgen voor vitale pijngeleiding (temperatuursveranderingen en prik pijn), non-peptiderge vezels (P2X3 bevattend) die non-vitale pijn (i.e. drukveranderingen) communiceren. De A-delta vezels (NF 200 bevattend) zorgen voor de snelle, acute, pijngeleiding.

In deze studie is gekeken naar de re-innervatie van epidermale huidvezels 12 weken na het overbruggen van een zenuwdefect met een donor zenuw. Deze studie laat het re-innervatie patroon zien van de voetzoolhuid van de rat, 12 weken postoperatief. Het totale aantal vezels is voor 70% ten opzichte van de controles teruggegroeid.

Onze bevindingen laten zien dat de vitale vezels eerder of in sterkere mate terug- groeien dan de non-vitale vezels. In literatuur was dit voorheen alleen nog maar een aanname. Nu is bewezen dat in een rattenmodel de acute pijngeleiding als eerste terugkomt. Verder is gebleken dat de gemyeliniseerde vezels veel sterkere re-innervatie hebben vergeleken met de andere vezels. Hiermee is, voor zover wij hebben kunnen nagaan, voor het eerst de regeneratie van de verschillende subgroepen van sensorische vezels van de huid in een zenuwregeneratie model gedemonstreerd.

De volgende stap werd gezet in **Hoofdstuk 6** waarbij we hebben gekeken naar de regeneratie van de sensorische vezels in de huid als de vene-spier interponaat met en zonder BMSC's werd gebruikt voor overbrugging van een zenuwdefect. De meest opmerkelijke bevinding is dat 12 weken na het herstel, de toevoeging van de BMSC's heeft bijgedragen aan een betere zenuwregeneratie. Met name het aantal gemyeliniseerde zenuwvezels, verantwoordelijk voor de snelle zenuwtransmissie, was groter wanneer de vene-interponaat werd gevuld met BMSC's.

Part II

De studie in **Hoofdstuk 7** is een retrospectieve studie waarbij gekeken is naar de prevalentie van koudeintolerantie in patiënten die een handfractuur hebben opgelopen. Aangezien koudeintolerantie een invaliderend probleem is bij patiënten, een prevalentie van 56% is beschreven in de literatuur, is een relevante vraag of deze prevalentie ook bij mensen met een handfractuur voorkomt. Om te bepalen of de patiënten koudeintolerantie hebben werd een vragenlijst ingevuld. 76 patiënten die een handfractuur hebben opgelopen tussen 2005 en 2006 hebben gereageerd en daarvan bleek 38% in meer of mindere mate last te hebben van koudeintolerantie. Deze hoge prevalentie heeft ons gestimuleerd om het onderzoek door te zetten en te kijken of de klachten verklaard kunnen worden met een abnormaal opwarmingspatroon of dat de oorzaak ergens anders gezocht moet worden.

Hoofdstuk 8 beschrijft een studie waar het patroon van de opwarming bij patiënten met een handfractuur is gemeten. Zowel patiënten met en zonder klachten van koudeintolerantie zijn gemeten. In totaal 18 patiënten en 13 gezonde mensen zijn gemeten ter controle. In beide groepen hebben we zowel normale als abnormale opwarming gevonden en daardoor is er geen correlatie tussen de koudeintolerantie klachten en een abnormale opwarming. Om die reden moeten we dus de klachten verklaren door verstoring in een ander mechanisme. Hierbij kan gedacht worden aan veranderingen in de sensitiviteit van het temperatuur-regulatoir systeem of andere neurobiologische mechanismen.

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Pap en Mam,
Onvoorwaardelijke steun en altijd bereid op iedere mogelijke manier te helpen. Jullie zijn mijn voorbeeld waarom ik altijd goed wil presteren en altijd denk dat het nog beter kan. Mam, de wake-up calls en je bezorgdheid geven aan hoe betrokken jij bent.
Pap, jouw hulp in de afgelopen jaren was onmisbaar, wat ben ik dankbaar dat jij ondanks je werk altijd tijd voor ons hebt.

Jolien,
Jij weet me altijd weer op mijn plek te zetten en juist daarom ben ik zo blij dat jij vandaag bij mij bent!

Curriculum Vitae

Tim Hendrik Jan Nijhuis was born on March 15th 1986 in Nijmegen, the Netherlands. After graduating from the Jeanne d'Arc College in Maastricht in 2004 (Gymnasium), he started medical school at the University of Rotterdam. During his study he worked as a student member on the medical ward of the department of plastic and reconstructive surgery of Professor S.E.R. Hovius. His interest in Plastic and Reconstructive Surgery was further strengthened by his first research project together with Ernst Smits. With the help of Jeanne Bart Jaquet and Ruud Selles they finished their first study quickly. After combining work, studies and research for 1½ years he decided to do a fellowship for one year in the Cleveland Clinic in Cleveland, the United States under supervison of Dr. Siemionow. Consequently he worked for a 1½ in Professor Hovius's laboratory to complete his thesis. Combining research and his internships he finished the final phase of his PhD and initiated a new project with the Mayo Clinic (Dr. Shin and Dr. Bishop). He has published his work in several peer-reviewed journals and has been awarded the best presentation at three conferences. He expects to finish his MD training in the beginning of 2013.

PhD Portfolio

Bridging the Gap in Relation to Nerve Injury

Summary of PhD training and teaching

Name PhD student: Tim Nijhuis

Erasmus MC, University Medical Center

Department: Plastic, Reconstructive and Hand Surgery

PhD period: September 2008 – December 2011

Promotor: Prof. SER Hovius

Supervisor: Dr. J.W. van Neck

PhD training	Year	Workload (Hours)
General courses		
– Principles of Research in Medicine and Epidemiology	2010	33 hrs
– Introduction to Data-analysis	2010	33 hrs
– Biomedical English Writing	2010	12 hrs
– Felasa C training	2009	80 hrs
Specific courses		
– Microsurgery; Mevr. JM Hekking Skillslab - Plastic and Reconstructive surgery	2009-2010	175 hrs
– Microsurgery basic training, M. Siemionow Microsurgery Laboratory, Cleveland Clinic, Cleveland, USA	2008-2009	
– Microsurgery advanced training, M. Siemionow Microsurgery Laboratory, Cleveland Clinic, Cleveland, USA	2008-2009	
Seminars and workshops		
– Methodology of patient related research and preparation of grant applications Consultation center for Patient related Research (CPO)	2011	10 hrs

PhD training	Year	Workload (Hours)
Oral Presentations		
– Presentation at the annual meeting of the Dutch Society for Hand Surgery (NVvH) *Amsterdam, The Netherlands,* *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* ***(awarded with best presentation)***	2011	20 hrs
– Presentation at the annual meeting of the American Society for Peripheral Nerve (ASPN) *Cancun, Mexico:* *The vein supported with muscle and bone marrow stromal cells compared to the nerve autograft as a natural conduit for bridging a 15mm nerve defect in rats*	2011	40 hrs
– Presentation at the 23rd Symposium for Experimental Research for all Surgical Specialism's (SEOHS) *Rotterdam, The Netherlands:* *The vein supported with muscle and bone marrow stromal cells compared to the nerve autograft as a natural conduit for bridging a 15mm nerve defect in rats* **(*awarded with best oral presentation*)**	2010	20 hrs
– Presentation at the 11the Triennial Congress of the International Federation of Societies for Surgery of the Hand *Seoul, South-Korea:* *A promising, novel technique using ultrasound-guided needle positioning for near-nerve stimulated myography in rats: preliminary results.*	2010	40 hrs
– Presentation at the European Conference of Scientists and Plastic Surgeons (ECSAPS) *Helsinki, Finland:* *The vein supported with muscle and bone marrow stromal cells compared to the nerve autograft as a natural conduit for bridging a 15mm nerve defect in rats*	2010	40 hrs
– Presentation at the annual research meeting of the Dutch Society of plastic Surgery (NVPC) *Maastricht, The Netherlands:* *The venous graft supported with Bone Marrow Stromal Cells as a natural conduit for bridging a 20mm nerve gap* ***(awarded with best oral presentation)***	2009	20 hrs
– Presentation at the European Conference of Scientists and Plastic Surgeons. *Rotterdam, The Netherlands:* *The venous graft supported with Bone Marrow Stromal Cells as a natural conduit for bridging a 20mm nerve gap*	2009	20 hrs
– Presentation at the Plastic Surgery Research Council (PSRC) 54th annual meeting. *Pittsburgh, USA:* *Review of current assessment techniques of sciatic nerve regeneration in a rodent model*	2009	20 hrs
– Presentation at the FESSH XIIIth (Federation of the European Societies for Surgery of the Hand) *Lausanne, Switzerland:* *Incidence and severity of cold intolerance in patients with hand fractures*	2008	20 hrs

PhD training	Year	Workload (Hours)
Other		
– Organizing “Annual Erasmus MC Plastic Surgery Meeting”	2010-2011	140 hrs
– Organizing the 17th Esser Course: Dupuytren’s Contracture in Rotterdam	2011	
Teaching		
Lecturing		
– Course “Anatomy of the Arm en Hand” (3th and 4th year students)	2009-2011	60 hrs
Supervising practical’s and excursions		
– Supervision Microsurgery course Microsurgery laboratory, Cleveland Clinic, Cleveland, USA	2008-2009	100 hrs
– Supervision Microsurgery course Skillslab, Erasmus MC, Rotterdam	2009-2011	100 hrs
– Course “Basics in suture techniques” (3th year students)	2009-2011	16hrs
– Course “Basics in suture techniques for animals” (FELASA C course particapants)	2009-2010	20hrs
Grants		
– Erasmus Trustfonds (€19.000)		
– Nuts-Ohra Foundation (€133.000)		
– Michaël van Vloten Fonds		