Chapter 11

Prerequisites for Mesenchymal Stem Cell Transplantation in Spinal Cord Injury

Sherif M. Amr

Additional information is available at the end of the chapter

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Abstract

We have aimed at distinguishing obligatory prerequisites for mesenchymal stem cell transplantation in spinal cord injury from those prerequisites which are unnecessary or are prerequisites that have to be further investigated. Obligatory prerequisites include the following. First, the site of injury is extensively gliotic, constituting an unsuitable medium for stem cell transplantation. It has to be dissolved by neurolyzing agents, chondroitinase ABC as an example. Second, stem cells need a suitable biomaterial scaffold for their proper integration. Third, the biomaterial scaffold necessitates a tissue filler harboring stem cells, other cells and neurotrophic factors in a combinatorial approach. Fourth, the efficiency of mesenchymal stem cells themselves has to be increased (by reducing oxidative stress-induced apoptosis, by hypoxic preconditioning, by modulating the extracellular matrix and by other measures). Prerequisites that have to be further investigated include the ideal source, mode, quantity, time point and number of injections of mesenchymal stem cells; which growth factors and cells to be used in the combinatorial approach; transforming mesenchymal stem cells into motor neuron-like cells or Schwann cells; increasing the homing effect of stem cells and how to establish a continuous drug and cell delivery system.

Keywords: spinal cord injury, mesenchymal stem cells, scaffolds, nerve grafting, neurotrophic factors, chondroitinase ABC, continuous drug delivery systems

1. Introduction

Traumatic spinal cord injury results usually from cervical and lumbar fractures; it may be associated with complete paraplegia. Regeneration after such an injury is fairly limited mainly due to the inhibitory milieu (the gliosis) within the spinal cord. Cellular therapeutic strategies may overcome this milieu by neuroprotection, immunomodulation, axon regeneration,

neuronal relay formation and myelin regeneration [1]. Clinically, in a meta-analysis on cellular therapy in traumatic spinal cord injury in humans published in 2012 [2], the authors reviewed eight bone marrow mesenchymal and hematopoietic stem cell studies, two olfactory ensheathing cell studies, one Schwann cell study and one fetal neurogenic tissue study. Three of these were Grade III and nine Grade IV level of evidence. It was concluded that improved preclinical studies and prospective, controlled clinical trials were needed. Nevertheless, ever since, the number of clinical trials have been increased. Mesenchymal stem cells, in particular, are easy to isolate, can be rapidly expanded in culture and can be cryopreserved without loss of potency [3, 4]. Clinical reports on their use have varied, starting from documenting their safety [5, 6] up to limited clinical efficacy [7], even partial or complete efficacy [8–11].

The aim of this review is to distinguish necessary prerequisites for effective mesenchymal stem cell transplantation in spinal cord injuries from those prerequisites which are unnecessary or are prerequisites that have to be further investigated.

2. Establishing a suitable niche

2.1. Dissolving the gliosis

Axonal regeneration following spinal cord injury is limited not only because central nervous system neurons have a poor intrinsic capacity for growth but also because injured axons encounter a series of inhibitory factors that are non-permissive for growth. These include myelin inhibitors [Nogo-A, MAG108 (myelin-associated glycoprotein) and OMgp109 (oligodendrocyte myelin glycoprotein)]; chondroitin sulfate proteoglycans (neurocan, versican, aggrecan, brevican, phosphacan and NG2); semaphorins and ephrins. In the central nervous system, laminin is replaced by netrins [12–15].

2.1.1. Chondroitinase ABC

Chondroitinase ABC [16–18] has improved recovery of function in synergy with mesenchymal stromal cells without [19] or with the addition of an acellular nerve allograft [20] or in synergy with brain-derived neurotrophic factor (BDNF) secreting mesenchymal stem cells [21]. Chondroitinase ABC should be thermostabilized with the sugar trehalose to reduce its temperature-dependent loss of activity [22]; it should be injected in high doses (50 or 100 IUs) [23–25], at multiple times [26–29] and be combined with cell transplantation and growth factor infusion [30, 31].

2.1.2. Other measures to overcome the gliosis

In a rat model of spinal cord contusion injury [32], infused *sialidase* has acted robustly throughout the spinal cord gray and white matter, whereas chondroitinase ABC activity has been more intense superficially, thus raising the possible consideration that it might be superior to chondroitinase ABC. Blocking myelin-associated inhibitors with *Nogo-A monoclonal antibodies or with Nogoreceptor competitive agonist peptide (NEP1-40)* has been shown to increase axonal regeneration [33]. Bone marrow mesenchymal stem cells with Nogo-66

receptor gene silencing have been used for repair of spinal cord injury [34]. *Blocking Rho-A with Rho inhibitor 'cethrin'* might overcome its effect; a synthetic membrane-permeable peptide mimetic of the protein tyrosine phosphatase σ, wedge domain can bind to tyrosine phosphatase σ and relieve chondroitin sulfate proteoglycan-mediated inhibition [35]. Chondroitin sulfate proteoglycans inhibition of phosphoinositide 3-kinase (PI3K) signaling is reversed by *cell permeable phosphopeptide (PI3Kpep)* [36]; *rolipram*, a phosphodiesterase4 inhibitor, can increase intracellular cAMP levels [33]; *taxol*, a microtubule-stabilizing agent, increases neurite outgrowth [37, 38].

2.1.3. Emerging role of heparin in lysing the gliosis

There is an emerging role of heparin in lysing of the gliosis, as reviewed elsewhere [39]. Both unfractionated and low molecular weight heparins have a fibrolytic (gliolytic) effect, can modulate astrocyte function and are used as lumen fillers. Astrocytes release a variety of trophic factors. These trophic factors include nerve growth factor, basic fibroblast growth factor, transforming growth factor-β, platelet-derived growth factor, brain-derived neurotrophic factor, ciliary neurotrophic factor and others. Astrocyte stress response and trophic effects are mediated by the fibroblastic growth factor family member, on which heparin exerts a profound influence [40–42].

2.2. Providing a suitable scaffold, both to bridge the gap and to harbor the cells

2.2.1. Biomaterial scaffolds in spinal cord injury

Biomaterial scaffolds in spinal cord injury have been reviewed elsewhere [43, 44]. Mesenchymal stromal cells have been grown onto *fibrin* scaffolds [45, 46]. The survival and neural differentiation of human bone marrow stromal cells have been tested on fibrin versus fibrin platelet-rich plasma scaffolds. The results have shown a clear superiority of platelet-rich plasma scaffolds, mainly after BDNF administration [47]. Mesenchymal stem cells have also been grown onto *collagen* scaffolds [48]. Rat adipose-derived stem cells have differentiated into olfactory ensheathing cell-like cells on collagen scaffolds by co-culturing with olfactory ensheathing cells [49]. *Acellular* spinal cord scaffolds [50, 51] and acellular muscle bioscaffolds [52] seeded with bone marrow stromal cells have promoted functional recovery in spinal cord-injured rats. Electroacupuncture has been found to promote the survival and differentiation of transplanted bone marrow mesenchymal stem cells pre-induced with neurotrophin-3 and retinoic acid in *gelatin* sponge scaffold after rat spinal cord transaction [53]. Human bone marrow mesenchymal stem cells and endometrial stem cells have been found to differentiate better into motor neurons on electrospun *poly(ε-caprolactone)* scaffolds [54]. Nogo-66 receptor gene-silenced cells have been transplanted in a poly(D,L-lactic-co-glycolic acid) scaffold for the treatment of spinal cord injury [55]. Bone marrow mesenchymal stem cells seeded in *chitosan*-alginate scaffolds [56] and biodegradable chitin conduit tubulation combined with bone marrow mesenchymal stem cell transplantation have reduced glial scar and cavity formation in spinal cord injury [57]. In a comparative study investigating the efficacy of allogeneic mesenchymal stem cell transplantation via simple intralesional injection versus the use of a poly (lactic-co-glycolic acid) scaffold or a chitosan scaffold, higher mesenchymal stem cell engraftment rates have been reported in the scaffold groups, particularly, in the chitosan scaffold group [58].

Injectable extracellular matrix *hydrogels* have been used as scaffolds for spinal cord injury repair [59]. Matrix metalloproteinase-sensitive, hyaluronic acid-based biomimetic hydrogel scaffolds containing brain-derived neurotrophic factor have been implanted [60]. Cell-seeded alginate hydrogel scaffolds have promoted directed linear axonal regeneration in the injured rat spinal cord [61]. Multichannel polymer scaffolds fabricated from positively charged oligo[poly(ethylene glycol)fumarate] hydrogel and loaded with either syngeneic Schwann cells or mesenchymal stem cells derived from enhanced green fluorescent protein transgenic rats have been successfully implanted into rat spinal cords following T9 complete transection [62]. Highly superporous poly(2-hydroxyethyl methacrylate) scaffolds with oriented pores [63] and highly superporous cholesterol-modified poly(2-hydroxyethyl methacrylate) scaffolds have been developed for spinal cord injury repair [64].

Three-dimensional culture can mimic the stem cell niche compared to conventional two-dimensional culture. Bone marrow-derived mesenchymal stem cells cultured in three-dimensional collagen scaffold have exhibited distinctive features including significantly enhancing neurotrophic factor secretions and reducing macrophage activations challenged by lipopolysaccharide [65]. A polyhydroxybutaryl-hydroxyvinyl-based three-dimensional scaffold for a tissue engineering and cell-therapy combinatorial approach for spinal cord injury regeneration has been developed [66]. A three-dimensional biomimetic hydrogel has been implemented to deliver factors secreted by human mesenchymal stem cells in spinal cord injury [67]. Bone marrow mesenchymal stem cells in a three-dimensional gelatin sponge scaffold have attenuated inflammation, have promoted angiogenesis and have reduced cavity formation in experimental spinal cord injury [68].

2.2.2. Prerequisites for the use of biomaterial scaffolds in spinal cord injury

Biomaterial scaffolds should be biocompatible, non-toxic, chemically stable, of known absorption and degradation kinetics matching the degree of in vivo cell/tissue growth and should have adequate surface for cell access, proliferation and cell differentiation [69, 70]. They *should meet macroengineering requirements* being of proper form [71, 72], design (shape) [73] and size (diameter) [74]. They should be supplied with macrogrooves [43, 75, 76] and have a wall thickness of 0.6 mm, a porosity of 80% and a pore size range of 10–40 μm [77–79]. They *should meet microengineering requirements*, microgrooves directing axonal growth [80–87]. Prestretch-induced surface anisotropy has been beneficial in enhancing axon alignment, growth and myelination [88]. Also, filament inclusion has been more effective for bridging long nerve defect gaps [43, 89, 90]; Schwann cell migration over gaps exceeding 18 mm is superior in the presence of filaments. Yoshii et al. [91, 92] have tested collagen microfilaments with diameters of 20 μm to repair long gaps (20 or 30 mm) in the rat sciatic nerve. Increasing fiber number (4000 versus 2000 filaments) has enhanced nerve regeneration. Thus, increasing the whole filament surface area by increasing their number and reducing their diameter (increased surface area-to-volume ratio) is also critical [89, 93, 94].

Scaffolds should fulfill nearly the same mechanical conditions of the recipient spinal cord, exerting incremental tensile forces on intact cord segments to promote axonal regeneration while unloading gliotic segments to reduce gliosis and harbor cellular transplants (**Figure 1a and b**). A scaffold should

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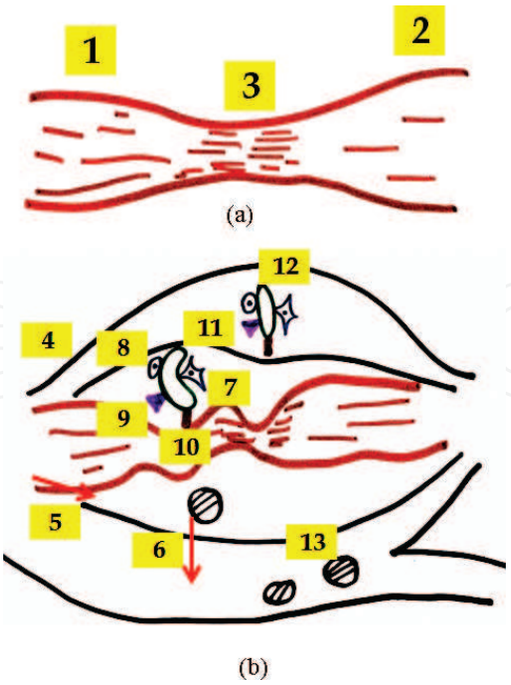

Figure 1. (a) How a spinal cord lesion looks like; (1) cranial spinal cord; (2) rostral spinal cord and (3) the gliotic segment. (b) A biomaterial scaffold (4) should fulfill nearly the same mechanical conditions of the recipient spinal cord, exerting incremental tensile forces (5—arrows) on intact cord segments to promote axonal regeneration while unloading gliotic segments (6—arrows) to reduce gliosis and harbor cellular transplants. In addition, it should meet macro- and microengineering requirements; it should provide adequate space for the interplay and manipulation of the different molecular pathways for axonal regeneration through lumen filling technology and it should meet requirements based on spatial distribution of neurotrophic factor gradients. Lumen filling technology allows for the incorporation and gradual local release of stem cells (7), accessory cells (8), molecular growth factors (e.g. BDNF, neurotrophin-3, etc.) (9) and neurolyzing agents (e.g. chondroitinase ABC) (10), either by combining them with a growth-supporting matrix in the lumen (11), by crosslinking (12) them to nerve conduit walls or by using microspheres (13) to deliver them. Growth-supporting matrices (11) in the lumen include hydrogel-forming collagen, fibrin, laminin, alginate, heparin and heparin sulfate. A natural and low-toxicity crosslinking agent (12), genipin, is commonly used.

possess sufficient toughness to resist compression or collapse, yet still be flexible and suturable [95]. A brittle scaffold that sustains little or no plastic deformation before fracture might break hampering axonal progression.

A scaffold should have an elastic modulus comparable with that of the recipient spinal cord. To approach appropriate mechanical properties, one strategy has been to form polymer composites with biopolymers such as chitosan [96], a polymer which has been established as being "softer" and biocompatible. The role of mechanical compliance in directing cell fate and function is a critical issue in material design [97–99]. A low elasticity and hierarchically aligned fibrillar fibrin hydrogel fabricated through electrospinning and concurrent molecular self-assembly process has been tested. Matrix stiffness and aligned topography have instructed stem cell neurogenic differentiation and rapid neurite outgrowth [100].

Scaffolds should provide adequate space for the interplay and manipulation of the different molecular pathways for axonal regeneration [80, 81, 101–103].

To provide adequate space and adherence for cells and molecules, biomaterial polymer nerve scaffolds should be porous [43]. Currently, ideal scaffolding should have 80–90% porosity

with a pore size of 50–250 μm. Its pores should be interconnected so as to provide physical support to cells and guide their proliferation and differentiation, also facilitating neovascularization [69, 104]. The porous structure can be stabilized by adding glutaraldehyde, polyethylene glycol, heparin or collagen, allowing the structure to become more resistant and to maintain elasticity. A natural and low-toxicity cross-linking agent, genipin, has been used to immobilize nerve growth factor, a neurotrophic factor, onto chitosan-based neural scaffolds to generate a novel nerve graft, which has been beneficial for peripheral nerve repair [105]. A novel method has been introduced for standardized microcomputed tomography-guided evaluation of scaffold properties in bone and tissue research [106].

Scaffolds should provide adequate space for lumen fillers Methods of lumen filling allow for incorporation of cells and molecular factors either by combining them with a *growth-supporting matrix* in the lumen, by crosslinking them to nerve conduit walls or by using microspheres to deliver them [107]. *Growth-supporting matrices in the lumen* include hydrogel-forming collagen, fibrin, laminin, alginate, heparin, and heparin sulfate.

Scaffolds should meet requirements based on spatial distribution of neurotrophic factor gradients.

Spatial molecular concentration gradients of nerve growth factor [108] and laminin [43, 109, 110] promote axonal sprouting. Thus, axonal growth can be hypothetically made to bridge the whole length of the neural gap by seeding the scaffolds with multiple nerve growth factor/laminin spatial concentration gradients [111].

3. Optimizing the therapeutic effect of mesenchymal stem cell transplantation

3.1. The ideal source for mesenchymal stem cells

Mesenchymal stem cells reside not only in various tissues of mesenchymal origin (e.g. bone marrow, adipose tissue, skin and peripheral blood) but also in perinatal sources (e.g. umbilical cord blood, umbilical cord matrix or Wharton's jelly, amniotic fluid and placenta) [112].

In a comparative study using mesenchymal stem cells extracted from both bone marrow and adipose tissue for spinal cord injury, animals receiving adipose tissue cells have presented higher levels of tissue brain-derived neurotrophic factor, increased angiogenesis, higher number of preserved axons and a decrease in the number of macrophages, suggesting the superiority of mesenchymal stem cells extracted from adipose tissue [113]. In another study, however, no difference has been found between animals receiving mesenchymal stem cells derived from bone marrow or adipose tissue, whether in terms of axonal regeneration, neuroprotection or functional recovery [114].

Mesenchymal stem cells obtained from perinatal sources can proliferate more rapidly and extensively than adult mesenchymal stem cells and are easily obtained after normal and cesarean births, with low risk of viral contamination. They may be used for allogenic transplantation because they act by suppressing immune response and are, therefore, considered non-immunogenic cells [112].

In a study comparing mesenchymal stem cells derived from fat, bone marrow, Wharton's jelly and umbilical cord blood for treating spinal cord injuries, dogs have been treated with only matrigel or matrigel mixed with each type of mesenchymal stem cells. Although there have been no significant differences in functional recovery among the mesenchymal stem cell groups, application of umbilical cord stem cells has led to more nerve regeneration, neuroprotection and less inflammation compared to other mesenchymal stem cells [115].

Central nervous system pericytes (perivascular stromal cells) have recently gained significant attention. These cells not only display a mesenchymal stem cell phenotype in vitro but also have similar in vivo immunomodulatory effects after spinal cord injury that are more potent than those of non-central nervous system tissue-derived cells [116].

3.2. Increasing the efficiency of mesenchymal stem cells and their influence on spinal cord regeneration

3.2.1. Influence of mesenchymal stem cells on spinal cord regeneration in general

Present around blood vessels, mesenchymal stem cells respond more readily to tissue damage [3]. The transdifferentiation capacity of mesenchymal stem cells into neuronal and glial lineages has been debated; transplanted mesenchymal stem cells do not differentiate into a neuronal fate, even if they display weak expression of NeuN (a neuronal marker) [3]. Mesenchymal stem cell-based cell therapy, even when applied during the chronic phase of spinal cord injury, leads to changes in a number of structural and functional parameters, all of which indicate improved recovery [117]. Mesenchymal stem cells promote repair in the injured cord by secreting growth factors that overcome the inhibitory environment of the lesion. These cells have anti-inflammatory, immunomodulatory, vascular promoting oxidative stress reducing and neuroprotective effects. They can secrete trophic factors thus exerting a paracrine effect that can stimulate axon regeneration contributing to functional recovery enhancement [112, 118]. Human mesenchymal stem/stromal cells suppress spinal inflammation in mice with contribution of pituitary adenylate cyclase-activating polypeptide [119]. Intrathecal transplantation of mesenchymal stem cells activates extracellular adjusting protein kinase1 and 2 in the spinal cord following ischemia reperfusion injury, partially improving spinal cord function and inhibiting apoptosis in rats [120].

Measures to increase the *efficiency of mesenchymal stem cells* include the following. *Replacing fetal bovine serum* has been proposed as a gold standard for human cell propagation [121]. *Mechanical fibrinogen-depletion* has been found to support heparin-free mesenchymal stem cell propagation in human platelet lysate [122]. A combination of *electroacupuncture* and grafted mesenchymal stem cells overexpressing tyrosine kinase C has been found to improve remyelination and function in demyelinated spinal cord of rats [123]. Arginine decarboxylase is a rate-limiting enzyme of agmatine synthesis and is known to exist in the central nervous system of mammals. Arginine decarboxylase-secreting human mesenchymal stem cells have been found to be more suitable candidates than human mesenchymal stem cell for stem cell therapy after spinal cord injury [124]. Heme oxygenase-1 is a stress-responsive enzyme that modulates immune response and oxidative stress associated with spinal cord injury. Functional recovery after spinal cord injury has been promoted by transplantation of mesenchymal stem cells

overexpressing heme oxygenase-1 [125]. *Hypothermia* is known to improve the microenvironment of the injured spinal cord in a number of ways. Neural cell transplantation has promoted the recovery of hind limb function in rats, and a combination treatment with hypothermia has produced synergistic effects [126]. *Extracorporeal shock wave* can introduce alteration of microenvironment in cell therapy for chronic spinal cord injury [127].

3.2.2. Peculiarities of bone marrow stromal cells in spinal cord regeneration

Bone marrow stromal cell transplantation has been shown to overcome the gliosis [3]. They have been reported to enhance neuronal protection and cellular preservation *via* reduction in injury-induced sensitivity to mechanical trauma. They can attenuate astrocyte reactivity and chronic microglia/macrophage activation. They have been found to infiltrate primarily into the ventrolateral white matter tracts, spreading to adjacent segments rostrocaudal to the injury epicenter. However, bone marrow stromal cell transplantation present certain issues. Migration beyond the injection site after intraspinal delivery is limited and inter-donor variability in efficacy and immunomodulatory potency might affect clinical outcome [4].

Measures to increase the *efficiency of bone marrow mesenchymal stem cells* include mainly measures to reduce oxidative stress-induced apoptosis, hypoxic preconditioning, measures to modulate the extracellular matrix and other measures.

Studies have demonstrated that the inhibition of the Notch1 pathway in bone marrow mesenchymal stem cells contributes to the differentiation of these cells. Research findings that certain antioxidants induce bone marrow mesenchymal stem cells to differentiate into neuronal cells suggest that bone marrow mesenchymal stem cell differentiation is related to the level of reactive oxygen species in cells. After bone marrow mesenchymal stem cell induction with the antioxidant β-mercaptoethanol, Western blotting and immunofluorescence have revealed gradual increases in the expression of Nestin (a neural stem cell-specific protein) and neuron-specific enolase but decreases in Notch1 expression. The decreased expression levels of Notch1 have correlated positively with changes in reactive oxygen species [128]. The effects of a calpain inhibitor (MDL28170) on increasing survival of bone marrow mesenchymal stem cells transplanted into the injured rat spinal cord have been investigated. The protective effects of MDL28170 on survival of bone marrow mesenchymal stem cells have inhibited the activation of calpain and stress-induced apoptosis [129]. Treatment with bone marrow mesenchymal stem cells combined with plumbagin may alleviate spinal cord injury by affecting oxidative stress, inflammation, apoptosis and the activation of the Nrf2 pathway [130]. Polydatin, a glucoside of resveratrol, has been reported to possess potent antioxidative effects and can used in combination with bone marrow mesenchymal stem cell for the treatment of spinal cord injury. Polydatin significantly protects bone marrow mesenchymal stem cell against apoptosis due to its antioxidative effects and the regulation of Nrf 2/ARE pathway [131]. Carvedilol, a nonselective β-adrenergic receptor blocker, has been reported to exert potent anti-oxidative activities. It has been shown that carvedilol protects cell death of H2O2-induced bone marrow mesenchymal stem cells partly through PI3K-Akt pathway, suggesting its use in combination with bone marrow mesenchymal stem cells to improve cell survival in oxidative stress microenvironments [132].

Hypoxic preconditioning effectively increases the survival rate of bone marrow mesenchymal stem cells following transplantation and increases their protective effect on injured tissues. Hypoxic preconditioning has upregulated the expression of hypoxia-inducible factor 1α in spinal cord tissues [133].

Cytokines and extracellular matrix can trigger various types of neural differentiation. To highlight the current understanding of their effects on neural differentiation of human bone marrow-derived multipotent progenitor cells, extracellular matrix proteins, tenascin-cytotactin, tenascin-restrictin and chondroitin sulfate, with the cytokines, nerve growth factor/brain-derived neurotrophic factor/retinoic acid, have been incorporated to induce transdifferentiation of human bone marrow-derived multipotent progenitor cells. Greater amounts of neuronal morphology have appeared in cultures incorporated with tenascin-cytotactin and tenascin-restrictin than those with chondroitin sulfate. It has been suggested that the combined use of tenascin-cytotactin, nerve growth factor /brain-derived neurotrophic factor/retinoic acid and human bone marrow-derived multipotent progenitor cells offers a new feasible method for nerve repair [134]. Fibronectin secreted by mesenchymal stem cells in the early stage has been found to accumulate on gelatin sponge scaffolds and promote neurite elongation of neuronal differentiating mesenchymal stem cells as well as nerve fiber regeneration after spinal cord injury [135].

Transplanted bone mesenchymal stem cells can be mobilized by erythropoietin toward lesion sites following spinal cord injury [136]. Propofol injection combined with bone marrow mesenchymal stem cell transplantation has improved electrophysiological function in the hindlimb of rats with spinal cord injury than monotherapy [137]. Combining bone marrow stromal cells with green tea polyphenols has attenuated the blood-spinal cord barrier permeability in rats with compression spinal cord injury [138]. Bone marrow stromal cells transplantation combined with ultrashortwave therapy has promoted functional recovery in spinal cord injury in rats [139].

Microtubule-associated protein 1B plays an important role in axon guidance and neuronal migration. Phosphatidylinositol 3-kinase and extracellular signal-regulated kinase 1/2 in bone marrow mesenchymal stem cells have been found to modulate the phosphorylation of microtubule-associated protein 1B via a cross-signaling network and have affected the migratory efficiency of bone marrow mesenchymal stem cells towards injured spinal cord [140]. Administration of valproic acid potentiates the therapeutic effect of mesenchymal stem cell therapy [141]. Interleukin-8 enhances the angiogenic potential of human bone marrow mesenchymal stem cells by increasing vascular endothelial growth factor production [142].

3.2.3. Peculiarities of adipose-derived stem cells in spinal cord regeneration

Human mesenchymal cells from adipose tissue have deposited laminin and have promoted regeneration of injured spinal cord in rats [143–146]. Transplanted during the acute and subacute phases after spinal cord injury, they have enabled the remodulation and regeneration of the lesion site, decreasing the importance of transplantation time in the treatment of spinal cord injury [145]. Chondroitinase ABC-adipose-derived stem cells constructed using lentiviral vector transfection have stably expressed chondroitinase ABC, and chondroitinase ABC expression

has significantly enhanced their migratory capacity [146]. Cytoplasmic extracts prepared from adipose tissue stromal cells have *inhibited H_2O_2-mediated apoptosis* of cultured spinal cord-derived neural progenitor cells and have improved cell survival. *Predifferentiation* of adipose tissue-derived stromal cells has promoted the protection of denuded axons and cellular repair. Such predifferentiated cells and hematopoietic stem cells have been successfully infused intrathecally [143]. Nevertheless, no evidence points to the superiority of neural differentiated adipose tissue-derived stromal over undifferentiated ones. *Allogenic* adipose-derived stem cells have improved neurological function in a canine model. All of the former evidence, however, is contradicted by a study in a rat C3–C4 hemisection in which adipose tissue-derived stromal cell transplantation has significantly reduced sprouting of the descending serotonergic fibers at the injured site [147].

Hypoxic preconditioning of adipose tissue-derived mesenchymal stem cells has increased their survival. Cotransplantation of such cells with engineered neural stem cells has improved both cell survival and gene expression of the engineered neural stem cells [4].

3.2.4. Peculiarities of human umbilical cord blood-derived mesenchymal stem cells in spinal cord regeneration

Human umbilical cord blood-derived mesenchymal stem cells (whether Wharton's jelly mesenchymal stem cells or human umbilical cord perivascular cells) may reverse spinal cord injury pathophysiology by *downregulating apoptotic genes and secreting neurotrophic factors* in few days; they may *transdifferentiate* toward neuronal and oligodendroglial phenotypes [3]. Intrathecal transplantation of human amniotic mesenchymal stem cells has promoted functional recovery in a rat model of traumatic spinal cord injury [148] and in a chronic constrictive nerve injury model [149]. Placental mesenchymal stromal cells have rescued ambulation in ovine myelomeningocele [150]. Umbilical cord-derived mesenchymal stem cell therapy for neurological disorders may act via inhibition of mitogen-activated protein kinase pathway-mediated apoptosis [115]. Through the effect on glial cells(suppression of activated astrocytes and microglia), proinflammatory (Interleukin-1β and Interleukin-17A) and anti-inflammatory cytokines (anti-inflammatory cytokine Interleukin-10), intrathecal injection of human umbilical cord-derived mesenchymal stem cells has ameliorated neuropathic pain in rats [151]. Also, neurotrophic factors have been expressed in the injured spinal cord after transplantation of human-umbilical cord blood stem cells in rats [152].

Preconditioning of umbilical cord mesenchymal stem cells in physioxic environment can enhance the regenerative properties of these cells in the treatment of rat spinal cord injury. In a study on umbilical cord, mesenchymal stem cells pretreated with either atmospheric normoxia (21% O_2) or physioxia (5% O_2) have grown faster, whereas physioxia has upregulated the expression of trophic and growth factors, including hepatocyte growth factor, brain-derived neurotrophic factor and vascular endothelial growth factor. This has been associated with a significant increase in axonal preservation and a decrease in the number of caspase-3+ cells and ED-1+ macrophages [153].

Calcitonin gene-related peptide, a neural peptide synthesized in spinal cord, contributes to homing of human umbilical cord mesenchymal stem cells. The PI3K/Akt and p38MAPK signaling

pathways have played a critical role in the calcitonin gene-related peptide-induced chemotactic migration of human umbilical mesenchymal stem cells [154].

Lavandula angustifolia has neuroprotective effects; it has potentiated the functional and cellular recovery with human umbilical mesenchymal stem cell treatment in rats after spinal cord injury [155]. The combined treatment with methylprednisolone and amniotic membrane mesenchymal stem cells after spinal cord injury in rats has potentiated the anti-inflammatory and anti-apoptotic effect of mesenchymal stem cell transplantation [156]. The neuroprotective effects of conditioned medium from cultured human CD34(+) cells have been similar to those of human CD34(+) cells and the conditioned medium has been found to enhance the neuroprotective effects of 17β-estradiol in rat spinal cord injury [157].

3.3. Inducing the transformation of mesenchymal stem cells into motor neuron-like cells or Schwann cells

A third method for optimizing the therapeutic effect of mesenchymal stem cell transplantation is inducing their transformation into motor neuron-like cells or Schwann cells [158–169]. Their differentiation into *motor neuron-like cells* has been induced through a pre-induction step using β-mercaptoethanol followed by 4 days of induction with retinoic acid and sonic hedgehog [158]. Motor neuron axonal sprouting has been induced by adding different concentrations of a nerve growth factor to the differentiation media. In another study [159], such cells have been tested for 2′,3′-cyclic-nucleotide-3′-phosphodiesterase and microtubule-associated protein 2, as well as to glial fibrillary acidic protein and beta III tubulin. Cells have been injected percutaneously into the spinal cord of paraplegic dogs for two times separated by a 21-day interval. *Optimal culture conditions* have been investigated as to the production of neural cells and neural stem cells [160]. β-Mercaptoethanol has been used as the main inducer of the neurogenesis pathway. Three types of neural markers have been used: nestin as the immaturation stage marker, neurofilament light chain as the early neural marker, and microtubule-associated protein 2 as the maturation marker. Results have shown that the best exposure time for the production of neural stem cells is 6 hours. It has also been demonstrated that LY294002, a small molecule inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway, can promote neuronal differentiation of mesenchymal stem cells cultured on polycaprolactone/collagen scaffolds [161]. Similarly, microRNA-124 has promoted bone marrow mesenchymal stem cell differentiation into neurogenic cells for accelerating recovery in the spinal cord injury [166, 169]. *Such induced motor neuron-like cells* have promoted axonal regeneration into the injured spinal cord, whether derived from bone marrow [162, 163, 168], human chorion [164] and placenta [167]. Their in vivo tracking by magnetic resonance has been possible in rabbit models of spinal cord injury [169].

3.4. Mode, quantity and number of injections; time point for injection age and donor variation; allo- and xenotransplantation

The mode, quantity and number of injections may influence the therapeutic effect of mesenchymal stem cell transplantation

3.4.1. Mode of injection

All methods for stem cell transplantation (intravenous, intrathecal, intramedullary, intranasal or skeletal muscle injection) are based on the homing effect, the ability of implanted stem cells to move to the injured area [170–180]. Mesenchymal progenitor cells have been injected *intravenously* in two models of cervical spinal cord injury, unilateral C5 contusion and complete unilateral C5 hemisection. Cells have been isolated from green fluorescence protein-luciferase transgenic mice and have been injected via the tail vein at D1, D3, D7, D10, or D14. Transplanted cells have been tracked via postmortem bioluminescence imaging. Cells have been found to accumulate in the lungs, irrespective of the time of injection or injury model. It has been proposed that they modulate the immune system via the lungs through secreted immune mediators [173]. The antioxidant and anti-inflammatory effects of intravenously injected adipose-derived mesenchymal stem cells have been proven in dogs with acute spinal cord injury [174]. Diffuse and persistent blood-spinal cord barrier disruption after contusive spinal cord injury has recovered following intravenous infusion of bone marrow mesenchymal stem cells [177]. Intravenous mesenchymal stem cell therapy has been effective after recurrent laryngeal nerve injury [179]. In a meta-analysis, the efficacy of intravenous bone marrow mesenchymal stem cell transplantation in spinal cord injury has been investigated. It has been concluded that the therapeutic window of intravenous bone marrow mesenchymal stem cell transplantation is wide [180]. The feasibility and safety of *intrathecal* transplantation of autologous bone marrow mesenchymal stem cells have been investigated in horses [175]. The *intranasal* delivery of bone marrow stromal cells to spinal cord lesions has been successfully tried out [176]. Stem cell injection in the *hindlimb* skeletal muscle has enhanced neurorepair in mice with spinal cord injury [178].

Although intrathecal is more effective than intravenous injection, it needs large stem cell numbers. Subarachnoid adhesions may prevent the cells from reaching the target site. The homing effect is absent in the chronic stage of spinal cord injury. Therefore, direct intramedullary injection into the injured site is the most effective method for delivering stem cells. Intramedullary injection proximal to the injured area is ideal for stem cell survival, but is hampered by volume effects caused by high tissue pressure and subsequent normal spinal cord damage. On the contrary, large volumes can be injected into the cavity area at the injured level. Injecting into the contused cavity may lead to resolution of the glial scar and may bridge for axonal regeneration. Therefore, Park et al. [171, 172] have injected into both the normal proximal spinal cord and the injured area. In addition, subdural stem cells have been applied in the hope the homing effect has been reinduced because of intramedullary injection.

3.4.2. Quantity, number and time point for mesenchymal stem cell transplantation

3.4.2.1. Quantity and number

Diversity of lesion models, animal types and route of cell administration influence the quantity of mesenchymal stem cells administered. Cell survival and enhancement in locomotor performance have been observed both after intravenous injection of one million cells in a

volume of 0.5 mL of DMEM in a model of balloon compressive injury in rats and after transplantation of 600,000 cells in a volume of 6 μL directly into the injury site after contusive injury in rats [112]. Other studies have advocated intrathecal administration from 100×10^6 up to 230×10^6 cells followed by an additional 30×10^6 cell administration at 3 months [5], or the administration of two or three intrathecal injections with a median of 1.2×10^6 mesenchymal stem cells/kg body weight [6]. In a phase III clinical trial, limited efficacy has been proven after injecting 1.6 × 10 autologous mesenchymal stem cells into the intramedullary area at the injured level and 3.2 × 10 autologous mesenchymal stem cells into the subdural space. Single mesenchymal stem cell application to intramedullary and intradural space has had a very weak therapeutic effect compared to multiple injections [7]; partial efficacy has been demonstrated in other trials [8–11]. Continuous improvement after multiple mesenchymal stem cell transplantations has been observed in a patient with complete spinal cord injury [181]. Multiple injections of human umbilical cord-derived mesenchymal stromal cells through the tail vein have improved microcirculation and the microenvironment in a rat model of radiation myelopathy [182].

3.4.2.2. Time point

Acute phase is defined as the first three days after spinal cord injury and chronic phase is defined as more than 12 months after spinal cord injury. Subacute phase is defined as the period between acute and chronic phase. In the acute phase, reactive oxygen-free radicals, excitatory transmitters, inflammatory molecules and hypoxia caused by hypoperfusion are cytotoxic to implanted stem cells. In the chronic phase, glial scar tissue acts as a physical barrier to axonal regrowth. Thus, it is difficult for implanted stem cells to survive in chronic spinal cord injury. In contrast, in the subacute phase, the inflammatory response is reduced and the glial scar formation has not formed. Therefore, the subacute phase seems to be an optimal phase in the respect of timing of stem cell application [170]. Experimentally, bone marrow-derived stem cells have been infused intravenously 10 weeks after spinal cord injury [183].

3.4.3. Age and donor variation, allo- and xenotransplantation

3.4.3.1. Age and donor variation

The potency of mesenchymal stem cells exhibits significant age and donor variation [3, 184–186]. A robust potency assay has been established based on pooling responder leukocytes to minimize individual immune response variability. It has highlighted significant donor variation of human mesenchymal stem/progenitor cell immune modulatory capacity and extended radioresistance [184, 185].

3.4.3.2. Allo- and xenotransplantation

The neuroprotective and immunomodulatory effects of *xenotransplantation* of adipose tissue mesenchymal stem cells in Lewis rats after lumbar ventral root avulsion have been proven [187]. The therapeutic effects of autologous and *allogenic* bone marrow-derived mesenchymal

stem cell transplantation have been established in canine spinal cord injury [188]. Immunosuppression of allogenic mesenchymal stem cells transplantation after spinal cord injury may improve graft survival [189].

3.4.4. Evaluating the therapeutic effect of mesenchymal stem cell transplantation

Although neurological evaluation of the spinal cord injured patient is usually conducted according to the International Standards for Neurological Classification of Spinal Cord Injury recommended by the American Spinal Cord Injury Association, it should be confirmed by electrophysiological studies (somatosensory evoked potentials and motor evoked potentials) and magnetic resonance imaging studies. Magnetic resonance imaging findings after stem cell therapy include widening of cord diameter, blurring of intramedullary cavity margin and appearance of fiber-like streak pattern in the injured spinal cord. Diffusion tensor imaging can perform accurate visualization and assessment of white matter tracts and is useful for the prediction of neurological recovery in spinal cord injury patients. Fiber continuity on diffusion tensor imaging not seen before stem cell therapy may be an indicator of axonal regeneration in stem cell therapy. Cell labeling techniques for *in vivo* visualization using biological indicators or contrast agents have helped monitoring the status of the transplanted stem cells in the body (survival, migration and exact location of implanted stem cells). Typical examples are supermagnetic iron oxide particle monitoring using magnetic resonance imaging and radionuclide monitoring using positron emission tomography or single-photon emission computed tomography [170, 190, 191].

4. Supplying neurotrophic factors and accessory cells

A combinatorial approach has been agreed upon for effective treatment of spinal cord injury [192–208].

The combination of *neurotrophic factors* such as BDNF and neurotrophin-3 has enhanced axonal regeneration and myelination [193]. Cyclic adenosine monophosphate (a neuronal stimulator) and neurotrophin-3 (neurotrophic factor) have been injected 5 days prior to a C4 transection at L4 to precondition the dorsal root ganglion soma. Bone marrow mesenchymal stem cells have been transplanted 7 days post injury. The effect of bone marrow mesenchymal stem cells on spinal cord regeneration has been augmented by modifying them to either express human brain-derived neurotrophic factor (BDNF) in an acute injury or neurotrophin-3 in a chronic injury model, by prestimulating them to secrete neurotrophic factors, e.g. by pretreating them with Schwann cell differentiating factors [3]. In an attempt to generate mesenchymal-derived differentiated neural cells expressing nerve growth factor or neurotrophin-3, mesenchymal stem cells have been infected with recombinant lentiviruses that express nerve growth factor both to induce their neural lineage genes and as a combinatorial approach [194]. Magnetic targeting of neurotrophin-3 gene-transfected bone marrow mesenchymal stem cells via lumbar puncture has enhanced their delivery to the site of injury and has significantly improved functional recovery and nerve regeneration compared

to transplanting neurotrophin-3 gene-transfected bone marrow mesenchymal stem cells without magnetic targeting system [195, 196]. Pulsed electromagnetic field exposure near the injured site and for 8 hours per day over 4 weeks has been suggested as a suitable protocol for directing the cells to the site of injury [197]. Electro-acupuncture has promoted the survival and differentiation of transplanted bone marrow mesenchymal stem cells pre-induced with neurotrophin-3 and retinoic acid in gelatin sponge scaffold after rat spinal cord transection [53, 198].

A combination of other trophic factors, including epidermal growth factor, fibroblast growth factor type 2 and platelet-derived growth factor have enhanced the survival of implanted cells. Likewise has been the addition of granulocyte macrophage-colony stimulating factor [4, 170]. Co-transplantation of bone marrow-derived mesenchymal stem cells and nanospheres containing FGF-2 has improved cell survival and neurological function in the injured rat spinal cord [199]. Human ciliary neurotrophic factor overexpressing stable bone marrow stromal cells have proved effective in a rat model of traumatic spinal cord injury [200]. Bone marrow mesenchymal stem cells combined with minocycline have improved spinal cord injury in a rat model [201]. Propofol has enhanced the therapeutic effect of bone marrow mesenchymal stem cell transplantation on spinal cord injury in rats [202].

The addition of accessory cells includes combining mesenchymal stem cells with neural progenitor cells [3], neural crest stem cells [203], olfactory ensheathing cells [204, 205] or Schwann cells [207, 208]. The effects of mesenchymal stem cell and olfactory ensheathing cell transplantation at early or delayed time after a spinal cord contusion injury in the rat have been compared. Mesenchymal stem cell grafting seems a better option than olfactory ensheathing cell grafting [206].

5. Establishing a continuous drug and cell delivery system

In spinal cord injury, the gap is usually extensive and associated with excessive scarring. The axonal growth cone would thus take years to reach the distal spinal cord. Consequently, the factors mentioned before have to be replenished continually.

This can take place through an intrathecal (possibly extradural) continuous cell and drug delivery system (catheter) [39, 209]. Catheter-related complications include tension headache, meningitis, fibrous track formation, catheter slippage, difficult catheter insertion and catheter blockage. Microsphere, nanosphere and nanoshell technology may help keep the catheter patent, dissolve fibrosis and replenish molecules and cells [43, 210–215]. Co-transplantation of bone marrow-derived mesenchymal stem cells and nanospheres containing FGF-2 has improved cell survival and neurological function in the injured rat spinal cord [199]. Controlling surface tension as well as hydrophobic and hydrophilic properties of the conduit lumen and the microspheres may help us fulfill the three aims described previously. One method to achieve the latter aim is using magnetic nanoparticle-incorporated human bone marrow-derived mesenchymal stem cells exposed to pulsed electromagnetic fields [190, 191, 197] (**Figure 2**).

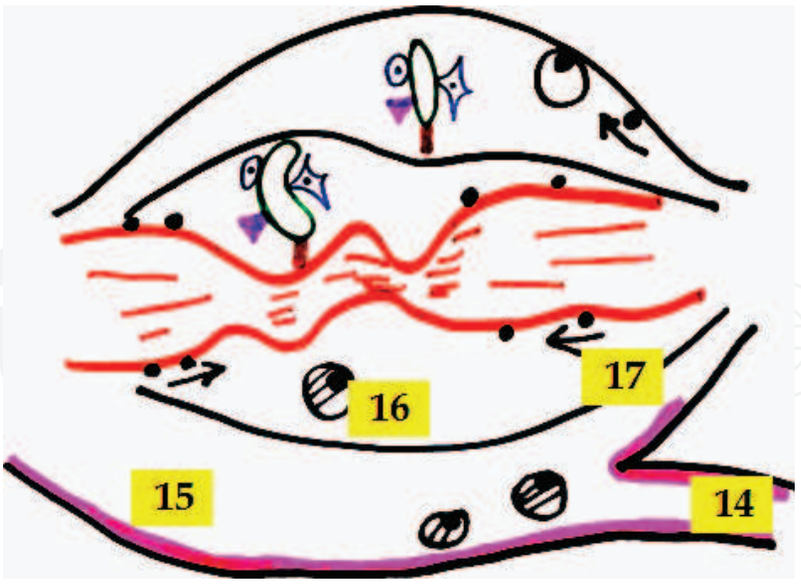

Figure 2. An intrathecal continuous cell and drug delivery system (catheter) (14) allows for the replenishment of stem cells, accessory cells, molecular growth factors and neurolyzing agents. To avoid catheter-related complications, it had better be lined with a biomaterial used for vascular grafts (15). Hydrophobic microsphere, nanosphere and nanoshell technology may also help keep the catheter patent, dissolve fibrosis and replenish molecules and cells. Magnetic nanoparticles (16) incorporated into microspheres may help guide the latter to the gliotic segment. After their release from microspheres, magnetic nanoparticles may be made to attach to the scaffold and to the intact cord segments and to apply tension on them (17—arrows), thus promoting axonal regeneration and enhancing engraftment and differentiation of transplanted cells.

6. Conclusion

We have attempted to identify the prerequisites for effective mesenchymal stem cell transplantation in spinal cord injuries. These fall into three categories (**Table 1**). The first category comprises those prerequisites, on which the literature is united. Research workers are thus obliged to follow them or provide a reasonable explanation for having not followed them.

The literature is unanimous on the following: (1) the gliosis has to be dissolved prior to mesenchymal stem cell transplantation (e.g. through chondroitinase ABC in high doses (50 or 100 IUs) and at multiple times); (2) a suitable scaffold has to be used; this scaffold should meet both macro- and microengineering requirements and should provide adequate space for lumen fillers; (3) the efficiency of mesenchymal stem cells themselves has to be increased (by reducing oxidative stress-induced apoptosis, by hypoxic preconditioning, by modulating the extracellular matrix and by other measures); (4) a combinatorial approach including growth factors, cellular transplants and neurolyzing agents has to be followed.

There are many issues, however, on which the literature is still not united. These fall into the second category. Among others, they include (1) the ideal source for mesenchymal stem cells, mode, quantity, time point and number of injections; (2) which growth factors and cells to be used in the combinatorial approach; (3) optimizing the therapeutic effect of mesenchymal stem cell transplantation by inducing their transformation into motor neuron-like cells or Schwann cells; (4) increasing the homing effect of stem cells (by calcitonin gene-related peptide). In the third category, more research has to be stimulated, e.g. as to how to establish a continuous drug and cell delivery system.

1. Establishing a suitable niche

1.1. Dissolving the gliosis

Category I (prerequisites, on which the literature is united)

Chondroitinase ABC in high doses (50 or 100 IUs) and at multiple times (at 0, 1, 2 and 4 weeks)

Category II (prerequisites, on which the literature is still not united)

- Heparins, sialidase
- Blocking myelin-associated inhibitors with Nogo-A monoclonal antibodies or with Nogoreceptor competitive agonist peptide (NEP1-40)
- Blocking Rho-A with Rho inhibitor 'cethrin'
- A synthetic membrane-permeable peptide mimetic of the protein tyrosine phosphatase σ can bind to protein tyrosine phosphatase σ and relieve proteoglycan-mediated inhibition
- Cell permeable phosphopeptide (PI3Kpep) reverses proteoglycans inhibition of phosphoinositide 3-kinase signaling in axons.
- Rolipram, a phosphodiesterase4 inhibitor, can increase intracellular cAMP levels
- Improving blood vessel formation might reduce cell death and promote angiogenesis within the injury zone
- Taxol, a microtubule-stabilizing agent, increases neurite outgrowth

1.2. Providing a suitable scaffold, both to bridge the gap and to harbor the cells

Category I (prerequisites, on which the literature is united)

- Scaffolds should meet macro- and microengineering requirements
- Scaffolds should fulfill the same mechanical conditions of the recipient spinal cord
- Scaffolds should provide adequate space for the different molecular pathways for axonal regeneration; they should be of ideal porosity
- Scaffolds should provide adequate space for lumen fillers
- Scaffolds should meet requirements based on spatial distribution of neurotrophic factor gradients

2. Optimizing the therapeutic effect of mesenchymal stem cell transplantation

2.1. The ideal source for mesenchymal stem cells

Category II (prerequisites, on which the literature is still not united)

Compared to stem cells of other mesenchymal origin (e.g. bone marrow, adipose tissue, skin), umbilical cord stem cells are superior

2.2. Increasing the efficiency of mesenchymal stem cells

Category I (prerequisites, on which the literature is united)

- Reducing oxidative stress-induced apoptosis
- Hypoxic preconditioning
- Modulating the extracellular matrix

Category II (prerequisites, on which the literature is still not united)

- Measures to reduce oxidative stress-induced apoptosis (arginine decarboxylase expressing cells; heme oxygenase-1 expressing cells; calpain inhibitor MDL28170; plumbagin; polydatin, a glucoside of resveratrol; carvedilol, a nonselective β-adrenergic receptor blocker)
- Measures during stem cell culture (replacing fetal bovine serum, mechanical fibrinogen-depletion)
- Measures during grafting (electroacupuncture, hypothermia, extracorporeal shock wave, propofol, green tea polyphenols, ultrashortwave therapy, valproic acid, IL-8)
- Measures increasing the homing effect and mobilization of stem cells (calcitonin gene-related peptide, erythropoietin)

2.3. Inducing the transformation of mesenchymal stem cells into motor neuron-like cells or Schwann cells

Category II (prerequisites, on which the literature is still not united)

2.4. Mode, quantity and number of injections; time point for injection; age and donor variation; allo- and xenotransplantation
Category I (prerequisites, on which the literature is united): intramedullary injection; injection during the subacute phase
Category II (prerequisites, on which the literature is still not united): all other issues
3. Supplying neurotrophic factors and accessory cells
Category I (prerequisites, on which the literature is united)
A combinatorial approach, including growth factors, cellular transplants and neurolyzing agents, has to be followed
Category II (prerequisites, on which the literature is still not united)
Which growth factors (epidermal growth factor, fibroblast growth factor type 2, platelet-derived growth factor, riluzole, minocycline, granulocyte-colony stimulating factor, BDNF, neurotrophin-3) and cells (embryonic stem cells, neural stem cells, induced pluripotent stem cells, neural crest stem cells, mesenchymal stromal cells, Schwann cells, olfactory ensheathing cells or macrophages) to be used in combination
4. Establishing a continuous drug and cell delivery system
Category III (prerequisites defective in the literature)

Table 1. Prerequisites for effective mesenchymal stem cell transplantation in spinal cord injuries.

List of abbreviations

Akt	Protein kinase B (PKB), a serine/threonine-specific protein kinase
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosine monophosphate
DMEM	Dulbecco's Modified Eagle Medium ED-1+ macrophages: antibody against cellular marker CD68 macrophages
FGF-2	Fibroblast growth factor type 2
LY294002	Morpholine-containing chemical compound that is a potent inhibitor of numerous proteins, and a strong inhibitor of phosphoinositide 3-kinases
MAG108	Myelin-associated glycoprotein
MDL28170	Calpain inhibitor III
NEP1-40	Nogoreceptor competitive agonist peptide
NeuN	Feminizing locus on X-3, Fox-3, Rbfox3, or hexaribonucleotide binding protein-3
NG2	Neural/glial antigen 2
Nogo-A	Reticulon-4, neurite outgrowth inhibitor
Nrf 2	Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2
Nrf 2/ARE pathway	The transcription factor Nrf2 (NF-E2-related factor 2) binds to the ARE, a cis-acting element called the antioxidant responsive element
OMgp109	Oligodendrocyte myelin glycoprotein
PI3K	Phosphatidylinositol 3-kinase
PI3Kpep	Cell permeable phosphopeptide: p38MAPK P38 mitogen-activated protein kinases
Rho-A	ras homolog gene family, member A

Author details

Sherif M. Amr

Address all correspondence to: sherifamrh@hotmail.com

The Department of Orthopaedics and Traumatology, Cairo University, Egypt

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