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Peripheral nerve regeneration: Experimental strategies and future perspectives ☆

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ABSTRACT

Peripheral nerve injuries represent a substantial clinical problem with insufficient or unsatisfactory treatment options. This review summarises all the events occurring after nerve damage at the level of the cell body, the site of injury and the target organ. Various experimental strategies to improve neuronal survival, axonal regeneration and target reinnervation are described including pharmacological approaches and cell-based therapies. Given the complexity of nerve regeneration, further studies are needed to address the biology of nerve injury, to improve the interaction with implantable scaffolds, and to implement cell-based therapies in nerve tissue engineering.

Contents

1. Introduction

The peripheral nervous system (PNS) has an intrinsic ability for repair and regeneration. Injuries are most commonly attributable to direct mechanical trauma, and less frequently, surgical resection secondary to tumour excision. Capacity for regeneration relates to age of patient,

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mechanism of injury and in particular to the proximity of the injury to the nerve cell body. Distal digital nerve injuries result in sensory loss to a fingertip and will regenerate well, whilst proximal brachial plexus avulsions are functionally devastating with impaired hand sensation, reduced motor function and frequently pain and cold intolerance [1]. Such injuries have a profound and permanent impact on the patient and their ability to perform activities of daily living, as well as preventing return to work.

Currently, the treatment of choice is meticulous microsurgical repair by tensionless epineurial sutures. In the presence of a nerve gap where end-to-end suturing is not possible, autologous nerve grafting remains the gold standard [2]; however, this sacrifices a healthy nerve, requires more extensive surgery and donor nerves are in finite supply. Nerve injuries should be repaired early with delayed repair demonstrated to be significantly detrimental to satisfactory sensory and motor recovery [3,4]. The principles in clinical treatment for nerve injury have not changed in the last 30 years despite substantially increased understanding of neuropathophysiology, and correspondingly clinical outcomes remain poor [5].

It has become apparent that a purely microsurgical approach to nerve repair will fail to address the complex cellular and molecular events of peripheral nerve regeneration. It is important to recognise that axonal injury has implications for the entire length of the neuron, as well as immediate functional consequences for the brain. Various factors have been implicated in the poor outcome of nerve regeneration: at the site of injury slow, insufficient and misdirected axonal outgrowth; at the target organ atrophy of muscle tissue and failure of reinnervation; in the brain rapid and longstanding cortical reorganisation [6]. Perhaps the single most important factor is the extensive cell death in the innervating neuronal pool [7,8], since the most fundamental neurobiological prerequisite to regeneration is that neurons are maintained in a viable form.

2. Neurobiology of peripheral nerve injury and regeneration

The PNS has far greater potential than the central nervous system for regeneration due mainly to the differences in response to injury of the respective glial cells [9]. The glia of the PNS, Schwann cells (SCs), convert to a regenerative phenotype thereby promoting the formation of a basal lamina and providing abundant cues to trigger neuronal regenerative response [10].

Following peripheral nerve injury, several molecular and cellular changes are observed at the level of the cell body (dorsal and ventral root), at the site of injury (proximal and distal stump) and in the target organs, each of which are discussed in this section.

2.1. The cell body

Perhaps the first signal received by the neuronal cell body following axonal injury is antidromic electrical activity in the form of a high frequency burst of action potentials, which can open calcium channels and initiate Jun-kinase cascades that influence transcription; however, the biggest determinant of neuronal survival is likely to be the withdrawal of target-derived neurotrophic support [11,12]. This leads to a profound response in both gene and protein expression [13,14]; the balance of these determines whether the neuron survives and attempts regeneration or results in apoptotic death [15]. Primary sensory neurons are significantly more vulnerable to apoptosis than spinal motoneurons, with 40% of dorsal root ganglion (DRG) neurons dying following injury [7]. Fortuitously, the time-course of neuronal death lends itself to a clinically relevant window of neuroprotective opportunity [16].

Specific to the field of urology, the major pelvic ganglia (MPG) and their related peripheral nerves are particularly susceptible to injury during pelvic surgery. Although the time-course and extent of neuronal death specific to MPG is not known, it should be expected to be similar to that described above. The molecular profiling of MPG neurons following cavernous nerve injury has been undertaken and demonstrates an upregulation in several genes associated with apoptosis, neuroprotection and regeneration [17]. The expression of these genes is likely influenced by the characteristics of the insult and the fate of the neuron will be determined by the balance of apoptotic/neuroprotective expression.

2.2. The site of injury

The distal stump of the injured nerve undergoes a series of molecular and cellular changes known as Wallerian degeneration (Fig. 1) [18]. Within a few hours, both the axon and the myelin in the distal stump degenerate and macrophages migrate to the site of injury and contribute to debris clearance [9,18,19]. In the first 24 h, SCs proliferate and switch from a myelinating to a regenerative phenotype and exhibit up-regulation of several molecules that assist the parallel degenerative and regenerative processes [18,19]. In particular, the denervated SCs down-regulate structural proteins such as protein zero, myelin basic protein and myelin-associated glycoprotein; whilst up-regulating cell adhesion molecules (CAM), — L1, neural CAM (NCAM), and glial fibrillary acidic protein, alongside 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) [20,21]. When the debris has been removed by the combined action of SCs and macrophages, SCs align forming columns called bands of Büngner. This forms a permissive environment rich in trophic factors, enabling guided axonal regeneration [22].

2.3. The target organ

Distal to the site of injury, there are several obstacles for the regenerating axon to overcome prior to successful reinnervation of the target organ. Misdirection towards the wrong target reduces functional outcome even with a good number of regenerated axons; however, this is checked by 'pruning' of growth cones that do not reach the correct target or lose support of their endoneurial tubes [23]. A lack of neuronal contact in the distal stump leads to chronically denervated SCs which down-regulate growth factors and enter a dormant state, unable to support axonal progression [4]. Similarly, the denervated target organ is exhausted of trophic factors, muscle fibres atrophy and satellite cells undergo apoptosis [24]. These responses bear a significant impact on functional recovery following proximal nerve injuries.

3. Experimental strategies and tissue engineering

3.1. Addressing neuronal survival

Surgical repair of the peripheral nerve is at best only partially neuroprotective and dependent upon very early repair within 24 h [25]. However, this is not always clinically feasible due to concomitant injuries or diagnostic delay, especially in those cases with very poor outcomes such as closed brachial plexus injuries. Therefore, an alternative approach to neuroprotection is required.

Exogenous replacement of growth factors can reduce neuronal loss experimentally; however, these are clinically problematic given the side-effect profile and unpredictable interactions between the various growth factors necessary for action on a heterogenous neuronal population [26,27]. More recently, two pharmacological agents, N-acetylcysteine (NAC) and acetyl-L-carnitine (ALCAR), have been shown to offer almost complete neuroprotection experimentally within a clinically pragmatic time-frame, and both are established as safe clinical pharmaceutical agents [15,28–30]. In animal models examining MPG neuroprotection following cavernosal nerve injury, the phosphodiesterase 5 inhibitor sildenafil has been demonstrated to promote a neurotrophic phenotype [31].

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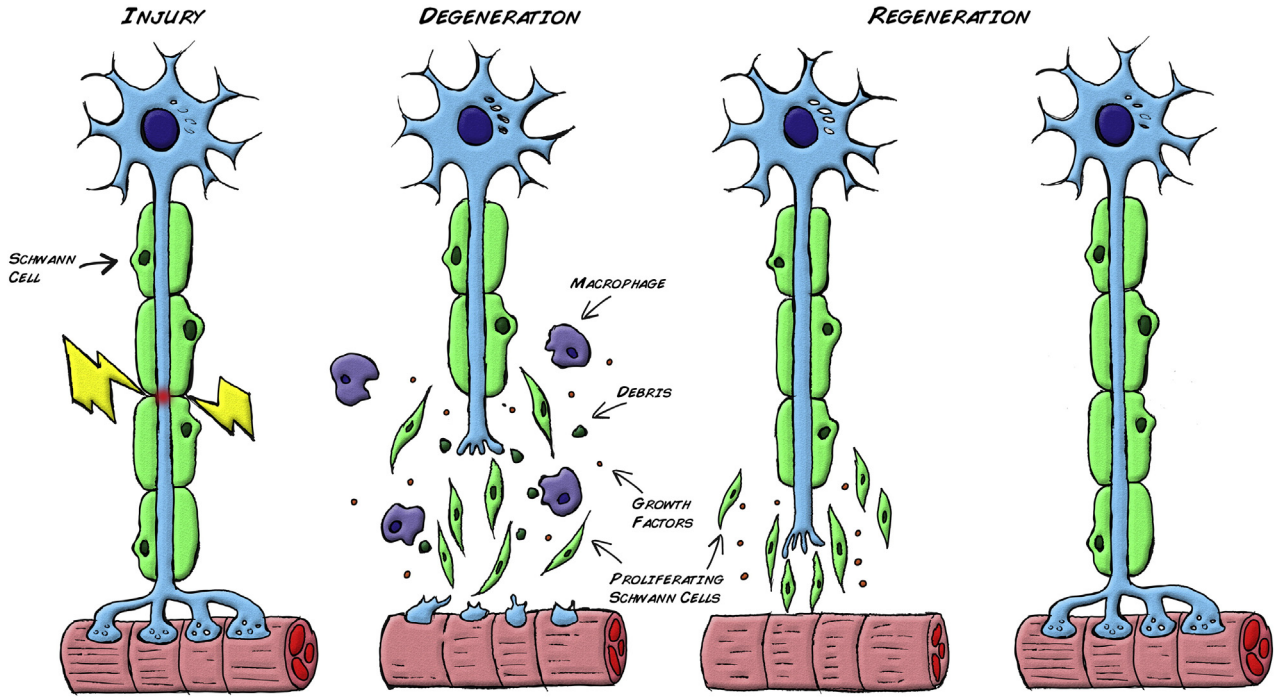

Fig. 1. Wallerian degeneration. Following injury, Schwann cells detach from the axons, start proliferating and help the recruited macrophages to clear the cellular and myelin debris. At the same time, expression of stimulating factors by SCs create a favourable environment for nerve regrowth towards the target organ.

An alternative and novel approach has been considered involving incorporation of cultured cells into biomaterial scaffolds, which combines nerve repair and enhanced axonal regeneration with neuroprotective therapies. Autologous SC therapy would require a nerve biopsy and prolonged culture times, therefore attention has shifted to exploiting stem cells as a clinically viable alternative. Adipose-derived stem cells (ASC) incorporated into a bioengineered nerve conduit significantly alter the gene expression of apoptotic mediators towards survival of the cell [32].

3.2. The site of injury

3.2.1. Nerve guidance scaffolds

In the case of a nerve gap, when tension-free neurorrhaphy is not possible, interposition of an autologous nerve graft is the current gold standard [1]. This provides structural support to guide axonal regeneration, preventing neuroma formation and fibrous tissue invasion; however, this mandates more extensive surgery with permanent loss of sensation at the donor site, likelihood of structural mismatch and a limited source of nerve grafts [33,34]. These problems have stimulated considerable investigation into manufacturing nerve guidance scaffolds in anticipation of promoting improved nerve regeneration through limitation of myofibroblast infiltration, reduction of scar formation and accumulation of neurotrophic factors in high concentrations [35]. However, commercially available devices (mostly biodegradable polymer or collagen-based hollow tubes) have failed to match the regenerative levels of autologous nerve grafting, they are limited to short defects (<2 cm) and show poor functional recovery [36].

Recent developments in the tissue engineering field have changed the concept of a passive scaffold that simply provided a protected space for nerve regeneration to an active environment model, which seeks to promote neural outgrowth and accelerate axonal regeneration (Fig. 2) [37]. Additional physiochemical and biological cues have been investigated and the experimental contribution of cell therapy is considered below (Section 3.2.3). The intraluminal surface topography of scaffolds can be modified to support cellular attachment, morphology and alignment [38]. Different topographical cues such as grooves, electrospun fibres, gels and films have been investigated to direct the outgrowth and migration of SCs and neuronal cells [39–42]. Furthermore, biomaterial chemistry can optimise cellular behaviour with short peptide sequences (e.g. RGD) facilitating cell adhesion on the surface of polymers [43]; and polymer modification by hydrolysis and aminolysis reactions enhance SC elongation and proliferation on polycaprolactone (PCL) films [44], whilst ECM molecules support SC attachment and stimulate excretion of neurite promoting factors [45].

3.2.2. Pharmacotherapy

Currently there are no clinically available pharmacological treatments for nerve injury. Nevertheless, several small molecules, peptides, hormones and growth factors have been suggested as potential candidates to improve nerve regeneration by reducing neuronal death following injury (Section 3.1) and by promoting the regeneration of the outgrowing axons. NGF enhances axonal outgrowth both when administered locally and systemically [46–48]. In a similar manner, BDNF and ciliary neurotrophic factor (CTNF) administration improves regeneration distances, re-myelination and functional recovery [49–52]. Axonal regeneration speed and functional outcomes can also be improved by local supply of insulin growth factor-1 (IGF-1), FGF or GDNF [53–56]. Neuregulin-1 (NRG1) signalling is a key player for axonal myelination during development, making it a putative target for re-myelinating therapies following injury [57]. Clinical administration of growth factors towards neuronal survival or axonal regeneration is not without problem; in particular the timing and dosage of treatment, the method of administration and release, interactions with other growth factors and the potent side-effect profiles as discussed above (Section 3.1). Indeed, if BDNF concentration is too high or badly timed, this could result in inhibition of axonal growth and even increased neuronal death [58,59]. A possible solution to this issue could consist in the exogenous modulation of endogenous growth factor expression by other pharmacological means [60], the delivery of growth factors by controlled release systems [61] or transplantation of growth factor expressing cells (Section 3.2.3).

Hormones are a promising alternative for pharmacological intervention in the treatment of nerve injuries. Neuroactive steroids, such as progesterone or allopreganolone, modulate SC physiology through

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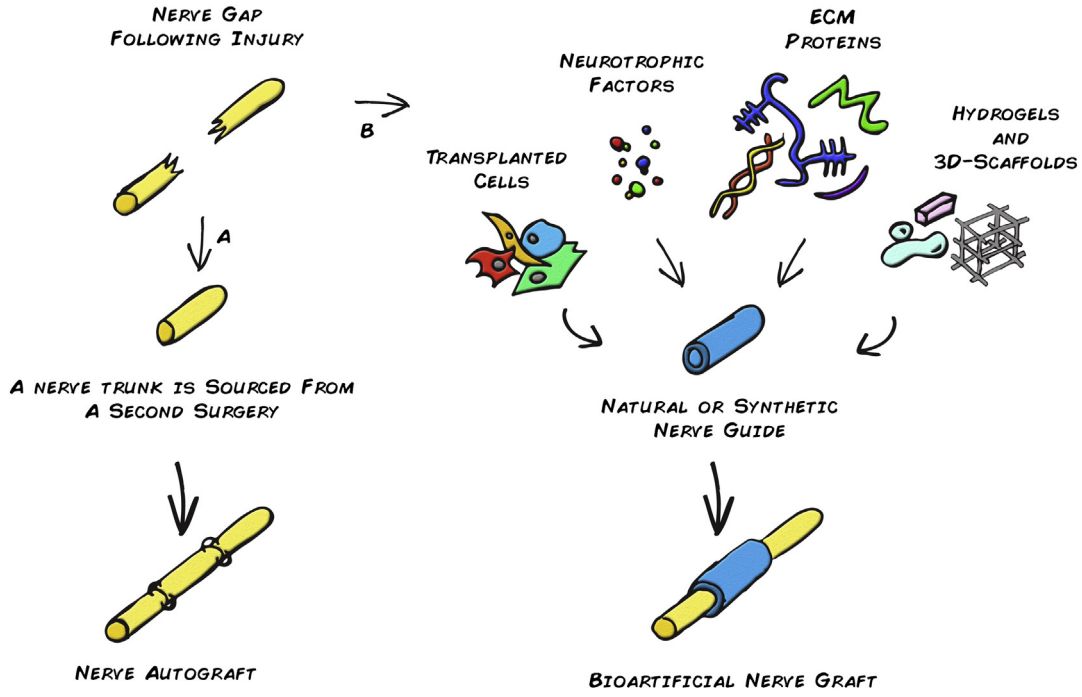

Fig. 2. Bioartificial nerve graft for nerve repair. As an alternative to autograft (A), the "gold standard" for nerve repair, the engineering of an artificial nerve graft (B) has been sought. Such graft will consist in a natural or synthetic nerve guide, which could be enriched with several factors to enhance axonal regrowth such as: 1) Transplantable cells, 2) neurotrophic factors or other pharmacological aids, 3) extra-cellular matrix (ECM) proteins and 4) hydrogels or 3D scaffolds as conduit fillers or cell/drug vehicles.

action on the expression of myelin proteins and SC differentiation [62,63]. Thyroid hormone and growth hormone have been shown to improve axonal myelination, myelin thickness and functional recovery in rat sciatic nerve injury models [64,65]. Finally, neurotransmitters such as γ-aminobutiric acid (GABA), adenosine triphosphate (ATP), glutamate and acetylcholine play key roles in neuronal-glia interaction, and their receptors (on neurons or SCs) have been proposed as potential targets for the development of pharmacotherapies for nerve repair [60]. In particular, both the metabotropic GABA-B receptors and the ionotropic $P2X_7$ receptor for ATP, have been shown to be key signalling pathways modulating SC development and differentiation towards SC myelinating and non-myelinating phenotypes, suggesting their ligands as promising tools to aid re-myelination following nerve injuries [66–68].

3.2.3. Cell therapy

Given the complex events that follow nerve injury and lead to nerve regeneration, the identifications of specific molecules or single targets to promote nerve repair has proven extremely challenging. A great hope in the field of regenerative medicine for nerve repair is the exploitation of the regenerative potential of cell-based therapies. This is of particular relevance especially for long gaps, where the use of nerve guides alone has failed to provide successful regeneration (Section 3.2.1).

Given the importance of SCs in PNS development and following injury, they have been utilised as transplantable cells in experimental nerve repair, and demonstrated improved regenerative outcomes [69–72]. Similarly, another source of specialised glia Olfactory Ensheathing Cells (OECs) can provide trophic support to regenerating nerves and contribute to the re-myelination process [73,74]. However, the sourcing of allogeneic or syngeneic SCs requires the sacrifice of a functional nerve, and both OECs and SCs have limited expansion capabilities, which hinders their use for neural tissue engineering [75]. For this reason there is a great interest in the study of alternative cell sources and stem cells represent the most promising avenue for the development of cell-based treatment for nerve repair.

Embryonic stem cells (ESC), neural stem cells (NSC), induced pluripotent stem cells (iPSC), and adult mesenchymal stem cells (MSC) from several niches have been the subject of several *in vitro* and *in vivo* studies investigating their suitability for nerve repair. Mouse ESC-derived neural progenitor cells promoted nerve repair in a rat sciatic nerve 10 mm gap as shown by histological, molecular and electrophysiological studies [76]. SC-like precursors can be generated from human ESC and have been shown to express myelin protein in models of peripheral nerve regeneration *in vitro* [77]. NSC showed results comparable to autografts when seeded in chitosan nerve guides to repair a 10 mm nerve gap [78], and they can be genetically engineered to overexpress GDNF or NT-3, which can potentially improve their regenerative potential [79–81]. iPSC can efficiently generate functional neural crest cells and have been used, in combination with FGF-incorporated gelatin microspheres, to repair 10 mm nerve gaps with 50% poly l-lactide (PLA) and 50% poly ε-caprolactone porous nerve conduits [82–84]. MSC can be found in most adult organs including bone marrow, adipose tissue, liver, dental pulp, skin and skeletal muscle where they control maintenance and repair of their tissue especially following injury [85–89].

Compared to ESC and iPSC, the use of MSC in regenerative medicine comes with less ethical implications regarding the sourcing of the cells, and the risk of teratoma formation or undesired cell differentiation. Bone marrow-derived MSC (BM-MSC) showed multipotential properties being able to generate cells from the mesodermal lineage [90–92], but also ectodermal or endodermal precursors [75,92,93]. SC-like BM-MSC [93,94] have been extensively studied in several *in vitro* and *in vivo* models of nerve regeneration, showing molecular and functional similarities to native SCs and representing one of the most promising sources of SC-alternatives for nerve repair [95]. Some of the problems related to the use of BM-MSC include the painful procedure for the harvest of marrow aspirates and the low yield of mononucleated colony forming units (CFU). By contrast, ASC obtained from the stromal vascular fraction (SVF) following digestion of adipose tissue contain 600× more CFU [96], and these cells proliferate faster and for longer durations [97,98]. ASC and BM-MSC have similar immunological profiles [99] and express the same cell surface markers [100–103]. Like BM-MSC they are multipotent and they can generate functional SC-like cells able to

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improve nerve regeneration *in vitro* and *in vivo* [104,105]. The use of undifferentiated and differentiated ASC for peripheral nerve repair has been recently reviewed [106,107]. Other promising sources of MSC for nerve repair are the skin [88], the umbilical cord [87] and the dental pulp [89], which can all differentiate to SC-like cells and improve peripheral nerve regeneration.

3.3. The target organ

Skin sensory receptors can be reinnervated effectively years after injury so if it is possible to preserve sensory neurons (Section 3.1) then recovery of sensation is possible. In contrast, denervated muscle progressively loses its ability to become reinnervated. Therefore, in addition to targeting regeneration of the nerve it is also necessary to prevent or minimize denervation-induced atrophy of the muscle. Experimental research suggests that intramuscular injections of various growth factors or stem cells could be a useful adjunct to the nerve repair. Furthermore, in situations when atrophy is extensive and the muscle can no longer receive the regenerated axons effectively it may be necessary to physically reconstruct the muscle. This is currently done with surgical muscle transfers but developments in the tissue engineering of skeletal muscle might one day be able to produce significant quantities of new tissue for transplantation.

3.3.1. Growth factors and cell therapy

IGF-1, a potent myogenic molecule, can be delivered effectively into injured muscle using a muscle specific non-viral vector [108]. In a rat laryngeal paralysis model, Shiotani et al. showed that injections of IGF-1 resulted in increased muscle fibre diameters, reduced motor endplate lengths and significantly increased percentage of endplates with nerve contacts [108]. The same research group subsequently demonstrated that the IGF-1 vector was equally efficacious when treatment was delayed after the nerve injury [109]. More recently, it was shown that intramuscular delivery of IGF-1 works by up-regulating the myogenic regulatory factors, myoD and myogenin [110]. Intramuscular injection of adeno-associated viral (AAV) vectors has also been used in a number of different experimental animal models and can correct various conditions such as muscular dystrophy and age-related atrophy [111]. In a rat median nerve injury model, intramuscular overexpression of vascular endothelial growth factor (VEGF) by means of AAV-VEGF vectors significantly reduced the progression of muscle atrophy [112]. Another study investigated the synergistic effects of an intramuscular injection of a mixture of growth factors (NGF, CNTF, GDNF) on functional recovery following rat sciatic nerve injury. Combined administration of these three factors increased muscle weights and promoted functional recovery [113]. The various growth factors might work through general systemic effects or boost the function of reinnervating motor neurons via their retrograde transport back to the neuron cell bodies in the spinal cord [114].

Muscle satellite cells are the intrinsic stem cells that help to rebuild muscle after neuromuscular system injury. Denervated muscle function can be improved by injecting exogenous in vitro expanded satellite cells [115]. Bacau and colleagues subsequently tested high-density injections of either adult myoblast or ASC into the denervated rat tibialis anterior muscle [116]. However, after 4 months the animals showed no benefits in terms of function or muscle morphology. In contrast, Halum et al. showed that autologous myoblasts injected into denervated rat laryngeal muscles, survive and fuse with the intrinsic fibres resulting in larger fibre diameters and volumes than those found in control animals [117]. Some animals showed functional improvements although there was no significant increase in muscle reinnervation as a result of the treatment [117]. ESC, differentiated into cholinergic neurons, form new neuromuscular junctions when injected into denervated rat gastrocnemius muscles, but their effect on preventing muscle atrophy is only short lived [118]. ASC differentiated into a SC-like phenotype reduce muscle atrophy when injected intramuscularly [119]. The cells might contribute to remodelling the neuromuscular junctions and/or they might fuse directly with the muscle fibres. Together these effects lead to functional improvements in a walking track test [119]. It is also likely that intramuscular injections of stem cells could boost muscle function through their expression of various growth factors. However, this hypothesis was not proven in a recent study [120]. Jiang et al. injected CNTF-expressing BM-MSC into denervated muscles and showed they could preserve muscle function and morphology but knockdown of CNTF did not abrogate these effects [120]. With regard to the clinical translation of these experimental studies, it is encouraging to note the results of a recent pilot study of nine brachial plexus patients with insufficient elbow flexion (partial denervation) treated with intramuscular injections of autologous BM-MSC [121]. Cell therapy produced significant decreases in muscle fibrosis, increased myofibre diameters and number of satellite cells and an increased capillary-to-myofibre ratio. Furthermore, patients showed increased motor unit amplitudes suggesting that the treatment could improve both muscle reinnervation and regeneration [121].

3.3.2. Tissue engineering new muscle

In cases when the endogenous regenerative capacity of the muscle is exhausted, replacement of the damaged tissue may be required. Any tissue-engineered muscle construct should be designed such that it has the appropriate structural and mechanical properties to promote rapid muscle recovery. The most basic requirement for a construct is a suitable cell source which can be easily cultured, expanded and differentiated into muscle lineage. Satellite cells or myoblasts are an obvious choice but their optimal growth properties have not been established and many other cell types including ESC, iPSC, pericytes and MSC have been tested [122]. Various types of scaffolds have been developed, many of which act to mimic the natural ECM of muscle, and they are typically made from resorbable substrates which remodel upon degradation [123]. As is the case for nerve conduits and the regeneration of axons, aligned nano- and micro-scale topographic features of polymer scaffolds provide for alignment of myoblasts and promote myotube assembly and organization into mature muscle fibres [124]. 3D gels such as collagen and fibrin and sheets of cells have all been used with varying degrees of success; one advantage of gels is that they can be used to release growth factors such as IGF-1 and VEGF, which augment myogenesis [123]. To enable cellular proliferation and differentiation the constructs need to be adequately vascularized and this has been addressed by the inclusion of endothelial cells but, if possible, surgical anastomosis with existing vasculature is the best approach [125]. Constructs also need to be able to support effective reinnervation and new 3D models of neuromuscular junction connectivity are informing design of these structures [126]. The recent advances in progenitor/stem cell biology and scaffold technologies suggest further progress can be made towards a clinically relevant tissue engineered muscle construct.

4. Future perspectives

4.1. Future clinical perspectives

Current peripheral nerve repair practice closely resembles the description by Gabriele Ferrara (1543–1627) of 400 years ago who detailed the procedure consisting of disinfection, appropriate identification of nerve stumps, a gentle suturing technique and limb immobilisation [127]. New concepts in technique were established in response to the injuries treated during World War II and included the use of autologous nerve grafts, primary and secondary repair [128]. In the 1960s, the incorporation of the operating microscope and nerve repair techniques which recognised the detrimental consequence of suture line tension, improved the technical aspect of nerve repair [129]. More recently, surgical techniques such as free muscle transfer and nerve transfer have been established in an effort to bypass the complex and inhibitory neurobiology of prolonged regeneration through a denervated

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distal stump towards the target organ. These novel methods can provide a substitute for some functions but will not deliver the outcomes that are sought for nerve-injured patients [130].

The next raft of clinical advancement is most likely to be delivered through greater understanding of the neurobiological implications of peripheral nerve injury and the experimental strategies described above. Many are approaching clinical application; however as novel interventions develop in other fields, this will open the door to alternative strategies in addressing peripheral nerve regeneration.

4.2. Future scientific perspectives

Given the multi-faceted aspects of peripheral nerve injury and the various clinical scenarios, combining pharmacological and molecular therapies with novel surgical intervention will be required. Using new nerve constructs together with molecular targeting strategies, for example developing scaffold materials to deliver drugs and silencing RNAs, should facilitate the intrinsic growth mechanisms. Approaches should focus not just on enhancing axonal regeneration but also target the SCs in the distal stump and the molecular reactions triggered in the denervated muscle. It should be recognised that many of the experimental advances made to date, remain limited to boosting regeneration over short distances, and new tissue engineered constructs should be able to support axon growth over longer distances, with the ultimate goal of matching the autologous nerve graft. A greater understanding of the structural anatomy and extracellular matrix of the peripheral nerve should give more informed construct design. For instance, the ability to mimic a bands of Büngner-like structure would more accurately replicate the architecture of the autologous nerve graft. As constructs are developed for larger defects, it will be necessary to ensure they are efficiently vascularized to maintain the viability of any transplanted cells. Computer and mathematical modelling could be useful tools in designing new constructs. Downstream, advanced 3D *in vitro* models should be used to more accurately mimic the anatomical and physiological properties of the peripheral nerve and to test these constructs. Finally large animal studies are required to determine the effectiveness of the constructs on the long nerve defects, which still remain a great clinical challenge.

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