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REVIEW

Extracellular vesicles: Revolutionizing targeted therapy for ischemic stroke

Ju Bai[a,b], Jingshu Yang[c], Wei Liu[b], Bin Han[d], Shaoyan Jiang[e,*], Jinping Sun[b,*], Hongzhao Qi[a,*]

[a]*Institute for Translational Medicine, The Affiliated Hospital of Qingdao University, College of Medicine, Qingdao University, Qingdao 266021, China*
[b]*Department of Emergency Medicine, The Affiliated Hospital of Qingdao University, Qingdao 266003, China*
[c]*School of Stomatology, Qingdao University, Qingdao 266023, China*
[d]*Department of Neurology, The Affiliated Hospital of Qingdao University, College of Medicine, Qingdao University, Qingdao 266003, China*
[e]*Department of Cardiology, The Affiliated Cardiovascular Hospital of Qingdao University, Qingdao 266000, China*

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Abstract Ischemic stroke (IS) remains a leading cause of global death and disability, with treatment effectiveness limited by its complex mechanisms, which include blood−brain barrier (BBB) disruption, neuroinflammation, excitotoxicity, oxidative stress, and cell death. This review summarizes emerging research on extracellular vesicle (EV)-based therapies for IS. EVs, sourced from animals, plants, and microbes, have unique benefits as natural nanocarriers, such as inherent BBB permeability, biocompatibility, and the ability to deliver multiple therapeutic cargos (like miRNAs, proteins, and drugs). We evaluate how EVs target key IS issues: (1) restoring BBB integrity by stabilizing tight junctions (TJs) and reducing matrix metalloproteinases (MMPs), (2) modulating microglia to reduce neuroinflammation, (3) decreasing excitotoxicity, (4) scavenging reactive oxygen species (ROS) to lessen oxidative stress, and (5) inhibiting apoptosis, ferroptosis, and other cell death pathways. Additionally, engineered EVs, such as antibody-conjugated or magnetically guided types, exhibit improved targeting and treatment accuracy, yielding promising results. Despite significant preclinical promise, clinical application faces challenges in standardization, scalable production, and delivery improvement. EVs offer a transformative, multi-targeted approach with the potential to overcome current limitations in IS treatment.

*Corresponding authors.
E-mail addresses: qihongzhao@qdu.edu.cn (Hongzhao Qi), sunjinping@qdu.edu.cn (Jinping Sun), Jsy18678957997@163.com (Shaoyan Jiang).
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1. Introduction

Stroke is a major contributor to global illness and death. According to projections by the World Stroke Organization-Lancet Commission on Neurology, the number of stroke-related deaths is expected to increase by 50%, rising from 6.6 million in 2020 to 9.7 million by 2050[1]. The occurrence of a stroke can be attributed to multiple factors, with ischemia responsible for approximately 87% cases[2]. IS occurs when an obstruction or narrowing of the cerebral arteries blocks blood flow to the brain. Currently, the main clinical approach for treating IS involves thrombolytic therapy with recombinant tissue plasminogen activator (r-tPA) or the use of endovascular procedures thrombectomy[3]. These therapeutic interventions restore cerebral blood flow and oxygen delivery to the ischemic brain; however, they have a significantly limited therapeutic window after a stroke begins, which restricts their clinical use. Additionally, the sudden revascularization can trigger a harmful cascade of events[4], intensifying secondary damage to brain tissue through oxidative stress, neuroinflammation, and secondary cytotoxicity, a pathophysiological process known as cerebral ischemia/reperfusion (CI/R) injury[5,6]. Additionally, neuroprotective agents are used in managing IS. One prominent example is vinpocetine, which demonstrates antioxidative, anti-inflammatory, and anti-apoptotic properties[7]. Clinical trials have examined its effectiveness in treating IS. Additionally, Edaravone, as an oxygen free radical scavenger, has been proven to alleviate neuronal and endothelial cell damage in IS[8]. Statins demonstrate anti-inflammatory and antioxidant properties, facilitate the stabilization of the BBB, and improve cerebral blood flow[9]. However, a standard limitation is the inadequate ability of these neuroprotective agents to cross the BBB, leading to limited therapeutic effectiveness. As a result, there is an urgent need to develop innovative pharmaceutical approaches or therapeutic strategies for managing IS.

EVs derived from biological sources have become an important area of interest in stroke therapy, drawing increasing attention from researchers and clinicians. Previous preclinical and early-phase clinical studies have shown that stem cell therapy is a safe and promising method for restoring neurological function following IS[10−12]. Several studies indicate that the therapeutic effect of stem cells is mainly due to EVs released from the administered cells. EVs show a level of versatility similar to that of cells. Importantly, abiotic EVs offer advantages in transport, storage, modification, and application, surpassing the capabilities of biotic cells[13]. To date, preclinical studies have shown the significant potential of EVs in treating IS, due to their antioxidative, anti-inflammatory, anti-apoptotic, neuroprotective, and immunomodulatory properties. For example, research by Xia et al.[14] revealed that small EVs derived from embryonic stem cells (ESC-EVs), which contain TGFB, Smad2, and Smad4 proteins, can activate the TGFB/Smad signaling pathway in $CD4^+$ T cells. This activation leads to the expansion of regulatory T cells (Tregs) and protects IS. Additionally, only a limited number of clinical trials have confirmed the effectiveness of EVs in treating IS. In particular, exosomes from human induced pluripotent stem cells (GD-iExo-003) and mesenchymal stem cells (MSCs) were administered to assess their potential in reducing disability in patients with acute IS (https://ichgcp.net/zh/clinical-trials-registry/NCT06138210). In summary, EVs offer significant potential for the clinical management of IS, possibly exceeding the effectiveness of traditional cell-based therapies.

Nevertheless, the mechanisms by which EVs influence the pathophysiology of IS and its therapeutic processes have not been thoroughly reviewed. A comprehensive investigation in this area is essential for advancing the clinical use of EVs in IS treatment. In this review, we systematically searched the Web of Science Core Collection for research articles and reviews on the use of EVs in IS, covering the period from July 2005 to July 2025, to gain a clear understanding of current research priorities and trends. Both qualitative and quantitative analyses were conducted using CiteSpace and GraphPad Prism 10. Our findings showed a total of 519 publications, reflecting a significant increase in studies on EVs in IS over the past twenty years (Fig. 1). A co-occurrence analysis of keywords identified the ten most common terms. Besides "ischemic stroke" and "extracellular vesicles," words like "angiogenesis" and "functional recovery" also appeared prominently.

Based on this framework, this review will first summarize the fundamental knowledge of EVs, focusing on the comparison of their characteristics from animal, plant, and bacterial sources. It will then examine the pathophysiological mechanisms of IS and systematically review the progress in applying EVs from different sources and engineered EVs to target various pathological aspects of IS, and discuss their mechanisms of action and targeting strategies. Simultaneously, the review will evaluate the current research landscape of EVs in IS treatment, providing insights into key issues such as scalable production, safety assessment, and clinical translation pathways. The goal is to move EV-based therapies from experimental research to clinical practice, ultimately unlocking their full potential in the clinical management of IS, which may surpass traditional cell therapies and offer new hope for IS patients.

2. The overview of EVs

EVs are typically defined as lipid bilayer-enclosed nanoparticles secreted by mammalian cells, enabling intercellular communication through the transfer of bioactive molecules, including proteins, nucleic acids, and lipids. These vesicles are essential to many physiological processes, thereby affecting cellular functions[15,16]. Within the immune system, EVs play a vital role in antigen presentation, immune regulation, and the modulation of inflammation responses[17,18]. In cancer-related settings, tumor-derived EVs promote cellular proliferation[19], invasion, and metastasis by transferring oncogenic molecules to adjacent cells and modulating the immune response in a way that supports tumor growth progression[20−22]. Accumulating evidence suggests that EVs are an emerging therapeutic platform with promising treatment outcomes in various pathological conditions[19,23−34]. EVs also have significant potential as diagnostic biomarkers for various

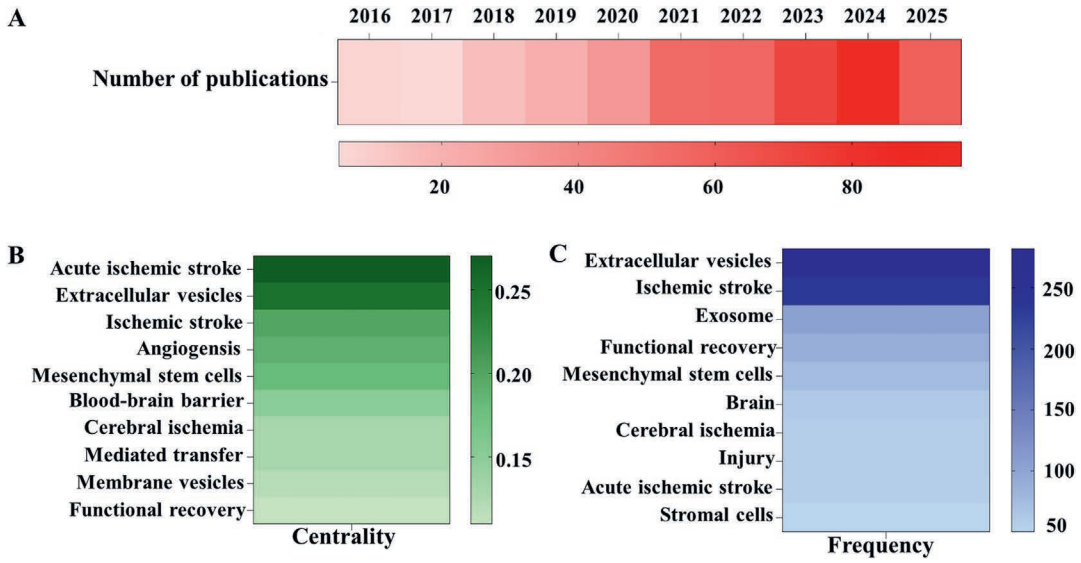

Figure 1 (A) Annual global publication volume from July 2005 to July 2025. (B) Keyword co-occurrence analysis reveals the betweenness centrality ranking of keywords. (C) Keyword co-occurrence analysis displays the top 10 most frequently co-occurring keyword pairs.

diseases[35−38]. Additionally, they can be designed to enable targeted drug delivery[39-42], enhancing the efficacy of drugs while minimizing their side effects.

2.1. Drug loading of EVs

EVs possess inherent advantages as drug delivery vehicles, owing to their stability[43], cargo-protective capacity[44], and low immunogenicity[45], which position them as promising tools in biomedical research and therapeutic applications. However, achieving high drug loading efficiency remains a significant challenge. Multiple strategies have been developed to address this limitation, varying in principle, efficiency, and applicability (Table 1[46−54]). The primary challenge lies in balancing encapsulation efficiency, vesicle integrity, and the activity of the encapsulated drug. Current methods include co-incubation, electroporation, sonication, and freeze-thaw cycles, each offering distinct advantages depending on the application. For example, co-incubation effectively loads small molecules such as acacetin while preserving high encapsulation efficiency and vesicle integrity[46]. In contrast, electroporation is more suitable for small RNAs (*e.g.*, miRNA and siRNA), thereby enhancing the efficacy of gene silencing in recipient cells. Sonication-extrusion facilitates controlled membrane fusion with liposomes, allowing for precise control of hybrid vesicle size[47]. Lipid fusion combines natural EV membranes with synthetic liposomes to improve hydrophobic drug loading capacity[48]. Surface conjugation covalently attaches pre-loaded nanoparticles to EV surfaces, substantially increasing drug payload. Emerging droplet-based co-extrusion, utilizing microfluidic platforms, also enables efficient fusion of EV-lipid nanoparticles[47]. Generally, method selection should follow a multi-factor framework: drug properties (such as hydrophilicity/hydrophobicity, molecular size, and stability) determine the core approach; therapeutic needs (like loading efficiency and dosage) direct optimization; and downstream uses (especially *in vivo*) require focusing on EV bioactivity and functional integrity. Accordingly, nucleic acid-based drugs are best loaded through electroporation or co-extrusion, while hydrophobic small molecules are best loaded through lipid fusion or co-incubation. High-dose delivery needs can be addressed through surface conjugation or lipid fusion.

2.2. The therapeutic potential of EVs

EVs are usually categorized into three primary groups based on their origin and size: exosomes, microvesicles (also known as ectosomes), and apoptotic bodies[55]. These different EV subtypes offer complementary therapeutic benefits due to their distinct biological traits and size ranges. Exosomes, the smallest subtype (30−150 nm) with high stability and the ability to cross the BBB, serve as an ideal platform for precise drug delivery. Modifying their surface and efficiently loading cargo improves their accumulation in ischemic areas, thereby enhancing therapeutic effectiveness. Microvesicles, with a broader size range (50−1000 nm) and the ability to carry various signaling molecules, act as long range signal transmitters. Membrane functionalization allows them to influence angiogenesis and inflammatory responses. Apoptotic bodies, the largest (1−5 μm) and abundant in "eat-me" signals, naturally target immune cells and can be engineered into biomimetic delivery systems to promote immune modulation and tissue growth repair[56,57]. Recent research has broadened the EV landscape beyond mammals, demonstrating that plant cells and bacteria also produce functional EVs[58,59]. Plant-derived exosome-like nanoparticles (50−500 nm) are involved in stress responses[60,61], intercellular communication[62], and pathogen defense[63]. Gram-negative bacteria produce outer membrane vesicles (OMVs, 20−300 nm)[64], which are enriched with outer membrane components and play key roles in virulence, biofilm formation, and modulating host immune responses. Gram-positive bacteria generate similar, though biogenetically different, membrane vesicles (MVs)[65]. Both plant and bacterial EVs share functional similarities with mammalian EVs in cargo delivery and communication. Their unique properties, including the ability to cross biological barriers, stability in circulation, and diverse bioactive cargo, make them promising for biotechnological and biomedical applications, such as therapeutic intervention, drug delivery, and

Table 1 Comparison of major drug loading methods for EVs.

Method		Principle	Advantages	Disadvantages/ potential Impacts	Ideal cargo	Ref.
Endogenous loading		Genetically engineering parent cells to overproduce a therapeutic biomacromolecule, enabling its encapsulation into emerging EVs through cellular mechanisms.	1. Maximizing drug activity retention; 2. Enabling a mild yet efficient loading process.	1. The technical process is complex; 2. The production cycle is lengthy; 3. Depending on specific parent cell types.	Therapeutic proteins (*e.g.*, FGF20, GDNF, cytokines, enzymes), nucleic acids, antigens (for vaccines), and reporter proteins (*e.g.*, GFP).	49−51
Electroporation		Using electrical pulses to temporarily create pores in the EV membrane for cargo loading.	High loading efficiency for small molecules.	May compromise membrane structural integrity and vesicle stability.	Small-molecule chemotherapeutic drugs (*e.g.*, DOX).	52,53
Physical fusion methods	Lipid fusion/EV-liposome hybridization	A specific application of physical fusion involves creating semi-synthetic hybrids by fusing EVs with synthetic liposomes.	1. Integrating the high-efficiency loading capacity of liposomes with EVs' targeting ability; 2. Combining parental cell functionality.	Complex process with unverified long-term stability.	siRNA, mRNA, and other nucleic acids.	48
	Sonication-extrusion fusion	A common method in physical fusion involves sonication pretreatment followed by sequential extrusion through membranes.	1. Integrating the high-efficiency loading capacity of liposomes with the targeting ability of EVs; 2. Combining parental cell functionality.	Complex process with unverified long-term stability.	Platform carrier for subsequent loading.	47
	Droplet-based co-extrusion fusion	A microfluidic method based on physical fusion, enabling precise fusion within droplets.	1. High-throughput and uniform particle production; 2. Highly controllable fusion process.	Equipment and process are complex.	Suitable for various LNP-loadable drugs (nucleic acids, small molecules).	47
Co-incubation		Depending on passive diffusion or membrane interactions for cargo loading.	1. Gentle process; 2. Preserving membrane integrity.	1. Limiting and variable loading efficiency; 2. Less effective for hydrophilic macromolecules.	Small hydrophobic drugs (*e.g.*, kaempferol) and certain lipids.	46
Surface conjugation/hybridization		Conjugating drug-loaded nanoparticles or therapeutic molecules onto the EV surface through chemical bonds or affinity interactions creates a conjugate or composite system.	1. High drug loading; 2. Combining functionalities of different modules.	1. May cause surface interference and affect natural targeting; 2. Structural control and metabolic behavior are complex.	Small molecule drugs (*e.g.*, DOX), proteins, and nucleic acids.	54

Table footnote: FGF20: Fibroblast growth factor 20; GDNF: Glial cell line-derived neurotrophic factor; GFP: Green fluorescent protein; DOX: Doxycycline.

vaccine development. Fig. 2 summarizes the current main applications of EVs in biology and medicine.

2.3. Summary of adverse effects of EVs

The diverse origins and functional versatility of EVs emphasize their significant potential as targeted therapeutic vehicles. However, it is crucial to recognize that the therapeutic effects of EVs are highly dependent on context, as their efficacy mainly depends on the specific cellular source and its compatibility with the pathophysiological environment[65−69]. This dependence manifests at two critical levels: on one hand, carefully engineered EVs can enable precise intervention in specific pathological processes[70,71]; on the other hand, naturally occurring EVs from

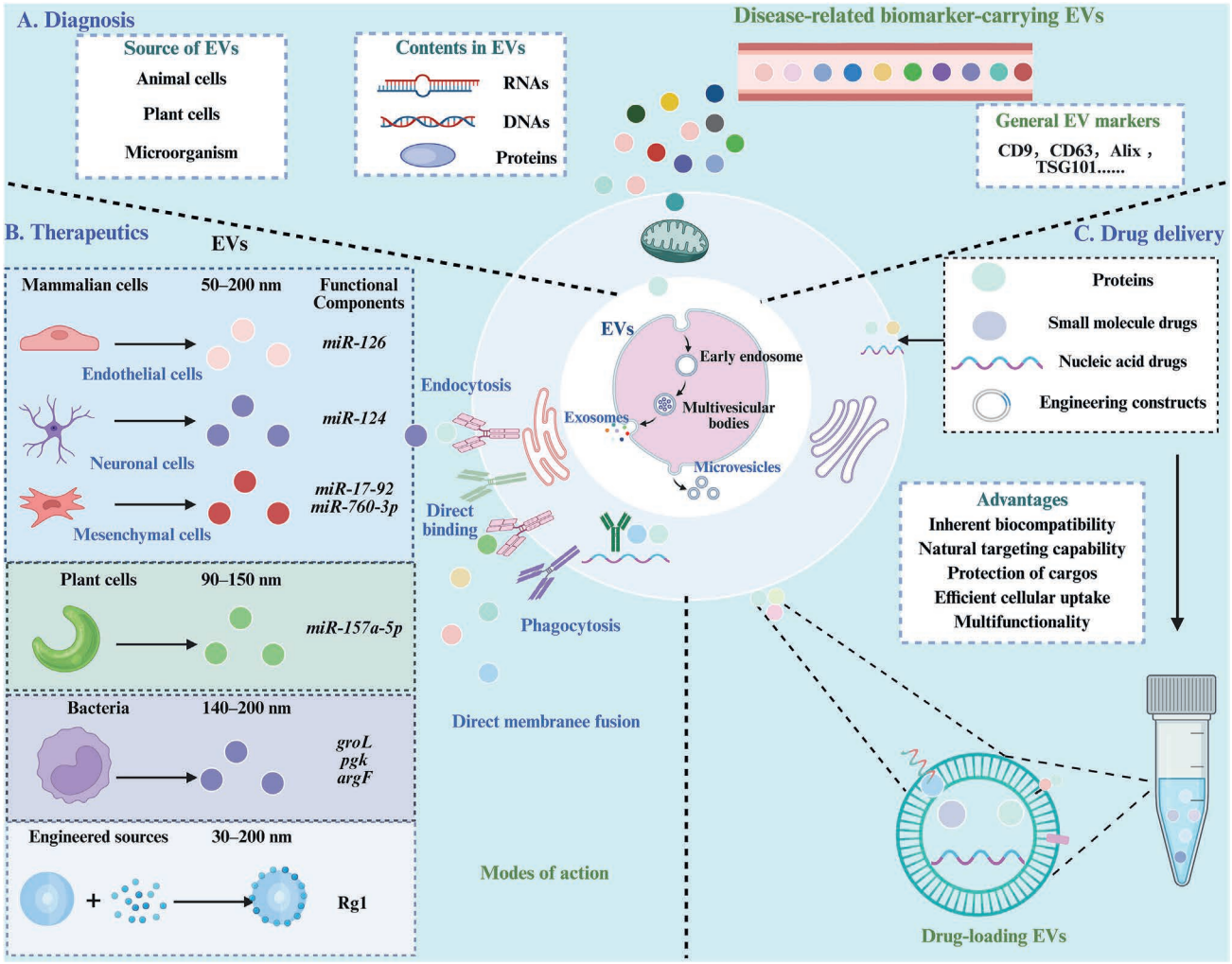

Figure 2 EVs in diagnostics, therapy, and drug delivery. (A) Sourced from animals, plants, or microbes, they carry biomarkers (RNA, DNA, proteins) for disease detection. (B) They exert therapeutic effects through endocytosis, membrane fusion, phagocytosis, or direct binding. (C) They deliver diverse cargo, including proteins, small molecules, nucleic acids, and engineered constructs. © By Biorender.

pathological states may transmit harmful signals that worsen disease progression. Studies show that EVs from damaged neurons can significantly inhibit the proliferation and repair functions of MSCs by transferring oxidative stress signals[35,72]. Similarly, EVs released by pro-inflammatory immune cells can exacerbate post-stroke neuroinflammation by carrying inflammatory factors, such as interleukin-1 beta (IL1B) and tumor necrosis factor-alpha (TNFA)[35,72,73]. Conversely, allogeneic EVs pose the risk of recognition and clearance by the host immune system[74,75]. To unlock the full therapeutic potential of EVs while effectively managing their associated risks, a comprehensive and systematic quality control framework must be developed. This involves choosing cell sources with well-defined safety profiles and avoiding cells in pathological conditions states[76,77]; optimizing parental cell conditions through hypoxic preconditioning, three-dimensional culture, or genetic engineering to produce EVs with specific therapeutic features; developing detailed EV safety standards through thorough cargo analysis (such as miRNAs, proteins, and lipids) and surface protein examination; and employing technologies like targeted peptide modification to reduce off-target effects and improve treatment efficacy specificity[78].

In summary, the complex pathological cascade of IS, including BBB disruption, neuroinflammatory responses, and neuronal death, offers multiple targets for EV-based therapies[79]. The actions of EVs vary from broad, synergistic modulation by natural vesicles to precise targeting of specific pathological sites through engineered systems. Moving these therapies from research to clinical practice requires equal focus on therapeutic effectiveness and safety, supported by standardized risk assessments, advanced engineering, and comprehensive preclinical studies. The upcoming sections will explain how EVs from different sources can be aligned with specific biological features, thereby connecting basic biological understanding with clinical application.

3. The pathophysiological processes of IS and the related treatment using EVs

To develop effective therapies based on EVs, it is crucial to harness and modify the abnormal pathophysiological features of IS. Therefore, a comprehensive understanding of these features is essential for advancing such therapeutic approaches. This section

offers an in-depth analysis of the various pathophysiological changes linked to IS (Fig. 3), including BBB disruption, glutamate excitotoxicity, neuroinflammation, oxidative stress, mitochondrial dysfunction, and cell death. Additionally, it will assess the use of EV-based therapies that target these specific pathophysiological alterations in the context of IS.

3.1. *BBB injury*

The BBB acts as a unique and tightly regulated boundary between the circulatory system and the central nervous system (CNS). It is made up of endothelial cells (ECs) located at the ends of astrocytes and pericytes, which are embedded within the basement membrane of capillaries[80]. The innermost lining of the BBB is composed of continuous, non-porous ECs sealed by tight junctions (TJs). These ECs are surrounded by pericytes, embedded within the capillaries, and by astrocytes, which cover the basement membrane. The complex interactions among ECs, pericytes, astrocytes, microglia, and neurons form the neurovascular unit (NVU), which is crucial for maintaining the functional integrity of the CNS. Unlike peripheral ECs, cerebral ECs create continuous monolayers without fenestrations, characterized by lower phagocytic activity and the presence of specific TJs[81]. TJs consist of three transmembrane proteins: mucin, occludin, and junctional adhesion molecule (JAM)[82]. Adhesive junctions consist of transmembrane proteins such as CDH5, which interact in a homophilic manner through their extracellular domains and connect to cytoplasmic domains linked to plaque proteins like CTNNB1, JUP, and CTNND1[83]. Junctional complexes, including adhesive junctions, form a continuous, zipper-like seal between neighboring ECs, acting as gatekeepers to regulate paracellular permeability. These structural features of the BBB enable the selective transport of substances from the blood to neural tissues[84]. They precisely control molecule exchange, maintaining brain equilibrium and shielding neural parts from toxin damage[85]. Therefore, the BBB acts as a complex barrier with physical, transport, metabolic, and immune components, maintaining a stable and precise environment within the CNS[86]. After IS, increased permeability through both paracellular and transcellular pathways, along with significant EC damage, allows blood-derived cells, chemicals, and fluids to infiltrate brain tissue *via* the compromised BBB. This breach disrupts water and ion balance in the brain, leading to cerebral edema. The subsequent infiltration of leukocytes further amplifies inflammation, worsening neuronal injury[87–90].

3.1.1. *The mechanism of BBB injury*

The disruption of the BBB during IS is a complex, multi-stage process driven by interconnected molecular and cellular mechanisms[91–97]. Initially, lack of oxygen and nutrients directly damages ECs, leading to the release of ROS and pro-inflammatory cytokines such as TNFA and IL1B[98–103]. These mediators further impair endothelial function and activate downstream pathways that weaken barrier integrity. A key factor in the early stage of

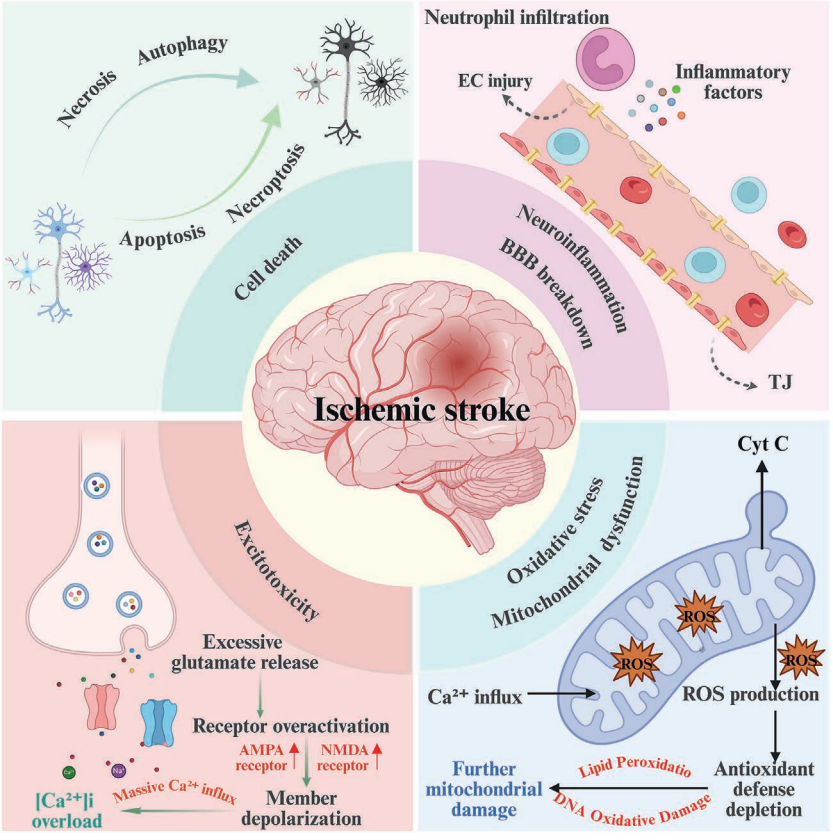

Figure 3 IS pathogenesis involves interconnected pathways: neutrophil infiltration damages the BBB through endothelial injury and inflammation; glutamate excitotoxicity causes calcium overload and depolarization; mitochondrial ROS production leads to oxidative stress and releases cytochrome *c* (Cyt C), resulting in neuronal death *via* necrosis, apoptosis, and necroptosis. © By Biorender.

ischemia-reperfusion (I/R) injury is Caveolin-1 (Cav-1), a scaffold protein essential for EC endocytosis. Focal brain I/R increases nitric oxide (NO) production, which promotes Cav-1 degradation. The reduction of Cav-1 then activates MMPs[104], especially MMP9, leading to the breakdown of TJ proteins such as zonula occludens-1 (ZO-1) and OCLN, which are essential for maintaining the BBB integrity[105−108]. The breakdown of TJ complexes is key to weakening BBB integrity. Typically, CLDN5 forms tightly packed strands at cell−cell junctions, with ZO-1 anchoring these proteins to the cytoskeleton, helping to prevent harmful molecules from passing through. Additionally, OCLN helps stabilize these junctions and adjusts barrier function permeability[85,109,110]. During IS, the behavior of TJs is significantly disrupted. In rat models of middle cerebral artery occlusion (MCAO), CLDN5 dissociates from cell membranes within 2 h of ischemia, a change associated with increased expression of Cav-1 in ischemic brain ECs (BECs)[111]. At the same time, levels of ZO-1 and OCLN decrease significantly, as shown in cerebral CI/R models. These changes weaken TJ clusters, transforming their continuous "bead-like" structures into broken lines. As a result, this disruption allows plasma components and inflammatory cells to leak into the brain tissue[112,113]. The activation of MMPs exacerbates damage to the BBB. During r-tPA thrombolysis following a stroke, r-tPA can directly activate MMP9, resulting in elevated levels of this enzyme[114]. Ischemic conditions stimulate the proteolytic activity of both MMP2 and MMP9, which degrade TJ proteins and the MCAO surrounding cerebral microvessels. This dual degradation process compromises endothelial adhesion, rendering the neurovascular unit vulnerable to infiltration by immune cells[115]. Research utilizing murine stroke models has demonstrated a significant increase in active MMP9 levels post-ischemia[116], which is associated with hemorrhagic transformation and the formation of edema. Furthermore, ischemia-induced inflammatory cascades upregulate the expression of endothelial adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), thereby facilitating leukocyte recruitment and further compromising the integrity of the barrier[117,118].

Beyond established mechanisms, neutrophil extracellular traps (NETs) have become key mediators of BBB disruption. Cerebral ischemia triggers extensive neutrophil infiltration and NET release through NETosis. These web-like structures, composed of decondensed chromatin DNA, histones, and granular proteins, worsen barrier damage by: (1) directly damaging ECs with histones[119], (2) causing mechanical disruption with physical structures, and (3) activating platelets and inflammatory pathways that sustain neuroinflammation[120]. As a result, targeting NETs provides a new therapeutic approach for protecting the BBB after IS. Due to their high specificity, effective NET clearance may require carefully designed EV strategies. Paradoxically, although reperfusion therapy is the primary clinical method to restore cerebral blood flow, it can also worsen BBB injury. During reperfusion, the renewed oxygen supply increases oxidative stress, leading to excess ROS that damage endothelial membranes and TJ proteins[121,122]. This phase also reactivates MMPs and inflammatory pathways, leading to secondary edema and leukocyte infiltration. Consequently, reopening the BBB after reperfusion presents a double-edged sword: it temporarily restores nutrient supply, yet simultaneously exacerbates neuroinflammation and tissue damage[123].

IS-induced BBB injury involves a complex network of parallel, self-reinforcing mechanisms, raising the core dilemma of broad multi-target intervention versus precise key pathway blockade (Fig. 4). EV therapy addresses this challenge through its inherent capacity for multi-target modulation and engineering, offering a flexible therapeutic solution.

3.1.2. EV-based BBB injury intervention for IS treatment

The disruption of the BBB caused by IS involves complex pathophysiological mechanisms. EV-based therapies are promising due to their various mechanisms of action. Medium to large EVs (m/lEVs) derived from ECs can transfer functional mitochondria to ischemic BECs, directly reducing oxygen-glucose deprivation (OGD)-induced ATP depletion and mitochondrial dysfunction. Research by Dave et al.[124] showed that m/lEVs not only incorporate into the recipient's mitochondrial networks to enhance ATP production but also decrease paracellular permeability when combined with heat shock protein 27 (HSP27). In mouse models, the intravenous injection of m/lEVs significantly reduced cerebral infarct volume, emphasizing mitochondrial rescue as a new strategy to protect the BBB. Beyond mitochondrial transfer, EVs also provide protective effects by modulating key signaling pathways. Zhang et al.[125] reported that MSC-EVs inhibit NF-κB signaling, which counters ischemia-induced MMP9 secretion and *ABCB1* upregulation in astrocytes, thereby reducing basement membrane degradation and BBB hyperpermeability. These findings collectively show that EVs help preserve BBB integrity through multiple mechanisms, including mitochondrial rescue and the regulation of key pathways. Mesenchymal stem cell-derived EVs (MSC-EVs) play an important role in maintaining BBB stability by regulating several pathways. Qiu et al.[126] demonstrated that MSC-EVs suppress astrocyte activation and neuroinflammation through the *miR-125b-5p*/TLR4/NF-κB axis, thereby reducing hemorrhagic transformation in r-tPA-treated stroke models. At the same time, ESC-EVs support the restoration of TJ proteins, such as ZO-1 and OCLN, which help maintain the barrier's integrity. Supporting these findings, Li et al.[127] studied the protective effects and mechanisms of small EVs (sEVs) from bone marrow MSCs (BMSCs) on BBB damage after IS. Their research showed that EV-based treatments decreased cerebral infarct size, reduced BBB permeability, lessened neuronal death, and improved blood flow and neurological function. Mechanistically, EVs increased the expression of TJ proteins ZO-1 and OCLN by suppressing the overexpression of Cav-1 in cerebrovascular ECs and its interaction with the autophagosome protein LC3B, thereby preserving the BBB integrity. Further *in vitro* experiments confirmed that Cav-1 promotes the degradation of TJ proteins by activating the autophagy−lysosomal pathway, and EVs reverse this process. The study revealed a new mechanism by which Cav-1-dependent autophagy disrupts BBB integrity after IS and showed that BMSC-EV therapy can protect BBB function by blocking this harmful cascade. Overall, these studies highlight the dual role of MSC-EVs in modulating both inflammatory responses and ECM remodeling. Engineered exosomes derived from M2 macrophages (M2exo) exhibit significant potential for targeted therapy. In a study by Wang et al.[128], M2exo@DNase, an engineered exosome designed to deliver DNase I, was developed to reduce pro-inflammatory IL1B levels and improve antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Transmission electron microscopy (TEM) analysis revealed that this treatment effectively reduces vascular gaps, restores TJ structures, and diminishes BBB damage in the infarcted brain regions of mice subjected to MCAO. These exosomes not only reduce neuroinflammation but also directly aid in

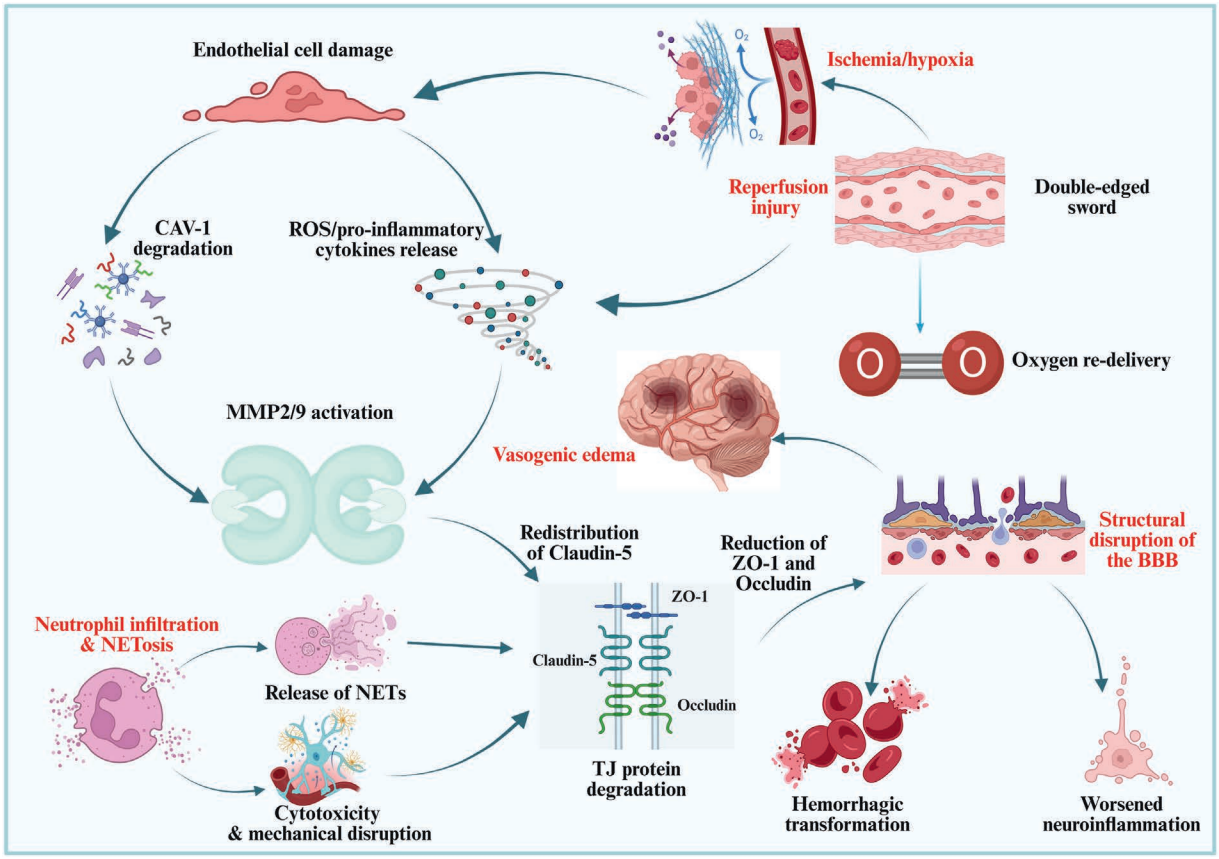

Figure 4 IS damages the BBB through endothelial injury, ROS, and inflammation. Upregulation of adhesion molecules promotes leukocyte infiltration and disrupts TJs, while MMP2/9 and ROS cause ECM breakdown and leakage, leading to edema, hemorrhage, and brain damage. © By Biorender.

repairing microvascular structures, thereby enhancing barrier protection. Microglial EVs are particularly effective at modulating the immune environment. Liu et al.[129] engineered platelet-mimetic exosomes (pEXO) carrying mannose-conjugated luteolin, which specifically target ischemic microglia and ECs. This method inhibits pro-inflammatory M1 polarization while promoting anti-inflammatory M2 phenotypes, thereby reducing infarct size and leakage of BBB damage. Furthermore, Pan et al.[130] demonstrated that interleukin-4 (IL4)-induced M2 microglial EVs suppress MMP3 and NF-κB p65 through *miR-23a-5p*, indirectly influencing neuron−glia interactions to preserve TJ protein levels. These findings offer new insights into multicellular repair mechanisms. Bone marrow stromal cell-derived small EVs (BMSC-sEVs) have unique advantages in controlling autophagy and TJ protein regulation. In a study by Li et al.[11], BMSC-sEVs were shown to inhibit *Cav-1*-dependent autophagy, reversing the degradation of TJ proteins ZO-1 and OCLN caused by OGD (Fig. 5). In rats with permanent MCAO, BMSC-EV treatment significantly increased ZO-1, CLDN5, and CD31 levels in the infarcted cortex, restoring BBB integrity. Importantly, whole-brain sections stained with 2,3,5-triphenyltetrazolium chloride (TTC), along with infarct volume analysis, showed that BMSC-sEVs greatly reduced cerebral infarction compared to the pMCAO group. Although ZO-1 and CLDN5 levels decreased after pMCAO, BMSC-EV treatment restored their expression. Western blot results supported these findings. Early ischemia−reperfusion also causes Cav-1 overexpression, which worsens TJ protein endocytosis. BMSC-sEVs help by inhibiting this pathway, thereby protecting barrier function and promoting long-term neurological recovery, demonstrating their targeted therapeutic potential.

Despite the mechanistic diversity of EVs from different sources, their main goal is to facilitate the comprehensive repair of the BBB. This is accomplished by stabilizing TJ proteins, inhibiting MMP activity, and modulating inflammatory signaling pathways. Innovations, such as platelet-mimetic surface modifications, enhance targeting to ischemic areas[131], while EVs-loaded microRNAs offer precise regulation of inflammatory responses[132,133].

3.2. *Neuroinflammation*

3.2.1. *The mechanism of neuroinflammation*

IS compromises the protective role of the BBB by abruptly stopping blood flow, leading to a cascade of inflammatory responses[134−137]. Within minutes to hours, the ischemic event activates intravascular leukocytes, causing endothelial and parenchymal cells to release pro-inflammatory mediators, which significantly worsen brain tissue damage[137].

This initial injury quickly develops into a biphasic immune response involving both local CNS and systemic responses activation[136]. Fig. 6 illustrates the process of neuroinflammation in IS. Within the first 6 h after the stroke, blood leaking into the parenchyma triggers activation of various pathways, including cytotoxic, excitotoxic, oxidative, and inflammatory processes. Microglia and astrocytes, as the first cells to respond to blood

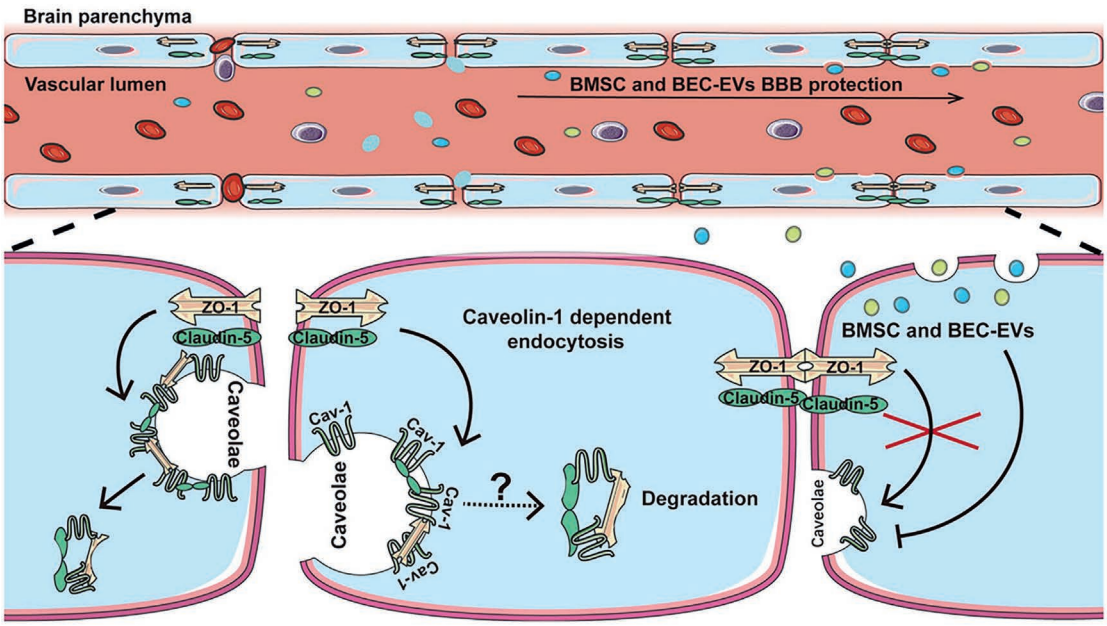

Figure 5 Schematic illustration of BBB structure and EV-mediated regulatory mechanisms: The upper panel shows the protective effects of BMSC/BEC-derived EVs on the BBB; the lower panel illustrates the dynamic regulation of ZO-1/Claudin-5 through Cav-1-dependent endocytosis to maintain barrier integrity. Reprinted with the permission from Ref. 11. Copyright © 2023 BioMed Central.

components in the tissue, trigger a critical sequence of events[138–140]. Microglial signaling promotes the recruitment of circulating macrophages and T cells, which subsequently release IL1B, TNFA, chemokines, and free radicals, all under the control of the transcription factor NF-κB[141–143]. NF-κB enhances the inflammatory response by increasing the production of adhesion molecules and inflammatory enzymes like inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (PTGS2), and phospholipase A2 (PLA2G4A)[144], along with additional cytokine components. These molecules, along with cellular debris from dying cells, create a self-perpetuating cycle of lymphocyte infiltration and ongoing neuroinflammation[145,146]. Notably, this inflammatory cascade increases the permeability of the BBB surrounding the ischemic core, allowing vasogenic edema to develop that worsens the mass effect and secondary ischemia. Consequently, these mechanisms drive inflammatory damage to the peri-infarct regions[146–148].

Neutrophils play a vital role as the first effectors in this pathological cascade. As the primary peripheral immune cells to infiltrate the brain, neutrophils reach their peak within 48 to 72 h after a stroke. This infiltration is mediated by TNFA, IL1B, IL8, and adhesion molecules, including VCAM1 and ICAM1[149,150]. In the context of sterile inflammation triggered by IS, damage-associated molecular patterns (DAMPs) released from necrotic cells persistently activate infiltrating neutrophils. This activation encourages them to initiate their innate immune responses, resulting in a significant release of ROS[151]. Paradoxically, this oxidative burst further compromises the structural integrity of the BBB, speeding up the progression of ischemic injury[152].

The neuroinflammatory cascade is not just a secondary event; it actively promotes neuronal death[153]. While the initial loss of blood flow following a stroke directly causes primary neuronal damage, the subsequent activation of neuroinflammatory pathways considerably worsens cell damage and death[154–158]. Extensive preclinical research has demonstrated that this secondary injury often surpasses the severity of the initial ischemic insult[136]. Therefore, prompt modulation of neuroinflammation has become a crucial therapeutic strategy, with the potential to decrease brain damage and enhance functional recovery after IS[159].

3.2.2. EV-based neuroinflammation regulation for IS treatment

Neuroinflammation plays a crucial role in worsening secondary brain injury after IS, often becoming more severe than the initial ischemic event. Therefore, timely modulation of this inflammatory response has become an important therapeutic goal. Recent research indicates that astrocytes, the most common cell type in the mammalian brain, have a dual role in managing neuroinflammation[160–163]. Astrocytes are known to release both pro-inflammatory mediators, like TNFA and IL6, which can lead to neuronal damage[162]. And anti-inflammatory mediators, such as IL10 and TGFβ, which help reduce neuroinflammation after IS. Therefore, regulating astrocyte functions to boost their anti-inflammatory effects is essential for promoting neurological recovery after IS. Recent research indicates that EVs (EC-EVs) from ECs may help decrease neuroinflammation by influencing astrocytic transcription factors such as activator protein 1 (AP-1). Signal transducer and activator of transcription 3 (STAT3), and NF-κB, thereby restoring their neuroprotective functions. Deng et al.[164] found that BMSC-derived exosomal *miR-138-5p* significantly inhibits neuroinflammation after IS. The mechanism involves negatively regulating the expression of *LCN2*, which in turn suppresses astrocyte apoptosis and the inflammatory response while promoting astrocyte proliferation. Additionally, Xin et al.[165] demonstrated that hypoxia-preconditioned microglial EVs (Hypoxia-EVs) significantly improve astrocyte dysfunction after stroke. These Hypoxia-EVs can restore the polarity of perivascular aquaporin 4 (AQP4), reduce pathological astrogliosis mediated by

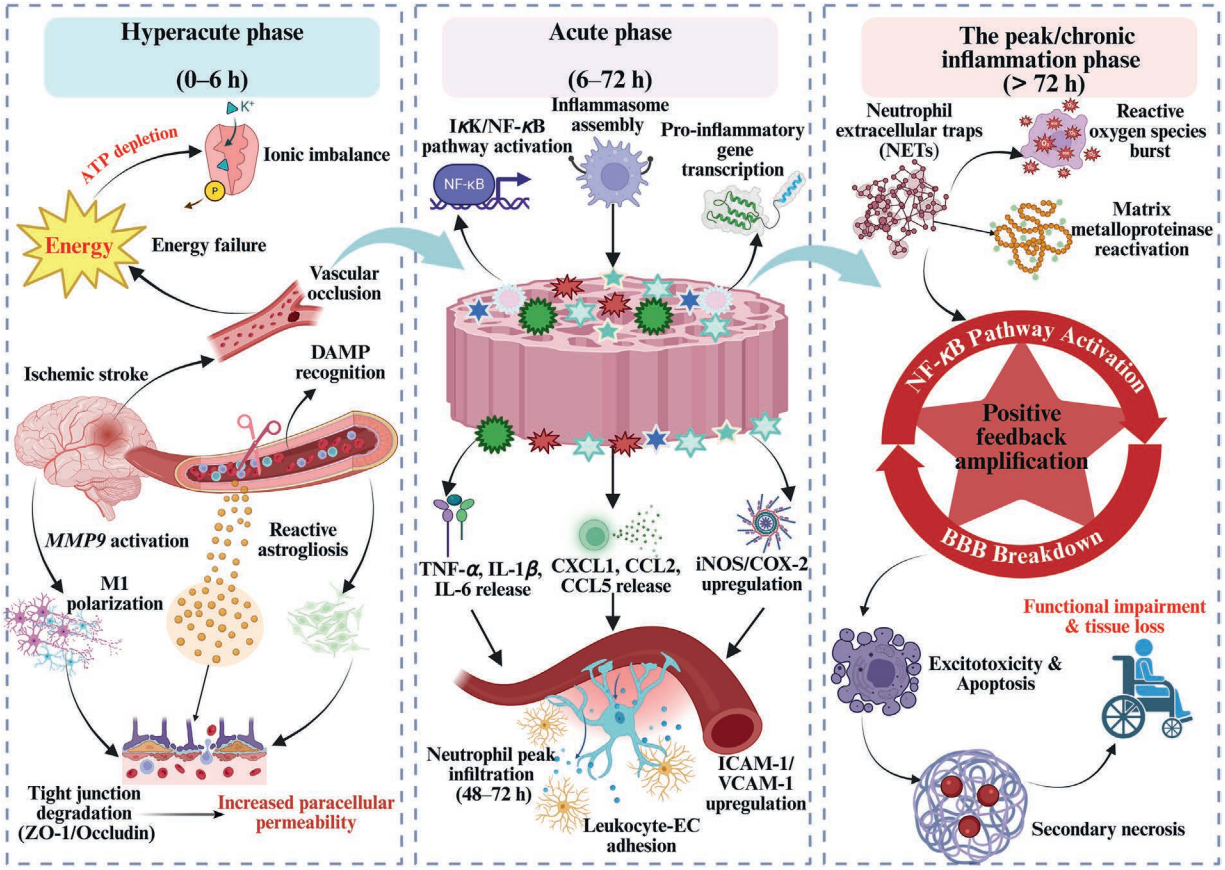

Figure 6 Post-ischemic neuroinflammation unfolds in three phases: the hyperacute phase (0−6 h), where ischemia leads to energy failure, ion imbalance, and vascular blockage. DAMP recognition activates *MMP9*, promotes microglial polarization, and triggers other processes, resulting in the breakdown of BBB TJs and increased permeability. The acute phase (6−72 h) sees activation of the IκK/NF-κB pathway and inflammasomes, causing the release of pro-inflammatory cytokines and chemokines. Neutrophil infiltration peaks, and leukocyte-endothelial adhesion occurs. The peak or chronic inflammation phase (>72 h) involves NETs formation, ROS burst, and *MMP* reactivation. NF-κB positive feedback further disrupts the BBB, leading to excitotoxicity, apoptosis, and secondary necrosis, which cause functional impairment and tissue loss. © By Biorender.

glial fibrillary acidic protein (GFAP), and lower pro-inflammatory cytokines such as TNFA and IL1B. This results in decreased neuroinflammation and alleviates cerebrospinal fluid (CSF) flow disruption. Mechanistically, Hypoxia-EVs are believed to deliver bioactive molecules, including microRNAs, that directly influence astrocyte phenotype by reversing their pro-inflammatory state and promoting cellular balance. These findings suggest that Hypoxia-EVs, by modulating communication between astrocytes and microglia, offer a promising new approach for neuroprotection following stroke. Qiu et al.[126] demonstrated that MSC-EVs have therapeutic effects on IS by influencing astrocytes. These MSC-EVs can cross the BBB and tend to accumulate in ischemic brain regions. Their presence reduces r-tPA-induced BBB disruption and hemorrhagic complications by decreasing astrocyte activation and inflammatory responses. Research shows that *miR-125b-5p*, delivered by MSC-EVs, is essential for maintaining BBB integrity by targeting Toll-like receptor 4 (TLR4) and inhibiting the NF-κB pathway in astrocytes. By suppressing astrocyte activation and related inflammation through the *miR-125b-5p*/TLR4//NF-κB pathway, MSC-EVs could serve as a new adjunct therapy for thrombolytic treatment in IS alongside r-tPA.

Microglia with anti-inflammatory traits play a crucial role in supporting brain recovery after IS by enhancing neuroprotective mechanisms. Therefore, promoting microglia to adopt an anti-inflammatory phenotype is essential in treating IS. For example, Jiang et al.[166] demonstrated that exosomes from adipose-derived stem cells (ADSCs) can deliver *miR-30d-5p*, which prevents autophagy-driven polarization of microglia to the M1 type, thereby reducing the area of cerebral infarction. Similarly, Zhang et al.[167] found that exosomes from human umbilical cord MSCs (hUMSCs) contain *miR-146a-5p*. This microRNA in hUMSC exosomes can decrease microglia-driven inflammation *via* the IRAK1/TRAF6 pathway after OGD. Animal studies have demonstrated that hUMSC exosomes significantly reduce infarct size, improve behavioral deficits, and induce a phenotypic switch in microglia. Their results indicate that hUMSC exosomes can reduce neuroinflammatory responses mediated by microglia, providing encouraging evidence for a cell-free therapy for IS. When IS occurs, neutrophils, the first peripheral immune cells to reach the brain, migrate toward the lesion sites through specific interactions between surface ITGB2/SELPLG and cerebral vascular endothelial ICAM1 and VCAM1. Building on this, Hu's team[71] developed a neutrophil-mimicking membrane vesicle delivery system using nitrogen cavitation technology, which maintains natural targeting abilities while reducing pro-inflammatory effects. Experimental results show that Rg1-loaded biomimetic

vesicles can accurately target ischemic lesions. When absorbed by microglia, these vesicles significantly enhance phagocytosis, inhibit the release of NETs, and reduce brain tissue damage. This delivery method offers new therapeutic insights for microglia-focused treatments by improving the post-stroke environment, supporting blood vessel repair, and advancing neurological recovery. In the early stage of IS, there is a temporary increase in anti-inflammatory M2 microglia, but their numbers quickly decline. Preventing this decline and controlling the inflammatory environment are essential for improving stroke recovery. Wang et al.[168] found that ferroptosis plays a key role in the rapid loss of M2 microglia. They used ADSC-derived exosomes (ADSC-Exo) to effectively decrease M2 microglia vulnerability to ferroptosis through the Fxr2/Atf3/Slc7a11 pathway, which suppresses inflammation and promotes neuronal survival. Both animal and cell studies demonstrated that M2-targeted exosomes (M2pep-ADSC-Exo) exhibit high specificity for M2 microglia, thereby preventing their ferroptosis and enhancing neurological function in stroke mice. Their research revealed that Fxr2 in ADSC-Exo decreases ferroptosis in M2 microglia by affecting Atf3/Slc7a11 levels, thereby reducing the inflammatory environment and aiding recovery after brain ischemia/reperfusion injury. These studies highlight a common treatment strategy: EVs from various cell sources, such as ADSCs, hUMSCs, and engineered versions, deliver specific miRNAs that inhibit key points in inflammatory signaling pathways, thereby guiding microglia toward a beneficial state. While this shared mechanism exists, the effectiveness and specificity of each EV type depend on their unique miRNA cargo and the pathways they utilize. For example, *miR-30d-5p* in ADSC-EVs primarily targets autophagy to suppress the pro-inflammatory M1 phenotype. At the same time, *miR-146a-5p* in hUMSC-EVs acts downstream to inhibit the IRAK1/TRAF6 pathway, thereby reducing the broad range of microglia-driven inflammatory responses. An advanced strategy involves engineering M2pep-ADSC-Exo, which maintains the anti-inflammatory properties of regular ADSC-EVs while providing improved targeting of M2 microglia. This directly protects this beneficial group from ferroptosis through the Fxr2/Atf3/Slc7a11 pathway. By intervening at multiple points, from initial polarization to stabilization, these EV-based strategies demonstrate complementary therapeutic potential. Selecting the EV source allows for customized treatments, whether aiming for broad M1 suppression, targeted enhancement of M2 microglia, or protecting M2 cells from inflammatory damage collapse. Additionally, modifying EV-producing cells can alter their EV composition, thereby conferring new functions. Jiang et al.[169] suggested using hypoxic pre-treatment of neural stem cells (NSCs) to simulate the low-oxygen conditions at IS sites. They collected exosomes from these pre-treated NSCs (H-EXO) for IS therapy (Fig. 7). The hypoxic environment altered the microRNA content in H-EXO. Their results demonstrated that H-EXO more effectively targets injured brain tissue than exosomes from regular sources of NSCs. After H-EXO treatment, inflammatory factors decreased significantly by Day 2, and by Day 4, levels were similar to those in control mice, with restored BBB integrity. MRI and TTC staining showed a substantial reduction in infarct size with H-EXO compared to MCAO. H-EXO also increased the number of endogenous NSCs in the injured area of the brain, enhancing the post-stroke environment and offering neuroprotection. In *Nestin-CreER* mice, hypoxic exosomes can stimulate the body's own NSCs, promoting nerve regeneration. While modifying cell culture conditions is a simpler way to change EV composition than engineering cells directly, this method still requires improvements in reproducibility and stability.

Previous sections explained how EVs from different cell sources, such as MSCs and NSCs, mediate key biological effects like neuroprotection and angiogenesis through specific miRNAs that regulate signaling pathways like the PTEN/Akt[170] pathway and the TLR4/NF-κB pathway[171]. These "source-EV-miRNA-pathway-efficacy" relationships form the core mechanism of this therapeutic approach. To make this complex information more intuitive and comparable, Table 2[170–184] provides a clear summary of these essential elements. This table not only highlights the functional differences of EVs from various sources but also helps readers quickly grasp the current research landscape and the future translational potential of the field.

Besides endogenous substances, external drugs can be directly incorporated into EVs to affect microglial cell polarization. For instance, exosomes from human neural stem cells (hNSCs)[185], known as hNSC-Exo, can be loaded with brain-derived neurotrophic factor (BDNF) to produce engineered exosomes BDNF-hNSC-Exo. These BDNF-hNSC-Exo have been shown to suppress microglial activation and reduce inflammation effectively, resulting in smaller infarct sizes and better neurological recovery function. Additionally, previous research suggests that macrophage-derived EVs can specifically target homotypic cells and influence the polarization of recipient macrophages[186]. Xu et al.[187] used exosomes derived from bone marrow-derived macrophages (BMDMs) loaded with 4-octyl itaconate. These exosomes, which effectively target macrophages and microglia (M/Ms), significantly reduce injury caused by MCAO. They achieve this by inhibiting M1 M/Ms activation and suppressing the release of pro-inflammatory cytokines, highlighting their potential for neuroprotective therapy in both prevention and treatment. Neutrophil-mediated inflammation provides a new therapeutic strategy. In ischemic regions, neutrophils congregate and release granular contents, forming a DNA-based network called NETs[188]. These NETs can promote inflammatory responses, contributing to tissue damage and vascular remodeling after a stroke. Breaking down NETs has been shown to reduce inflammation and improve neurological function recovery. However, the primary enzyme that degrades NETs, DNase 1, has a short half-life in the bloodstream and low efficiency crossing the BBB. To address this, Wang et al.[128] developed a $CD206^+$ M2-like macrophage-derived exosomal (M2exo) system to deliver DNase 1, aiming to enhance IS therapy (Fig. 8). The M2exo system can cross the BBB through endocytosis and tends to accumulate in the ischemic region. To evaluate the NET-inhibitory effects of M2exo@DNase 1, Neutrophil extravasation was observed using two-photon confocal microscopy. In the MCAO mouse model, Ly6G-labeled neutrophils extensively infiltrated the peri-infarct cortex, while they were nearly absent in sham controls. Immunostaining confirmed widespread neutrophil presence across the hemispheres in MCAO brains. Unlike in sham brains, further analysis showed intense H3Cit labeling in ischemic brains, indicating NET formation after neutrophil infiltration. The exosomal delivery system was essential for boosting DNase 1's ability to cross the BBB, aiding in the clearance of intraparenchymal NETs. Treatment with M2exo@DNase 1 did not change $Ly6G^+$ neutrophil counts but significantly decreased H3Cit expression. Additionally, SYTOX-labeled extracellular DNA fibers were nearly eliminated after treatment. The study showed an 82.5% reduction in NETs compared to MCAO controls. TTC staining revealed that DNase 1 and M2exo alone reduced infarct volumes by 12.9% and 39.9%,

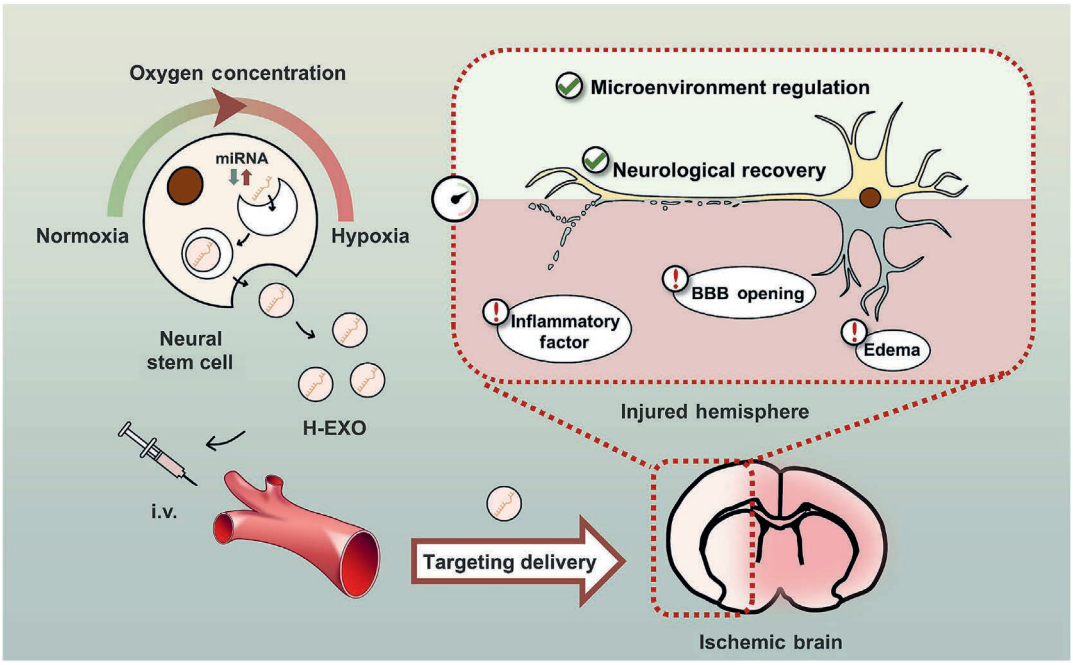

Figure 7 H-EXO, miRNA-enriched exosomes derived from hypoxic neural stem cells, target the ischemic hemisphere after intravenous delivery. They reduce neuroinflammation, strengthen BBB integrity, decrease edema, and promote neural repair and functional recovery through modulation of the microenvironment. Reprinted with the permission from Ref. 169. Copyright © 2023 Springer Nature.

respectively. The combined M2exo@DNase 1 therapy achieved a 71.2% reduction. Microglial polarization analysis showed minimal expression of $CD16/32^+$ (M1) or $CD206^+$ (M2) markers in sham-operated mice, indicating a resting M0 state. MCAO significantly induced $CD16/32^+$ M1 polarization, but M2exo treatment shifted polarization toward a $CD206^+$ M2 phenotype. ELISA results confirmed higher levels of TNFA and IL6 (M1 markers) and lower levels of IL10 and TGFB (M2 markers) in MCAO mice. Treatment with M2exo@DNase 1 increased IL10 and TGFB levels by 2.1-fold and 1.3-fold, respectively, compared to MCAO alone. Mechanistic studies suggest that *miR-124* promotes M2 polarization. M2exo secretes anti-inflammatory agents, cytokines. Encouraging microglia to adopt the M2 phenotype allows them to exert neuroprotective effects. Additionally, DNase 1 released by M2exo can degrade NETs, reduce inflammation, and support vascular remodeling. By inducing M2 polarization and clearing NETs in microglia simultaneously, M2exo@DNase 1 offers a promising approach for tissue repair after I/R injury.

3.3. Excitatory toxicity

3.3.1. The mechanism of excitatory toxicity

Excitotoxicity is increasingly recognized as the key molecular mechanism in post-IS situations, emphasizing glutamate and *N*-methyl-D-aspartate receptors (NMDARs)[189]. Although glutamate is an essential excitatory neurotransmitter in the mammalian CNS that facilitates neural communication, its harmful role becomes evident when its levels rise excessively[190]. This abnormal increase triggers excitotoxic cascades that are crucial during acute hypoxic-ischemic injury, especially in stroke[191]. The development of excitotoxicity begins with an energy deficiency. Ischemic injury causes ATP depletion, which significantly impairs the function of glutamate transporters, leading to a substantial buildup of glutamate outside neurons[192]. As a result, this excess glutamate overstimulates NMDARs on postsynaptic membranes, causing a large influx of sodium and calcium ions into the cell neurons[193–196]. Sodium entry mainly causes reversible neuronal swelling; however, the major harmful transition is characterized by calcium overload, which leads to irreversible excitotoxicity damage[197,198]. The brain's susceptibility to decreased blood flow causes rapid ATP loss in neurons during ischemic events, disrupting energy-dependent ion pumps like Na/K-ATPase (NKA) and Na^+/Ca^{2+} exchangers (NCX)[199]. This failure increases glutamate release and hinders its reuptake, disrupting normal metabolic processes and leading to severe intracellular calcium buildup overload[200].

Calcium is a vital intracellular messenger that influences numerous cellular functions, including excitability, exocytosis, motility, transcription, and apoptosis. During the first 30 min of ischemia, intracellular calcium levels spike sharply, providing a potential window for treatment[201]. The effects of this calcium overload are complex: Ca^{2+} activates destructive enzymes like calpain, which aids in proteolysis, and neuronal nitric oxide synthase (NOS1), which promotes cell death[202]. Additionally, calcium enters mitochondria through uniporters and exchangers, causing mitochondrial damage and dysfunction, the release of apoptotic factors, DNA damage, and ultimately neuronal death[203].

The neurotoxicity associated with NMDAR activation goes beyond calcium entry. The cytoplasmic tail of NMDARs interacts with key signaling molecules such as death-associated protein kinase 1 (DAPK1), nNOS, and cyclic AMP response element-binding protein (CREB), increasing neuronal damage[204]. Importantly, the origin and location of calcium entry influence its toxicity. Calcium influx through synaptic NMDARs is notably more damaging than influx *via* extrasynaptic NMDARs or voltage-gated calcium channels (VGCCs). Although VGCCs can

Table 2 Summary of therapeutic mechanisms of EVs from different cellular sources in IS *via* miRNA.

EV Source	Key miRNA	Main target/signaling pathway	Primary therapeutic effects in IS	Ref.
ADSC-exo	*miR-760-3p*	Targeting CHAC1	Reducing neuronal iron loss	172
BV2-M2-sEV	*miR-124*	Regulating the AAK1/Notch1 signaling pathway	Promoting the differentiation and proliferation of NSCs	173
BV2-M2-sEV	*miR-124*	Regulating the *miR-124*/STAT3 signaling pathway	Reducing glial scar formation	174
BV2- M2-EVs-RVG29	*miR-221-3p/miR-423-3p*	Targeting the p38/ERK signaling pathway	Modulating apoptosis-related pathways, thereby promoting neuronal survival and improving outcomes	175
BV2-M2-EVs	*miR-23a-5p*	Directly targeting Olig3 to promote OPC differentiation	Promoting functional recovery and remyelination	176
BV2-M2-EVs	*miR-25-3p/miR-93-5p*	Suppressing the expression of TGFBR, PTEN, and FOXO3	Increasing NSC proliferation and neuronal differentiation, and reducing infarct volume after IS	177
MSC-EXs	*miR-132-3p*	Targeting ECs to downregulate RASA1, thereby upregulating Ras and PI3K phosphorylation	Alleviating cerebrovascular ROS production, BBB dysfunction, and brain injury.	178
MSC-EVs	*miR-125b-5p*	Targeting TLR4 to inhibit NF-κB signaling in astrocytes	Maintains BBB integrity by inhibiting NF-κB signaling in astrocytes	171
Neuronal-EVs	*miR-98*	Targeting the platelet-activating factor receptor to inhibit microglial phagocytosis	Reducing neuronal death and infarction, and improving neurological function in rodent stroke models	179
DPSC-EVs	*miR-877−3p*	Regulating the *Bclaf1*/Trp53 signaling pathway	Attenuating cerebral I/RI and reducing neuronal apoptosis, thereby promoting early neurological recovery	180
HUVEC-EV	*miR-155-5p*	Modulating the c-Fos/AP-1 signaling pathway	Protecting astrocytes against OGD injury and promoting neurological recovery	181
EPCs- EVs	*miR-126*	Regulating the PIK3R2/PI3K/AKT signaling pathway	Promoting angiogenesis, thereby reducing infarct volume, improving cerebral perfusion, increasing microvascular density, and lowering neurological deficit scores	182
LEVs	*miR-101a-3p*	Regulating the *miR-101a-3p*/c-Fos/TGF-β axis	Inhibiting neuronal apoptosis, thereby protecting against ischemic brain injury	183
RVG-FGF20-EVs	*miR-181b-5p*	Mediating the PTEN pathway	Promoting neuroplasticity and functional recovery	170
HuMSC-exo	*miR-1228-5p*	Inhibition of TRAF6 expression and activation of the TRAF6-NOX1 axis	Improving mitochondrial function and cognitive behavior	184

Abbreviations: CHAC1: Cation transport regulator homolog 1; AAK1: AP2 associated kinase 1; STAT3: Signal transducer and activator of transcription 3; p38/ERK: P38 mitogen-activated protein kinases/Extracellular signal-regulated kinases; Olig3: Oligodendrocyte transcription factor 3; OPC: Oligodendrocyte precursor cell; TGFBR: Transforming growth factor beta receptor; PTEN: Phosphatase and tensin homolog deleted on chromosome 10; FOXO3: Forkhead box O3; ECs: Endothelial cells; TLR4: Toll-like receptor 4; PIK3R2/PI3K/AKT: Phosphatidylinositol 3-kinase regulatory subunit 2/phosphatidylinositol 3-kinase/AKT kinase (protein Kinase B); c-Fos: Cellular FBJ murine osteosarcoma viral oncogene homolog; TRAF6: TNF receptor-associated factor 6; TRAF6-NOX1: TRAF6-nicotinamide adenine dinucleotide phosphate H (NADPH) oxidase 1.

sometimes lead to toxic calcium levels, they do not consistently cause neuronal damage, emphasizing the complex relationship between calcium toxicity, entry pathways, and downstream effects factors[205,206].

The complex nature of NMDARs is reflected in their dual functions. Receptors containing the GluN2B subunit are heavily associated with excitotoxicity and cell death during ischemic events, while those with GluN2A are linked to protective effects. Furthermore, activation of synaptic NMDARs usually promotes neuronal survival, unlike the mostly harmful effects of extrasynaptic activation. This apparent difference between synaptic and extrasynaptic locations, along with their GluN2A and GluN2B subunit compositions, highlights the potential for these receptors to mediate both neuroprotection and neurodegeneration in stroke. Ultimately, excessive NMDAR activity leads to high intracellular calcium levels, accelerating cell death pathways[207]. Therefore, the excitotoxic cascade in ischemia mainly begins with postsynaptic cell death mechanisms triggered by the glutamate-NMDAR pathway. Studies show that abnormal calcium entry is crucial in neuron death caused by ischemia[208−210]. Blocking this entry could help reduce calcium overload and offer neuroprotection. However, although calcium antagonists have shown promise in animal studies, their effectiveness in human trials has been limited[211]. This gap underscores the need for more comprehensive research on calcium ion behavior and regulation to gain a deeper understanding of excitotoxicity and develop more effective treatments. As a result, targeted interventions that block harmful NMDAR-mediated calcium entry offer hope for reducing neuronal damage in ischemia[212]. Fig. 9 illustrates the mechanism of excitotoxicity in IS.

3.3.2. *EV-based excitotoxicity reduction for IS treatment*

Glutamate receptor antagonists and calcium channel blockers have shown promise in reducing excitotoxicity and providing

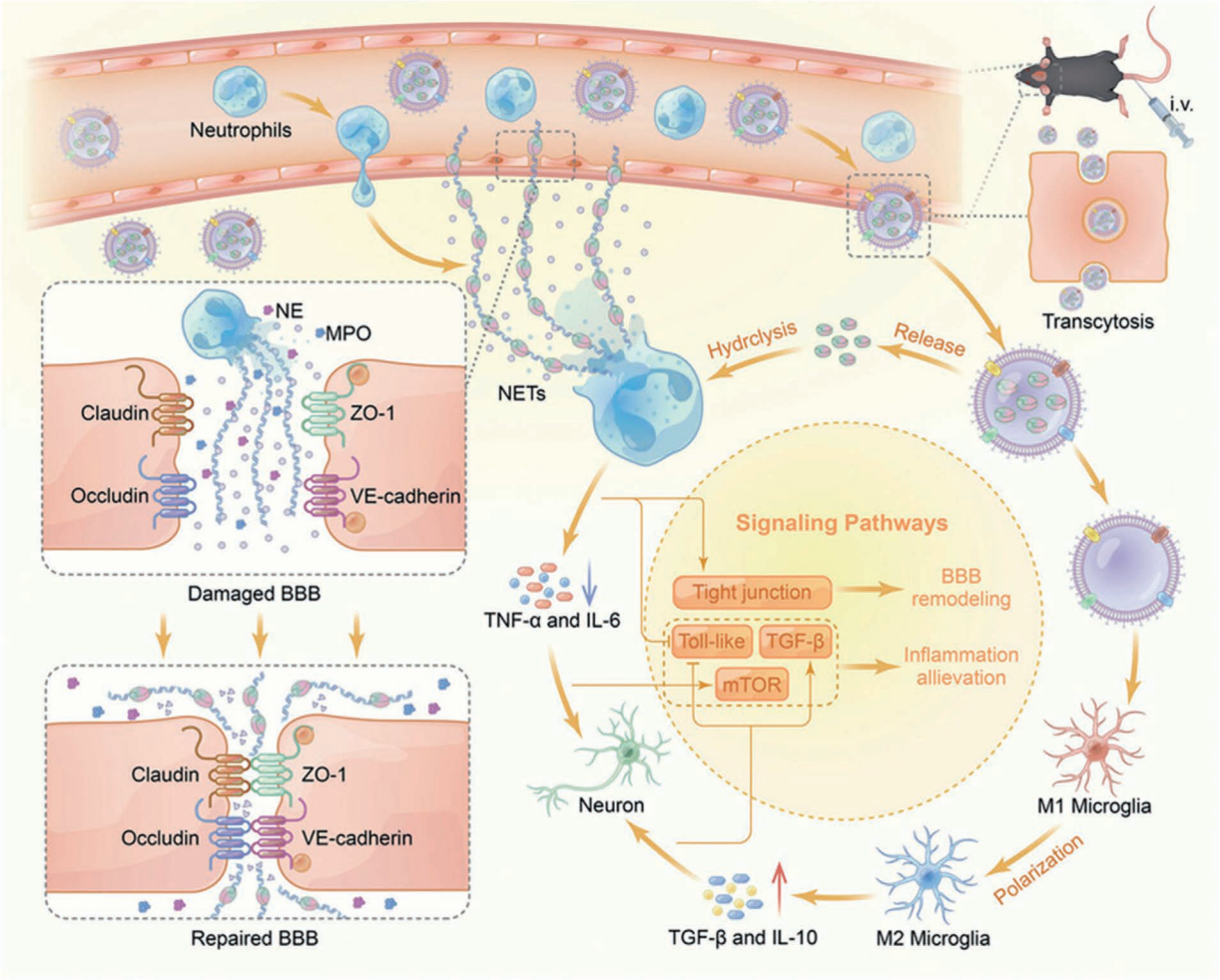

Figure 8 Exosomes from M2 macrophages have therapeutic effects in IS by reducing NET formation. M2exo@DNase1 targets and breaks down NETs, decreases inflammation, promotes BBB repair, and provides neuroprotection through M1−to−M2 microglial polarization by downregulating Toll/mTOR signaling and upregulating TJ/TGFB pathways. Reprinted with the permission from Ref. 128. Copyright © 2024 Wiley.

neuroprotection in treating IS. However, current therapies face challenges such as limited duration of effectiveness, suboptimal targeting, and inadequate control over drug release. Therefore, the search for innovative, effective, and safe strategies to modulate excitotoxicity and achieve more precise and sustained neuroprotection remains a key focus for future IS research. Evidence shows that activated microglia produce excessive NO *via* iNOS, causing neurotoxic effects. Elevated NO levels can inhibit essential enzymes in neuronal mitochondria, thereby triggering neuronal apoptosis. The Jin team conducted a comprehensive study on microglial iNOS expression following IS[179]. Their results showed that *microRNA-98* (*miR-98*), released by neurons *via* EVs, significantly suppressed iNOS expression on the third day after ischemia. This suppression confirms that *miR-98* reduces neurological damage in both mouse and rat models after IS. Further analysis showed that administering *miR-98* agomir significantly increased the number of Nissl-positive cells, indicating its neuroprotective effect against ischemia-induced neuronal injury in the cortex and striatum. Additional experiments showed that *miR-98* agomir reduced cerebral infarct size and enhanced neurological function in rodent models. Furthermore, in a model of lipopolysaccharide (LPS)-induced microglial inflammation, overexpression of *miR-98* effectively suppressed microglial activation. In summary, these results indicate that EV-derived *miR-98* reduces iNOS production by inhibiting the platelet-activating factor receptor (PAFR), thereby effectively mitigating inflammation-related damage.

Meanwhile, Zeng et al.[213] examined the complex mechanisms by which EVs and exosomes indirectly influence excitotoxicity through the regulation of mitochondrial quality control and autophagy in cerebral ischemia−reperfusion injury (CIRI). The study reveals that hyperactivation of dynamin-related protein 1 (Drp1) disrupts the breakdown of p62-labeled autophagosomes *via* the RIP1/RIP3 pathway, leading to the secretion of undegraded autophagosomes as pathological exosomes. These abnormal exosomes induce inflammatory responses, such as releasing TNFA, which further damages mitochondria by causing fragmentation and increasing ROS levels. This creates a harmful cycle: ROS impairs autophagy, which causes more release of pathological exosomes. Blocking excessive Drp1 activation reduces these pathological exosomes, lowers inflammatory cytokines, and indirectly decreases inflammation excitotoxicity. This process

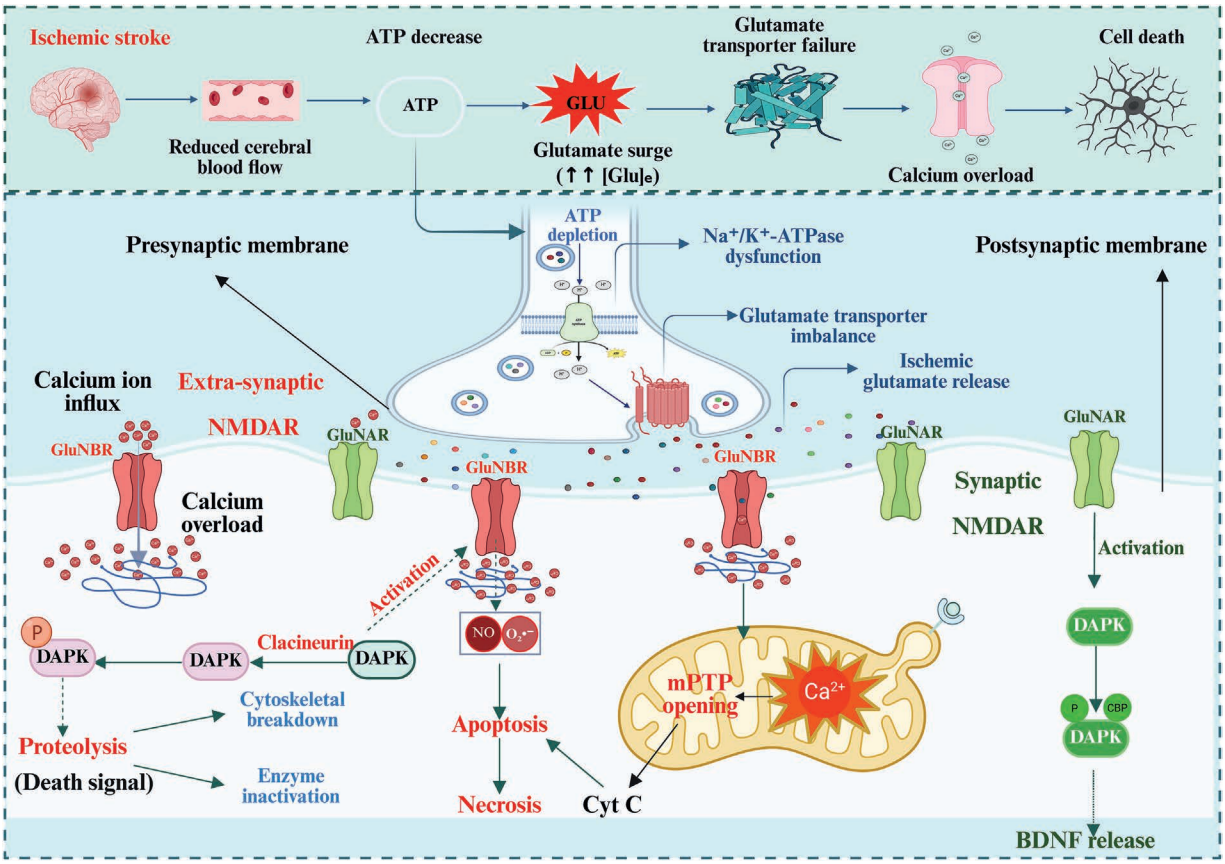

Figure 9 Ischemic excitotoxicity results from energy failure-induced glutamate buildup and NMDA receptor overactivation, leading to calcium overload. Presynaptically, Ca^{2+} activates DAPK, disrupting structure and signaling; postsynaptically, it causes mitochondrial failure and decreases BDNF. Continuous ionic and glutamate transport failure sustains neuronal death. © By Biorender.

involves reducing glutamate release caused by inflammation, preserving astrocytes' ability to uptake glutamate, and preventing overactivation of NMDARs. Under normal conditions, EVs help protect neurons by aiding in the removal of damaged mitochondrial components through controlled autophagy and reducing mitochondrial ROS leakage. This maintains mitochondrial calcium buffering, prevents calcium overload, supports ATP production for ion pump activity, and enhances antioxidant defenses. These processes are primarily controlled by three key factors: Drp1-Ser616 phosphorylation, which regulates mitochondrial fission and fusion; the activity of the RIP1/RIP3 pathway, which links autophagy failure to inflammation; and the integrity of p62-mediated autophagic flux, which controls the accumulation of misfolded proteins. Although this study mainly highlights the harmful roles of EVs in disease conditions, the inhibitory effects of normal EVs on excitotoxicity can be inferred by examining these pathological mechanisms in reverse. These insights offer a new understanding of EVs' complex role in modulating excitotoxicity during cerebral ischemia injury. As a result, EVs provide significant benefits in reducing post-stroke excitotoxicity through various neuroprotective mechanisms. By delivering therapeutic microRNAs, such as *miR-98*, EVs can inhibit microglial iNOS expression and NO-related neurotoxicity while preserving synaptic AMPA receptor density. Furthermore, their unique ability to regulate mitochondrial quality control through modulating Drp1 phosphorylation and p62-dependent pathways of autophagy enables simultaneous targeting of ROS buildup, calcium overload, and energy deficits.

3.4. Oxidative stress injury

3.4.1. The mechanism of oxidative stress injury

Oxidative stress is a well-established primary factor contributing to brain injury following cerebral ischemia[214]. It significantly impacts the pathological process through complex and diverse mechanisms, severely disrupting normal cellular physiology, causing irreversible structural damage, and ultimately leading to necrosis and apoptosis[214,215]. During a stroke, neurons face ischemic-hypoxic conditions, experiencing severe oxidative attacks on DNA that cause neuronal damage. This damage then triggers signaling pathways linked to cell death, leading to a chain reaction of programmed cell death that results in significant neurological consequences dysfunction[216,217].

A significant contributor to this damage is the substantial rise in ROS and reactive nitrogen species (RNS) within the brain following IS, similar to a destructive storm[218,219]. Although ROS are essential for basic biochemical functions and maintaining redox balance under normal conditions, excessive production that exceeds the body's regulatory ability can turn them into powerful agents of destruction. Under conditions of pathological oxidative stress, ROS severely target lipids, proteins, and DNA, creating a significant threat. These highly reactive molecules attack neurons

and glial cells, triggering a cycle of cellular redox stress that results in DNA damage and mitochondrial dysfunction, ultimately leading brain cells towards apoptosis[220,221]. This vulnerability is exacerbated by the inherent features of brain tissue, which are high in unsaturated fatty acids and lipids but low in antioxidant capacity, rendering it particularly susceptible to ROS-induced damage. Furthermore, the production of ROS and the resulting mitochondrial damage form a self-sustaining cycle during stroke, thereby increasing tissue damage[222].

Under typical physiological conditions, a robust defense mechanism is present. A variety of antioxidant enzyme systems, including SOD, catalase, and GSH-Px, act as vigilant protectors, forming a formidable barrier against free radicals and carefully maintaining cellular oxidative balance. SOD, as a key free radical scavenger, plays an essential role by effectively removing superoxide radicals and preventing the invasion of oxygen-free radicals. It provides significant protection against ischemic brain injury, especially during I/R, by decreasing peroxidation products and boosting cortical anti-hypoxic responses capacity[223,224]. SOD and GSH-Px show a synergistic relationship, working together to scavenge radicals and prevent oxidative damage[224–227]. However, ischemic insult significantly disrupts this balance. After IS, the activities of SOD and GSH-Px decrease sharply, leading to considerable mitochondrial structural and functional damage, which worsens brain injury and neurological deficits. This decline in antioxidant enzyme activity, combined with the brain's natural deficiency in antioxidants, allows ROS to multiply unchecked, thereby heightening mitochondrial damage and becoming major contributors to stroke progression.

The condition often worsens significantly after reperfusion. Studies show that the sudden return of blood flow usually doesn't save the damaged tissue and instead makes the situation worse, leading to a more dangerous state known as CIRI[228–230]. This complex process involves multiple mechanisms, including oxidative stress, inflammation, energy imbalance, and mitochondrial dysfunction, which start during ischemia but become much more severe during reperfusion. During CIRI, oxidative stress peaks to a critical level. When oxidant production greatly exceeds the body's ability to clear them, ROS build up significantly, causing the oxidation of DNA, lipids, and proteins, which accelerates cell death and worsens neurological damage and dysfunction[231,232]. Reperfusion is characterized by a surge in ROS, such as hydroxyl radicals, superoxide anions, and hydrogen peroxide, which have a high affinity for membrane lipids. This interaction triggers lipid peroxidation, produces harmful peroxides, increases membrane permeability, and severely damages structural integrity. Further exacerbating the damage, interactions between ROS and RNS generate powerful oxidants, such as peroxynitrite, which further exacerbate tissue injury[233–235].

A thorough understanding of how ROS interacts with antioxidant defenses can form the basis for more targeted therapeutic strategies to reduce brain injury and improve neurological outcomes. Research in this area not only clarifies the pathophysiology of stroke but also offers vital insights for developing innovative treatments and drugs. Fig. 10 shows how oxidative stress injury occurs in IS.

3.4.2. EV-based oxidative stress elimination for IS treatment

The suppression of oxidative stress is essential for effective neural repair after a stroke. Zhang et al.[236] addressed a significant challenge in stem cell therapy for stroke, specifically the low survival rate of transplanted cells in the microenvironment caused by oxidative stress from I/R injury. By co-transplanting NSCs with their secreted exosomes, the researchers showed that exosomes can significantly improve the transplant environment through three main mechanisms: (1) directly reducing ROS-mediated oxidative damage; (2) regulating oxidative stress-related pathways *via* miRNAs found in the exosomes; and (3) supporting the survival and differentiation of transplanted NSCs in the infarcted area. Although exosome treatment alone did not yield significant therapeutic results, its primary role in specifically reducing oxidative stress led to notably better neurological recovery in the co-transplantation group compared to NSC transplantation alone. This study provides mechanistic evidence supporting the role of exosomes as enhancers of stem cell therapy, with a particular emphasis on their crucial role in mitigating the oxidative stress microenvironment through miRNA-regulated networks.

Building on these findings concerning exosome-mediated regulation of oxidative stress, Ma et al.[237] demonstrated that *Rab27a*-dependent exosomes confer protection against cerebral ischemia by mitigating oxidative stress through the NOX2/ROS pathway. Ischemic conditions were found to increase *Rab27a* expression and exosome secretion in peri-infarct regions, with *Rab27a* knockout (*Rab27a-KO*) mice showing higher vulnerability to ischemic damage. Exosomes from wild-type mice (EXWT) had significantly stronger, dose-dependent antioxidant effects compared to those from *Rab27a*-deficient mice. Additionally, EXWT more effectively restored cerebrovascular function in *Rab27a-KO* mice. These findings suggest that *Rab27a*-mediated exosome secretion is a new therapeutic target for IS.

To further clarify the molecular mechanisms of EV-mediated neuroprotection, Sun et al.[238] suggested using EVs derived from the probiotic Lactobacillus reuteri (LrEVs) for the treatment of IS. The primary mechanism involves the dual suppression of oxidative stress damage (Fig. 11). LrEVs achieve highly efficient BBB penetration through peptidoglycan-specific targeting of the upregulated Toll-like receptor 2 (TLR2) in ischemic brain regions. The researchers studied the neuroprotective effects of LrEVs in a rat model of middle cerebral artery occlusion/reperfusion (MCAO/R). TTC staining was used to evaluate cerebral infarction in the rat brains, showing a significant infarction area of 47.9% in MCAO/R rats. 24 h after injection, exosomes from ECs (EC-EXO) reduced the infarct area to 30.1%, while LrEVs treatment proved more effective, decreasing the infarct volume to 21.3%. Moreover, LrEVs-treated MCAO/R rats showed significantly fewer neurological deficits compared to both the MCAO/R and EC-EXO groups. To further investigate the antioxidant capacity of LrEVs, intracellular ROS levels were measured in SH-SY5Y cells subjected to oxygen-glucose deprivation/reperfusion (OGD/R) using the DCFH-DA probe. The OGD/R treatment significantly increased ROS production. However, EC-EXO did not show a significant ROS-scavenging effect. In contrast, lactobacillus-derived EVs (LrEVs) effectively reduced OGD/R-induced ROS production, leading to a significant decrease in fluorescence intensity. *In vivo* assessments of ROS levels in infarcted brain tissues supported these findings: MCAO/R rats exhibited higher ROS levels compared to sham controls, while LrEVs, unlike EC-EXO, showed significant ROS clearance, effectively decreasing ROS in the tissues. Malondialdehyde (MDA) assays also indicated that both OGD/R and MCAO/R significantly increased MDA levels *in vitro* and *in vivo*. While EC-EXO slightly decreased MDA levels, LrEVs treatment significantly lowered MDA levels, emphasizing their protective role against oxidative stress in ischemic brain conditions. Bio-TEM imaging of mitochondrial

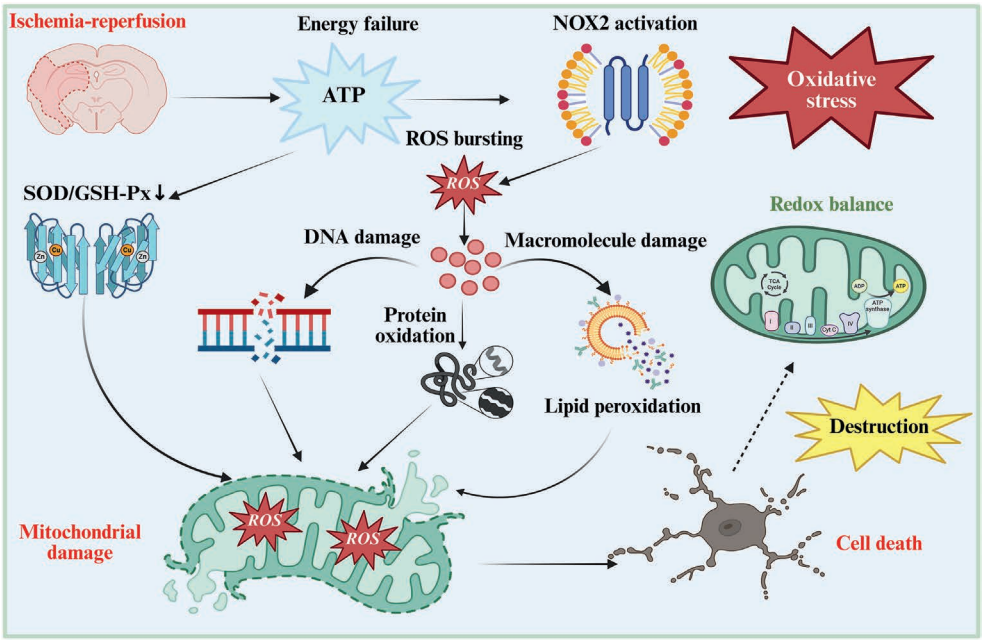

Figure 10 I/R injury involves energy failure and weakened antioxidant defenses, leading to a NOX2-driven ROS burst. This results in oxidative damage to biomolecules and mitochondrial dysfunction, which sustains a self-amplifying ROS cycle, causing irreversible cell death. © By Biorender

morphology showed that control samples had intact double-membrane structures with well-defined cristae. In contrast, OGD/R caused cristae disruption, swelling, and partial dissolution of the membrane. While EC-EXO partially alleviated these mitochondrial abnormalities, LrEVs significantly preserved mitochondrial function integrity. Mechanistically, LrEVs not only directly scavenge excess ROS but also modulate microglial polarization through the MAPK and NF-κB signaling pathways, thereby effectively reducing post-ischemic oxidative stress injury. These findings suggest a new therapeutic approach using probiotic-derived vesicles for targeted delivery and ROS removal in treating neural damage injuries.

Furthermore, the study by Zhao et al.[239] explained the main way human neural stem cell-derived exosomes (hNSC-Exos) reduce oxidative stress in CIRI by influencing the mitophagy pathway. The study showed that hNSC-Exos effectively activate the PINK1/PARKIN signaling pathway, promoting the removal of damaged mitochondria and significantly decreasing ROS levels. RNA sequencing revealed a significant increase in lysosomal activity under oxidative stress conditions, supporting the role of hNSC-Exos in maintaining intracellular redox balance by enhancing mitophagy. Experimental findings confirmed that hNSC-Exos can cross the BBB to target damaged areas, improve neurological deficits, and reduce cerebral infarction volume in MCAO rats. Compared to common clinical antioxidant drugs, hNSC-Exos show better targeting abilities and higher bioavailability. This study provides crucial evidence for the development of exosome-based antioxidant therapeutic strategies. Pan et al.[240] demonstrated that exosomes derived from endothelial progenitor cells enriched with ACE2 (ACE2-EPC-Exos) significantly reduce oxidative stress injury after ischemia in aged mice through the *miR-17-5p*/PTEN/PI3K/*Akt* signaling pathway. Their findings show that ACE2-EPC-Exos dramatically increase *miR-17-5p* expression, decrease PTEN protein levels, and activate the PI3K/*Akt* pathway. This results in a significant reduction of ROS levels in the ischemic brain area and improved mitochondrial function. Compared to traditional EPC-derived exosomes, those enriched with ACE2 display notably higher antioxidant activity. To further improve the brain-targeting ability of EVs, Hu et al.[184] developed a magnetic nanoparticle (Spion)-modified exosome delivery system (Spion-Ex). Spion-Ex was observed to upregulate *miR-1228-5p*, thereby suppressing the TRAF6/NOX1 signaling pathway and significantly lowering ROS levels. This intervention effectively restored the structural integrity and function of mitochondria, including the membranes and cristae, after ischemic events. Additionally, Spion-Ex facilitated about a threefold increase in hippocampal exosome accumulation through magnetic guidance, without causing hepatorenal toxicity. This targeted delivery system presents a novel therapeutic approach for addressing cognitive impairment following stroke.

It is essential to acknowledge that certain types of EVs may contribute adversely to oxidative damage. Huang et al.[241] demonstrated that OGD/R treatment significantly elevated ROS levels in N2A cells and umbilical cord MSCs (UC-MSCs) in a time-dependent manner, while simultaneously decreasing ATP content, SOD activity, and total antioxidant capacity. Notably, the conditioned medium derived from injured neurons and their secreted EVs further intensified oxidative stress in UC-MSCs, as evidenced by increased ROS levels and decreased antioxidant indicators. This detrimental effect was more pronounced under OGD/R conditions and exhibited a concentration-dependent pattern for EVs. Mechanistic investigations have demonstrated that inhibiting neuronal EV secretion *via Rab27a*-siRNA leads to a significant reduction in oxidative stress levels within umbilical cord-derived MSCs (UC-MSCs). Notably, hypoxic preconditioning significantly enhances the resilience of UC-MSCs to oxidative stress by upregulating the expression of hypoxia-inducible factor 1A (*HIF1A*). Hypoxia-preconditioned UC-MSCs exhibit lower

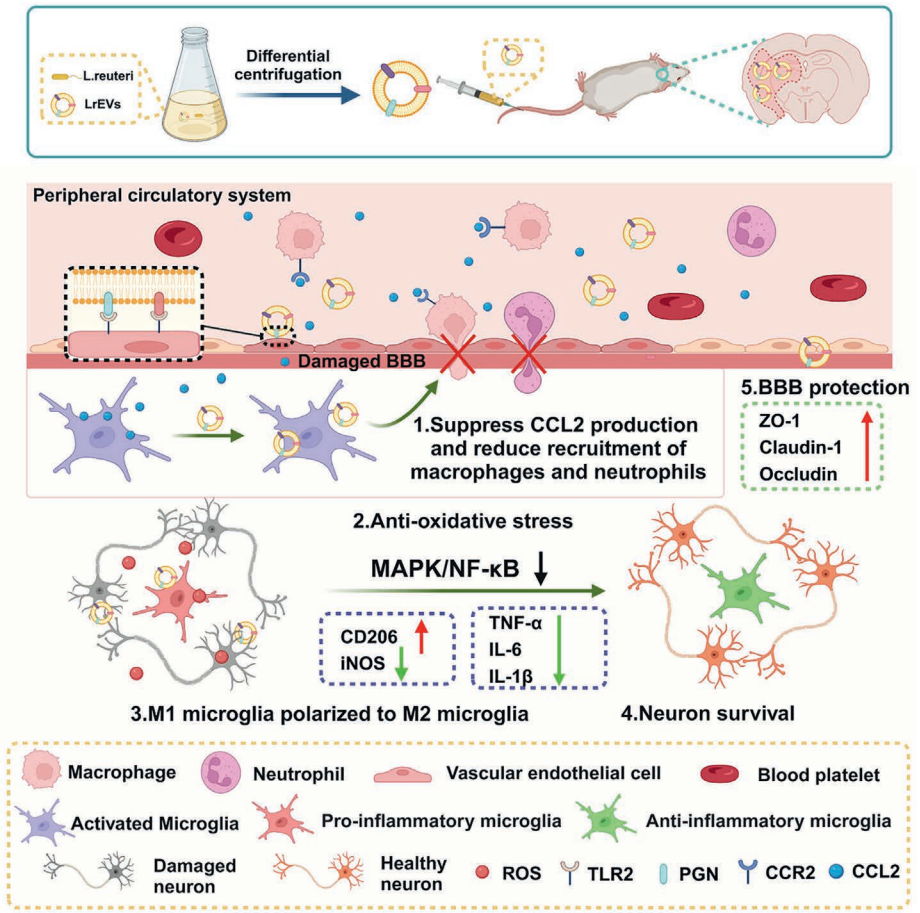

Figure 11 LrEVs cross the BBB *via* PGN-TLR2 interaction on brain ECs. In the ischemic brain, they protect neurons by scavenging ROS and modulating microglial polarization through the MAPK/NFκB pathways. At the same time, LrEVs suppress CCL2 expression, reduce infiltration of peripheral macrophages and neutrophils, and help maintain BBB integrity. Reprinted with the permission from Ref. 238. Copyright © 2025 American Chemical Society.

levels of ROS and an increased antioxidant capacity, even when exposed to conditioned media from injured neurons or elevated concentrations of EVs. These findings suggest that strategies targeting the suppression of EV release from injured neurons or the augmentation of antioxidant defenses through hypoxic preconditioning may be crucial for enhancing the survival of transplanted MSCs. This study highlights the critical role of oxidative stress regulation in optimizing stem cell therapy for stroke, providing empirical evidence to support the development of microenvironment-targeted interventions.

EVs can also serve as a means to deliver exogenous drugs aimed at mitigating oxidative stress in the treatment of IS. Guo et al.[242] demonstrated that quercetin (Que) possesses the ability to scavenge ROS due to its distinctive molecular structure; however, its limited water solubility poses challenges for clinical application. To overcome this limitation, the researchers developed a GAP43 antibody-modified plasma exosome delivery system (mAb GAP43-Exo), which notably enhanced the stability and brain-targeting efficacy of Que. In both OGD-treated SH-SY5Y cells and MCAO/R rat models, Que/mAb GAP43-Exo exhibited significant antioxidant properties. These included: 1) a marked reduction in intracellular ROS levels and ROS accumulation in the ischemic penumbra; 2) activation of the Nrf2/HO-1 signaling pathway, leading to the upregulation of antioxidant proteins such as NQO-1, HO-1, SOD1, and GPx1; 3) promotion of Nrf2 nuclear translocation, thereby enhancing cellular antioxidant defenses. Mechanistic investigations revealed that this delivery system, facilitated by mAb GAP43, enables the targeted enrichment of Que in GAP43-overexpressing damaged neurons, thereby more effectively alleviating oxidative stress injury. These findings confirm that Que/mAb GAP43-Exo exerts its antioxidant effects *via* a dual mechanism: directly scavenging ROS and simultaneously activating endogenous antioxidant pathways. This represents an innovative, targeted therapeutic strategy for I/R injury.

In conclusion, recent advancements highlight the significant potential of EV-based therapies for IS, as they exert a multifaceted regulation of oxidative stress. EVs derived from NSCs, probiotics, and EPCs, as well as engineered EV systems, demonstrate superior neuroprotective effects by directly scavenging ROS, activating endogenous antioxidant pathways, modulating microglial polarization, and restoring mitochondrial function. Innovative targeted delivery systems, such as TLR2-mediated recognition and magnetic nanoparticle guidance, significantly enhance EV accumulation in ischemic regions while maintaining excellent BBB penetration. Compared to conventional antioxidants, EV-based approaches offer distinct advantages through their multi-mechanistic actions that concurrently address oxidative damage and facilitate tissue repair.

3.5. Cell death

3.5.1. The mechanism of cell death

Research has clearly demonstrated that multiple cell death pathways contribute to the development of IS[243,244]. These pathways include endogenous and exogenous apoptosis, necroptosis, autophagy, ferroptosis, and phagocytic cell apoptosis, which collectively promote neuronal loss[5]. Endogenous apoptosis is characterized by specific mechanisms such as calpain-mediated[245], ROS-mediated[246], and DNA damage-mediated pathways[247], whereas exogenous apoptosis can result from neural cascade reactions. Necroptosis, notably different from apoptosis, is a regulated form of cell death[248], with cerebral ischemia frequently inducing necroptosis of neurons within the vulnerable ischemic area penumbra.

The role of autophagy in IS is notably complex, characterized by its dualistic nature[249]. This evolutionarily conserved process involves the sequestration of organelles and cytoplasmic macromolecules within membranes to form autophagosomes, which subsequently undergo lysosomal degradation and recycling. Although autophagy generally plays a protective and pro-survival role, surpassing a critical threshold can paradoxically cause cell death[250]. The main regulatory pathways that induce neuronal autophagy during ischemia include the mechanistic target of rapamycin (mTOR) signaling pathway[251], HIF1A signaling[252], and endoplasmic reticulum (ER) stress[253].

A specific pathway that has attracted significant scholarly interest is ferroptosis. During cerebral ischemia, energy depletion causes ATP exhaustion and disrupts cellular ion gradients, leading to membrane depolarization and excessive release of glutamate[254]. The activation of glutamate receptors then triggers phospholipase activity, which breaks down membrane phospholipids to release arachidonic acid, while also inhibiting the lipid peroxide repair function of glutathione peroxidase 4 (GPX4)[255]. Furthermore, the buildup of intracellular redox-active iron works together with these processes, ultimately causing neuronal death through the mechanism of ferroptosis[256].

Concurrently, the apoptosis of phagocytic cells represents a distinct mechanism of cellular clearance. Cerebral ischemia causes an overload of intracellular Ca^{2+}, depletion of ATP, and bursts of ROS[257]. Microglia, serving as the brain's primary phagocytes, continuously monitor the health of neurons. They recognize "eat me" signals, such as the externalization of phosphatidylserine (PS) and the exposure of calreticulin, which are displayed by damaged cells[258,259]. Although the phagocytosis of dead or dying neurons offers anti-inflammatory benefits, adverse effects happen when microglia engulf healthy neurons, causing their demise[260,261]. Significantly, this process can be blocked by inhibiting phagocytosis. During ischemic events, surviving neurons often exhibit reversible PS exposure due to oxidative stress, primarily activating phagocytosis through the MERTK/MFGE8 pathway in microglia, ultimately leading to the loss of functional neurons[262,263]. Notably, experimental interventions that inhibit phagocytosis, such as complement inhibition, have demonstrated the potential to preserve neurons in the penumbra, thereby underscoring phagocytosis regulation as a promising therapeutic target[264].

The complex interactions of these different cell death pathways after IS (Fig. 12) pose significant challenges for treatment. However, an EV-based therapeutic approach might provide a new and promising solution intervention.

3.5.2. EV-based therapies for cell death

The survival of neurons is essential for maintaining brain homeostasis and functional integrity after IS. Recognizing this, Zhang et al.[265] demonstrated that EVs derived from OGD-pretreated microglia promote neuroprotection and functional recovery. Their research revealed that these EVs are enriched with TGFB1 and exert their beneficial effects in a TGFB1-dependent manner by targeting neurons and ECs, thereby activating the Smad2/3 signaling pathway. This finding not only clarifies a specific molecular mechanism but also highlights the therapeutic potential of microglial EVs in stroke recovery. Besides microglial sources, MSC-derived EVs also show therapeutic promise. Kim et al.[266] demonstrated that EVs and nanovesicles (NVs) from MSCs act as effective therapeutic agents for IS. By employing iron oxide nanoparticles (IONPs) for targeted delivery to the ischemic region, comprehensive *in vivo* and *in vitro* experiments elucidated the neuroprotective mechanism of MSC-derived nanovesicles (MNVs) in ischemic brain injury (Fig. 13). *In vivo* experiments showed that immunofluorescence analysis revealed a significantly higher density and proportion of MAP2-positive neurons in the MNV (+MF) group compared to the PBS and MNV (−MF) groups, thus confirming the effectiveness of MNV in promoting neuronal growth and survival. *In vitro* mechanistic studies revealed that MNV modulates microglial function through multiple pathways. Firstly, qPCR analysis showed that MNV treatment significantly reversed LPS/hypoxia-induced changes in apoptosis-related gene expression, characterized by a decrease in *Bax* and an increase in *Bcl-2*. CCK-8 assays further confirmed that MNV substantially improved microglial survival under stress conditions. Secondly, MNV treatment resulted in a significant decrease in M1 phenotype markers (iNOS, IL1B, and TNFA) and an increase in M2 phenotype markers (Arg-1, CD206, and IL10), indicating that MNV promotes the shift of microglia from a pro-inflammatory M1 state to an anti-inflammatory M2 state. Most notably, TUNEL assays showed a significant reduction in apoptotic cells (green fluorescence) in the MNV (+MF) group, with quantitative analysis indicating much lower levels of neuronal apoptosis compared to the control groups. This study demonstrates the effectiveness of MNVs in inhibiting neuronal apoptosis in ischemic brain regions through targeted delivery methods. The findings clarify the dual protective roles of MNVs in ischemic brain injury: directly preventing neuronal apoptosis and promoting cell survival, while also indirectly providing neuroprotection by regulating the apoptosis-survival balance of microglia and supporting the M1/M2 phenotype switch.

These insights provide a vital theoretical foundation for developing new therapeutic strategies for ischemic brain injury. The vesicles release pro-angiogenic and anti-apoptotic factors, which significantly promote cerebral angiogenesis and inhibit programmed cell death. Importantly, they also influence macrophage polarization toward an anti-inflammatory phenotype. Overall, these effects work together to decrease infarct size and enhance motor function, offering an innovative intervention strategy that combines vascular regeneration with anti-apoptotic mechanisms.

To improve the accuracy of EV therapy, various engineering methods have been employed. In a notable study, Tian et al.[267] developed a targeted delivery system by effectively conjugating the c(RGDyK) peptide to the surfaces of EVs using bioorthogonal reactions, resulting in the formation of cRGD-Exo. In a transient middle cerebral artery occlusion (tMCAO) mouse model, intravenous administration of these engineered EVs resulted in their

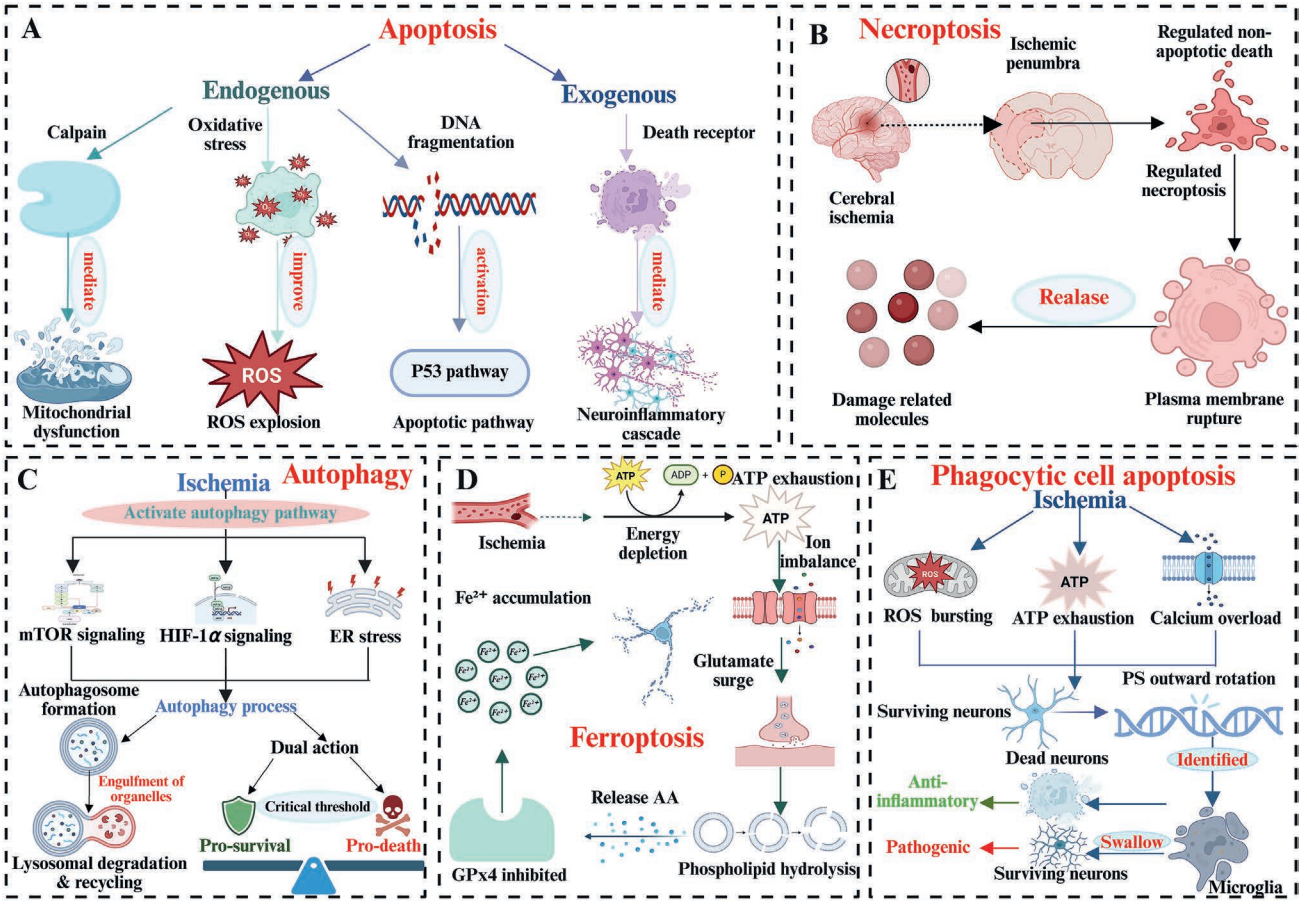

Figure 12 Ischemic injury involves five main cell death pathways. (A) Apoptosis through both intrinsic (mitochondrial/ROS/P53) and extrinsic (death receptor-mediated) pathways; (B) Necroptosis *via* regulated necrosis and membrane rupture; (C) Autophagy with dual roles, controlled by *mTOR/HIF1A*/ER stress; (D) Ferroptosis triggered by GPX4 inhibition and phospholipid peroxidation; (E) Phagoptosis characterized by phosphatidylserine exposure and microglial engulfment. © By Biorender.

specific accumulation in the ischemic lesion. Additionally, the incorporation of curcumin into the system (creating cRGD-Exo-Cur) significantly reduced inflammatory responses and neuronal apoptosis within the ischemic region. This study supports a novel EV-based approach for targeted drug delivery to the ischemic brain, confirming the viability and effectiveness of intravenous treatment for IS.

Stem cell-derived small EVs (sEVs) offer a promising therapeutic approach. Current research shows that embryonic stem cell-derived EVs (ESC-EVs) significantly improve stroke outcomes after intravenous administration. In a tMCAO model, Xia et al.[14] demonstrated that ESC-sEV intervention significantly inhibited leukocyte infiltration and neuronal apoptosis, thereby reducing cerebral infarct volume and improving long-term neurobehavioral outcomes and tissue integrity. Notably, the mechanism of ESC-EVs extends beyond direct cytoprotection. The study demonstrated that ESC-EVs facilitate the remodeling of the immune microenvironment by selectively enhancing regulatory T cells (Tregs), thereby overcoming the limitations of traditional cell therapies. This discovery provides a crucial theoretical foundation for developing cell-free, sEV-based drug delivery systems, marking a significant step toward precise immune regulation in the treatment of IS.

Furthermore, the study by Wu et al.[268] showed that EVs derived from hypoxic neurons, referred to as HypEVs, exhibit neuroprotective properties through a mechanism mediated by the fused in sarcoma (FUS) protein. The primary function of these vesicles is to inhibit mitochondria-related processes, such as apoptosis. FUS enhances the uptake of HypEVs and promotes the transfer of mitochondrial mRNA, thereby supporting neuronal survival. The knockdown of FUS significantly reduces the anti-apoptotic effects of HypEVs, highlighting FUS as the key molecule responsible for their neuroprotection function. These findings provide significant insights into the neuroprotective mechanisms mediated by HypEVs and underscore the potential therapeutic applications of Hypoxia-EV-based interventions for ischemic injury. In contrast, the study on HypEVs identifies a more mechanistically precise strategy, where the neuroprotective effect is attributed mainly to the specific action of the FUS protein in facilitating mitochondrial mRNA transfer, thereby inhibiting apoptosis.

Furthermore, targeting specific cell death pathways, like ferroptosis, requires the development of innovative delivery methods. The research team, led by Pan[269], addressed this issue by designing a biomimetic drug delivery system using bacterial elements, specifically OMVs. They carefully encapsulated the ferroptosis inhibitor pioglitazone (PGZ) within OMVs, forming OMV@PGZ nanoparticles. This system cleverly utilizes the natural chemotactic properties of neutrophils toward pathogen-

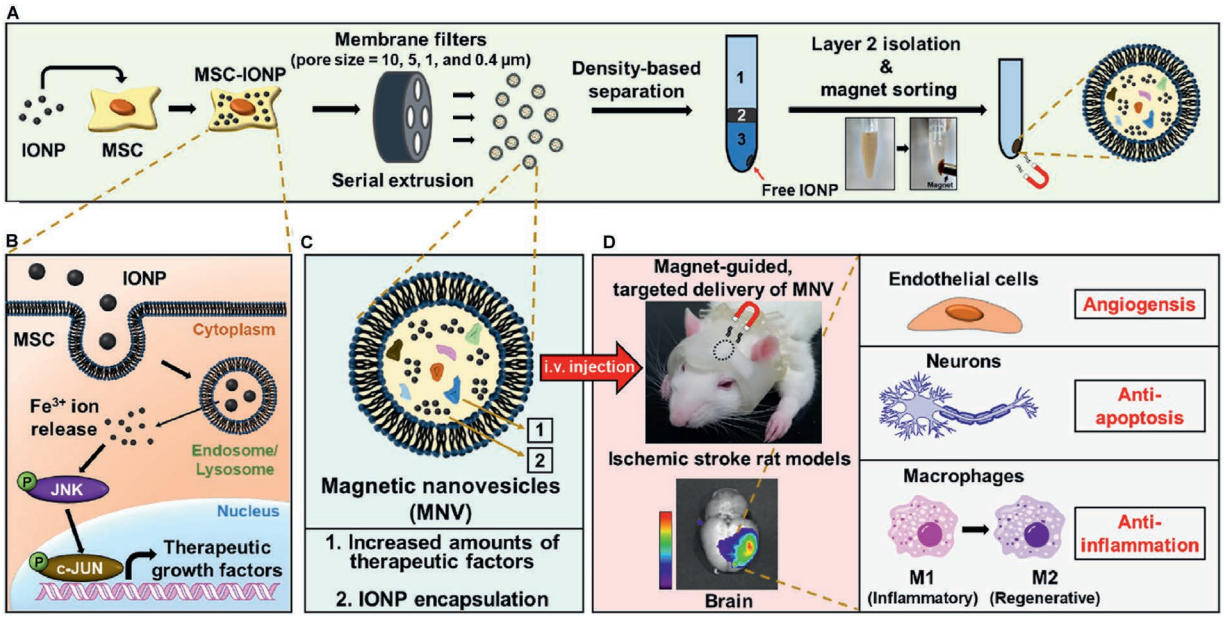

Figure 13 Schematic diagram of IS treatment using MSC-derived magnetic nanovesicles (MNVs). (A) Preparation of MNVs from MSCs preloaded with iron oxide nanoparticles (IONPs). (B) Intracellular upregulation of therapeutic growth factors in IONP-loaded MSCs. (C) MNVs loaded with increased levels of therapeutic factors and IONPs. (D) Magnetic helmet-guided targeted delivery of MNVs to the ischemic lesion in a rat model, followed by therapeutic actions on ECs, neurons, and macrophages within the affected area. Reprinted with the permission from Ref. 266 and adapted. Copyright © 2020 Elsevier.

associated molecular patterns (PAMPs) present on OMVs, facilitating targeted transport across the BBB to ischemic regions. Experimental findings revealed that OMV@PGZ provides neuroprotection through dual mechanisms: (1) inhibition of *NLRP3* inflammasome activation, thereby reducing neuroinflammation, and (2) disruption of ferroptosis pathways, leading to decreased lipid peroxidation damage. These effects collectively lead to a notable reduction in post-reperfusion infarct sizes and better clinical outcomes. The schematic diagram (Fig. 14) demonstrates the therapeutic potential of OMV@PGZ nanoparticles for IS. Quantitative histopathological analysis showed that OMV@PGZ treatment not only significantly increased the number of Nissl-stained neurons and the density of Luxol Fast Blue (LFB)-stained myelin but also substantially decreased the average optical density following stroke in murine subjects' brains. This neuroprotective effect was confirmed by microtubule-associated protein-2 (*MAP-2*) immunohistochemistry, which showed significantly larger infarct volumes in saline-treated controls compared to OMV@PGZ-treated mice, indicating the treatment's effectiveness in reducing long-term tissue damage. Neuroimaging and pathological correlations revealed notable differences: MRI-measured infarct volumes were 46% in saline controls and 41% in the OMV-only group, while PGZ and OMV@PGZ treatments reduced infarction to 26% and 14%, respectively. This finding was consistently confirmed by TTC staining, which showed the superior effectiveness of OMV@PGZ in decreasing infarct volume across all experimental cohorts. Furthermore, using single-nucleus RNA sequencing (snRNA-seq), the study identified the oligodendrocyte-specific transcription factors Pou2f1 and Nrf1 as collaborative regulators in neural repair, thereby providing essential molecular targets for the development of future therapies that modulate ferroptosis. This research underscores the unique advantages of immune cell-mediated strategies for targeted brain therapy interventions.

To systematically present the current research landscape of EV-based therapies for IS, we have compiled relevant findings in Table 3[11,12,59,71,124,126,165,174,175,179,182,236,238,265,268–282]. Based on the systematic analysis, the EV-based therapeutic strategies reviewed in this paper exhibit highly focused characteristics in terms of "targeted pathological processes". Although the sources and mechanisms of various EVs are diverse, their core interventions precisely target the three key axes of post-stroke cascade injury: BBB disruption, neuronal apoptosis/death, and neuroinflammation. In-depth analysis reveals an inherent homing logic between the cellular source of EVs and their pathological targets. For example, EC-derived EVs naturally tend to home to and repair damaged BBB structure and function[11]; Immune/glial cell-derived EVs are primarily involved in regulating neuroinflammation, glial cell activation, and glial scar formation[165,174]; while stem cell-derived EVs demonstrate greater pleiotropic effects, capable of simultaneously intervening in multiple processes such as angiogenesis, apoptosis inhibition, and inflammation regulation[270–272]. This "source-target" correspondence reflects a rational design based on biological principles. More importantly, comparative analysis indicates that the research paradigm in this field is undergoing a strategic shift from leveraging the inherent affinity of natural carriers, such as the broad-spectrum protection of Momordica charantia ELNs against BBB disruption and apoptosis[273], to endowing them with precise pathological targeting capabilities through engineering strategies, *e.g.*, Rg1@NCM specifically reprograms microglia to clear neutrophils[71].

Our in-depth analysis reveals that optimizing EV-based therapy for IS is far more than a simple choice of carrier. It is an integrated design challenge that requires strategic balance across two critical

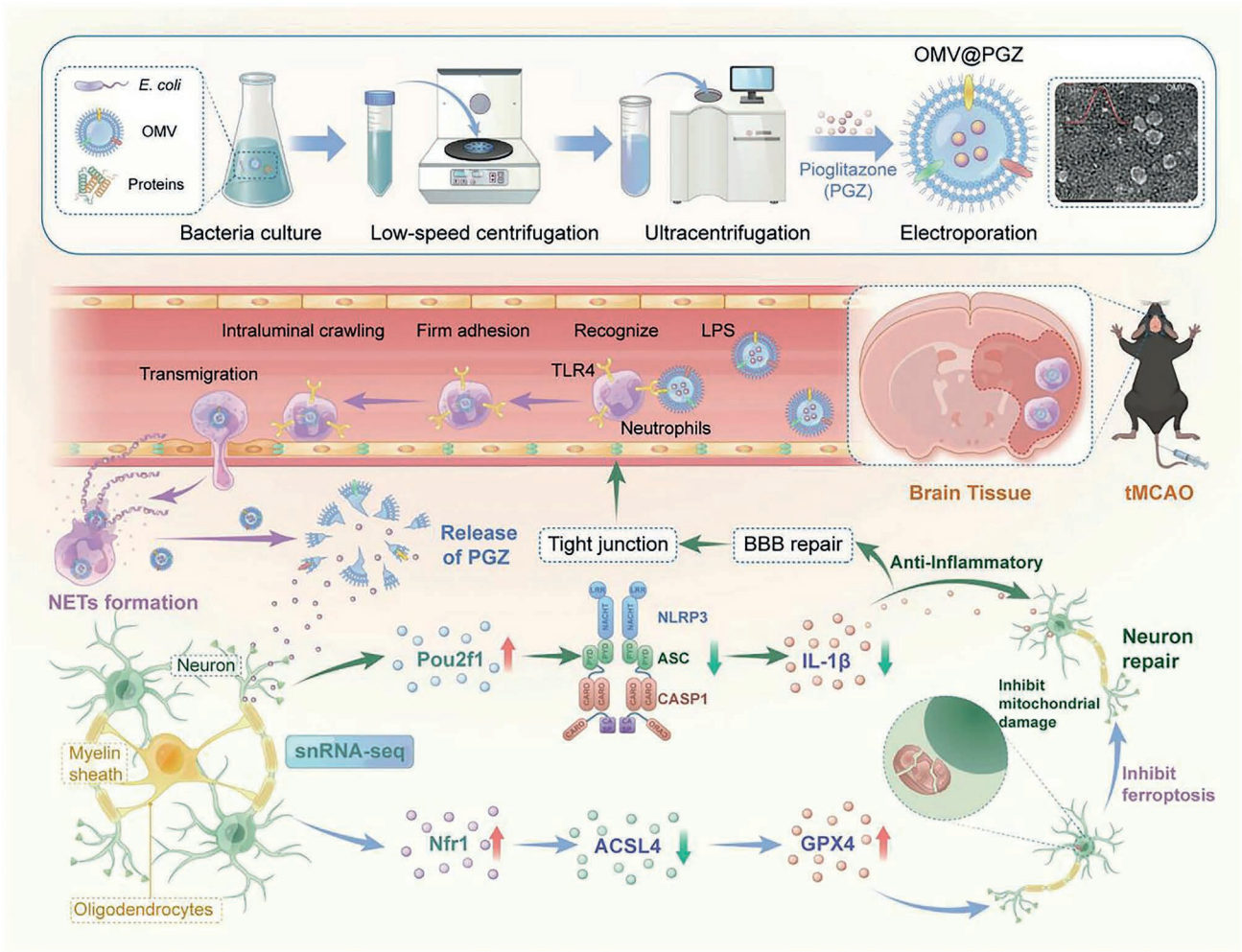

Figure 14 Bacteria-derived OMVs assist neutrophils in hitching a ride to boost IS therapy. (A) Diagram of OMV@PGZ nanoparticles for targeted IS treatment (OMV extracted by differential centrifugation, PGZ loaded *via* electroporation; TLR-mediated neutrophil targeting to lesions, dual-pathway inhibition of NLRP3 inflammasome and ferroptosis). Reprinted with the permission from Ref. 269. Copyright © 2023 Wiley.

dimensions: "therapeutic breadth versus precision" and "delivery efficiency versus feasibility".

First, within the therapeutic paradigm dimension, the core tension lies in the choice between multi-pathway coordinated intervention and single-node precision strike. Natural EVs, such as those derived from MSCs, represent a systems therapy. Like natural messengers carrying multiple tools, through their inherent, unmodified molecular cargo, such as miRNAs, proteins, and lipids, they can simultaneously and synergistically act on multiple interconnected pathways, including inflammation regulation, angiogenesis, anti-apoptosis, and neuroprotection. This pleiotropic nature is particularly suited for the remodeling of the complex and dynamically changing post-stroke microenvironment, aiming to holistically reverse the pathological state and promote endogenous repair[270–272]. However, the broad scope of their action may also lead to reduced efficacy and off-target risks. In contrast, engineered EVs represent the paradigm of precision medicine. Through active surface engineering, *e.g.*, conjugation of targeting peptides such as RVG or RGD, or cargo engineering, such as loading specific siRNA, mRNA, or small molecule drugs, they are designed as pharmacological precision-guided munitions. This strategy aims to efficiently intervene at a key, validated pathological node within the stroke cascade to maximize therapeutic efficacy while minimizing side effects[71,175]. Therefore, the choice of therapeutic paradigm is essentially a strategic decision based on an understanding of the disease stage and the core pathological mechanisms. The acute phase may require rapid precision strikes, while the recovery phase benefits from long-term environmental remodeling.

Second, within the delivery efficiency dimension, the efficiency of EV delivery to the brain fundamentally depends on the route of administration. We conducted a systematic analysis of data in Table 3 to quantitatively compare, from a pharmacokinetic perspective, how different "modes of administration" shape "targeting efficiency". The results indicate that the administration route is a key variable determining the hierarchy and potential of EV brain delivery efficiency, and its selection involves a core trade-off among targeting precision, operational feasibility, and the clinical translation pathway. Intra-arterial (IA) injection, leveraging its first-pass effect and local high-concentration advantage, demonstrates the highest immediate targeting efficiency. Data show that its brain accumulation can be approximately 2.5-fold higher than that of intravenous (IV) injection, representing the current peak of delivery efficiency[175]. However,

Table 3 EV-based therapeutic strategies for IS: sources, pathological targets, and mechanisms.

EV source		Isolation method	Disease modeling type	Targeting efficiency	Mode of administration	Targeted pathological process	Mechanism of action	Therapeutic effect	Ref.
Biotype Animal	Cell type HEK293T	UC	PT	Not quantified	Tail-i.v. injection	Neurological deficits	1. Efficient delivery of circSCMH1 2. Regulation of neural plasticity 3. Anti-apoptotic effect and anti-inflammatory actions 4. BBB penetration and lesion enrichment	1. Functional recovery 2. Reduced infarct volume 3. Increased synaptogenesis	274
	BMSC/BEC	UC	pMCAO	Not quantified	Tail-i.v. injection	1. BBB disruption 2. Hyperpermeability in acute IS	1. Preserve BBB integrity 2. Anti-inflammatory regulation 3. Endothelial protection	1. Improved neurological function 2. Suppressed secondary injury	11
	BV2	PEG UC	MCAO	Not quantified	Femoral vein injection	1. Impaired angiogenesis 2. Neuronal apoptosis 3. Inflammatory response	1. Promote vascular regeneration 2. Anti-apoptosis 3. Microenvironment modulation	1. Reduced infarct volume 2. Improved cerebral blood perfusion 3. Enhanced neurological functional recovery	265
	BV2	UC PEG	MCAO	Not quantified	Tail-i.v. injection	1. AQP4 water channel protein depolarization 2. Impaired glymphatic (cerebrospinal fluid) flow 3. Astrogliosis 4. Initiation and exacerbation of neuroinflammation	1. Stabilize AQP4 polarity 2. Restore CSF flow 3. Suppress astrogliosis 4. Attenuate neuroinflammation	1. Reduced brain edema 2. Improved neurological function	165
	BV2	UC	tMCAO	Implied but not quantified	Tail-i.v. injection	Post-stroke glial scar formation	1. *miR-124* delivery 2. Glial scar reduction 3. Promote axonal regeneration 4. Neuroinflammation modulation	1. Nervous structural recovery 2. Functional improvement 3. Molecular evidence	174

	BV2	UC	tMCAO	1. IA injection yielded ∼2.5-fold higher brain EV accumulation than i.v. 2. RVG29 modification boosted *in vitro* neuronal uptake from 41.02% to 71.22%.	IA injection(identified as optimal)/i.v. injection/IN route	Neuronal apoptosis	1. TGN peptide binds transferrin receptor on the BBB ECs 2. Suppresses microglial activation 3. Reduce neuronal apoptosis 4. Deliver SOD/CAT to scavenge ROS 5. Stimulate angiogenesis	1. Infarct volume reduction 2. Neurological function recovery 3. Motor function improvement 4. Long-term neuroregeneration	175
	EPC	Commercial EV isolation kit (Umibio, UR52121).	pMCAO	Not quantified	Tail-i.v. injection	1. Angiogenesis 2. Brain perfusion	1. Promote vascular regeneration 2. Anti-apoptosis 3. Anti-inflammation 4. Neuroprotection	1. Infarct volume reduction 2. Neurological recovery	182
	NSC	UC	MCAO/R	Not quantified	Lateral ventricle injections (ICV)	1. Post-ischemic microenvironment (inflammation, oxidative stress) 2. Neuronal death 3. Glial scar formation	1. Reduce inflammation 2. Alleviate oxidative stress 3. Enhance NSC survival and differentiation 4. Inhibit neuronal apoptosis	1. Promoted neural remodeling 2. Neural structure recovery 3. Functional improvement 4. Microenvironment modulation	236
	Neurons	UC	PT	Uptake of HypEVs is significantly higher than NorEVs (no precise percentage provided)	Stereotactic injection/ Intranasal administration	1. Neuronal damage 2. Loss of neurite integrity 3. Mitochondrial dysfunction 4. Apoptosis	1. FUS-enhanced BBB penetration 2. Anti-apoptotic 3. Shifts microglia to M2 phenotype 4. Promote angiogenesis:	1. Neurological recovery 2. Reduced brain edema 3. Molecular effects 4. Increased synaptophysin	268
	Neuron	UC	tMCAO	Not quantified	ICV injection	1. Microglial phagocytosis of stressed-but-viable 2. Neurons neuroinflammation	1. Neuron-to-microglia communication *via* EVs 2. Delivers *miR-98* to recipient microglia 3. Targets and inhibits PAFR in microglia 4. Suppresses microglial phagocytosis of stressed-but-viable neurons	1. Reduced neuronal phagocytosis 2. Infarct volume reduction 3. Functional recovery 4. Anti-inflammation	179

(*continued on next page*)

Table 3 (*continued*)

EV source		Isolation method	Disease modeling type	Targeting efficiency	Mode of administration	Targeted pathological process	Mechanism of action	Therapeutic effect	Ref.
	DPSCs	UC	tMCAO	Not quantified	Tail-i.v. injection	1. Neuronal apoptosis 2. Neurodegeneration	1. Intercellular communication 2. Targeted delivery of *miR-877-3p* to neurons 3. Inhibition of the apoptotic pathway 4. Anti-apoptotic effect	1. Enhanced neuronal survival 2. Reduced infarct volume 3. Accelerated functional recovery 4. Molecular evidence	275
	REN	UC	MCAO/R	Lesion/ contralateral fluorescence intensity ratio = 7.84	Tail-i.v. injection	1. Post-ischemic neuroinflammation microglial overactivation 2. Pro-inflammatory cytokine release	1. Targeted delivery of anti-inflammatory miRNAs 2. Inhibition of the MAPK signaling pathway 3. Attenuation of neuroinflammation 4. Preservation of BBB integrity	1. Functional recovery 2. Reduced cerebral infarct volume 3. Attenuated neuronal death 4. Molecular & cellular effects	276
	ADSC	UC	tMCAO	Ipsilateral hemisphere fluorescence intensity: 3.51-fold vs. native EVs, 1.72-fold vs. RGD-single modified EVs	i.v. injection	1. BBB disruption, neuronal apoptosis 2. Neuroinflammation 3. TJ protein degradation	1. Targeted delivery and BBB penetration to ischemic region 2. Microglia-mediated anti-inflammatory effects 3. Angiogenesis and cerebral blood flow restoration	1. Reduced infarct volume 2. Promote the recovery of neurological function 3. Promote neural repair	270
	ADSC	UC	tMCAO	Not quantified	Tail-i.v. injection	1. Microglial M1 overactivation 2. Neuroinflammation 3. White matter damage 4. Cerebrovascular angiogenesis deficiency 5. Neuronal impairment	1. Induction of microglial M2 polarization 2. Creation of an anti-inflammatory milieu 3. Comprehensive tissue repair	1. Functional recovery 2. Pathological improvement 3. Reduction in infarct volume	271
	IPSC	UC	MCAO	Not quantified	Tail-i.v. injection	1. BBB aging 2. BBB disruption 3. Neuroinflammation 4. Neuronal/ oligodendrocyte death 5. TJ protein (Claudin5, ZO-1) loss	1. Rejuvenate senescent BBB 2. Alleviate cellular senescence 3. Anti-inflammatory & antioxidant effects	1. BBB restoration 2. Reduced infarct volume 3. Functional recovery 4. Reversed aging phenotypes	277

						4. Promote neurogenesis		
BEC	UC	MCAO	m/lEVs vs sEVs: 68% vs 13% (MitoT + cells, 150 μg protein/ well, 72 h co-culture).	i.v. injection	1. IS BEC mitochondrial dysfunction 2. BBB TJ disruption 3. Increased paracellular permeability 4. Cerebral endothelial cell death	1. Mitochondrial transfer for energy restoration 2. Anti-inflammatory effects 3. Anti-apoptotic effect 4. BBB protection 5. Antioxidant effect	1.Neuroprotective effect 2. Improves long-term neurological damage 3. Alleviates neurological dysfunction	124
HBEC/MBEC	UC	MCAO	~1.3-fold higher uptake of mBEC-MVs than hBEC-MVs (homotypic vs. heterotypic) at 60 μg/well	Tail-i.v. injection	1. Ischemic BEC mitochondrial dysfunction 2. Reduced cellular ATP 3. BBB disruption; cerebral infarction 4. Neurological deficit	1. Attenuate mitochondrial dysfunction 2. Antioxidant effects 3. Anti-apoptosis 4. Immune modulation	1. Infarct volume reduction 2. Functional recovery 3. Mitochondrial recovery 4. Long-term protection	278
BMSC	UC	MCAO	Not quantified	Tail-i.v. injection	Post-ischemic angiogenesis impairment	1. Promote vascular regeneration 2. Anti-apoptotic effects 3. BBB improvement 4. Anti-inflammatory	1. Reduced infarct volume 2. Improved cerebral blood perfusion 3. Accelerated neurological recovery	279
ADMSC	PEG UC	MCAO	Not quantified	Femoral vein injection	1. Excessive autophagy 2. P53-BNIP3 pathway activation 3. Neuronal death 4. Cerebral infarction 5. Neurological deficit	1. Suppress excessive autophagy 2. Anti-apoptotic effects 3. Promote angiogenesis 4. Neuroprotection and repair	1. Reduced infarct volume 2. Improved neurological function	12
MSC	PEG UC	MCAO	Not quantified	Tail-i.v. injection	1. Brain tissue remodeling 2. Anti-inflammatory 3. Pro-angiogenic effects	1. Enhance cerebral angiogenesis 2. Reduce neuroinflammation 3. Brain remodeling 4. Alleviate oxidative stress	1. Angiogenesis and blood flow restoration 2. Reduced infarct volume 3. Neurological recovery 4. Long-term neuroprotection	280
MSC	Anion exchange Chromatography	MCAO/R	Not quantified	Intranasal administration	1. Neuroinflammation 2. Neuronal apoptosis 3. Impaired endogenous repair	1. Activation of BDNF/TrkB signaling 2. Promote vascular	1. Neurological recovery 2. Reduced infarct volume	281

(*continued on next page*)

Table 3 (*continued*)

EV source		Isolation method	Disease modeling type	Targeting efficiency	Mode of administration	Targeted pathological process	Mechanism of action	Therapeutic effect	Ref.
						4. Structural damage to neural networks after IS.	regeneration 3. Anti-inflammatory effects 4. Reduction of glial scarring	3. Enhanced synaptic plasticity and neuronal survival 4. Long-term neuroprotection	
	HuMSCs	Sequential centrifugation	tMCAO	The ipsilateral/contralateral brain fluorescence intensity ratio reached ~3.5-fold at 48 h post-injection.	i.v. injection	1. Neuroinflammation 2. Oxidative stress 3. Neuronal death 4. Impaired angiogenesis	1. Promote M2 polarization 2. Phagocytic clearance 3.Stimulate angiogenesis ($CD31^+$) and neurogenesis (DCX^+) 4. Reduce neutrophil infiltration	1. Reduced infarct volume 2. Improved neurological function 3. Long-term functional recovery	272
	MSC	UC	MCAO	Not quantified	Tail-i.v. injection	1. Astrocytes 2. The TLR4/NF-κB pathway 3. Inflammatory effects	1. Protection of BBB integrity 2. Mitigation of r-tPA toxicity 3. Anti-inflammatory effects 4. Reduction of neuronal apoptosis	1. Reduced BBB permeability 2. Reduced infarct volume 3. Improved neurological function 4. Long-term benefits	126
Microorganism	MsbB mutant *E. coli* W3110 (low endotoxin)	UC	tMCAO	OMV-hitchhiked neutrophils increased by approximately 3.1-fold	Tail-i.v. injection	Post-IS injury (neuroinflammation & ferroptosis)	1. Neutrophil-mediated BBB penetration 2. Suppression of ferroptosis 3. Anti-inflammatory effects 4. Oligodendrocyte repair 5. Reduce I/R injury	1. Reduced infarct volume 2. Improved motor function 3. Prolonged survival	269
	Lactobacillus plantarum	UC	tMCAO	Not quantified	Tail-i.v. injection	Neuronal apoptosis	1. miRNA delivery-mediated gene regulation 2. Suppression of neuroinflammation 3. Preservation of BBB integrity 4. Promotion of neuroregeneration 5. Inhibition of	1. Reduction in infarct volume 2. Improvement in neurological function 3. Long-term functional restoration	59

							neuroinflammation and oxidative stress		
	Gut probiotic lactobacillus reuteri	UC	MCAO/R	*In vitro* BBB model: 26.8% *trans*-endothelial penetration at 6 h	Tail-i.v. injection	1. Post-ischemic oxidative stress 2. Hyperactivated immune microenvironment	1. ROS scavenging 2. Immune modulation 3. Shift microglia from M1 ($CD86^+$) to M2 ($CD206^+$) phenotype 4. BBB protection 5. Neutrophil/ infiltrating leukocyte reduction	1. Reduced infarct volume 2. Neurological function 3. Lower oxidative stress 4. Anti-inflammatory effects 5. Enhanced neuron survival	238
Plant	Panax notoginseng	1. UC 2. SEC	tMCAO	Not quantified	Tail-i.v. injection	1. Cerebral CI/R injury 2. Neuroinflammation	1. Microglia polarization shift 2. Anti-ferroptosis: 3. Oxidative stress reduction 4. BBB protection 5. Anti-inflammatory effects	1. Reduced infarct volume 2. Improved neurological function 3. Enhanced neuroprotection	282
	Momordica charantia	UC	MCAO	Not quantified	Tail-i.v. injection	1. BBB disruption 2. Neuronal apoptosis.	1. Inhibition of MMP9-mediated BBB disruption 2. Activation of AKT/GSK3B signaling pathway 3. Anti-inflammatory and antioxidant effects	1. Reduced cerebral infarct volume 2. Protected neuronal survival 3. Improved neurological function 4. Maintained BBB integrity	273
Engineering-derived EVs	Rg1@NCM	Nitrogen cavitation	tMCAO	8 times higher with Rg1@NCM than with free Rg1	Tail-i.v. injection	1. Neuroinflammation 2. Inefficient microglial phagocytosis of neutrophils 3. NETs formation	1. Microglial phagocytosis reprogramming 2. Polarization to an anti-inflammatory phenotype 3. Neutrophil clearance 4. BBB protection	1. Reduced infarct volume 2. Improved neurological function 3. Decreased neuroinflammation 4. Enhanced phagocytosis of neutrophils by microglia	71

Table footnote: BMSC: Bone marrow mesenchymal stem cell; EC: Endothelial cell; BEC: Brain endothelial cell; EPC: Endothelial progenitor cell; HUVEC: Human umbilical vein endothelial cell; NSC: Neural stem cell; DPSC: Dental pulp stem cell; REN: Renewed neural progenitor cell; ADSC: Adipose-derived stem cell; IPSC: Induced pluripotent stem cell; HBEC: Human brain endothelial cell; MBEC: Mouse brain endothelial cell; ADMSC: Adipose-derived mesenchymal stem cell; EEV: Engineered extracellular vesicles; UC: Ultracentrifugation; PEG: Polyethylene glycol precipitation; IA injection: Intra-arterial injection; i.v. Injection: Intravenous injection; IN route: Intranasal route.

Table 4 Registered clinical trials of EV-based interventions for IS.

Trial Identifier	Phase	EV source	Intervention/method	Primary outcomes	Status	Registry & Link
NCT06138210	Phase I (dose escalation & expansion)	Human iPSCs	GD-iExo-003 i.v. injection, once daily for 7 days Dose: 2, 4, 8 $\times$ 10^9 particles/kg	Incidence of dose-limiting toxicity (DLT)	Recruiting	https://clinicaltrials.gov/study/NCT06138210
NCT06319742	Observational	Patient serum	EV surface antigen profiling Multiplex flow cytometry (MACSPlex) NTA, WB, ELISA	Diagnostic accuracy (sensitivity & specificity for TIA *vs.* stroke mimics)	Recruiting	https://clinicaltrials.gov/study/NCT06319742
NCT06612710	Early-stage exploratory	Human iNSCs	NouvSoma001 i.v. infusion, once daily for 7 days Dose: 4, 8, 16 $\times$ 10^9 particles/kg	Safety, tolerability, and MTD determination	Recruiting	https://clinicaltrials.gov/study/NCT06612710
NCT05645081	Observational	Patient plasma/serum (endothelial)	Diagnostic testing Quantification of endothelial-EVs Prothrombin time measurement	Diagnostic accuracy and correlation with stroke risk	Recruiting	https://clinicaltrials.gov/study/NCT05645081
NCT06995625	Phase I (dose escalation)	Allogeneic Wharton's jelly MSCs	SNE-101 i.v. infusion, once daily for 5 days Dose: 4.8, 9.6, 19.2 $\times$ 10^{10} particles	Safety, tolerability, and MTD determination	Not yet recruiting	https://clinicaltrials.gov/study/NCT06995625
NCT06871800	Observational biomarker discovery	Patient serum (multi-cellular source EVs)	EV SPRi profiling Longitudinal serum collection (T0, T1, T2) Surface marker analysis (CD31, CD171, IB4, *etc*.)	Functional recovery (change in modified Barthel index)	Recruiting	https://clinicaltrials.gov/study/NCT06871800
NCT07145294	Phase I/II exploratory	Human iPSC	Intranasal iEV Twice a week for 12 weeks 2 mL (2×10^{10}/mL) per time	Neurological & cognitive improvement (NIHSS, mRS, ADL, MoCA, MMSE)	Recruiting	https://clinicaltrials.gov/study/NCT07145294

Table footnote: Human iPSCs: Human induced pluripotent stem cells; GD-iExo-003**:** Intravenous exosomes derived from human induced pluripotent stem cell; Human iNSCs: Human induced neural stem cells; NouvSoma001: Intravenous administration of human-induced neural stem cell-derived EVs; SNE-101: Allogeneic wharton's jelly-mesenchymal stem cell-derived EVs; iEV: Induced pluripotent stem cells-derived small EVs(sEVs).

due to its highly invasive nature, it is largely confined to specific research contexts or potential interventional therapy scenarios. IV injection, as the most prevalent systemic administration route, exhibits the broadest efficiency range. Its performance is highly dependent on the engineering modifications of EVs. For instance, RGD dual-modification can increase ipsilateral targeting efficiency by 3.51-fold[270]. Femoral vein injection, in principle, belongs to the peripheral venous system alongside the tail vein injection, and its targeting efficiency logic is similar. Intracerebroventricular (ICV) injection bypasses the BBB, enabling direct cerebrospinal fluid delivery. Its targeting efficiency is theoretically very high locally, but is strictly confined to the periventricular region, making uniform distribution across the entire ischemic lesion challenging. The connotation of its efficiency emphasizes local bioavailability rather than active targeting following systemic administration. In contrast, intranasal (IN) administration, as a completely non-invasive route, offers the significant advantage of bypassing the BBB and accessing the brain *via* neural pathways (olfactory/trigeminal). However, quantitative *in vivo* targeting data in stroke models remain relatively scarce. Existing reports primarily focus on *in vitro* model penetration rates (*e.g.*, 26.8%)[238], and their actual *in vivo* enrichment ratio and temporal kinetics require further elucidation. In summary, different administration routes constitute a continuous spectrum from ultra-high precision but highly invasive (IA/ICV) to systemically optimizable and well-balanced (IV), and further to non-invasive and convenient but efficiency yet to be proven IN.

Ultimately, developing effective EV-based therapies requires deep strategic alignment and trade-offs between these two major dimensions: the therapeutic paradigm (systems remodeling versus precision strike) and the administration route (the efficiency-feasibility spectrum). Future breakthroughs may depend on developing intelligent delivery systems capable of temporal or spatial specificity in targeting the aforementioned multiple pathological components, thereby achieving synergistic repair of the complex post-stroke microenvironment. Clinical translation strategies must be based on specific therapeutic windows, pathological mechanisms, drug properties, and patient status, making data-driven, precise decisions within this multidimensional framework.

4. Current clinical landscape of EV-based therapies for IS

Research on EV-based therapies for IS has reached a pivotal stage in clinical translation, with the development of a dual-track approach that emphasizes both therapeutic exploration and diagnostic advancement. This emerging field is gradually linking basic research with clinical practice through systematic trials. Table 4 summarizes registered clinical trials investigating EV-based diagnostic and therapeutic methods for IS.

In terms of therapy, several trials have shown promising results. NCT06138210, the first Phase I trial of human iPSC-derived exosomes (GD-iExo-003), completed a 7-day safety assessment after intravenous injection, establishing a key foundation for future studies. NCT06612710 and NCT06995625 use human iNSC-derived NouvSoma001 and Wharton's Jelly mesenchymal stem cell-derived SNE-101, respectively, to explore the optimal therapeutic window through dose-escalation studies. Notably, the

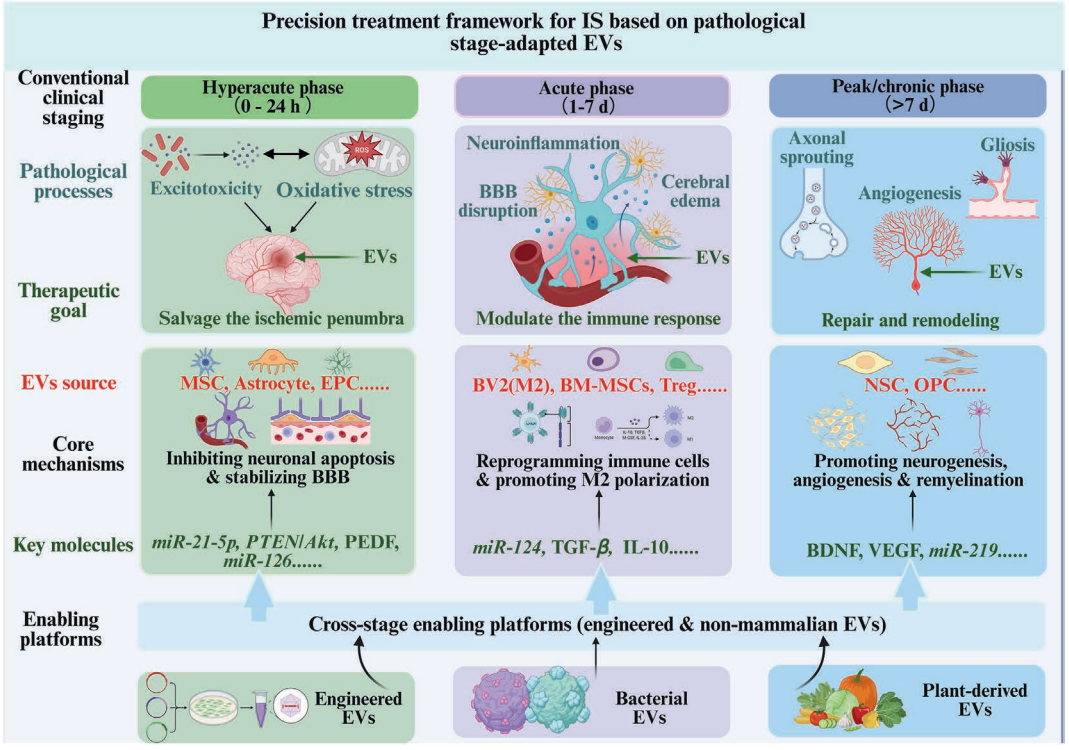

Figure 15 Framework for EV therapy in IS by stage. Hyperacute phase (0−24 h): EVs derived from sources such as MSCs target excitotoxicity and oxidative stress to salvage the ischemic penumbra; Acute phase (1−7 days): EVs derived from sources like BV2 cells regulate neuroinflammation and the BBB, promoting M2 polarization; Chronic hase (>7 days): EVs derived from sources such as NSC support neural repair and vascular regeneration; Cross-stage platform: Engineered EVs and EVs derived from microbial and plant sources. © By Biorender.

NCT07145294 trial introduces an innovative intranasal delivery method, overcoming traditional intravenous limitations. Over 12 weeks, it evaluates improvements in neurological and cognitive functions, offering a new approach for stroke rehabilitation.

In diagnostics, EVs demonstrate significant potential as liquid biopsy biomarkers. NCT06319742 aims to improve the accuracy of diagnosing transient ischemic attacks through EV surface antigen profiling. NCT05645081 investigates the relationship between endothelial-derived EVs and stroke risk. NCT06871800 employs a longitudinal design, establishing correlation models with functional prognosis by monitoring changes in EV surface markers over time.

Although EV-based therapies show promising therapeutic potential, they face several interconnected challenges in clinical translation. Most evidence comes from small exploratory trials, which are hindered by the lack of standardized preparation protocols, unified quality control standards, and consistent dosing regimens. Additionally, an incomplete understanding of mechanisms of action and the absence of reliable predictive biomarkers further impede progress in clinical development.

Furthermore, achieving optimal therapeutic efficacy requires pathology stage-specific treatment strategies. We suggest a "timing-source-efficacy" framework (Fig. 15) to guide this method: during the hyperacute phase (0−24 h), EVs from sources such as MSCs[178], delivering cargo, including *miR-21-5p*, should be prioritized to counteract excitotoxicity and oxidative stress, thereby salvaging the ischemic penumbra. As the disease progresses to the acute phase (1−7 days), characterized by dominant neuroinflammation, EVs derived from M2 macrophages exhibit a greater ability to modulate the immune microenvironment and reduce secondary damage through mediators such as *miR-124*[174]. In the chronic phase (more than 7 days), when tissue repair and remodeling become crucial, EVs from NSCs emerge as particularly well-suited, promoting neurovascular regeneration through neurotrophic signals like BDNF and VEGF[171].

In conclusion, addressing these challenges and validating this framework requires coordinated future efforts. Key priorities should include conducting large-scale multicenter randomized controlled trials, establishing standardized EV characterization systems, and utilizing multi-omics technologies to identify reliable biomarkers. Ultimately, creating personalized treatment strategies based on this pathology-driven framework is essential for advancing EV therapies from experimental research to definitive clinical use.

5. Conclusions and perspectives

EVs represent a transformative frontier in IS therapy, demonstrating substantial potential to address the complex pathophysiology of cerebral ischemia. Their intrinsic biocompatibility and ability to cross the BBB, and the capacity to deliver diverse bioactive molecules, enable multifaceted therapeutic effects. EVs derived from stem cells, plants, bacteria, and engineered sources effectively target core pathological processes: they enhance BBB integrity by stabilizing TJ proteins and inhibiting MMPs; attenuate neuroinflammation by reprogramming microglia and degrading NETs; mitigate excitotoxicity through miRNA-mediated regulation; and reduce oxidative stress *via* direct (ROS scavenging and activation of endogenous antioxidants) pathways. Innovations in EV engineering, such as magnetic targeting, antibody-directed delivery, and biomimetic coatings, have further enhanced their targeting accuracy and accumulation in ischemic lesions, expanding the scope of therapy specificity.

However, moving from strong preclinical results to clinical use requires a clear assessment of several key challenges. While mammalian EVs are often seen for their low immune response, the safety of non-mammalian and engineered versions needs careful examination. Potential risks, such as immune activation triggered by bacterial OMVs or the long-term biodistribution and bioaccumulation of synthetically modified EVs, must be thoroughly assessed and investigated. The advancement of sensitive imaging methods (*e.g.*, bioorthogonal chemistry, innovative radionuclide labeling techniques) is crucial for accurately tracking the biodistribution and pharmacokinetics of EVs. Concurrently, engineering strategies must be integrated to extend circulation time, such as surface functionalization to evade immune clearance or the adoption of biomimetic camouflage technologies. Furthermore, the safety of combining EVs with established therapies, such as thrombolytics (*e.g.*, r-tPA), requires scrutiny to prevent any synergistic increase in bleeding risk, despite early evidence suggesting EV-mediated protection against hemorrhagic complications. Meanwhile, overcoming technical bottlenecks in scalable production and standardization remains crucial. The industry must move beyond low-yield methods, such as ultracentrifugation, and adopt scalable alternatives, including microfluidic separation and tangential flow filtration. Optimized serum-free culture systems should support these to ensure consistency from batch to batch. This effort must be paired with strict quality control metrics that specify critical attributes, such as particle size distribution, purity (*e.g.*, protein-to-particle ratio), and standardized bioactivity assays, to ensure therapeutic potency and reproducibility.

Looking ahead, the clinical application of EV-based therapies needs progress in several areas. Foundational research should identify ideal treatment time frames, dosing schedules, and dependable biomarkers for patient stratification. Concurrent technological innovation must concentrate on developing next-generation EV systems, including stimuli-responsive EVs for spatiotemporal control and hybrid synthetic-EV vectors to improve stability and loading capacity.

An auspicious approach involves the use of synergistic therapeutic strategies. Emerging evidence shows the potential of combining EVs with neuromodulation or rehabilitation interventions. For example, exercise-derived EVs act as "exercise mimetics" to boost neuroplasticity, while combining them with physical training offers complementary advantages: EVs deliver molecular-level neuroprotection and promote plasticity, whereas rehabilitation encourages activity-dependent synaptic circuit strengthening. The exploration of novel EV sources, including plant and microbiome-derived EVs, for integration with other neuroprotective agents further enhances this synergistic potential.

Ultimately, the field's progress will depend on the results of early-phase clinical trials. However, long-term success relies on building strong infrastructure, including EV biobanks and clear regulatory frameworks, as well as effectively addressing challenges related to safety, manufacturing scalability, and standardization. Through these coordinated efforts, EV-based therapies could develop into multi-targeted platforms that advance stroke treatment beyond just acute recanalization, moving toward genuine brain repair and lasting functional recovery.

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Author contributions

Ju Bai, Jingshu Yang, and Wei Liu were responsible for drafting the initial manuscript. Bin Han was responsible for drafting the initial manuscript and acquiring research funding. Shaoyan Jiang and Jinping Sun were responsible for reviewing and editing the manuscript as well as conceptualizing the study. Hongzhao Qi was responsible for reviewing and editing the manuscript, acquiring research funding, and conceptualizing the study. All authors have read and approved the final manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used DeepSeek-R1 in order to improve the readability and language of the article. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Conflicts of interest

The authors declare no conflicts of interest.

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