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Review Article

Mesenchymal stem cell-derived extracellular vesicles: emerging concepts in the treatment of spinal cord injury

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Abstract: Spinal cord injury (SCI) is a prevalent central nervous system disease with a high disability rate, leading to the loss of motor and sensory nerve function. Due to the complex pathophysiology of SCI, more effective clinical treatment strategies are needed. Research has indicated the considerable potential of extracellular vesicles (EVs) derived from mesenchymal stem cells (MSC-EVs) as a cell-free therapy in SCI repair and regeneration due to their ability to regulate immune cell activity and stimulate damaged neuron regeneration. Moreover, applying MSCs and engineered EVs can fully exploit the potential of MSC-EVs in spinal cord repair. Here, we outline the pathological process of SCI and its current clinical treatment status, summarize the latest MSC-EVs research and its pretreatment and engineering strategies in SCI treatment, and explore MSC-EVs application prospects.

Keywords: Spinal cord injury, mesenchymal stem cells, extracellular vesicles, engineering, treatment

Introduction

Spinal cord injury (SCI) is a severe central nervous system (CNS) disorder with high disability and mortality rates and is recognized as one of the greatest threats to human health [1, 2]. SCI is estimated to permanently disable more than 27 million people worldwide, with approximately 77,000 new cases each year [1, 3, 4]. Current clinical treatments for SCI include pharmacotherapy, surgical decompression, hemodynamic therapy, and electrical stimulation. However, these methods do not completely slow SCI's pathological progression [3-5]. Thus, an urgent need exists for a novel SCI treatment approach [6].

Transplantation of mesenchymal stem cells (MSCs) is a promising therapeutic approach for SCI treatment, but direct transplantation of stem cells brings safety and ethical concerns [10, 11]. MSCs, or multipotent stromal cells, are a class of pluripotent stem cells belonging to the mesoderm, mainly found in connective tissue and organ mesenchyme [7]. MSCs have a strong proliferative capacity with multiple differentiation potentials and immunomodulatory functions and are also "seed cells" for tissue damage repair [8, 9]. Notably, growing evidence suggests that MSCs' therapeutic properties are more likely to derive from paracrine effects, particularly from extracellular vesicles (EVs) [12, 13]. EVs are essential mediators of intercellular communication and are involved in many pathological processes [14, 15]. The therapeutic potential of MSCs derived from EVs (MSC-EVs) in SCI has received increasing attention in recent years [16-18].

Here, we review the pathophysiology and current status of SCI treatment. Then, we describe EV biogenesis and summarize the roles and mechanisms of MSC-EVs in SCI repair. Furthermore, we review the application and challenges of pretreatment methods and engineering strategies being used to improve the therapeutic potential of MSC-EVs in SCI.

The pathophysiology of SCI

Most spinal cord injuries are caused by contusion or impingement [6]. Changes in spinal canal shape or volume lead to physical deformation of spinal cord tissue, compressing blood

vessels and axons and triggering a cascade of pathological processes [1, 19, 20]. In the acute phase of SCI (typically within hours to days after injury), bleeding from ruptured blood vessels causes tissue ischemia and localized spinal cord swelling [21, 22]. Damaged cells release adenosine triphosphate (ATP) that act on purinergic receptors of various immune cells, inducing the immune cells to converge on the damaged area [23, 24]. Microglia (innate immune cells of the CNS) exert phagocytosis in the injured tissue to prevent injury spread. However, microglia also mediate excessive immune responses. Subsequently, tissue reperfusion-induced oxidative stress and glutamate release lead to the death of neighboring neurons and glial cells [25, 26].

Furthermore, a dramatic imbalance in the ionic homeostasis of the tissue microenvironment activates enzymes such as lipid oxidase and lipid accumulation, mediating the occurrence of cellular lipid peroxidation and expanding areas of tissue necrosis [19, 22, 26].

Later in the acute phase of SCI, the hemostatic response also brings about a series of cascade reactions to stop hemorrhage from broken vessels while also bringing about a potent inflammatory response stimulus (e.g., chemokines and eicosanoids released by platelets) to glial cells [27, 28]. The subacute phase occurs days to weeks after injury, where the incomplete pathological tissue and inflammatory microenvironment activate a variety of cells (including astrocytes, fibroblasts, oligodendrocytes, pericytes, and macrophages), triggering a cascade of secondary damage that further exacerbates injury and inflammation [21, 29-32]. Later in the subacute phase, cross-linked interactions between reactive glial cells and non-neural cells form scar tissue [33, 34]. Simultaneously, extracellular matrix (ECM) molecules, such as chondroitin sulfate proteoglycans (CSPGs), block the outgrowth and regeneration of damaged axons [20, 35, 36].

SCI's chronic phase exhibits a gradual decrease in the local acute inflammatory response and a diminishing of glial cell proliferation. Additionally, the spinal cord tissue at the injury site may become atrophied, softened, cystic, or even spinal cavernous [21, 30, 36]. At the periphery of the damaged area, distinct scar tissue or spinal cord compression forms by glial cells, fibroblasts, and ECM molecules. While limiting inflammation, scar tissue can prevent axonal lengthening [31, 35]. Lack of axonal lengthening results in long-term loss of spinal cord function and affects the physiological function of the trunk and extremities below the site of spinal cord damage [29, 37-39].

Current status of SCI treatment

According to the Clinical Neurorestorative Therapeutic Guidelines for Spinal Cord Injury (version 2019) proposed by the International Association of Neurorestoratology and the Chinese Association of Neurorestoratology, the primary strategies for SCI neurorepair are pharmacotherapy, surgical intervention, and electrical stimulation therapy [3]:

(a) Pharmacotherapy: Early high-dose methylprednisolone (MP) therapy was once considered to benefit neurorepair in SCI's acute phase. However, various clinical trials have shown that MP therapy may have serious side effects, such as pneumonia and wound infections [40, 41]. Accordingly, high-dose MP therapy is no longer recommended for routine use for acute SCI [4, 42]. Pharmacological treatments, such as gangliosides and acidic fibroblast growth factor, may benefit SCI, but additional clinical data are needed to confirm their use [43, 44].

(b) Surgical intervention: Spinal cord decompression and internal fixation surgery can be immediately performed post-injury, within an appropriate window, to reduce secondary injury and prevent further destruction of spinal cord tissue. However, many patients are usually unable to undergo surgery promptly due to transportation, preoperative examination, and preparation [5, 45].

(c) Electrical stimulation therapy: In SCI's chronic phase, local neuromuscular or peripheral nerve electrical stimulation may improve and induce axonal regeneration. However, this therapy is still being further explored for therapeutic effects [46-48].

The pathological changes of SCI are incredibly complex. Clinical treatment focuses on alleviating the imbalance of the microenvironment caused by secondary injury in the acute and/or subacute phase and protecting the surviving

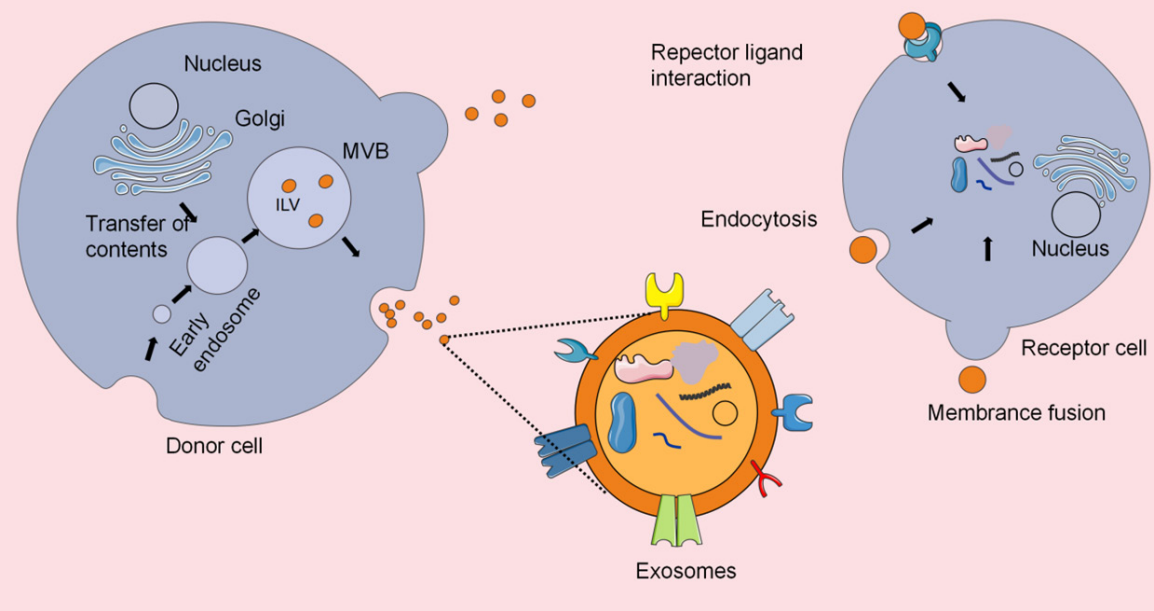

Figure 1. The biogenesis and uptake of exosomes. Exosomes are generated via the endocytic pathway and are released into the extracellular space. Cellular contents such as proteins, lipids, metabolites, small molecules, DNA, RNA, and cell surface proteins can be internalized into extracellular vesicles (EVs) via endocytosis and membrane invagination. The EVs released by donor cells can interact with recipient cells and induce biological responses within the recipient cells. The process occurs via an interaction with cell surface proteins or receptors or through internalization via endocytosis or membrane fusion.

neurons. However, the current treatment options are ineffective in changing the underlying pathology of SCI [49]. Therefore, searching for novel, more effective, and safe therapies is an urgent clinical need.

Overview of MSC-EVs

With strong immunomodulatory and tissue regenerative potential, MSCs effectively treat various refractory diseases, including SCI [50, 51]. MSCs influence tissue damage repair primarily through paracrine effects. One of the most essential paracrine effectors of MSCs is EVs. EVs are nanoscale-sized vesicles composed of phospholipid bilayer membranes that deliver bioactive components [7, 12, 16].

Depending on their size and biogenesis, EVs can be classified into three major categories: apoptotic bodies [52], microvesicles, and small EVs, known as exosomes [53]. Apoptotic bodies are the largest subpopulation of EVs and are released by cells undergoing programmed cell death. Microvesicles are 200-1000 nm in diameter and arise from the plasma membrane surface by means of outgrowth [54]. Exosomes are produced by the endocytic pathway and have an average diameter of 30-200 nm.

Exosome formation is a complex process involving the formation of endosomes and intracellular multivessel bodies (MVBs) and ends in their release [55]. First, the cytoplasmic membrane forms early endosomes via the endocytic pathway. Early endosomes subsequently fuse and exchange with other organelle material to form late endosomes. Late endosomes further develop into MVBs [56]. During this process, intraluminal vesicles form and accumulate in MVBs with the help of Golgi complexes. Finally, intraluminal vesicles form exosomes after fusing with the plasma membrane; exosomes are then released into the extracellular environment [57]. When exosomes are released, they can be internalized into recipient cells by three potential mechanisms: endocytosis, direct fusion (with plasma membrane), or receptor-ligand interactions [53, 58]. Exosomes may contain many components depending on the parental cells' specific physiological or pathological state, including DNA, RNA, lipids, proteins, and metabolites [59, 60]. Thus, exosomes reflect the parental cells' metabolic state and function. Exosomes then reach recipient cells and exchange molecular information [15] (**Figure 1**).

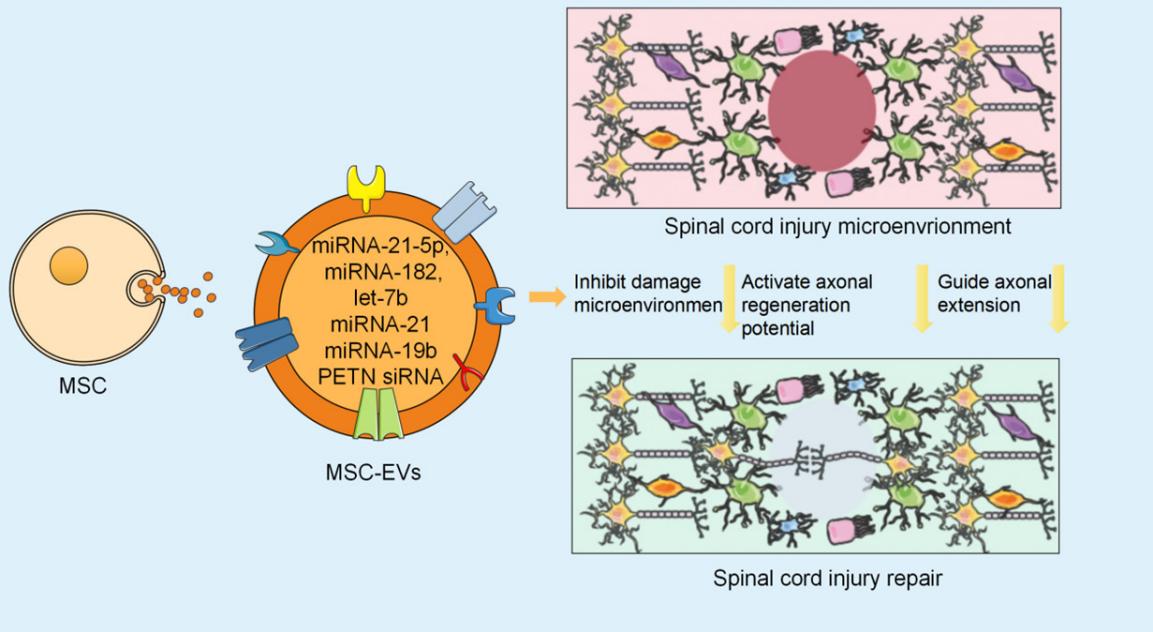

Figure 2. Natural mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) in spinal cord injury (SCI) repair. The utilization of MSC-EVs in SCI restoration is achieved through the transportation of active molecules like miRNA, which suppresses the detrimental microenvironment, activates the regenerative potential of surviving neurons, and guides axonal regeneration to alleviate SCI.

Many studies have found that MSC-EVs can repair a wide range of tissue damage and may be a novel "non-cellular" therapeutic strategy superior to cell therapy [12, 61]. Additionally, the expression profiles of MSC-EV components from multi-omics applications of active RNA, protein, and lipid components have been gradually revealed. We now know that MSC-EVs play essential therapeutic roles in diabetes, tissue recovery, immunomodulation, and neuroprotection by regulating gene expression of receptor cells [62-66]. Mechanically, enriched proteins of MSC-EVs play irreplaceable roles in tissue repair and regeneration, including in Wnt4 [67], 14-3-3ζ [68, 69], glutathione peroxidase 1 [70], casein kinase 1δ (CK1δ), β-transducin repeats-containing proteins [71], E3 ubiquitin-protein ligase NEDD4 [72] and Beclin-1 [73], among others. In summary, MSC-EVs have promising applications in various refractory diseases and tissue damage repair. The following section will focus on the latest advances in MSC-EVs in treating SCI.

Therapeutic role of MSC-EVs in SCI

Natural MSC-EVs in SCI

The use of MSC-EVs in SCI repair has been gaining increased attention, both independently and in conjunction with bioactive molecules [74]. Typically, these treatments focus on three key areas: (1) the inhibition of inflammation in the microenvironment of the spinal cord to decrease barriers of axonal germination [75], (2) the activation of the axonal regeneration potential of impaired neurons or endogenous neural stem cells to reconstruct damaged neural circuits and facilitate functional recovery [76], and (3) the use of MSC-EVs with biomaterials to supply both graft and nutritional support for guided axonal regeneration [77] (**Figure 2**).

Inhibition of the damaged microenvironment: The first step in regenerating damaged CNS neurons is early growth cone generation that guides axonal regeneration [78]. However, damaged neurons form structures called retraction bulbs, and axonal outgrowth ceases due to the poor microenvironment of the damaged spinal cord [34]. The spinal cord microenvironment comprises glial cells, immune cells, pericytes, endothelial cells, extracellular matrix, and neurotransmitters [39].

It has been well established that MSC-EVs can modulate various cell types of the spinal cord microenvironment to improve microenvironments that otherwise are not conducive to neu-

ral regeneration [79]. Zhao et al. found that MSC-EVs suppressed inflammation in the spinal cord microenvironment by blocking the activation of the nuclear factor-κB signaling pathway and the microglia complement system. Microglia are the first immune cells to activate and mediate inflammation after SCI [80]. Also, various miRNAs of MSC-EVs origin (e.g., miRNA-21-5p, miRNA-182, and let-7b) have been found to promote macrophage polarization toward the anti-inflammatory phenotype M2 alleviating SCI by targeting and inhibiting toll-like receptor signaling pathway [81-83]. Macrophages in the peripheral circulation are recruited in response to vascular rupture and inflammation caused by spinal cord damage. They further exacerbate the inflammatory response of the spinal cord microenvironment and are detrimental to axonal regeneration [81]. Lastly, MSC-EVs were found to protect pericytes, critical components of the neurovascular unit, thus promoting neuronal survival and nerve fiber extension by inhibiting cell scorching and improving blood-spinal cord barrier integrity; MSC-EVs ultimately improved motor function in spinal cord injured rats [84, 85].

Activation of axonal regeneration potential: Multiple transcriptional programs are repressed in most highly differentiated neurons in mammals, thus limiting their regenerative potential [86]. The mammalian target of the rapamycin (mTOR) signaling pathway activates neonatal CNS neurons to maintain cell growth, metabolism, and protein synthesis. However, in adult CNS neurons, enhanced expression of phosphatase and tensin homolog deleted on chromosome ten (PTEN) activity, a negative regulator of mTOR, considerably reduces mTOR signaling levels, leading to diminished neural regeneration [17].

MSC-EVs have been demonstrated to inhibit PTEN by delivering miRNA-21 and miRNA-19b promoting axonal growth and neuronal survival [86, 87]. Conversely, activation of endogenous neural stem cells in the spinal cord to differentiate toward mature neurons can reconnect parts of the neural circuits and restore neural function. Additionally, MSC-EVs have been suggested to promote the activation of proliferating endogenous neural stem cells by activating the ERK pathway, as evidenced by a considerable increase in activation of spinal SOX2 + GFAP + and SOX1 + KI67 + cells, among others [88-90].

Combining biomaterials to guide axonal extension: MSC-EVs can also be combined with biomaterials such as hydrogels for SCI repair applications [91]. Gel biomaterials can guide axonal extension toward the lesion region to slow-release carriers and enhance the utilization efficiency of MSC-EVs [92]. For example, Wang et al. encapsulated MSC-EVs in an injectable (FE@EVs) for in situ drug delivery in SCI rats. FE@EVs achieved up to 56 days of EV release in vivo and considerably improved motor function recovery in rats after SCI [77]. Additionally, to mimic the conductive properties of neural tissue, Fan et al. developed a conductive hydrogel composed of gelatin methacrylate and polypyrrole loaded with MSC-derived exosomes (GMPE). GMPE induced the differentiation in a mouse spinal cord hemisection model of neural stem cells to mature neurons in vitro and showed considerable neuroregenerative benefits after implantation [93]. Accordingly, the combined application of MSC-EVs and biomaterials may serve as an in vivo treatment for SCI (**Figure 3**).

Pretreating and engineering strategies to enhance the therapeutic potential of MSC-EVs for SCI

Despite the tremendous therapeutic potential of natural MSC-EVs for SCI, many inherent limitations still limit their large-scale application in SCI therapy. For example, the decreased stemness and replicative senescence of MSCs under conventional culture conditions lead to a decrease in the number and therapeutic efficacy of their secreted EVs [94, 95]. Additionally, due to the inherent properties of natural EVs, MSC-EVs are poorly targeted to SCI injury sites in vivo after intravenous administration [96, 97]. Therefore, improving the production of MSC-EVs and enhancing their targeting ability is essential for their clinical application [98].

Pretreating methods: The therapeutic potential of MSC-EVs is closely related to their cellular status. Changing culture conditions or drug pretreatment are effective strategies to improve MSC-EV yield and therapeutic efficacy [99]. For example, hypoxic culture was found to mimic the “stem cell niche” environment of MSCs in vivo, maintaining the stemness of MSCs and

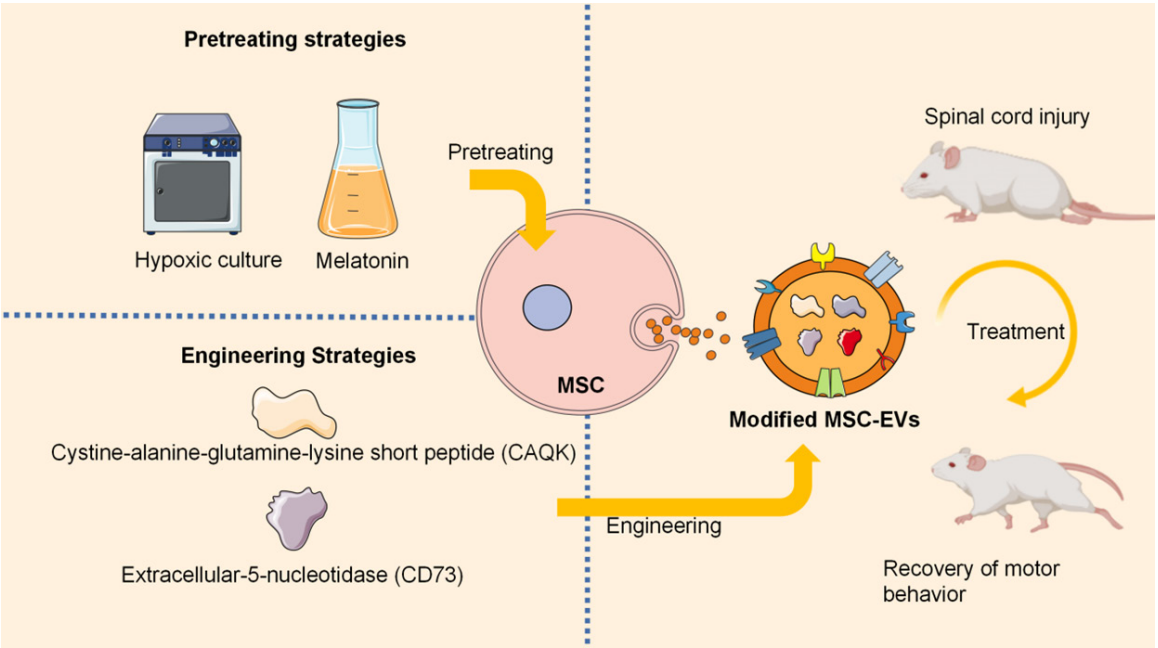

Figure 3. Pretreating and engineering strategies to enhance the therapeutic potential of mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) for spinal cord injury (SCI) repair. Hypoxia and melatonin pretreatment alter the contents of MSC-EVs and thus improve their SCI efficacy. cystine-alanine-glutamine-lysine short peptide (CAQK) and extracellular-5-nucleotidase (CD73) engineering strategies can improve the expected efficacy of MSC-EVs.

enhancing their proliferation capacity [100]. Also, MSC-EVs from a hypoxic culture delivered microRNA molecules involved in neuroregeneration and inflammation regulation (e.g., miRNA-511-3p and miRNA-499a-5p) and effectively promoted SCI repair [101, 102]. Liu et al. found that melatonin (MT) pretreatment (an amine-like hormone secreted by the brain's pineal gland with free radical scavenging and antioxidant effects [103]) decreased the expression of methyltransferase 3 in MSCs, inhibited m6A methylation, and maintained the stability of ubiquitin-specific protease 29 (USP29) mRNA in the cells. They also found that MT-pretreated MSC-EVs stabilized nuclear-like factors by delivering USP29, which regulated microglia/macrophage polarization and promoted recovery of motor behavior in SCI mice [103]. These studies demonstrate the benefits of diverse pretreatment methods for MSCs and MSC-EVs. Designing novel combinations of MSC pretreatment methods for different pathological stages or degrees of injury in SCI may provide more effective treatment strategies for SCI in the future [98].

Engineering strategy: MSC-EVs have also been modified by engineering means such as content piggybacking or membrane modification to enhance spinal cord targeting and SCI therapeutic efficacy [104, 105]. Recently, it has been found that cystine-alanine-glutamine-lysine short peptide (CAQK) can specifically target CSPGs at SCI-damaged sites [106]. Accordingly, Wang et al. constructed CAQK peptide-modified MSC-derived exosomes (EXO-C@P) loaded with a TNF-α-responsive self-feedback CRISPR/Cas9 system. The team successfully delivered EXO-C@P by intravenous injection to the SCI-impaired site. EXO-C@P was phagocytosed at the site by activated immune cells (e.g., macrophages and neutrophils) and carried out cellular-level gene editing, resulting in the secretion of soluble mTNFR1 to neutralize TNF-α and reduce inflammation; the system facilitated recovery from SCI [106].

Additionally, as extracellular-5-nucleotidase (CD73) can catabolize excess ATP to adenosine after SCI and alleviate the damaged microenvironment, Zhai et al. engineered MSC-EVs overexpressing CD73 ($CD73^+$ MSC-EVs). $CD73^+$ MSC-EVs improved SCI by reducing extracellular ATP in spinal cord tissue and activating the A2bR/cAMP/PKA pathway [107]. As more therapeutic molecules for spinal cord and neuronal

Table 1. Therapeutic role of MSC-EVs in SCI

Origin of EVs	Target cells	Cargos in EVs	Mechanism	Ref.
Natural Mesenchymal stromal cells-derived extracellular vesicles (MSC-EVs)	Microglia	/	Inhibite activation of the microglia complement system and NF-κB signaling pathway	[80]
	Macrophages	miRNA-21-5p, miRNA-182, or let-7b	Promote macrophage polarization toward M2 (anti-inflammatory phenotype)	[81-83]
	Pericytes	/	Inhibite cell scorching and improving blood-spinal cord barrier integrity	[84, 85]
	Neurons	miRNA-21 or miRNA-19b	Promote axonal growth and neuronal survival	[86, 87]
	Neural stem cells	/	Promote the activation of proliferating endogenous neural stem cells by activating the ERK pathway	[88-90]
F127-polycitrate-polyethyleneimine hydrogel (FE@EVs)	Neurons	/	Improve motor function recovery in rats after SCI	[77]
Gelatin methacrylate (GM) and polypyrrole (PPy) loaded with MSC-derived exosomes (GMPE)	Neurons	/	Induce the differentiation of neural stem cells	[93]
MSC-EVs from a hypoxic culture	Neurons	miRNA-511-3p or miRNA-499a-5p	Improve neuroregeneration and inflammation regulation	[101, 102]
Melatonin (MT) pretreated MSC-EVs	Microglia/macrophage	/	Regulate microglia/macrophage polarization and promoted recovery of motor behavior in SCI mice	[98]
Cystine-alanine-glutamine-lysine short peptide (CAQK) modified MSC-derived exosomes (EXO-C@P)	Macrophages and neutrophils	TNF-α self-feedback CRISPR/Cas9 system	Carry out cellular-level gene editing, resulting in the secretion of soluble mTNFR1 to neutralize TNF-α and reduce inflammation	[106]
Engineered MSC-EVs overexpressing CD73 ($CD73^+$ MSC-EVs)	Microglia	CD73	Reduce extracellular ATP in spinal cord tissue and activating the A2bR/cAMP/PKA pathway	[107]

cells are uncovered, engineering technologies will load multiple combinations of molecules into MSC-EVs. The combined application of membrane modification and content piggybacking technologies is expected to address MSC-EV neural targeting while improving the enrichment of active molecules [108-110]. Thus, pretreatment and engineering strategies are expected to be the propellers of MSC-EVs in SCI treatment.

Conclusions and outlook

Due to the weak regenerative capacity of the CNS in adult mammals, recovery of neurological function after SCI is rather limited [1, 5]. The pathological process of SCI is also complex and lengthy, with the recovery of spinal cord tissue subject to a combination of factors. Despite the availability of therapeutic tools to reduce mortality in SCI patients, functional recovery after SCI remains a great challenge for current medicine [4, 28]. A series of experiments have demonstrated that MSC-EVs can easily cross the blood-spinal cord barrier, improve the poor injury microenvironment of the spinal cord, activate the regenerative potential of damaged neurons, and combine with biomaterials to guide axonal extension at the injured site [18, 87, 111]. These conclusions suggest that MSC-EVs are an expected novel, non-cellular therapy for SCI treatment. Additionally, pretreatment methods like hypoxia and engineering strategies like membrane modification could considerably optimize MSC-EV therapeutic potential in SCI [98] (**Table 1**).

Certainly, MSC-EVs or modified MSC-EVs in SCI treatment need more study to elucidate their mechanisms [13, 112]. Also, further clinical trials are required to validate the efficacy and safety of MSC-EVs in humans [111, 113]. Likewise, establishing a set of standardized processes for the isolation and purification of MSC-EVs and the concentration and mode of administration are the keys to upscaling applications [114, 115]. Future studies that address these issues will provide a comprehensive theoretical basis for the clinical translation of MSC-EVs in SCI treatment, providing direction and hope for SCI clinical treatment [116, 117].

Disclosure of conflict of interest

None.

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