Asian Journal of Pharmaceutical Sciences 19 (2024) 100965

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Research Article

# Extracellular vesicles for delivering therapeutic agents in ischemia/reperfusion injury

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ARTICLE INFO

*Article history:*
Received 7 May 2024
Revised 8 June 2024
Accepted 29 June 2024
Available online 4 September 2024

*Keywords:*
Extracellular vesicles
Extracellular vesicle engineering
Ischemia/reperfusion injury
Nanocarrier
Drug delivery

ABSTRACT

Ischemia/reperfusion (I/R) injury is marked by the restriction and subsequent restoration of blood supply to an organ. This process can exacerbate the initial tissue damage, leading to further disorders, disability, and even death. Extracellular vesicles (EVs) are crucial in cell communication by releasing cargo that regulates the physiological state of recipient cells. The development of EVs presents a novel avenue for delivering therapeutic agents in I/R therapy. The therapeutic potential of EVs derived from stem cells, endothelial cells, and plasma in I/R injury has been actively investigated. Therefore, this review aims to provide an overview of the pathological process of I/R injury and the biophysical properties of EVs. We noted that EVs serve as nontoxic, flexible, and multifunctional carriers for delivering therapeutic agents capable of intervening in I/R injury progression. The therapeutic efficacy of EVs can be enhanced through various engineering strategies. Improving the tropism of EVs *via* surface modification and modulating their contents via preconditioning are widely investigated in preclinical studies. Finally, we summarize the challenges in the production and delivery of EV-based therapy in I/R injury and discuss how it can advance. This review will encourage further exploration in developing efficient EV-based delivery systems for I/R treatment.



## 1. Introduction

Ischemia can affect diverse organs, leading to conditions including ischemic stroke (IS) and myocardial infarction (MI). To prevent irreversible tissue damage in an ischemic organ, restoring blood supply to it is essential. Paradoxically, rapid reperfusion of the infarcted organ can induce cell death while exacerbating the extent of injury—a phenomenon known as ischemia/reperfusion (I/R) injury [1]. Since its initial recognition in the 1960s, I/R injury has been observed in various conditions, including acute myocardial infarction,

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Peer review under responsibility of Shenyang Pharmaceutical University.

https://doi.org/10.1016/j.ajps.2024.100965

stroke, and organ transplantation. I/R exacerbates tissue damage and contributes to numerous pathologies [2]. Currently, it is one of the leading causes of disability and death, accounting for approximately 30% of deaths in patients with ischemia [3]. Several therapeutic approaches have been suggested to enhance organ resilience against I/R injury. In the clinical treatment of myocardial I/R injury, primary percutaneous coronary intervention and thrombolytic agents are pivotal steps in addressing ischemic damage [3]. Certain ischemic preconditioning strategies have been suggested to mitigate tissue injury. Nonetheless, their feasibility for clinical application remains limited [4]. In ischemic stroke, small molecule drugs such as neuroprotective agents and antioxidants are currently undergoing preclinical investigation [5–8]. However, owing to the complexity of the pathological processes and underlying mechanisms involved in I/R injury, the therapeutic benefits of these approaches remain limited [5]. Additionally, the lack of distribution specificity and potential side effects further restrict the clinical applications of these treatments. Currently, neither pharmacological nor non-pharmacological interventions have proven entirely effective in shielding organs from I/R injury. Consequently, preserving organ function remains a challenge for physicians, necessitating the need for a synergistic, highly specific, and nontoxic therapy.

The emergence of extracellular vesicles (EVs) represents a promising avenue for I/R therapy. EVs are nanoscale heterogeneous particles characterized by a lipid bilayer-enclosed structure and membrane proteins comparable to cell membranes [9]. Moreover, EVs contain various bioactive molecules in their lumen, including nucleic acids and proteins [10]. After secretion, EVs can circulate in body fluids and it can subsequently be internalized by recipient cells. Substantial evidence indicates that EVs are implicated in various physiological processes, such as immune regulation and angiogenesis, as well as pathological processes, including neurological disorders [11–13]. The unique capacity of EVs to transfer bioactive cargo and regulate cell conditions inspires further research into their potential for delivering therapeutic agents in disease treatment. The diverse cargo carried by EVs can regulate multiple signaling pathways in recipient cells, effectively modulating cell states and intervening in disease progression. Additionally, EVs present several advantages as drug delivery vehicles. First, EVs can acquire a similar repertoire of surface receptors as their parent cells and inherit similar homing patterns [14]. For example, endothelial colony-forming cell-derived exosomes can target the ischemic kidney *via* exosomal CXC chemokine receptor type 4 (CXCR4) [15]. Specially, EVs can penetrate the blood-brain barrier (BBB), enabling them to effectively transport therapeutic agents to cerebral injury sites [16,17]. The stable structure of EVs shields encapsulated cargo from degradation, while their presence in body fluid exhibits nontoxicity and low immunogenicity. Thus, EVs have emerged as a promising therapeutic option for various diseases, such as rheumatoid arthritis [18], neurodegenerative diseases [19], cardiovascular disorders [10], and cancer [20]. In recent years, numerous studies have highlighted the potential of EVs in I/R treatment. They can bolster the functional recovery of injured tissue by stimulating angiogenesis, influencing immune cell polarization, and preventing cell apoptosis [21,22].

While native EVs have demonstrated the capacity to elicit functional responses in target cells, they encounter challenges in areas including active targeting and bioactivity. To address these challenges and boost EV therapeutic efficacy, several engineering strategies have been devised. Besides their inherent properties, EVs can be engineered to target specific tissues or carry exogenous substances. Therefore, this review aims to provide an overview of the pathological process involved in I/R injury and the biophysical properties of EVs. Subsequently, the potential of EVs derived from diverse cell origins for delivering therapeutic agents and the challenges associated with native EVs are discussed in this review. The findings could help elucidate the pivotal roles of modification strategies in improving EV targeting ability and modulating their contents. We also examine and compare various modification strategies in bioengineered EVs. Finally, the challenges of EV-based therapy in I/R injury and avenues for advancing it are discussed.

## 2. Overview of I/R injury

Understanding the pathophysiology of I/R is crucial for optimizing the efficacy of EV-based therapy. I/R injury occurs when blood flow to an organ is restricted and subsequently reestablished. For example, IS arises from sudden blood flow interruption in the middle cerebral artery, while myocardial I/R injury is triggered by coronary vessel obstruction [23]. The prompt restoration of blood supply to the ischemic area through thrombolytic approaches represents the most effective clinical intervention. However, reperfusion itself can exacerbate cellular damage—a phenomenon known as I/R injury. Despite variations in the underlying causes of these diseases, accumulating evidence suggests a common pathophysiology underlying I/R disease progression. The fundamental pathophysiology of I/R includes oxidative stress, inflammation, and microvascular dysfunction [2]. Recent studies indicate several signaling pathways, such as RNA and protein profile alterations, are associated with I/R injury progress and recovery. These proteins and RNA are involved in cell survival, immune regulation, and angiogenesis, suggesting potential therapeutic targets for I/R.

The overall injury caused by I/R comprises two components: ischemic and reperfusion injuries. Ischemia restricts oxygen availability and nutrient supply to the organ, resulting in a hypoxic microenvironment. Hypoxia can impair the electron transport chain in mitochondria and induce anaerobic metabolism [24]. Anaerobic metabolism leads to reduced production of cellular antioxidative agents and adenosine triphosphate (ATP), resulting in an accumulation of reactive oxygen species (ROS) [25]. Xanthine oxidoreductases play a central role in the ROS production. The decrease in ATP levels leads to the dysfunction of calcium pumps, consequently activating calcium-dependent protease. These proteases can convert xanthine dehydrogenase into xanthine oxidoreductase. Upon restoring blood flow to ischemic tissue, xanthine oxidoreductase catalyzes hypoxanthine conversion to uric acid in the presence of

elevated oxygen levels [26], accompanied by massive ROS production. ROS accumulation is toxic to cell metabolism and can activate cell death programs. Hypoxia in vascular tissue also impairs the endothelial barrier and increases vascular permeability. Additionally, I/R injury induces sterile inflammation. This inflammation is marked by the accumulation and infiltration of inflammatory cells alongside the production of pro-inflammatory cytokines [27]. In myocardial I/R injury, monocytes are recruited from a splenic reservoir to the injured tissue, where they differentiate into macrophages [28,29]. Macrophages can polarize into M1/M2 phenotypes based on the inflammatory state. The M1 macrophage phenotype is associated with pro-inflammatory traits, while the M2 phenotype is linked to tissue healing and can downregulate M1 activation-related cytokines [30]. Moreover, vascular endothelial cells (ECs) and microvascular dysfunction contribute to I/R injury. Cardiac microvascular endothelial cells (CMECs) swell, promoting leukocyte-endothelial cell adhesion and platelet-leukocyte aggregation. This impedes blood and nutrients diffusion, further exacerbating microvascular dysfunction [31]. Additionally, the gap junction structure between CMECs becomes leaky, resulting in microvascular leakage [32] and, consequently, dysfunction of endothelial cell barriers.

Regarding cerebral I/R injury, significant alterations are observed in microRNA (miRNA) expression profiles. A study revealed an increase and decrease in 15 miRNAs and 44 miRNA expression in cerebral I/R injury, respectively [33]. Several miRNA groups have been implicated in regulating cell survival, inflammation, and angiogenesis [34,35]. For example, miR-188-5p expression increases in cerebral I/R injury. It regulates the pathological network by interacting with the RNA-binding protein Lin28. Silencing miR-188-5p mitigates neuronal cell death and inflammation in cerebral I/R injury [34]. Other miRNAs, such as miR-143, miR-125b, miR-181b, miR-210, and miR-4732-3p contribute to angiogenesis following I/R injury [36–38]. Furthermore, miR-1-3p, miR-22-3p, miR-31, miR-137, and miR-206 are crucial in suppressing cell apoptosis [39–42]. miR-124-3p, miR-126, and miR-223-3p contribute to alleviating inflammatory response [43–45]. Although proteins have been studied less extensively than microRNAs, they are also associated with I/R progression. For instance, heat shock protein 90 can be activated by hypoxia, which is associated with the suppression of the complement system, thereby regulating the inflammatory response in I/R [46,47]. Collectively, these miRNAs and proteins involved in I/R injury pathology have crucial clinical implications, serving as potential therapeutic targets. Additionally, accumulating evidence indicates that these essential miRNAs and proteins are selectively transported in exosomes [48,49]. Hence, EVs intrinsically possess the potential to regulate I/R pathological development.

## 3. Properties of EVs

EVs are heterogeneous, lipid-bilayer-enclosed vesicles that play crucial roles as transfer factors in various biological processes. Various cell types secrete EVs under normal or pathological conditions. Owing to their distinctive composition and biological functions, EVs are utilized as carriers for delivering therapeutic agents.

The term “Extracellular Vesicles” comprises a heterogeneous population [50]. Based on their size and biogenesis, EVs can be categorized into four major subtypes: exosomes (30–150 nm), microvesicles (50–1,000 nm), apoptotic bodies (800–5,000 nm), and oncosomes (1–10 mm) [51]. However, this classification remains contentious. The International Society for Extracellular Vesicles suggests categorizing EVs into two main groups: small EVs (sEVs, $<$ 200 nm in diameter) and large EVs (lEVs, $>$ 200 nm in diameter) [50]. Regardless of the classification method, the size of EVs is a vital parameter in their characterization. The *in vivo* fate of EVs is also shaped by their size, lEVs ($>$ 200 nm) tend to accumulate in the liver and spleen, while sEVs can traverse leaky endothelial barriers and accumulate in ischemic tissue [52,53].

Proteomic evidence indicates that tetraspanins, particularly CD63, CD81 and CD9, are highly expressed in EVs derived from various donor cells [54]. These tetraspanins typically serve as molecular markers of EVs. Moreover, EVs carry parent cell-specific signatures, enabling specific interactions with target cells. EV cavities encapsulate various proteins and RNAs, including miRNA, long noncoding RNAs (lncRNA), transfer RNA and ribosomal RNA [55]. These cargos are fundamental for intercellular communication, reflecting the physiological properties of the parent cells. EVs originating from different cell sources exhibit significant variations in the miRNA types and quantities. However, the mechanism underlying EV cargo sorting remains incompletely understood [56].

Once EVs reach their recipient cells, they can be internalized *via* various mechanisms, including micropinocytosis, phagocytosis, clathrin-mediated endocytosis (CME), and possibly direct fusion [57,58]. Several factors, including the size of EVs, can influence the routes of internalization. Studies suggest that sEVs (up to 200 nm) are primarily internalized *via* CME, while lEVs tend to be taken up by cells through macropinocytosis and phagocytosis [59]. The surface components of EVs also influence their internalization. Proteins on EV membranes, such as tetraspanins, lectins, and integrins, participate in EV uptake by interacting with membrane receptors on target cells [57,60]. Therefore, preserving the intact membrane EV structure is essential. Other factors, including surface charge and the proteins and glycoproteins on target cells, are likely to affect the uptake routes of EVs [61]. However, further research is warranted to understand the mechanisms underlying EV cellular uptake. After endocytosis, EVs reach multivesicular endosomes, releasing their contents by fusing with the endosome membrane. However, in most cases, multivesicular endosomes are targeted to lysosomes. Alternatively, EVs that enter receptor cells *via* membrane fusion can release their contents directly into the cytoplasm. This direct fusion route is more efficient for cargo delivery. Once the cargo enters the cell, it can regulate the physiological state of the target cells. EVs have been found to contribute to cell survival, inflammation, neurogenesis, angiogenesis, and fibrosis regarding I/R injury, depending on their origin and composition (Fig. 1) [36,48,62].

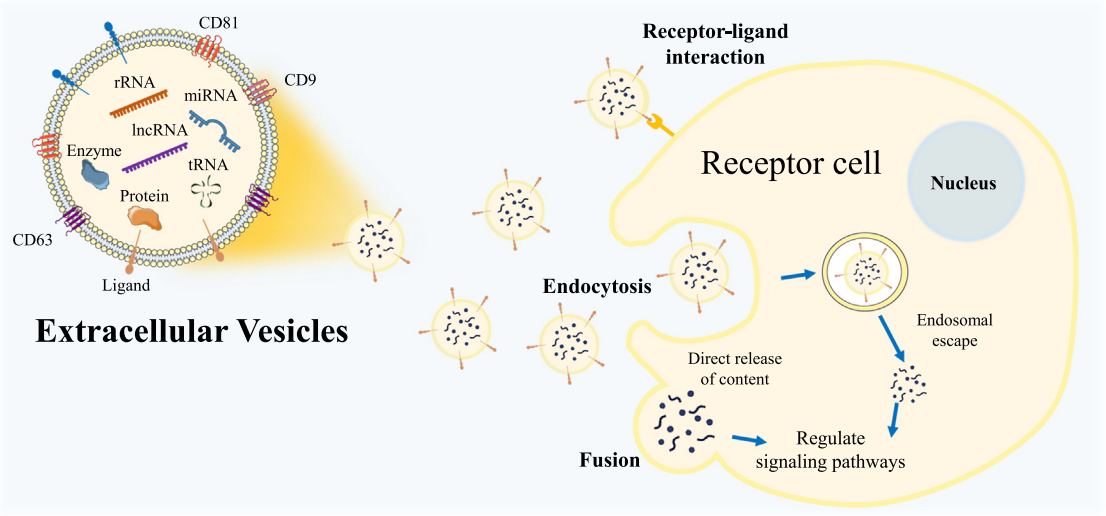


**Fig. 1 – The structure of extracellular vesicles (EVs) and proposed mechanisms of EV uptake by target cells.**

## 4. Native EVs for delivering therapeutic agents in I/R injury

EV biophysical properties have inspired further investigation into their therapeutic applications. Acting as natural carriers of genetic material, EVs have a highly stable structure that can protect therapeutic agents from endogenous enzymatic degradation. As heterogeneous vesicles, EVs are characterized by low immunogenicity and high biocompatibility. Compared to cell-based therapy, such as stem cell implantation, EVs circumvent the risks associated with tumorigenicity and abnormal tissue growth [63]. EV therapeutic potential is primarily attributed to their ability to transport proteins and RNAs. Furthermore, EVs can be derived from varying parent cells depending on the treatment intent. EVs derived from different cell origins carry diverse cargo that reflects the physiological state of the parent cells. Here, we describe the therapeutic effects of native EVs from different parent cells and the distinct cargo they carry (Table 1).

### 4.1. *EVs from stem cells*

Stem cell-based therapy is a vital component of regenerative medicine for ischemic heart disease and ischemic stroke, exhibiting marked potential in tissue repair. Our previous research revealed the therapeutic potential of stem cell-based therapy in IS [64]. Stem cells were initially assumed to exert their therapeutic effects by engrafting at the injured site and subsequently differentiating into mature functional cells. However, recent studies have highlighted the paracrine capability of stem cells [65,66]. As paracrine factors of stem cells, EVs exert similar biological functions in restoring tissue microenvironment homeostasis [20,67]. Additionally, compared to whole cell-based therapy, EVs offer superior advantages, such as lower immunogenicity and the inability to form tumors directly [65]. Therefore, stem cell-derived EVs serve as potential cell-free alternatives to whole-cell therapy. Their therapeutic effects are extensively investigated in ischemic heart disease and ischemic stroke.

Currently, mesenchymal stem cell-derived EVs (MSC-EVs) are the most extensively researched stem cell-derived EVs. MSC-EVs demonstrate therapeutic potential in anti-inflammation, angiogenesis, neuroprotection, and functional recovery by delivering bioactive cargo (Fig. 2A). For example, MSC-EVs are enriched with therapeutic microRNAs, such as miR-125a-5p and miR-200a-3p, which alleviate I/R injury [68,69]. For example, miR-125a-5p, one of the most highly expressed miRNAs in MSC-EVs, protects the heart against I/R injury by modulating macrophage polarization (Fig. 2B–2D). Additionally, miR-125a-5p regulates the proliferation and migration of vascular ECs, indicating an angiogenic effect (Fig. 2E) [70]. Proteins are another crucial component of EVs in injury recovery. MSC-EVs encapsulate growth factors secreted by MSCs, such as hepatocyte growth factor and vascular endothelial growth factor (VEGF), to regulate vascular remodeling and EC proliferation [22,71]. Proteomic analysis revealed significant clustering of glycolytic enzymes such as GAPDH, PGK, PGM, ENO, PKm2 and PFKFB3 in MSC-EVs, potentially compensating for glycolytic deficits and facilitating ATP production in I/R injury [72]. However, protein cargos in MSC-EVs are less studied than miRNA, warranting further investigation into their therapeutic effects.

### 4.2. *EVs from endothelial cells*

ECs are crucial for vascular homeostasis, acting as barriers within the vasculature [73]. ECs release EVs that transmit messages to recipient cells. In ischemic injury, endothelial cell-derived extracellular vesicles (EEVs) mitigate tissue damage and modulate inflammatory cells in peripheral blood [74]. For example, EEVs alleviate cardiomyocyte cell death through the activation of the ERK1/2 pathway in a myocardial I/R injury model [75]. Further research revealed that EEVs exert their protective effect by directly delivering RNA and protein cargo. A study demonstrated that EVs derived

**Table 1 – Native EVs for treatment of I/R injury.**

| EV source | Type | Cargo | Disease | Model | Function | Ref. |
|---|---|---|---|---|---|---|
| ADSC | sEV | miR-221,miR-222, miR-31 | MI/R | Mouse | Inhibit cardiomyocyte apoptosis, promote angiogenesis and tube formulation | [155] |
| ADSC | sEV | miR-22-3p, miR-25, miR-31, miR-760-3p, | MCAO/R | Mouse | Inhibit neural cell apoptosis, neuron ferroptosis and autophagic flux | [41,156] |
| BMSC | sEV | miR-132, miR-486-5p, miR-25-3p, lncR-Mir9-3hg | MI/R | Mouse | Inhibit cardiomyocyte apoptosis, suppress cardiomyocyte ferroptosis, suppress cytokine expression | [157,158] |
| BMSC | Exosome | miR-150-5p, miR-193b-5p, miR-455-3p, lncR-ZFAS1, lncR-KLF3 | MCAO/R | Rat | Reduce neural cell pyroptosis, oxidative stress and inflammation, promote M2 macrophage polarization | [159] |
| ESC | sEV | TGF-$\beta$, Smad2, Smad4, | MCAO/R | Mouse | Modulate neuroinflammation, | [160] |
| EC | EV | miR-129, MMP-2, MMP-9 | MI/R | Mouse | Relieve inflammatory injury, inhibit cardiomyocyte apoptosis, improve migration and tubulogenesis | [77] |
| EC | sEV | miR-206, miR-1-3p, miR-126 | MCAO/R | Mouse | Inhibit neural cell apoptosis, promote M2 macrophage polarization, induce capillary tube formation | [42,44] |
| Serum | Exosome | miR-124-3p, HSP70 | MCAO/R | Mouse | Attenuat BBB deterioration BBB, prevent mitochondria damage | [43] |
| Serum | EV | miR-21-5p, miR-23a, miR-130a-3p, miR-142, miR-223-3p, miR-765, EGF, IGF-1 | MI/R | Mouse | Stimulate angiogenesis, inhibit cardiomyocyte apoptosis, reduce immoderate autophagy | [161] |

MI/R, myocardial ischemia/reperfusion injury; MCAO/R, middle cerebral artery occlusion reperfusion.

from human umbilical vein endothelial cells carry miR-129, which mitigates inflammatory injury in mice myocardial I/R models [76]. EEVs contain a profile of proteins that are related to antioxidants, glycolysis, cellular redox homeostasis, and calcium homeostasis. Additionally, the cardioprotective proteins in EEVs can repair the cellular proteome of I/R-injured human cardiac myocytes toward their uninjured state [77].

### 4.3. *EVs from plasma*

Circulating exosomes are crucial in the cardioprotective effect of remote ischemic preconditioning. Exosomes collected from plasma during the late phase of remote ischemic preconditioning demonstrate protective effects in myocardial I/R injury [78]. Studies show the therapeutic potential of plasma-derived EVs in myocardial and cerebral I/R injury. This protective effect is not attained through internalization by primary cardiomyocytes. Moreover, the heat shock protein 70 (HSP70) on the EV surface stimulates endothelial toll-like receptor 4 (TLR4) signaling through receptor-ligand interaction [79]. This TLR4 pathway activation in cardiomyocytes triggers downstream signaling involving ERK1/2 and p38MAPK, phosphorylating cardioprotective effector proteins [79]. The interaction between HSP70 and TLR4 improves lateral migration across the BBB in cerebral I/R injury [80]. Additionally, exosomal HSP70 effectively inhibits ROS-mediated mitochondrial apoptosis [80,81]. These findings suggest the crucial therapeutic role of exosomal HSP70 in I/R injury. Several miRNAs in plasma-derived EVs associated with therapeutic functions, such as miR-130a-3p and miR-126a-3p, have been identified [82,83]. miR-130a-3p carried by plasma-derived EVs reduces excessive autophagy in cardiomyoblasts by inhibiting ATG16L1 [83].

These studies highlight the potential of native EVs in treating ischemic disease. EV function and content are determined by the parent cells. Nevertheless, the specific EV type optimal for I/R treatment remains undetermined. EVs, as key carriers of bioactive cargo, can modulate pathological processes of I/R injury. However, their potential benefits in clinical settings remain incompletely achieved. Hence, certain limitations must be addressed before EVs can be translated into practical applications. For example, the extent of functional improvement is limited following the systemic injection of unmodified EVs [84]. EVs tend to accumulate in the organs of the reticuloendothelial system (RES), with the highest concentrations found in the liver, lungs, kidneys, and spleen [10]. Consequently, only a few exosomes concentrate in the injured areas during cerebral and myocardial ischemia following systemic administration. Moreover, the cargo profile of natural EVs is influenced by parent cell conditions, leading to uncontrollable therapeutic outcomes [85]. These challenges could be addressed through the application of engineering technologies aimed at enhancing the properties of native EVs.

## 5. Engineering strategies of EVs in I/R therapy

The components and structure of EVs allow extensive modification. Optimizing their tissue targeting and bioactive cargo loading can significantly enhance the therapeutic benefit of EVs (Fig. 3). Several established modification

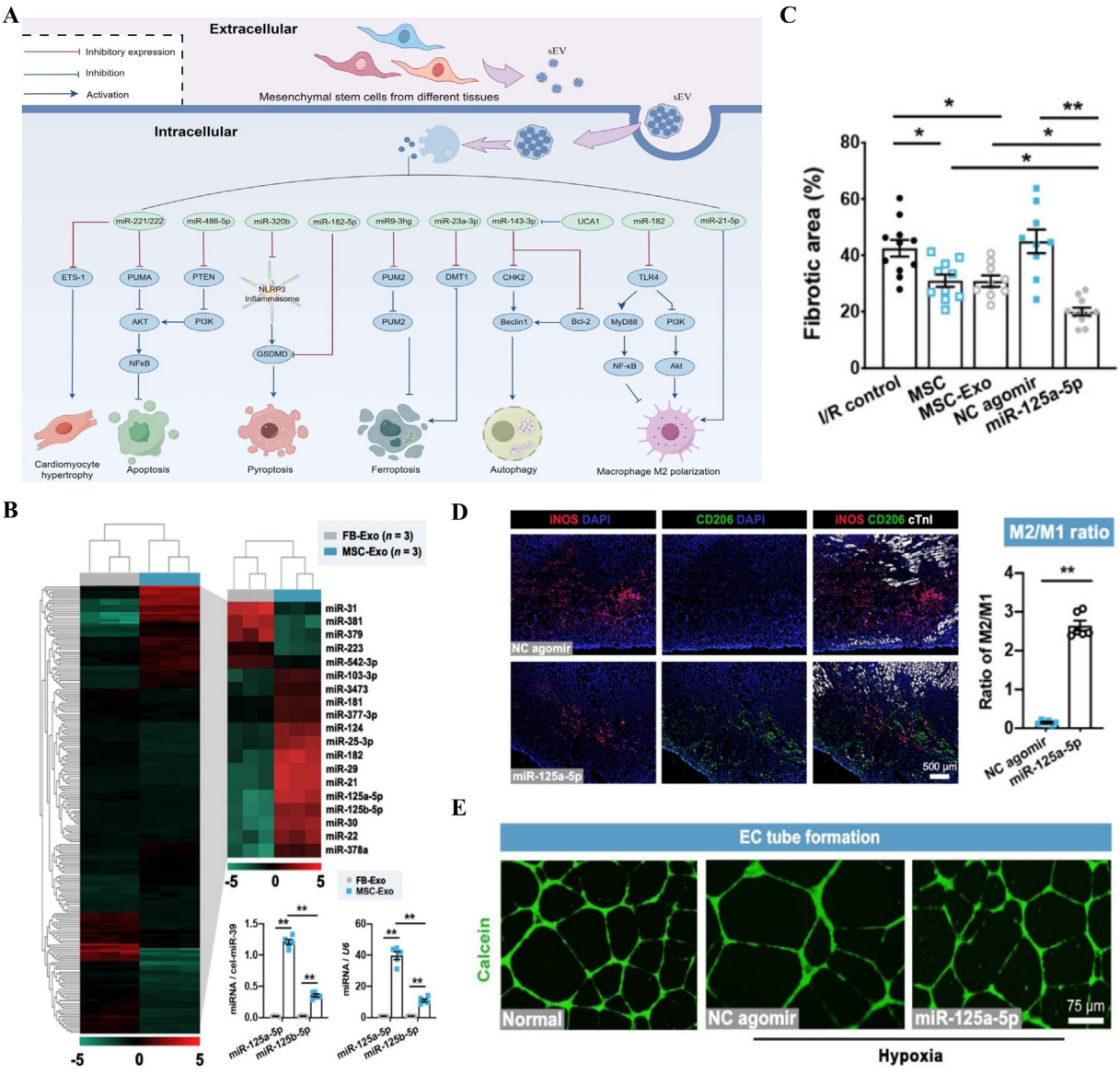


**Fig. 2 – Native MSC-EVs in I/R injury: (A) Schematic diagram of the mechanisms involved in native MSC-EVs treating I/R injury. Reprinted with permission from [154]. Copyright 2023, PMC. (B) The microRNA expression patterns of MSC-Exos are characterized by enrichment of miR-125a-5p compared to exosomes derived from mouse cardiac fibroblasts (FB-Exos). (C) MSC-Exo reversed the I/R-induced adverse myocardial remodeling. (D) miR-125a-5p regulated the polarization of macrophages by declining M1 macrophage phenotype and increasing M2 phenotype. (E) The tube formation of endothelial cells is facilitated by miR-125a-5p. Reprinted with permission from [68]. Copyright 2023, The author(s).**

strategies used for liposomes and nanoparticles, such as cell membrane coating and homing peptide conjugation, can also be applied to EVs (Table 2). Modulating bioactive cargo can be achieved by preconditioning the source cells or loading exogenous substances.

### 5.1. *Cell membrane fusion*

Cell membrane-coating is a commonly utilized technique in nanoparticle modification [86]. Unlike traditional ligand conjugation, cell membrane modification is superior in replicating highly complex cellular functionalities. Thus, cell membrane fusion proves suitable for multifunctional modification and is applicable in complex biological systems [87]. Membrane fusion involves mixing EVs with isolated cell membranes *via* mechanical forces [86]. Co-extrusion is a commonly used method for creating membrane-hybrid EVs, which is achieved by co-incubating EVs with isolated cell membranes and extruding them several times through a polycarbonate membrane. A minimal amount of cargo loss may occur during membrane fusion, potentially owing to deformation during extrusion. Some membrane fractions may remain on the filter, leading to potential functional protein loss. Limiting the number of extrusion rounds can reduce this loss of functional components. Selecting appropriate EVs and cell membrane proportion ensures the integrity and

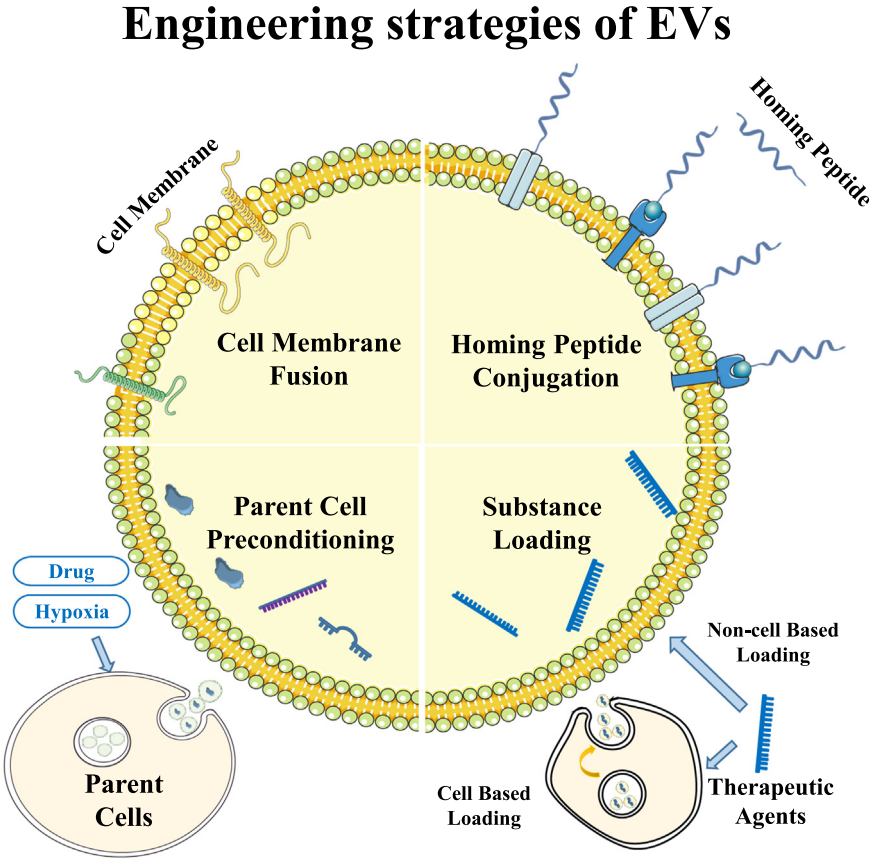


**Fig. 3 – Major engineering strategies of EV modification.**

**Table 2 – Modification to improve the tropism of EVs.**

| Modification | EV source | Method | Disease | Model | Outcome | Ref. |
|---|---|---|---|---|---|---|
| Platelet membrane | MSC | Membrane fusion | MI/R | Mouse | Enhance cellular uptake and targeting; shift macrophage polarization at the injury site | [90,91,92] |
| Macrophage membrane | MSC | Membrane fusion | MI/R | Mouse | Enhance targeting by responding to inflammatory signal | [162] |
| Monocyte membrane | MSC | Membrane fusion | MI/R | Mouse | Enhance targeting and retention | [88] |
| Monocyte membrane | cMac | Membrane fusion | MI/R | Mouse | Enhance targeting and immune evasion | [94] |
| RGD-ACDCRGDCFC | HEK293T | Lentiviral delivery | MCAO/R | Mouse | Enhance targeting | [99] |
| CSTSMLKAC | MSC | Lentiviral delivery | MI/R | Mouse | Enhance targeting and accumulation | [100] |
| LAMP2B-WLSEAGPVVTVRALRGTGSW | CDC | Lentiviral delivery | MI/R | Mouse | Enhance targeting and retention | [163] |
| LAMP2B–APWHLSSQYSRT | HEK293T | Vector transfection | MI/R | Mouse | Enhance targeting | [164] |
| c(RGDK) | MSC | Click chemistry | MCAO/R | Mouse | Enhance targeting | [101] |
| CSTSMLKAC | CDC | DOPE-NHS linker | MI/R | Rat | Enhance targeting | [104] |
| CSTSMLKAC | CDC | DMPE-PEG-STVDN linker | MI/R | Rat | Enhance targeting | [102] |

cMac, cardiac-resident macrophage; RGD, arginine -glycine-aspartic acid; CDC, cardiosphere-derived stem cell; LAMP2B, lysosome-associated membrane glycoprotein 2B.

stability of membrane-hybrid EVs [88]. After fusion, these EVs take on features of parent cells, exhibiting enhanced targeting abilities (Fig. 4A) The fused membrane-EVs displayed a slight size increase and a lower zeta potential compared to unmodified EVs.

Platelet membranes are extensively used for modification in treating I/R injury [89]. Platelets are activated in myocardial I/R injury and can adhere to the injured vessel through platelet surface glycoproteins such as integrin $\alpha 2/\beta 1$ (GPIa/IIa) [90]. Therefore, coating MSC-EVs with platelet membrane

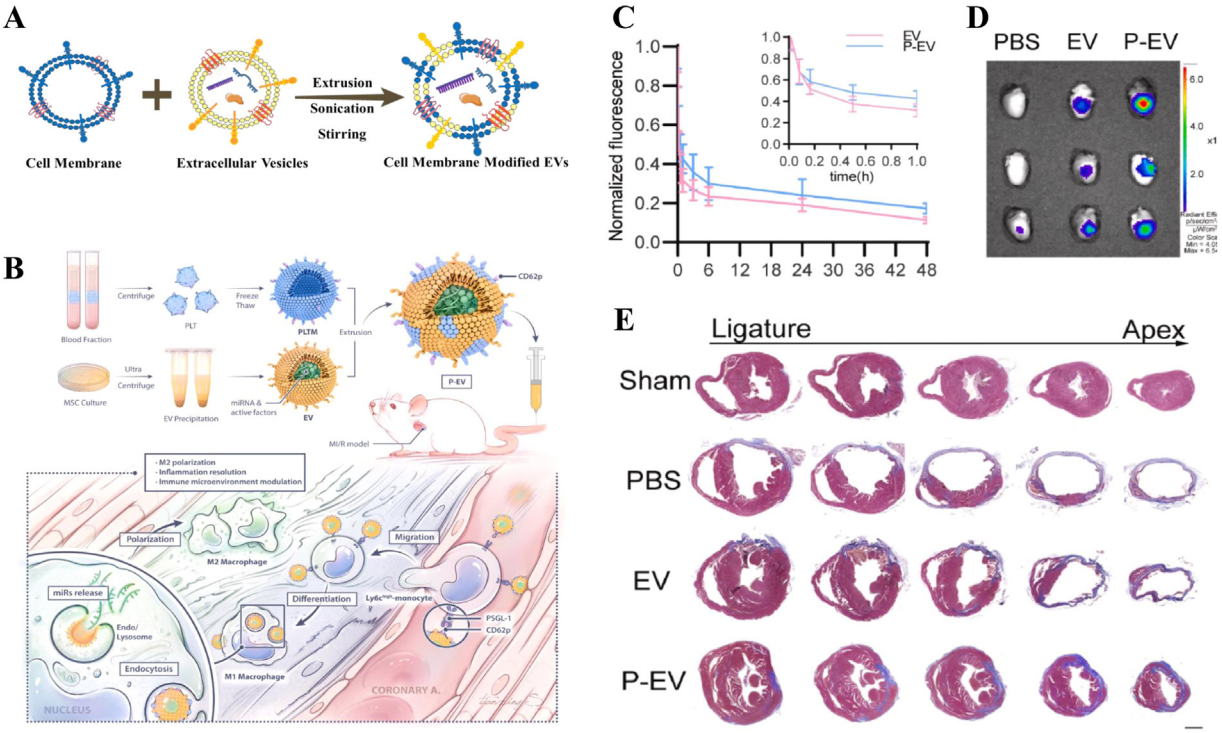


**Fig. 4 – Modification of EVs by cell membrane: (A) Schematic diagram of cell membrane fusion on EVs. (B) Schematic diagram of P-EVs in ischemic myocardium for immune regulation. (C) Pharmacokinetics curve of P-EVs. (D) The infarcted hearts at 3 h post injection with PBS, DiD-labeled EVs or P-EVs after 72 h reperfusion. (E) Cardiac repair of EVs and P-EVs in I/R injured heart. Reprinted with permission from [91]. Copyright 2022, Elsevier.**

confers targeting abilities to the hybrid EVs while maintaining the regenerative function of MSC-EVs [90,91]. Additionally, modifying platelet membranes enhances cellular uptake by endothelial cells and reduces macrophage clearance through the signaling protein CD47 [92]. Another study on platelet membrane-fused EVs demonstrates therapeutic benefits through immunomodulation. Platelet membrane-fused MSC-EVs (P-EVs) bind to pro-inflammatory Ly-6Chigh monocytes *via* P-selection (Fig. 4B). At the myocardial I/R site, P-EVs can undergo *in-situ* endocytosis by M1 macrophages differentiated from monocytes and reprogram them into anti-inflammatory M2 macrophages [91]. These findings suggest that platelet membrane fusion modification enhances the tissue-homing ability and therapeutic efficacy of EVs (Fig. 4C–4E). However, a potential concern with using platelet membranes is the risk of thrombosis, necessitating further attention to such adverse effects of platelet membrane modification.

Modifying with monocyte membranes provides another potential strategy for disease-specific targeting in I/R injury [93]. Leukocytes infiltrate the injured area following I/R injury as part of the inflammatory response. MSC-EVs modified by monocyte membranes can simulate the recruitment of circulating monocytes after myocardial I/R injury, thereby demonstrating enhanced targeting efficiency. This improved adhesion and migration of the modified EVs are partly due to the adhesive molecules LAF1/Mac1/VLA4 on the monocyte membrane [88]. Furthermore, the expression of CD47 on the monocyte membrane helps evade phagocytosis by the mononuclear phagocyte system, thereby avoiding immune clearance and enabling extended circulation in the bloodstream [94].

Surface modification with cell membranes confers EVs with multifunctionality, thereby enhancing the specificity and efficacy of EV-based therapy in I/R injury. Understanding the cell membrane components will facilitate the development of cell membrane-fused EVs. For example, immunosuppressive cells, such as regulatory T cells (Tregs), express functional ligands on their surface. Several membrane proteins, including Neuropilin-1 and CTLA-4, contribute to Treg-mediated immunosuppression. Therefore, a strategic combination of Treg cell membranes and EVs can be expected to enhance the anti-inflammatory effects of I/R injury treatment [93,95,96]. Concurrently, advancements in cell membrane isolation and fusion precision are necessary for enhancing the controllability of cell membrane fusion.

### 5.2. *Homing peptide conjugation*

Homing peptides are small molecules that can specifically interact with target proteins [97]. Injured tissue shows high expression of certain proteins, such as integrin $\alpha v\beta 3$ and growth-associated protein-43 (GAP43), after I/R [98,99]. Specific epitope-targeting homing peptides can be obtained through phage display. Table 2 summarizes various methods of homing peptide conjugation. In bioengineering, parent cells undergo genetic modification to express particular peptides,

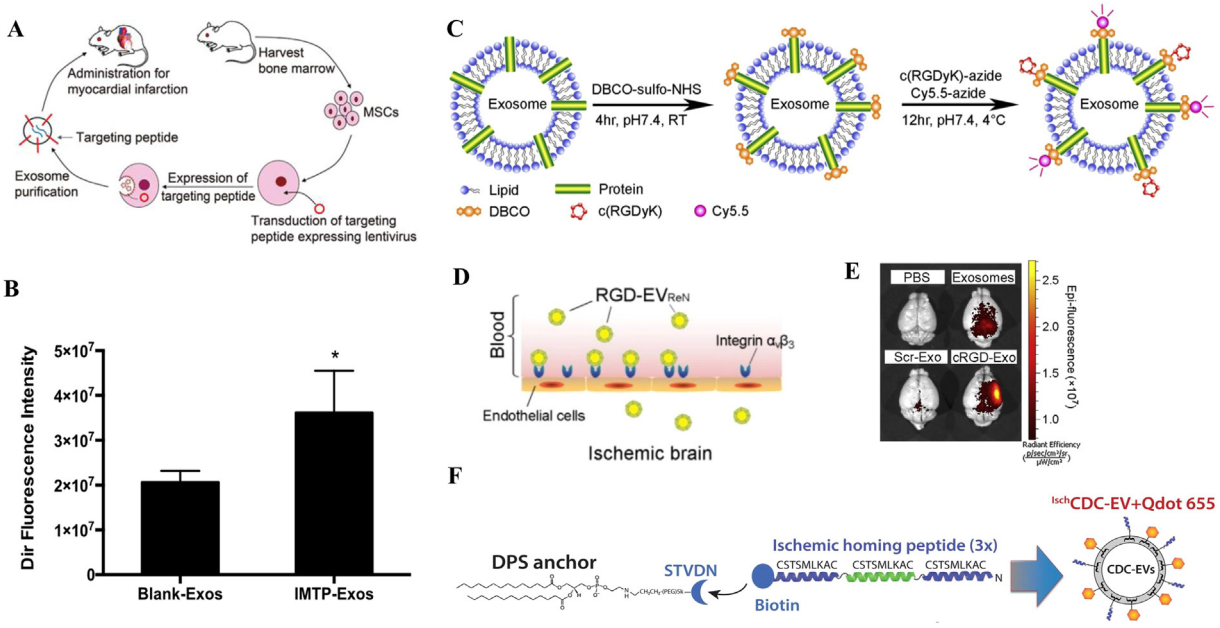


**Fig. 5 – Homing peptide conjugation on EVs: (A) Schematic diagram of conjugating ischemic myocardium-targeting peptide CSTSMLKAC on EVs through lentiviral delivery. (B) Accumulation of signal in the myocardial ischemic region after treatment with IMTP-Exos and blank-Exos. Reprinted with permission from [100]. Copyright 2018, Wiley. (C) Schematic diagram of conjugating c(RGDyK) and Cy5.5 fluorophor to exosomal amine groups within a two-step reaction. (D) Schematic diagram of RGD-EVReN binding to the integrin αvβ3 on activated endothelial cells of I/R injured brain following intravenous administration. Reprinted with permission from [101]. Copyright 2018, Elsevier. (E) Representative NIRF images of MCAO/R mice brains after administration of PBS, Cy5.5-labeled exosomes, Scr-Exo or cRGD-Exo. (F) Schematic diagram of ischemic peptide membrane cloaks. Reprinted with permission from [102]. Copyright 2018, The Author(s).**

which are subsequently displayed on secreted EVs. For example, Wang et al. used lentiviral delivery to fuse the ischemic myocardium-targeting peptide CSTSMLKAC (IMTP) onto MSCs, resulting in IMTP-exosome production. This significantly enhanced the specificity of EVs targeting the ischemic myocardium [100] (Fig. 5A and 5B). This approach allows for the reproducible production of EVs with desired properties. However, creating modified cell lines is time-consuming and costly. Additionally, controlling the peptide density remains challenging.

Homing peptide conjugation can be achieved through chemical methods. This approach is less time-consuming than genetic editing and more suitable for clinical applications. Functional peptides can be incorporated into membranes *via* a lipid anchor. Tian et al. used click chemistry (azide-alkyne cycloaddition reaction) to conjugate the cyclo(Arg-Gly-Asp-d-Tyr-Lys) peptide [c(RGDyK)] to the surface of MSC-derived exosomes (Fig. 5C). c(RGDyK)-conjugated exosomes can selectively bind to integrin αvβ3, which is predominantly expressed on cerebral vascular ECs following ischemia (Fig. 5D and 5E) [101]. Furthermore, Tian et al. generated a recombinant fusion protein (RGD-C1C2) that self-associates with phosphatidylserine on the EV membrane. This modification was more efficient, with RGD-C1C2 peptide decoration achieved through a 15-min incubation [99]. In another study, researchers developed a modular EV membrane anchoring platform by conjugating streptavidin (STVDN) with glycerol-phospholipid-PEG (DMPE-PEG). DMPE-PEG is inserted into vesicle membranes as an anchor, allowing any biotinylated molecule (*e.g.*, homing peptide CSTSMLKAC) to be displayed on the membrane (Fig. 5F) [102]. Another common conjugation method for EVs is lipid insertion. This conjugation method has been applied on modifying EV for osteoarthritis and cancer therapy[103]. Owing to the high fluidity of the vesicle membrane, homing peptides coupled with a lipid linker can penetrate the EV membrane and anchor on the surface. Distearoyl phosphoethanolamine (DSPE) and dioleoylphosphatidylethanolamine N-hydroxysuccinimide (DOPE-NHS) are commonly used linkers for modification [103,104]. This conjugation method is simple and applicable to virtually all EV types, making it a potential approach for homing peptide conjugation in I/R injury treatment.

Various conjugation methods have been used to decorate EVs with functional ligands, each with its advantages and disadvantages. Future research will focus on enhancing the controllability of the modification, including the location and density of the conjugated peptide.

### 5.3. *Parent cell preconditioning*

The cargo in EVs is significantly influenced by the condition of the parent cells. Different treatment conditions and cultural environments can modulate the characteristics of these cells. Preconditioning with bioactive substances has been shown to improve the therapeutic efficacy of MSC-based therapy. Therefore, preconditioning parent cells is a potential method to enhance EV functions by modulating encapsulated cargo

**Table 3 – Precondition on parent cells to modulate EV cargos.**

| Precondition method | EV source | Cargo change | Disease | Model | Outcome | Ref. |
|---|---|---|---|---|---|---|
| Hypoxia | NSC | Upregulate miR-148a-3p, miR-30e-3p, miR-146a-5p, miR-25-3p, miR-26b-5p, miR-320-3p, miR-103-3p and miR-99a-5p | MCAO/R | Mouse | Regulate inflammatory microenvironment and promote nerve regeneration | [109] |
| Hypoxia | ADSC | Upregulate miR-224-5p | MI/R | Mouse | Ameliorate cardiomyocyte apoptosis and pyroptosis | [165] |
| Hypoxia | MSC | Upregulate miR-126-3p, miR-140-5p, let-7c-5p and downregulate miR-186-5p, miR-370-3p, miR-409-3p | MCAO/R | Mouse | Enhance endothelial growth, migration and tube formation | [110] |
| Cyclic HR | C2C12 | Upregulate miR-182-5p | MCAO/R | Mouse | Enhance accumulation in the ischemic hemisphere and angiogenesis | [166] |
| KLF2-transduction | HUVEC | Upregulate miR-24-3p | MI/R | Mouse | Alleviate inflammation | [167] |
| TNF-$\alpha$ | MSC | Upregulate miR-21-5p | RI/R | Mouse | Modulate inflammatory microglia and alleviate apoptosis | [168] |
| Atorvastatin | MSC | Upregulating long non-coding RNA H19 | MI/R | Rat | Improved cardiac function and promote blood vessel formation | [111] |
| Hydrogen sulfide | MSC | Upregulate miR-7b-5p | MI/R | Mouse | Enhance neuroprotective and anti-inflammatory effects | [169] |
| TSA | MSC | Upregulate miR-223-5p | MI/R | Rat | Reduce monocyte infiltration and enhance angiogenesis | [113] |

HR, hypoxia-reoxygenation; KLF2, krüppel-Like Factor 2; TSA, Tanshinone IIA.

[105]. Various preconditioning strategies, including hypoxia, cytokines, and pharmacological substances, have been widely employed (Table 3).

Hypoxia is the most extensively investigated preconditioning strategy. Research has shown that EVs derived from hypoxia-stimulated cells exhibit enhanced functions in angiogenesis, neuroprotection, and tissue regeneration [106,107]. Previous research substantiated that hypoxia preconditioning of MSCs can upregulate the hypoxia-inducible factor 1-alpha (HIF-1$\alpha$) in exosomes, thereby stimulating angiogenesis in vascular ECs [108]. Additionally, hypoxic preconditioning was employed on neural stem cells (NSCs) to generate hypoxic exosomes (H-EXOs) (Fig. 6A). In the middle cerebral artery occlusion (MCAO) mouse model, H-EXOs repaired the damaged BBB induced by I/R injury, creating a more conducive environment for neurological recovery (Fig. 6B) [109]. The enhanced function of hypoxic EVs can be attributed to the alteration of their cargo. Previous studies have demonstrated that hypoxic MSC-derived EVs exhibit higher levels of miR-126, which is associated with angiogenesis. MicroRNAs (miR-148a-3p, miR-146a-5p, and miR-103–3p) in exosomes are selectively upregulated by hypoxia (Fig. 6C). Proteome analysis revealed that EVs derived from hypoxic MSCs are rich in growth factor pathway–associated proteins and extracellular matrix proteins/proteases, while showing a decrease in oxidative metabolism-associated proteins [110]. Additionally, pro-angiogenesis factors, such as VEGF, EGF, FGF, monocyte chemoattractant protein 2, and monocyte chemoattractant protein 4, are significantly upregulated in EVs derived from hypoxia-preconditioned cells [107].

Cytokines or pharmacological substances can be used in donor cell preconditioning, enhancing the expression of protective miRNA in EVs. Ning et al. utilized atorvastatin (ATV)—a commonly prescribed lipid-lowering drug for coronary heart disease—to pretreat MSCs. Exosomes derived from ATV-preconditioned MSCs ($MSC^{ATV}$-Exo) exhibited enhanced angiogenesis and cardioprotective effects in a rat acute MI model (Fig. 6D). This functional enhancement is mediated by lncRNA H19 and miR-139-3p upregulation in $MSC^{ATV}$-Exo (Fig. 6E) [111,112]. Similarly, Li et al. used tanshinone IIA (TSA)—a potential pharmacological agent for treating myocardial I/R injury—in MSC preconditioning. The expression of cardioprotective miR-223-5p increased significantly in TSA-MSCexo, thereby attenuating myocardial damage following I/R injury effectively [113]. In another study, oridonin-preconditioned bone marrow mesenchymal stem cells (BMSCs) were utilized. The derived EVs increased autophagy activation and showed enhanced protective effects [114].

In summary, donor cell preconditioning strategies have demonstrated several favorable outcomes in experimental investigations. Various methods have been explored to enhance therapeutic outcomes. However, before implementing these in clinical settings, developing standardized methods and identifying optimal culture conditions for parent cell preconditioning is crucial.

### 5.4. *Substance loading*

Table 4 summarizes the various methods used to package therapeutic agents into EVs. These methods fall into two

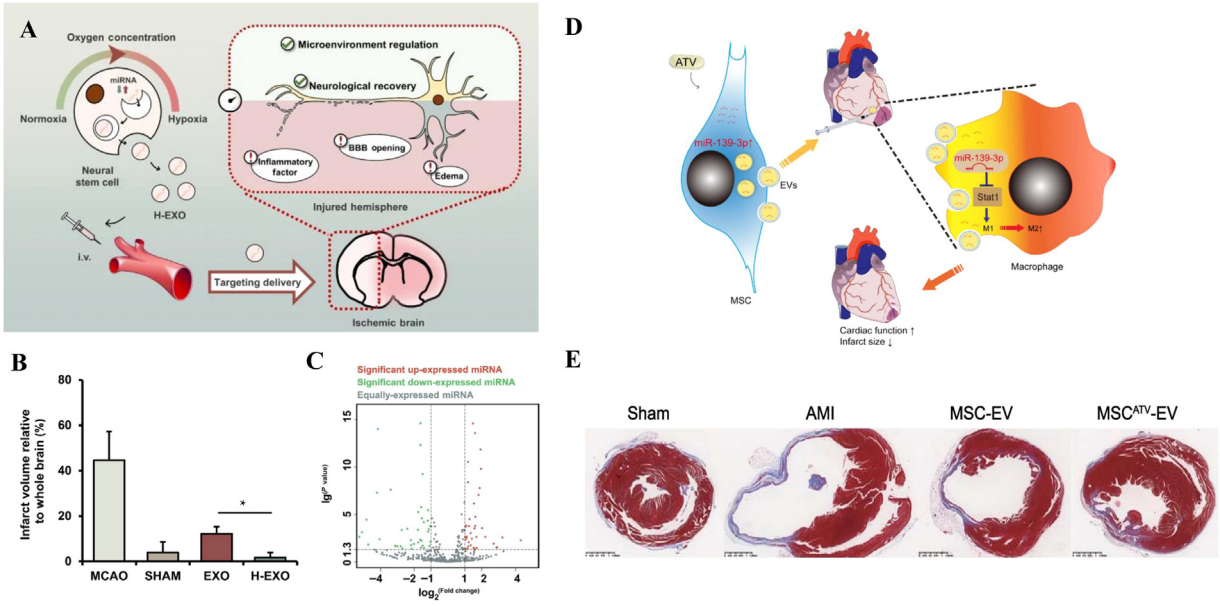


**Fig. 6 – Modification by precondition on parent cells. (A) Schematic diagram of exosomes generated from hypoxic preconditioned NSCs (H-EXO) for specifically delivering therapeutic miRNA to the ischemic region in brain. (B) The infarct volume after EXO and H-EXO treatment. (C) Volcanic spot of miRNA expression with distinct alteration. Reprinted with permission from [109]. Copyright 2022, Springer. (D) Schematic diagram of atorvastatin precondition to enhance the therapeutic effect of MSC-EVs in acute MI through increasing the expression of miRNA-139-3p. (E) Masson trichrome staining of 4 weeks following MSC$^{ATV}$-EVs treatment in MI. Reprinted with permission from [112]. Copyright 2023, BMC.**

**Table 4 – Substance loading to modulate EV cargos.**

| Method | Substance | EV source | Disease | Model | Outcome | Ref. |
|---|---|---|---|---|---|---|
| Electroporation | miR-126 | CPC | MI/R | Rat | Reduce infarct size and improve myocardial function | [85] |
| Sonication | Heptapeptide | macrophage | MCAO/R | Rat | Reduce mitochondrial damage | [170] |
| Co-incubation | BDNF | NSC | MCAO/R | Rat | Inhibit the activation of microglia and promote the differentiation of endogenous NSCs into neurons | [171] |
| Parent-cell transfection | miR-126 | ADSC | MI/R | Rat | Enhance microvasicular generation and migration; reduce cardiac fibrosis | [119] |
| Parent-cell transfection | miR-21 | HEK293T | MI/R | Mouse | Enhance anti-apoptotic effect | [172] |
| Parent-cell transfection | BDNF | MSC | MCAO/R | Mouse | Increase neurogenesis, angiogenesis and synaptic plasticity | [173] |
| Parent-cell transfection | AAV9-SERCA2a | HEK293T | MI/R | Mouse | Improve cardiac remodeling and function | [174] |

BDNF, brain-derived neurotrophic factor; AAV9-SERCA2a, adeno-associated virus9- sarcoplasmic reticulum calcium ATPase 2a.

categories: non-cell-based and cell-based loading (Fig. 7A). Non-cell-based loading involves introducing exogenous substances into EVs by enhancing the permeability of the EV membrane through methods such as electroporation, sonication, or heat shock [115,116]. This approach is highly flexible, allowing for the customization of cargo within EVs [85]. For example, Sruti et al. employed electroporation to encapsulate endothelial-specific miR-126 cargo into cardiac c-kit+ cell-derived EVs (CPC-sEVs) (Fig. 7B). The modified EVs retain their membrane structure for cellular uptake while carrying customized cargo, enhancing reparative effects in myocardial I/R injury [85] (Fig. 7C). In another study, miR-126 was loaded into CPC-sEVs using thin-film hydration (TFH)—a well-established and high-efficient method in synthetic nanoparticle [117]. However, loading genetic material often requires disrupting the EV membrane with mechanical force. This process may compromise the integrity of the EV membrane and denature membrane proteins [118]. Since the membrane structure and stability are crucial for protecting the lumen cargos, improper loading processes can damage exosomes [115]. For non-cell-based substance loading, EVs can also be fused with liposomes. Drugs can

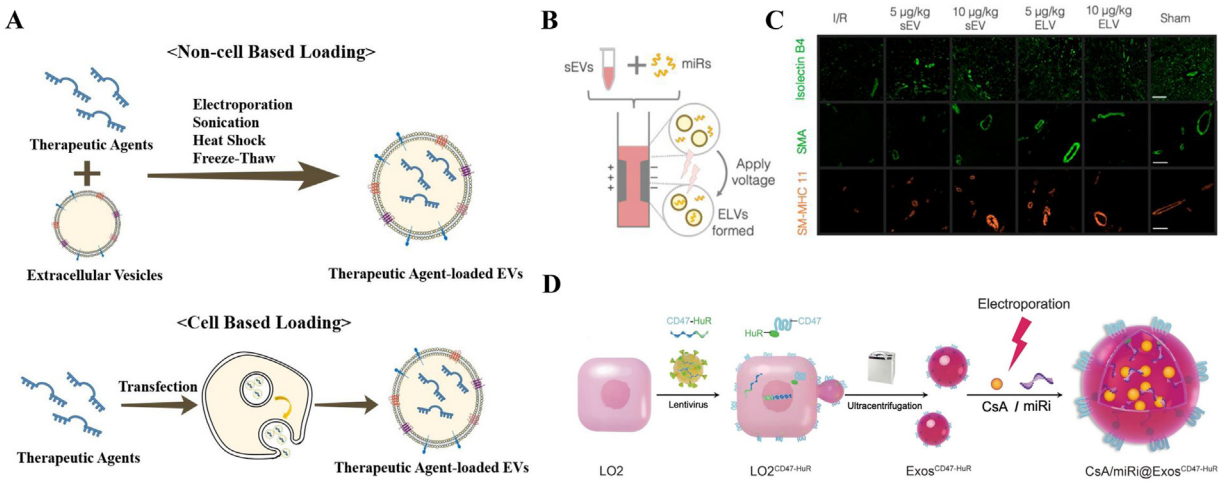


**Fig. 7 – Modification by substance loading. (A) Schematic diagram of substance loading of EVs. (B) The process of synthesizing ELV, using sonication to remove cargo and electroporation to load miR-126. (C) After 28 d of treatment, miR-126+ ELVs enhance the formation and size of blood vessels. Reprinted with permission from [85]. Copyright 2023, ACS. (D) Schematic illustration of CsA/miRi@ExosCD47-HuR preparation. Reprinted with permission from [123]. Copyright 2024, PMC.**

be loaded into EVs through co-extrusion with liposomes. However, its loading efficiency remains a significant challenge.

Genetic material can also be loaded into EVs through a cell-based approach by modifying donor cells to express specific proteins or RNA, which are then encapsulated into the EVs. This method minimizes damage to the EVs, which has been extensively studied. For example, EVs obtained from adipose-derived stem cells (ADSCs) overexpressing miR-126 demonstrated a reduction in myocardial damage by inhibiting myocardial cell apoptosis and promoting angiogenesis following I/R injury [119]. In another study, Hyun-Ji et al. used synthetic miRNA inhibitors to knockdown the expression of miR-192-5p and miR-432-5p in CPCs. This reduction in miRNA expression correlated with a decrease in miRNA content in CPC-sEV. The therapeutic efficacy of miR-192-5p/miR-432-5p-depleted sEVs is enhanced in a myocardial I/R model [120]. To further enhance the controllability of indirect loading, an extensive understanding of the precise mechanisms by which genetic cargoes are sorted into EVs is necessary.

Both loading methods aim to optimize the cargo carried by EVs and explore their potential as nanocarriers. The choice of loading method primarily depends on the types and properties of the therapeutic agents involved. Transduction and transfection of parental cells are suitable for loading large biomolecules, including therapeutic proteins and nuclear acids [121]. The agents loaded using the cell-based method should have low toxicity to parent cells. Additionally, the cell-based loading method avoids exposing therapeutic agents in solution, which can influence cargo stability and activity. Non-cell-based loading methods, including simple incubation, are commonly used for loading small RNAs (<1 kDa) or lipophilic drugs that can easily permeate the EV membrane [118].

While various substance-loading methods are available, challenges associated with loading efficiency need to be addressed. The ratio of the EV lumen to the overall EV volume is limited, resulting in a constrained loading capacity of EVs [118]. Therefore, strategies to increase the intravesicular volume of EVs would enhance their loading capacity. Optimizing loading parameters, such as drug concentrations, can also improve loading efficiency. Additionally, controlling the amount of drugs loaded into EVs is challenging. Loaded exogenous substances might interact with endogenous cargo, potentially leading to a loss of functional cargo within EVs. Thus, precise quantification of loaded cargo is necessary for developing substance-loaded EVs [122].

In summary, various engineering strategies can be applied to EVs derived from different cell origins (Table 5), as follows: (1) Cell membrane fusion enables EVs to specifically target injured areas or be taken up by specific recipient cells. This modification strategy is ideal for multifunctional enhancements and can be applied within complex biological systems; (2) Homing peptide conjugation enables EV to interact with specific targets in injured tissues. The conjugation can be achieved through genetic engineering or chemical reactions; (3) Preconditioning of parent cells can influence the cargo loaded into EVs. Common preconditioning methods include hypoxia, cytokines, and pharmacological substances; (4) Loading substances into EVs is an efficient method for customizing their cargo. It is crucial to understand the nature and mechanism of the cargo to be loaded. This loading process can be achieved through non-cell-based and cell-based methods.

Each engineering strategy has its own set of advantages and disadvantages. When selecting the most suitable engineering strategy, it is essential to thoroughly consider the treatment goals, cost factors, and potential risks involved. Combining these modification methods can lead to increased synergies and flexibility in EV-based therapy. For example, Liu et al. functionalized human liver cells (LO2) with a CD47-HuR fusion protein to produce CD47-HuR-reprogrammed

**Table 5 – Comparison of common EV engineering strategies and their benefits/disadvantages.**

| Modification | Method | Benefits | Disadvantages | Outcome | Ref. |
|---|---|---|---|---|---|
| Cell membrane fusion | Mix EVs with isolated cell membranes *via* mechanical forces | Replicate complex cellular functionalities; suitable for multifunctional modification; easy to operate | Potential functional cargo loss;r isk of thrombosis (using platelet membrane) | Enhance targeting abilities;e nhance cellular uptaker educe immune clearance | [90,87] |
| Homing peptide conjugation | Genetic editing of parent cells | Allows for reproducible production of desired EVs; less interference to EV integrity | Time-consuming; cost-consuming | Enhance targeting abilities | [163] |
| | Chemical modification | Less time-consuming | Potential interference to EV surface components | | [99,101] |
| Parent cell preconditioning | Precondition parent cells with bioactive substances | Easy to operate; high efficiency; less interference to EV integrity | Lack of standardized methods | Enhance functions in angiogenesis, neuroprotection, and tissue regeneration | [109,112] |
| Substance loading | Non-cell-based loading | Well-established;c ustomization of loaded cargo | Potential interference to EV integrity; limited loading efficiency; potential interference to endogenous cargo | Enhance functions in angiogenesis, neuroprotection, and tissue regeneration | [85] |
| | Cell-based loading | Customization of loaded cargo;l ess interference to EV integrity | Time-consuming; potential interference to endogenous cargo | | [175] |

exosomes (Fig. 7D). The CD47 protein emits a "do not eat me" signal to macrophages, allowing the exosomes to evade immune surveillance. Additionally, through electroporation, the Cyclosporin A (CsA) and mitochondrial transcription factor A (TFAM) targeting microRNA inhibitor (miRi) were encapsulated into CD47-HuR-reprogrammed exosomes. Reprogramming of exosomes allowed for the specific delivery of CsA and microRNA inhibitors to the ischemia/reperfusion injured area, maintaining mitochondrial homeostasis and reducing ROS production [123].

## 6. Challenges and prospects

The potential of EVs to deliver therapeutic cargo for recovering from I/R injury has been demonstrated in preclinical studies. Currently, several clinical trials involving EV-based therapy (generally MSC-EVs) are underway for IS treatment [124–126]. However, despite these promising developments, several crucial challenges exist that need to be addressed to facilitate the widespread clinical translation of EVs.

### 6.1. *Challenges in production*

The low yield and poor reproducibility in EV production inhibit its widespread application. A single cell typically secretes 50 EVs per min [127]. Producing a clinical-grade quantity of EVs requires long-term passaging, which may influence the condition of the cells. Increasing EV secretion through physical stimuli (*e.g.*, high-frequency ultrasound and electric treatment) or environmental factors (*e.g.*, hypoxia and acidity) can help alleviate this challenge [128]. Furthermore, the production of EVs, particularly sEVs, faces challenges related to separation and purification methods. The most commonly used method for separating EVs is ultracentrifugation, which isolates substances based on density and size. This process requires extended periods of centrifugation, limiting its widespread applications [129]. Additionally, ultracentrifugation cannot separate specific EV populations, and impurities such as co-purifying protein aggregates often contaminate the samples [129]. The heterogeneity of EVs and the presence of co-isolates make maintaining batch-to-batch consistency challenging. Developing standardized, reproducible, and cost-effective isolation methods is significant for ensuring quality control in EV production. Alternative methods, including size exclusion chromatography (SEC) and tangential flow filtration (TFF), offer advantages over the conventional ultracentrifugation-based isolation method in terms of reproducibility and purity [130]. With the advancement of nanotechnology, microfluidics has emerged as a promising technique for optimizing EV isolation. Microfluidic devices manipulate fluid flow through a network of microchannels and can separate EVs based on their size, density, or surface antigens [131]. Microfluidics offers unique advantages in terms of high efficiency, purity, and yield. However, its widespread applications still require more large-scale experimental data to support its efficacy [132].

Quality control is a crucial concern in the production of EVs. The endogenous cargo and bioactivity of EVs

are significantly influenced by culture conditions such as cell passage number and cell density [133]. Consequently, considerable uncertainties regarding the quality of EVs for clinical use exist [129]. Moreover, owing to a lack of analytical tools, identifying critical quality attributes (CQAs) of EVs remains challenging. Consequently, developing advanced analytical techniques capable of characterizing EVs at a single EV-level, such as super-resolution microscopy, is warranted [134]. Besides technological limitations, adherence to Good Manufacturing Practices (GMP) is essential for ensuring product quality and minimizing risks. It is crucial to establish guidelines for the manufacturing and in-process control of EVs.

To reduce batch-to-batch variation and improve EV yield, researchers are increasingly exploring EV mimics and synthetic EVs. EV-mimics are exosome-like nano-vesicles generated through extrusion, ultra-sonication, or freeze-thawing [135]. They demonstrate structural and functional similarities to natural EVs, and their generation efficacy is enhanced > 100 times [127,136,137]. For example, stem cell-derived bio-responsive EV-mimics for MCAO treatment were developed. These mimics retained high substance-loading capability and effectively delivered payloads to the injured cerebrum [19,138]. Synthetic EVs involve assembling various small molecules, including lipids and proteins, into intricate structures to replicate the functionalities of natural EVs. The lipid, protein, and RNA composition of synthetic EVs can be precisely controlled, enhancing the quality of administration [139]. EV mimics or synthetic EVs can collectively overcome challenges associated with low yield, scale-up production, and quality control in EV-based therapy. However, the immunogenicity, bioactive cargo, and therapeutic effect of these EV alternatives still require further assessment.

Identifying suitable sources for EV manufacturing and clinical use is another challenge that requires attention. EVs obtained from exogenous sources could be advantageous for large-scale production. However, the potential immunogenicity of exogenous EVs needs to be approached cautiously. Exogenous EVs may express heterogeneous MHC class I proteins on their surface, potentially triggering an immune response in patients [140]. Additionally, protein contaminants in EV samples can trigger inflammatory reactions. In contrast, EVs obtained from autologous sources offer advantages in terms of immunocompatibility. However, autologous sources, including biological fluids and autologous cells, are limited. While extensive research has focused on mammalian cell-derived EVs, recent research suggests that plant-derived exosome-like nanoparticles (PENs) could serve as potential nanocarriers [141]. Accumulating evidence indicates that PENs can be produced in large quantities from renewable plant resources, addressing the demand for large-scale manufacturing [142]. Moreover, plants do not harbor human pathogens, and PENs have demonstrated no toxicity in preliminary clinical studies. There is still no report on PENs inducing immune responses, demonstrating their immunocompatibility [143]. PENs can transport bioactive cargo into animal cells, and their therapeutic potential has been shown in recent studies. For example, a study indicated that PENs derived from momordica charantia can inhibit ischemia-reperfusion-induced disruption to the BBB and reduce infarct sizes in MCAO/R rats [144]. As emerging therapeutic modalities, further investigations are needed to understand the mechanisms, characterization, and side effects of PENs [145].

Storage conditions significantly influence the bioactivities and structure of EVs. Currently, the most widely used approach involves preserving samples at −80 °C in phosphate-buffered saline (PBS). However, a systematic study investigating the influence of storage on EVs has revealed that storing them at −80 °C results in a time-dependent decrease in EV concentration and purity, along with an increase in EV size and size variability [146]. Optimizing storage conditions, including reducing freezing and thawing cycles or altering the storage buffer, can mitigate the influence on EV properties [147]. Emerging methods, such as lyophilization with cryoprotectant, also show significant promise for preserving EV integrity during storage. However, no consensus exists on the optimal condition for preserving EVs for clinical applications. Further investigation is needed to improve preservation methods and enhance the stability of EV samples [148].

### 6.2. *Challenges in delivery*

The delivery of EVs in therapeutic applications faces several challenges. Generally, EV-based therapy is administered through intravenous injection. However, the low retention rates and limited presence of EVs in the injured area restrict their long-term protective influence [149]. A significant portion of injected EVs are cleared by mononuclear phagocytes, leading to a short half-life [123]. Some studies have proposed novel methods of EV delivery, including catheter-based intracoronary and open-chest intramyocardial deliveries, particularly for myocardial I/R injury [150]. These approaches have shown improved therapeutic outcomes. However, systemic administration remains a less invasive, safer, and more suitable approach for clinical treatment. To address the challenge of EV retention, biomaterial-based approaches have been developed. For example, EV-releasing hydrogels incorporate EVs into the hydrogel matrix, enabling sustained release of EVs at injury sites [149,151,152]. Additionally, certain EV-releasing hydrogels can respond to pathological changes, such as variations in ROS concentration, thereby offering stimuli-responsive EV-based therapy. Furthermore, Hu et al. developed an exosome-eluting stent (EES) for vascular recovery following ischemic injury. MSC-EVs are coated onto drug-eluting stents and released at the injured site after stent implantation. This process promotes angiogenesis and M2 macrophage polarization at the ischemic site, suggesting improved vascular healing and tissue repair. [153]. However, the safety, stability, and therapeutic effects of these novel delivery methods still require further examination in large-animal studies and clinical trials.

Despite these challenges, the biocompatibility, low-immunogenicity, and flexibility of EVs highlight their strong potential for delivering therapeutic agents. Innovations in therapeutic strategies and technologies are actively overcoming these challenges in the clinical translation of EVs. Therefore, continued exploration and further investigation

into stable and efficient EV-based delivery systems are encouraged.

## 7. Conclusion

Owing to their unique biophysical properties, non-toxicity, and flexibility, EVs play crucial roles in transmitting therapeutic agents for the treatment of ischemia/reperfusion injury. EVs can encapsulate and deliver multiple therapeutic molecules to recipient cells, thereby regulating the pathological process. While native EVs offer advantages as carriers for delivering therapeutic agents, inherent limitations such as limited tropism and low efficacy hinder their applications. Researchers have recently concentrated on modifying natural EVs to improve their targeting ability and manipulate their loaded cargo. These engineered EVs exhibit improved functions and demonstrate better therapeutic outcomes. Despite the robust therapeutic potential shown by native and engineered EVs in preclinical studies, challenges still exist in translating them into clinical applications. To address these constraints, advanced technologies, such as synthetic EVs and EV-releasing hydrogels, have been developed. Ultimately, native and engineered EVs offer promising approaches for modulating the intricate pathophysiology of I/R injury, potentially improving the therapeutic outcomes of patients affected by it.

## Conflicts of interest

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

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (U22A20383, 82003668); the Natural Science Foundation of Zhejiang Province (LD22H300002, LQ21H300002); and Ningbo Technology Innovation 2025 Major Special Project (2022Z150).

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