Nanovector-mediated drug delivery for spinal cord injury treatment

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SPINAL CORD INJURY PHYSIOPATHOLOGY

Spinal cord injury (SCI) represents the most frequent disabling injury between the diseases of the spine. An estimated 2.5 million people worldwide live with SCI and more than 180,000 new injuries are reported each year.[1] The persisting SCI has a great impact on the quality of life of the affected persons and also represents a heavy burden for the society in terms of loss of income and healthcare costs. The acute spinal cord trauma results from a primary injury due to contusive, compressive, or stretch insult and it is followed by a multifactorial secondary injury, which worsens the clinical course.[2] The result is a motor dysfunction below the level of the lesion, as well as the development of chronic pain syndromes, loss of sensation, and change in sexual function. The knowledge of the physiopathology and of the mechanisms underlying SCI has increased greatly in recent decades owing to prolific preclinical research. These mechanisms are often conceptualized as being either intrinsic, related to the specific inability of neuron to regenerate itself in the central nervous system (CNS), or extrinsic in nature, related to molecules and/or physical barriers that inhibit axon regrowth after the injury. Following SCI, one of the main factors responsible for the absence of axonal regeneration is the lack of an appropriate cell body response in terms of a proper prosurvival gene expression. Indeed, the limited axonal regeneration produced after a lesion in the spinal cord is in part related to a lack of gene expression of different regeneration-associated proteins (GAP-43, c-jun, α-tubulin, CAP-43, NCAM, cAMP,

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CREB, ATF3, or STAT3)[3,4] and/or a limited trophic support [nerve growth factor (NGF); brain-derived growth factor (BDNF); neurotrophin 3 (NT-3); glial cell-derived neurotrophic factor (GDNF); vascular endothelial growth factor (VEGF); insulin growth factor 1 (IGF-1)].[5] In addition, different evidences have shown that also a permissive environment appears to be crucial to encourage axon regrowth, whereas an adverse environment is generated in the spinal cord after injury. The specific inhibition found in the CNS environment is mainly produced by myelin structure, glial scar, and inflammation. In particular, it has been demonstrated that the expression of specific inhibitory proteins by myelin (myelin-associated glycoprotein, nogo, and oligodendrocyte myelin glycoprotein) causes cytoskeletal rearrangements in neurons, leading to growth cone collapse and inhibition of neurite growth (reviewed by Yiu and He[6] and Nash et al.[7]). Moreover, the initial insult causing SCI is followed by consolidation of barriers aimed to limit a widespread degeneration. This includes the development of a glial scar around the injury site constituted mainly of astrocytes, oligodendrocytes, and microglia. This glial scar produces chemical signals, such as chondroitin sulfate proteoglycans (CSPGs) and keratin sulfate proteoglycans (KSPGs), tenascin, semaphorin 3, and keratin, that are able to inhibit the axonal sprouting.[8–11] This hostile milieu is also exacerbated by the inflammation caused in response to the mechanical primary injury to the spinal cord. Microglia are rapidly activated undergoing morphological/molecular changes and neutrophils, macrophages, and lymphocytes migrate into the injury site, initiating the inflammatory cascade that mainly characterizes the *secondary injury*.[12–16]

Two main strategies of treatment have been proposed to counteract the neuropathological evolution of SCI, identified as neuroprotective or neuroregenerative. Neuroprotection is focused on preventing the widespread degeneration associated with the secondary injury, limiting the damage, whereas neuroregeneration aims to rewire the damaged neuronal connections sustaining axonal regeneration and/or to reinstate neuronal loss by recellularization in the injured tissue. So far different neuroprotective therapeutic strategies have been adopted such as antioxidants, anti-inflammatory and antiapoptotic compounds, or drugs able to repair damaged membranes.[17] On the other hand, regenerative strategies have been focused on exogenous growth factor supplement, blockade of myelin-associated inhibitors, reactivation of regenerative associated proteins, remyelination, treadmill-based training, and electrical stimulation.[18]

NANOBIOMATERIALS AS A NOVEL DELIVERY TOOL IN SCI

Conventional drug delivery directed to the damaged spinal cord is severely limited by the physical obstruction due to the blood spinal cord barrier (BSCB). Indeed, most therapeutic compounds are not able to cross the BSCB when administered by oral intake or intravenous infusion.[19] Alternatively, intrathecal delivery by catheters and implanted minipumps have been proposed.[20] However, several disadvantages are associated with the latter delivery techniques, such as side effects of the surgery that required them and pump refilling.[21] Recently, new approaches to overcome these limitations have been proposed, such as an original use of nanobiomaterials. In line with this strategy, relevant advances have been made in developing hydrogels and nanoparticles (NPs) as new smart tools able to deliver *in situ* a wide range of molecules (drugs, antibodies, and neurotrophins) and cells.

Hydrogels

Hydrogels are three-dimensional nanostructured networks of hydrophilic homopolymers, copolymers, or macromers cross-linked to form insoluble polymeric matrices.[22] Because of their soft and elastic nature, hydrogels result as injectable biocompatible and degradable biomaterials that can be loaded with different drugs or cells.[23] Their swelling kinetics, mechanical properties, and degradation profiles can be tuned, as well as the drug release rates. Their similarity with the native extracellular matrix (ECM) makes them a valuable tool not only for drug delivery but also as supporting material for tissue regeneration.[19] Different factors make hydrogels the ideal delivery tool for SCI. Hydrogels, with proper *in situ* gelation, are able to bond directly to the host, filling the space and directly taking contact with cells. Furthermore, the property of *in situ* formation of the hydrogel allows a more precise positioning of this delivery tool, avoiding more invasive surgery, which could induce a further damage to the injured spinal cord.[23] Moreover, hydrogels can be used as scaffolds, being permissive to cellular migration, thus allowing glial cells and axons to penetrate into the biomaterial. Hydrogels can be classified according to their nature as natural, synthetic, or a combination of the two.[24] Complete overviews about the use of hydrogels in SCI could be found in both Refs 24 and 25. Recently, different preclinical studies demonstrated that hydrogels could be engineered to release neurotrophins directly into the damaged spinal cord.[24–26] It has been demonstrated that supplementary administration of exogenous neurotrophins such as NT-3, NT-4/5,

GDNF, NGF, and BDNF facilitates neuroprotection and neuroregeneration in SCI.[5] Several methods to supply trophic factors have been tested such as direct injection into the spine, systemic administration, intrathecal infusion pump, genetically modified cells, and adenoviral vectors.[5] However, these techniques present different disadvantages such as unfeasibility to cross the BSCB, pump refilling, lack of control of the duration of transgene expression, and reduced cellular survival after implantation. To sustain the *in situ* delivery of growth factors different hydrogels have been studied and they showed different advantages in comparison to conventional methods. Hydrogels are able to provide a constant and tailorable delivery of the loaded growth factors directly into the injury site. This represents a relevant advantage given the short *in vivo* half-life of them.[23,27] Several neurotrophins such as NT-3,[28,29] GDNF,[30] fibroblast growth factor-2 (FGF-2),[31] and BDNF[32] have been delivered through hydrogels *in situ*, demonstrating a preserved bioactivity and tailorable release over time. Cellular transplantation could provide an alternative method for the release of a great amount of trophic factors in the injury site. Indeed, even if the real mechanism of action of stem cells is not yet known, one hypothesis is that they act through the release of trophic factors [i.e., BDNF, NGF, VEGF, and hepatocyte growth factor (HGF)[33,34]]. Stem cell therapy is now considered a relevant therapeutic approach that has been extensively investigated in a wide range of different neurologic diseases, including SCI.[35] Independently from the cell source, relevant limitations remain in stem cell delivery when administered directly into the damaged tissue, such as a widespread diffusion outside the injury site, poor stem cell survival due to the adverse environment, adverse immunity response, and uncontrolled differentiation after transplantation.[36] To overcome these limitations, with the aim to increase cell survival and cell integration within the nervous tissue, hydrogels represent an emerging strategy. Indeed, the filling and bridging of the cavity could be achieved by polymeric materials used as scaffold for stem cells. Different studies have demonstrated the feasibility of seeding different stem cells within hydrogels to be later implanted in the injured spinal cord. Among them, Li et al. engineered an injectable hydrogel system based on thiol-functionalized hyaluronic acid and thiol-functionalized gelatin, which can be cross-linked by poly(ethylene glycol) diacrylate as a supportive niche to provide a regenerative permissive environment for transplanted oligodendrocyte progenitor stem cells (OPCs). They demonstrated that transplanted OPCs within the hydrogels enhanced survival, oligodendrogenic differentiation, and remyelination in a demyelinated animal model.[37] Moreover, Sykova et al. tested hydrogels based on derivatives of 2-hydroxyethyl methacrylate or 2-hydroxypropyl methacrylamide seeded with bone marrow stem cells. They obtained a reduction of the lesion at 35 days postimplantation in an SCI rat model and higher scores in the motor behavior evaluations.[38] Furthermore, new hydrogels are already under investigation as nerve guidance channels to address axonal regeneration and to prevent the ingrowth of fibrotic tissue.[39] As previously mentioned, one of the main properties of this kind of nanostructured material is the ability to mimic the ECM, providing a contact guidance for tissue regeneration.[24] Indeed, hydrogel scaffolds could be permissive to cellular migration, thus allowing glial cells and axons to penetrate, sustaining tissue regeneration. Several studies evaluated the efficacy of therapeutic hydrogels loaded with drugs, cells, or biological molecules in an acute SCI paradigm, but there are also few studies demonstrating that hydrogels delivery is able to improve the motor function in chronic SCI.[40,41] In parallel, *in situ* treatment has also been evaluated in the chronic phase of SCI by different investigators such as Hejcl et al. who used an hydrogel Arg-Gly-Asp-*N*-(2-hydroxypropyl)-methacrylamide seeded with mesenchymal stem cells (MSCs) and implanted into the lesion of an SCI rat model. They showed a statistically significant improvement in the group treated with both hydrogel and MSCs compared with the control group. Furthermore, infiltration of blood vessels, astrocytes, and myelinated axons into the hydrogel was observed.[40] Alternatively, Woerly et al. demonstrated that neurogel implanted 3 months after a severe injury improved the behavioral score of the treated SCI animal group, showing a reparative effect at 7 months in the injury site.[42] However, further studies are needed to evaluate promising results of therapeutic hydrogels in chronic model of SCI, and an accurate investigation of the biomaterial proposed is necessary to demonstrate the complete long-term safety and tolerability of hydrogels once implanted in the injured spinal cord.[23]

Nanoparticles

In recent years, the nanomedicine has provided many innovations and has been increasingly applied in drug development, underlining the importance of NPs in biomedical applications. Polymeric NPs show relevant potential advantages in pharmacological delivery by enhancing drug targeting and concentration in the injury site, reducing side effects, limiting drug catabolism, and slowing release of drugs over

time. Polymeric NPs can present different sizes, can have hydrophilic/lipophilic features, and can be functionalized with different molecules to meet several therapeutic needs.[43–45] Furthermore, NPs are considered a vehicle for targeted therapies because of their capacity to pass biological barriers, entering and diffusing within cells.[46–49] Recently, different nanostructured materials have been characterized as delivery tools in SCI, such as polymeric NPs, micelles, and nanowires.[50] Different preclinical studies demonstrated that NPs represent a promising drug delivery alternative for SCI treatment. Chemically conjugated NPs composed of ferulic acid and glycol chitosan, systemically administered in an SCI rat model, were able to reach the lesioned spinal cord showing neuroprotection and functional restoration.[51] Furthermore, intravenously injected NPs containing prostaglandin E(1) were able to reduce the lesion cavity volume and promoted the recovery of motor dysfunction.[52] A preclinical study using GDNF loaded in poly-lactic-*co*-glycolic acid (PLGA) NPs, directly injected into the damaged spinal cord to target neural and glial cells, demonstrated an increase of neuronal survival and an improvement of motor locomotion.[53] In addition, nanovectors were used to optimize the delivery of anti-inflammatory drugs. This is the case of methylprednisolone (MP) loaded in PLGA and administered *in situ*. It showed a higher pharmacological efficacy compared with the conventional systemic administration, reducing tissue damaging and inflammation, and improving behavioral outcome in an SCI rat model.[54] Furthermore, a micellar structure of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) has been used as a delivery vehicle able to increase the bioavailability of MP in the injured spinal cord.[55] Also the delivery of cerebrolysin (a mixture of different neurotrophic factors such as BDNF, GDNF, NGF, and ciliary neurotrophic factor) was able to reduce spinal cord water content, leakage of plasma proteins, and neuroprotection when carried by NPs.[56] The direct injection of NP suspension into the injury site is one attractive alternative compared with systemic administration. Intravenous administration of NPs could have different contraindications to the treatment of the SCI, such as limited or unequal distribution of NPs in the target tissues owing to BSCB,[19] and relevant NPs uptake by circulating macrophages in the liver and/or spleen.[57] However, NPs intrathecally injected without any support very often leave the zone of injection.[58,59] According to these critical issues, several studies suggested to associate hydrogels with NPs, providing a localized targeted therapy able to maximize the efficacy of neuroprotective agents.[31,60,61] Kang et al. showed that FGF-2 encapsulated in PLGA NPs and embedded in a biopolymer blend of hyaluronan and methylcellulose was able to enhance the endogenous angiogenic response once implanted into the damaged spinal cord.[31] Moreover, *in situ* delivery was optimized encapsulating MP in PLGA-based NPs subsequently embedded in an agarose hydrogel and implanted into the site of the contusion, showing a significant reduction of the early inflammation.[61]

Interestingly, nanomaterials themselves have been recently recognized as having a neuroprotective efficacy.[62] Intravenously injected micelles composed of self-assembled monomethoxy poly(ethylene glycol)–poly(D,L-lactic acid) diblock copolymer effectively recovered locomotor function and reduced both the lesion volume and the inflammatory reaction in SCI rats.[63] Moreover, local administration of poly(ethylene glycol) reduced oxidative stress and repaired nerve membranes, leading to the restoration of the nerve potential conduction and increasing behavioral outcome in SCI models of guinea pig.[64,65] A superior neuroprotective efficacy has also been demonstrated using nanowired material as a delivery tool in SCI. Tian et al. showed that TiO_2 nanowires were able to increase the bioavailability of neuroprotective and anti-inflammatory compounds (Acure Pharma synthesized compounds), improving their efficacy. This was likely due to the higher concentration of drugs available in the injured tissue.[66] NPs result to be a very interesting delivery tool also for a cell-targeted therapy given their ability to enter in specific cells, exploiting specific receptors or permissive pathways.[67] Once internalized, NPs may act as a drug depot within cells, protecting therapeutic compounds from degradation or efflux and delivering therapeutic doses with a sustained-release drug profile.[68] An interesting application of this innovative cell-targeted delivery regards the pharmacological modulation of microglia/macrophages. New evidence both *in vitro* and *in vivo* suggest that NPs can be selectively internalized by a specific endocytotic/phagocytic activity of the macrophagic cells after different insults, exploiting them as Trojan horses.[57] It is well known that microglia/macrophages assume phagocytic activity after traumatic stimuli[69] and this makes NPs a potential tool for drug targeting. Recently, this approach has been used by Cerqueira et al. and Papa et al. Cerqueira et al. demonstrated that surface-engineered carboxymethyl chitosan/polyamidoamine dendrimer NPs were able to deliver MP into glial cells, specifically microglial cells, allowing a controlled and selective release of MP in the injury site.[70] Alternatively, Papa et al. demonstrated a therapeutic approach able to treat selectively inflammatory cells using

NPs loaded with a well-known anti-inflammatory drug (minocycline). Specifically, they showed that both non-biodegradable poly(methylmethacrylate)[48] and biodegradable poly-ϵ-caprolactone (PCL)[49] NPs were captured exclusively by microglia/macrophages. Furthermore, minocycline-loaded PCL-based NPs were able to modulate the activation of microglia/macrophages *in vitro* and *in vivo*, reducing their proliferation and turning them from a round-shape phagocytic-like phenotype to a more arborized resting phenotype with low CD68 staining (inflammatory marker). In addition, they showed that a selective delivery into the microglia/macrophages was more efficient when compared with a free delivered minocycline in the injured tissue, demonstrating that an increased availability of the drug specifically in those cells is responsible for the potentiation of the pharmacological activity (Figure 1).

However, a critical issue in using NPs is the safety of the nanostructured material proposed as biomedical tools. Indeed, biocompatibility and efficacy can be influenced by minor variation in different parameters that characterize the nanomaterial, such as size, shape, chemistry, solubility, and surface area,[71] suggesting that a deeper investigation is mandatory in these terms before being translated into clinical trials and medical practice.[48,49]

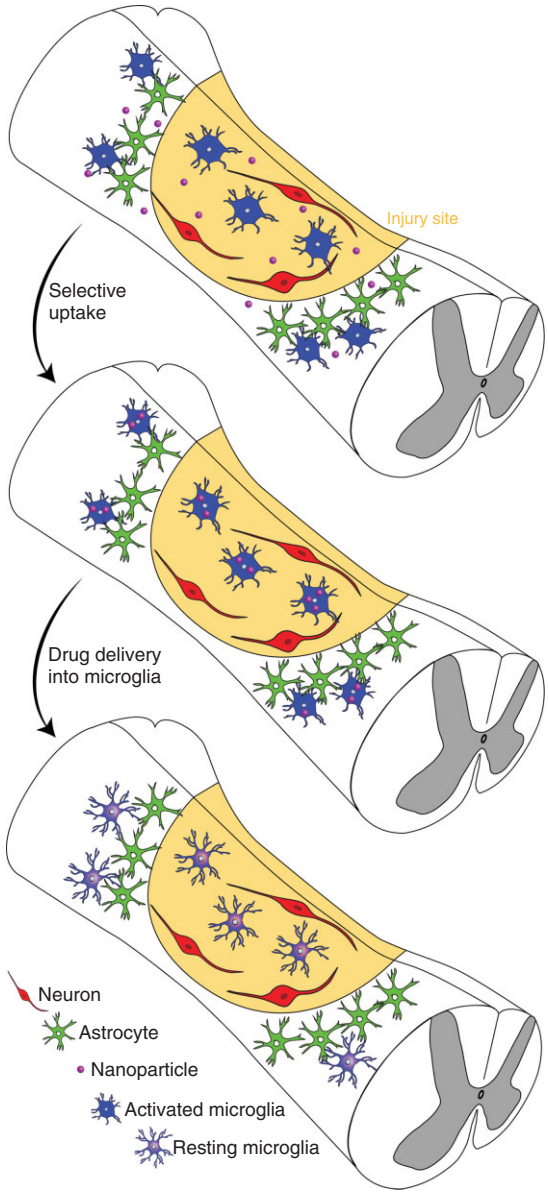

FIGURE 1 | Selective uptake of polymeric-based nanoparticles (NPs) into microglia/macrophages in spinal cord injury (SCI). Specific polymeric NPs (poly-ϵ-caprolactone, PCL) are selectively absorbed by activated microglia/macrophages in the injury site. An anti-inflammatory drug (minocycline) is encapsulated inside the NPs and selectively delivered into microglia/macrophages turning them from a round-shape phagocytic-like phenotype to a resting phenotype (anti-inflammatory effect).[49]

COMBINATORIAL THERAPY BY USING NANOBIOMATERIALS IN SCI

Although the concept of secondary injury in SCI is experimentally well supported, clinical trials with neuroprotective or neuroregenerative agents have been disappointing.[72] One reason could be that several therapeutic approaches were directed to just one physiopathological mechanism, whereas SCI is characterized by a temporal development of different biochemical pathways of degeneration and it is reasonable to think that more targets should be modulated over time. Toward this direction, recent research has focused its attention on multitherapeutic compounds able to target multiple mechanisms involved in the secondary injury.[19] Accordingly, different studies proposed new smart nanostructured biomaterials to deliver combinatorial therapies *in situ* demonstrating that these tools are safe and efficient as a multitherapeutic approach in SCI (Figure 2). Hwang et al. used this strategy to bridge the lesion cavity in a hemisection model of SCI.[73] They applied a polymeric scaffold constituted by PCL loaded with neuronal stem cells and NT-3. Moreover, in the full combinatorial strategy, they also applied chondroitinase ABC (chABC), which is able to digest chondroitin sulfate proteoglycans at the interface between spinal cord and implanted scaffold, counteracting scar formation and increasing axonal growth. This combinatorial strategy was able to improve the behavioral outcome of treated injured rats in comparison to single treatment.[73] A similar strategy has been pursued by using a combination of human fetal neural stem

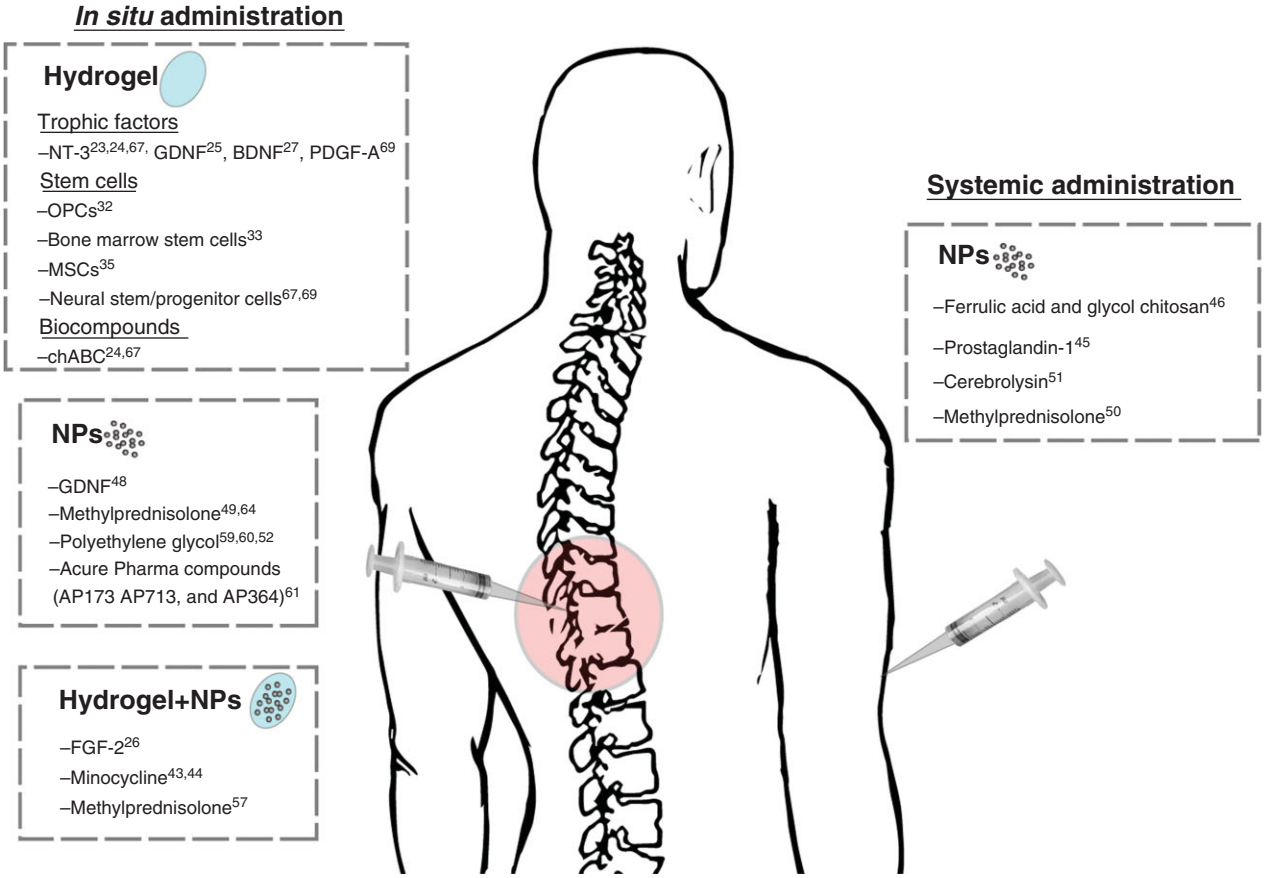

FIGURE 2 | Schematic overview of nanovector-mediated delivery strategies to treat spinal cord injury (SCI).

cells loaded within hydrogel modified with serotonin. Preclinical tests using this multitherapeutic treatment demonstrated a reduced astrocytic reactivity and tissue atrophy together with the possibility to increase the ingrowth of axons and blood vessels into the scaffold 1 month after implantation.[74] A combinatorial approach with stem cells has been also proposed and characterized by Mothe et al. They tested the survival and the efficacy of adult brain-derived neural stem/progenitor cells injected within a hydrogel blend of hyaluronal and methyl cellulose covalently modified with recombinant rat platelet-derived growth factor-A. They demonstrated an improved behavioral recovery compared to rats transplanted with only stem cells, showing an improved graft survival, a significant reduction of cavitation, and an increased oligodendrocytic differentiation.[75] Furthermore, a sustained delivery of both Rho GTPases and BDNF for 2 weeks, using an agarose hydrogel scaffold embedded with lipid microtube, was able to promote axonal growth over the scar tissue deposited in the injured site in a hemisection rat SCI model.[32] This work demonstrates that altering simultaneously multiple axonal responses to inhibitory cues is a promising approach to sustain spinal cord regeneration. A regenerative multitherapeutic treatment was also proposed by Bellamkonda's group demonstrating that the delivery of both thermostabilized chABC and NT-3, using a hydrogel–microtube scaffold system, enhanced axonal sprouting and functional recovery after SCI.[29]

CONCLUSION

The physiopathology of SCI is the result of an unexpected primary lesion which is followed by secondary degenerative pathways. Different mechanisms of the secondary degeneration have been clearly investigated and each of them can play a role in determining the progressive loss of nervous tissue and motor performances. Unfortunately, different potential therapeutic strategies have led to modest improvement of the locomotor outcome in preclinical studies showing even less relevant results when translated as clinical treatments. One reason for these disappointing results could be found in the limited feasibility to treat the spinal

cord by systemic drug administration (BSCB restrictions) reducing the potential efficacy of the treatment. Moreover, different therapeutic designs involved just a pharmacological treatment directed to a single neurodegenerative mechanism, not considering SCI as an evolving multifaceted pathology. Accordingly to these critical issues, different strategies have been developed to optimize the pharmacological treatment of the damaged tissue in SCI and the most promising could come from the nanoscaled delivery tools (hydrogel and NPs). These devices show different advantages compared with conventional administration (intravenous and oral delivery). On one hand drug-loaded hydrogels can be implanted intrathecally and remain temporally localized in the spinal cord. Here, they are able to control drug levels within a desired range, reducing side effects. On the other hand, NPs can be used to deliver drugs selectively into specific cells of the spinal cord, paving the base for a cell-targeted therapy. In addition, nanostructured materials could be the right answer to the multitherapeutic clinical needs. Indeed, a therapeutic combinatorial treatment is a new challenge in the pharmacological management of SCI and simultaneous drug delivery through nanovectors could represent a considerable opportunity to synergy the efficacy of multitherapeutic treatments against the multifactorial mechanisms of the secondary injury.

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