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Application of mesenchymal stem cells for treating spinal cord injury in dogs: Mechanisms and their therapeutic efficacy

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ABSTRACT

Despite progress in the treatment of spinal cord injury (SCI), recovery of the spinal cord with normal motor and sensory activities remains a challenge due to the complex anatomy of the spine, and its limited regeneration potential in mammals. Recently, the clinical application of mesenchymal stem cells (MSCs) in SCI led to promising results in both human and veterinary medicine. The mechanism by which MSCs might promote wound healing of SCI has been extensively investigated. Previous reports have suggested that transplanted MSCs enhance the numbers of neurons and glial cells, prevent neuronal apoptosis, inhibit inflammation, stimulate vascular angiogenesis, axonal myelination, and neuro-regeneration at the lesion site. It has also been reported that therapeutic applicability depends on the source of derivation of MSCs and their differentiation capability into specific cell lineages at the transplanted site. Therefore, this review is focussed on precise mechanisms by which transplantation of MSCs promote functional recovery and also addresses the challenges to improve the therapeutic efficacy of MSCs for treating SCI in dogs.

Keywords: Central nervous system, Dogs, Mesenchymal stem cells (MSCs), Neurotrophic factors, Spinal cord injury

Spinal cord injury (SCI) is a serious nerve tissue injury that affects both humans and animals like horses, dogs, etc. Apart from a traumatic injury, the intervertebral disc prolapse is most common in dogs (Van den Berg *et al.* 2010). Based on the published reports, the neuropathology of SCI can be classified into two phases: (1) mechanical damage/ trauma, and (2) secondary injury after mechanical damage/ trauma. The mechanical damage to the spinal cord causes immediate cell death and vascular injury with disruption of the blood-brain barrier at the lesion site. It is followed by immediate extravasation of inflammatory cells to the injury site (Fracaro *et al.* 2020). Matrix metalloproteinases, other proteolytic and oxidative enzymes, and proinflammatory cytokines that are produced by infiltrating neutrophils and macrophages, along with resident microglia, induce a reactive process of secondary cell death in the tissue periphery to the lesion site due to DNA damage, protein oxidation, and mitochondrial malfunction (Kjell and Olson 2016, Ahuja *et al.* 2017). The secondary damage continues in the days and years after SCI, which may lead to an increase in cavity and cyst formation at the centre of the lesion site, further exacerbating neurological dysfunction (Fracaro *et al.* 2020). Some pieces of evidence suggested extensive migration of macrophages and astrocytes to the lesion site in chronic cases (Wright *et al.* 2012). Astrocytes secrete a wide range of cytotoxic extracellular matrices and form a physical barrier at the periphery of the SCI lesion site, that walls off or encapsulates the lesion from that of healthy tissues (Zweckberger *et al.* 2016). This wall is called a glial scar, which contributes to an environment that is inhibitory to axonal regeneration (Fracaro *et al.* 2020). Hence, SCI treatment should aim at the prevention of secondary tissue damage followed by restoration of the neuronal circuit, and re-establishment of a damaged axon and synaptic connection. Stem cell-based therapy using MSCs is gaining popularity worldwide as an alternative for treating SCI. MSCs have been investigated extensively in dogs as model animal (Bhat *et al.* 2018), but the therapeutic application of MSCs in SCI is still in its infancy due to uncertainty in the restoration of physiological normalcy, especially motor sensory nerve sensations.

Mesenchymal stem cells and their therapeutic advantages

MSCs are multipotent stem cells (Prockop *et al.* 2003), having an extensive differentiation potentiality into trilineage cells like embryonic stem cells (Ilic and Polak 2011). MSCs reside in a stromal compartment of bone marrow (BM), first identified by Fridenstein and his colleagues in 1970 (Friedenstein *et al.*1970). Apart from BM, this cell can be isolated from almost all post-natal tissues and organs of adult as well as fetal tissues, eg. adipose tissue, umbilical cord blood, amniotic fluid, fetal lung, Wharton jelly (Caplan 2007, Somal *et al.* 2016), brain (Uccelli *et al.* 2011), fat, placenta, dental pulp, tendon, synovial

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membrane and skeletal muscle (Phinney and Prockop 2007, Schipani and Kronenberg 2009, Mili *et al*. 2018).

Isolation of a homogenous MSCs population is a pre-requisite for post-transplantation safety in clinical applications. A wide variety of surface markers have been identified by advanced molecular techniques for distinguishing different types of stem cells (Prasajak *et al.* 2014). However, there are no canine-specific markers that have been identified so far for the characterization of dog MSCs (Bakker *et al.* 2013). The surface markers used for the characterization of dogs' MSCs are presented in Table 1.

MSCs are the best choice for cell-based therapy using stem cells in regenerative medicine because of the advantages given below.

• MSCs are relatively easy to harvest from different sources and have no ethical issues (Lu *et al.* 2006). The proliferation rate of MSCs is very rapid, their ability to differentiate into trilineage cells-like embryonic stem cells (Ilic and Polak 2011) and they can still possess the ability for trilineage differentiation even after cryopreservation at -80°C (Kotobuki *et al.* 2004).

• The unique therapeutic advantage of MSCs is their immunogenic property. Host immunogenic effect is exerted by expressing low MHC class I antigens with a lack of MHC class II with release of paracrine factors of immunogenic property (Asari *et al.* 2009, Shi *et al.* 2012).

• MSCs also possess homing properties and can migrate towards the site of injury (Zachar *et al.* 2016, Mortada and Mortada 2018).

• MSCs secrete paracrine/neurotrophic factors that have a promising therapeutic value in terms of anti-inflammatory, anti-apoptotic, anti-oxidative, vascular angiogenesis, axonal growth, and neuroregeneration (Seo *et al.* 2009, Quertainmont *et al.* 2012, Liang *et al.* 2014, Song *et al.* 2014).

Mechanisms underlying therapeutic potentiality of MSCs in SCI

The precise mechanism by which transplantation of MSCs promotes functional recovery after SCI, is still unclear. The majority of data available describes how MSCs transplantation can influence the wound healing of SCI (Fig. 1) and therefore, based on the published reports, the regenerative effects of transplanted MSCs on wound healing of SCI can be classified into two, viz. direct effect through recruiting new neurons and glial cells at the lesion site; and indirect effect by preventing neuronal apoptosis, reducing inflammation and glial scar, and stimulating vascular angiogenesis, axonal myelination, and neuroregeneration. Many authors agreed that transdifferentiation of transplanted MSCs into neuronal and glial cells at the SCI lesion site is the main underlying neuronal regeneration mechanism (Ryu *et al*. 2012, Chen *et al.* 2015, Gao *et al.* 2019). It is possible due to an intrinsic transdifferentiation potentiality of these cells (Tondreau *et al.* 2004, Blecker *et al.* 2017, Melo *et al.* 2017, Mili *et al.* 2021). Further, Wu *et al* (2018) reported that transplanted MSCs also differentiated into a perineurium-like sheath. This sheath protects the neuronal

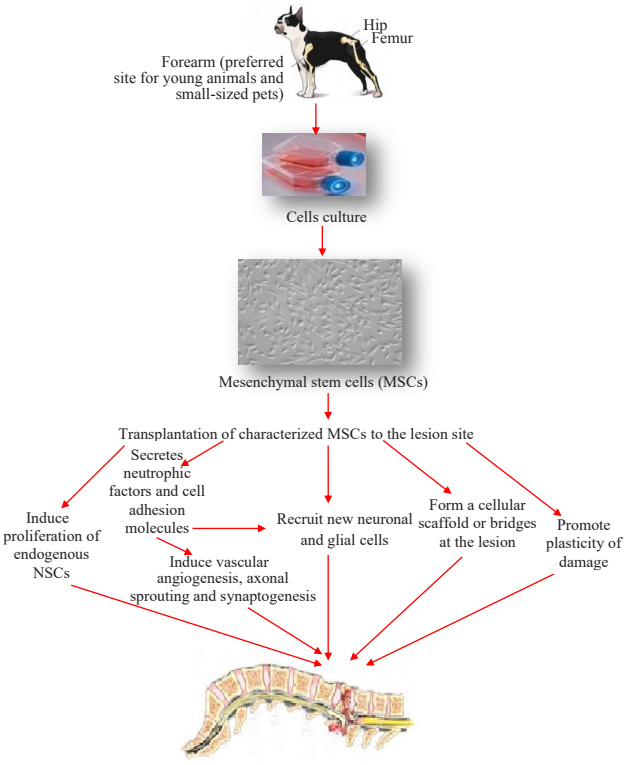

Fig. 1. The mechanism by which MSCs promote wound healing of SCI.

Table 1. Markers use for the characterization of dog MSCs

Source of MSCs	Surface markers	References
Bone marrow	Positive marker: CD-44, CD-73, CD-90, CD-105, CD-29, MHC-1, STRO-1 Negative Marker: CD-14, CD-34, CD-45, CD-146, MHC-II	Kamishina *et al.* 2006, Ryu *et al.* 2012, Hodgkiss-Geere *et al.* 2012, Takemitsu *et al.* 2012, Mili *et al.* 2018
Umbilical cord blood	Positive marker: CD-44, CD-73, CD-90, CD-105 Negative marker: CD-14, CD-34, CD-45	Ryu *et al.* 2012, Kang *et al.* 2012
Adipose tissue	Positive marker: CD-44, CD-73, CD-90, CD-105, CD-29, CD-140a, CD-9, CD-8a Negative: CD-14, CD-34, CD-45, CD-117, CD-146	Vieira *et al.* 2010, Martinello *et al.* 2011, Ryu *et al.* 2012, Swiech *et al.* 2019
Wharton Jelly	Positive marker: CD-44, CD-73, CD-90, CD-105 Negative marker: CD-14, CD-34, CD-45	Ryu *et al.* 2012
Muscle	Positive marker: CD-90, CD-44	Kisiel *et al.* 2012

cells of the SCI lesion site from oxidative damage. Thus, transplanted MSCs could bring about the restoration of an SCI milieu that is required for the growth, and proliferation of the newly recruited cells and resident neuronal stem cells (NSCs). In contrast, several studies have reported that the main therapeutic effects of transplanted MSCs are produced by their secretory neurotrophic factors. Transplanted MSCs secrete a broad range of neurotrophic factors at the SCI lesion site in response to the surrounding environment (Wright *et al.* 2012, Teixeira *et al.* 2013, Hofer *et al.* 2016). These neurotrophic factors influence neighbouring cells and regulates multiple biological processes such as preventing neuronal apoptosis, inflammation and glial scar, vascular angiogenesis, axonal myelination, and neuroregeneration, which are required for wound healing of SCI (Fracaro *et al*. 2020).

MSCs play a pivotal role in inflammatory, proliferative, and remodeling phases of wound healing, and their involvement encourages healthy physiological functioning towards successful healing after the event of SCI (Wright *et al.* 2012, Bhat *et al.* 2018). Fracaro *et al.* (2020) reported that MSCs also secrete a wide range of anti-inflammatory cytokines that directly inhibit the inflammation at the SCI lesion site. It has also been proposed that transplanted MSCs inhibits the release of pro-inflammatory cytokines such as tumour necrosis factor α (TNFα), interferon-gamma (IFN-γ), and IL-6 by the host cells (Boido *et al.* 2014). These mechanisms produce sufficient levels of anti-inflammatory cytokines at the lesion site. At the same time, other secretory neurotrophic factors such as TIMP metallopeptidase inhibitor 1, VEGF, HGF, PDGF, IL-6, IL-8, BDNF, NGF, GDNF, bFGF and EGF that directly encourages vascular angiogenesis, nervous tissue neuroregeneration including the formation of new synapses and myelination (Wright *et al.* 2012, Teixeira *et al.* 2013, Hofer *et al.* 2016). In addition, monocyte chemoattractant protein-1, and granulocyte-macrophage colony-stimulating factor of MSCs act as a neuroprotector by recruiting more number of monocytes during inflammation for enhancing clearance of myelin debris, neurite extension, axonal regeneration, and remyelination at the SCI lesion site (Bouhy *et al.* 2006).

Transplanted MSCs can also activate resident NSCs from their dormant state, bringing their differentiation into relevant neurons/glial cells, regulate scar formation, prevent cyst formation, and secrete some neurotrophic factor to promote repair and regeneration (Donnelly *et al.* 2012, Sabelstrom *et al.* 2013). As such, resident NSCs cannot reinstate the loss of neurons (Sabelstrom *et al.* 2013). Activated resident NSCs differentiate maximum into astrocytes at the SCI lesion site (Wright *et al.* 2012). Astrocytes secrete neurotropic factor that helps in the restoration of the blood-brain barrier of the CNS, inhibits glial scar and cyst formation (Sabelstrom *et al.* 2013). Simultaneously, a few transplanted MSC forms a plug or a bridge-like appearance or a cellular scaffold at the lesion site (Li *et al.* 2015). The plug is formed as a result of interaction between MSCs with host immature glial cells, nerve fibre outgrowth (Hofstetter *et al.* 2002), and many cell adhesion surface protein molecules such as Integrin-1 and Integrin-2, Netrin-4 (Neuhuber *et al.* 2005), Robo1, and Robo 4 (Uccelli *et al.* 2011), which contributes to the decrease of cavitations at the lesion site. Thereby, transplanted MSCs promote axonal regeneration or encourage functional plasticity by establishing a microenvironment, which supports axonal growth by abrogating the inhibitory influence of the glial scar.

Current status of MSCs for treating SCI in dog

MSCs derived from different origins have been used for treating SCI in both preclinical and clinical cases in dogs (Table 2). Several studies have shown that both autologous and allogeneic transplantation of MSCs is feasible, safe, and successful to some extent in terms of functional recovery of motor-sensory nerve sensations. This could be due to the dynamic variability of the neuropathological outcome after SCI and the differentiation potentiality of MSCs. The majority of MSCs transplanted in animal models undergoing SCI have been conducted in the acute phase (Lim *et al.* 2007, Jung *et al.* 2009, Park *et al.* 2012, Ryu *et al.* 2012, Kim *et al.* 2015, Wu *et al.* 2018, Khan *et al.* 2018, Swiech *et al.* 2019). Also, there are several studies conducted on chronic complications of SCI in dogs (William *et al.* 2011, Penha *et al.* 2014, Sarmento *et al.* 2014, Besalti *et al.* 2016, Kim *et al.* 2016, Maciel *et al.* 2017, Bhat *et al.* 2018, Krueger *et al.* 2019). In the acute to sub-acute cases of SCI, the mechanism by which transplanted MSCs might induce wound healing after SCI, differs from the chronic cases. It will be important to study these effects in future using MSCs to improve functional recovery of SCI in dogs.

The therapeutic applicability of MSCs depends on sources of derivation and their differentiation capability into specific cell lineages at the transplanted site (Jeon *et al.* 2016). Bone marrow is the primary source of multipotent MSCs. Bone marrow-derived MSCs (BM-MSCs) have the highest differentiation potentiality into neurons and astrocytes (Melo *et al.* 2017, Mili *et al.* 2018, 2021). However, BM harvesting is an invasive procedure and quite painful to animals (Colleoni *et al.* 2009). On the other hand, adipose tissue-derived MSCs (AD-MSCs) and umbilical cord-derived MSCs (UB-MSCs) are relatively easy to obtain with the minimum trauma and can be expanded *in vitro* (Somal *et al.* 2016). Further, Somal *et al.* (2016) reported that Wharton›s jelly-derived MSCs (WC-MSCs) were superior in terms of growth characteristics, proliferation, and trilineage differentiation potentiality compared to amniotic fluid, amniotic sac, and cord blood. This finding suggested that WJ-MSCs might give better therapeutic applicability and may be an ideal source for cell-based therapy in regenerative medicine. Therefore, it will be important to study the following issues to enable the definition of the best source for derivation of MSCs, cell number, route of infusion, and the number

Table 2. Preclinical and clinical studies for treating SCI in dog using MSCs derived from different sources

Type of experiment	Types of cell and route of administration	Therapeutic efficacy	References
Preclinical trial: Experimentally induced SCI in healthy adult Mongrel dogs (n=5).	Intralesional injection of allogenic UCB-MSCs @ 1×10^6/dog with or without human granulocyte colony-stimulating factor at the lesion site.	A significant improvement was recorded in the nerve conduction velocity based on the somatosensory evoked potentials in both groups. The weight-bearing ability of the pelvic limbs was improved from 10 to 50% in both groups after eight weeks.	Lim *et al.* (2007)
Preclinical trial: Experimentally induced SCI in adult Beagle dogs between 1 to 4 years old (n=10).	Intrathecal injection of autologous and allogenic BM-MSCs @ 10×10^6/dog at the lesion site.	The dogs treated with autologous BM-MSCs were able to stand and lift their trunk after five weeks. Whereas, there was only a sensory response in dogs treated with allogenic BM-MSCs.	Jung *et al.* (2009)
Clinical trial: A male Boxer crossbred dog 6 months old with a complete paraplegia, total loss of motor and sensory functions due to automobile accident (n=1).	Intralesional engraftment of autologous bone Marrow Mono Nuclear Cells (BM-MNCs) with thermoreversible gelation polymer @ 20×10^6/dog at the lesion site followed by intravenous transplantation of 4.16×10^6 on day 19.	The motor and sensory nerve activities and pelvic gait movements were observed on day 53. The dog resumed the normal pelvic gait movements without recurrence of the neurological disorders on day 180.	William *et al.* (2011)
Preclinical trial: Experimentally induced SCI with a complete pelvic limb paralysis in healthy dogs between 2–3 years old (n=6).	Intralesional engraftment of AD-MSCs with matrigel at the lesion site.	A significant improvement in terms of functional recovery after eight weeks in treatments groups. Further, one dog showed weight-bearing ability with a coordinated fore and hind limb gait.	Park *et al.* (2012)
Preclinical trial: Experimentally induced SCI in adult Beagle dogs (n=4).	Intralesional injection of allogenic BM-MSCs, AD-MSCs,UCB-MSCs, Wharton's jelly-derived MSCs @ 6×10^6/dogs at the lesion site.	A significant improvement in terms of functional recovery after eight weeks in treatments groups. However, there was more induced nerve regeneration and anti-inflammation activities in dogs treated with UCB-MSCs.	Ryu *et al.* (2012)
Clinical trial: SCI due to herniated intervertebral disk at T2 to L5 vertebrae. Dogs were between the ages of 2-4 years old (n=4).	Intralesional injection of autologous BM-MSCs @ 5×10^6/dog at the lesion site.	All dogs exhibited progressive recovery of the panniculus reflex and diminished superficial and deep pain response. Further, there was a remarkable improvement in moments of three dogs after 18 months.	Penha *et al.* (2014)
Clinical trial: Dogs were selected from private veterinary hospital and classified as chronic SCI as per the Olbey score (n=7).	Intramedullary injection of allogenic foetal bone marrow stem cells @ 1×10^6/dog at the lesion site.	The locomotors and a sensory activity were observed on day 90. The dogs showed hind limb movement and were able to take small steps without any support. Five dogs showed pain reflexes and defecation on day 90.	Sarmento *et al.* (2014)
Preclinical trial: Experimentally induced SCI in Beagle dogs between the ages of 2-3 years old (n=4).	Intravenous infusion of AD-MSCs @ 10×10^6/dog at the lesion site for three successive days.	Intravenous infusion of AD-MSCs was safe and enhanced motor activities were recorded.	Kim *et al.* (2015)
Clinical trial: The dogs (n=13) were paraplegic, lacking deep pain perception (DPP) due to external trauma. The ages of the dogs were between 2.5 months to 8 years.	Intralesional injection of autologous neurogenically-induced bone marrow-derived mesenchymal stem cells (NIBM-MSCs) @ 5.0×10^6/dog at the lesion site.	Improvement in gait, nociception, proprioception, somatosensory evoked potentials, and motor evoked potentials in 2 dogs, only gait improvement in 6 dogs, and no improvement was recorded in 5 dogs.	Besalti *et al.* (2016)

Type of experiment	Types of Cell and Route of Administration	Therapeutic Efficacy	References
Clinical trial: A total of 25 dogs with no deep pain perception due to the acute thoracolumbar intervertebral disc.	Intralesional injection of allogenic AD-MSCs @10 × 10^6/dog at the lesion site.	The result revealed that 56.6% of dogs were fully recovered with a normal neurologic state, 22.2% of dogs regained with deep pain perception (DPP), but still with mild ataxia and 22.2% did not regain DPP or the ability to walk without support till 6 months follow up.	Kim *et al.* (2016)
Clinical trial: Chronic SCI dogs between the ages of 6-7 yrs and one dog aged 12 years old (n=6).	Intralesional injection of ^{99m}Tc-labeled allogenic AD-MSCs at the lesion site.	The results revealed the improvement of locomotion in three dogs and one dog was able to walk without support.	Maciel Escalhao *et al.* (2017)
Clinical trial: Dogs were selected on the basis of signs of neurological disorders. Paraplegia was reported in all dogs. The dogs were between the ages of 1–10 years old (n=44).	Intralesional injection of allogenic BM-MSCs locally @ 1 × 10^6 cells/dog.	The results revealed that 50% of the BM-MSCs transplanted dogs showed more than 75% recovery as per Olby score (2010).	Bhat *et al.* (2018)
Preclinical trial: Experimentally induced SCI (completed paralysis) in Beagle dogs between the ages of 6 to 8 months old (n=6).	Implantation of BM-MSCs overexpressing Schwann cells with gelatin at the lesion site.	Restoration of neuronal circuit of the paralyzed limb in dogs treated with BM-MSCs over expressing Schwann cells.	Wu *et al.* (2018)
Preclinical trial: Experimentally induced SCI in Beagle dogs between the ages of 1 to 2 years old (n=4).	Intralesional injection of allogenic AD-MSCs and BDNF-overexpressed AD-MSCs with heme oxygenase-1 (HO-1) at the lesion site after 1 week induced SCI.	A significant improvement of hind limb moments with a higher BBB score was observed in dogs treated with BDNF-overexpressed AD-MSCs with heme oxygenase-1 compared to dogs treated with only AD-MSCs.	Khan *et al.* (2018)
Clinical trial: Paraplegic dogs of age between 1-10 years old	Transplanted allogenic AD-MSCs by a lumbar puncture at the lesion site @ 1.2 × 10^6 /dog (n=9) and another group (n=10) a combined therapy of both low-intensity electrical stimulation and AD-MSCs.	In AD-MSCs treated group, three dogs showed motor improvement, four dogs showed low motor improvement and two dogs showed no improvement. Whereas in combined therapy, two dogs showed motor improvement, four dogs showed low motor improvement and four dogs showed no improvement.	Krueger *et al.* (2019)
Preclinical trial: Acute paraplegia resulting from a Hansen type I disc herniation in the thoracolumbar region (T3-L3) in adult dogs (n=11).	Intralesional injection of AD-MSCs @ 10 × 10^6 cells/dog AD-MSCs.	Reported faster locomotor recovery and return of normal urinary function at 3 months in the treatment group.	Swiech *et al.* (2019)

of infusions that may lead to the development of the best suitable cell-based therapeutic strategy using MSCs for the treatment of SCI in both humans and animals.

• The selection for the best source of derivation of MSCs for treating SCI, route of administration and time window.

• Standardization of dose rate of MSCs for intralesional and intravenous infusion as per the SCI lesion size.

• Optimization of dose and combination of paracrine or neurotrophic factors for co-transplantation with MSCs.

• Standardization of use of biomaterials for delivery, engraftment or sedation of paracrine or neurotrophic factors with MSCs at the lesion site.

• A standard procedure of post-transplantation therapeutic management for SCI.

Conclusion

MSCs are a promising cell-based therapy to treat SCI; however, the efficacy in terms of functional recovery of motor-sensory nerve sensations is highly variable. The clinical data indicates that MSCs can be used to treat patients with SCI without any immediate serious

complications. Therefore, some challenging concepts like reducing neural cell death, restoring healthy neural cells, reducing glial scarring, stimulating axonal regeneration, and remodeling the injury niche needs to be addressed to improve the therapeutic efficacy of MSCs for treating SCI in dogs.

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