The Role of Schwann-like Cells Differentiated from Adipose-Derived Mesenchymal Stem Cells *in vitro* versus Adipose-Derived Mesenchymal Stem Cells in the Regeneration of Sciatic Nerve Injury in Rats

Thesis
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INTRODUCTION

There is a high incidence of peripheral nerve injuries throughout the world, which represents a major economic problem for the society. Despite surgical advances, functional recovery of these patients is relatively poor ***(Liu et al., 2013).***

Several approaches have been proposed to exert beneficial effects on peripheral nerve regeneration, including application of an electrical field, administration of neurotrophic factors, and use of autologous nerve grafts ***(Pan et al., 2006; Pan et al., 2007; Yang et al., 2012)***.

Recently, cell based therapy has been proposed as an efficient method for regenerating injured nerves. Transplantation of Schwann cells (SCs) or stem cells of various origins which differentiate towards a Schwann cell-like phenotype, could stimulate peripheral nerve repair ***(Fuhrmann et al., 2010; Lopatina et al., 2011)***.

SCs are key regulators of the regeneration process of nervous tissue. They release neurotrophic factors, form myelin sheath, provide structural support, and guidance for peripheral nerve regeneration following injury ***(Wiberg et al., 2003; Lopatina et al., 2011; Lui et al., 2013)***.

But in reality, the isolation of an adequate number of SCs for clinical practice is confronted with donor morbidity and low cell yield ***(Bunge et al., 2003; Zaminy et al., 2013)***. Their

autologous transplantation is highly traumatic and they are difficult to expand in vitro ***(Lopatina et al., 2011)***.

That's why alternative sources of neuronal stem cells are now proposed. Under appropriate conditions, mesenchymal stem cells (MSCs), whatever their origin, may selectively differentiate not only into mesenchymal lineages but also endodermal and ectodermal cell lineages, in vitro ***(Zavan et al., 2010; Anghileri et al., 2013)***.

The adult MSCs were first discovered in the bone marrow. But bone marrow aspiration is excessively painful for patients and yield low number of harvested cells ***(Bonefield et al., 2010; Zavan, 2010)***. While adipose-derived stem cells (ASCs), can be easily obtained and expanded extensively in vitro for use in autologous cell therapy ***(Lopatina et al., 2011)***.

Currently, co-culture with SCs or addition of glial growth factors and specific chemicals are often utilized for Schwann-like cell differentiation of ASCs. However, these methods are usually complicated and expensive. In the current study, we will administer trophic factors secreted from rat sciatic nerve leachate, after being cut and soaked in culture medium, to rat ASCs, hence induce the ASCs differentiation into SCs in vitro ***(Li et al., 2009; Liao et al., 2010; Liu et al., 2013)***.

In the present study, the role of ASCs and ASCs differentiated into Schwann-like cells will be assessed in a rat model of crushed sciatic nerve injury.

AIM OF THE WORK

The current study aims to differentiate ASCs into Schwann-like cells in vitro, and then assess the role of the differentiated Schwann-like cells versus the ASCs in the regeneration of crushed sciatic nerves in male adult albino rats.

HISTOPATHOLOGY OF PERIPHERAL NERVE INJURY & REGENERATION

The peripheral nervous system (PNS) was shown to have an intrinsic ability for repair and regeneration. Most commonly, injuries were caused by direct mechanical trauma, and less frequently, surgical resection secondary to tumor excision ***(Faroni et al., 2015)***.

Capacity for regeneration was affected by the age of the patient, the severity of injury and the proximity of the injury to the nerve cell body. Distal digital nerve injuries, resulting in sensory loss to a fingertip, regenerated well, while proximal brachial plexus avulsions were functionally devastating with impaired hand sensation, and frequently pain and cold intolerance ***(Faroni et al., 2015).***

Clinically useful injury grading systems were developed to allow correlation of the microscopic changes occurring after nerve injury and patient symptomatology. These grading systems were developed by Seddon ***(Seddon, 1943)*** and Sunderland ***(Sunderland, 1990) (Fig. a)***.

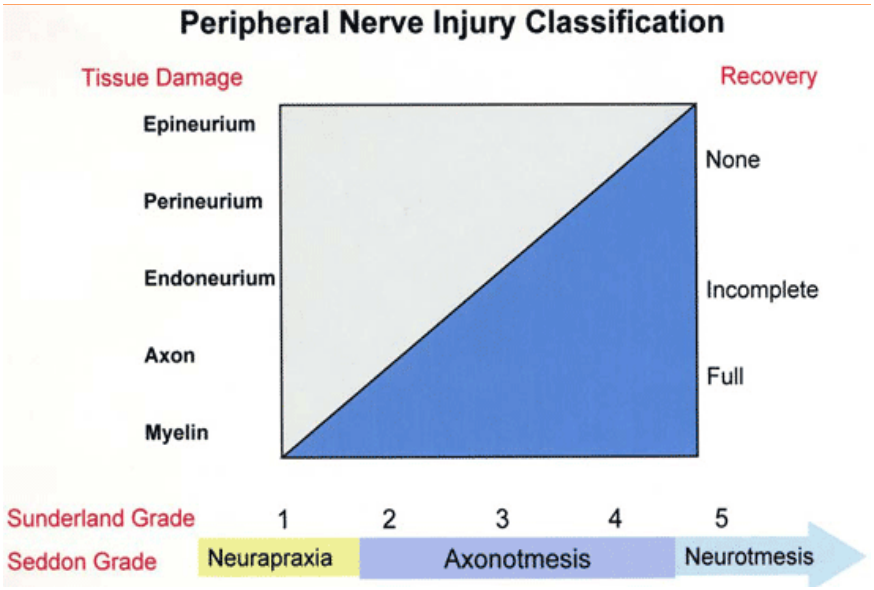

Figure (a): Graph illustrating the Sunderland and Seddon peripheral nerve injury (PNI) grading systems. Gradations in both systems are associated with the anatomical extent of injury and with the chance of a spontaneous recovery of function after trauma.

Seddon divided nerve injuries by severity into three broad categories: neurapraxia, axonotmesis, and neurotmesis.

Neurapraxia, the mildest injury type, was explained as transient functional loss without affecting nerve continuity. This symptom was thought to be due to a local ion-induced conduction block at the injury site, although alterations in myelin structure have also been found.

Axonotmesis was described when there was complete interruption of the nerve axon and surrounding myelin while the surrounding mesenchymal structures including the perineurium and epineurium, stayed preserved. Axon and

myelin degeneration occurred distal to the point of injury, causing complete denervation.

The hope of recovery is excellent in such injuries because of the remaining uninjured mesenchymal latticework that provides a path for subsequent sprouting axons to reinnervate their target organ.

Neurotmesis involved disconnection of the whole nerve including the surrounding epineurium & perineurium. Functional loss was found to be complete and recovery without surgical intervention, did not often occur because of scar formation and loss of the mesenchymal guide that properly directs axonal regrowth.

Sunderland's classification system, furtherly divided the three injury types described by Seddon into five categories according to severity. A first-degree injury equivalent to Seddon's neurapraxia and a second-degree injury equivalent to axonotmesis. Third-degree nerve injuries were described when there was disruption of the axon (axonotmesis) and partial injury to the endoneurium. This categorization placed a third-degree between Seddon's axonotmesis and neurotmesis. Dependent on the extent of the endoneurial damage, functional recovery could be possible. Sunderland divided Seddon's neurotmesis into fourth- and fifth-degree injuries. In a fourth-degree injury, all portions of the nerve were disrupted except the epineurium. Recovery was not possible without surgical

intervention. Similarly, a fifth-degree injury was described when the nerve was completely disrupted. This classification is still used nowadays ***(Faroni et al., 2015)***.

The PNS has a much greater potential for regeneration than the central nervous system mainly due to the differences in response to injury of the respective glial cells ***(Chen et al., 2007)***. The glia of the PNS, Schwann cells (SCs), were shown to convert to a regenerative phenotype promoting the formation of a basal lamina and enhancing neuronal regenerative response ***(Geuna et al., 2009)***.

Nerve Degeneration:

Following peripheral nerve injury, several molecular and cellular changes were observed at the level of the cell body (dorsal and ventral roots), at the site of injury (proximal and distal stumps) and in the target organs.

Changes in neuronal cell bodies and in nerve fibers proximal to the site of injury were shown to depend on the severity of the injury as well as the proximity of the injured segment to the cell body. SCs inevitably degraded along the proximal segment near the area of injury, §axons and myelin were reduced in diameter. This proximal degradation could be minimal, ranging from the injury site back to the next node of Ranvier, or extending all the way back to the cellular body. If the cellular body degenerated in severe trauma, the entire

proximal segment would undergo Wallerian degeneration and phagocytosis ***(Burnett and Zager, 2004; Faroni et al., 2015)***.

Within 6 hours of the injury, the nucleus was seen migrating to the periphery of the cell, and Nissl granules, rough endoplasmic reticulum, broke down and dispersed. This process was called chromatolysis. Simultaneously, there was a brisk proliferative response of perineuronal glial cells, mostly signaled by the process of chromatolysis. Glial cell processes extended to the affected neuron and interrupted its synaptic connections, to isolate the neuron for its recovery phase ***(Burnett and Zager, 2004).***

A series of changes, known as Wallerian degeneration, were shown at the distal segments of severed nerve axons within 12–72 hours ***(Fig. b)***. Both the axon and the myelin in the distal stump degenerated, hence, macrophages would migrate to the site of injury and contribute to clear the debris ***(Burnett and Zager, 2004; Chen et al., 2007)***.

In the first 24 hours, SCs proliferated and switched from a myelinating to a regenerative phenotype and exhibited upregulation of several molecules that assist both the parallel degenerative and regenerative processes. The denervated SCs downregulated structural proteins such as protein zero (P0), myelin basic protein and myelin-associated glycoprotein. Simultaneously, there was upregulation of cell adhesion molecules (CAMs), neural CAMs (NCAMs), and glial fibrillary

acidic protein (GFAP), along with growth factors: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF) and neurotrophin 3 (NT-3) ***(Jessen et al., 2008; Griffin et al., 2013)***.

Such surface receptors and cytoskeletal molecules were found to be necessary to mediate and transduce efficient interactions between SCs, axons and the extracellular matrix, for a successful regeneration process ***(Previtali et al., 2004).***

When the debris have been removed by the combined action of SCs and macrophages, SCs aligned forming columns called bands of Büngner. This formed a suitable environment rich in trophic factors, and guided the sprouting axons regeneration ***(Scheib et al., 2013)***.

In the severe injuries causing complete nerve avulsion, the nerve ends became a swollen mass of disorganized SCs, capillaries, fibroblasts, macrophages, and collagen fibers. Regenerating axons reached the swollen bulb of the proximal stump and encountered enormous barriers to further growth. Many axons formed whorls within the collagen fibers of the scar tissue or were turned back along the proximal segment or out into the surrounding tissue ***(Burnett and Zager, 2004)***.

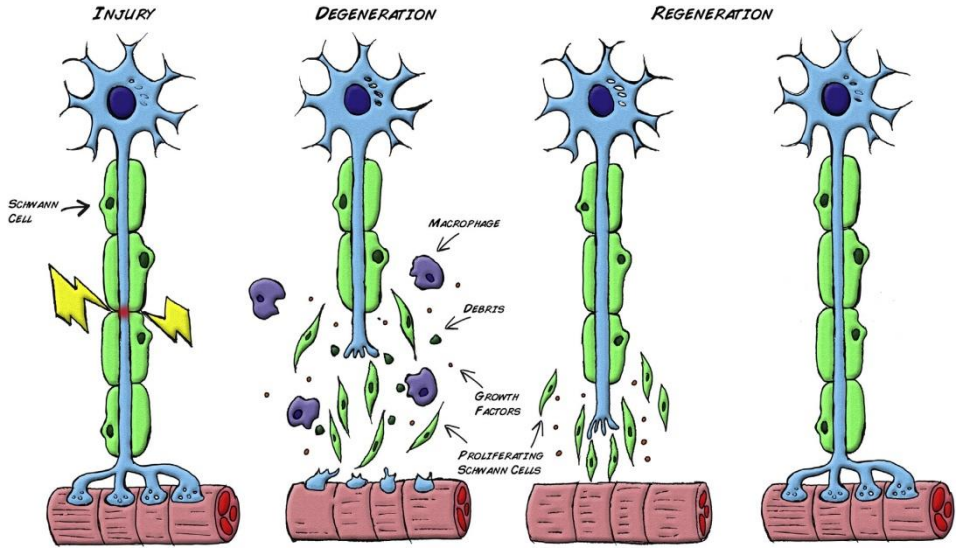

Figure (b): Wallerian degeneration. Following injury, SCs detach from the axons, start proliferating and help the recruited macrophages to clear the cellular and myelin debris. At the same time, expression of stimulating factors by SCs create a favorable environment for nerve regrowth towards the target organ.

Distal to the site of injury, there were several obstacles for the regenerating axon to successfully reinnervate the target organ. Misdirection towards the wrong target reduced functional outcome even with a respectable number of regenerated axons. A lack of neuronal contact in the distal stump lead to chronically denervated SCs which down-regulated growth factors and entered a dormant state, unable to support axonal progression ***(Allodi et al., 2012).***

Similarly, the denervated target organ was exhausted of trophic factors, muscle fibers atrophied and apoptosis occurred in satellite cells. These responses had a significant impact on

functional recovery following proximal nerve injuries ***(Gordon et al., 2011)***.

Nerve Regeneration:

Regarding nerve regeneration, in severe injuries, it began only after Wallerian degeneration had terminated its course, but in mild injuries the regenerative and repair processes began almost immediately. For first and second-degree injuries (neurapraxia and axonotmesis), restoration of function was the rule. This occurred early by reversal of conduction block or late by axonal regeneration. In more severe nerve injuries in which endoneurial tubes were disrupted, regenerating axons were no longer surrounded by their original sheaths, thus they failed to reinnervate their proper end organs ***(Chen et al., 2007).***

The sequence of regeneration was histologically divided into levels: the neuronal cell body; the segment between the cell body and the injury site; the injury site itself; the distal segment between the injury site and the end organ; and finally the end organ ***(Faroni et al., 2015)***.

At the level of the neuronal cell body, the earliest signs seen were visible changes indicating the reversal of chromatolysis. The nucleus returned to the cell center and nucleoproteins reorganized into the compact Nissl granules. The metabolic machinery was reprogrammed so that the cell would be able to produce the great amount of protein and lipid

needed for axonal regrowth during the regeneration phase ***(Burnett and Zager, 2004; Faroni et al., 2015)***.

A complex interaction was seen between the cell body and the regenerating axon tip. Axoplasm arised from the proximal axon segment and cell body. Components of axoplasmic transport supplied materials from the cell body to the sites of axonal regeneration. The rate of increase in protein and lipid synthesis in the cell body influenced the rate of growth and the final caliber of the regenerating axon ***(Chen et al., 2007)***.

At the site of injury, the first signs of axon regrowth were seen as early as 24 hours post-injury, and were delayed for weeks in more severe injuries. The axonal regrowth would reach a rate of 1–2 mm/day. However, reported rates of regeneration varied broadly from 0.5 to 9 mm per day **(*Hadlock et al., 2005).***

This variability was due to several factors: the species, the distance from the cell body to the advancing axon tip, the method and severity of nerve injury, the techniques for measuring regeneration, the duration of denervation and finally the condition of the peripheral tissue**s (*Hadlock et al., 2005).***

A significant overlap was found between the timing of degeneration and regeneration. For example, in mild injuries in which there was no significant delay in regeneration across the

injury site, the growth cone at the advancing axon tip encountered the debris of Wallerian degeneration in the distal segment. Interestingly, the debris didn't stop the regenerative process, because the growth cone was shown to secrete a protease that can help dissolve the material blocking its path ***(Jessen et al., 2008; Griffin et al., 2013)***.

In very proximal injuries in which there was a considerable delay before the advancing axon tip could reach the distal segment, the empty endoneurial tubes would decrease in diameter by time, leading to slowing of the axonal regrowth ***(Griffin et al., 2013).***

Moreover, in severe nerve injuries that disrupted the endoneurial tubes, enormous obstacles faced the regenerating axons on their way to the injury site, allowing regenerating axon sprouts to wander into surrounding tissue, and scarring inevitably occurred which impeded the regeneration and misdirected axon sprouts into functionally unrelated endoneurial tubes ***(Faroni et al., 2015)***.

Axons that successfully entered the endoneurial tubes in the segment distal to the injury site would have a good chance of reaching the end organ, given their environment had good growth conditions. The distal regeneration rate was slower if the endoneurial tubes were disrupted, because axon sprouts had to first find their way into the tubes before advancing. The specialized growth cone at the tip of each axon sprout was

shown to have multiple filopodia that adhered to the basal lamina of the Schwann cell and used it as a guide ***(Scheib et al., 2013)***.

End organ underwent characteristic histological changes with nerve degeneration and subsequent reinnervation. Muscle fibers atrophied quite rapidly and cell nuclei took a central rather than the normal peripheral position. The synaptic folds of motor endplates were preserved for at least 1 year after denervation ***(Bozkurt et al., 2007 & 2008)*.**

If a functionally unrelated end organ was reached or if the end organ had already undergone degeneration, further development of the axon and remyelination would not occur. If the entry of regenerating axons into the distal segment was delayed, the axons had to enter endoneurial tubes of smaller diameter. This shrinkage made it more difficult for axon sprouts to locate and enter endoneurial tubes, but this did not stop axonal regrowth once sprouts were inside the tubes, due to the elastic properties of the endoneurium ***(Faroni et al., 2015).***

Concerning muscle tissue, tremendous proliferation of fibroblasts also characterized the histological picture of denervation. New collagen was deposited in both the endo- and perimysium. Atrophied fibers were separated by thick connective tissue, so that the overall internal pattern of muscle architecture was preserved. Unfortunately, incomplete motor recovery commonly occurred after moderate to severe nerve