Title: Advances in Management of Spinal Cord Injury Using Stem Cell-Derived Extracellular Vesicles: A Review Study

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Abstract

Introduction: Spinal cord injury (SCI) is characterized by serious both motor and sensory disability of the limbs below the injured segment. It is the most traumatic disorder among central nervous system (CNS) conditions that not only leads to psychological and physical harm to patients also results in a dramatic loss in life quality. Many efforts have been developed to find a therapeutic approach for SCI; however, an effective treatment has yet to be found. The lack of effective treatment approach and rehabilitation of SCI underscores the need to identify the novel approaches. Tissue engineering associated with stem cells has been recently introduced as an effective treatment approach for traumatic SCI. Although, low survival rates, immune rejection, cell dedifferentiation, and tumorigenicity have been addressed for tissue engineering. Regenerative medicine is an interdisciplinary field developing and applying tissue engineering, stem cell (SC) therapy, SC-derived extracellular vesicles therapy that aims to provide reliable and safe ways to replace the injured tissues and organs. The application of mesenchymal stem cells-derived extracellular vesicles (MSC-EVs) has been recently attracting attention to improve central nervous system dysfunction such as SCI, mainly through promoting neurogenesis and angiogenesis.

Conclusion: This study has summarized the efficacy of MSC-EVs, including exosomes, alone or in combination with scaffolds in treatment of SCI and has then discussed the therapeutic outcomes observed in SCI experimental models following treatment with MSC-EVs alone or loaded on scaffolds in particular collagen-based scaffolds.

Keywords: SCI, Exosomes, Stem Cells, Tissue Engineering

Introduction

CNS disorders especially SCI still remain among the most challenging pathologies having no or limited therapeutic strategies (Ebrahimi-Barough et al., 2015). This traumatic disorder is characterized by serious both motor and sensory disabilities of the limbs below the injured segment leading to physical and psychological harm to patients as well as a dramatic loss in life quality (Liu et al., 2021). Despite the current treatments such as hemodynamic therapy, surgical decompression, corticosteroids, and invasive spinal cord pressure monitoring, the function of the injured spinal cord has not been completely restored, and it is urgent to find a new method for treating SCI (Hawryluk et al., 2015; Fehlings et al., 2012; Kwon et al., 2009; Ryken et al., 2013; Squair et al., 2017; Streijger et al., 2018; Wutte et al., 2019; Yang et al., 2019). Regenerative medicine is an interdisciplinary field developing and applying tissue engineering, SC therapy, and stem cells-derived extracellular vesicles therapy to provide reliable and safe ways to replace the injured tissues and organs (Shirian et al., 2016; Norouzi-Barough et al., 2020; 2021), subsequently results in restoring the cellular function that is lost because of several conditions such as SCI (Liu et al., 2021; Wutte et al., 2019).

The sources of transplanted cell including adult SCs, induced pluripotent SCs (iPSCs), and embryonic SCs (ESC), are considered as the main factors involved in regenerative medicine. MSCs are isolated easily from various tissues including, amniotic fluid and membrane, Wharton's jelly, adipose tissue, dental tissues, endometrium, limb bud, placenta, menstrual blood, peripheral blood, bone marrow, salivary gland, fetal membrane, synovial fluid foreskin, and sub-amniotic umbilical cord lining membrane (Ullah et al., 2015; Najafi et al., 2020). MSCs have low immunogenicity and immunoregulatory properties so they have been widely used as promising transplanted cells for a broad range of defects and diseases, approximately 1000 clinical trials, by regenerative medicine approaches (Ross et al., 2018, Five Decades Later) such as the regeneration of the damaged neuronal cells (Guy and Offen, 2020).

Despite the advantages of differentiation of MSCs to the neuronal cells with neuron-like functions as well as immuno-modulating properties, some limitations were addressed for their implantation. The therapeutic effects of MSCs have been not depended on the physical existence of the transplanted cells within the damaged sites of tissues (Guy and Offen, 2020; Eleuteri et al., 2019). In addition, few fractions of the transplanted MSCs are localized to the injured tissues and site

(Guy and Offen, 2020). To overcome these limitations, several extracellular vesicles and intercellular communication have been recently introduced (Liu et al., 2019). Therefore in this review study the current data regarding the efficacy of the existing MSC-derived EVs in targeting inflammatory and non-inflammatory processes to improve the therapeutic outcomes in experimental models of SCI has summarized.

Underlying Mechanisms involving in the pathophysiology of SCI

Primary and secondary injuries are two consecutive processes involving in pathology of SCI. Primary injury is an irreversible process, which is defined as the mechanical injury to the spinal cord (Kumar et al., 2017). Mechanical injuries result in rupture and destruction of the axonal membranes and the release of a variety of powerful axonal regeneration inhibitors decomposition products from the myelin sheath, such myelin-associated glycoprotein, as neurite outgrowth inhibitor protein A, and chondroitin sulfate proteoglycan oligodendrocyte myelin glycoprotein (Fournier et al., 2001; GrandPré et al., 2000; Beller and Snow, 2014). The secondary progressive injury is started with delay and is generally more complicated than the primary injury (Uldred et al., 2016, Javdani et al., 2019).

Scarring and inflammatory responses to SCI

Inflammatory responses and scarring are associated with SCI include a complex wide variety of cells and cellular activities, which vary based on timing, injury type, and spatial distribution (Orr and Gensel, 2018). The destruction of the blood spinal cord barrier leads to release inflammatory cytokines by inflammatory cells (Uldred et al., 2016). Inflammation-induced secondary injury after SCI includes electrolyte abnormalities and release of excitatory amino acids and reactive oxygen species (ROS) that, in turn, lead to edema, ischemia, apoptosis, and cell necrosis at the injured site (Liu et al., 2021). However, secondary injury responses by microglia, astrocytes, endothelial cells, pericytes, Schwann cells, meningeal cells, fibroblasts, and other glial cells not only potentiate SCI damage also facilitate endogenous repair (Orr and Gensel, 2018).

Extracellular vesicles (EVs)

EVs are the lipid bilayer membrane-enclosed organelles containing various biomolecules involving in the variety of complex intercellular communication systems. They are released from all cell types into their surrounding environment and are important vehicles for paracrine delivery

of therapeutic agents (Veziroglu and Mias, 2020). They are classified into three main subtypes including shedding microvesicles (MVs), exosomes (Exos), and apoptotic bodies (ABs) that are mainly differentiated and classified by their size, biogenesis pathway, cargo, and markers (Norouzi-Barough et al., 2020;2021).

Moreover, based on the latest guidelines (MISEV2018) the following criteria for the classification of EVs have been recommended: I): biochemical makeup (CD63⁺/CD81⁺-EVs, Annexin A5-stained EVs, etc.), II): physical properties of EVs including density (low, middle, high, with each range defined) and size (“small EVs” (sEVs, 50 to 200 nm) and “medium/large EVs” (m/lEVs, more than 200 nm), III): definition of conditions or cell of origin (hypoxic EVs, podocyte EVs, and large oncosomes) (Théry et al., 2018).

Among all EVs, only MVs and Exos (50–1000 nm) mimic the biological activity of parent cells and horizontally transfer the bio-molecules such as RNA fragments (rRNA, lncRNAs, mRNAs, tRNA, piRNAs, miRNAs, RNAs and Y- vault RNAs,) (Sadovska et al., 2015), DNA (genomic DNA, mtDNA) (Williams et al., 2014), lipids, and proteins, to the local microenvironment and target cells, and subsequently mediate tissue regeneration through various mechanisms (Roefs et al., 2020). Therefore, EVs have been explored to act as potential therapeutic agents in a wide range of neuronal diseases associated with inflammatory responses and neuronal apoptosis such as SCI.

Neural stem cells (NSCs) and NSCs-derived small extracellular vesicles (NSC-sEV) in SCI

NSCs have been reported to have the ability to self-renew and differentiate to astrocytes, oligodendrocytes, and neurons (Tang et al., 2017). The unique neuroprotective function post-NSC transplantation has been widely studied that promotes functional recovery after acute SCI (Cheng et al., 2016; Kokaia and Lindvall, 2018). Indeed, NSC transplantation mediates the promotion of regeneration, neurogenesis, plasticity and suppresses the neuroinflammation (Cheng et al., 2016; Kokaia & Lindvall, 2018). However, low survival rate owing to ischemia and immune rejection has been addressed the clinical application of direct NSC for the treatment of SCI (Rong et al., 2019). Therefore, to overcome these limitations, NSC-sEVs have been recently reported to regenerate the SCI (Rong et al., 2019).

NSC-sEV treatment has been reported potentially to inhibit neuroinflammation, attenuate neuronal apoptosis, reduce microglia activation, and experimentally promote functional recovery at an early stage of SCI by promoting autophagy (Rong et al., 2019). Rong et al (2019) reported that NSC-sEV therapy of SCI rat model inhibit neuroinflammation and reduce neuronal apoptosis depends on autophagy induction. sEVs produced by NSCs also showed therapeutic efficacy against inflammatory, ischemic, and neurodegenerative diseases (Stevanato et al., 2016). The anti-inflammatory effect of NSC-sEVs has been also contributed to down-regulate the NO production by activated microglia involving in inflammation following traumatic SCI. The expression levels of pro-inflammatory cytokines are considerably suppressed in SCI pretreated with NSC-sEVs (Rong et al., 2019).

MSC-Derived Exosomes

Exosomes are mostly secreted by MSCs (Yu et al., 2014). The tetraspanins as the common exosomal surface markers (CD9, CD63, and CD81) are expressed by MSC-Derived Exosomes. MSC-Derived Exosomes also express ALG-2 interacting protein X, heat shock proteins (HSP)-60, HSP-70, and HSP-90, adhesion molecules (CD73, CD44, and CD29), and tumor susceptibility gene 101 (Tsg101) (39)(Colombo et al., 2014). They also carry various biomolecules such lipids, nucleic acids (microRNAs (miRNAs)), and proteins (Zaharie et al., 2014). Several MSCs-Derived Exosomes have been reported to have potential promoter for functional recovery (FR) and their contents can serve as biomarkers in SCI (2) (Liu et al., 2021) that have summarized in this study.

Exosomes Derived From Pericytes (P-sEVs)

Pericytes are contractile cells that encircle the endothelial cells (Simonavicius et al., 2012). Pericytes are important compounds of the astrocytes, endothelial cells (ECs) in neurovascular unit, and neurons (Dore-Duffy and Cleary, 2011). They take part in the control of integrity and permeability of microvascular, pressure of microcirculation, participate in angiogenesis, and regulating ECs differentiation by paracrine signaling and direct physical contact (Orehhov et al., 2014). ECs are able to take up exosomes of pericytes due to the special relationship between endothelial cells and pericyte that play role in the mediation of ECs function. P-sEVs reduce pathological changes and improve the blood flow, the motor function, and oxygen deficiency after SCI. P-sEVs improve the endothelial ability to protect the blood-spinal cord barrier, regulate blood flow, and decrease edema. The down-regulated expression of Bax, MMP2, HIF-1 α , and,

Aquaporin-4, as well as the up-regulated the expression of bcl-2 Claudin-5 by P-sEVs has led to inhibit apoptosis (Yuan et al., 2019). Therefore, they have been suggested to have therapeutic prospects for apoptosis and regulate the microcirculation after SCI (Yuan et al., 2019).

Exosomes derived from human placenta (hP-sEVs)

MSCs derived from human placenta have low immunogenicity and their effectiveness is attributed to secretion of factors. hP-sEVs have been shown to improve neurologic function in mice SCI model. hP-sEVs have proangiogenic effects on endothelial cells leading to promote the angiogenesis in mice SCI model after injury (Zhang et al., 2020).

Exosomes derived from bone marrow MSCs (BM-sEVs)

Astrocytes play an important role in the process of SCI, as they can inhibit or promote the CNS recovery. A1 astrocytes that are induced by neuroinflammation rapidly are formed after SCI. The inhabitation of their neurotoxic effects on synapses, myelin, and neurons may be a promising strategy for treatment of SCI (Liddelow et al., 2017). Following SCI, BM-MSC and BM-sEVs reduce SCI-induced A1 astrocytes via down-regulation of phosphorylated NF κ B p65. They exert anti-inflammatory and neuroprotective effects with the therapeutic effect when injected intravenously (Wang et al., 2018). Not only the secondary injury induced by inflammation in SCI is regulated by the NF- κ B pathway (Conti et al., 2003) but also is extensively activated by a variety of pro-inflammatory cytokines such as TNF- α and IL-1 as well as ROS (Kaltschmidt & Kaltschmidt, 2009). SO inhibition of the NF- κ B signaling pathway can promotes FR after SCI (Wang et al., 2018).

Autophagy is critical for the protection against SCI and BM- sEVs have been shown to attenuate neuronal apoptosis by promoting autophagy *in vitro* and after SCI and improve functional behavior and recovery of motor function in SCI rat model (Guo et al., 2019).

Human umbilical cord-derived Exosomes (hUC-sEVs)

The modulation of inflammation is obtained after the injection of hUC-MSCs and hUC-EVs in rat SCI model that can be contributed to their potential to reach the injured spinal cord tissue and directly interact with microglia. The capability of hUC-EVs to interact with rat microglia has been addressed *in vitro* using microglia cultures. Since, hUC-EVs have been shown to reduce the

induction of expression levels of IL-1 β and IL-6 in rat microglia cultures in response to LPS, they might directly interact with the activated microglia at their early activation and modify the cytokine expression profile toward a milder pro-inflammatory response and inhibit the mRNA expression level of pro-inflammatory cytokines during secondary injury in a SCI (Romanelli et al., 2019). The hUC-EVs also contribute to the recovery of motor function by inhibiting the formation of scars (Romanelli et al., 2019). It has been suggested that hUC-sEVs can improve activities of aged MSCs and enhance their function by down-regulating apoptotic protease activating factor 1 (Apaf1) having pro-apoptotic activity (Zhang et al., 2020).

Human endometrial-derived Exosomes (hUC-sEVs)

Endometrium has been recently introduced as an available source of MSCs that can be isolated without anesthesia and adverse effects (Ebrahimi-Barough et al., 2015). Despite therapeutic potential of human endometrial MSCs as well as the biological role of hE-sEVs has been previously established; the effectiveness of hE-sEVs for treating SCI has been yet known. However, hE-sEVs have been shown to increase proliferation, migration, and angiogenesis of HUVECs *in vitro* (Taghdiri Nooshabadi et al., 2019).

Epidural fat-Mesenchymal stem cells derived Exosomes (EF-sEVs)

Intravenous injection of EF-sEVs has been shown to inhibited NLRP3 inflammasome activation and reduced the expression of inflammatory cytokines (Huang et al., 2020). In addition, systemic administration of EF-sEVs in SCI rat model results in up and down-regulated the expression levels of antiapoptotic Bcl-2 and proapoptotic protein Bax, respectively (Huang et al., 2020).

Exosome loaded on scaffolds

In contrast of local implantation of MSC-sEVs that is a promising therapeutic strategy, the efficacy of exosomes has been reported to be limited by the poor presence at lesion site of the spinal cord when are injected systematically (Li et al., 2020). Therefore an effective delivery strategy using a three-dimensional biocompatible scaffold has been demonstrated to achieve for the efficient retention, effective delivery, release, and integration of exosomes in the injured spinal cord (Li et

al., 2020). A variety of materials are capable to carry the transplanted cells for treating SCI. However, based on our knowledge biocompatible 3D scaffolds for adhesive sEVs delivery remains poorly investigated. Li et al. (2020) have recently shown the local delivery of hP-sEVs by using an adhesive HA hydrogel.

Discussion

Individuals with SCI usually suffer from permanent neurological and non-neurological disorders and deficits, while therapeutic efficacies are limited (Bourassa-Moreau et al., 2013). At present, no clinical trials of MSC-sEVs have been conducted to regenerate the SCI. However, stem cell therapy as well as exosome derived stem cells isolated from different sources seems to be an attractive option for treating SCI. An injured spinal cord is poorly regenerated due to insufficient repair of endogenous cells, the weakened ability of axonal growth, and the presence of inhibitory molecules at the injured site (Wu et al., 2015; He and Jin, 2016; Jure et al., 2017). Overcoming these obstacles would lead to find an ideal strategy for treatment of SCI.

To date, the therapeutic efficacy of the exosomes derived from stem cells for treating an injured spinal cord tissue has been mainly demonstrated by several mechanisms including anti-inflammatory effects, regulating inflammation, angiogenesis promotion, and axon formation, maintaining the integrity of the blood–spinal cord barrier, and inhibiting apoptosis (Kumar et al., 2017; Ren et al., 2020; Rong et al., 2019; Zhang et al., 2020). The exosomes increase A2 astrocytes playing neuro-protective role by up-regulating the expression of neurotrophic factors. In contrast, they decrease the A1 astrocytes that are rapidly formed post-SCI, and have neurotoxic effects. The exosome reduce the A1 astrocytes by inhibition of nuclear translocation of NF- κ B p65 (Wang et al., 2018).

It has been shown that the exosomes can be used as valuable carriers for targeting miRNAs (miRNA-21, miRNA, 29, miRNA-133b, miRNA-126, miRNA-216) at the SCI site. The miRNA-29, miRNA133b, and miRNA126 promote the axonal regeneration. The miRNA-216 and miRNA-21 promote microglia polarization and reduce neuronal apoptosis, respectively (Ren et al., 2020, Huang et al., 2020). In general miRNAs plays an important role in the recovery of motor function, reduces neuronal axonal regeneration, mediates inflammation, neuronal differentiation, growth, and apoptosis (61).

The anti-inflammatory effects of exosomes are attributed to multiple effects of the exosomes. They directly interact with the activated and polarized microglia and down-regulate the mRNA expression level of pro-inflammatory cytokines such as IL-1 β and IL-6 during secondary injury (Romanelli et al., 2019). The nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome is an important protein complex-inflammasome that activates the resident immune cells leading to neuroinflammation and secondary injury of SCI (Zhou et al., 2016). It has been shown that the activity of the NLRP3 inflammasome is increased in SCI models (Zhou et al., 2016). Functional recovery occurred by inhibiting activation of the NLRP3 inflammasome in a rat model of SCI (Jiang et al., 2017). Intravenous injection of EF-sEVs inhibits the activation of NLRP3 inflammasomes and reduces inflammation. Sun et al. (2018) found that hU-sEVs increase the levels of the anti-inflammatory cytokines IL-4 and IL-10 and reduce the levels of the pro-inflammatory cytokines IL-6, TNF- α , interferon- γ , and granulocyte colony-stimulating factor. The EVs sources and their action mechanisms are shown in Fig. 1.

Conclusion

Although the treating SCI has been extensively studied, the effective strategy has yet to be found for restoring lost function. The treatment of SCI remains a great challenge and hinders the development of treatments leading to a full recovery due to the pathological process of SCI is very complex. Exosomes derived from stem cells alone or loaded on various biocompatible scaffolds such as collagen hydrogel or other ECM-based scaffold can be considered as the good drug carriers that has great potential for treatment of SCI.

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Figure legends

Fig. 1: Most common sources of MSCs are bone marrow, adipose tissue, and umbilical cord. Extracellular vesicles (EVs) including exosomes and microvesicles are released from different cell types including MSCs, and mediate the beneficial effects of MSCs on target cells. MSC-EVs reduce neuroinflammation via inhibiting the activation of astrocytes and microglia and switching to an anti-inflammatory phase. They also attenuate neuronal apoptosis, and promote angiogenesis, neurogenesis, and functional recovery in SCI patients. **B**) EVs can be derived from the patient's MSCs (autologous) or from healthy donors (allogenic) and then expand *in vitro*. In the next step, EVs are extracted and purified by various protocols based on different protein markers, sizes, and density of EVs. Isolated EVs following quality control can be administrated directly, or with biocompatible 3D scaffolds.