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Review

Next generation of neurological therapeutics: Native and bioengineered extracellular vesicles derived from stem cells

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ABSTRACT

Extracellular vesicles (EVs)-based cell-free therapy, particularly stem cell-derived extracellular vesicles (SC-EVs), offers new insights into treating a series of neurological disorders and becomes a promising candidate for alternative stem cell regenerative therapy. Currently, SC-EVs are considered direct therapeutic agents by themselves and/or dynamic delivery systems as they have a similar regenerative capacity of stem cells to promote neurogenesis and can easily load many functional small molecules to recipient cells in the central nervous system. Meanwhile, as non-living entities, SC-EVs avoid the uncontrollability and manufacturability limitations of live stem cell products *in vivo* (*e.g.*, low survival rate, immune response, and tumorigenicity) and *in vitro* (*e.g.*, restricted sources, complex preparation processes, poor quality control, low storage, shipping instability, and ethical controversy) by strict quality control system. Moreover, SC-EVs can be engineered or designed to enhance further overall yield, increase bioactivity, improve targeting, and extend their half-life. Here, this review provides an overview on the biological properties of SC-EVs, and the current progress in the strategies of native or bioengineered SC-EVs for nerve injury repairing is presented. Then we further summarize the challenges of recent research and perspectives for successful clinical application to advance SC-EVs from bench to bedside in neurological diseases.

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1. Introduction

Neurological disorders are the leading cause of mortality and morbidity worldwide [1]. Neurons in the mature central nervous system (CNS) are difficult to regenerate after injury and contribute to permanent neurological impairments. Over the past half-century, despite significant developments and innovations in pharmacological interventions, surgical procedures, and rehabilitation therapies, they cannot provide a permanent cure but only mitigate symptoms and delay progression [2]. The crucial of neurological impairment repair is the regrowth of damaged nerve cells [3]. The conventional view holds that endogenous neurogenesis mainly occurs in the fetal stage but rarely in mature CNS [4]. Although several lines of evidence have suggested that endogenous neurogenesis could also occur in the hippocampus's dentate gyrus and the striatum's subventricular zone in adult brain [5], it is insufficient to induce adequate tissue repair after brain damage [6]. Moreover, the complexity of the adult brain's structure led to the lack of understanding of pathological mechanisms, and many potential therapeutic targets remain unknown, further hindering the development of small molecule-based drugs or recombinant proteins [7]. Therefore, finding an effective strategy to cure neurological disorders is crucially important.

While there are no effective therapies available [8], stem cell transplantation has shown promising therapeutic effects in promoting neurogenesis and facilitating nerve repair after injuries, such as Alzheimer's disease (AD), Parkinson's disease (PD), stroke, spinal cord injury (SCI), cerebral palsy (CP), traumatic brain injury (TBI) and oncological diseases (glioblastoma) [9–11]. Stem cells may exert neuroprotective effects by neurogenesis, angiogenesis, antiapoptosis, immunomodulation, and inflammation. However, with the conduct of clinical trials of stem cells, the live stem cells' quality control *in vitro* and safety assessment *in vivo* limit its clinical application, such as the difficulties in reliable cell type characterization, precise scalable methods for cell production and purification, storage efficiency, transport stability, heterogeneity among different batches, the viability maintenance of stem cell during the infusion process, delivery and integration into the desired target tissue [12], the tumorigenicity [13], the immune incompatibility [14], and ethical issues [15] associated with applications. Recent studies in neurological disease models have implied that these therapeutic effects of stem cells are due to the paracrine mechanism, especially the extracellular vesicles (EVs) [16,17]. These EVs from stem cells (SC-EVs) are proposed to harbor regenerative capacity to promote neurogenesis [18] and can cross the blood-brain barrier (BBB) to deliver small molecules to the CNS, which may be exploited for neurological disorders therapy. Although further studies are needed to confirm that SC-EVs can substitute for stem cell therapy, delivering SC-EVs rather than stem cells themselves may present a clinically attractive therapeutic option.

Initially, EVs were regarded as a mechanism for transporting intracellular waste to the extracellular space only, while they have emerged as major mediators for intercellular communication today. SC-EVs, as a kind of unique EVs from stem cells, can mediate the exchange of nucleic acids, lipids and proteins between cells and participate in both homeostatic and pathological processes [19,20]. In the CNS, SC-EVs can mediate interactions between neurons and oligodendrocytes and participate in neurogenesis and synapse formation. This unique intercellular communication is essential for all stages of CNS development and incorporates signals over short distances (*i.e.*, cell-to-cell) or wide-spreading throughout the brain via cerebrospinal fluid (CSF). SC-EVs have promising prospects as personalized medicines for neurological disorders, but before SC-EVs are established as an effective therapeutic tool, their homogeneity and stability must be ensured; thus, several strategies are needed to explore the clinical application of SC-EVs and enhance their potential therapeutic effects: (i) development of platforms to mass produce SC-EVs to meet practical clinical needs at a reduced cost; (ii) enhancement of SC-EVs detection methods to provide pharmacokinetic information in order to define the optimal dose, dosing frequency, and tissue distribution; and (iii) improvement of modified strategies of SC-EVs to enhance their biological functions and to optimize their accumulation at the intended target sites. To unlock the clinical potential of SC-EVs for neurological applications, pre (engineering the parent cells of SC-EVs)- and/or post (direct modification of SC-EVs)-isolation innovative engineering techniques, which can modulate the intrinsic characteristics of native SC-EVs, need to be developed (Fig. 1). Researchers need to put more effort into tapping the therapeutic potential of SC-EVs through innovating methods and integrating decades of previous experience to advance cell-free therapy progress.

In this review, we first describe the biophysical properties of SC-EVs and the therapeutic potential of SC-EVs for neurogenesis. To expand the scope of SC-EVs' clinical application, engineering strategies for yield and bioavailability improvement are comprehensively discussed. Then, we provide an overview of the progress and challenge for SC-EVs translation from bench to bedside in CNS diseases, thereby promoting interdisciplinary integration and the progress of industrialization and medicine to help with the widespread the clinical practice of the SC-EVs as soon as possible.

2. SC-EVs' biophysical properties

The term "extracellular vesicles" refers to lipid membrane-enclosed extracellular structures secreted by most, if not all, cells. According to EVs' biogenesis and biophysical/biochemical characteristics, EVs can be divided into exosomes, microvesicles, and apoptotic bodies. Usually, exosomes, with a diameter of 50–200 nm, formed intracellularly and secreted in the extracellular space via cytosolic ejection after multivesicular bodies (MVB) fused with the cell surface; microvesicles, with a diameter of 50–1000 nm, formed by outward budding of the plasma membrane [21]; and apoptotic bodies (500 nm-2 μm) formed by blistered and distinct membrane-enclosed vesicles of dying cells at the end of apoptosis [22]. Owing to the complexity involved in identifying the biogenesis of EVs, the International Society for Extracellular Vesicles (ISEV) recommended categorizing them

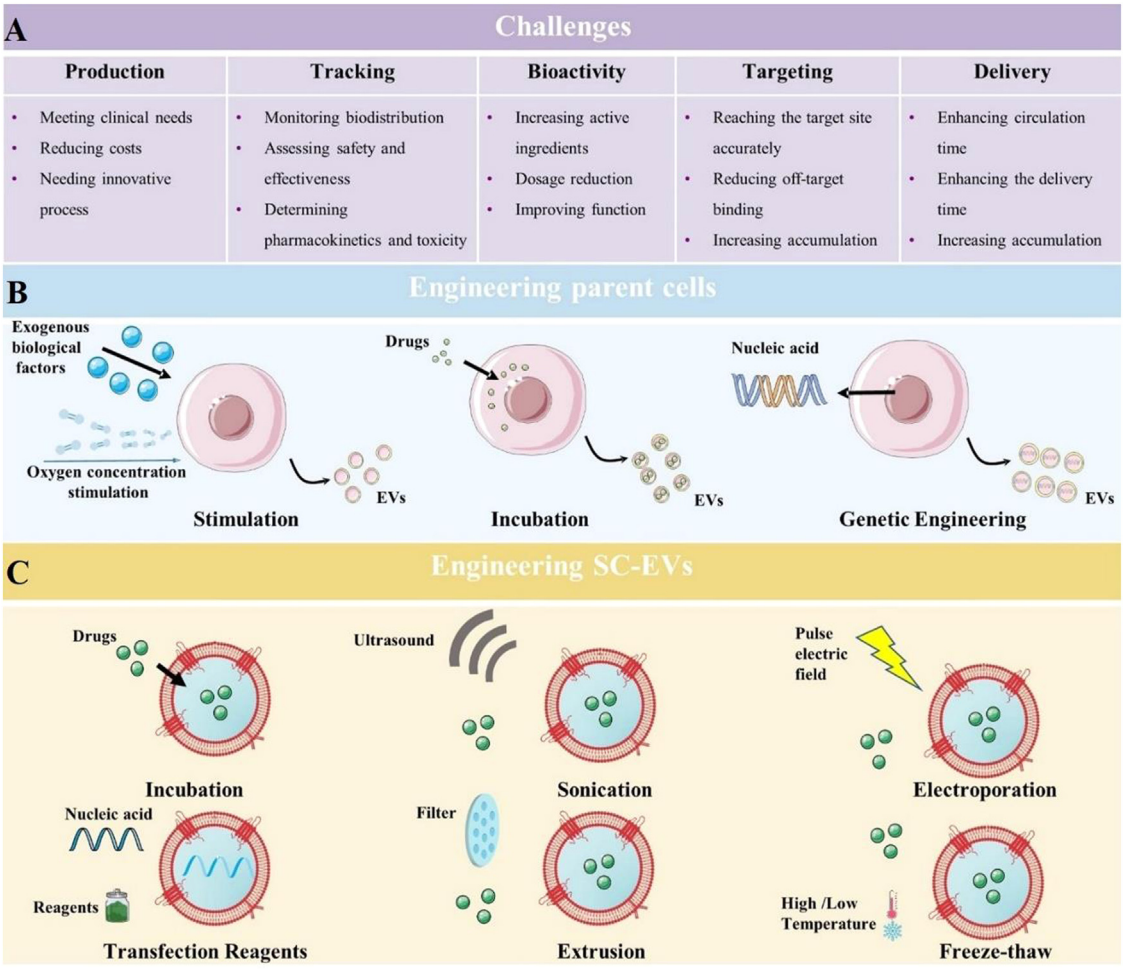

Fig. 1 – Classes of bioengineered SC-EVs:(A) Native SC-EVs as neurological therapies have their unique challenges in the clinical application. (B, C) these challenges have facilitated the development of bioengineered SC-EVs for improved therapeutic function: the modification of SC-EV-producing parent cells (B) and the design of SC-EVs after their isolation(C).

into two groups- small extracellular vesicles (sEVs <100 nm) and large extracellular vesicles (lEVs >200 nm) [23]. In this review, we use "EVs" as an umbrella term for exosomes and microvesicles (50–1000 nm) because the distributions of the size of both subsets overlapped and the biological origin of EVs is not conclusive in many studies.

SC-EVs' biophysical properties are similar to EVs of non-stem cell origin. They all have the typical bilayer membrane structure and specific biomarkers, such as the tetra-transmembrane proteins (CD9, CD81, and CD63), cytoplasmic proteins tumor susceptibility gene 101 (TSG101), apoptosis-linked gene 2-interacting protein X (ALIX), Ras-related protein GTPase Rab, etc., which are intimately linked in EVs biogenesis, membrane fusion, and cargo transfer [24,25]. Still, SC-EVs also has its own uniqueness, like size and composition, which may influence their biological function.

The size of SC-EVs is highly heterogeneous due to the complex nature of their biogenesis [26]. For example, the diameter of mesenchymal stem cell-derived EVs (MSC-EVs, about 300 nm) is larger than that of neural stem cell-derived EVs (NSC-EVs, about 200 nm) [27]. Compared with the larger EVs, the smaller EVs are more easily absorbed by target cells and communicate more rapidly with the intracellular [28]. In addition, in CNS, the smaller EVs might easily cross the BBB and perform the biological function, which is crucial for peripheral circulation administration.

SC-EVs' membranes are an important manifestation of their function and partially mediate the neurological therapeutic effects. As vesicle-like substances of cellular origin, SC-EVs' membranes are rich in specific lipids and proteins, just like their parental cell plasma membranes. For instance, the membrane-bound protein neuropilin-1 (NRP1) is a novel enriched surface biomarker for human MSC-EVs, which regulates cell migration, invasion, chemotaxis, and angiogenesis [29]. CD63 is highly prevalent in MSC-EVs [30], and CD63-positive EVs are more likely to recognize neurons and glial cells in the brain [31], partly explains the targeting role of MSC-EVs in the CNS.

SC-EVs' contents, especially proteins and miRNAs, are the major functional subjects. Proteomics analysis and miRNA sequencing of SC-EVs and non-SC-EVs showed great differences in the composition, especially in the proportion of each composition [29–32]. For example, in non-SC-EVs, the two most highly expressed EVs' proteins are Syntenin-1

and ALIX [32], in NSC-EVs, they are Hemopexin and Serum albumin [32], while in EVs from embryonic stem cells (ESC-EVs), they are Wnt10b and Notch ligand Delta-like 4 (DLL4) [33]. Similarly, highly significant differences in miRNA expression levels were uncovered. The research shows that miR151a-3p, miR320a, miR-21–5p, miR4443, miR365a-5p, and miR-744–5p are the most highly expressed in non-SC-EVs [32]. On the other side, different contents, including miR148a, miR532–5p, miR378, and let-7f, targeted to transcription factors to modulate angiogenesis and adipogenesis, were preferentially found in MSC-EVs [18]. While miR-1246, miR-4488, miR-4492, miR-4508, and miR-4516 as the five most enriched miRNA were identified in NSC-EVs [34]. In addition, ESC-EVs are highly enriched in mRNAs of several pluripotent transcription factors (Oct-4, Rex-1, Nanog, SCL, and GATA-2), and more importantly, these mRNAs can be delivered by ESC-EVs to the target cells and translated into the corresponding proteins [35]. These differences may lead to the unique and different effects of SC-EVs in treatment.

In conclusion, SC-EVs are emerging as suitable candidates for cell-free therapies due to their unique biological properties. Moreover, these properties can be modified according to bioengineering tools to be more suitable for practical clinical needs in the context of neurological diseases, as described in Section 4.

3. Native SC-EVs for neurological disorders

The first study confirming the potential therapeutic applications of SC-EVs was published in 2010 [36]. Since then, large amounts of studies have shown the beneficial roles of SC-EVs in neurological disorders, such as stroke [37], TBI [38], SCI [39], AD [40], PD [41], etc. Presently, SC-EVs' optimal dosing regimen remains unknown. Investigators have evaluated the effects of exogenous SC-EVs in neurological disorders using different administration strategies (intravenous, intraarterial, intracerebral, and intranasal route) in multiple animal models (mouse, rat, pig, and rhesus monkey). Due to the uniqueness of the brain anatomy (natural barrier-BBB between the blood and the brain parenchyma [42]), nasal administration and stereotactic brain injection may yield higher retention of SC-EVs in the brain. Given the vastly different functions and sources of EVs, the administered dose of SC-EVs for animals varies widely (as shown in Table 1). Next, we will describe the effects and potential therapeutic applications of different types of SC-EVs in neurological disorders.

3.1. *EVs derived from neural stem cells*

NSC-EVs have shown neuroprotective benefits in many neurological disorders, primarily by improving the immune microenvironment, inhibiting brain cell apoptosis, inducing vascular remodeling and regeneration, and alleviating inflammation to protect damaged nervous tissue.

Stroke is the major cause of neurological dysfunction [43]. The neuroprotective effects of NSC-EVs on stroke have been demonstrated in rodent models of different ages and mammalian models. NSC-EVs promote recovery of damaged brain tissue and function during the acute phase of infarction, effectively reduce microglia density and neuronal apoptosis [44], and promote neuronal axon elongation and angiogenesis by stimulating nuclear translocation of NF-E2-related factor 2 (Nrf2) [45]. Even missing the clinical thrombolytic time window of recombinant tissue plasminogen activator (rt-PA), NSC-EVs can significantly reduce infarct size, improve brain atrophy, and modulate systemic immune responses to promote stroke recovery [27]. In a procine model, NSC-EVs promoted a reduction in cerebral lesion volume and edema, protected white matter integrity, and restored behavior and mobility after stroke [46,47].

Spinal cord injury (SCI) and traumatic brain injuries (TBI) are considered as devastating traumatic injuries [2]. After SCI, activation of autophagy can reduce tissue damage. NSC-EVs activate autophagy by increasing Beclin-1 expression through 14–3–3t protein [48], inhibit neuronal apoptosis and neuroinflammation, and promote early functional recovery [49]. In addition, NSC-EVs promote angiogenesis in spinal cord microvascular endothelial cells (SCMECs) by transferring VEGF-A, thus accelerating microvascular regeneration and tissue healing [50]. Interestingly, the repairment of TBI mediated by VEGF showed gender differences. In male rats, NSC-EVs indirectly mediated neural tissue repair after TBI by enhancing VEGF activity and increasing the migration of endogenous NSCs to the lesion site. However, this phenomenon was not found in female rats, which may be due to an undefined interaction between NSC-EVs and estrogen [51].

Dementia is a grave social problem. NSC-EVs are capable of conferring cognitive impairment protective effects via various mechanisms. AD is the most common cause of dementia, and the deposition of amyloid β (Aβ) plaques have long been thought to be the initiating factor in AD development [52]. NSC-EVs may prevent synaptic loss in the brain by reducing Aβ accumulation and inhibiting microglia activation by suppressing intracerebral inflammation [53]. However, another study showed that despite no Aβ plaque levels reduction, NSC-EVs could rescue cognitive deficits by enhancing mitochondrial function and reducing pro-inflammatory cytokine levels [54]. Furthermore, NSC-EVs could also produce effective and profound neuroprotection for Radiation cognitive dysfunction, the severe side effect of intracranial radiation therapy for CNS malignancies, by reducing neuroinflammation through miR-124 [55] and providing extensive nutritional support to affect the irradiated brain [56].

3.2. *EVs derived from mesenchymal stem cells*

MSC-EVs of varying origins (*e.g.*, bone marrow, umbilical cord, and adipose) have been demonstrated unique beneficial effects in neurological disorders. The potential therapeutic applications of MSC-EVs include: (i) enhancing neurogenesis and angiogenesis, reducing apoptosis, (ii) promoting neurite remodeling and synapse growth [57], and (iii) reducing inflammatory response by suppressing microglia and inflammatory factors [58,59]. The benefits have been mostly ascribed to MSC-EVs acting on different recipient cell types via the transfer of non-coding RNAs (particularly miRNAs, as shown in Fig. 2) and bioactive proteins.

Table 1 – Native SC-EVs for neurological disorders.

Cells	Injury	Outcomes	Model	Administration route	Administration time	Dose of SC-EVs	Refs.
H-NSCs	Stroke	Promoting the survival of post-H/R injury neurons and inhibiting neuronal apoptosis	NA	NA	NA	NA	[45]
R–NSCs	Stroke	Reducing microglial density, neuronal apoptosis and improving cerebral functional recovery	Rat MCAO	IC	2h	30 ug/rat (BCA)	[44]
H-NSCs	Stroke	Improving the cellular, tissue, and cerebral functional outcomes	Mouse TE	IV	2 h/14 h/38h	NA	[27]
H–NSCs	Stroke	Improved neural tissue preservation and cerebral functional levels	Porcine MCAO	IV	2 h/14 h/24h	2.7 E10± 10% /kg/procine (NTA)	[46]
M-NSCs	SCI	Reducing neuronal apoptosis, inhibiting neuroinflammation, and promoting cerebral functional recovery	Rat SCI	IV	0h	200 μg/rat (BCA)	[49]
M-NSCs	SCI	Promoting cerebral functional behavior recovery	Rat SCI	IV	0h	200 μg/rat (BCA)	[48]
M-NSCs	SCI	Promoting microvascular regeneration and tissue healing	Mouse SCI	IV	0.5 h/3 h/6 h/9 h/12h	200 μg/mouse (BCA)	[50]
H-NSCs	RICD	Abrogating RICD and neuroinflammation	Mouse 9 Gy head-only irradiation model	RO/SI	2d	6.7E6 part/mouse (NTA)	[55]
H–NSCs	AD	Reducing dense core Aβ plaque accumulation and microglial activation, protecting against synaptic loss and improving cerebral cognition	Mouse 5xFAD accelerated transgenic model	NA	NA	2.25E7 part/mouse (NTA)	[53]
M-NSCs	AD	Enhancing mitochondrial function, Sirt1 activation, synaptic activity, decreasing inflammatory response and rescuing cognitive deficits	Mouse TG	SI	BIW for 4 weeks	200 μg/mouse (BCA)	[54]
R-BMSCs	Stroke	Resisting inflammation and apoptosis	Rat SAH	IV	NA	200 μg/rat (BCA)	[60]
R-BMSCs	Stroke	Promoting neurogenesis and angiogenesis and improving behaviors	Rat tMCAO	IV	NA	30 μg/rat (BCA)	[61]
R-BMSCs	Stroke	Suppressing oligodendrocytes apoptosis	NA	NA	NA	NA	[62]
NA-MSCs	Stroke	Regulating neurite outgrowth	Rat tMCAO	NA	NA	NA	[63]
NA-MSCs	SCI	Suppressing the apoptosis of neuron cells	Rat SCI	IV	NA	NA	[64]
R-BMSCs	SCI	Resisting apoptosis and promoting motor function	Rat SCH	IV	1h	100 μg/rat (BCA)	[65]
R-BMSCs	SCI	Attenuating neurological damage	Rat SCRI	IV	NA	5E10 part/rat (NTA)	[66]
R-BMSCs	AD	Reducing Aβ deposition area and contents, rescuing AD hippocampal neurons, and improving cognitive function	Rat AD	SI	5d	30 μg/rat (BCA)	[67]
R-BMSCs	AD	Ameliorating astrocytic inflammation and improving cognitive impairment	Mouse APP/PS1	NA	NA	NA	[70]
H-ESCs	Stroke	Modulating neuroinflammation and protecting against ischemic stroke	Mouse MCAO	IV	2h	1E9 part/mouse (NTA)	[73]
M–NPCs	Stroke	Enhancing poststroke BBB integrity and attenuating inflammatory cell recruitment	Mouse MCAO	IV	0 h/6h	10 μg/mouse (BCA)	[71]
H-USCs	RTT	Improving behavior, motor coordination, and cognition and promoting the differentiation of NSCs	Mouse RTT	SI	QOD for 3 weeks	NA	[76]

H: human; R: rat; M: mouse; NA: not available; BMSCs: bone marrow-derived mesenchymal stem cells; H/R: hypoxia/reoxygenation; SCH: spinal cord hemisection; SCRI: spinal cord ischemia-reperfusion injury; RICD: radiation-induced cognitive dysfunction; SAH: subarachnoid hemorrhage; RTT: Rett syndrome; MCAO: middle cerebral artery occlusion; tMCAO: transient middle cerebral artery occlusion; TE: thromboembolic; TG: transgenic; IV: intravenous; IC: intracardial injection; IN: intranasal; RO: retro-orbital vein injection; SI: stereotactic system for intracerebral delivery; BIW: twice a week; QOD: every other day; part: particles; BCA: bicinchoninic acid; NTA: nanoparticle tracking analysis.

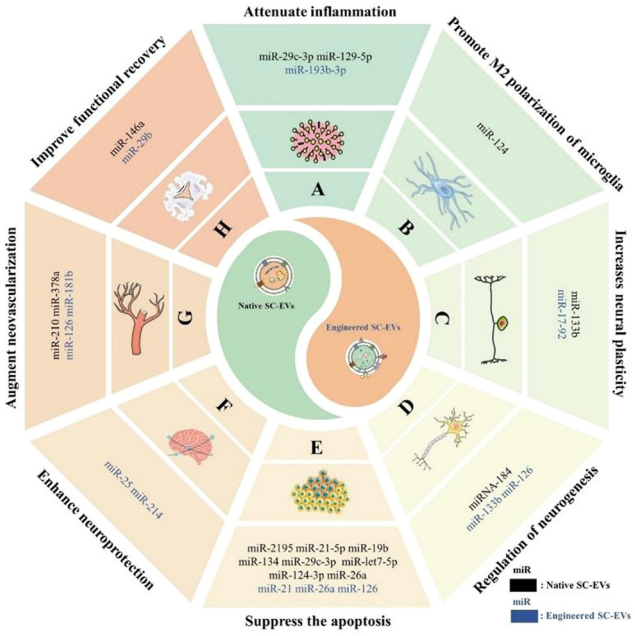

Fig. 2 – Role of miRNAs in native and engineered SC-EVs. SC-EV-miRNAs have been associated with many important biological processes involved in repairing neurological damage: (A) attenuate inflammation, (B) promote M2 polarization of microglia, (C) increases neural plasticity, (D) regulation of neurogenesis, (E)suppressing neuronal apoptosis, (F) enhance neuroprotection, (G)augmenting neovascularization, and (H) improve functional recovery. miR: microRNA.

In hemorrhagic stroke, MSC-EVs could improve neurological functions, reduce brain edema and maintain the integrity of BBB through the anti-inflammation and antiapoptotic effects mediated by miRNA129–5p, which inhibit the activity of the HMGB1-TLR4 pathway (high-mobility group box 1 protein- Toll-like receptor-4) [60]. Similarly, MSC-EVs could promote neurological recovery [61], suppress oligodendrocytes apoptosis [62], and promotes neurite outgrowth [63] after ischemic stroke through the therapeutic efficacy mediated by miRNA-184, miRNA-21, miR-134, and miR-133b. Therefore, MSC-EVs may be novel potential therapeutic agents for ischemic stroke treatment.

Also, recent findings have demonstrated that MSCs-EVs could exert neuroprotective function after SCI by promoting angiogenesis, attenuating apoptosis, and inhibiting the inflammatory response. Multiple miRNAs might mediate these repair effects. For example, miR-21/miR-19b could suppress neuron apoptosis by downregulating the expression of *PTEN* [64]. miR-21–5p could suppress apoptosis and inflammation by targeting the *FasL* [65]. miR-124–3p could promote M2 macrophage polarization and ameliorate the nerve injury by negative regulating *Ern1* [66]. After being injected via the tail vein, most of the MSCs-EVs could be directed to the lesioned site and incorporated into neurons, and the regulation of the MSCs-EVs miRNA can be a promising strategy for clinical therapy of SCI.

Furthermore, MSC-EVs showed remarkably improvement of cognitive behavioral disorders and reduction of Aβ-amyloid plagues and the levels of inflammatory cytokines in AD rats [67]. MSC-EVs can play a critical role in AD through various regulatory mechanisms. Multiple studies have confirmed that MSC-EVs could effectively reduce extracellular and intracellular Aβ-amyloid levels *in vitro* because they carry an important Aβ degradation enzyme, neprilysin [68], and protect neurons against Aβ-induced oxidative stress and synapse damage [69]. Meanwhile, MSC-EVs can transfer miR-146a into astrocytes to improve cognitive impairment and deliver miR-29c-3p to neurons to inhibit BACE1 expression and activate the Wnt/β-catenin pathway, thereby playing a therapeutic role in AD [67,70] .

3.3. *EVs derived from other stem cells*

Excepting NSC-EVs and MSC-EVs, EVs derived from multiple stem cells have active roles in treating neurological diseases. For example, EVs derived from neural progenitor cells (NPC-EVs) attenuate inflammatory cell recruitment in ischemic stroke by inhibiting the NF-κB signaling pathway, which leads to the reduction of ATP-binding cassette subfamily B member 1 transporter (ABCB1) and matrix metalloproteinase-9 (MMP-9) activation, protects BBB integrity [71], and improves the nerve regeneration and neurological recovery [72]. ESC-EVs also have extraordinary regenerative capacity. In stroke models, ESC-EVs induced a significant increase in regulatory T cells (Tregs) by activating the TGF-β/Smad signaling pathway, thereby attenuating neuroinflammation and long-term neurological deficits and tissue loss [73]. ESC-EVs could also reverse hippocampal neural stem cell senescence to attenuate cognitive impairment by regulating the myelin transcription factor 1 (MYT1)- egl-9 family hypoxia inducible factor 3 (Egln3)- sirtuin 1 (Sirt1) axis through the delivery of SMADs [74]. The potential for treating neurological disorders was also demonstrated in EVs derived from human urine stem cells (USC-EVs). USC-EVs could promote histone deacetylase 6 inhibition by mediating microRNA-26a transfer, thereby reducing neurological deficits and promoting neurogenesis in rats with ischemic stroke [75]. Similarly, in the Rett syndrome (RTT) model, USCs-EVs promoted early neurogenesis, differentiation of NSCs in the lateral subventricular zone, and significantly increased doublecortin positive cells by delivering miR-21–5p to regulate the Eph receptor A4 (EphA4) /TEK axis [76]. In SCI, USC-EVs can activate PI3K/AKT signaling pathway by delivering Angiopoietin Like 3 (ANGPTL3) to promote angiogenesis and enhance motor functional recovery [77].

In conclusion, these studies demonstrated the therapeutic potential of native SC-EVs derived from different kinds of stem cells for neurological disorders. NSC-EVs and MSC-EVs are the main therapeutic subjects for neurological diseases. Similar therapeutic benefits exist between them, involving different combinations of mechanisms to induce neural repair, such as promoting neurogenesis, vascular regeneration, and immunomodulation. However, we do not exactly know which kind of SC-EVs are more suitable for certain neurological diseases. Although some studies have confirmed that NSC-EVs are more effective than MSC-EVs in

treating ischemic stroke, such results may not represent that MSC-EVs are less beneficial than NSC-EVs in the treatment of other neurological disorders. Moreover, due to ethical and source issues of NSCs, MSC-EVs seem to cover a broader application in neurological diseases, such as multiple sclerosis [78], epilepsy [79], PD [80], Duchenne muscular dystrophy [81], autoimmune encephalomyelitis [82], glioma [83], etc. Therefore, over the next few years, the main research direction might be to identify the most suitable SC-EVs for the specific neurological disease and then to accelerate the progression of clinical translation. Currently, two clinical trials registered in NIH investigate the safety and efficacy of allogeneic MSC-EVs for stroke (NCT03384433) and dementia (NCT04388982). However, the therapeutic efficacy of native SC-EVs does not adequately meet current clinical needs due to some critical limitations (poor overall yield [84], low bioactivity [85], weak targeting [86], and short half-life [87]), which have been demonstrated in Fig. 1. As described in subsequent sections, these limitations might be overcome by modifying native SC-EVs using engineering strategies.

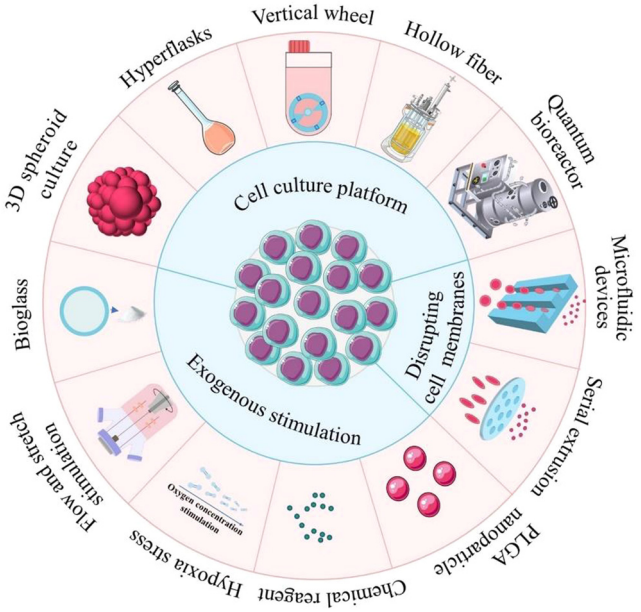

Fig. 3 – Principles of the different methods that can maximize the yield of SC-EVs: There are three ways to increase the yield of SC-EVs. (1) optimizing the cell culture platform (*e.g.*, 3D cultures); (2) increasing the yield of EVs per cell via physical and chemical stimulation; and (3) disrupting the cell membrane to form biological vesicles which are enriched with cell membrane components and intracellular content.

4. SC-EVs' bioengineering

Considering the practical limitations of native SC-EVs for neurological disorders (*e.g.*, yield limitations, low bioactivity, weak targeting, and rapid clearance *in vivo*), researchers are working to develop bioengineering platforms for SC-EVs to enhance their therapeutic effects. Combining the inherent biological advantages of SC-EVs with advanced design approaches could contribute to the establishment of more efficient and powerful therapeutic SC-EVs. Recently, an assortment of bioengineering strategies has been developed to modify SC-EVs. Bioengineered SC-EVs, as a rule, can be generated by two different approaches. One is to modify the SC-EV-producing parent cell, resulting in the subsequent secretion of SC-EVs with a specific cargo molecule of interest inside. The other approach is the direct modification of SC-EVs after their isolation. These efforts have led to the enhancements of SC-EVs' production, biological activity, targeting, and delivery in treating neurological disorders.

4.1. *Increasing production*

For SC-EVs to become a candidate for cell-free therapy as a safer and more cost-effective clinical application, it is essential to achieve large-scale production, especially for source-restricted stem cells. There are three commonly used methods of SC-EVs modification. (i) optimizing the cell culture platform (*e.g.*, three-dimensional (3D) cultures); (ii) increasing the yield of EVs per cell via exogenous stimulation; and (iii) disrupting the cell membrane to form biological vesicles which are enriched with cell membrane components and intracellular content (Fig. 3).

4.1.1. *Changing cell culture platform*

Optimizing the cell culture platform (*e.g.* hyperflasks [88], spinner flasks [84], vertical wheel [89], hollow fiber [90,91] and Quantum bioreactor [92]) to increase the available surface area of cells in a limited space to operationally reduce culture time and cost and achieve maximal EVs yield (Table 2). The culture platform mentioned above allows for multiple consecutive collections of supernatants from extracted EVs compared to conventional cell culture, and enhances nutrient exchange to facilitate SC-EVs production; in addition, the scalability of the culture platform and the ability to monitor and adjust culture parameters allows for better fine-tuning of SC-EVs production. For instance, MSCs in 3D culture with hollow fiber bioreactor increased total SC-EVs' production up to 19.4-fold [90], while tangential flow filtration (TFF) combined with 3D cultured MSCs can further improve the yield of MSC-EVs up to 100-fold [84]. Moreover, compared with traditional flat tissue culture methods, cells cultured in 3D spheres of stem cells are in a non-adherent state, which can increase the yield of EVs, and the smaller the size of the cellular 3D spheres, the more SC-EVs are generated [93].

4.1.2. *Exogenous stimulation*

Exogenous stimulation can also increase SC-EVs' production by upregulating SC-EVs yield per cell. Studies have demonstrated that mechanical stress stimulation can boost the yield of SC-EVs [94,95]. It was recently determined that EVs production was 40.7-fold higher than static conditions by exerting flow stimulation on dental pulp or adipose-derived stem cells in 3D culture, which maybe mediated by YAP mechano-sensitivity [94]. Chemical stimuli can likewise improve the yield of SC-EVs [96]. The influence of oxygen environment on EVs production is uncertain,

Table 2 – Comparison of different production strategies of SC-EVs.

Methods	Cells	Fold increase	Comparison to native SC-EVs	Refs.
Vertical wheel	H-BMSCs, H-ATMSCs, H-UCMSCs	3-fold	Similar Zeta potential, but different morphology protein contents and size distribution.	[89]
Hollow fiber	H-UCMSCs	19.4-fold	Similar morphology, size distribution and protein markers. EVs were more effective.	[90]
Hollow fiber	H-UCMSCs	7.5-fold	Similar morphology, size distribution and protein markers; higher EVs activity.	[91]
TFF+Microcarrier-based 3D culture	H-UCMSCs	140-fold	Similar morphology, size distribution, but distinct proteome.	[84]
Methyldopamine and nore-pinephrine	H-BMSCs	3-fold	Different size distribution, proteome.	[102]
LPS	H-UCMSCs	1.37-fold	Similar size distribution but different protein contents.	[96]
EP4 Antagonist-Elicited	H-BMSCs	2-fold	Similar morphology, size distribution, but distinct proteome.	[101]
Hypoxia stress	M-BMSCs	1.3-fold	Similar morphology, size distribution and protein markers; higher EVs activity.	[97]
Flow or stretch stimulation	H-DPSCs, H-DSCs	40.7-fold	Similar morphology, size distribution, but distinct proteome.	[94]
Mechanical stimulation caused by a rotary cell culture system	H-UCMSCs	4-fold	EV composition, especially lncRNA H19, was upregulated, which enhanced the therapeutic effect.	[95]
3D spheroid culture	H-BMSCs	2 to 3-fold	NA	[93]
Microcarrier-based 3D culture	H-UCMSCs	20-fold	Proteomics analysis showed 357 high-abundance proteins detected in all exosome variants and 21–369 low-abundance proteins unique to an exosome variant.	[84]
PLGA nanoparticle	NA-BMSCs	2-fold	Similar size distribution, different Zeta potential.	[103]
Bioglass	H-BMSCs	2-fold	The ability of promoting ECs vascularization was enhanced	[104]
Serial extrusion (10 μm, 5 μm and 1 μm)	NA-ASCs	30–fold	Similar morphology, size distribution and protein markers.	[105]
Serial extrusion (10 μm, 5 μm and 1 μm)	H-BMSCs	8-fold	Similar size and morphology.	[106]
Centrifugation-based filtration (10 um, 5 um)	M-ESCs	250–fold	Similar morphology, but size distribution and distinct proteome.	[109]
Slicing living cells in microchan nels	M-ESCs	100–fold	NA	[110]

H: human; M: mouse; BMSCs: bone marrow-derived mesenchymal stem cells; ATMSCs: adipose tissue-derived mesenchscells; DPSCs: dental pulp stem cells; ASCs: adipose-derived stromal cells; NA: not available.

stimulation by hypoxic conditions or serum deprivation can also increase EVs yield by 1.3–5 fold [97,98], while serum deprivation combined with hypoxia increased SC-EVs production by 10-fold [99]. Nevertheless, another study showed that oxygen conditions (1, 5 and 18% air oxygen) did not influence the production of SC-EVs [100]. A possible reason for this conflicting result is that SC-EVs yields could significantly be affected by the multiple culture conditions (Oxygen concentration, hypoxia time, cell type, etc.). The prostaglandin E_2 receptor-4 antagonist GW627368X elicited a twofold increase in the production of MSC-EVs compared with dimethyl sulfoxide control [101]. The treatment of combined MSCs with N-methyldopamine and norepinephrine also increased EV production 3-fold without altering the intrinsic regenerative effects of MSC-EVs [102]. Moreover, recent research has linked that the internalization of exogenous nanoparticles and biomaterials can significantly increase SC-EVs numbers. Internalization of positively charged iron oxide nanoparticles (NPs) encapsulated in poly lactid-glycolide acid (PLGA) and polyethyleneimine increase MSC-EVs production about 2.5-fold [103]. 45S5 Bioglass® (BG) is broadly used as a biomaterial since its ion products can create a biofriendly and mild microenvironment for MSCs. MSC-EVs production has multiplied 5-fold with BG ion products related to upregulating the expression of neutral sphingomyelinase-2 (nSMase2) and Rab27a [104].

4.1.3. *Disrupting cell membranes*

Disruption of cell membranes is another strategy to obtain EVs in large quantities. In practice, EVs produced by cell membrane disruption are referred to as 'artificial nanovesicles' (ANVs). The most commonly used method of cell disruption is extrusion. Cells are concentrated in a small amount of 1 × phosphate-buffered saline (1 × PBS) and serially extruded through a filter with micro-sized pores (10, 5, 1 μm), using a mini-extruder. With this technique, the yield of ANVs is ~30 times higher than that of naturally secreted SC-EVs [105,106]. Furthermore, ANVs produced via extrusion had higher antioxidant activity than those loaded by incubation or freeze-thawing methods when loaded with

catalase *in vitro* [107]. However, the safety of this extrusion method needs to be further verified. Evidence that prolonged extrusion can alter the zeta potential of ANVs, which may lead to significant cytotoxicity after EVs were extruded 31 times to load porphyrins [108]. However, another group found no cytotoxicity or physical alteration when EVs were extruded 10 times to load peroxidase [107]. This phenomenon may be related to the kind of stem cells and the type and quantity of cargo loaded.

To further standardize the production of ANVs, researchers introduced a device that forced MSCs through a sterile membrane with 5–10 μm pores by centrifugation to achieve better control compared with extrusion. During this process, MSCs are ruptured by centrifugal force and reassembled into ANVs with a yield 250 times higher than the quantity of naturally secreted EVs [109]. In addition, the mass production of ANVs can be achieved by developing microfluidic systems. The microfluidic system generates plasma membrane fragments by mincing living cell membranes with a silicon nitride (SixNy) microblade (500 nm in thickness), which spontaneously self-assembled into ANVs of ~100–300 nm in diameter. The method showed higher yields (100-fold) than native EVs, and these ANVs can be loaded with exogenous materials to enhance delivery to recipient cells [110].

Despite no uniform consensus regarding optimal technology for SC-EVs production, the continuous innovation of methods to increase SC-EVs yields create conditions for its clinical application. However, the clinical application of SC-EVs is still faced with significant challenges. Firstly, from a pharmaceutical perspective, the diversity of production methods makes it more challenging to control the quality of SC-EVs during the manufacturing process. Secondly, all the subtle variations in parent cell culture conditions (cell passaging, cell inoculation density), time and frequency of EVs collection, different EVs isolation and purification methods, EVs storage conditions will vary the composition of SC-EVs [111–113]. Finally, the uniqueness of EVs size makes the process of sterilization and/or removal of additives more challenging [114]. Therefore, there is a need for continued improvement and innovation in preparing clinical-grade SC-EVs with precise quality control and compliance with manufacturing specifications, ultimately creating a uniform SC-EVs manufacturing process using current Good Manufacturing Practices (GMP). This will facilitate the implementation of clinical trials for neurological disorders and eventually contribute to the transition from stem cell to SC-EVs therapy.

4.2. *Improving bioactivity*

The bioactivity of SC-EVs (including native and engineered EVs) is important for their potential application in nerve injury repair. The biological activity of native SC-EVs is limited by the complex composition and low percentage of active ingredients due to the secretion mechanism of EVs, which affects their practical application as a treatment for neurological diseases. The bioactivity of SC-EVs can be enhanced by modulating certain parameters in SC-EVs-producing parent cells or by modifying the SC-EVs directly.

4.2.1. *Modification of stem cells*

EVs-secreting stem cells can be modulated to improve the bioactivity of SC-EVs by exogenous stimulation during cell culture, such as hypoxia [85,115,116], inflammation [117], cytokines [78,118–120], custom lipid supplements [121], chemical agents [122], collagen scaffolds [123], and herbal tonics [124]. Upon being exposed to EVs collected from MSCs cultured under hypoxia conditions, the microglia phenotype shifted from M1 to M2 phenotype *in vitro*, and the EVs promoted recovery of functional behavior after SCI in a mouse model. These effects were determined to be mediated by miR-216a-5p in MSC-EVs [116]. Immunomodulatory factors can also modulate the bioactivity of SC-EVs. EVs from MSCs, which stimulated with the secretome of lipopolysaccharide (LPS)- or Aβ-activated microglia, were more effective at inhibiting brain inflammation, demyelination, and improving memory and anxiety-like behavioral dysfunction in mice models of neuroinflammation [117]. 3D-SC-EVs may also have better bioactivity compared with conventional culture. 3D-culture presents a more realistic physiological milieu through more native cell-cell and cell-matrix interactions, along with mechanical strain and position [125]. MSC-EVs with 3D cultures showed 7-fold more potent in transferring small interfering RNAs (siRNA) to neurons [84,125].

In addition, stem cells can be genetically engineered to enhance the bioactivity of SC-EVs by transfecting with lentivirus [126–129] and exogenous nucleic acids, especially modulated miRNAs [130,131], miRNA mimics [132–135], miRNA antagonists [63], shRNA [136], lncRNAs [137], and plasmid DNA [138–140], as shown in Table 3. For example, EVs from MSCs which transfected with phosphatase and tensin homolog pseudogene 1 (PTENP1)-short hairpin RNA (shRNA) upregulated miR-19b and miR-21 expression but inhibited PTEN expression suppressed the neuronal apoptosis and facilitated recovery after SCI [136]. Likewise, EVs derived from microRNA-199a-overexpressing MSCs can also inhibit the proliferation, invasion, and migration of glioma cells by down-regulating ArfGAP with GTPase domain, ankyrin repeat, and pH domain 2 (AGAP2) [141].

4.2.2. *Modification of SC-EVs*

Direct modification of SC-EVs is another important approach to enhance their biological activity. Membrane-permeabilizing strategies, such as incubation, sonication, extrusion, freeze-thaw procedures, saponification, electroporation, and chemical transfection, can load exogenous materials into SC-EVs which have been widely used in medicine with varying degrees of success in neurological interventions [142–144]. For example, connective tissue growth factor (CTGF) is known to participate in the initial activation of glial cells at injury sites. CTGF overexpression accelerates the proliferation of the glial scar, which impedes axonal migration and regeneration [145]. Loading CTGF-siRNA into MSC-EVs by electroporation not only effectively inhibited the CTGF expression, but these MSC-EVs thwarted neuronal apoptosis and quenched inflammation, inhibited the formation of the glial scar and promoted the recovery of locomotor function following SCI [142]. Myelin-specific DNA aptamer (LJM-3064 aptamer) is often used as a targeting ligand and therapeutic

Table 3 – Modulation of SC-EV bioactivity for neurological applications.

	Source	Methodology	Animal model	Outcomes	Administration route	Administration time	Dose of SC-EVs	Refs.
Engineering of SC-EV-producing parent cells	M-BMSCs	Hypoxia-preconditioned	Mouse APP/PS1	Ameliorating cognitive decline and regulating inflammatory responses	IV	BIW for 4 months	150 μg/mouse (BCA)	[85]
	H-UCMSCs	Hypoxia-preconditioned	Rat MCAO	Attenuating the post-stroke brain damage and improving the neurological outcome	IV	2h	150 μg/rat (BCA)	[115]
	M-BMSCs	Hypoxia-preconditioned	Mouse SCI	Improving function and shifting microglial M1/M2 polarization	IV	NA	200 μg/mouse (BCA)	[116]
	M-ADSCs	LncGm37494	Rat SCI	Shifting microglial M1/M2 polarization	IV	NA	200 μg/rat (BCA)	[137]
	R-BMSCs	miR-133b	Rat CA	Amplifing neuronal cell survival	IC	NA	200 μg/rat (BCA)	[131]
	H-MSCs	Inflammatory-educated	NA	Enhancing immune regulatory and fighting neuroinflammation.	NA	NA	NA	[117]
	H-BMSCs	IFN-γ	Mouse EAE	Reducing demyelination, decreasing neuroinflammation, and improving functional outcomes	IV	NA	150 μg/mouse (BCA)	[78]
	H–NSCs	IGF-1	Rat SCI	Resisting inflammation and apoptosis and promoting the neuroprotective effects	IV	NA	100 μg/rat (BCA)	[118]
	H-NSCs	IFN-γ	Rat MCAO	Improving function and promoting the neuroprotective effects	SI	24 h	4E9 part/rat (NTA)	[120]
	H-BMSCs	IL-1	Mouse SE	Reducing the inflammatory response and improving the cognitive performance	SI	NA	7.5 μg/mouse (BCA)	[119]
	HFM-MSCs	Formulated lipid supplementation (Refeed®)	NA	Increasing cell migratory capability	NA	NA	NA	[121]
	M-BMSCs	Hydrogen sulfide-modified	Mouse HI	Having neuroprotective and anti-inflammatory effects and improving HI-induced cognitive impairments	IC	24 h	100 μg/mouse (BCA)	[122]
	H-MSCs	3D collagen scaffolds	Rat TBI	Improving functional recovery, promoting endogenous angiogenesis and neurogenesis, and reducing neuroinflammation	IV	NA	100 μg/rat (BCA)	[123]
	R-MSCs	Buyang Huanwu decoction	Rat MCAO	Elevating angiogenesis in rat brain	IV	NA	400 μg/kg/mouse (BCA)	[124]
	R-BMSCs	miR-21	Rat SCI	Inhibiting apoptosis	IV	24 h	1E5 ug/rat (BCA)	[130]
	R-BMSCs	miR-133b	Rat SCI	Promoting the regeneration of axons and improving the recovery of hindlimb locomotor function	IV	24 h	100 μg/rat (BCA)	[132]
	H-BMSCs	miR-26a	Rat Depression	Restraining apoptosis in hippocampal tissues and boosting hippocampal neuron proliferation	IV	NA	100 μg/rat (BCA)	[134]
	R-BMSCs	miR-126	Rat SCI	Promoting angiogenesis and neurogenesis, and attenuating apoptosis	IV	30 min	100 μg/rat (BCA)	[135]
	R-BMSCs	miR-17–92	Rat MCAO	Increasing neural plasticity and functional recovery	IV	24h	100 μg/rat (BCA)	[138]
	R-ADSCs	miR-181b	NA	Promoting the angiogenesis	NA	NA	NA	[139]
	R-BMSCs	miR-124	Rat TBI	Promoting M2 polarization of microglia and improving hippocampal neurogenesis and functional recovery	IV	24h	100 μg/rat (BCA)	[140]
	R-BMSCs	miR-133b	Rat MCAO	Improving neural plasticity and functional recovery	IV	24h	100 μg/rat (BCA)	[126]
	R-BMSCs	miRNA-29b	Rat SCI	Improving functional recovery	IV	1h	100 μg/rat (BCA)	[127]
	R-BMSCs	miR-25	Rat SCI	Enhancing neuroprotection	IT	1 d before SCI.		[128]
	R-BMSCs	miR-214	Rat CPB and DHCA	Conducting powerful neuroprotection	SI	1 d before CPB and DHCA	30 ug/rat (BCA)	[129]
Direct modification of SC-EVs	M-BMSCs	miR-193b-3p electroporation	Mouse SAH	Alleviating neurobehavioral impairments and neuroinflammation	NA	NA	NA	[133]
	R-BMSCs	CTGF-siRNA electroporation	Rat SCI	Inhibiting the formation of glial scar and ameliorating behavioral dysfunction	IV	QD for 5 d	200 ug/rat (BCA)	[142]
	M-BMSCs	Covalent conjugation	Mouse EAE	Suppressing inflammatory response and lowering demyelination lesion region	IV	1, 3, 6,12, 15, 18 d	200 μg/mouse (BCA)	[146]

H: human; R: rat; M: mouse; BMSCs: bone marrow-derived mesenchymal stem cells; UCMSCs: Umbilical cord mesenchymal stem cells; ADSCs: adipose-derived stem cells; HFM: human fetal membranes; CA: cardiac arrest; EAE: experimental autoimmune encephalomyelitis; SE: status epilepticus; MCAO: middle cerebral artery occlusion; HI: hypoxic-ischemic; SAH: subarachnoid hemorrhage; DHCA: deep hypothermic circulatory arrest; CPB: cardiopulmonary bypass; IFN-γ: interferon-gamma; IGF-1: insulin-like growth factor 1; IL-1: interleukin 1; shRNA: short hairpin RNA; IV: intravenous; IC: intracardial injection; IT: intrathecal injection; SI: stereotactic system for intracerebral delivery; part: particles; BCA: bicinchoninic acid; NTA: nanoparticle tracking analysis; NA: not available.

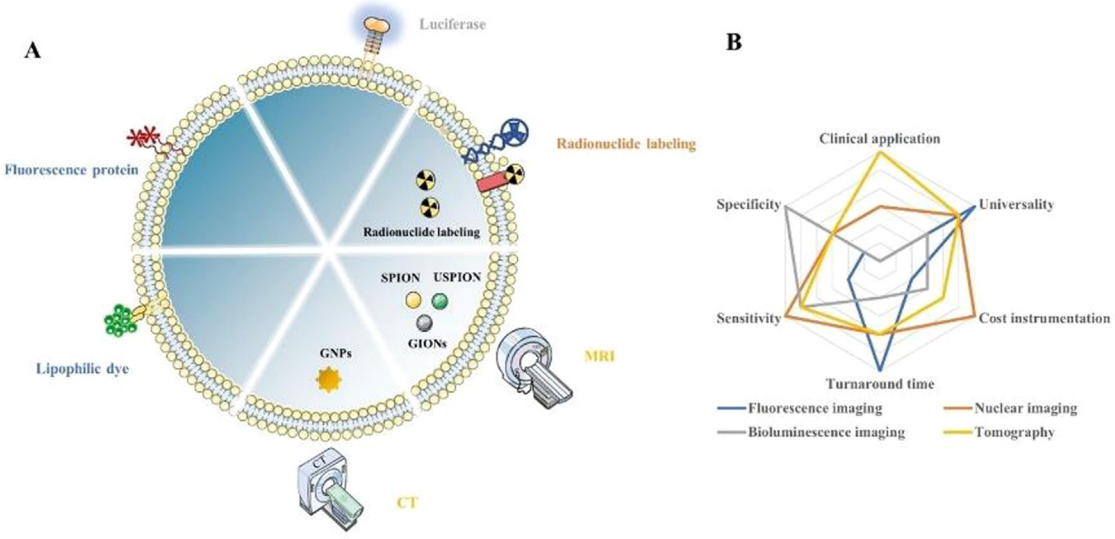

Fig. 4 – Different strategies for labeling of extracellular vesicles: (A)Reported strategies for tracking SC-EVs can be grouped into the following imaging techniques: fluorescence, bioluminescence, nuclear, and tomography. (B)Each technique has its own unique advantages. MRI: magnetic resonance imaging, CT: computed tomography, GNPs: Gold nanoparticles, GIONs: Gold Iron Oxide Nanoparticles, SPION: Superparamagnetic iron oxide nanoparticles, USPION: ultrasmall Superparamagnetic Iron Oxide Nanoparticles.

agent because it shows a considerable affinity for myelin and remyelination induction. The carboxylic acid-functionalized LJM-3064 aptamer was covalently conjugated to the amine-containing molecules on the EV surface through 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide (EDC/NHS) chemistry. The aptamer-EVs bioconjugate could promote cell proliferation *in vitro*, inhibit inflammatory response, and reduce the demyelination lesion region in a rodent model [146].

These preclinical studies suggest that changing the biological activity of SC-EVs greatly enhances the efficacy of complex neurological disease treatment. These results provide an important experimental reference for the design of precision medicine studies, especially in selecting treatment strategies in the follow-up clinical trials. But, further investigation for large clinical trials is needed to clarify the efficacy and safety before its clinical application.

4.3. *Changing biodistribution and targeting*

The major challenges to the clinical application of SC-EVs involves the conundrum of the unspecific distribution of SC-EVs after *in vivo* administration and whether they can reach the target site accurately. Recently, efforts to analyze EVs biodistribution and targeting have become an essential step in preclinical studies and represent significant maturation of the EVs field.

4.3.1. *Biomedical imaging strategies*

Biodistribution is a critical parameter for SC-EVs for therapeutic purposes, as it allows for estimation of on-target as well as off-target effects of SC-EVs. The study of biodistribution of SC-EVs relies on the development of bioimaging technology, which could assess SC-EVs accumulation in the intended target tissue and non-desired sites, confirm acute and long-term dynamic changes and desired function, determine pharmacokinetics and toxicity, and assess interaction between SC-EVs and host cells in a non-invasive manner. Especially for brain disease, the visualization results of SC-EVs reaching the damaged brain area through the BBB and the brain's complex structure are essential. Therefore, non-invasive biomedical imaging strategies for EVs tracking in neurological disorders have been extensively explored in recent years, such as fluorescence, bioluminescence, nuclear, and tomography (Fig. 4). Fluorescent proteins are suitable for tracing *in vitro* [147], while fluorescent dyes are ideal for short-term tracing *in vivo* [148,149]. Bioluminescence is a highly sensitive, high-contrast tracking method with no background fluorescence virtually in mammals [150]. Nuclear imaging offers exceptionally high sensitivity and ultra-high tissue penetration and is the most accurate method for tracking EVs for quantitative biodistribution studies, including pharmacokinetic analysis [147]. Tomography is considered an efficient and non-invasive imaging technique and is convenient for clinical studies, which is suitable for longitudinal imaging of EVs over time [151]. Each imaging modality has its own advantages, and the appropriate tracking method can be tailored according to the purpose of SC-EVs tracking. Likewise, the tracking of SC-EVs is also critical for the optimization of treatment protocols.

Based on the development of numerous tracing methods, the biodistribution of EVs just gets to be elucidated. Current studies have shown that the distribution of exogenous EVs

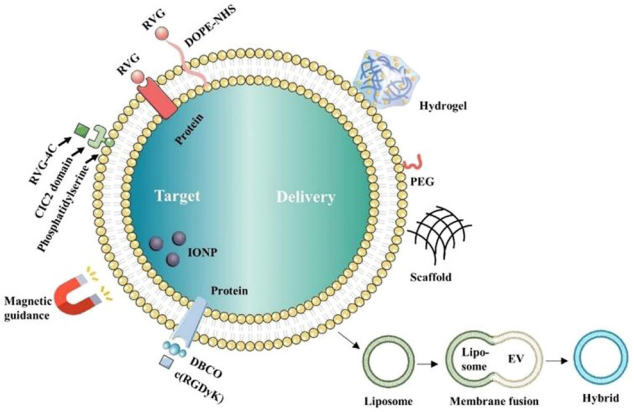

Fig. 5 – Engineering methods for enhanced SC-EVs targeting and delivery: The biological molecular cargo can be loaded into SC-EVs through specific strategies. IONP: iron oxide nanoparticles, RVG: Rabies virus glycoprotein, C1C2: C1 and C2 domains of lactadherin, DOPE-NHS: dioleoylphosphatidylethanolamine N-hydroxysuccinimide, DBCO: dibenzocyclooctyne, c(RGDyK): cyclo(Arg-Gly-Asp-D-Tyr-Lys).

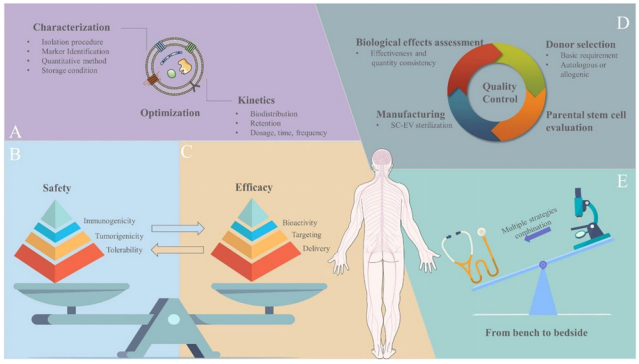

Fig. 6 – Challenges for SC-EV from bench to bedside applications. Challenges remain in the use of SC-EVs before their establishment as an effective clinical therapeutic tool, including but not limited to (A) standardization of preparation and generalization of dosing regimens, (B and C) balancing safety and efficacy, and (D) ensuring precise quality control for SC-EVs; (E) Multiple strategies should combinate from bench to bedside.

administered intravenous is mainly concentrated in highly-perfused organs with well-developed phagocytic systems (*e.g.*, liver, lungs, kidneys, and spleen), regardless of the origin of EVs or the recipient species [152]. The biodistribution of SC-EVs is influenced by many factors, such as origin and physicochemical properties, the route and regimen of administration, and the kinds of animal models [151,153]. For example, NSC-EVs have better brain targeting than MSC-EVs in stroke mouse model [27]. And, intra-arterial and intranasal administration is superior to intravenous transplantation for EVs accumulation in the brain, which is critical for treating neurological disorders [154]. This kind of non-specific accumulation of SC-EVs might increase adverse effects and create uncertainty for future clinical treatment. Moreover, although SC-EVs can cross the BBB more easily than stem cells, the efficiency in delivery of SC-EVs still needs to be improved. To retain a larger amount of SC-EVs in the brain, SC-EVs have been engineered to target the lesion site specifically in the brain to enhance the accumulation and improve more precise therapeutic effects (Fig. 5).

4.3.2. *Targeting strategies*

Several modifications of parental stem cell methods have been developed to target the brain. It has been suggested that lysosome-associated membrane glycoprotein 2b (Lamp2b) is EV's membrane protein, and Rabies virus glycoprotein (RVG) is a specific neuron peptide which can explicitly bound to acetylcholine receptors [155]. MSCs transfected with plasmids encoding Lamp2b-RVG could highly express RVG on the membrane, thus, targeting the ischemic zone after stroke to improve brain injury by promoting neural progenitor cells to acquire a neuronal phenotype at the site of infarction [86]. At the same time, glycosylation modification of Lamp2b could avoid the degradation of Lamp2b and improve the stability and expression of the peptide [156]. MSCs treated with iron oxide nanoparticles (IONP) could secrete IONP-rich EVs (IONP-EVs), which carried more therapeutic growth factors than untreated MSC-EVs. Under exogenous magnetic guidance, IONP-EVs can effectively accumulate in the damaged spinal cord compared to untreated human MSC-EVs [157]. Furthermore, the fusion of different cell membranes can also improve EVs targeting. The macrophage membrane-fused exosome-mimetic nanovesicles (MF-NVs) fabricated by extruding macrophage membrane and MSCs simultaneously have more targeting molecules to injured sites in SCI mouse than normal MSC-EVs [158].

Direct modification of SC-EVs allows more precise physical or chemical modification based on EVs surface proteins, which can target the exact site of the injured brain. For instance, 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine-N-hydroxysuccinimide (DOPE-NHS) is a hydrophobic molecule and can be inserted directly into the membrane of EVs. MSC-EVs conjugate with neuron-targeting peptides RVG through the DOPE-NHS linker have better targeting of cortex and hippocampus in AD transgenic mice [143], and could mitigate the inflammatory response and improve learning and memory function meanwhile. Another group generated a recombinant fusion protein by fusing the arginine-glycine-aspartic acid (RGD)—4C peptide (ACDCRGDCFC) to the phosphatidylserine-binding domain of lactadherin (C1C2) (RGD-C1C2 protein), which could bind to integrin $\alpha_v\beta_3$ on the vascular endothelial cells in the ischemic brain. This RGD-C1C2 protein can readily self-assemble into the SC-EVs' membrane, and target lesioned areas of ischemia after intravenous administration and strongly inhibit the inflammatory response [144]. The cyclo(Arg-Gly-Asp-D-Tyr-Lys) [c(RGDyK)] peptide also has a high affinity for integrin $\alpha_v\beta_3$, which could be bounded to SC-EVs by simple, rapid and bioorthogonal copper-free azide alkyne cycloaddition (click chemistry) [159]. In addition, curcumin [159] and cholesterol-modified miR-210 [160] have also been shown to increase the targeting of RGD-EVs.

In conclusion, engineered design can improve the brain targeting of SC-EVs, enhance therapeutic efficiency and diminish the off-target side effects while encapsulating therapeutic cargo. When administering SC-EVs, the modification method of SC-EVs must be carefully selected based on the tissue tropism and SC-EVs' biodistribution.

4.4. *Extending half-life*

Optimal therapeutic results from EVs administration require high concentration and continuous release of EVs in the lesion area. Although it is widely believed that SC-EVs can evade the immune system due to their biofilm structure compared with stem cells, exogenous SC-EVs can be rapidly cleared from the circulation by liver and spleen macrophages when administered systemically. The clearance of native EVs from the circulation often happened within 10 min after intravenous injection in mice [161]. So, efforts to strengthen the circulation time of SC-EVs are important. PEGylation is a common method to extend the half-life of EVs in circulation. Coating EVs with polyethylene glycol (PEG) can reduce the recognition by the immune system [161]. Fusion of EVs with PEGylated liposomes alters their membrane properties and achieves "bio camouflage", thereby reducing their interaction with macrophages. Furthermore, this fusion strategy enables efficient EVs loading, which can contribute to the development of EVs drugs with adaptive activity [87]. Some membrane proteins also have immune-evasion properties. CD47 is the "Self-tagged" transmembrane protein and is expressed in MSCs [162]. The extracellular domain of CD47 could bind with the signal-regulated protein-alpha (SIRPα) on the macrophage membrane and activate the "do not eat me" signal, thereby evading phagocytosis by macrophage [163].

Furthermore, apart from enhancing the cycle time of SC-EVs, another alternative approach is to extend the action duration of SC-EVs. Incorporating therapeutic EVs into various biomaterials is a common approach to achieving precise targeting and sustaining delivery in the brain while minimizing the potential adverse effects of non-targeted peripheral delivery methods [164] (Fig. 5). Many researches have been conducted to incorporate EVs into biomaterials (*e.g.*, hydrogels or scaffolds). Hydrogels are a commonly employed delivery material that has shown promising results. For example, the PPFLMLLKGSTR peptide-modified HA hydrogel allows for continuous MSC-EVs release over 11 d in the damaged site of the spinal cord [39]. At the same time, the structural and functional integrity of engineered MSC-EVs in photo-crosslinkable alginate hydrogels containing fibronectin remain unchanged for a long time after delivery when applied to a calvarial defect model [165]. MSC-EVs encapsulated by blending PEG hydrogels through a rapid click-through reaction can release MSC-EVs extendedly for up to 1 month [166]. Excepting the hydrogel material, the structural support of porous scaffolds is essential for the slow release of the SC-EVs. A collagen scaffold constructed with a novel bispecific peptide (BSP) can effectively retain MSC-EVs within the scaffold, avoiding flushing body fluids and extending the half-life of MSC-EVs [167].

5. Ongoing challenges and future perspectives

Over the last few decades, substantial progress has been made toward understanding the biological properties and potential therapeutic applications in neurological disorders of SC-EVs. Now, we can better understand the composition of SC-EVs, their roles in intercellular communication within the same or different tissues *in vivo*, and some of the mechanisms that influence the pathological development of neurological disorders. As a result of these advances, several clinical trials of SC-EVs as therapy have been registered for treating neurological disorders (NCT04388982, NCT04202783, NCT03384433, NCT04202770). The list is relatively small at the moment but will undoubtedly grow by orders of magnitude in the coming years. In addition, engineering methods can enhance the therapeutic potential of SC-EVs to increase yield, bioactivity, stability, and targeting to control the composition of their cargo, improve their transport and delivery *in vivo*, and control the space and time of their release at the target sites through the use of various biomaterials. Moreover, novel bionanomaterials, such as dendrimers, liposomes, and two-dimensional inorganic nanosheets, have emerged to provide viable approaches for treating neurological diseases. However, these bionanomaterials have huge heterogeneity, and the exact mechanisms of their interaction with the complex ecological environment *in vivo* are not fully understood through artificial fabrication, which may hinder their further clinical translation. Moreover, since SC-EVs are derived from living stem cell entities, they have a complex combination of contents and precise ratios, which are difficult for biomimetic delivery vehicles to simulate *in vitro*. In the future, engineered SC-EVs will undoubtedly represent a promising platform for applying cell-free, powerful and customizable therapies to treat neurological diseases.

Several challenges remain to enable the translation of SC-EVs and engineered SC-EVs based neurotherapy to clinical practice (Fig. 6). Firstly, the preparation and quality control system of SC-EVs conforming to GMP level needs to be established to ensure the consistency and reproducibility of SC-EVs products and the stability of SC-EVs storage and transportation. On this basis, reduce the implementation cost of SC-EVs in treating neurological diseases. Secondly, the exact therapeutic mechanisms of SC-EVs for neurological diseases need to be clarified and select the types of SC-EVs rationally according to the etiology and pathogenesis. Even we can try to use the joint application of different SC-EVs when adequate considering the interactions of different SC-EVs. Thirdly, SC-EVs need to address the challenges inherent in delivering complex therapeutic molecules to specific tissues at specific times and concentrations and minimizing off-target effects. Finally, the immunogenicity and toxicity of allogeneic SC-EVs remain important risk factors for adverse reactions. Therefore, safety issues related to the possible side effects of SC-EVs must be considered.

However, current challenges and concerns should not obscure the tremendous therapeutic potential of SC-EVs. The advancement of SC-EVs in the field of the neurogenesis undoubtedly establishes a new paradigm for treating

neurological diseases, paving the way for clinical repairing nerve damage one day.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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