International Journal of Nanomedicine

Dovepress
Taylor & Francis Group

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REVIEW

Advanced Therapeutic Approaches Based on Small Extracellular Vehicles (sEVs) For the Regeneration of Spinal Cord Injuries

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Abstract: Spinal cord injury (SCI) is severe damage to part of the central nervous system (CNS) that can result in impaired sensory and motor function, significantly impacting the quality of life for patients and creating a substantial economic burden on society. The process of SCI involves both primary and secondary injury, with the latter being a series of heightened responses triggered by the initial damage. The complex nature of SCI's pathological mechanisms has made it challenging to develop effective treatment strategies in clinical settings. Small extracellular vesicles (sEVs) are membrane-bound vesicles with a size range of ≤200 nm, released from cells into extracellular spaces. These vesicles are heterogeneous and can originate from various intracellular compartments, including endosomal and non-endosomal sources. A growing body of evidence points to the potential of sEVs in repairing SCI. This review explores the preparation, functions, routes of administration, advantages, challenges, and advanced therapies for sEVs. It also examines the mechanisms through which various types of sEVs can promote healing in SCI and assesses the effectiveness of combining sEVs with other treatment approaches. Furthermore, the review discusses the opportunities and obstacles associated with using sEVs to repair SCI.

Keywords: neuroinflammation, mesenchymal stem cells, MSC, angiogenesis, glial scar formation, nerve regeneration

Introduction

SCI is a drastic and pernicious condition damaging the CNS. It causes both sensory and motor impairments, leading to a high level of disability and even death.[1–3] Unfortunately, the incidence of SCI is on the rise, with morbidity and mortality rates increasing annually. Approximately 10.4–83 out of 1 million individuals globally experience SCI each year.[4] SCI continues to burden society significantly and poses numerous challenges to the medical field. Following an SCI, a series of pathophysiological changes occur at the injury site.[2] This includes an inflammatory response, neuronal cell death, and the formation of cavities and scars. These processes ultimately inhibit the regeneration of axons, further complicating the recovery process. The ability of the spinal cord (SC) to regenerate after a SCI is very limited due to the lack of flexibility in the central nervous system and the restricted ability for neurons to regrow.[2]

According to the International Society for Extracellular Vesicles (ISEV) guidelines, the term extracellular vesicles (EVs) represents a broad category (30 to 1000 nm) that includes all types of membrane-bound vesicles released from cells.[5] Generally, they are characterized by the encapsulation of various biomolecules and the inability to replicate independently. On the other hand, the popularized use of "exosomes" (Exos) is defined by their endosomal origin, formed by the inward budding to achieve their multivesicular bodies. This particle fuses with the plasma membrane to release the exosomes into the extracellular space. However, the biogenesis of exosomes is not often studied, resulting in numerous disputes based on the unverifiable properties related to the EV origins. Therefore, the nomenclature, sEV is more

Received: 11 February 2025
Accepted: 12 May 2025
Published: 12 June 2025

appropriate as it includes all EVs at sizes of ≤200 nm. This alternative description allows inclusivity and consistency across many research groups, overcoming many ambiguities. Hereafter, the manuscript shall employ the term sEVs except for the explicitly assigned abbreviations in the reviewed articles.

The sEVs contain a variety of bioactive molecules, such as nucleic acids and proteins, that play a crucial role in cellular communication.[6] Significantly, sEVs exhibit exceptional stability, biocompatibility, the ability to penetrate biological barriers, and low immunogenicity, making them ideal for tissue repair.[7–9] Moreover, these nanoparticles can transport novel functional proteins produced by genetically engineered cells.[10,11] Additionally, sEVs can serve as carriers for delivering small molecules or nucleic acids to target specific cell types or tissues with precision.[5,12,13] Although stem cells have been studied extensively for decades, even acquiring promising results as a treatment for SCI, various challenges have hindered the bench-to-bedside translation. Thus, the sEVs that already possess the qualities of stem cells are further advantaged by the simpler configuration, equal allogeneic tolerance, and potential for more cost-effective production, encouraging more technological maturation and promising clinical developments for SCI. This review aims to provide a comprehensive overview of the current understanding of sEVs, their biogenesis, molecular composition, their roles in cellular communication, the latest advances in their classification, and potential applications in diagnostic and therapeutic strategies.

Mechanisms of Action of sEVs in SCI Regeneration

Reduction of Inflammation and Modulation of Immune Response

Neuroinflammation is a major hallmark of SCI, contributing to secondary tissue damage and preventing regeneration.[11,14] The sEVs derived from neuroprotective cells like mesenchymal stem cells (MSCs) have been shown to suppress pro-inflammatory immune cell activation (eg, microglia and macrophages), reduce pro-inflammatory cytokine release, and enhance debris clearance at the injury site.[15] The study examined platelet-rich plasma-derived sEVs (PRP-Exos), demonstrating their ability to stabilize the blood-spinal cord barrier (BSCB) and reduce neuroinflammation in SCI. PRP-Exos were found to restore tight junction integrity in endothelial cells under hypoxic conditions, modulate the NF-κB signaling pathway, and promote neural recovery in an SCI model. These findings suggest that PRP-Exos could be an effective therapeutic strategy for SCI by protecting the BSCB and alleviating inflammation.[15]

Aging has been shown to affect sEVs cargo and SCI recovery. In a study of young adults (YA) versus aged mice with SCI, the sEVs from aged SCI mice induced higher secretion of pro-inflammatory cytokines and neuronal apoptosis. Interestingly, sEVs from young animals exhibited rejuvenating effects in aged mice. Profiling of the sEVs cargo revealed distinct miRNAs (eg, miR-145-5p; 2-fold, $P<0.0001$), proteins (eg, Ccl2; $P<0.0001$) and lipid profiles (eg, triacylglycerols; $P<0.05$) significantly affected in the aged versus YA SCI models. These findings suggest that sEVs may mediate SCI-induced brain dysfunction in aged animals, highlighting the importance of plasma sEV-mediated signaling and chronic release of pro-neuroinflammatory factors in SCI pathology.[16]

A promising approach for SCI treatment involves using M2 microglia-derived Exos (M2-Exos). The M2 microglia cells are known for modulating inflammation in the CNS to mitigate secondary neurodegeneration symptoms and subsequently, facilitate recovery through the secretion of neurotrophic factors through sEVs. However, the biodistribution involving homing and controlled release of sEVs remains a challenge of this therapy. Recently, these sEVs were successfully incorporated into electroconductive hydrogels and used to promote SCI repair. In vitro, M2-Exos significantly promoted neural stem cell growth and axon regeneration, exhibiting large density ($61 \pm 3.61\%$; $P<0.001$) and length (201.67 ± 10.41 μm; $P<0.001$) in the dorsal root ganglion as compared to the control group. They facilitated the polarization of microglia from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. In vivo, M2-Exos significantly enhanced neuronal and axonal regeneration after 8 weeks post-treatment, improving Basso-Beattie-Bresnahan motor function score (BBB: 8 vs 2; $P<0.0001$) in SCI vs control group. These results suggest that M2-Exos can synergize with electroconductive hydrogels to accelerate SCI rehabilitation.[12]

A study utilized genetically engineered M2-Exos, modified with viral macrophage inflammatory protein II (vMIP-II) and lysosomal-associated membrane protein 2b (Lamp2b), to target the injured SC and modulate inflammation. These modified sEVs effectively inhibited key pro-inflammatory signaling pathways (eg, MAPK and Akt), reduced pro-

inflammatory cytokines (eg, IL-1β, TNF-α), and promoted anti-inflammatory cytokine production (eg, IL-4, Arg1). Additionally, the M2-Exos facilitated the polarization of microglia/macrophages to the M2 phenotype. The combination of targeted delivery and anti-inflammatory effects resulted in improved histological and functional recovery in SCI animals. These findings suggest that vMIP-II-Lamp2b-M2-Exos offers a targeted and effective approach to SCI therapy.[8]

The sEVs carry microRNAs (miRNAs) that play a key role in regulating inflammation. miRNA array analysis revealed that miR-124-3p was the most significantly enriched microRNA. sEVs carry microRNAs (miRNAs) that play a critical role in regulating inflammation. Notably, miR-124 has been shown to suppress microglial activation and promote a shift toward a neuroprotective phenotype, highlighting its therapeutic potential in neuroinflammatory conditions.[17,18] It was demonstrated that neuron-derived sEVs promoted functional recovery in SCI models by inhibiting the activation of M1 microglia and A1 astrocytes, both in vivo and in vitro. miRNA array analysis revealed that miR-124-3p was the most abundant miRNA in neuron-derived sEVs. It was identified that MYH9 was the target gene of miR-124-3p, and a series of experiments confirmed the miR-124-3p/MYH9 axis. Furthermore, it was found that the PI3K/AKT/NF-κB signaling pathway was likely involved in modulating microglial activation through miR-124-3p. Neuron-derived sEVs, enriched with miR-124-3p, represent a promising minimally invasive approach for SCI treatment. By modulating neuroinflammation through the miR-124-3p/MYH9 axis and PI3K/AKT/NF-κB signaling, neuron-derived sEVs provided a novel therapeutic strategy for SCI recovery.[9]

A novel approach to SCI treatment involved melatonin-enhanced sEVs (MExos), which was shown to promote a more pronounced transition of microglia from the M1 (pro-inflammatory) to the M2 (anti-inflammatory) phenotype compared to unmodified sEVs. miRNA profiling of the MExos revealed elevated levels of miR-138-5p, which was found to downregulate SOX4, a gene that impedes M2 polarization and anti-inflammatory cytokine production. The inhibition of SOX4 facilitated the conversion of microglia to the M2 phenotype, suggesting that MExos could modulate inflammation and promote tissue repair. This mechanism positions MExos as a promising therapeutic strategy for SCI.[19]

Collectively, these studies highlighted the potential of sEVs as a therapeutic tool in SCI treatment. The sEVs derived from neuroprotective cells like MSCs and M2 microglia can modulate inflammation, promote tissue repair, and improve recovery. Innovations such as the use of platelet-rich plasma, electroconductive hydrogels, and genetic modifications to enhance targetability further improve the efficacy of sEV-based therapies. Additionally, the use of miRNAs, like miR-138-5p, provides new insights into the molecular mechanisms underlying microglia polarization and neuroinflammation regulation, positioning sEVs as a promising strategy for SCI rehabilitation (Figure 1).

Promotion of Neuroprotection and Survival of Neurons

The sEVs derived from certain cell types, including MSCs or neural stem cells (NSCs), contain neurotrophic factors like brain-derived neurotrophic factor (BDNF),[20] nerve growth factor (NGF), and glial cell-derived neurotrophic factor (GDNF).[7,13,21,22] These factors support neuronal survival and function, protecting neurons from secondary injury and promoting their health in the early stages of SCI. SCI often leads to irreversible motor, sensory, and autonomic dysfunction. Despite advances in medical treatments, effective therapies for SCI remain limited. sEVs derived from MSCs or NSCs have been extensively studied for their regenerative potential in SCI.

Bone marrow-derived MSC (BMSC-derived) sEVs carrying miR-26a-5p have shown great promise in promoting axonal regeneration and functional recovery following SCI.[20] The mechanism involves miR-26a-5p repressing EZH2 expression, which in turn increases BDNF and TrkB expression, enhances cyclic AMP response element binding protein (CREB) phosphorylation, and boosts potassium chloride cotransporter 2 (KCC2) expression. These changes promote neuronal survival and protect against secondary injury after SCI. In vivo and in vitro studies have demonstrated that BMSC-derived sEVs can significantly reduce inflammation, promote cell proliferation, and enhance axonal regeneration, offering a potential therapeutic strategy for SCI.[7]

The sEVs derived from epidermal growth factor receptor-positive NSCs (EGFR+NSCs) have been shown to promote neurite regrowth and improve neurological function in SCI models. These sEVs carry miR-34a-5p, which stabilizes microtubules and induces autophagy, leading to enhanced neuronal regeneration. Local administration of EGFR+NSC-derived sEVs has been demonstrated to improve both structural and functional recovery in SCI mice, making them a promising approach for SCI therapy.[21]

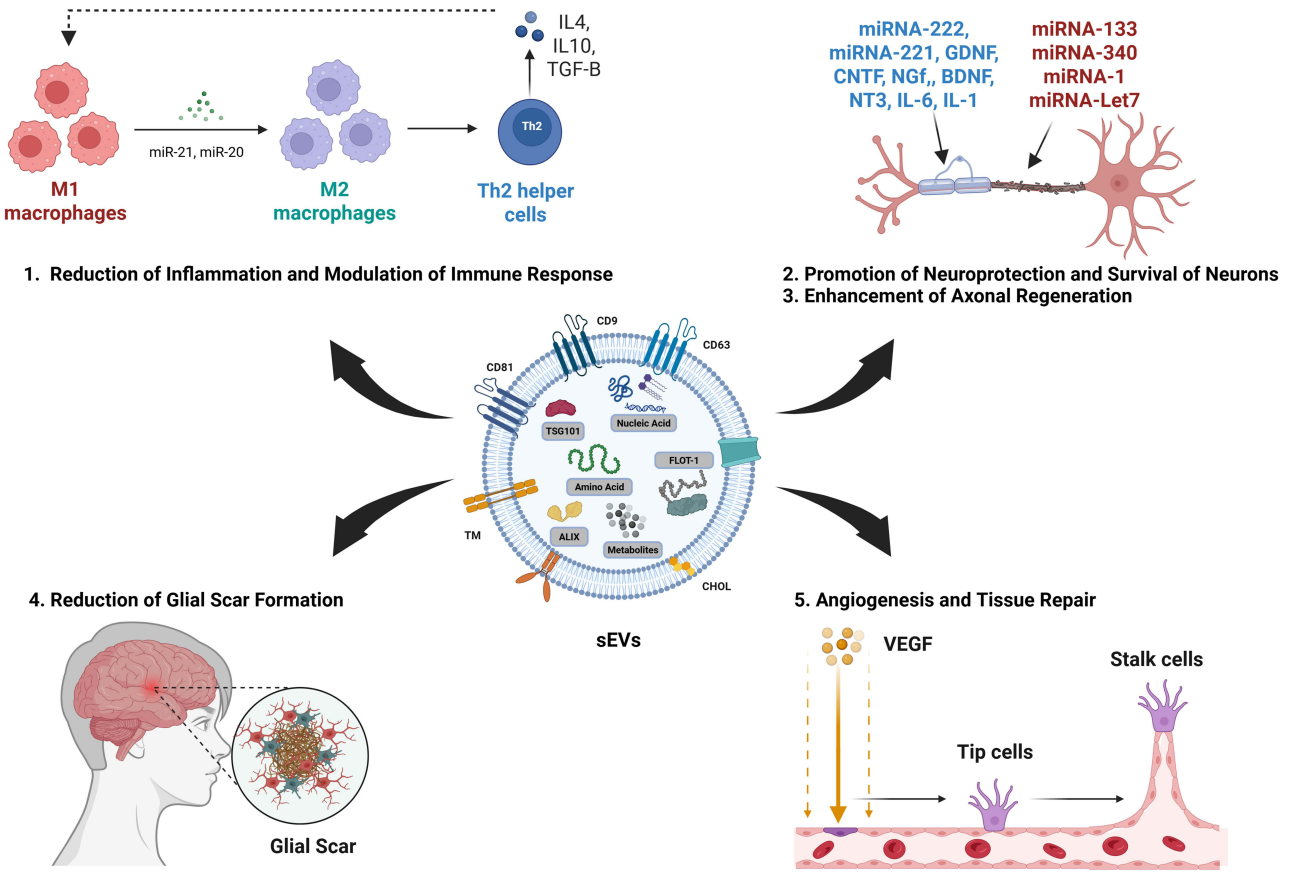

Figure 1 Mechanism of action of sEVs in promoting SC regeneration after SCI: (1) The sEVs alleviate symptoms by modulating the neuroinflammatory response to SCI. (2, 3) Simultaneously, the sEVs prevent neural cell death via inhibiting pro-apoptotic signaling pathways and improve neural cell proliferation, including axonal repair of injured cells. (4) The immunomodulatory and regenerative action prevents glial scar formation. (5) Hemorrhaged blood vessels from SCI trauma are recovered through the formation of new blood vessels (angiogenesis).

Human umbilical cord mesenchymal stem (hUCMSCs) cell-derived sEVs loaded with brain-derived neurotrophic factor (BDNF-Exo) have shown significant therapeutic potential. These sEVs deliver BDNF to the affected brain regions, crossing the blood-brain barrier (BBB) upon intravenous administration. In vitro, BDNF-Exo protects dopaminergic SH-SY5Y cells from apoptosis and ferroptosis induced by 6-hydroxydopamine (6-OHDA). In vivo, BDNF-Exo enhances neuronal survival, promotes neuronal cytoskeletal stability, and activates the Nrf2 signaling pathway, providing neuroprotection against oxidative stress and neuronal degeneration. These findings suggest that BDNF-Exo may serve as a promising treatment strategy.[23]

In Methylmalonic acidemia (MMA)-induced injury models, plasma-derived sEVs have shown neuroprotective effects by reducing neuronal apoptosis, normalizing the expression of apoptosis-related proteins (eg, Bcl-2 and Bax), and improving synaptic protein expression (eg, Syp-1). These sEVs also promote functional recovery in terms of learning and memory by crossing the BBB and targeting neuronal pathways involved in apoptosis and synaptic function. The potential of plasma-derived sEVs as a therapeutic strategy for MMA lies in their ability to modulate miRNA cargo and key signaling pathways involved in neuronal survival.[24]

Hypoxic-preconditioned MSC-derived sEVs (H-sEVs) showed significant therapeutic potential in SCI by reducing oxidative stress, preventing neuronal apoptosis, and promoting functional recovery. In a rat model of SCI, H-sEVs improved motor function as assessed by BBB scores, gait analysis, and neuroelectrophysiological monitoring. Histological analysis revealed reduced lesion size and enhanced neuronal survival following H-sEV treatment. H-sEVs also reduced oxidative stress markers, including superoxide dismutase (SOD) and malondialdehyde (MDA), while upregulating sirtuin 1 (SIRT1), a key regulator of cellular stress responses. Moreover, H-sEVs' anti-apoptotic effects

were linked to the activation of SIRT1 and potentially the Nrf2/HO-1 signaling pathway. These findings highlight H-sEVs as a promising cell-free therapy for SCI, with the potential for broader application in neurodegenerative diseases where oxidative stress and neuronal apoptosis are key factors.[25]

The sEVs derived from MSCs, NSCs, and plasma carry bioactive molecules like miRNAs and neurotrophic factors, which can regulate key molecular pathways involved in neuronal survival, regeneration, and functional recovery. Further research into the precise mechanisms by which sEVs exert their therapeutic effect and the optimization of sEV-based delivery systems will be crucial for translating these findings into clinical applications.

Enhancement of Axonal Regeneration

One of the major challenges in SCI is the inability of axons to regenerate across the injury site.[26] sEVs from various stem cell sources have been shown to stimulate axonal growth by releasing growth factors, including NGF and BDNF, and by reducing the inhibitory effects of the extracellular matrix (eg, chondroitin sulfate proteoglycans) that typically impede axon regeneration.[13,27] sEVs may modulate signaling pathways involved in axonal growth. The exosomal proteins and RNAs can activate ERK and PI3K/AKT signaling pathways that promote axonal growth and sprouting.[28]

In a study, sEVs derived from MSCs, with or without transforming growth factor beta 1 (TGF-β1) treatment (T-EVs), were evaluated for their therapeutic effects on SCI. T-EVs enhanced NSC proliferation and anti-apoptotic activity in vitro. When administered to SCI mice, T-EVs promoted a shift from M1 to M2 microglial polarization, alleviating neuroinflammation and supporting residual cell survival in the acute phase. Additionally, T-EVs increased the number of endogenous NSCs (eNSCs) near the injury site, stimulating neurite outgrowth, axonal regrowth, remyelination, and locomotor recovery during the chronic phase. Notably, in Rictor−/− SCI mice, the therapeutic effects of T-EVs were diminished, suggesting that T-EVs activate eNSCs through the mTORC2/Rictor pathway. These findings highlighted the potential of T-EVs as a promising therapeutic approach for SCI, particularly by targeting the mTORC2/Rictor signaling pathway to enhance eNSC activation and neurogenesis.[13]

Another study developed a novel bioactive hydrogel functionalized with Arginyl-Glycyl-Aspartic acid (RGD) peptides loaded with bone marrow stromal cell-derived sEVs (BMSC-Exos) and GDNF. The hydrogel, composed of methacrylic anhydride-grafted sodium alginate and acrylate RGD, was crosslinked with BMSC-Exos and GDNF to form a composite material. The hydrogel exhibited favorable drug-release properties and biocompatibility. When implanted into the SCI site in rats, the hydrogel effectively modulated the inflammatory response, promoted SC neuron and axon regeneration, and improved motor function recovery. These findings highlighted the potential of BMSC-Exo and GDNF-loaded bioactive hydrogels as a promising therapeutic strategy for SCI repair.[13]

One study investigated the role of BMSC sheets in SCI repair and their underlying mechanisms. BMSCs were isolated from C57BL/6 mouse bone marrow, and sheets were formed when the cells reached 100% confluence. sEVs derived from these BMSCs were isolated and characterized, and their content of NGF was quantified. NSCs were co-cultured with NGF-overexpressing BMSC-derived sEVs (Exos-oe-NGF), which promoted NSC differentiation, axonal regeneration, and functional recovery in vitro and in vivo. The BMSC sheet-loaded sEVs significantly enhanced NSC differentiation into neurons, facilitated axonal regeneration, and improved motor function recovery in SCI mice. These results suggest that Exos-oe-NGF-loaded BMSC sheets offer a promising therapeutic strategy for SCI repair by promoting neurogenesis and tissue regeneration.[29]

Reduction of Glial Scar Formation

After SCI, glial scarring (formed by reactive astrocytes) at the injury site forms a barrier to axonal regeneration (Figure 1). sEVs derived from certain sources, such as MSCs, have been shown to reduce astrocyte activation and glial scar formation. MSCs sEVs carry signaling molecules that influence the transformation of reactive astrocytes into a less inhibitory, more supportive phenotype. The sEVs cargo, including miR-21 and miR-222, has been shown to modulate the behavior of astrocytes, promoting a repair-oriented phenotype and reducing scar formation, which may help facilitate axonal growth across the injury site.[30]

In several studies, sEVs treatments have demonstrated a reduction in glial scar formation. In one model, sEV-loaded Gelfoam was implanted at the lesion site in SCI rats, leading to significant improvements in motor function as assessed

by BBB scores and gait parameters. sEVs treatment enhanced nerve regeneration, remyelination, and synapse formation, as indicated by increased expression of Neurofilament Protein (NF200), Myelin basic protein (MBP), and Growth-Associated Protein 43 (GAP43). It also reduced the upregulation of GFAP and Chondroitin Sulfate Proteoglycans (CSPGs), which are associated with glial scar formation, while decreasing pro-apoptotic and inflammatory markers.[15]

Intrathecal injection of human placental MSCs-derived sEVs (HPMSCs-Exo) in the acute phase of SCI in female rats significantly improved functional recovery over 6 weeks. Compared to controls, HPMSCs-Exo treatment reduced neuronal apoptosis, decreased GFAP expression, and increased NF200 levels at the injury site. Additionally, sEVs treatment prevented cavity formation and preserved tissue integrity. These results demonstrate the neuroprotective and anti-apoptotic potential of HPMSCs-Exos, suggesting them as a promising therapeutic strategy for SCI. Early intrathecal injection of sEVs accelerates recovery, with myelogram imaging serving as an effective method to confirm injection accuracy and assess the subarachnoid space in animal models.[31]

Additionally, Schwann cell-derived sEVs (SCDEs) were found to reduce CSPG accumulation and promote axonal growth by modulating protein tyrosine phosphatase receptor sigma (PTP-σ) activity via the Rho/ROCK signaling pathway. In this study, it was found that PTP-σ levels and CSPG deposition increased during glial scar formation after SCI. However, following SCDE injection, CSPG deposition decreased, PTP-σ expression was elevated during axonal growth at the injury site, and motor function improved. Importantly, using Rho/ROCK inhibitors alongside SCDEs blocked the reparative effects on scar tissue. These findings proved that SCDEs reduce PTP-σ activation via the Rho/ROCK pathway, inhibiting scar formation and promoting functional recovery after SCI.[32]

In an innovative approach, a hydrogel combining sEVs from cortical neurons derived from human induced pluripotent stem cells (iPSCs) and decellularized extracellular matrix (dECM) from hUCMSCs demonstrated excellent cytocompatibility and promoted a pro-regenerative microenvironment. The Exo-dECM hydrogel not only improved motor function recovery but also activated neural stem cells, promoting axon regeneration and preserving tissue integrity. After eight weeks, significant activation of endogenous neural stem cells, axon regeneration, and remyelination were observed, along with functional recovery and preservation of urinary tissue in SCI rats. These results suggested that the Exo-dECM hydrogel is a promising therapeutic approach for SCI treatment.[22]

In a study, rats treated with sEVs from hUCMSCs exhibited improved motor function compared to SCI rats. Transcriptomic analysis of BV2 microglia revealed that the NF-κB/MAPK signaling pathway might mediate the effects of hUCMSCs-derived sEVs. Specifically, sEVs inhibited the phosphorylation of P38, JNK, ERK, and P65 in BV2 microglia and SCI rat tissues. Additionally, sEVs treatment reduced microglial apoptosis, inflammatory responses, and reactive oxygen species (ROS) production both in vitro and in vivo. These findings suggest that hUCMSCs-derived sEVs protect against SCI by modulating the NF-κB/MAPK pathway, offering potential as novel therapeutic targets for SCI treatment.[33] The studies cited here mainly focus on the role of sEVs in modulating neuroinflammation, with less emphasis on glial scar formation specifically. While reducing inflammation and promoting neuronal protection through pathways like NF-κB/MAPK is critical in SCI recovery, these processes also indirectly influence glial scar formation, as inflammation plays a key role in scar tissue development. These studies collectively underscore the potential of sEV-based therapies to reduce glial scar formation and enhance functional recovery following SCI.

Angiogenesis and Tissue Repair

Angiogenesis, the process of new blood vessel formation, plays a critical role in tissue repair and regeneration, particularly following SCI, where it helps restore oxygen and nutrient supply to damaged tissues (Figure 1). sEVs derived from various sources, including endothelial progenitor cells, MSCs, and other angiogenic cells, have shown promise in promoting angiogenesis and improving SCI outcomes. These sEVs are rich in angiogenic factors such as vascular endothelial growth factor (VEGF), which can stimulate blood vessel growth and support tissue regeneration.[34]

One study investigated the effects of sEVs derived from human amniotic MSCs (hAMSCs) on neurological recovery in a rat model of traumatic spinal cord injury (TSCI). Administered two hours after injury via tail vein injection, hAMSC-derived sEVs significantly reduced inflammation, apoptosis, and oxidative stress. Notably, sEV treatment improved blood-brain barrier integrity, promoted angiogenesis, enhanced axonal regeneration, and reduced lesion volume. These findings suggest that hAMSC-derived sEVs could offer a promising cell-free therapeutic approach for acute TSCI.[35]

Another study focused on sEVs derived from human placenta MSCs (hPMSCs) and their proangiogenic effects in SCI. In vitro, hPMSCs-derived sEVs promoted tube formation and migration of human umbilical vein endothelial cells (HUVECs). In vivo, intrathecal injection of these sEVs significantly increased vessel formation and connectivity within the spinal cord, improving both sensory and locomotor function in SCI mice. These results highlight the potential of hPMSCs-sEVs as a promising strategy for promoting angiogenesis and improving SCI outcomes.[36]

In a study, sEVs derived from epidermal growth factor receptor-positive NSCs (EGFR+NSCs) were shown to enhance neurite regrowth and improve neurological function in SCI mice. miRNA sequencing revealed that miR-34a-5p, a key component of EGFR+NSCs-derived Exos, played a crucial role in this process. miR-34a-5p inhibited the expression of HDAC6 in neurons, promoting microtubule stabilization and autophagy, which contributed to SCI repair. This study underscores the therapeutic potential of NSC-derived sEVs in enhancing neural regeneration following SCI.[21]

Furthermore, sEVs derived from M2 macrophages (M2-Exos) have been shown to significantly enhance angiogenic activity in spinal cord microvascular endothelial cells (SCMECs) in vitro. When delivered via a hydrogel for sustained release, M2-Exos promoted vascular regeneration and functional recovery in SCI mice. Proteomic analysis revealed that the ubiquitin thioesterase OTULIN was highly enriched in M2-Exos and played a pivotal role in their pro-angiogenic effects. OTULIN activated the Wnt/β-catenin signaling pathway by inhibiting the ubiquitination of β-catenin, which in turn triggered the expression of angiogenesis-related genes. Inhibition of Wnt/β-catenin signaling with ICG001 attenuated the pro-angiogenic effects of M2-Exos, both in vitro and in vivo. These findings suggest that M2-Exos can promote vascular regeneration and neurological recovery after SCI by transferring OTULIN to activate the Wnt/β-catenin signaling pathway.[21]

Together, these studies demonstrated the therapeutic potential of sEVs derived from various cell types, including hAMSCs, hPMSCs, EGFR+NSCs, and M2 macrophages, in promoting angiogenesis, neural regeneration, and functional recovery following SCI. These findings support the use of sEV-based therapies as a promising, cell-free strategy for enhancing tissue repair and recovery in SCI. A summary of the mechanisms of action of sEVs in SCI regeneration has been presented in Table 1, and a diagrammatic representation is shown in Figure 1.

Table 1 Mechanisms of Action, Regenerative Content, and Signaling Pathway of EVs-Based Therapy in SCI Regeneration

Mechanism	Regenerative Content	Pathway	Ref
1. Reduction of Inflammation and Modulation of Immune Response	sEVs from neuroprotective cells (eg, MSCs, M2 microglia) reduce neuroinflammation, promote microglial polarization from M1 to M2, and enhance debris clearance. Platelet-rich plasma-derived sEVs (PRP-sEVs) stabilize the BSCB and modulate NF-κB. sEVsomal miRNAs (eg, miR-124) suppress microglial activation and promote neuroprotective phenotypes.	NF-κB Signaling	[12,15,16]
2. Promotion of Neuroprotection and Survival of Neurons	sEVs from MSCs and NSCs carry neurotrophic factors (eg, BDNF, NGF) and miRNAs (eg, miR-26a, miR-34a) that support neuronal survival, promote regeneration, and reduce apoptosis. sEVs from hUCMSCs-BDNF (BDNF-Exo) cross the BBB activating Nrf2 pathways to protect neurons from oxidative stress. Hypoxic-preconditioned MSC-derived sEVs (H-sEVs) reduce oxidative stress.	Nrf2 pathways	[7,21,23]
3. Enhancement of Axonal Regeneration	sEVs from MSCs, NSCs, and BMSCs promote axonal growth and regeneration by activating pathways like ERK and PI3K/AKT. sEVs can reduce extracellular matrix inhibitors (eg, CSPGs), promote neurite outgrowth, and aid remyelination. TGF-β1-treated sEVs (T-EVs) stimulate neural stem cell proliferation and axonal regrowth. sEVs-loaded hydrogels enhance axonal regeneration.	ERK and PI3K/AKT Signaling	[13,29]

(*Continued*)

https://doi.org/10.2147/IJN.S522028

Table 1 (Continued).

Mechanism	Regenerative Content	Pathway	Ref
4. Reduction of Glial Scar Formation	sEVs from MSCs and Schwann cells (SCs) reduce glial scar formation by modulating reactive astrocytes and promoting a repair-oriented phenotype. miR-21 and miR-222 in MSC-derived sEVs reduce scar formation, facilitating axonal growth. SCDEs reduce CSPG accumulation, while inhibiting Rho/ROCK signaling promotes scar reduction and functional recovery.	Rho/ROCK Signaling	[15,22,31,37]
5. Angiogenesis and Tissue Repair	sEVs derived from endothelial progenitor cells, MSCs, and M2 macrophages promote angiogenesis by delivering angiogenic factors like VEGF. M2-sEVs activate Wnt/β-catenin signaling to enhance vessel regeneration. sEVs from hAMSCs and hPMSCs improve blood-brain barrier integrity, promote angiogenesis, and improve functional recovery after SCI.	Wnt/β-catenin signaling	[21,24,36]

Note: Key signaling pathways involved in regenerative mechanisms (NF-κB, Nrf2, PI3K/AKT, Rho/ROCK, Wnt/β-catenin).
Abbreviations: MSC, Mesenchymal Stem Cell; NSC, Neural Stem Cell; BMSC, Bone Marrow-Derived MSC; PRP, Platelet-Rich Plasma; hUCMSC, Human Umbilical Cord Mesenchymal Stem Cell; BDNF, Brain-Derived Neurotrophic Factor; CSPG, Chondroitin Sulfate Proteoglycan; SCDE, Schwann Cell-Derived Exosome; T-EVs, TGF-β1-treated Extracellular Vesicles; H-sEVs, Hypoxic-preconditioned MSC-derived sEVs; hAMSC/hPMSC, Human Amniotic/Placenta-Derived MSCs; BSCB, Blood-Spinal Cord Barrier.

Sources of sEVs for SCI Therapy

The sources of sEVs for SCI therapy are diverse, with various cell types being explored for their ability to produce sEVs with beneficial therapeutic properties. The following sections highlight some of the key sources of sEVs under investigation and summarize their respective therapeutic benefits.

Mesenchymal Stem Cells (MSCs)

Among the various sources, MSC-derived sEVs are one of the most widely studied sources due to their immunomodulatory, anti-inflammatory, and regenerative properties. MSC sEVs have been shown to promote axonal regeneration, reduce inflammation, and enhance tissue repair in SCI models. MSC-derived sEVs have demonstrated potent regenerative effects in SCI models. These sEVs are rich in bioactive molecules, including growth factors (eg, VEGF, BDNF), cytokines, and miRNAs that can enhance tissue repair, reduce inflammation, and promote neuroprotection.[38] Different types of MSC-derived sEVs, their cellular sources, and therapeutic potential are summarized in Figure 2 and Table 2, illustrating the breadth of evidence supporting their use in SCI therapy.

Neural Stem Cells (NSCs)

Building on the promise shown by MSC-derived sEVs, sEVs derived from NSCs (NSCs-sEVs) have also emerged as a powerful candidate for SCI therapy. Due to their inherent neurogenic potential, NSC-derived sEVs are promising for direct neuronal repair. These sEVs contain a variety of growth factors and miRNAs that can support the survival, differentiation, and regeneration of neurons in the injured SC.[46] NSCs-sEVs enhanced angiogenesis in SC microvascular endothelial cells (SCMECs), with vascular endothelial growth factor A (VEGF-A) identified as a key pro-angiogenic factor enriched in the sEVs. Downregulation of VEGF-A in NSCs-sEVs impaired their angiogenic effects, highlighting the critical role of VEGF-A in this process. NSCs-sEVs significantly improved microvascular regeneration, reduced spinal cord cavity formation, and promoted functional recovery in SCI mice, as evidenced by improved Basso mouse scale scores.[46]

Induced Pluripotent Stem Cells (iPSCs)

In addition to naturally occurring stem cells, iPSCs offer a unique advantage by being patient-specific and highly versatile. iPSC-derived sEVs are another emerging source, offering the advantage of being patient-specific and capable of regenerating multiple cell types. sEVs derived from iPSCs promote neuronal and glial cell repair and reduce inflammation. The study aimed to investigate the effects and molecular pathways of iPSCs-sEVs in SCI mice. Characterization of iPSCs-sEVs was performed using transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and

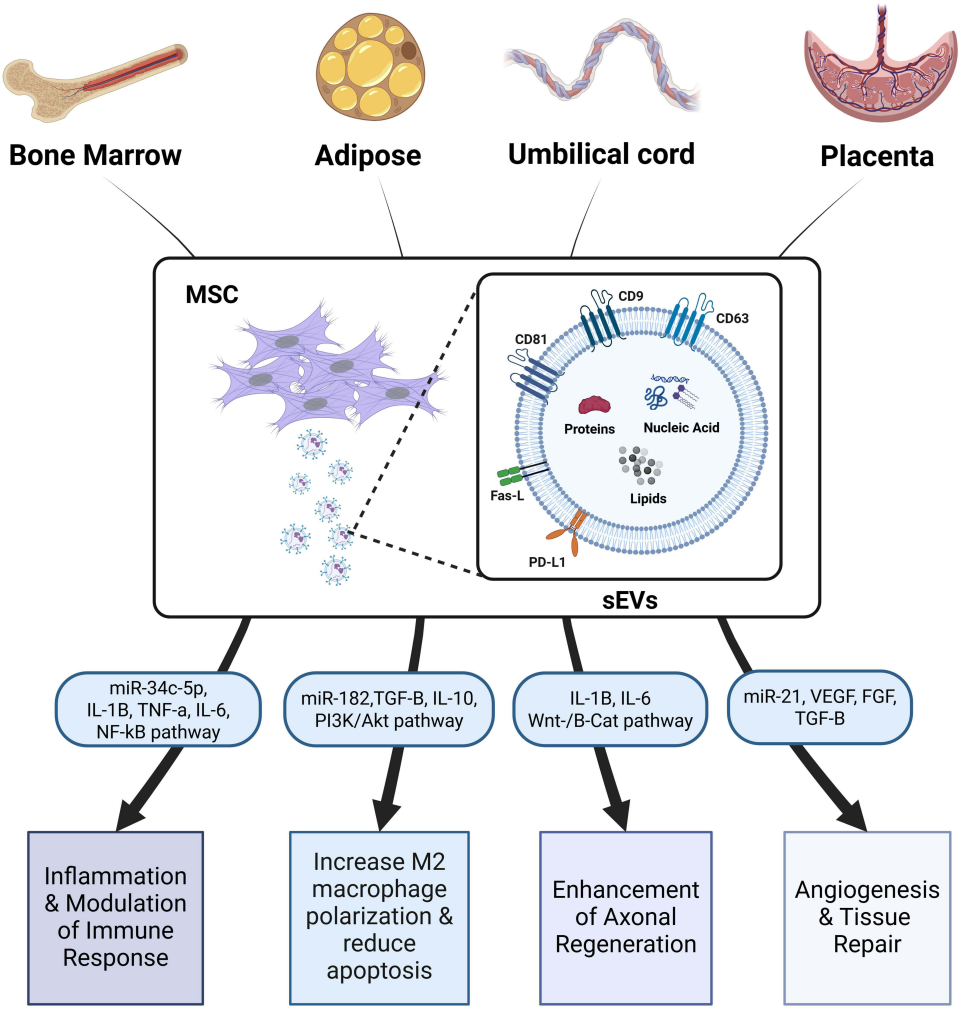

Figure 2 Impact, Source, and Therapeutic Outcome of MSC-derived sEVs. The different sources of MSCs and their isolated sEVs maintain their biological markers (eg, CD9, CD63, and CD81) but may possess varying vesicle cargo (eg, proteins, nucleic acid, and lipids), serving as alternatives for more specific or directed therapeutic outcomes.

Western blotting. The iPSCs-sEVs improved motor function in SCI mice, as assessed by Basso Mouse Scale (BMS) scores and H&E staining, while also promoting a shift from M1 to M2 macrophage polarization and modulating inflammatory factors in LPS-treated bone marrow-derived macrophages (BMDM) in vitro. miR-199 b-5p was identified as a key functional component of iPSCs-Exo, targeting hepatocyte growth factor (Hgf) and influencing macrophage polarization. Overexpression of miR-199b-5p promoted M2 polarization and neural regeneration in SCI, with rescue experiments confirming that miR-199b-5p-induced polarization and SCI recovery occurred through the regulation of Hgf and the PI3K signaling pathway.[47]

Endothelial Progenitor Cells (EPCs)

Further expanding the range of cell sources, endothelial progenitor cells (EPCs) have also been investigated for their pro-angiogenic and neuroprotective properties. sEVs derived from EPCs (EPC-EVs) have angiogenic properties and can promote the formation of new blood vessels in SCI, improving the recovery of injured tissue. The study investigated the effects of miR-210-loaded EPC-EVs (miR210-EPC-EVs), miR-210 was found that protects cerebral endothelial cells by reducing oxidative stress. miR210-EPC-EVs were generated by transfecting EPCs with a miR-210 mimic, and middle cerebral artery occlusion (MCAO) was used to induce acute IS in C57BL/6 mice. miR210-EPC-EVs or EPC-EVs were administered via tail vein injection. Results showed that miR210-EPC-EVs significantly reduced infarct volume, improved neurological deficit scores, and decreased cell apoptosis and oxidative stress compared to EPC-EVs.

https://doi.org/10.2147/IJN.S522028

Table 2 The Various sEV-Derived Therapies for SCI, Therapeutic Intervention, Key Modulators, and Potential Outcomes Promoting Regeneration in SC

Therapeutic Effect	Mechanism	Outcome	References
Anti-oxidative Stress	Modulation of oxidative stress	MSC-sEVs reduce oxidative stress by elevating antioxidant activity (eg, SOD, GSH) and counteracting ROS.	[39,40]
Anti-apoptotic Effects	Inhibition of apoptosis	MSC-sEVs inhibit neuronal apoptosis via miRNA-rich cargo (eg, miR-21, miR-134), targeting apoptotic pathways (eg, FasL, PI3K/Akt).	[41]
Regulation of Autophagy	Inhibition of excessive autophagy	MSC-sEVs modulate autophagic signaling, promoting neuronal survival by balancing autophagic processes and inhibiting autophagy-mediated damage.	[42,43]
Inhibition of Reactive Astrocytes	Suppression of neurotoxic astrocyte activation	MSC-sEVs inhibit A1 astrocytes activation through NF-κB signaling, reducing neurotoxic inflammation in SCI.	[44]
Axon Regeneration	Promotion of axonal growth	MSC-sEVs promote axon regeneration by enhancing neurotrophic signaling pathways (eg, ERK1/2, STAT3), as well as reducing glial scar formation.	[45]

Notes: Major signaling pathways involved in therapeutic effects, such as PI3K/Akt, NF-κB, ERK1/2, and STAT3.
Abbreviations: SOD, Superoxide Dismutase; GSH, Glutathione; ROS, Reactive Oxygen Species; miR-21, miR-134; FasL, Fas Ligand; A1 astrocytes, a neurotoxic reactive astrocyte phenotype.

Furthermore, miR210-EPC-EVs upregulated the phosphorylation of PI3K, VEGFR2, and TrkB in the brain, and these effects were partially blocked by specific inhibitors of the VEGFR2/PI3K and TrkB/PI3K pathways.[48]

Another study explored the role of vascular endothelial cells in the polarization of microglia/macrophages and the underlying mechanisms. BV2 microglia and RAW264.7 macrophages were pretreated with lipopolysaccharide (LPS) and then exposed to conditioned medium from the bEnd.3 endothelial cell line (ECM). ECM treatment promoted M2 polarization of microglia/macrophages, improved mitochondrial function, and reduced ROS production in vitro. These effects were reversed by GW4869, a secretion inhibitor, suggesting that sEVs within the conditioned medium contributed significantly to the observed benefits. However, it is important to note that conditioned media is a complex mixture that includes not only sEVs but also soluble proteins, metabolites, and other vesicles, and the role of sEVs was inferred indirectly using GW4869. To further clarify this, in vivo experiments using purified sEVs derived from bEnd.3 cells demonstrated enhanced motor rehabilitation and M2 polarization of microglia/macrophages after SCI, confirming the therapeutic potential of sEVs. Mechanistically, ubiquitin-specific protease 13 (USP13), which deubiquitinates and stabilizes the NF-κB inhibitor IκBα, was identified as a key mediator of these effects. Inhibition of IκBα with betulinic acid (BA) attenuated the therapeutic effects of sEVs, further supporting the involvement of the USP13/IκBα pathway.[10] A summary of various sources of sEV-derived therapies for SCI intervention, based on the parent cell source and their therapeutic effects, has been reported in Table 3.

The structural and molecular composition of sEVs plays a critical role in determining their functional properties and therapeutic potential. The composition of sEVs can vary based on the type of cell, its physiological state, and the surrounding environment. However, sEVs contain several common constituents. EVs, including sEVs, are commonly classified based on their size, biogenesis, and molecular markers, as recommended by the ISEV MISEV2018 guidelines.[68] A concise summary of the structural composition of sEVs is presented in Table 4. Gurunathan et al have reviewed that the tetraspanins (eg, CD9, CD63, CD81), beyond being known as markers for sEVs, possess a natural curvature that factors into the shape of sEVs and is crucial for facilitating cellular uptake.[69,70] While the mechanisms are not completely understood, proteins such as ALIX and TSG101 were shown to influence the selection and encapsulation of ubiquitinated proteins as cargo. Therefore, it can be considered a potential target in the context of disease-signaling sEVs from infected or injured cells.[69] Other known molecular proteins, like heat shock proteins (HSPs), also serve as molecular chaperones for numerous biological processes, most commonly for stress and immune responses.[70] Annexins and some metabolic enzymes have also been shown to remodel intra- (self-) and intercellular membranes of target cells, modulating cellular uptake of sEVs.[71] Phospholipids, cholesterol, ceramide and sphingomyelin make up the bulk of lipid-

Table 3 The Summary of MSC-Derived sEVs, Their Therapeutic Effect, Mechanism of Action, and Key Findings Promoting Neuroprotection and Neuroregeneration

sEV Source	Therapeutic Effects in SCI	Key Findings	References
Mesenchymal Stem Cell-Derived sEVs	Promotes angiogenesis, axonal regeneration, and reduce apoptosis.	MSC-derived sEVs (MSCs-sEVs) reduce inflammation (IL-1β, TNF-α, IL-6) scar formation and promote functional recovery and neuroprotection.	[48,49]
Bone Marrow MSC-Derived sEVs	Neuroprotective, reduce scar size, stimulate macrophage phagocytosis, and axonal regeneration.	Reduce A1 astrocytes via NF-κB activation. CircRNA administration in BMSCs-sEVs mitigates apoptosis and inflammation.	[40,50]
Umbilical Cord MSC-Derived sEVs	Reduce apoptosis, promote axonal growth, improve angiogenesis, modulate immune response (M1→M2 polarization).	Enhance antifibrotic activity and decrease inflammation (IL-1, IL-6). Activate Wnt/β-catenin pathway for regeneration.	[44,51]
Adipose Tissue MSC-Derived sEVs	Limit NLRP3 inflammasome activation, reduce inflammatory cytokines (IL-1β, TNF-α), improve neuronal survival.	ADSC-sEVs reduces inflammatory cascade in rat SCI models. Increase M2 macrophage polarization and reduce apoptosis.	[39,52]
Placental MSC-Derived sEVs	Promote neuroprotection, angiogenesis, and neuronal regeneration.	Increase endothelial cell tube formation and promote neural stem cell expression (SOX2+GFAP+). Improve sensory-motor function post-SCI.	[53,54]
Neural Stem Cell-Derived EVs (NSC-sEVs)	Enhance angiogenesis, reduce spinal cord cavities, promote motor function recovery.	NSC-sEVs loaded with VEGF-A promotes angiogenesis. MiR-374-5p/STK-4 axis reduces apoptosis and promotes neuronal repair.	[46,55,56]
Induced Pluripotent Stem Cell-Derived sEVs	Improve motor function by modulating M1→M2 macrophage polarization, reduce inflammation.	iPSC-sEVs accelerate SCI recovery, reducing inflammation and improving functional recovery.	[47]
Schwann Cell-Derived sEVs (SCDEs)	Stimulate axonal growth, reduce glial scar formation, enhance autophagy.	SCDEs stimulate regeneration and reduce PTEN activity, boosting functional recovery.	[57,58]
Macrophage-Derived sEVs	Modulate immune response, promote tissue repair, reduce inflammation (M2 polarization), enhance angiogenesis.	M2-sEVs induces angiogenesis via OTULIN and Wnt/β-catenin signaling. sEVs-berberine reduces neuronal apoptosis and promotes recovery.	[59,60]
Pericyte-Derived sEVs	Improve blood flow, support angiogenesis, reduce ischemic damage.	Pericyte-sEVs promotes blood flow recovery and enhances functional recovery in SCI models.	[61]
Microglia-Derived sEVs (MG-sEVs)	Reduce neuroinflammation, promote axonal development, protect neurons from apoptosis.	MG-sEVs activates Keap1/Nrf2/HO-1 antioxidant pathways for neuroprotection. miR-151-3p controls apoptosis in SCI models.	[45,62]
Neuron-Derived sEVs	Enhance axonal growth, protect neurons from oxidative stress, and promote functional recovery.	Neuron-sEVs supports axon regeneration and oligodendrocyte maturation. miR-124-3p suppresses neurotoxic microglia activation.	[63,64]
Astrocyte-Derived sEVs	Potential to reduce neuroinflammation, support neuronal regeneration.	sEVsomal miR-148a-3p reduces inflammation and improves neurological function in TBI models.	[65,66]
Regulatory T Cell-Derived sEVs	Modulate immune response, promote recovery by reducing microglia pyroptosis.	Treg-sEVs reduces inflammation, supporting recovery in SCI models.	[67]

(*Continued*)

Table 3 (Continued).

sEV Source	Therapeutic Effects in SCI	Key Findings	References
Platelet-Derived EVs (PD-sEVs)	Promote anti-inflammatory effects, modulate immune pathways, and improve neuronal survival.	PD-sEVs with dexamethasone improves SCI symptoms by targeting Bax/Bcl2 and TNF-α/IL-10 signaling.	[20]
Vascular Endothelial Cell-Derived sEVs	May enhance immune response and functional recovery by promoting microglia/macrophage polarization.	USP13-loaded VEC-sEVs boosts M2 microglia/macrophage polarization and stabilizes inflammatory pathways in SCI.	[10,48]

Notes: indicate specific proteins, genes, cytokines, microRNAs, or pathways (eg, IL-1β, TNF-α, miR-124-3p, VEGF-A, OTULIN), critical signaling pathways or molecules relevant to SCI pathology and repair (eg, NF-κB, Wnt/β-catenin, NLRP3, Keap1/Nrf2/HO-1, PTEN). M1→M2 indicates the phenotypic shift of macrophages or microglia from pro-inflammatory (M1) to anti-inflammatory (M2). $SOX2^{+}GFAP^{+}$ denotes co-expression of neural stem cell and astrocytic markers.
Abbreviations: MSC, Mesenchymal Stem Cell; sEVs, Small Extracellular Vesicles; BMSC, Bone Marrow MSC; ADSC, Adipose-Derived Stem Cell; NSC, Neural Stem Cell; iPSC, Induced Pluripotent Stem Cell; Treg, Regulatory T Cell; PD-sEVs, Platelet-Derived Small EVs; VEC, Vascular Endothelial Cell; SCI, Spinal Cord Injury; TBI, Traumatic Brain Injury.

Table 4 Structural Composition of EVs, the Active Component, and Underlying Therapeutic Activity for the EVs for SCI

Active Component	Classification	Therapeutic Activity	References
Tetraspanins	Protein-derived	Transmembrane proteins (eg, CD9, CD63, CD81, CD82) involved in biogenesis, cargo sorting, and membrane fusion.	[69,70]
Heat Shock Proteins (HSPs)	Protein-derived	HSP70 and HSP90, involved in cellular stress responses and protecting cargo molecules from degradation.	[70]
Alix & TSG101	Protein-derived	Components of ESCRT apparatus, involved in intraluminal vesicle formation and sEV release.	[69]
Annexins	Protein-derived	Calcium-binding proteins that play a role in membrane remodelling and fusion during sEV biogenesis.	[71]
Metabolic Enzymes	Protein-derived	Includes proteases and phosphatases, modulating the extracellular environment and influencing recipient cell behavior.	[71]
Phospholipids	Lipid-derived	Include phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine, contributing to membrane integrity.	[73]
Cholesterol	Lipid-derived	Maintains membrane fluidity and stability.	[73]
Ceramide & Sphingomyelin	Lipid-derived	Involved in membrane organization and signaling processes.	[72]
Nucleic Acids	Lipid-derived	Includes miRNA, mRNA, and noncoding RNAs, which can be transferred to recipient cells, influencing gene expression and cellular functions.	[72]
Transmembrane Receptors	Research-driven (modified)	Receptors like TLR and TNF, incorporated into designed sEVs to enhance target efficacy.	[74]

Notes: Active biomolecular components such as proteins, lipids, or nucleic acids contribute to the therapeutic activity of sEVs. Protein-derived components include tetraspanins (eg, CD9, CD63, CD81, CD82), heat shock proteins (HSP70, HSP90), Alix, TSG101, annexins, and metabolic enzymes. Lipid-derived components include phospholipids (phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine), cholesterol, ceramide, sphingomyelin, and nucleic acids (eg, miRNA, mRNA, noncoding RNAs). Transmembrane receptors such as TLR and TNF may be incorporated into engineered sEVs to enhance targeting and therapeutic efficacy.

based molecules responsible for membrane integrity and stability.[72,73] However, most of the active constituents are represented by the nucleic acids and transmembrane receptors in sEVs, which have been thoroughly researched as potential biomarkers, therapeutic targets, or subjects of genetic modifications.[72,74]

Method of sEVs Synthesis for SCI Treatment

EVs secreted or isolated from cells can be pre-loaded with therapeutic agents and subsequently taken up by the parent cells through methods such as transfection, activation, and other techniques. Various strategies for loading drugs into sEVs have been explored, including incubation, extrusion, transfection, electroporation, sonication, and saponin-assisted loading, among others. These approaches are being used to introduce pharmaceuticals into EVs for potential therapeutic intervention in SCI. A concise summary of the sEVs synthesis techniques for SCI therapy, techniques used, advantages, challenges/limitations as demonstrated in previous studies, has been presented in Table 5.

Methods such as transfection were shown to have minimal impact on sEVs' stability by leveraging the cells' machinery to develop biologically compatible constructs. Depending on the drug's properties or the cell's response to the drug, it may significantly affect the cargo.[75,76] Incubation or co-incubation is also relatively inexpensive and simple to deploy, but can often yield low purity due to suboptimal loading capacity.[77,78] Otherwise, transiently deforming the membrane through saponin-assisted electroporation (pore) and sonication of the drug can be combined to sEVs on top of its original cargo without significant interference. However, the reconstitution of sEVs could risk aggregation or a minorly altered structure thereafter.[76,79–81] Alternatively, synthetic routes such as the extrusion method compensate for low drug loading capacity by artificially shaping larger vesicles.[82] Compared to the previous methods, direct manipulation of sEVs may cause premature release of drugs, resulting in cytotoxicity or off-target side effects.

Table 5 Summary of the EV Synthesis Method and Techniques to Improve the Regenerative Potential, the Methodology, Advantages, Limitations, and Challenges of Advanced Techniques for SCI Treatment

Technique	Methodology	Advantages	Challenges/Limitations	References
Transfection	Loading therapeutic molecules (eg, nucleic acids, proteins) using biological or chemical agents.	Stable loading, efficient packaging, and overexpression of specific proteins on exosomal membrane.	Variable efficiency, potential changes in gene expression, and toxicity of reagents.	[75,76]
Incubation	Direct incubation of drugs with sEVs or donor cells.	Simple and cost-effective.	Suboptimal loading efficiency, potential aggregation, and limited drug compatibility.	[77]
Co-incubation	Simultaneous loading of RNA and hydrophobically modified siRNAs into sEVs.	Improved loading efficiency and therapeutic cargo encapsulation.	May still require optimization for large-scale applications.	[78]
Saponin-assisted Loading	Use of saponins to create pores in sEV membranes for drug loading.	Does not alter size or zeta potential significantly.	Hemolytic toxicity, requiring purification steps.	[79]
Sonication	Ultrasound treatment to deform exosomal membranes, facilitating drug entry.	Increased loading efficiency compared to incubation, suitable for small molecules and large macromolecules.	sEVsome aggregation, potential structural damage.	[80,81]
Extrusion	Forcing sEVs through a porous membrane for uniform drug distribution.	Controlled, reproducible, and consistent loading.	Potential cytotoxicity with some drugs.	[82]
Electroporation	Application of an electric field to create pores in sEV membranes for large molecule loading.	Effective for large nucleotide payloads like siRNAs and miRNAs.	Aggregation, RNA precipitation, reduced loading efficiency, and membrane damage.	[76]

Notes: This table summarizes commonly used techniques for loading therapeutic agents into sEVs. Transfection uses biological or chemical agents to incorporate proteins or nucleic acids, enabling stable expression, but may introduce cytotoxicity or gene expression changes. Incubation and co-incubation are straightforward, cost-effective methods, but often have lower loading efficiencies or limited compatibility with certain drugs. Saponin-assisted loading increases membrane permeability without major structural alterations but may induce hemolytic toxicity. Sonication and extrusion physically disrupt sEV membranes to enhance drug entry and distribution, although they may lead to vesicle aggregation or cytotoxicity. Electroporation is efficient for large RNA molecules but may cause RNA precipitation, membrane damage, and reduced encapsulation efficiency.

https://doi.org/10.2147/IJN.S522028

sEV-based therapies hold great potential for SCI by offering a targeted, less invasive approach to enhance tissue regeneration and functional recovery (Figure 3). Recent developments in nanobased therapy, particularly through sEVs-loaded nanoparticles or nanogels, offer a promising frontier.[83] These nanocarriers can be engineered to contain regenerative cargo such as neurotrophic factors, antioxidants, and anti-apoptotic agents, enhancing their efficacy in targeting damaged tissues. Tailored sEVs can also be engineered to cross barriers such as the BSCB, delivering therapeutic payloads directly to the injury site. Nakazaki et al demonstrated that IV administration of MSC did not traffic to the injury site, but was accomplished by MSC-sEVs, which were also taken up by M2 macrophages, cascading towards lower inflammation and improved functional recovery.[84] Additionally, through genetic engineering techniques, sEVs can be customized to carry specific RNA and proteins, achieved via methods like transfection or transformation, further enhancing their therapeutic potential. Surface functionalization of these sEVs with binding ligands, antibodies, and peptides allows for targeted delivery to specific cell types or tissues, thereby increasing treatment precision and effectiveness. The integration of cutting-edge gene editing technologies, such as CRISPR, into sEVs therapy not only allows for the correction of genetic defects but also enhances the therapeutic outcomes in various diseases.[85] Moreover, biomaterial scaffolds, including those created via 3D printing technologies, provide structural support for sEVs, facilitating localized and sustained release of therapeutic agents.[86] The approach of combination therapies, which includes electrical and mechanical stimulation alongside hydrogel-based systems, offers synergistic effects that can significantly improve healing and regeneration. Finally, integrating stem cell therapy with sEVs delivery further

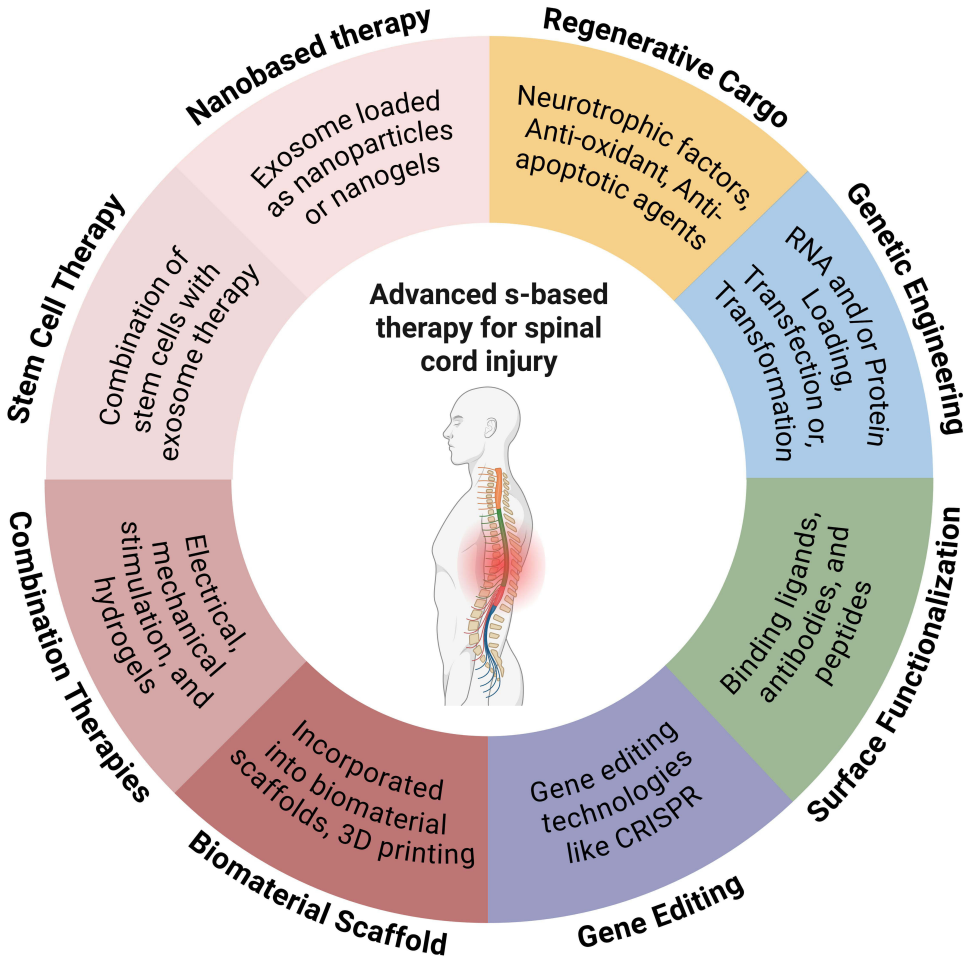

Figure 3 Advanced methods used to improve the activity of sEV-based therapy for SCI. Various studies have shown interest in diversifying or modifying the cargo of sEVs for targeted delivery of active compounds. Others have also demonstrated improvement in the surface functionalization of sEVs by gene or genetic editing to ease barrier permeability and surface interaction with target cells. Combination therapies are also well-established that involve either co-administration of sEVs with cells, incorporation of sEVs into scaffolds for controlled release, or other functional materials.

potentiates the regenerative capabilities of these treatments, harnessing the intrinsic healing properties of stem cells augmented by sEVs functions, and paving the way for innovative and more effective medical treatments. These approaches have the potential to surpass traditional treatments, addressing the complex challenges of SCI and improving patient outcomes.

The Benefit of sEVs-Based Delivery Systems

sEV-based delivery systems have emerged as a promising therapeutic strategy. These systems leverage the unique properties of sEVs to target and treat the complex cellular and molecular processes involved in SCI (Figure 4).

Targeted Delivery and Tissue Penetration

One of the most significant properties of sEVs for SCI is their ability to cross biological barriers, including the BSCB, which is like BBB.[87] sEVs derived from stem cells naturally exhibit a capacity to target damaged neurons and glial cells within the SC. Through surface proteins or engineered ligands, sEVs can be modified to enhance their ability to bind to specific receptors on target cells, such as neurons, oligodendrocytes, or microglial cells, making them ideal for site-specific delivery of therapeutic cargo (Figure 4). One study presented an autologous plasma sEVs (AP-Exo)-based biological scaffold for treating SCI, which addresses the challenges of axon regeneration and BSCB limitations. The AP-Exo scaffold was loaded with neuron-targeting peptide (RVG) and growth-promoting peptides (ILP and ISP) for targeted delivery to injured neurons. This approach significantly enhanced axon regrowth, over 30 times greater than naïve treatments, reestablishing intraspinal circuits and promoting motor function recovery in SCI mice. Additionally, HP-Exo loaded with the same peptides showed no liver or kidney toxicity in ex vivo studies, confirming the safety of this method. The results demonstrated the promising potential of AP-Exo-based and HP-Exo personalized treatment for SCI, combining efficacy and safety while expanding the use of autologous sEVs and combinatory peptides in regenerative medicine for SCI recovery.[88]

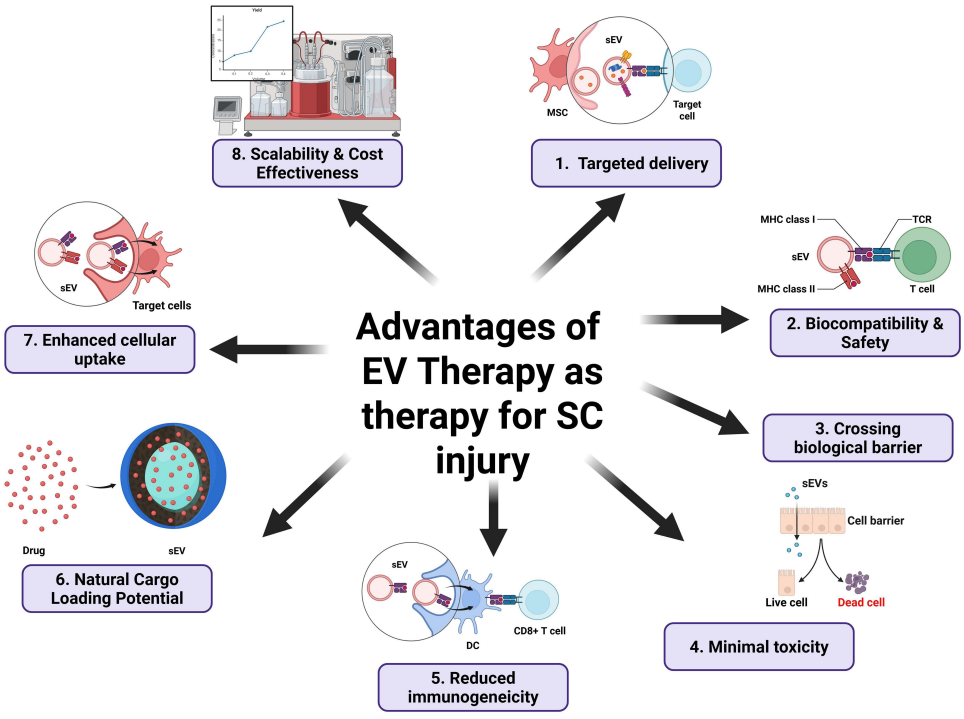

Figure 4 Advantages of sEV therapy aiding SC regeneration. Compared to other therapeutic models including stem cells, sEV-based therapy holds numerous advantages that include: (1) receptor-ligand targeted delivery; (2) strong biocompatibility and safety data; (3) crossing of most biological barriers; (4) minimal-to-no toxicity; (5) low immunogenicity or allogeneic tolerance; (6) excellent potential as drug delivery system; (7) innate cell fusion abilities and; (8) opportunities for up-scaled and cost-efficient improvements for clinical manufacturing setup.

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Biocompatibility and Safety

sEVs are composed of lipids, proteins, and other molecules that resemble the natural cellular membrane, which makes them highly biocompatible. This biocompatibility reduces the risk of immune rejection or toxicity when administered as part of SCI therapy. Studies have demonstrated that sEV-based therapies for SCI have low immunogenicity and do not cause systemic toxicity (Figure 4), making them an attractive choice for regenerative medicine.[21,89] While sEVs show promise in promoting neuroregeneration and immune modulation after SCI, their biocompatibility and safety profile warrant careful consideration, particularly when moving toward clinical applications. sEVs are universally biocompatible, but their immunogenicity, toxicity, and biodistribution are influenced by several factors, including their cellular origin, surface markers, and isolation methods.[62,90] Utilizing autologous cells (eg, patient-derived stem cells or somatic cells) to produce sEVs minimizes the risk of immunogenicity and cross-species transmission.[91] For instance, sEVs derived from induced pluripotent stem cells (iPSCs) generated from a patient's somatic cells provide a highly personalized and potentially safer therapeutic option.[92] Culturing well-characterized, non-tumorigenic, and pathogen-free donor cells under controlled conditions allows for more consistent EV production and quality control. Cell sources like mesenchymal stromal cells (MSCs) and endothelial cells are widely used due to their favorable safety profile.[93,94] The synthetic nanoparticles, sEVs acquire a protein corona upon interaction with biological fluids, which can alter their biodistribution, uptake, and immune recognition.[95] Understanding and potentially engineering this corona by pre-coating sEVs or controlling their exposure environment could improve biocompatibility and targeting. Techniques such as genetic modification of donor cells, click chemistry, or membrane fusion allow for the customization of sEV surfaces to enhance targeting, reduce immunogenicity, or incorporate stealth features (eg, PEGylation), making EVs safer and more effective.

Crossing Biological Barriers Efficiently

Furthermore, their ability to naturally home to specific tissues and cross biological barriers, like the blood-brain barrier, adds to their utility while maintaining a safe profile. A feature that significantly enhances their potential as delivery vehicles for therapeutic agents. This capability is particularly useful since the BBB, a highly selective and protective barrier, prevents most drugs and large biomolecules from entering the brain tissue. sEVs defined by their small size and natural composition can interact with and permeate through cellular barriers without interfering with their structural integrity. This allows them to deliver therapeutic molecules directly to brain cells, offering a potent strategy for treating neurological disorders that are otherwise challenging to manage due to the restrictive nature of the BBB. A study by Zhou et al deduced that human milk-borne sEVs penetrate the brain, accumulate intensely in the hippocampus, cortex, and cerebellum, preserving neural recovery capacity, spatial learning, and memory retention in seizure-induced mouse models.[96] Besides the brain, their ability to cross other barriers, such as the intestinal barrier or the placental barrier, opens up possibilities for non-invasive delivery routes and treatments for a wide range of conditions, ensuring that therapeutic agents can reach their target sites effectively and safely.[97,98] This other unique characteristic of sEVs holds immense promise for advancing drug delivery technologies and creating more effective treatments for diseases that require targeted intervention across these biological barriers.

Minimal Cytotoxicity

sEVs are also promising tools in biomedical applications due to their minimal toxicity, making them ideal for therapeutic use.[99] sEVs are naturally produced by cells and mimic their signaling mechanisms, which significantly reduces the risk of adverse immune responses that are common with synthetic delivery systems. Their biocompatibility is a critical determinant as it ensures that do not elicit harmful systemic effects, ensuring safer and long-term treatments. This quality is particularly important in treatments involving frequent dosing regimens, such as in chronic diseases or regenerative therapies. Thus, sEVs stand out in the landscape of drug delivery and regenerative medicine as an effective means of therapy with minimal risk of toxicity to the patient.

Reduced Immunogenicity

sEVs are also known for their low immunogenicity, which is a vital component for clinical applications.[100] The low immunogenic profile of sEVs means they can be administered into the body without eliciting strong immune responses such as inflammation or immune rejection. This feature is particularly beneficial when considering treatments involving repeated doses over time, as there is minimal risk of the body developing antibodies against the sEVs. This characteristic arises from the inherent properties of MSCs themselves, which are known for their immunomodulatory effects. MSCs can modulate immune responses by interacting with various immune cells, inhibiting the proliferation of T cells, and inducing regulatory T cells and M2 macrophage polarization, all of which contribute to an anti-inflammatory environment.[84,101,102]

Cargo Delivery

sEVs can encapsulate and deliver a variety of bioactive molecules, such as neurotrophic factors, microRNAs, proteins, and enzymes. (i) Neurotrophic Factors: Proteins such as BDNF, NGF, and GDNF have been shown to promote neuronal survival, stimulate axonal regeneration, and support functional recovery in SCI models.[7,21,22] (ii) MicroRNAs (miRNAs): Small non-coding RNAs, such as miR-133b and miR-146a, are loaded into sEVs to regulate gene expression in target cells. These miRNAs play roles in inflammation, cell survival, and neuroprotection, which are critical in SCI recovery.[15,22,31] (iii) Proteins and Enzymes: sEVs deliver enzymes that break down scar tissue (eg, matrix metalloproteinases), anti-inflammatory cytokines (eg, interleukin-10), or other bioactive molecules that modulate the local microenvironment after SCI.[20,103]

Enhanced Cellular Uptake

sEVs possess inherent properties that facilitate enhanced cellular uptake. sEVs are enveloped by a phospholipid bilayer like that of cell membranes, enabling them to merge effortlessly with cellular membranes. This natural affinity facilitates direct fusion with the plasma membrane of target cells, allowing the contents of sEVs to be efficiently transferred into the cytoplasm. sEVs display surface molecules (eg, tetraspanins – CD9, CD81, and CD63) that can specifically interact with target cells, initiating receptor-mediated endocytosis.[104] In some cases, sEVs derived from stem cells carry integrins that bind selectively to receptors on endothelial or inflammatory cells, promoting tissue-specific uptake, particularly in injured or inflamed environments like the spinal cord. In addition to fusion and receptor-mediated pathways, sEVs can be internalized via macropinocytosis, a non-selective form of endocytosis in which extracellular fluid and particles are engulfed by the cell.[105] This pathway is less selective but can significantly contribute to the bulk uptake of sEVs, particularly in responsive cell types.

In SCI, the local microenvironment plays a critical role in modulating sEV uptake. Following injury, the BSCB becomes compromised, enhancing the permeability to circulating factors, including EVs. This disruption, coupled with the infiltration of inflammatory cells and upregulation of adhesion molecules, creates a window of opportunity for targeted delivery of therapeutic sEVs.[106] sEVs bearing ligands for receptors upregulated in SCI (eg, ICAM-1 or VCAM-1) may preferentially accumulate at the injury site.[107] To further improve brain and spinal cord targeting, researchers are exploring membrane engineering and genetic editing strategies. One widely used method is the expression of targeting peptides, such as the rabies virus glycoprotein (RVG) peptide, on sEV surfaces by genetically modifying parent cells to express peptide-fusion constructs with EV membrane proteins like Lamp2b or CD63.[108] This enhances sEV transport across CNS barriers and increases uptake by neurons and glial cells. Other approaches include click chemistry-based surface modifications, enabling the post-production attachment of ligands, and the use of stimuli-responsive coatings that release cargo in response to environmental cues,[109] such as the oxidative stress or acidity characteristic of injured spinal tissue. Furthermore, the formation of a protein corona on sEVs in vivo can alter their interaction with target cells.[95] Controlling the corona, either through in vitro manipulation or surface shielding techniques, may provide an additional avenue for tuning EV biodistribution and enhancing therapeutic precision in SCI. Altogether, the natural and engineered properties of sEVs offer a multifaceted platform for targeted and efficient cellular delivery in the context of SCI. Their ability to navigate biological barriers and interact with cell-specific receptors positions them as promising candidates for regenerative and immunomodulatory therapies in the CNS.

Scalability and Cost-Effective Clinical Manufacturing

One of the primary challenges in scaling up sEV production is the need for large-scale cell culture systems. Advances in bioreactor technology, such as the use of stirred-tank reactors, hollow-fiber systems, and suspension cultures, allow for the mass production of cells that secrete sEVs,[111–113] each system poses unique challenges. For instance, stirred-tank reactors offer scalability but may induce shear stress, potentially affecting vesicle integrity. Hollow-fiber bioreactors, while providing high surface area and compartmentalized environments conducive to sEV harvest, can suffer from clogging and are more complex to maintain. Suspension cultures simplify scaling but often require genetic or media adaptation to maintain sEV yield and functionality. In terms of downstream processing, tangential flow filtration and ultracentrifugation are commonly used, yet their efficiency and selectivity still face limitations when handling large volumes.[113,114] Ultracentrifugation is labor-intensive and time-consuming, whereas filtration systems need to be finely tuned to prevent loss of vesicles or contamination by protein aggregates and other extracellular particles. Continuous bioprocessing represents a promising shift from traditional batch processes, allowing for steady-state production and potentially higher yields. However, integrating continuous production with real-time monitoring and quality control is still under development. Furthermore, the automation of sEV isolation and purification remains in early stages. While automation reduces labor and enhances reproducibility, current systems are often cost-prohibitive and lack flexibility across different sEV-producing cell types. The implementation of fully closed and integrated systems offers a compelling solution to reduce contamination and processing time. Yet, these systems require significant capital investment and robust standardization to meet regulatory expectations, especially in clinical-grade production. Finally, although economies of scale could ultimately reduce the cost per unit of sEVs[115] current cost-benefit ratios remain a barrier without assured therapeutic demand. This underscores the need for parallel advancements in clinical validation, regulatory frameworks, and market development.

Effect of Various Factors on sEVs Bioactivity

The production and therapeutic effects of sEVs are influenced by various extracellular factors, including culture conditions, biochemical agents, mechanical stimuli, and environmental enrichment. The low-density culture conditions increase sEVs production, while hypoxic preconditioning enhances angiogenesis. Biochemical factors like IGF-1 and BDNF can enhance sEVs output from MSCs and enrich their content with factors that promote neuroprotection and functional recovery. Moreover, advanced techniques such as 3D culture systems, vertical-wheel bioreactors, and low-intensity pulsed ultrasound (LIPUS) further optimize sEVs yield and biological activity (Figure 5 and Table 6). These approaches offer promising strategies for enhancing therapeutic applications in SCI and other neurodegenerative diseases. A summary of these factors and their impact on the SCI regeneration mechanism is presented in Table 6.

Challenges and Strategies Related to sEV-Based Delivery for SCI Treatment

Although sEVs are considered ideal candidates for targeted drug delivery with minimal toxicity, they face significant challenges in real-world applications. These challenges primarily involve the complexities of their synthesis, scaling up production, and storage, which limit their potential for therapeutic use in SCI. sEV-based therapies face several significant challenges, including issues with production, purification, stability, and transport. A summary of the challenges and strategies related to sEV-based delivery for SCI treatments is reported in Table 7.

Heterogeneity of sEV Cargo

sEVs can be derived from various biological fluids (like blood, urine, or cell culture media) and different cell types. This variability can affect the composition, size, and function of the sEVs, leading to less consistent results in therapeutic applications. Since sEVs carry a diverse array of biological molecules, including proteins, lipids, RNA, and DNA, this complexity could difficult the characterization and standardization of cargo for consistent therapeutic outcomes.[138] Identifying which components are therapeutically active is essential for efficacy and safety.

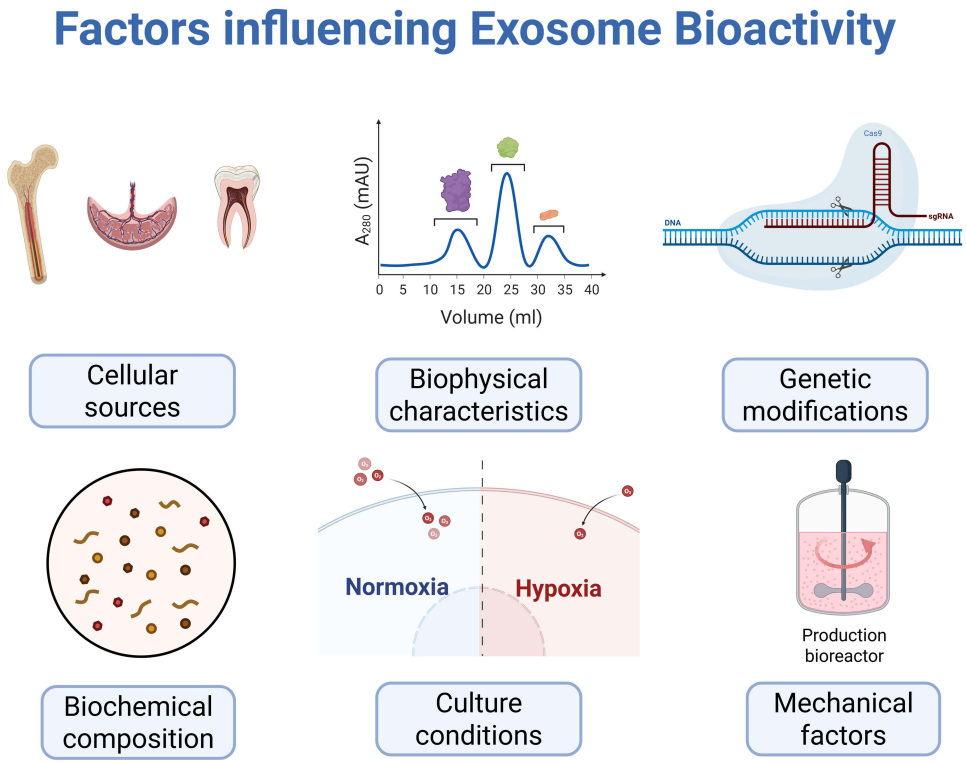

Figure 5 Factors affecting the sEV's bioactivity. Despite the advantages of sEVs, some factors could hinder or influence sEVs biological and functional qualities. These include the type of cells the sEVs were derived from, which could affect the biophysical or biochemical compositions. Depending on culture conditions or setup, environmental factors, including temperature, oxygenation, and mechanical tolerance, may alter the cells or sEV properties directly. Gene modification has also become commonplace, although many protocols have yet to be optimized.

Biodistribution and Delivery Challenges

sEVs typically range from 30 to 150 nm in diameter, which is far below the resolution limit of conventional microscopy. Techniques such as electron microscopy become essential to achieve the necessary resolution, but are not applicable for live imaging or dynamic tracking in biological systems.[139] Effective imaging of sEVs also requires labeling them with fluorescent probes or other markers. However, ensuring that these labels do not alter the vesicles' natural properties or functionalities remains a significant challenge. Over-labeling or the use of complex labels could potentially interfere with the sEVs' ability to interact with target cells or tissues. Measuring the exact number of sEVs reaching a target site is

Table 6 Biochemical, Mechanical, Cultural, and Genetic Factors and Their Impact on Therapeutic Outcome and Molecular Mechanism of EVs

Factor	sEVsome Source	Therapeutic Function of sEVsomes	Molecular Mechanism	References
Biochemical factors: LPS (Pro-inflammatory)	MSCs	Reduced pro-inflammatory cytokine secretion	AKT1/AKT2 phosphorylation via NF-κB signaling	[116]
Biochemical factors: IFN-γ, TNF-α	BMSCs	Anti-inflammatory effects, targeted to inflammation sites	Upregulation of COX2/PEG2, cytokine modulation	[117]
Biochemical factors: Fe3O4 (Iron oxide nanoparticles)	BMSCs	Enhanced angiogenesis and targeting injured sites	miR-21-5p ↑, VEGF, HIF-1α ↑ via PI3K/AKT & ERK1/2	[118,119]

(*Continued*)

https://doi.org/10.2147/IJN.S522028

Table 6 (Continued).

Factor	sEVsome Source	Therapeutic Function of sEVsomes	Molecular Mechanism	References
Culture condition: High Glucose	SCs	Exacerbated sciatic nerve impairment in diabetic mice	N/A	[120]
Culture condition: Cell Density (High vs Low)	MSCs	High-density: Contact inhibition, quiescence; Low-density: Activates paracrine signaling pathways	Higher sEV secretion at 60–90% confluence (preferred for collection)	[121]
Culture condition: Hypoxic Preconditioning	hUCMSCs	Modifies gene expression and proteome, upregulates HIF-1α, VEGFA, and KDR, influencing angiogenesis	sEVsomes from hypoxia-preconditioned hUCMSCs show increased HIF-1α content, enhancing pro-angiogenic effects (VEGF overexpression)	[122]
Culture condition: 3D Culture System	UCMSCs	Mimics in vivo conditions more closely, enhancing paracrine signaling	20-fold increase in sEV production with microcarrier-based 3D system; combined with TFF, production amplified by 7x	[123]
Mechanical Factors: Hollow-fiber Bioreactor	UCMSCs	High yield, enhanced biological functions, promoted cell proliferation and migration	TGF-β1, Smad2/3 signaling activation	[124]
Mechanical Factors: Vertical-wheel Bioreactor	BMSCs, ADMSCs, UCMSCs	Increased sEV concentration and productivity	Increase expression of markers	[125]
Mechanical Factors: Low-intensity Pulsed Ultrasound (LIPUS)	SCs	Enhanced nerve regeneration via sEV miRNA changes	PI3K-Akt-FoxO signaling pathway	[126]
Genetic factors: miR-133b	MSCs	Promoted axon regeneration and recovery of hindlimb function	Activation of ERK1/2, STAT3, and CREB signaling pathways	[127]
Genetic factors: miR-125a	BMSCs	Reduced inflammation, promoted M2 polarization	Negative regulation of IRF5 expression	[128]
Genetic factors: miR-499a-5p	MSCs	Reduced neuronal apoptosis after OGD/R	Inhibition of JNK3/c-jun signaling pathway	[129]
Genetic factors: miR-26b	ADMSCs	Suppressed cell autophagy in injured SCs	Downregulation of Kpna2	[130]
miR-672-5p	M2 Microglia	Inhibited neuronal pyroptosis and promoted functional recovery	Suppression of AIM2/ASC/caspase-1 signaling pathway	[131]
miR-199a-3p/145-5p	UCMSCs	Promoted PC12 cell differentiation, facilitated spinal cord functional recovery	NGF/TrkA↑	[44]

Notes: This table outlines the therapeutic functions and mechanisms of various sEV. Biochemical factors like LPS and inflammatory cytokines (eg, IFN-γ, TNF-α) modulate NF-κB and COX2/PEG2 signaling. Fe3O4 nanoparticles enhance angiogenesis through miRNA modulation and VEGF upregulation. Culture conditions like high glucose exacerbate nerve injury, while hypoxic preconditioning and 3D culture systems enhance sEV production and promote angiogenesis through HIF-1α and VEGF pathways. Mechanical factors like bioreactors and low-intensity pulsed ultrasound (LIPUS) improve sEV yield and promote cell migration and nerve regeneration via PI3K-Akt-FoxO signaling. Genetic factors such as miRNAs (eg, miR-133b, miR-125a) regulate axon regeneration, M2 polarization, and neuronal apoptosis by modulating various signaling pathways like ERK1/2, STAT3, and JNK3/c-jun.

crucial for dose verification and understanding therapeutic outcomes but remains technically demanding. Yet, current imaging modalities may not offer the quantitative precision needed for detailed analysis. Gupta et al comprehensively reviewed 64 pre-clinical studies and reported dose selection that was independent of EV pharmacokinetics or biodistribution trends due to the lack of tracking and quantitative strategies.[140]

Table 7 Challenges Associated with EV Treatment, the Cause, Impact, and Strategy to Overcome These Challenges to Enhance EV Penetration and Activity

Challenge	Problem	Impact	Solution/Strategy	References
BSCB Barrier	BSCB hinders EVs entry into the spinal cord.	Reduces the ability to deliver EVs effectively to the injury site.	Use of targeting ligands or bioengineering to enhance EVs penetration across the BSCB.	[132]
Spinal Cord Microenvironment	Altered post-SCI environment complicates precise targeting of injury sites.	Inconsistent EVs delivery, reducing treatment efficacy.	Tailored EVs formulations to adapt to the post-SCI microenvironment (eg, tissue composition, inflammation).	[133]
Off-Target Effects	Non-specific delivery triggers potential immune responses or unintended interactions.	Increased risk of toxicity, immune reactions, or adverse effects.	Incorporating receptor-specific ligands or biomimetic approaches for targeted delivery.	[133]
EVs Surface Modifications	Modifying EVs surfaces (eg, g peptides or ligands) to enhance targeting.	Enhances efficacy but complicates synthesis or requires complex modifications.	Engineering EVs with specific ligands (eg, Lamp2b, C1C2, CD9, transferrin) for targeted delivery.	[134]
Transport Efficiency	Complex delivery routes and fluid dynamics hinder efficient transport.	Low targeting efficiency, limiting therapeutic outcomes.	Use of microfluidic devices and biomimetic systems for precise control of EVs delivery.	[135,136]
Imaging & Tracking	Difficulty in visualizing sEVs uptake and intracellular fate in real-time.	Lack of detailed information on sEVs distribution and efficacy in vivo.	Use of advanced imaging techniques (eg, super-resolution microscopy, live-cell imaging).	[137]

Notes: This table highlights the major challenges in sEVs delivery for SCI treatment. BSCB Barrier poses a significant challenge for EVs to penetrate the spinal cord, the spinal Cord microenvironment post-SCI complicates precise targeting, off-target effects result in potential immune reactions or toxicity. sEVs surface modifications offer targeted delivery using specific ligands like Lamp2b or transferrin. Transport efficiency is hindered by complex fluid dynamics. Lastly, imaging and tracking difficulties in visualizing sEVs uptake and intracellular fate.

Delivering sEVs also poses several challenges related to the routes of administration.[141] While intravenous administration allows sEVs to circulate throughout the body, it also raises challenges regarding the specificity of targeting. sEVs can become diluted or may preferentially accumulate in organs like the liver, spleen, or lungs, rather than the target tissue. While effective at targeting CNS tissues, intrathecal or intracerebroventricular injection routes are invasive and carry risks of infection, hemorrhage, and injury to nervous tissues. Oral delivery of sEVs faces the challenge of degradation within the harsh gastrointestinal tract environment. Delivering sEVs via inhalation is an attractive option through efficient nebulization of sEVs without damaging their integrity, deep lung deposition, and the potential for immune reactions in the lung tissue.

Clinical Production Inconsistencies

The production process is also hindered by sEVs heterogeneity, scaling difficulties, and contamination, limiting consistency and purity. Certain purification methods, such as ultracentrifugation, yield low purity and can damage sEVs. Additionally, sEVs are sensitive to environmental conditions, making stability and long-term storage much desired.[133] Transport to the injury site is further complicated by the BSCB and the dynamic nature of the SC post-injury, reducing targeting precision. To overcome these hurdles, strategies such as bioengineering, targeted surface modifications (eg, Lamp2b, CD9), microfluidic devices, and advanced imaging techniques are being explored to enhance sEVs delivery, stability, and therapeutic efficacy for SCI treatments (Figure 3).[132]

Regulatory Hurdles

sEVs are divided between classifications of biological drugs, cell therapies, and drug delivery systems. Establishing a clear regulatory definition for sEVs is challenging due to their varying origin, complex composition, and diverse

functions.[143–145] To begin with, defining and measuring the purity or potency of sEVs is essential for regulatory approval. However, given the diverse molecular contents of sEVs, selecting appropriate biomarkers for these characteristics is complicated. For sEVs, the traditional pharmacokinetic and pharmacodynamic models may not be completely applicable, hence, alternative approaches are required to review the biodistribution, clearance, immunogenicity, and therapeutic effects.[145] Regulatory bodies require decisive and evidence-based criteria for what constitutes an acceptable level of purity and how potency shall be assessed based on the intended therapeutic effect. This ambiguity has continued to complicate the regulatory approval process as it affects how these therapies should be evaluated and approved.

Limitations and Future Perspectives

Although sEV-based therapies for SCI show substantial promise, several challenges must be addressed. A deeper understanding of the composition of sEVs from various sources is crucial, as this will help optimize their therapeutic potential. Additionally, the standardization of isolation and characterization methods is needed to ensure consistent and reproducible results. The optimization of delivery routes and doses is essential for overcoming biological barriers and ensuring that sEVs effectively reach the injured tissues. Furthermore, long-term safety, potential side effects, and immune responses require thorough investigation to guarantee the feasibility of clinical applications. Despite these challenges, progressive advancements in sEV engineering, such as genetic modification, biomaterial incorporation, and external cue-based delivery methods like magnetic guidance and ultrasound, offer promising solutions to improve targeting specificity and delivery efficiency. Future research, including preclinical studies and clinical trials, will be crucial for overcoming these limitations and achieving successful clinical translation of sEV-based therapies for SCI.

Conclusion

sEV-based therapies for SCI hold significant promise, offering potential solutions for inflammation reduction, neuroprotection, and neural repair. EVs have demonstrated the ability to reduce glial scarring and fibrosis, providing a safer alternative with lower risks of immune rejection and tumorigenesis. Their stability, ease of storage, and ability to cross biological barriers enhance their practicality for clinical use. Additionally, EVs can be engineered to carry specific therapeutic agents and target injured tissues more effectively. These factors make them a compelling therapeutic option. However, addressing the limitations discussed and advancing the understanding of sEVs will be key to realizing their full therapeutic potential. Continued research and innovative strategies are essential to ensure that sEV-based therapies become a viable and effective treatment for SCI, offering hope for more effective and targeted interventions in the future.

Abbreviations

SCI, Spinal Cord Injury; CNS, Central Nervous System; MSC, Mesenchymal Stem Cells; EV, Extracellular vesicles; sEV, Small Extracellular Vesicle; Exo, Exosomes; PRP-Exos, Platelet-rich plasma-derived sEVs; BSCB, Blood-Spinal Cord Barrier; M2-Exos, M2 microglia-derived Exos; BBB, Basso-Beattie-Bresnahan; vMIP-II, Macrophage Inflammatory Protein II; Lamp2b, Lysosomal-associated membrane protein 2b; MExos, Melatonin-enhanced sEVs; BDNF-Exo, Brain-derived neurotrophic factor-exosomes; VEGF, Vascular endothelial growth factor; GDNF, Glial cell-derived neurotrophic factor; BMSC, Bone marrow-derived MSC; hUCMSCs, Human umbilical cord mesenchymal stem cells; 6-OHDA, 6-hydroxydopamine; MMA, Methylmalonic acidemia; SOD, Superoxide dismutase; MDA, Malondialdehyde; TGF-β1, Transforming growth factor beta 1; RGD, Arginyl-Glycyl-Aspartic acid; MBP, Myelin basic protein; NF200, Neurofilament Protein; GAP43, Growth-Associated Protein 43; CSPGs, Chondroitin Sulfate Proteoglycans; SCDEs, Schwann cell-derived sEVs; iPSCs, Induced pluripotent stem cells; ROS, Reactive oxygen species; HUVECs, Human umbilical vein endothelial cells; TEM, Transmission electron microscopy; NTA, Nanoparticle tracking analysis.

Acknowledgment

The Faculty of Medicine, Universiti Kebangsaan Malaysia, Malaysia, Grant No FF2019/542/1, supported this work, and the authors also would like to acknowledge the support provided by the University of Maryland, Baltimore, and the National Institute of Health (NIH), USA.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by the Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, with Grant No. FF-2019-542/1.

Disclosure

Yogeswaran Lokanathan reports grants from Ministry of Education, Malaysia, grants from Moleac Pvt Ltd, grants from Medixcell Sdn. Bhd., grants from Ming Medical Services Sdn. Bhd., during the conduct of the study. The authors declare no other conflicts of interest in this work.

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