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REVIEW

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Neuroangiogenesis potential of mesenchymal stem cell extracellular vesicles in ischemic stroke conditions

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Abstract

Ischemic stroke (IS) is a life-threatening condition in humans with high morbidity and mortality rates in developing and industrialized countries. The occlusion of blood-supporting vessels by thrombus or emboli can contribute to massive brain cell damage, neurological deficits, and long-term disability, and in more severe conditions, results in sudden death. Current therapeutic strategies, along with rehabilitation, in part, but not completely, can restore the integrity and function of the brain. These features necessitate the advent of novel therapeutic protocols for yielding better regenerative outcomes in IS patients. In past decades, the discovery of stem cells and byproducts has led to promising results in in vitro settings and pre-clinical studies. Extracellular vesicles (EVs) are nano-sized particles released from various cell types, for instance, mesenchymal stem cells (MSCs), with certain signaling biomolecules, growth factors, and cytokines involved in cell-to-cell communication. A great plethora of studies have pointed to the fact that EVs with specific cargo can distribute easily in different parts of the body, making them appropriate therapeutics under different pathological conditions. The current review articles aimed to highlight the neuroangiogenesis properties of MSC EVs in IS conditions. How and by which mechanisms MSC EVs can orchestrate the process of nervous system regeneration is at the center of debate. We think that the current article can help us better understand MSC EVs' function in the restoration of brain function under IS conditions in terms of neurogenesis and angiogenesis.

Keywords Ischemic stroke, Extracellular vesicles, Neuroangiogenesis, Therapeutics, Brain, Regeneration

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Introduction

IS is a leading cause of long-term neurologic deficits, such as cognitive disabilities, and in more severe cases, can contribute to human death. Based on the released statistics, nearly 34% of afflicted individuals die within one year after the occurrence of IS [1]. In IS patients, certain brain functions such as motor sensors, memory capacity, speech, and senses are irreversibly impaired [2, 3]. Thrombolysis-based medications, such as intravenous (IV) administration of recombinant tissue plasminogen activator (rtPA) along with thrombectomy, are the main conventional IS treatment to resolve clots and restore blood perfusion into the ischemic areas [4, 5]. However, applying rtPA is possible about 4.5 h post-IS to reduce side effects such as hemorrhage and intracerebral hemorrhage (ICH) [6, 7]. Besides, many IS patients cannot reach the hospitals in the golden time to receive the therapeutics, reducing rtPA efficacy. Factors such as age, genetic makeup, comorbidities, extent of ischemic changes, and clots vary the therapeutic effectiveness of rtPA [8]. Data confirmed that blood flow re-establishment in rtPA-administered patients can also increase the possibility of oxidative stress and pro-inflammatory response [9].

Along with these statements, the emergence and development of de novo medications and protocols are mandatory for obtaining higher therapeutic efficacy and longer durability. In this regard, stem cells and their byproducts are novel and valuable platforms for alleviating various pathologies in humans and animals [10, 11]. It has been indicated that rapid and in-time blood re-establishment partially reduces the pathological changes after IS [12]. Therefore, angiogenesis-based strategies are charming platforms in IS patients [13]. Stem cells can foster nascent blood formation via direct differentiation into vascular cells, or produce angiocrine with arrays of pro-angiogenesis factors, leading to enhanced blood perfusion [14]. Despite the significant impact of stem cells in revascularization-based therapies, the possibility of immune rejection, activation of alloreactive immune cells, difficulties associated with isolation, purification, etc., has caused biologists and clinicians to use stem cell secretome [15]. The existence of harsh microenvironments such as severe hypoxia and active inflammation can diminish transplanted stem cell viability and retention time at the site of injection [16, 17]. Besides, the systemic administration of stem cells causes noticeable off-target efficiency due to massive vascular beds in specific organs such as hepatic tissue, pulmonary tract, spleen, etc. On the other hand, a prominent inflammatory response at the stroke site and involvement of adjacent vascular cells, especially in small-sized units, can increase the possibility of occlusion and reduction of cerebral blood flow [18]. In recent years, stem cell biologists and clinicians have shifted their interest toward stem cell secretome and soluble components such as EVs. These particles harbor several concentrated signaling biomolecules and cytokines and can distribute easily in all biofluids. Specifically, EVs maintain a communication bridge and chemical language between the juxtaposed cells or cells in remote sites in a paracrine manner [19, 20]. Interestingly, the absence or low levels of major histocompatibility complex (MHC) class I and II antigens potentiate EVs to regulate the activity of NK cells, T lymphocyte proliferation, and immune system responses [21, 22]. Along with these features, ability to distribute, and cross the biological barriers, lack of dose-dependent toxicity, and possibility of long-term repetitive injection are some advantages of EVs compared to direct stem cells use in clinical settings [10, 23]. Besides, EVs do exhibit tumorigenic potential and can be produced in large scales without the critical ethical issues [10].

In this review, we summarized the recent findings related to the application of EVs, mainly exosomes (Exos), under ischemic conditions following IS. How and by which mechanisms EVs can orchestrate neuroangiogenesis is at the center of debate. Novel and recent strategies in the application of EVs in terms of IS were also discussed.

Pathophysiology of IS

Different factors, such as cardiac embolism, local vasculitis, artery-to-artery embolism, infections, etc., are attributed to the occurrence of IS [24]. In response to IS, severe hypoxic conditions or ischemia are mighty due to the sudden decrease of O_2, glucose, and ATP, leading to bioenergetic stress conditions in neurons [25]. Along with the progression of ischemia-related pathologies, brain malfunction and deficits follow, especially in blood vessel-nourishing areas [26]. The neurotoxicity is exacerbated by simultaneous cation and anion imbalance, in which abnormal Na^+, K^+, and Ca^{2+} levels result in glutamate release. Of note, the attachment of glutamate to its cognate receptors, namely N-methyl-D-aspartate receptors (NMDARs), leads to subsequent neuron toxicity and death [27, 28]. From morphological aspects, the neurons juxtaposed to the ischemic core are characterized by axons and soma disappearance, while interface penumbra neurons are relatively active with some cytopathies such as Nissl bodies disintegration, swelling, etc. [29, 30]. Following IS, microglia acquire an inflammatory phenotype (M1 type) and release several cytokines and free radicals, such as reactive oxygen species (ROS), to scavenge the necrotic neuron remnants (Fig. 1) [31]. The uncontrolled and excessive ROS and oxidant contents cause progressive damage to the brain parenchyma, recruitment of immune cells, and delivery of digesting

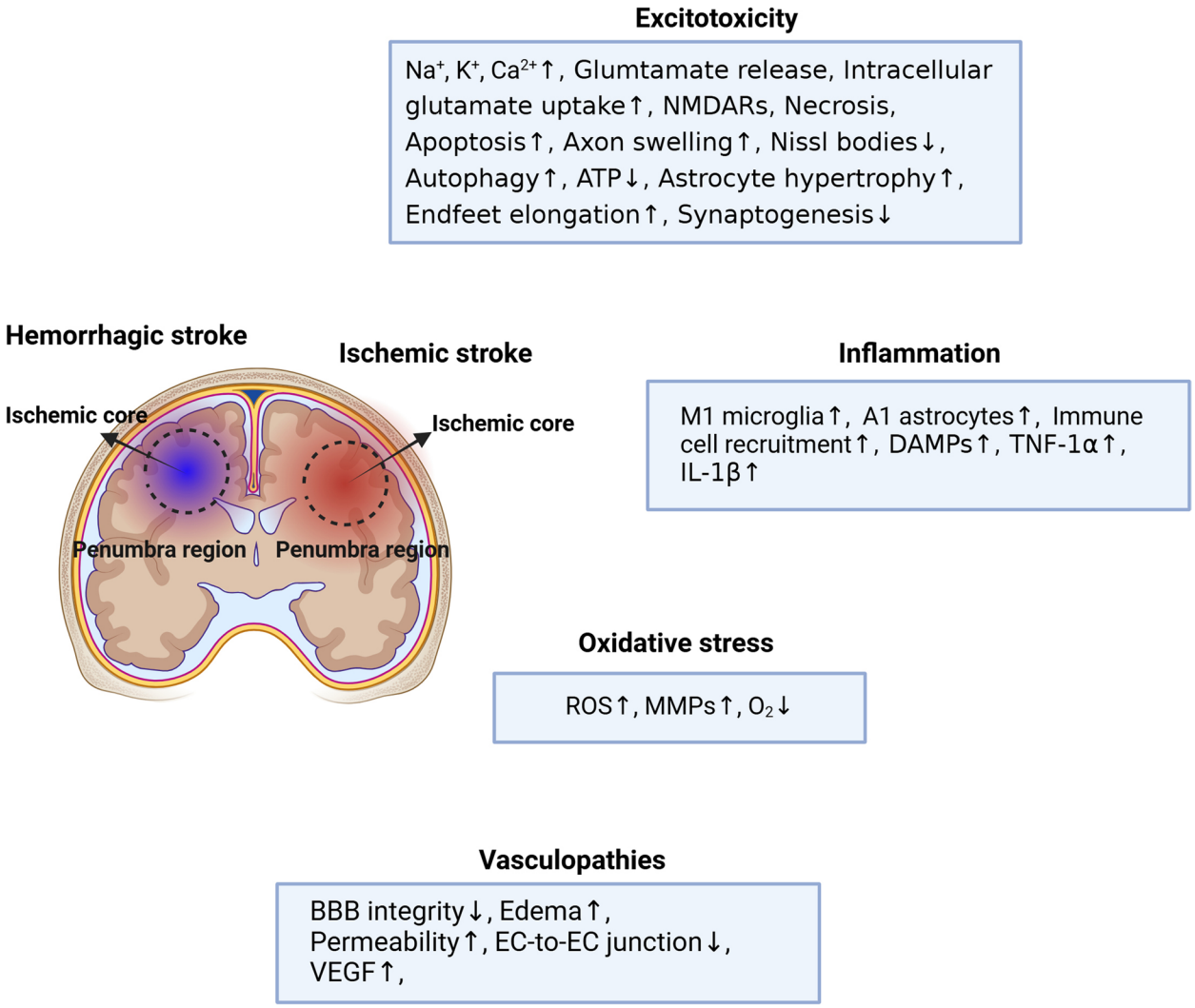

Fig. 1 Different damaging mechanisms lead to the progression of pathologies after IS and hemorrhagic stroke in the brain parenchyma. Created by BioRender's web-based software

enzymes such as metalloproteinases (MMPs), resulting in aberrant brain tissue remodeling [32]. Besides, ROS leakage and glutamate excitotoxicity in adjacent healthy and relatively injured neurons can contribute to mitochondrial dysfunctions and activation of both programmed (apoptosis, autophagy, etc.) or unprogrammed (necrosis) cell death mechanisms [33]. It seems that autophagy and apoptosis are dominant cell death mechanisms in penumbra neurons due to their access to relatively minimal levels of O_2 and glucose. By contrast, excessive necrotic changes are remarkable cellular changes in neurons of the ischemic core [34–36]. In the presence of glutamate, the activation of NMDARs with the GluN2B subunit provokes the phosphatase and tensin homolog (PTEN) signaling pathway and thereby stimulates cell death [37]. The release of damage-associated molecular pattern molecules (DAMPs) from injured neurons recruits and triggers immune cells [38]. Of course, the up-regulation of several adhesion molecules in the luminal surface of microvascular endothelial cells (ECs) helps the tethering of circulatory immune cells and extravasation into the brain parenchyma [39]. By that time, certain immune cell types such as neutrophils, T lymphocytes, and activated microglia accumulate in ischemic sites with a concomitant increase of local interleukin 1-β (IL-1β), IL-6, tumor necrosis factor-alpha (TNF-α), etc. Even though the compensatory cell reactions in brain astrocytes cannot prevent the extension of ischemia-related pathologies. For instance,

astrocytes'morphological adaptions such as hypertrophy, and elongation of endfeets within minutes after IS. In progressive cases, further activation and proliferation of astrocytes increase the possibility of glial scar formation in collaboration with microglia, ECs, and fibroblasts to fill the damaged regions after scavenging neuron remnants. The extension and condensation of scar units can prohibit neuronal regrowth and axonal extensions [40–42]. Due to the existence of chronic tissue remodeling, the glial scar is a bottleneck limiting the efficiency of therapeutic protocols in IS patients [43]. Further release of ATP and CpG-rich DNA frustrates the local microglia to produce a large amount of pro-inflammatory cytokines [44]. The occurrence of IS can lead to the loss of neurons after necrotic changes, and the release of these cytokines by microglia and recruited immune cells distorts the physiology of other neurons via the regulation of metabolism and protein synthesis, leading to impaired synaptogenesis and postponed neurologic recovery [45]. The extension of ischemic foci response following IS can affect the barrier function and integrity of the blood–brain-barrier (BBB), resulting in inward leakage of blood components into the brain parenchyma [46].

BBB is considered a selective neurovascular barrier that separates the brain parenchyma from blood components. The BBB barrier is composed of a multiplicity of cells, such as ECs, pericytes, and glia (astrocytes, microglia, and oligodendrocytes) with a supporting basal layer between both vascular cells and neuronal cells [47, 48]. In response to local cytokine levels following ischemic changes, the physical connection between the BBB cells weakens, coinciding with the recruitment of immune cells from the blood side to the brain [49]. Of course, in juxtaposed microvascular units to ischemic foci, BBB disintegration occurs in distinct and separate phases, 6–12 h, 2–4 days post-IS due to active neuroinflammation [50]. The local and limited angiogenesis response is promoted by BBB ECs, causing permeability and edema from 1 week to 1 month post-IS [51–53]. Along with these changes, astrocytes are stimulated and acquire an inflammatory phenotype (A1 type) with the potential to produce and release various inflammatory cytokines and proteases, *i.e.*, MMPs, resulting in further BBB leakage [54]. Like astrocytes, the number of inflammatory microglia, M1 type, increases following IS. These cells can also release inflammatory cytokines such as IL-6, IL-1β, and TNF-α, leading to the loosening of the EC-to-EC junction [55]. The production of VEGF and other angiogenesis-related factors following IS and hypoxia exacerbate BBB structure disruption [56]. The continuity of hypoxia or massive ischemia stimulates the production of angiogenesis factors to afford blood supplementation into the injured sites. Therefore, the density of these factors, along with the progression of brain parenchyma injury and accumulation of inflammatory factors, intensifies the BBB interface loss (Fig. 2) [56].

EVs biogenesis

EVs, nano-sized and membrane-bound vehicles, are produced by almost all eukaryotic cells to maintain cell-to-cell paracrine activity and synchronized cell behavior via the transfer of various signaling compounds between the donor and recipient cells [57]. Based on size and origin, EVs are three main types: Exos, microvesicles (MVs), and apoptotic bodies, and play critical roles in both physiological and pathological conditions [58]. Apoptotic vesicles range between 1000–5000 nm and are produced in cells that undergo apoptotic changes via cell membrane blebbing. These EV types are eliminated by neighboring cells or local immune cells without the stimulation of inflammation [59]. Microvesicles are generated by the budding of cell membranes with an average size between 100–1000 nm, with several concentrated cytokines [60]. In the latter type, Exos ranging between 40–250 nm are produced via the activity of the endosomal system with an intricate molecular mechanism [61]. This system contains early endosomes that encompass internalized Exos from other cells. With the maturation of early endosomes to late endosomes, numerous intraluminal vesicles (ILVs) are generated via the invagination of endosome membrane and involvement of several molecular machineries such as tetraspanins, ESCRT complex, and accessory proteins [62]. Irrespective of factor types, the function of endosomal machinery leads to the sequestration and enrichment of targeted compounds into the ILVs [63]. In the next step, late endosomes mature into multivesicular bodies (MVBs) with numerous ILVs containing several factors from different subcellular compartments (Fig. 3) [64]. MVBs can be guided toward lysosomal degradation or fuse cell membranes to release their content into the extracellular matrix (ECM), where ILVs are hereafter known as Exos [65].

Exos easily distribute in biofluids and transfer the cargo to neighboring cells or cells in the remote site [66, 67]. Even, the bidirectional transfer of Exos has been documented through the BBB from the blood side to the cerebrospinal fluid and vice versa [68, 69]. It is possible that Exos can cross the EC barrier to reach the brain parenchyma or be retained within the brain microvascular cells. Exos primarily use transcytosis to cross the BBB via engaging specific glycoproteins, which are recruited after the stimulation of EC receptors [70]. During the transcellular entry, the activation of endocytosis is followed by the formation of MVBs, and subsequent exocytosis at the abluminal surface [71]

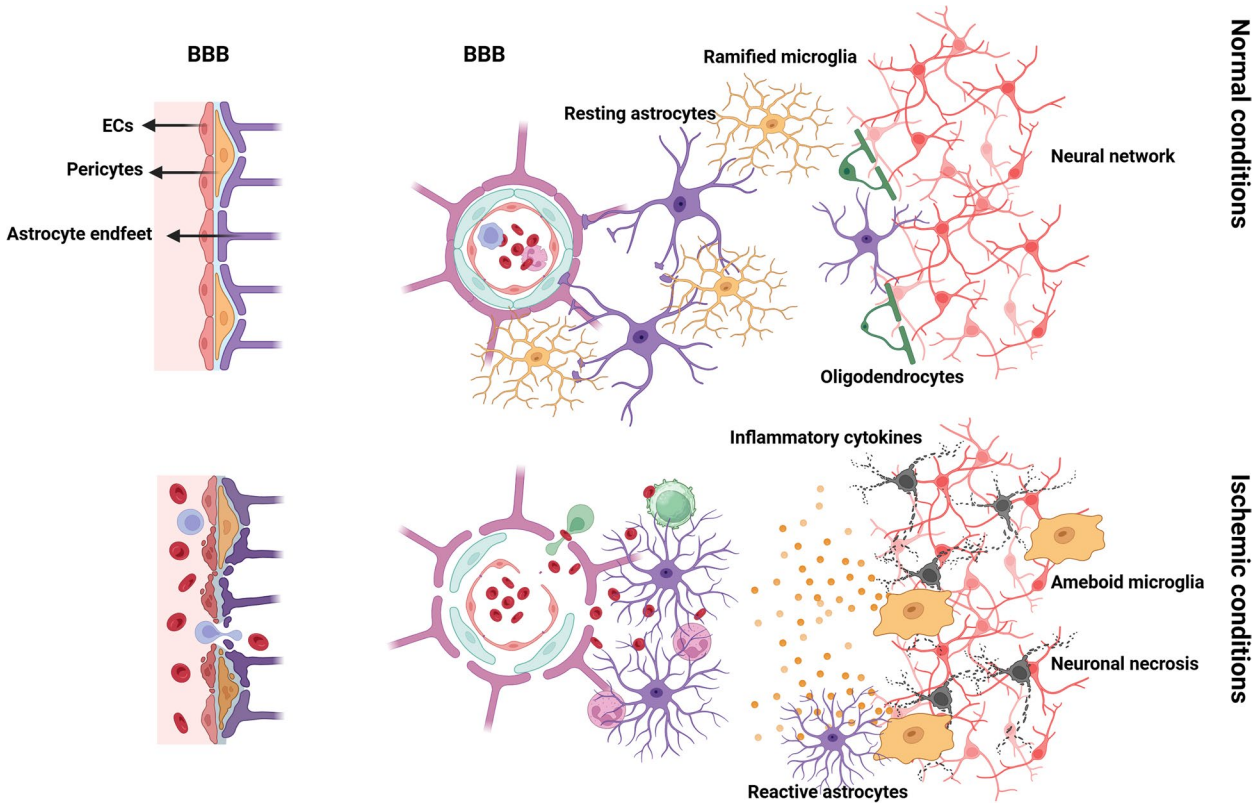

Fig. 2 The occurrence of ischemic conditions can contribute to the loss of BBB integrity and recruitment of systemic immune cells into the brain parenchyma. After the induction of neuronal injury and activation of glia cells, the local density of pro-inflammatory cytokines influences the BBB structure in the proximity, resulting in the loosening of EC-to-EC connections, EC-to-pericyte intercommunication, and loss of astrocyte endfeet. These conditions increase the possibility of extravasation and activation of local glia cells. Created by BioRender's web-based software

(Fig. 4). The ability of Exos to cross the healthy BBB via paracellular route is not common because of tight junctions restricting the passage of large-sized molecules unless the occurrence of pathological conditions and inflammatory responses can loosen the EC-to-EC connection and facilitate the bilateral transfer of Exos [72]. Emerging data have highlighted the significant role of EVs in neuron-to-neuron and neuron-to-glia, neuron-to-EC interactions within the brain parenchyma [73, 74]. Considering the inherent capacity to harbor biological macromolecules such as proteins, lipids, and genetic materials makes them valid and magical therapeutic tools in terms of IS [75]. Besides their therapeutic roles, the progression of IS-related tissue injury is also promoted by these biological nanovesicles, indicating their eligibility to afford several biological responses in normal and abnormal conditions [76, 77]. For instance, inflammatory microglia and astrocytes contain diverse pro-inflammatory cytokines with the potential to trigger subsequent inflammation and neuronal death [78]. The uptake of these inflammatory EVs by BBB ECs after IS loosens the EC-to-EC connection and thus BBB integrity [79]. The entry of active astrocyte EVs into the circulation initiates an acute phase protein response and further immune cell recruitment into the damaged sites [80]. Of course, it should be kept in mind that the metabolic status of the parent cell can pre-determine the inflammatory and/or anti-inflammatory properties of released EVs. In type A2 astrocytes, EVs diminish the lactate dehydrogenase levels and apoptotic changes in neurons, resulting in activation of brain tissue reparative mechanisms post-IS [81].

Due to the ability of stem cells to secrete several growth factors and cytokines, their EVs are enriched with certain cargoes that can promote the healing process in the injured sites [82]. For example, MSCs and neural stem cells (NSCs) EVs protect the injured neurons post-IS via the regulation of neurogenesis and inflammation [83]. The promotion of angiogenesis via EVs is another mechanism that helps the synaptic plasticity and restoration of learning and memory function following IS [84]. These features highlighted the regenerative potential of EVs, especially Exos, in the context of IS.

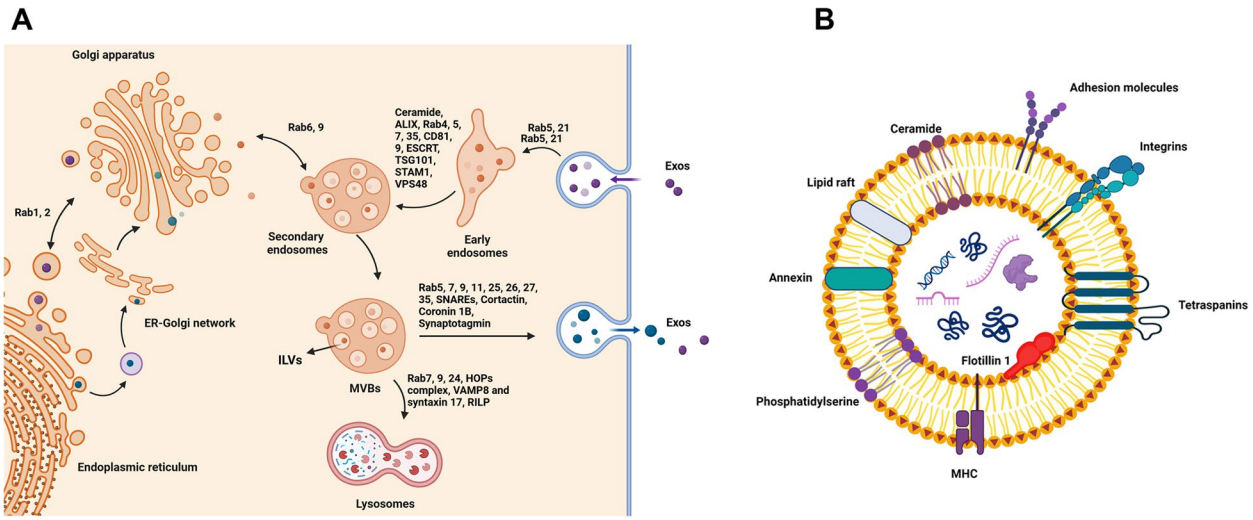

Fig. 3 Exo biogenesis (**A**). Different compartments related to the endosomal system are actively involved in the generation of Exos and their release into the ECM. Inside the endosomes, numerous ILVs are produced via the direct invagination of the membrane. Along with this step, several signaling molecules from the trans-Golgi network are sequestered and packed inside the ILVs. Some of the internalized exogenous Exos are directly guided to the endoplasmic reticulum to release their contents. The newly generated Exos inside the host cells are directed toward enzymatic degradation by lysosomes or fuse with cell membranes to release the ILVs, hereafter known as Exos. General Exo structure (**B**). Exos possess a lipid membrane bilayer with transmembrane ligands and receptors originating from host cells. Inside the exosomal lumen, several signaling molecules such as lipids, peptides, polypeptides, and genetic elements are sequestrated via the activation of different effectors inside the cytosol. Created by BioRender's web-based software

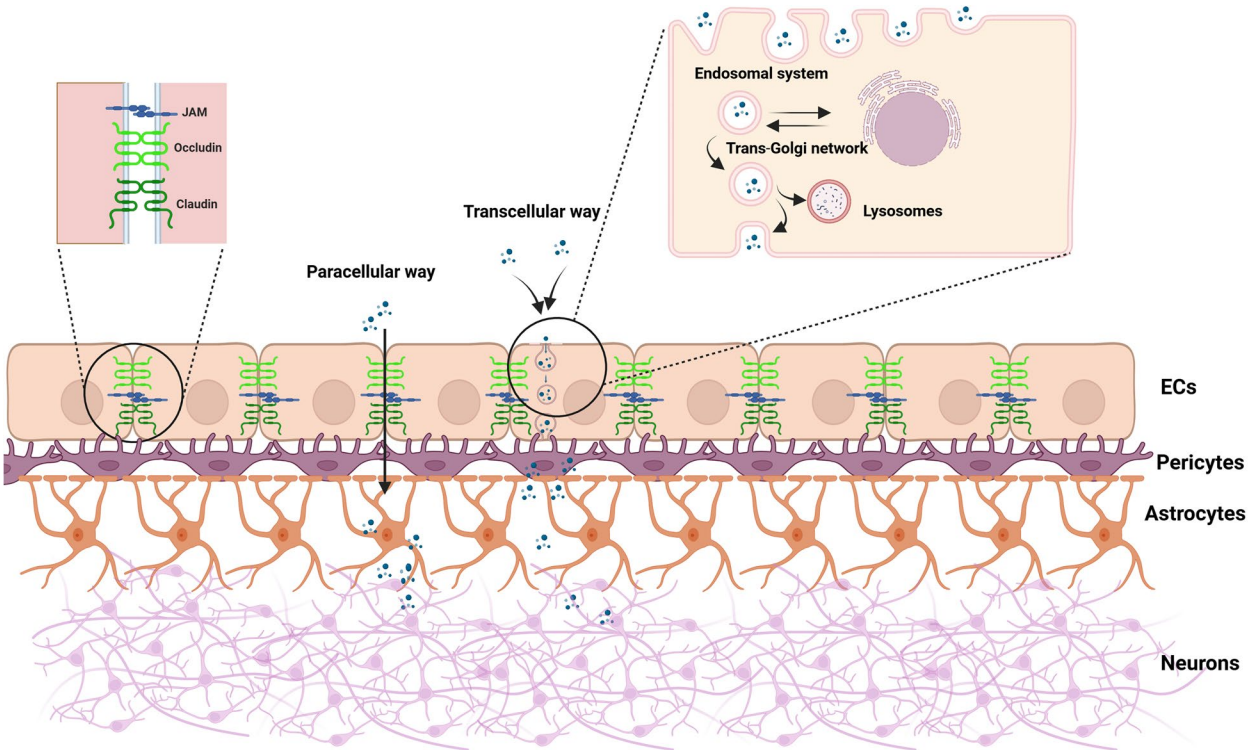

Fig. 4 Circulating Exos can cross the BBB via the endothelial layer, either paracellular or transcellular way. Exos can directly pass the endothelial layer from the luminal to the abluminal surface without any changes. Some fractions of Exos can be guided toward lysosomal degradation. The trans-Golgi network can send nucleic acids and proteins via newly generated ILVs to the abluminal surface. Created by BioRender's web-based software

EVs in preclinical studies

For clinical purposes and basic science experiments, MSCs can be isolated from different tissues, in which bone marrow, umbilical cord, and adipose tissues are the most common sources [85]. Obtaining BM-MSCs from humans is a painful and invasive procedure, but due to their immunosuppressive properties, low immunogenicity, differentiation capacity, and homing potential, these cells are the primary choice for clinical use [86]. Along with bone marrow, adipose tissue is the most abundant and feasible source of MSCs. Of note, adipose tissue is readily accessible and can be obtained as the byproduct of both therapeutic and cosmetic surgery. It seems that the bioactivity and morphological properties of adipose tissue MSCs are similar to bone marrow counterparts. Adipose tissue MSCs exhibit long-term stability in culture systems with the potential to commit to multiple cell lineages [87]. MSCs isolated from neonatal tissues like the umbilical cord have a lower risk of immune rejection, high proliferative potential, and the ability to differentiate into multiple lineages. They can be cryopreserved for future use at the time of care [88]. Irrespective of their origin, MSCs and secretome have been widely used to alleviate several pathological conditions in the central nervous system (CNS) and other tissues (Table 1) [89]. Of note, MSCs support the regeneration of injured tissues in various ways, including direct cell differentiation, soluble factor secretion, and the production and release of EVs [90, 91]. An experiment conducted by Xu et al. showed neurological function recovery via the increase of fiber length and number within the CNS in IS mice receiving MSC EVs. Based on the obtained data, significant neuro-angiogenesis properties were achieved along with reduced inflammatory response (IL-1β) [92]. Research suggests that upon the administration of MSC EVs, the local intensity of certain factors, such as BDNF and VEGF, increased in animals subjected to MCAO. These effects were related to the modulation of Zeb2/Axin2 signaling axis, in which the administration of Zeb2/Axin2-enriched MSC EVs significantly elicited the regenerative outcomes via concomitant increase of BDNF, NGF, VEGF, and modulation of OX10, Wnt/β-catenin, and endothelin-3/EDNRB signaling axes. Under such conditions, the migration of neural progenitor cells from the SVZ and SGZ of the dentate gyrus is increased and leads to neuroplasticity. However, neuroplasticity is different in these regions, and aberrant migration can occur following CNS injury. The data suggest that MSC EVs can modify the neurogenic niches to promote neurogenesis and angiogenesis in IS models, but the upregulation of trophic factors and increased cell migration is not enough to demonstrate improved neuroangiogenesis [93]. These data show that MSC EVs can regulate neuroangiogenesis in IS rodents. Huang et al. proved that MSC EVs can also exert benefits in the regulation of glutamate excitotoxicity, which is common in IS [94]. In response to MSC EV treatment, the expression of Glutamate Transporter-1 (GLT-1) is stimulated in rat astrocytes with oxygen–glucose deprivation/reperfusion (OGD/R) injury via the activation of miR-124/mTOR signaling cascade. Data confirmed that miR-124 exhibits the potential to increase cellular distribution of GLT1 via the inhibition of mTOR, and down-regulation of PS6 [94]. Of note, the regulation of intracellular free radical contents in hypoxic neurons can make them resistant to ischemic injury. In support of this statement, it was suggested that MSC EVs can blunt the toxic effects of 100 μM H_2O_2 on rat hippocampal neurons [95]. Luo and colleagues showed that MSCs can produce EVs with higher levels of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) under hypoxic conditions. The uptake of MSC EVs by hippocampal neurons can reduce the intracellular markers associated with lipid peroxidation (4-Hydroxynonenal↓) and protein oxidation (Dityrosine ↓). Along with the above-mentioned changes, the increase of stress-associated factors such as iNOS, HMGB1, HO-1, and Nrf2 was controlled in stressed neurons after incubation with MSC EVs [95]. In a similar work performed by Yang and Chen, they found that incubation of OGD/R-treated mouse BV-2 microglia with MSC EVs led to increased antioxidant capacity (SOD↑, and MDA↓), reduced inflammation (IL-1β↓, IL-6↓, and TNF-α↓) and apoptotic changes. These effects would be associated with the direct interaction of ZFAS1 with miR-15a-5p to downregulate its expression. Such a phenomenon led to accelerated healing properties in IS rodents, coinciding with reduced inflammatory response, oxidative stress, and infarct region [96].

Source cell preconditioning is another strategy to alter the EV cargo to obtain better regenerative outcomes. For instance, in a study, the injection of EVs of hypoxia-conditioned adipose tissue MSCs (AD-MSCs) led to the improvement of cognitive function in IS mice via acceleration of M1 to M2 phenotype shifting via circ-Rps5 [112]. In addition to necrotic changes and apoptosis, other cell death mechanisms have been found in brain ischemic areas, like ferroptosis. Hong et al. declared that circBBS2, containing umbilical cord MSC EVs and reduced ferroptosis (GPX4↑, GSH content↑, MDA↓, and SLC7 A11↑) in hypoxia/reperfusion-treated human SH-SY5Y by the inhibition of miRNA-494. It is thought that SLC7 A11 can increase the entry of cysteine into neurons to increase GSH levels [113]. A study showed that

Table 1 Some studies related to the application of EVs in IS models

EV source	EVs	Target cells	Outcome	Ref
MSCs	BDNF-loaded EVs	Neurons	Functional behavior↑, neural regeneration↑, angiogenesis↑, infarct volume↓, and synaptic plasticity↑	[97]
MSCs	Naïve EVs	Astrocytes	Inflammation-induced cognitive deficits↓, neurological function↑, calcium signaling and mitochondrial function↑, and reactive astrogliosis↓	[98]
MSCs	Naïve EVs	Neurons	Leukocyte infiltration (neutrophils, macrophages, and monocytes) into the ischemic zone↓	[99]
UC-MSCs	Naïve EVs	Microglial cells	Microglial-mediated neuroinflammation↓, IRAK1/TRAF6 signaling pathway↓, miR-146a-5p↑	[100]
BM-MSCs	Naïve EVs	Microglial cells	EV lncRNA H19↑, M1 microglia markers↓, M2 microglia markers↑	[101]
AD-MSCs	Naïve EVs	HUVECs	LPS-induced inflammation↓, ROCK1 and PTEN pathways↑, Enhanced angiogenesis (miR-132↑, and miR-146a↑), endothelial cell proliferation↑, and tube formation↑	[102]
AD-MSCs	Naïve EVs rich in miR-26a	Neurons	Neuronal damage↓, KLF9-mediated regulation of TRAF2/KLF2 axis	[103]
AD-MSCs	Naïve EVs	Neurons	Brain injury↓, EV miR-22-3p↑, and the inhibition of miR-22-3p increased neuronal apoptosis	[104]
BM-MSCs	Tetramethylpyrazine-loaded EVs	Brain neuronal cells and endothelial cells	Neuronal apoptosis↓, neuronal cell survival↑, angiogenesis↑, and inflammation↓	[105]
AD-MSCs	Naïve EVs	Primary neurons	EV miR-25-3p↑, p53-BNIP3 activity↓, and neuronal death↓	[106]
iPSC-derived MSCs	Naïve EVs	Endothelial cells	Endothelial cell proliferation↑, migration↑, and tube formation↑, STAT3-dependent autophagy pathways↑	[107]
MSCs	Treated with lithium	Neuronal cells	Neuronal survival↑, apoptosis↓, neurological outcomes↑, infarct size↓, and modulation of TLR4 signaling pathways↑	[107]
AD-MSCs	Naïve EVs	Macrophages	M2 macrophage phenotype↑	[108]
AD-MSCs	Naïve EVs	Neurons	EV miR-31↑, neurological function↑, TRAF6↓, IRF5↓, neuronal apoptosis under ischemic conditions↓	[109]
hiPSC-MSCs	Naïve EVs	Neurons	Infarct volume↓, spontaneous movement abilities↑, angiogenesis↑ (up-regulation of VEGF and CXCR4)	[110]
Hypoxia-primed MSCs	EVs	Neurons and endothelial cells	Infarct volumes↓, neurological function↑, VEGF↑, and blood vessel formation↑	[111]

Abbreviations NLRP3 Nucleotide-binding domain of inflammasome signaling family protein 3, *BM-MSCs* Bone marrow-MSCs, *AD-MSCs* Adipose tissue-derived-MSCs, *BDNF* Brain-derived neurotrophic factor, *HUVECs* Human umbilical vein endothelial cells, *CXCR-4* C-X-C chemokine receptor type 4, and *VEGF* Vascular endothelial growth factor

the depletion of circBBS2 by genetic elements blunted the therapeutic effects of MSC EVs in rats with cerebral ischemic/reperfusion injury, with concomitant neuronal necrosis and loss [114].

Based on previously published data, MSC EVs exhibit neuroangiogenesis potential and various biological activities such as anti-inflammatory, anti-fibrotic, etc. [115–118]. The application of xenogeneic EVs can yield regenerative outcomes similar to autologous and allogenic EV sources. For example, in a published study, incubation of LPS-treated mouse astrocytes with human MSC EVs (~ 10 μg EV protein) led to reduced astrogliosis (C3↓, CD81↓, GFAP↓) via the regulation of Nrf2-NF-κB signaling pathway [119]. Chen and co-workers suggested that the simultaneous IV injection of xenogeneic minipigs AD-MSCs (1.2×10^6 cells) plus AD-MSC EVs (~ 100 μg EV protein) in rats with acute brain ischemia led to better regenerative outcomes, indicated with significant

restoration of sensorimotor function [120]. Several weeks after injection, the local density of VEGF, CXCR4, and SDF-1α can improve the regeneration of the ischemic zone [120].

As aforementioned, EVs, especially Exos over MSC administration, are the bulk biodistribution in different biofluids due to nanosized features [121]. MSC EVs can cross the BBB barrier, enter brain parenchyma, and internalize into the injured and healthy neurons, making them suitable platforms for CNS diseases, especially ischemic conditions [122]. Using EV biogenesis and abscission, MSCs can produce and secrete different amounts of cytokines such as VEGF-A, bFGF, placental growth factor (PGF), IL-6, angiopoietin-1, Notch 2, VCAM-1, and TGF-β2, which are actively involved in the angiogenesis phenomenon. Certain factors, like Notch and VEGF-VEGFR pathways with synergistic activity, can intensify the process of de novo blood vessel formation (Fig. 5) [123]. Of course, different signaling pathways are influenced via MSC EVs in terms of angiogenesis potential. For example, the modulation of the NF-κB signaling pathway is among biomolecules that participate in the angiogenesis process in the presence of MSC EVs [124]. Under ischemic conditions, the sequestration of specific factors such as EGFR, FGF, PDGF, and NF-kB has been shown to increase in MSC EVs [124]. The existence of an inflammatory response and release of several cytokines at the site of ischemia can loosen the physical connection of BBB cell components, leading to the internalization of systemically administrated EVs [125]. In a study, the systemic injection of ~100 μg MSC EVs in MCAO rats via the tail vein led to increased neuroangiogenesis properties (BrdU↑, vWF↑, DCX↑, synaptophysin↑, SMI-31↑), resulting in functional recovery [126]. Upon the occurrence of ischemic changes, the activation and/or inhibition of certain signaling pathways contributes to subsequent pathological outcomes. It was found that excessive autophagic response not only does not promote CNS recovery but also can exacerbate cellular injury after ischemia [107]. In an experiment conducted by Xia et al., they found that induced pluripotent stem cell (iPSC)-derived MSC EVs can accelerate angiogenesis via the proliferation of ECs at the site of ischemia ($CD31^+/EdU^+$ ECs). Data confirmed that iPSC-MSC EVs can upregulate STAT3, leading to the regulation of autophagic response (LC3-II/LC3-I ratio↓, Beclin-1↓, and P62↑) [107]. These data revealed the possible relationship between the angiogenesis potential in IS and the activity of the autophagy

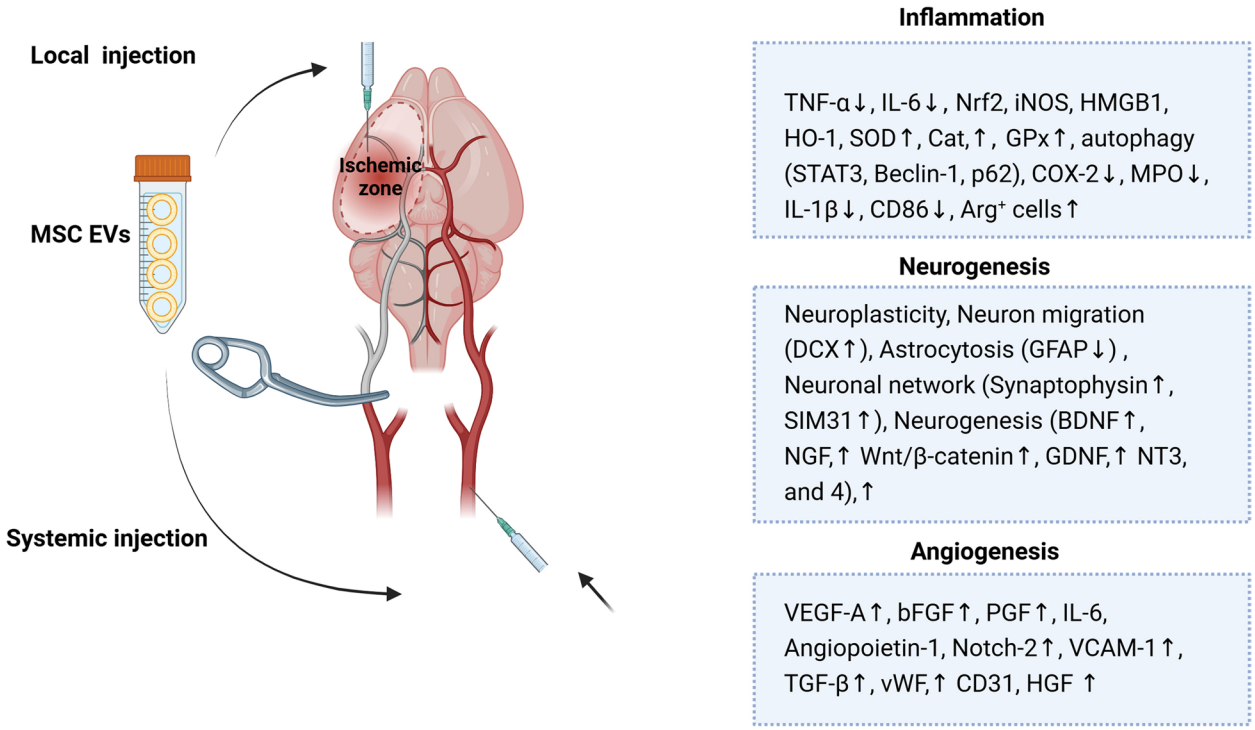

Fig. 5 Different underlying mechanisms by which MSC EVs reduce pathological conditions after the occurrence of ischemia, and reduce inflammation and inflammatory factors. MSC EVs can also reduce astrocytosis and increase neural networks. Through EV biogenesis and abscission, MSCs produce and secrete a variety of cytokines, including VEGF-A, bFGF, placental growth factor (PGF), IL-6, angiopoietin-1, Notch 2, VCAM-1, and TGF-β2, which are associated with angiogenesis. Certain factors, like Notch and VEGF-VEGFR pathways with synergistic activity, can intensify the process of de novo blood vessel formation. Created by BioRender's web-based software

signaling pathway. To reduce the off-target properties of MSC EVs following administration, Young et al. tried to increase the delivery efficiency using magnetic navigation [127]. A study suggested that the incubation of parent MSCs with 40 μg/ml iron oxide nanoparticles led to the release of magnetic particle-bearing EVs. The injection of 200 μg of magnetic particle-bearing EVs was done via the tail vein in the MCAO rats and the injected particles were guided toward the target site using the magnet helmet [127]. An experiment confirmed the on-target accumulation of VivoTrack 680-labeled magnetic EVs (~ 2.9 times) in the brain parenchyma, resulting in enhanced anti-inflammatory (TNF-α↓, IL-1β↓, MPO↓, and Cox-2↓, $GFAP^+$ cells↓, $CD86^+$ cells↓, Arg-1^+ cells↑), angiogenic (expression of FGF2↑, Ang-1↑, HGF↑, VEGF↑, TGF-β3↑ genes, and vWF^+ ECs), and neuroprotective (BDNF↑, GDNF↑, NT3, and NT4↑) effects. Based on the obtained data, magnetic EVs can promote the phenomenon of blood vessel formation via the modulation of the c-Jun N-terminal kinase signaling pathway (Fig. 6) [127].

Data have also indicated that MSC EVs harbor certain genetic factors, such as miRNAs with the potential to exert therapeutic effects on the brain ischemic sites [126, 128, 129]. Previous data have confirmed the existence of miR-21-5p, miR-184, miR-210, miR-29 b-3p, miR-140-5p, etc., with putative effects on angiogenesis, neurogenesis, apoptosis, and local immune cell activity within the brain parenchyma [124, 126, 128–134]. Geng et al. modified ADSCs-EVs to overexpress miRNA-126, and their result showed promotion in functional recovery of rats after MCAO, significantly increasing the expression of vWF and doublecortin as markers of ECs and neuroblasts [135]. In addition, Xin et al. reported that the EV-mediated transfer of miRNA-17–92 clusters improved functional recovery and neural plasticity following stroke [136]. In a subsequent study, miRNA-17–92 was shown to play a role in downregulating the expression of the PTEN gene and activating the PI3 K/Akt/mTOR pathway, which explains this recovery. By activating this pathway, axon extension and myelination are enhanced, and electrophysiological responses are improved [137].

In the context of IS, VEGF can act as a double-edged sword. Within 3 h after the onset of IS in animals, transcription of VEGF is stimulated and reaches maximum levels around 24 h. Based on the obtained data, the expression of VEGF is active for up to 7 consecutive days [138]. To stimulate the vascularization, the production and release of various proteases such as collagenase, plasminogen activators (PA), and PA inhibitor-1 are essential steps along with the activation of VEGFRs. It is also possible that VEGF can recruit several intracellular effectors, such as focal adhesion kinase, with the potential to regulate EC viability and exert neuroprotective effects. Despite these angiogenesis properties, VEGF can also loosen the EC-to-EC integrity within the ischemic area with concomitant MMP-9 activity, leading to the internalization of VE-cadherin and loss of BBB integrity [139, 140]. Commensurate with these descriptions, in strategies based on VEGF, the balance of these effects should be carefully considered.

EV engineering for regenerative purposes

EV engineering has been used for sophisticated modification of targeted and efficient delivery of regenerative compounds into injured sites, especially ischemic brain foci [141]. The control of neuroinflammation, increased neurogenesis, angiogenesis, etc., using engineered EVs was at the center of attention (Table 2) [142].

Endogenous cargo loading

Cargo loading is a fundamental aspect of EV engineering that allows for incorporating therapeutic molecules into EVs [148]. In this approach, parental cells are primed to overexpress/downregulate specific molecules, altering their contents inside the EV lumen [149]. For instance, the increase of microRNA-17–92 clusters in MSCs led to the production of EVs with higher regenerative properties in stroke rats via the regulation of NSC proliferation, neural plasticity, and angiogenesis [136]. miR-133b-expressing MSCs release EVs with profound effects on neuronal remodeling and apoptotic changes in IS rats [150]. It seems that EVs' application scope is not limited to a certain cell lineage. In an experiment, endothelial progenitor cells (EPCs) expressing miR-126 had the potential to produce EVs with higher angiogenesis properties. The injection of miR-126-enriched EVs led to enhanced neurological function and reduced necrotic areas in brain parenchyma [151].

Exogenous cargo loading

In contrast to endogenous cargo loading, exogenous cargo loading uses various modalities to incorporate therapeutic cargo into isolated and purified EVs [149]. In incubation with membrane-permeabilizing agents, chemical reagents such as saponin are used to temporarily increase membrane permeability, and certain compounds are directed into the EV lumen. Using this technique, it is possible to load proteins and small molecules. In the freeze–thaw cycles method, the targeted molecules are loaded into EVs using cycles of freezing and thawing steps. Unfortunately, the formation of ice crystals can damage the EV membrane during the freezing and thawing cycle, resulting in reduced charging efficiency [152]. Electroporation is another approach for the loading of targeted molecules into EVs. In this approach, cargo loading is done using electrical pulses

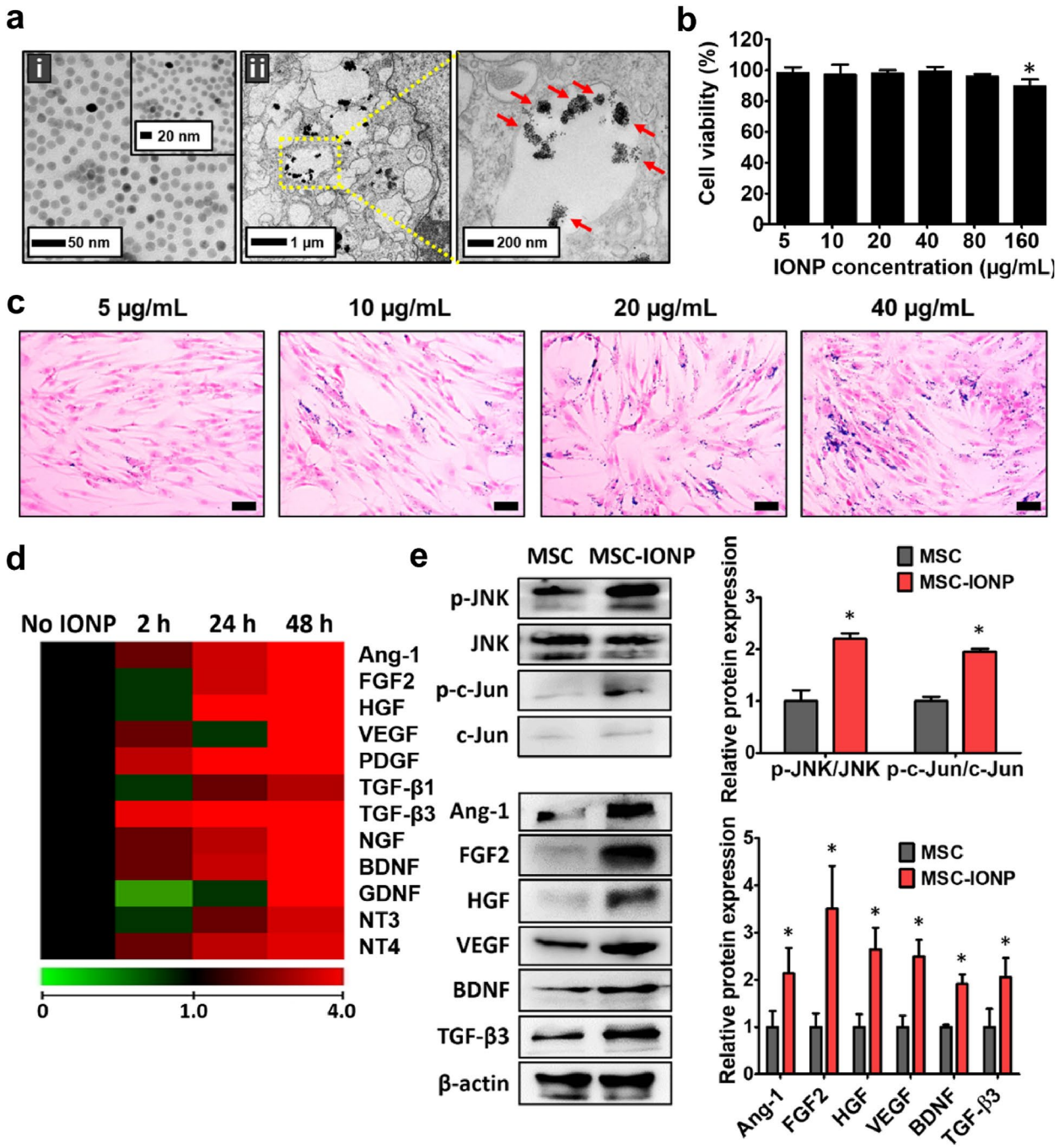

Fig. 6 The regenerative properties of iron oxide nanoparticles (IONP) on MSC angiogenic, neuroprotective, and anti-inflammatory properties. The internalization of IONPs by MSCs indicated with TEM images for representing IONPs (**ai**)**,** and after being internalized inside the MSCs (**aii**). The particles were accumulated inside the endosomes (**aii**; dotted yellow box). The survival rate of MSCs 16 h after being incubated with different concentrations of IONPs (**b**; $n = 5$; $^*p < 0.05$). Dose-dependent uptake of IONPs by MSCs was indicated using Prussian blue staining (**c**). Blue-colored spots are identical to IONPs (Scale bars: 100 μm). The expression of angiogenesis (Ang-1, FGF2, HGF, VEGF, and PDGF), neuroprotective (NGF, BDNF, GDNF, NT3, and NT4), and anti-inflammatory (TGF-β1 and TGF-β3) factors in IONP-treated MSCs at hours 2, 24, and 48 using real-time PCR analysis (**d**: $n = 3$). Western blotting for monitoring protein levels of phosphorylated JNK, c-Jun, and different factors in MSCs 48 h after treatment with IONPs (**e**: $^*p < 0.05$). In panels d and e, MSCs were exposed to 40 μg/ml IONPs ($n = 3$). Copyright 2020; [127]. Biomaterials

Table 2 Some studies related to the application of engineered EVs in preclinical studies

Cell source	Engineering method	Target cell	Outcome	Ref
BM-MSCs	MiR-145 enriched EVs	Neurons	Infarct area in MCAO rats↓, apoptosis↓, and cell cycle arrest↓	[143]
BM-MSCs	Zeb2/Axin2-Enriched EVs	Neurons	Functional recovery↑, spatial memory↑, nerve function↑, number of neurons in the subventricular zone, and the cortical area↑	[93]
BM-MSCs	Encapsulated miR-132 EVs	Neurons	Acvr2b expression↓, phosphorylated-Smad2 (p-Smad2)/c-Jun signaling pathway↓	[144]
MSCs	MiR-17–92 enriched EVs	Neurons	Axon-myelin remodeling↑, electrophysiological properties of neurons↑, and fiber density↑	[137]
MSCs	Iron oxide nanoparticles (IONP)-harboring MSC	Neurons	Angiogenesis↑, anti-inflammatory ↑, and anti-apoptosis response↑, infarction volume↓, and motor function↑	[127]
MSCs	Overexpressing miR-132-3p EVs	Endothelial cells and neurons	ROS production↓, apoptosis↓, blood–brain barrier disruption↓, RASA1 ↓ and Ras/PI3 K/Akt/eNOS signaling pathway↑	[145]
BM-MSCs	CXCR4-overexpressing EVs	Microvascular endothelial cells	Proliferation↑	[146]
BM-MSCs	mir-138-5p overexpressing	Astrocytes	LCN2↓, survival rate↑, and apoptosis↓	[147]
BM-MSCs	Plasmid (pCAG-GFP-miR-17–92)	Neural progenitor cells	Neural plasticity↑, functional recovery following IS↑, AKT)/mTOR/GSK3β signaling pathway↑	[136]

with transient pores in the EV membrane. It is thought that this approach is suitable for the loading of genetic elements such as small RNAs. The possibility of cargo accumulation is high in this approach [153]. The induction of membrane pores is also possible using sonication for fostering cargo loading [154].

EV surface modification

EV surface modification is done to increase their targeting efficiency and regenerative outcomes [155]. In this regard, two main techniques, genetic engineering and chemical modification, are available. Like the endogenous loading method, genetic engineering involves the manipulation of parent cells to produce certain proteins/peptides onto the EV membrane [156]. In an experiment, engineered EVs decorated with neuron-specific rabies viral glycoprotein (RVG) peptide were subjected to an electroporation method to increase luminal miR-124. RVG-decorated EVs had the potential to easily cross the BBB and deliver miR-124 to injured neurons in IS mice, leading to reduced infarct volume and improved functional recovery [157]. In another study, engineered EVs with c(RGDyK) peptide on their surface were loaded with curcumin and injected into IS rats. Data indicated the engineered EVs successfully targeted the ischemic brain region, causing reduced inflammatory response, cellular apoptosis, and improved neurological functionality [158]. Using chemical modification, it is possible to attach specific compounds to the EV surface without manipulating parent cells [158].

Targeted delivery strategies have been extensively used to increase the orientation of EVs toward target sites and improve therapeutic efficacy [159]. In this scenario, ligand-mediated targeting and physical targeting are two commonly used techniques for obtaining higher delivery efficiency [160]. The ligand-mediated targeting encompasses the decoration of the EV surface with specific ligands or peptides to increase the internalization rate in specific tissues and cell lineages. Using a chemical technique, the cyclo (Arg-Gly-Asp-D-Tyr-Lys) peptide was conjugated to the surface of MSC EVs. In vivo, data in IS rats confirmed enhanced targeting efficiency via the endothelial barrier and restoration of brain functional outcomes [158]. The physical targeting method applies external stimuli or guidance systems to enhance EV delivery to the brain ischemic sites. To this end, EVs were loaded with supermagnetic iron oxide nanoparticles and guided to the target site by using an external magnetic field as a guiding system [127].

It is thought that engineered EVs have advantages over conventional and unmodified drug delivery systems in terms of IS [161]. The BBB crossing properties, controlled and sustained release, reduced immunogenicity, multifunctional capabilities, and preservation of cargo bioactivity are most dominant in modified EVs [162]. Of note, the EV immunogenicity depends on the specific modification approaches. Studies have proven that HEK293 T cell EVs bearing therapeutic molecules induce minimal immune responses in vivo without significant toxicity and cytokine production even after repeated administration [163]. Using certain modification strategies, it is also possible to reduce immunogenicity via the induction of immune checkpoint molecules like PD-L1 on EV surfaces. By the addition of a specific molecular signature, such as CD47 on EV surface, circulating EVs can be easily engulfed by macrophages [164].

Different biological effects can also be controlled using multiple engineering procedures in the EV system to

yield higher regenerative outcomes. The simultaneous load of neuroprotective, angiogenetic, and anti-inflammatory agents can help us in better control of pathological conditions after IS. For instance, the MSC EVs loaded with miR-216a-5p and miR-210, and brain-derived neurotrophic factor (BDNF) can simultaneously regulate the inflammatory response and neurogenesis via systemic injection in IS mice, leading to improved brain function [165].

EVs-loaded hydrogels for the restoration of brain injuries

Three-dimensional (3D) hydrophilic hydrogels are effective carriers for delivering large doses of EVs to target tissues, especially brain parenchyma [58]. Hydrogels can be used as supporting scaffolds for on-target and sustained release of drugs, therapeutics, and various cells into the injured sites [166]. Despite these advantages, designing and fabricating distinct hydrogels with appropriate physicochemical features for cell encapsulation and delivery is challenging [167]. To be specific, various parameters like biocompatibility, mechanical properties, permeability, and the capability to support cell viability, efficient function, and biological properties should be supported by grafting hydrogels.

Engineered hydrogels for EV loading

Recent progress in the fabrication of engineered hydrogels has led to the development of biomimetic materials with ECM-like properties [168, 169]. In one approach, specific motifs can be incorporated into the polymeric structure of hydrogels for the regulation of stem cell bioactivities such as dynamic growth, differentiation, migration, and self-renewal. In this scenario, numerous reinforced hydrogels with specific chemical features have been used for encapsulation and delivery of various cell lineages [170]. In the context of CNS and the existence of a prolonged healing process, it is essential to fabricate and use scaffolds with the potential to release EVs for long periods without detrimental effects on their integrity and bioactivity [58]. Therefore, several modification strategies are available to improve hydrogels' physicochemical properties, reciprocal interaction with cells, and signaling biomolecules [171]. Using specific chemical reactions, it is possible to attach covalently and non-covalently certain functional groups to the polymer backbone, facilitating the incorporation of various nanoparticles, peptides, and biomolecules [172]. For instance, RVG peptides have been linked to hydrogels via electrostatic and covalent interactions. RVG-bearing hydrogels can suitably attach to acetylcholine (AChR) or gamma-aminobutyric acid (GABA) receptors on the brain microvascular endothelial cell (EC) surface, leading to the activation of receptor-mediated endocytosis across the BBB [173, 174]. The decoration of the EV surface with RVG derivatives was shown to enhance BBB crossing via transcytosis. It has been indicated that RVG peptides can be incorporated into hydrogels by forming nanocomposites through the self-assembly of cationic RVG peptides with negatively charged molecules like EVs, or by electrostatically assembling RVG with oxidatively degradable arginine-grafted polymer (PAMABP) [175]. Moreover, by using PLGA (polylactide-co-glycolide) covalently bonded with RVG, encapsulated molecules can be delivered into the CNS [176]. It should not be forgotten that biomimetic natural or synthetic polymer-based hydrogels formed via different chemical and physical crosslinking methods create a stable 3D network with high aqueous content and functional properties [177]. Among the various natural composites, collagen, gelatin, alginate, fibrin, hyaluronic acid, and methylcellulose have been applied for developing biocompatible hydrogels [178]. Of note, it is often mandatory to modify the composites to improve mechanical stability and toughness. The use of semi-synthetic hydrogels consisting of both natural and synthetic polymers through various chemical or physical crosslinking can enhance the release characteristics of hydrogels. By adjusting the physical and chemical properties of semi-synthetic hydrogels, including hydrophilicity, we can use different types of these hydrogels for encapsulating various biomaterials [179]. Compared to non-covalently formed hydrogels, the inherent stability of covalently crosslinked hydrogels makes them suitable for prolonged delivery purposes in in vivo and in vitro conditions [180, 181]. Importantly, the physicochemical features of these hydrogels, including pore size, degradation rate, and water content, are significantly influenced by the density or concentration of crosslinked bridges, which is, per se, crucial for loading, releasing, and preserving EV function [182]. Commensurate with these descriptions, it is essential to apply an appropriate crosslinking technique to ensure cytocompatibility with cellular systems. Thus, developing engineered hydrogels with appropriate ECM-mimetic properties, such as high biocompatibility, tunable stiffness, porosity, and degradation rates, enables the best modulation of mechanical and biochemical signals, thereby creating an optimized microenvironment for stem cell interaction [183]. Besides to abovementioned features, hydrogels should exhibit injectability and self-healing properties that undergo reversible inter/intramolecular interactions, resulting in the promotion of appropriate interactions with EVs and prolonging their bioactivates [170, 184, 185]. Data confirmed that these hydrogel types exhibit great potential for EVs delivering into the brain injury sites. Having the injectability properties and filling irregular cavities, it facilitates higher

regenerative outcomes within the CNS. In an experiment conducted by Wang et al., they used multifunctional hydrogel based on F127-polycitrate-polyethyleneimine (FE) with excellent injectability, self-healing, and thermo-responsive properties to significantly enhance the healing process in spinal cord injury (SCI) [186]. Data confirmed that the incorporation of DiR-labeled MSC EVs into FE hydrogel and orthotopic injection into SCI rats led to a controlled and sustained release of EVs for up to 56 days [186]. Analyses indicated the reduction of fibrotic scar (laminin↓, NG-2↓, and neurocan↓), $CD68^+$ macrophages, iba-1^+ microglia, and apoptosis (Bcl-XL↑, Bax↓, cleaved-caspase-3↓ and cytochrome↓), and increase of remyelination (myelin basic protein-2↑), axonal regeneration (NF-200↑) [186]. Considering the fact that the existence of electrostatic interactions between EV surface and different polymer chains can also increase the loading capacity, several key factors, including polymer content, pH, temperature, and crosslinking procedures should be monitored during the fabrication of each hydrogel type [187, 188]. Taken together, the type and intensity of EVs integration within 3D polymer hydrogels are at the center of attention for conducting sophisticated and smart delivery approaches in terms of brain injuries especially IS.

EV-loaded hydrogels for IS alleviation

As mentioned above, the immunosuppressive properties of MSCs from different sources, *i.e.,* bone marrow (BM-MSCs), can reduce the inflammation after IS [189]. The release of neurotrophic factors, immunomodulatory cytokines, proangiogenesis biomolecules, and other growth factors by BM-MSCs makes them relatively suitable cells for the alleviation of IS conditions. Of note, BM-MSC EVs can inhibit neuronal death and improve neurological function after cerebral I/R injury [190]. In response to inflammation and exposure to IL-1β or other cytokines, BM-MSCs can produce EVs with putative immunomodulatory effects. In support of this notion, Zhang et al. fabricated an injectable supramolecular thermosensitive hybrid hydrogel to assess delivery, retention time, and regenerative properties of IL-1β-primed MSC EVs in a rat model of MCAO (Fig. 7) [191]. To this end, a supramolecular branched polymer (HDU) was fabricated using hyaluronic acid methacrylate, 2-(3-(6-methyl-4-oxo-1,4-dihydropyrimidin-2-yl) ureido) ethyl methacrylate, and di (ethylene glycol) methyl ether methacrylate, followed by physical incorporation of silk fibroin (SF). The injection of DIR-labeled MSC EV-bearing HDU/SF hydrogel into the ischemic zone led to an increase in retention time up to 28 days, while in MCAO rats that received direct EVs, the fluorescent signals disappeared after 7 days. IL-1β-primed MSC EVs incorporated HDU/SF hydrogel can release the laden EVs to inflamed mouse microglia BV2 cells in in vitro conditions in which the levels of proinflammatory markers such as CD68, CD32, TNF-α, and IL-1β were reduced, indicating the superiority immunomodulatory effects of MSC EVs after being exposed to inflammatory cytokines. In the MCAO rats, the neuronal loss was diminished in the group that received IL-1β-primed MSC EVs incorporated HDU/SF hydrogel [191]. In another work conducted by Liu et al., they prepared BM-MSC EV-loaded hyaluronan-collagen (DHC-BME) hydrogel for the induction of neuroangiogenesis in rats with experimentally induced traumatic brain injury (Fig. 8) [192]. Data showed the stimulatory effect of DHC-BME hydrogel to increase the local $CD31^+$ and vWF^+ ECs, α-SMA^+ pericytes, indicating enhanced angiogenesis at the site of injury. Besides, EV-loaded hydrogel simultaneously promoted the neurogenesis via the induction of local neural stem cell migration ($nestin^+$ cells), and differentiation into immature (Tuj-1^+ cells), and mature neurons ($NeuN^+$ cells), cholinergic lineage ($ChAT^+$ cells), and oligodendrocytes (MBP^+ cells) 14 days after injection [192]. Along with these changes, the local $GFAP^+$ and $vimentin^+$ astrocyte numbers were significantly reduced and coincided with proper axonal growth and synapse formation ($GAP43^+$, $PSD95^+$, SYN^+, and $MAP2^+$ cells) [192]

In addition to direct EV loading within the supporting hydrogels for the stimulation of the healing process under ischemic conditions, it is also possible that the EV-producing MSCs have been incorporated into the hydrogel and transplanted into the injured sites. Of course, the applied hydrogels should possess certain physicochemical properties to support the dynamic growth and paracrine activity of loaded MSCs. For instance, Pei et al. investigated the neuroprotective effects of BM-MSC EV-loaded hydrogel/nanofiber composite consisting of core polymer GelMA and shell polymer PCL, for the alleviation of IS changes in a rat model [193]. Data showed that the regenerative potential of MSCs incorporated into hydrogel was due to enhanced migration properties and paracrine activity via the EVs. Of note, the brain edema, neurological deficits, and inflammatory response (Iba1^+ microglia↓, $GFAP^+$ astrocytes↓) were significantly reduced, leading to the restoration of injured $nestin^+$ neurons at the site of ischemia [193]. These features coincided with the stimulation of neovascularization and blood supplementation via local increase of $CD31^+$ endothelial lineage. It was suggested that most of the restorative effects are closely related to the release of EVs, especially Exos, into the ischemic area, harboring specific genetic factors such as miR–206–3p mediating the neuroangiogenesis properties via engaging the PI3 K/AKT signaling pathway [193]. Upon the activation of the PI3 K/AKT signaling pathway, the target effectors such as

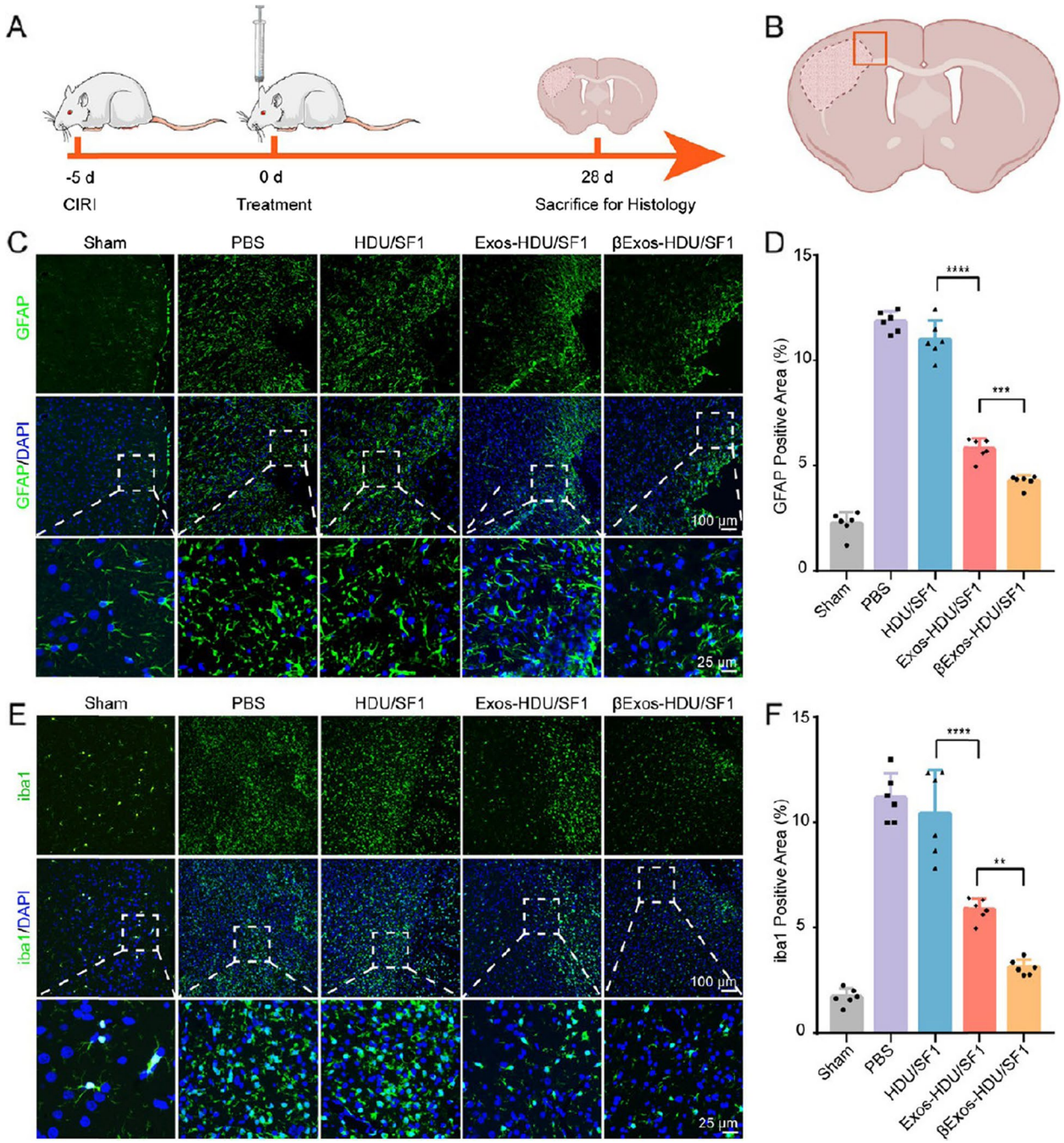

Fig. 7 Monitoring the delivery efficiency of IL-1β-primed MSC EVs incorporated HDU/SF hydrogel in the dynamic activity of astrocytes and microglia in the ischemic penumbra zone of the MCAO rats (**A-F**). Schematic diagram of the current experiment (**A**). The while-colored square indicates the selected area for further histological analysis (**B**). Astrocytes and microglia are labeled with green-colored GFAP (**C**) and iba1 (**E**) antibodies, respectively. The nuclei were stained using blue-colored DAPI. Data confirmed the reduction of astrocytosis (**D**) and microgliosis (**F**) in the ischemic penumbra in rats that received EVs incorporated HDU/SF hydrogels. Of note, these effects were more evident in the group that ischemic zone filled with IL-1β-primed MSC EVs incorporated HDU/SF hydrogel, showing the superior anti-inflammatory properties of MSCs pre-treated with inflammatory cytokines compared to non-treated MSCs (n = 6). One-Way ANOVA. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Copyright 2023. [191]. ACS Applied Materials & Interfaces. Cerebral ischemia – reperfusion injury: CIRI; Phosphate-buffered saline: PBS; and βExos; IL-1β-primed MSC EVs; Exos: EVs were isolated from non-treated MSCs

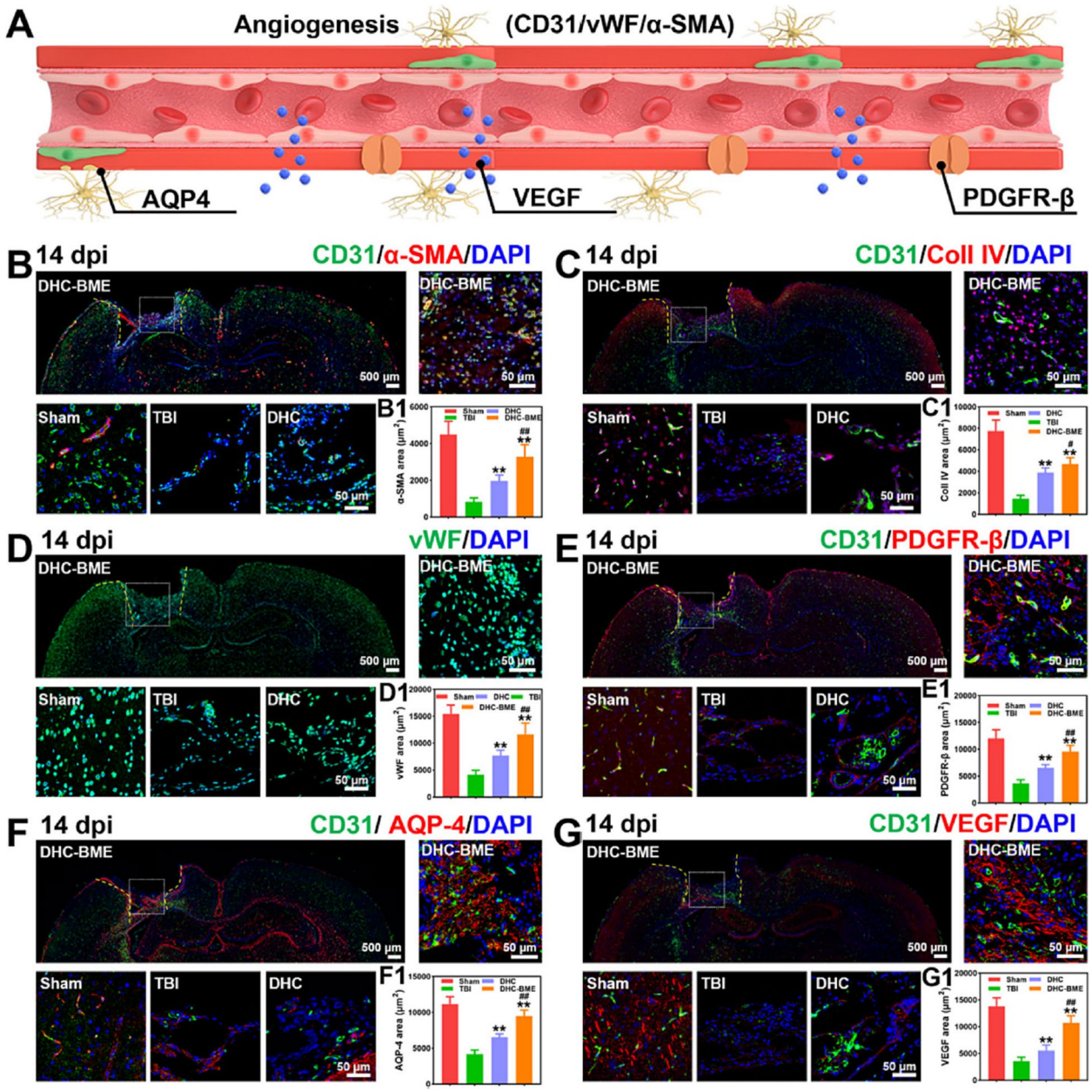

Fig. 8 Monitoring the angiogenesis properties of BM-MSC EV-loaded hyaluronan-collagen (DHC-BME) hydrogel in TBI rats after 14 days (**A-G**). Schematic illustration associated with the angiogenesis properties of EV-bearing hydrogel (**A**). Immunofluorescence staining for detection of local angiogenesis at the site of injury by monitoring green fluorescent $CD31^+$ ECs, and red colored α-SMA^+ pericytes (**B**). Data confirmed the significant increase of α-SMA^+ pericytes in TBI rats that received DHC-BME compared to the other TBI groups (**B1**). The integrity of vascular units was investigated using double CD31/Col IV staining (**C**). The vascular integrity was profoundly enhanced in the DHC-BME rats (**C1**). Monitoring the number of green fluorescent vWF^+ ECs to indicate typical angiogenesis properties at the site of injury (**D**). Based on the data, the number of vWF^+ ECs was significantly increased in DHC-BME rats compared to sham, TBI, and EV-free hydrogel groups (**D1**). Double green fluorescent CD31 and red colored PDGFR-β staining (**E** and **F**). Double CD31/AQP4 staining (**F**) for monitoring the vascular unit maturation. Data revealed a significant increase in PDGFR-β (**E1**) and AQP4 (**F1**) positive cells in the DHC-BME group. Double CD31/VEGF staining to assess the angiogenesis potential (**G**). Measuring local VEGF content per field (mm^2) (**G1**). While dashed areas stand for the close-up regions. Two-way ANOVA. $^{**}p < 0.01$ vs TBI group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs DHC group. Copyright. 2023. [192]. Carbohydrate Polymers

VEGFR2 and VEGFA are also initiated, which are mainly involved in the process of angiogenesis via regulating EC function [193]. Along with recent advances in the fabrication of various supporting hydrogels for on-target MSC EV delivery, novel platforms such as microneedles have also been developed for sustained release and topical introduction of EVs into ischemic tissues [194]. In this scenario, Zhang and co-workers used a mouse MSC EV-loaded GelMA microneedle patch in MCAO rats. Data confirmed the reduction of glial scarring (GFAP↓), apoptotic changes ($TUNEL^+$ cells↓), inflammation (IL-6↓ and IL-10↑), and an increase of angiogenesis ($CD31^+$ cells↑) after 28 days, offering a potential new avenue for clinical applications of microneedle patches [194]. It was suggested that the placement of microneedle patches on the surface of the ischemic area can help the sustained release of loaded MSC EVs into the deep layer of brain tissue, concomitant with the degradation rate.

Clinical application of EVs

While most EV research is associated with preclinical studies, just a few clinical trials have been done in IS patients. For instance, Wang and co-workers injected 4×10^9 NSC EVs per kg of body weight (NouvSoma001) in a single-center, randomized, open-label, placebo-controlled, dose-escalation trial (NCT06612710). In another phase 1 multicenter, randomized, double-blinded, placebo-controlled, dose-escalation trial, induced pluripotent stem cell (iPSC) Exos (GD-iExo-003) were administered intravenously for the treatment of 29 IS patients (NCT06138210). The clinical trial study with registered code NCT05158101 also monitored the safety and therapeutic efficiency of allogenic UC-MSC Exos via single intranasal administration in IS patients. To the best of our knowledge, preliminary data on the effectiveness of EV/Exo therapy have not yet been provided. Based on the previous evaluation of IS rodent models, it was suggested that stem cell EVs and especially MSC EVs can reduce the pathological conditions and neurological dysfunction, indicating a de novo therapeutic option in CNS ischemic conditions and its translation into stroke patients [195].

Limitations and challenges

The delivery methods of EVs into the brain parenchyma are one of the most challenging issues in terms of IS [196]. The selection of delivery approach is done based on anatomical features, the extent of IS, EV type, and their cargo, and the general patient's condition [75]. Intranasal, intravenous, and local injection are commonly available approaches for introducing EVs into the CNS [197]. Among them, nasal delivery is a non-invasive route and can help the administered EVs reach the brain via the olfactory bulb [198]. Due to the bulk vascularized niche in the nasal mucosa, EVs quickly enter the CNS system and exert their regenerative properties [199]. Besides, nasal delivery can circumvent several side effects reported in systemic delivery. Nasal cavities with small geometries cannot hold higher EV dosages, and thus repeated doses are mandatory [200]. The EV uptake via the nasal cavity is solely associated with general tissue features. For instance, congestion (hyperemia), mucosal layer integrity, and individual anatomical variations can affect EV uptake. Besides, repeated doses of EVs can contribute to nasal sensitivity, irritation, and mucosal layer injury [201]. It is logical to hypothesize that the nasal route is a relatively suitable approach for delivering EVs into the anterior brain parts rather than the posterior regions. In circumstances with massive brain injuries, delivery of EVs via the systemic route (intravenous injection) seems logical [202]. In this approach, the rate of injection and dose of EVs are controllable, leading to fewer side effects and higher safety [203]. The introduction of EVs via the systemic route is relatively invasive and does need sterile conditions and well-experienced personnel [204]. It has been thought that intravenously administrated EVs are rapidly cleared from the bloodstream via the activity of allo-reactive immune cells or macrophages. These features often necessitate multiple doses to yield the optimal regenerative outcomes [205]. In contrast to nasal and systemic delivery methods, local injection of EVs yielded maximum EV concentration at the site of injury. Despite this property, local injection is highly invasive and needs intricate surgical steps and sterile conditions [206]. The existence of pain and discomfort at the site of injection needs intensive care and follow-up [207]. However, the precise delivery of EVs to the deeply injured sites is challenging and should be done by specialists. Besides, the possibility of infection, inflammation, and iatrogenic tissue injury should not be neglected. In some cases, direct injection causes EV aggregation, and thus the torrent of recruited immune cells can scavenge the injected EVs from the target site, leading to low retention time [208]. Other non-common approaches such as intraarterial and intrathecal injection, and transcranial magnetic stimulation (TMS)-mediated delivery have been used in some studies [209, 210]. However, more studies should be done to show their proficiency in the context of IS.

MSCs gained a great deal of interest due to their safety profile, availability, ease of isolation, ease of expansion, availability from a variety of tissues, homogeneity in culture, and ease of identification [211]. But due to inherent heterogeneity in content and size, it is difficult to use a standard protocol for obtaining EVs from biological fluids with high GMP grade and clinical applications [10, 212]. However, the Minimal Information for studies of

EVs (MISEV) guidelines provide some guidance, these features can contribute to batch-to-batch inconsistency [213, 214]. Of note, parameters such as cell lineage and number of passages, specific culture medium and supplements, and O_2 and pH levels, etc., have a profound impact on the paracrine activity of parent cells and regenerative properties of EVs [215]. Finding suitable parent cells for EV isolation and purification is another limiting issue for the treatment of IS cases. The metabolic status of host cells can affect the cargo of EVs and thereby regenerative outcomes [216]. Along with these descriptions, the lack of reliable and consistent outcomes is common in clinical settings. The necessity for repeated bullous in different pathological conditions needs large-scale production of EVs, while the commonly used techniques are time-consuming and yield low contents of EVs [217, 218, 223]. Besides, the lack of guidelines for monitoring the safety and efficiency of EVs makes their application difficult in the clinical setting. In this regard, the use of clear and rigid guidelines related to EV production, phenotyping, and preparation for clinical uses should be developed [219–221]. For providing off-the-shelf EVs, the storage temperature and preserving EV integrity and therapeutic effects for prolonged periods are critical issues [222]. Manufacturing cGMP-grade EVs is possible in certain bioreactors with more regenerative properties and large scales with fewer man-made technical errors. In the laboratory setting, the culture of parent cells in serum-free or chemically defined media can help to collect xenobiotic-free EVs [211].

Conclusion

MSC EVs exhibit remarkable potential in treating IS cases in various preclinical studies. These particles can potentially deliver therapeutic cargo into the injured sites and promote neuroangiogenesis via engaging several molecular and cellular mechanisms. It is suggested that future studies should focus on the identification of exact molecular mechanisms by which MSC EVs promote CNS regeneration under ischemic conditions. The delivery of EVs using sophisticated delivery approaches is mandatory for higher on-target efficiencies. The engineering of EVs via tissue engineering approaches and biotechnological techniques can help to yield better regenerative outcomes.

Abbreviations

AChR	Acetylcholine
BBB	Blood-brain-barrier
BM	Bone marrow
BDNF	Brain-derived neurotrophic factor
CAT	Catalase
CNS	Central nervous system
DAMPs	Damage-associated molecular pattern molecules
ECs	Endothelial cells
EPCs	Endothelial progenitor cells
Exos	Exosomes
ECM	Extracellular matrix
EVs	Extracellular vesicles
GABA	Gamma-aminobutyric acid
GelMA	Gelatin Methacrylate
GLT-1	Glutamate Transporter-1
GPx	Glutathione peroxidase
iPSCs	Induced pluripotent stem cells
IL-1β	Interleukin 1-β
ICH	Intracerebral hemorrhage
ILVs	Intraluminal vesicles
IV	Intravenous
IS	Ischemic stroke
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
MMPs	Metalloproteinases
MVBs	Multivesicular bodies
NSCs	Neural stem cells
NMDARs	N-methyl-D-aspartate receptors
OGD/R	Oxygen-glucose deprivation/reperfusion
PTEN	Phosphatase and tensin homolog
PA	Plasminogen activators
RVG	Rabies viral glycoprotein
ROS	Reactive oxygen species
SOD	Superoxide dismutase
3D	Three-dimensional
rtPA	Tissue plasminogen activator
TMS	Transcranial magnetic stimulation
TNF-α	Tumor necrosis factor alpha

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Authors' contributions

"B. M., S. A., P. H., P. N.M., F. V. N., B. Y., S. A. C., S. R., A. M., E. S., and H. S. collected data and prepared the draft. R. R. and M. K. conceptualized the study and edited the final manuscript. M. M. acquired funding. "

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Data availability

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Declarations

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Competing interests

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