Spinal cord regeneration: A brief overview of present scenario and a sneak peek into the future

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Abstract

Central nervous system (CNS) portrays appreciable complexity in developing from a neural tube to controlling major functions of the body and orchestrated co-ordination in maintaining its homeostasis. Any insult or pathology to such an organized tissue leads to a plethora of events ranging from local hypoxia, ischemia, oxidative stress to reactive gliosis and scarring. Despite unravelling the pathophysiology of spinal cord injury (SCI) and linked cellular and molecular mechanism, the over exhaustive inflammatory response at the site of injury, limited intrinsic regeneration capability of CNS, and the dual role of glial scar halts the expected accomplishment. The review discusses major current treatment approaches for traumatic SCI, addressing their limitation and scope for further development in the field under three main categories- neuroprotection, neuro-regeneration, and neuroplasticity. We further propose that a multi-disciplinary combinatorial treatment approach exploring any two or all three heads simultaneously could alleviate the inhibitory milieu and ameliorate functional recovery.

Keywords: Central nervous system**,** spinal cord injury, neuroprotection, neuroregeneration, neuroplasticity

Introduction

The development of the nervous system initiates with the formation of neural plates during the gastrulation stage of embryonic development; the plate further bends and folds to form a neural tube, which develops with time into the brain and spinal cord, compositely called the central nervous system of the body[1]. The brain and spinal cord reside in cranial cavity and spinal canal, respectively, and are surrounded by three meninges- duamater, arachnoid, and piamater[2]. The spinal cord consists of an inner core of gray matter surrounded by white matter and acts as relay centre between brain and rest of the body, also modifying and integrating signals as they travel to and from the brain and thus controlling the sensory, motor, autonomic functions of the body. Any kind of damage to the spinal cord leads to loss of intricate communication between brain and body and hence forth loss of sensory, motor, and autonomic (sexual, urinary, intestinal, and cardiovascular) functions depending on level and extent of injury. The damage can be congenital[3] as most of the congenital abnormalities like spinal bifida or could be acquired as in traumatic injury, infections, neoplastic interventions or neurodegenerative diseases[4,5]. According to the World Health Organization (WHO), approximately 25 million people are affected with some kind of spinal cord injury, and 90% of those are traumatic cases. Traumatic injury results in most severe consequences as they initiate with the fracture of vertebrae, which compress the spinal parenchyma or may cause partial or complete transection leading to permanent neurological deficit for the rest of the life of patient. Cellular and molecular niche at the site of injury is a major player for its repair or regeneration, which unfortunately is nonconductive and hostile in case of spinal cord [6-7]. Decompression surgeries could only provide some symptomatic relief after spinal cord injury. Although neuroregenerative therapies, neuroprotective pharmacological interventions, and stem cell transplantation could re-establish the lost neuronal functions but none of the approach could make its place in the clinics. Thus, a more comprehensive and

multi-disciplinary approach is the need of the hour to combat the condition successfully. This review is an attempt to understand the different treatment approaches, their limitations and speculations for ideal treatment approach.

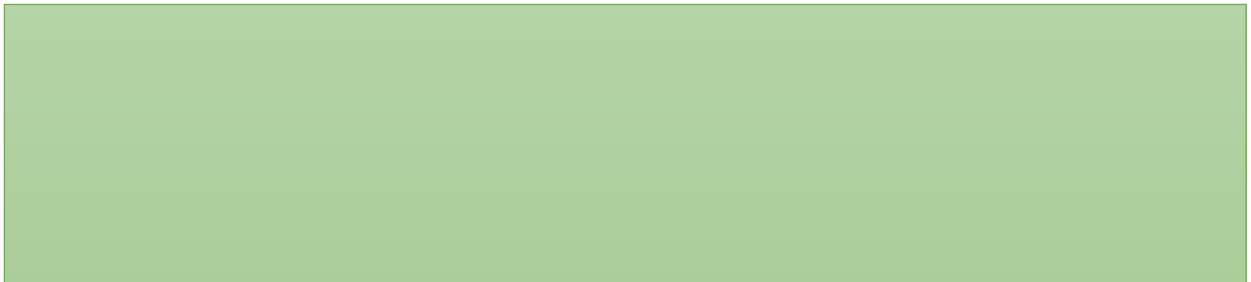
Pathology of spinal cord injury

The hallmark of any living system pathology is its intricate circuitry with time, which divides the progression of traumatic SCI temporally into three phases: acute, intermediate, and chronic phase, and thus, any therapeutic intervention must synchronize rationally with the temporal progression of the injury. The primary injury at the lesion epicenter initiates a continuum of irreversible reactions sharing congruency with standard wound repair mechanism. The acute phase is marked by cell death and inflammation which progresses with cell replacement and repair during sub-acute phase ultimately resolving with the formation of glial scar during chronic phase[8]. The disruption of blood brain barrier and necrosis of intrinsic cells leads to ischemia, hypoxia, extravasation of immune cells and vasogenic edema during primary phase of injury[9]. The inflammation continues during the secondary phase of injury creating cytotoxic environment comprising cytokines, free radicals, and excitatory neurotransmitters detrimental for tissue growth[10], which is demarcated by glial scar separating non-functional lesion core from potentially functional adjacent neural tissue[11]. The axonal damage could propagate as either irregular varicosities or axonal spheroids. Thus, these sequences of events offer great opportunity for therapeutic intervention, targeting hypoxia, ischemia in acute, inflammatory mediators in intermediate and hypertrophic astrocyte in chronic phase to modulate the repair process after injury. Examining the functionality of injured tissue **(Box1)**[12], SCI can be categorized into two types- complete or incomplete. The complete injury is marked by absence of any neuronal connectivity across the lesion site and thus complete loss of motor control or sensory perception below the lesion site. Incomplete injury spare certain amount of neural tissue across the lesion and thus

imparting neuronal connectivity and circuit reorganisation potential through supraspinal or propriospinal sources.

Comprehensive overview

Appreciable research on varied aspects of spinal cord regeneration has been explored all over the globe, obtaining some positive results in-vitro and in-vivo, where a few therapies could make into clinical trials. Designing any meticulous treatment relies to a great extent on understanding the lesion architecture varying from microscopic damage to severe contusion or transection injury with different forms of axonal growth possible from true regeneration to sprouting. Such variability at severity level, axonal growth type and functional recovery assessment complicates the formulation of single standard treatment approach. The prominent aspects to focus are hostile extrinsic environment[13,14], intrinsic incapability of neurons to grow[7,15] and restructuring the lost/disrupted synaptic circuitry[16]. Addressing these aspects, the treatment strategy can be categorized as: a) neuroprotection- modulating inhibitory/toxic milieu, b) neuroregeneration- bridging the lesion site by augmenting neurogenesis and, c) neurorehabilitation- enhancing circuit reorganization and synaptic connectivity[17-18] as represented in **Figure 1**. The approach undertaken to catalyze functional recovery varies with the injury type- contusion injury holds certain degree of spared functional tissue that can rejuvenate its functionality by appropriate neuroprotective therapeutics or neurorehabilitation-based training, contrasting to transection injury creating a gap potentiating the need for neurogenesis and denovo connection connecting rostral and caudal segment to facilitate the transmission of information. Thus, the mode and time of therapeutic intervention must rationalize the injury type and phase of pathology. Aiming to restore lost functionality, these approaches differ slightly in their therapeutic target and expected outcome as further discussed.



Box1- Neurological impairment assessment and initial stabilization- The extent of neurological impairment is evaluated over 28 dermatomes and 10 paired myotomes including external anal sphincter and assigned grade A to E (A= no sensory or motor function and E= intact cord) based on criterion laid by AIS (American spinal injury association- international standards) for neurological classification of SCI. The detailed view of injured site through X-Ray, CT-Scan, fMRI assist in efficient prognosis followed by initial respiratory, cardiovascular and hemodynamic stabilization, surgical decompression and administering different anti-inflammatory drugs.

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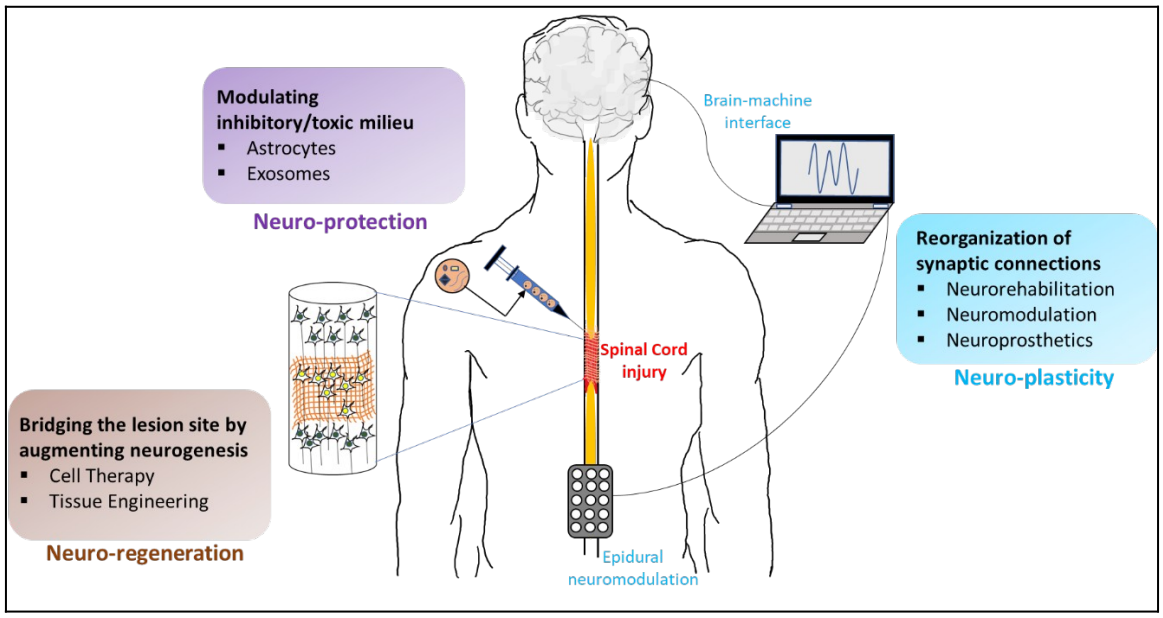

Figure 1: The three major approaches for the treatment of spinal cord injury i.e., a) neuroprotection b) neuro-regeneration, and c) neuro-plasticity are depicted in the figure as discussed in the review

1. Neuroprotective therapy

It includes protecting the injured though functional tissue from inhibitory milieu (further damage). The target of such therapy could either be cellular players at lesion or hostile milieu as discussed below.

1.1 Targeting the astrocytes- Any sort of trauma to spinal cord invites plethora of inflammatory cascades that resolves with time into a remodelled tissue yielding mature scar at the lesion site. The scar divides the lesion site into three compartments, each with distinct

cellular composition; (a) an inner lesion core also called as fibrotic or mesenchymal scar majorly composed of non-neural extrinsic cells, (surrounded by) (b) glial scar constituted by astrocytes, microglia and other glial cells which is continuous with (c) adjacent functional yet reactive neural tissue[19-20]. The glial scar formation is a unique event specific to CNS injury and even more fascinating is its constituent astrocytes, which though conventionally proved to be detrimental for axonal regeneration, amazed the neuroscientist with its neuroprotective influence over injured milieu. Astrocytes exert extremely influential role in CNS, from synaptic organisation during development to overcoming oxidative stress in adulthood or recruiting and restricting inflammation during injury. They secrete a range of molecules like thrombospondins, and hevin, modulating synaptic plasticity to sulphated proteoglycans restricting unintended connections[22-21]. An injury initiates continuum of irreversible reactions, termed as reactive astrogliosis leading to transformation of astrocytes from native to reactive state exerting a neuroprotective effect that soon proliferate and transforms into scar forming astrocytes inhibiting axonal regeneration[22,23]. Attenuating this transformation from reactive to scar forming astrocytes *via* targeting integrin *N*-cadherin pathway led to accelerated axonal growth, but can the cells depicting an array of diverse functions be resolved with such simplistic approach[24]. Thus, most evidence supports the fact that ablating astrocytes has expanded lesion cavity, increased death of neurons, increased demyelination, and worsened the functional outcome[8]. One of the controversial studies supporting the assertive functionality of astrocytes towards regeneration comes from Anderson's lab. It has been demonstrated that ablating the acute or chronic astrocytes led to no increase in axonal outgrowth, hence positing astrocyte's optimistic role in axonal growth[25]. The study was questioned by scientist like Jerry Silver, where undermining the role of other glial cells or inhibitory factors over the results obtained was strongly challenged[26]. Some recent study does explore the functional heterogeneity among astrocyte subpopulation where

neuroinflammation induced A1 astrocytes secreted neurotoxin. However, ischemia induced A2 astrocytes exerted a neuroprotective effect, thus paving the way for tremendous therapeutic opportunities inherited in astrocytes[27]. These cells exert decisive over the success of scaffold or cell transplantation at the lesion site. SC matrigel bridge implicated that the astrocytes process could either align along the brain stem axon escorting the regenerating axons from inhibitory milieu or form a dense boundary inhibiting their regeneration [28]. Studies suggest the synergistic effect of astrocytes over transplanted exogenous cells leading to the up-regulation of axonal growth-promoting genes and associated signaling pathways[29]. With the advent of advanced genetic tools, re-engineering astrocytes to induce neurogenesis and further functionalization of these neuroblast into specific neuronal sub-types with single transcription factor has been achieved[30]. These recent studies surely prompt us to conclude that astrocytes are inherited with a diverse array of functions and potent therapeutic potential, thus contradicting the tradition notion of being a mean to restrict inflammation or inhibit regeneration[31]. It is relevant to mention that glial scar and astrocytes are not synonyms. Therefore, various other biological[32] (NG2 glia) and biochemical factors[33] (CSPG, Nogo) have an influential role to play towards regeneration that contradicts the notion of spontaneous regeneration on ablating astrocytes[26]. With such a conclusion comes an even intimidating challenge of manifesting beneficial postulates of astrocytes over its detrimental influence.

1.2 Overcoming the inhibitory milieu- Besides re-engineering the cellular candidates, the prominent inflammatory events like macrophage infiltration, microglial activation, and astrocytes forming the scar are being targeted for different therapeutic interventions. Some compounds unveiled with therapeutic potential are discussed in **Box2**, in which the recent entity to be added is exosome.

Exosomes as neurorestorative therapy- Intercellular communication has traditionally been believed to mediate through transport channels, direct ligand receptor signalling, gap junctions or tunnelling nano or microtubes[34]. It is only recently that membrane vesicles have emerged as an intriguing mode of communication between cells, although their observation dates back to 1960s in literature. Cells give rise to variety of extracellular vesicles (EVs), both *via* endosomal pathway or budding of plasma membrane termed as microvesicles (ectosomes), microparticles, exosomes, oncosomes etc[34]. Right from elucidating their function in immune cells activation to determining RNA and protein as their trafficking constituents, arduous effort by scientists unveiled that these membrane vesicles ensured protected and directed transfer of information in spatiotemporally organised manner echoing the parental cells and acting as cell biopsy[35]. With advancing nanotechnology, the nano vesicles secreted by cells termed exosomes grabbed the lime light, complying with the saying "size does matter"[36]. Their synthesis initiates with the invagination in endosomal membrane (late endosome) giving rise to vesicle (intra luminal) filled with specific protein, lipids and nucleic acids, which further pinches off into endosomal space, giving rise to multivesicular bodies (MVBs) filled with intraluminal vesicles (ILVs,)[37,38]. These MVBs fuse either with plasma membrane or lysosomes, constituting secretory or degradative pathway, where secretory pathway leads to release of ILVs termed as exosomes and degradative leads to

Box 2: Pharmacotherapy- The pathophysiology of SCI is intricately linked with inflammation, the onset and resolution of which is crucial for survival and functional recovery. Administration of Maresin 1 (Mar1- a highly conserved SPM) lead to effective catabasis and efferocytosis in a contused mouse[158]. Optimistic results with Simvastatin in ameliorating oxidative stress and melatonin targeting astrogliosis and microgliosis, paved way for their future investigation as neuroprotective agents[159]. The inhibition of BET proteins [epigenetic readers for inflammatory genes] *via* JQ1 led to improved functional recovery, elucidating the intricate crosstalk between epigenetics and SCI pathology[160]. Oral treatment with glycyrrhizin (isolated from Glycyrrhiza glabra root) induced the polarisation of macrophages towards anti-inflammatory M2 subtype leading to improved functional recovery[161]. Some other compounds like curcumin[162], substance P, melatonin[163] etc. are been mined to demonstrate their efficacy in ameliorating inhibitory milieu.

discarding the cargo[34]. The third possibility of MVBs acting as a temporal storage compartment also exists[39]. Exosomes initially observed in supernatant of cultured sheep erythrocytes, are uniform lipid bilayered nanovesicles with some unique lipid, protein and miRNA composition. Some intriguing aspects of exosomes such as nano dimension, low immunogenicity, non-toxicity, permeability for physiological barriers, stability in circulation and its ability to modulate autocrine or paracrine signalling, claims it to be excellent delivery vehicle which can be engineered with desired cargo[40-41]. The fate upon release may follow either fusion with plasma membrane or endocytosis by target cells[42]. The complexity does arise with diversity in population of exosomes, the influence of microenvironment in their packaging and their participation in propagation of inflammation. Still, their constitutive and regulated release suggest an evolutionary conserved mechanism of intercellular communication[43]. The intricate morphology and functional integrity in nervous system requires precisely controlled shuttling of genetic and molecular codes between neurons and glial cells[44]. Nanovesicles secreted in CNS contribute not only towards survival, plasticity or immune regulation but also assist in propagating proinflammatory signals in diseased condition, securing their candidature as efficient non-invasive biomarker for diagnosis of diseases[45-46]. The functionality of exosomes is reported to be dependent on microenvironment present during their biogenesis, context dependent and cell type specific. One of the fascinating studies exploring the crosstalk between neurons and astrocytes *via* exosomes stated that intraperitoneal injection of RAR beta agonist induced release of PTEN in exosomes. These PTEN-exo were taken up by astrocytes, which altered their morphology from mesh like entangled processes to elongated tunnelling bridge, ablating scar formation and enhancing regeneration. Such intercellular communication through exosomes that could assist in modulating inhibitory milieu and augmenting regeneration mediating by different indirect pathways certainly demand further investigation[47]. One of the extensively explored

secretome profile belongs to mesenchymal stromal cells (MSCs), where the therapeutic efficacy of their paracrine trophic factors paved way for cell free therapy[48,49]. From exploring the conditioned media to studying the secreted cytokines or growth factor, MSCs have been central research idea for SCI[49,50]. The arduous efforts by various neuroscientists have narrowed down the search to extracellular vesicles (EVs), in particular exosomes[51] which not only rescued the dislodging incapability and tumorigenic potency of MSCs, but also depicted comparable efficacy in exerting neuroprotective influence[52-53]. The therapeutic efficacy of MSCs are mostly inherited in exosomes when compared to their differentiating potency or cell-cell contact is now a scientific fact than mere observation[54-55]. The exosomes vary in their molecular signature depending on the source of MSCs. The conditioned MSCs exosomes (such as hypoxia induced exosomes) have been proved to be beneficial in the context of gait recovery than normal conditioned exosomes[56]. These optimistic conclusions do specify the significance and specificity of composition of exosomes which are mostly inherited in mRNA and miRNA packed within, that could shuttle between different cells. These uniquely packed exosomal shuttle RNA can be transferred between different cells and translated using host active protein synthesis machinery, hence dynamically modulating the proteomics of the recipient cell[57]. These studies did pave way for exosome engineering with desired miRNA[46] [miR-17-92[58], miRNA-133b[59]], and siRNA [PTEN-siRNA, siRNA-ASC][60,61] to augment their therapeutic efficacy over injured spinal cord niche. Thus, the journey that once started as nanovesicles has reached on fabricating "designer exosomes" making its way to clinical trials[62], but the field still lag behind due to lack of standard isolation protocol, largescale manufacturing and quality control and fractionating this entity based on biogenesis or composition. Unveiling the molecular mechanism and refining safety and efficacy of the therapy, decoding and encrypting desired pro-regenerative messages with designer exosomes surely paves way for future elaborative research.

2. Neuro-regeneration

The term neuro-regeneration implies regrowth of transacted axon through the lesion and hence in no sense should be confused with sprouting i.e. branching from uninjured intact axon[17]. The dichotomous behaviour of lesion cavity, although being inhibitory towards regeneration, does provide a transplantation site for cellular graft. These grafts not only modulate the inhibitory milieu by secreting trophic factors but also assist in formation of denovo circuit replacing the lost tissue[63,64]. Some pioneering work from Cajal's laboratory demonstrated that transplanting peripheral nerve graft into injured spinal cord led to efficient regeneration concluding that CNS lacked specific chemoattractant to induce axonal regeneration[65]. Supporting the conclusion, David and colleagues posited that CNS niche either lacked specific cues for regeneration or exhibited cues that inhibits axonal growth[66]. Exploring the difference in regeneration capacity of neonate and adult rats, team led by Bregmann concluded that at birth the transplant (fetal spinal cord) can function both as bridge and relay by enhancing functional synapsis but only as a relay during adulthood[67]. Reier underscored these critical observations postulating the significance of modulating the inhibitory milieu with the suitable implant (PNS or spinal grafts) that assisted in relaying supraspinal information caudal to lesion site and hence the circuit reconstruction[68]. Transplanting Schwann cells, Richard Bunge successfully demonstrated regeneration in inured CNS[69]. These strong conclusions of 20th century with simple experimental plans and contemporary techniques sustain the present innovative research, equipping CNS to be no longer an incurable conundrum. Cell based therapy came out as an advancement over nerve grafts due to their injectable and genetically modulating avenue posing limited damage to spared tissue and enhanced trophic support[64]. Advent of iPSCs[70] and other cell types like MSCs[71], SCs[69], NSPCs[72], ESCs, OPCs , OECs[73] are reported to assist nerve regeneration, each

with its own pros and cons[74]. The two routes to achieve efficient nerve regeneration can be augmenting native stem cells or injecting/implanting exogenous cells at

injured loci to reconstruct lost circuitry where genetic manipulation of these cells to enhance their therapeutic efficacy has also been explored as discussed in **Box 3**.

Box 3: Genetic interventions- Expressing several transcription factors to enhance regeneration is been practised owing to limited intrinsic capacity of CNS to regenerate. The rationale behind selecting the appropriate factor dates back to 1984, with the emergence of concept of "conditioning lesion"[164,165]. DRG neurons with pseudo unipolar structure extend a central and peripheral branch sharing a common cell body. Peripheral branch being capable of regenerating, transacting a peripheral branch a priori or concomitantly to central transaction initiates regenerative cascades that assist not only regeneration of peripheral but also central axons. The common cyton thus act as transcriptional hub for expressing "regeneration associated genes (RAGs)" that would not be expressed following central lesion only. Several studies involving co-deletion of SOCS 3 and PTEN led to activation of STAT3 and mTOR resulting in robust collateral sprouting, blocking Ryk-Wnt signalling coupled with neurorehabilitation and activation of epigenetic factors like PCAF *via* retrogradely induced pERK induced regeneration and functional recovery[166-167]. Such genetic[168,169] and epigenetic factors[170-171] have been extensively reviewed where a major setback comes from these transcription factors to be cell type specific and expressed under strict temporal regulation in cell. It is equally important to mention that a repressive chromatin landscape is a major impediment leading to failure of overexpressed transcription factors, hence lack of pro-regenerative epigenetic regulation is now being explored extensively to initiate regeneration after CNS lesion[8].

2.1 Augmenting the endogenous stem cells- The CNS seat neural stem cells at the subventricular zone of lateral ventricles, subgranular zone of hippocampal dentate gyrus in brain and spinal cord ependyma or parenchyma[75-76]. Exploring ependymal cells as stem cells, several studies report injury induced activation and differentiation of these cells into oligodendrocytes and majorly into astrocytes constituting the glial scar. Their close proximity to sprouting axons argued against their growth detrimental nature[77]. The epSPCi (ependymal

stem progenitor cells injury) differentiated into motor neurons, augmenting functional recovery, acting as a backup to self-repair during adulthood[78,79]. These observations were strongly challenged by Ren and colleagues suggesting that contribution of ependymal cells is mostly restricted to resealing the central canal, and they possess limited migration or differentiation capacitance[80]. Such contrasting reports do interrogate the candidature of ependymal cells as stem cells, thus modulating the inhibitory lesion core to enhance endogenous neurogenesis seems an intelligent option. Fabricating advanced scaffolds mimicking the native extracellular matrix (ECM) that could transform the inhibitory lesion core into growth permissive niche has been reported *via* implanting CBD-Fab collagen scaffolds (bovine aponeurosis) that supported migration and maturation of NSCs into GABAergic and dopaminergic neuron improving motor functions[81]. F-SAP (functional-self assembling peptides) conjugated with growth factors led to the formation of neuronal interconnections by recruiting eNSCs (endogenous neural stem cells) and OLs (oligodendrocytes) in rats with complete transection[82]. Injecting nanofiber hydrogel and injectable hydrogel conjugated with decellularized (porcine brain) ECM augmented neurogenesis[83,84]. Although these reports promise significant results, the efficiency of number of cells migrating, differentiating and aiding in reconstructing the functional circuit cannot be strictly regulated. This major limitation can be addressed by injecting optimized number of cells sufficient to rebuild lost circuitry.

2.2 Injecting exogenous cells- Injecting exogenous cells in optimized numbers to restore the lost connections has been explored by several labs all over the globe, and series of studies from Mark Tunszyski's team yields optimal results. Implanting embryonic neural stem progenitor cells embedded with growth factor in a fibrin matrix led to exuberant growth of transacted axons forming bidirectional functional relays, being the first study to demonstrate growth of corticospinal tract (CST) axons[85]. On replicating the same experiment, Steward

reported the same exuberant growth but with different ectopic colonies formed at distant location from transplant site[86,87]. The axons derived from such proliferating bodies might form erroneous connections leading to neuropathic pain and dysreflexia[88]. Such colonies are still the bottle neck for scientists questioning the clinical efficacy of therapy. Taking the study ahead, Tunszyski's lab reported robust CST regeneration (without ectopic colonies) with E14 rat spinal cord derived neural progenitor cells when provided with caudalized graft (not rostralized) and graft being in contact with injured tract. Such results were not achieved with Schwann cells or BMSCs expressing BDNF or NT3, consolidating the efficacy of neural progenitor cells[89]. Examining these results in NHP (non-human primates), the group revealed optimization of immunosuppression protocol, fibrin thrombin concentration for matrix and drainage of CSF was required to ensure the bioactivity of scaffold in the rhesus monkey. The results for the first time reported improved motor function of monkeys given a prolonged for maturation (as compared to rats) of the graft[90]. The group revealed the phenotypic fate of dissociated NPCs to assemble in organotypic dorsal horn like domain of spinal cord which was preferentially innervated by specific host sensory neurons into topographically and functionally appropriate target. The study paved the way for spatial engineering of graft interfacing relevant domain in the intact spinal cord[91]. Several other reports like transdifferentiated hADSCs into motor neurons and transduced NSCs (overexpressing Wnt 4) led to efficient regeneration[92,93]. Although the approach seems simplistic, the implantation site, injured niche with tapering gradient of localization and migration cues and variability in physiological effects of different cells, their mode of injection and time of intervention influence the engraftment, migration, and maturation dynamics of injected cells complicating the comparative study of functional recovery[94-95]. Although several clinical trials have been initiated[69,96,97], several challenges like locating the cells to desired loci, preventing anoikis, and ensuring the appropriate quantity of viable cells are still central questions to be answered.

The application of magnetic field has ameliorated diffusion of injected cells; the approach needs further optimization for obtaining intended results[98-99]. One of the critical aspects of nerve regeneration is providing a bridge that connects rostral and caudal ends of lesion, acts as substrate for transacted/injected axons to adhere and grow, transforming growth refractory injury site into growth permissive substrate thus, paving way for implanting an engineered construct known as scaffolds with appropriate cells, tropic and trophic cues to reconcile the lesion site.[100-101]

2.3 Tissue engineering accentuating cell therapy- Tissue engineering is one of the rapidly emerging fields[102] and its summation with cell therapy and recent technologies (3D printing) imparts conviction to succeed over any orchestrated regenerative chore[103,104]. Biomaterial scaffolds constitute the heart of this approach and hence the most appropriate choice of biomaterial and its fabrication into high fidelity scaffolds becomes the two Cartesian co-ordinates determining the success to attain intended results[105]. Nerve tissue engineering is one of the challenging fields to pursue owing to intrinsic complexity and variability over functional assessment[106]. Our lab has been working in this arena and has successfully demonstrated the regeneration of sciatic nerve *via* conducting cryogels and aligned nerve guidance conduits[107-108]. Injecting the cells might represent less invasive approach but shear stress on cells during injection, lack of native ECM and leakage or reflux of cells at the injection site interrogate the efficacy of the therapy. Addressing these limitations, Marquardt and colleagues published the fabrication of SHIELD (Shear-thinning Hydrogel for Injectable Encapsulation and Long-term Delivery) encompassing thixotropic-plug flow mechanics, rapid self-healing and stiffening property and cell adhesive ligand to allow anchorage dependent Schwann cells to successfully adhere to material. The injectable hydrogel undergoes dual crosslinking; firstly, ex-situ (physical crosslinking) which further disrupts on application of force during injection allowing the gel to shear thin and in-situ secondary cross

linking due to body temperature. The mechanism significantly assured membrane protection and massive (700%) improvement in Schwann cell viability, consequently leading to improved morphology of cells, reduction in lesion cavity and reductive astrogliosis and improved functional efficacy when implanted in contusion injury rat model[109,110]. The technological advancement led to significant improvement in spatial resolution and architecture of scaffolds. Recently microscale continuous printing projection-version of 3D printing assuring 1-micron resolution led to designing of photocurable 3D biomimetic hydrogel loaded with NSCs resulting in efficient functional recovery. Although technological advancements paved way for biomimetic tissue with high spatial resolution, the significance of elastic moduli, topographical and biochemical cues, mechano-transduction and vascularization of scaffolds leading to efficient bio-integration cannot be undermined.[111-112] It is equally important to mention that reconciling the inhibitory milieu with scaffolds might not always yield the intended outcome. Several studies reporting negative results with dystropihic axonal ends, scaffold scarring due to fibrosis, immature vasculature impeding the bio-integration of scaffold and factors like collagenous rift disconnecting the scaffold has halted the desired regeneration[113-114,115].Thus, though the field has witnessed tremendous progress with episodes of unprecedent success, the host compatibility and functional recapitulation close to native tissue does interrogates its clinical efficacy.

3. Neuroplasticity

Neuroplasticity can be conceptualise as ability of CNS to reorganise its synaptic connections through collateral sprouting, synaptic remodelling or regeneration of transacted axons in response to learning, experience and even after injury[17]. Homeostatic plasticity mostly leads to erroneous connections resulting in spasticity, dysreflexia and neuropathic pain thus, emerging fields like neuroelectronic paved way for remodelling such connections into functional circuitry[116-117,118]. Recent reports provide evidence that even anatomically complete

spinal cord injury contains certain spared tissue[119,120] that paves way for spontaneous recovery either through propriospinal detour circuit[121] or muscle spindle feedback[122] leading to reconstruction of denovo circuit facilitating to and fro communication through the cord. Precision in replicating the exact sculpture is consolidated by studies reporting regeneration but deficit in functional recovery[123]. With the increasing advent of machine learning and next generation neural interfaces, modulating neuronal activity has seen a pragmatic rise over couple of decades. Neuromodulation coupled with neurorehabilitation via neuroprosthetics[124] are some intriguing aspects harnessing the functionality of spared tissue bypassing the injury[117] (Refer to **Box4**).

Box 4: Environmental enrichment (EE)- Introduced by Donald Hebb, EE is an experimental paradigm augmenting hippocampal neurogenesis, synaptogenesis and dendritic branching, thus affecting brain development and plasticity[172,173]. Recent studies do suggest an association between EE and epigenetic programming, leading to proprioceptive sprouting below injury. Further studies would explore the mechanism behind gene-environment interactions leading to improved functional outcome[174].
Neuromodulation aims at augmenting neuronal activity *via* electrical or chemical means to excite, inhibit or modulate neural connections leading to recanalization of spinal cord[175].
Neuro-rehabilitation is an essential methodology to stimulate neuronal plasticity, residual connections and circuit reorganisation for functional outcome[175].
Neuroprosthetics are assistive devices interfacing brain and spinal cord to enhance activity dependent plasticity[176]. Classical example is BMI (brain machine interface)[177-178] with a key idea to record and decode cortical activity in brain and deploy the information to drive a robotic arm or stimulate the muscles directly (FES)[119]. The neuro-rehabilitation protocol must encompass a neuroprosthesis[116,176,179] that perturbs propulsion and enhance balance in a severe SCI.

Next generation neuroprosthetics (NPs) are designed to include multitarget, repetitive electrical and chemical stimulation conceptualise as closed loop wearable or implantable NPs[119]. Conjugating or following up neuromodulation with neurorehabilitation leads to functional remodelling of synaptic circuits *via* maintaining neural excitability after its activation and consolidating the acquired circuit reorganisation through training or task dependent plasticity[119,125]. Such modulation of neural circuitry does strengthen the Hebbian

Box 5: Neuronal cytoskeleton- The cytoskeleton of neuron is majorly comprising of

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better functional outcome.

Neuro-rehabilitation accentuating neuromodulation- Conjugating neuro-rehabilitation (training) with electrochemical neuromodulation, lumbosacral reorganisation enabled full weight bearing possible in rats with complete spinal cord transection[126]. The neurorehabilitation was further integrated with robotic interface and over-ground training which improved the efficacy of detour circuit and enhanced functional outcome[127]. Changes in neuromodulation protocol from manually tuned to feed-back (system input system output SISO) loop restored the complex locomotion in paralysed rats. Mimicking the spatiotemporal modulation pattern of motor neuron *via* spatial selectivity and temporal precision over specific flexor and extensor hotspots yielded EMG curve similar to intact rats[128,129]. The efficacy of electrical modulation protocol is coded in its variable like frequency, amplitude where preventing collision of EES and naturally encoded proprioceptive signals lead to enhanced motor neuron activity. Although spinal cord leads to disruption of transmission of nerve impulses, the cortical activity can be manifested to control voluntary movements. Recording the cortical activity, decoding the signals through various algorithms to further operate and control neuroprosthetics equipped with several electrodes enabled grasping in primates[130] and activated wrist and arm muscles in human patients[131]. The concept led to fabrication of wireless BSI (brain spine interface) where binary ON/OFF and proportional BSI linking cortical activity to frequency of stimulation, recovery was attained but in the presence of stimulation[132,133]. Leveraging the intact sensory circuitry in incomplete injury led the group to design a demultiplexed BCI (brain computer interface) where the spared circuitry was utilized to decode and demultiplexed the information to control FES (functional

electrical stimulation) and sensory feedback achieving improved sensory and motor functions[120]. Robotic-neuroprosthetic interface[134] has witnessed great advancement with the designing of multidirectional trunk support system[135] and adaptive multidirectional gravity assist refining the field to advance interface devices that restored kinetic and kinematic of gait pattern in ecological settings[136]. Integrating such advance gravity assist rehabilitation with neuromodulation, reorganisation of residual cortico-reticulo spinal circuitry with persistent functional improvement is reported[137]. Linking such advanced prosthesis with spatiotemporally controlled EES protocol enable the patient to not only walk but adjust their stride length and speed. Such advancement in the field undoubtedly showcased promising results but most of these technologies demand handling *via* expert technician which confine them mostly to lab environments. Recently designed voice controller watches with inertial measurement unit enabled patient to walk and cycle in natural settings, addressing the translation limitation of the field[138,139]. Successful integration of electronic implant within the soft neural tissue due to mechanical mis-match is also increasingly been addressed by designing systems like electronic duramater (edura) which being mechanically compatible led to decrease in deformation at implant site[140]. Different case studies and follow up are reported demonstrating the efficacy of electrical modulation accentuating training-based neurorehabilitation still, clinical trial with larger sample size and personalised protocols and studies exploring connection between neuronal activity and chromatin accessibility[141-142] warrant future investigation[143].

Future prospective

The CNS is a complex multifaceted nervous structure encompassing high density local circuits to long range connectivity seeking automaticity to interdependence where mild circuitopathy could yield drastic functional deficits[116]. Advanced in the field of computer, electronics, and communication industry has led to the development of significant

breakthroughs in the field; still, these high-end therapies remain confined to sophisticated research laboratories. Keeping pace with flourishing technology is as essential as refining current treatment protocols to obtain clinical impact[144]. The results of therapy do vary with age[145,146] or physical factors like exercise[147,148], thus influencing the recovery. Contemplating different treatment approaches through flow chart depicted in **Figure 2**, we surmise future therapy based on a combinatorial approach that acts synergistically with the most relevant injury model, emphasizing temporal window of intervention, translational efficacy, ethical and economic constraints.

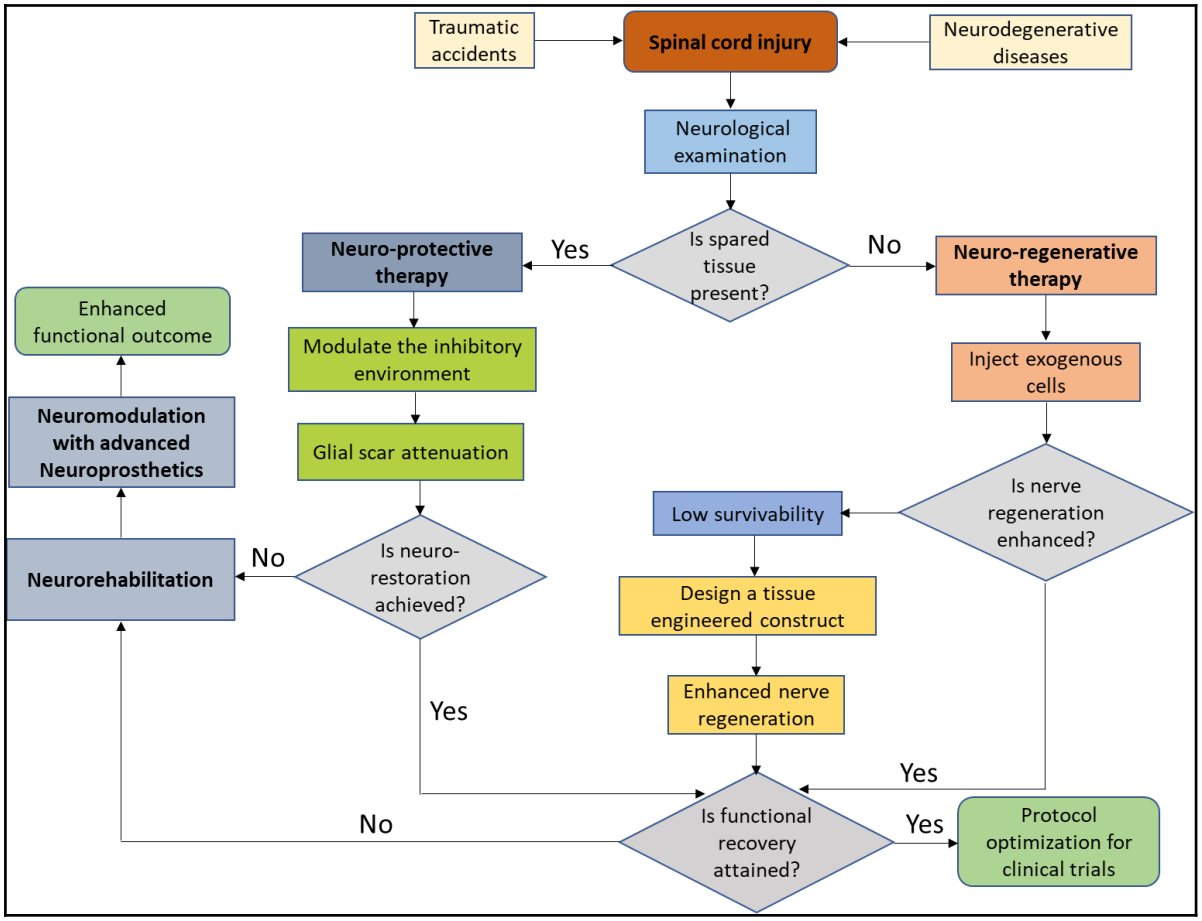

Figure 2: Flow chart depicts the various routes to achieve efficient recovery after spinal cord injury after thorough neurological examination

Is combinatorial therapy the panacea? If yes, in what permutation and combination?

Discussing the three broad spectrums for spinal cord repair, it is inevitable that each approach assists in tackling the issue; however, the increasing consensus suggests these approaches to act synergistically rather than individually. If the neuroprotective effect is combined with neuroregeneration[149,150] or neuroregeneration with neurorehabilitation[151] or all three together, it might lead to some unprecedented results. Although pragmatic, combinatorial therapy is complex and must consider the interactions between different mechanisms as many such failed attempts remain confine to lab record books[144]. Certain combinatorial approaches such as conjugating pre-degenerate peripheral nerve with BMSCs and ChABC (chondroitinase) and treating hiPSC derived NS/PC with gamma-secretase inhibitor depicted enhanced functional outcome when compared to single therapy alone[152,153]. Still, it is important to mention that certain failed attempts reporting undesirable results by combining different approaches are also evident[17,154] . SCI is associated with an immense plethora of inflammatory response, which is intricately orchestrated with temporal control; hence the time of intervention too becomes a variable for further assessing the functionality of results. Will acutely subjected pharmacotherapy and sub-acute cell therapy act synergistically to restore the lost function or additive effect of sub-acute to chronic neuro-rehabilitation with sub-acutely transplanted bioengineered scaffolds harvest maximum output. It is essential to consider that an increasing number of interventions might lead to competition over the same target substrate, and unravelling the molecular mechanism backing these interactions is undoubtedly an adventurous feat. With several amalgams possible, extensive research and immense resource is the need of the hour to provide desired momentum to current therapeutic approaches. Keeping pace with technological innovation, are some emerging concepts of engineered exosomes, biomimetic construct and advanced neurorehabilitation which are on verge to replace most of the conventional approaches as depicted via humorous conversation in **Figure 3**. We therefore suggest a combinatorial therapy that is temporally resolved and

spatially linked with the progression of injury pathology to be the panacea but the exact permutation and combination of each contributing therapy remains to be deciphered, paving the way for another episode of the arduous shot by dedicated cohorts of neuroscientists.

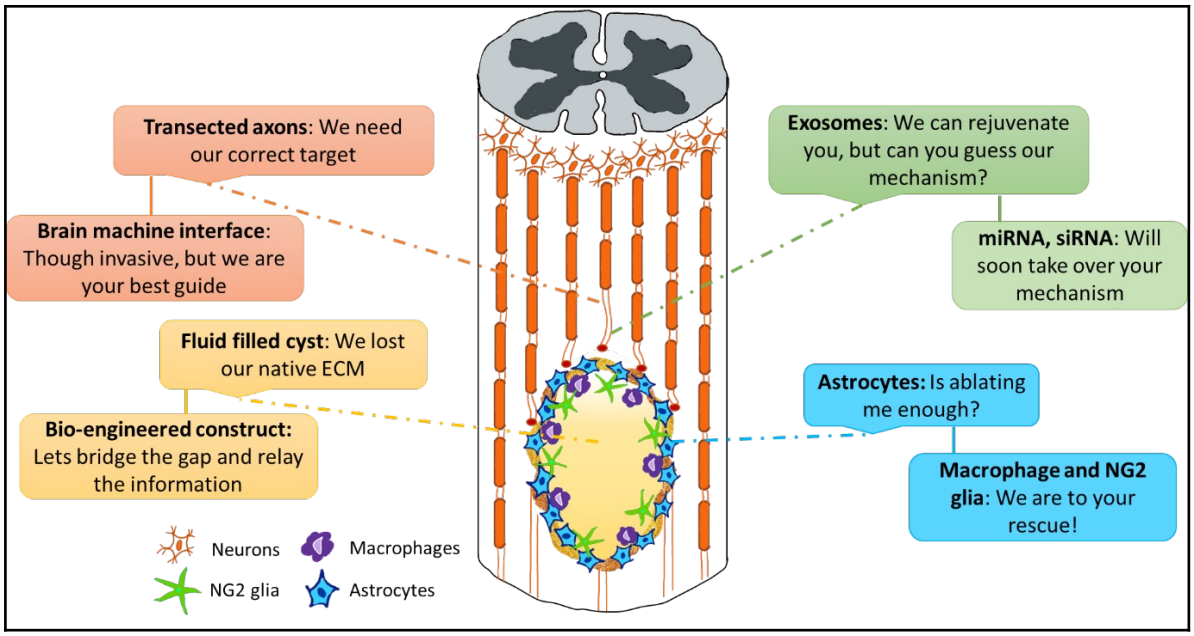

Figure 3: The figure depicts the conversation among major cellular players and therapeutic targets at spinal cord lesion site

Will the preclinical model suffice?

Transection injuries may be the ideal approach for evaluating any regenerative chore, which can be precisely replicated. However, they differ from the clinical scenario as contusion or crush injuries majorly constitute the clinical paradigm leaving a variable degree of spared tissue, creating inconsistent replica among different subjects[155]. Such limitations can be addressed by scholars being diligent in documenting the lesion extent with precision, clarity in temporal frame work for intervention and authenticity in unravelling the molecular interaction governing the recovery[156,144]. Rodent model though extensively used for scrutiny of any approach, differ anatomically and functionally from the human. NHP (non-human primates) being a better alternative demand magnificent cost and recalcitrant ethical issues[116]. An underpowered study with an inadequate number of subjects never reported negative

results or overrepresented positive results leads to episodes of misinterpretation and pseudo conclusion[156]. Heterogeneity at both intrinsic (motivation, concurrent illness) and extrinsic (level, the extent of the injury) factors pose a limitation to the reliability of results[63]. Repetitive scrutinization in different laboratories, evaluation in non-human primates, multi-centred, and randomized controlled experiments might lead to transparency in outcomes and clinical conduction. Albeit some of the approaches are in clinical trial after successful pre-clinical examination, the divergence over the injury model needs some standard criterion.

Are we into clinics?

Striving against the battle, clinical trials over the globe impart hope to prescribe the best medication for the injury soon certainly. Clinical trials with NeuroRegen scaffold combined with MSCs (Phase I) and NSCs (Phase II) have been initiated (NCT 02688049). The study has been furthered by designing another clinical trial where the combinatorial approach of epidural electrical stimulation with scaffold (NCT 03966794) will be assessed in acute or chronic SCI patients. Deciphering the role of PMZ-1620 (Sovateltide) as an anti-apoptotic and antioxidant agent, a prospective, multicentre, randomized, double-blinded, parallel, saline controlled phase II clinical study (NCT 04054414) has been planned to depict the efficacy of the compound in augmenting native NSCs towards neurogenesis when administered intravenously. Phase II, III clinical study is intended to evaluate the first-in-human administration of Neuro Cells (NCT 03935724), which are autologous stem cells modulating secondary inflammation (https://clinicaltrials.gov/). Clinical research must be designed keeping economic constraints, and technical variables in mind as some initiatives (Stem Cell Inc.) were abandoned due to financial burden. Japan recently becomes the first country to issue (conditional) government approval for stem cell therapy called Stemirac. After receiving and expanding MSCs from patients, the cells will be intravenously infused into patients in the sub-acute phase of injury[157].

Conclusion

Several decades of untiring research has now paved the way for a new era of neuroelectronics, exploration of functional heterogeneity among cells at lesion niche, and cell-free therapy after being guided by studies with impressive functional outcomes to witnessing episodes of occasional withdrawal. SCI is a debilitating disease where the success of therapy is intimately linked with patient's motivation to conquer the pathology and generous consolation from family and friends. It is depressive to accept that most patients still prefer wheelchair than elaborative treatment awaiting their active participation in medical institutes. The advancement of technology from in-vivo live cell imaging to CRISPR-Cas enabling genomic editing has now made it possible to observe dynamic changes induced by injury to repair mechanism following therapeutics. This review attempts to understand the multifaceted problem with three distinct approaches aimed towards restoring the native tissue property *via* different routes. Albeit each route leads to the same destination, the precise and intricate roadmap (combinatorial therapy) still needs to be deciphered. The roadmap has several signals (time of intervention) and intersections (one approach complimenting others), leading to the striking outcome if selected wisely.

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ES & AS conceptualized and wrote the initial draft of review article. **AK** provided supervision, funding and review.

Declaration of competing interests

The authors declare that they have no known competing financial interests.

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