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REVIEW ARTICLE

Roles of Extracellular Vesicles in Cerebral Protection of Ischemic Stroke

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Abstract

Stroke is the second-leading cause of death worldwide and exhibits a high disability rate. Ischemic stroke accounts for approximately 80% of all stroke cases. Inflammatory responses induced by innate immunity are involved in all stages of stroke-related injury, including early cerebral-infarction tissue repair and regeneration after ischemia. Toll-like receptors are the main receptors involved in innate immunity. Toll-like receptors specific antagonists inhibit neuroinflammation by reducing overproduction of inflammatory mediators. But there are still some limitations, such as affecting protein clearance and myelination. Extracellular vesicles are widespread and distributed in various body fluids, carry and transmit important signal molecules, affect the physiological state of cells and are closely related to the occurrence and progress of many diseases. In the present review, we summarize recent findings regarding the mechanisms by which extracellular vesicles act as signaling vectors to regulate cellular crosstalk between neurovascular units and further discuss the therapeutic effects of extracellular vesicles derived from mesenchymal stem cells on brain injury. Collectively, our review may provide novel insights into further elucidating pathogenesis and cerebral-protective measures of ischemic stroke.

INTRODUCTION

Ischemic stroke refers to ischemic and hypoxia caused by cerebral vascular embolism, which leads to ischemic injury of brain tissue. Ischemic stroke is characterized by high morbidity, disability, recurrence, and mortality rates. In addition, poststroke complications such as post-stroke epilepsy (PSE) and poststroke depression can seriously affect the quality of life of patients and induce a heavy economic burden upon their families and society on the whole (Zelano *et al.* 2020; Villa *et al.* 2018).

In the process of hypoxia and ischemia, all supporting cells closely related to neurons also have different degrees of damage. Hence, simply dealing with neuronal injury cannot fundamentally promote recovery of neurological function. Therefore, researchers have proposed the concept of neurovascular unit (NVU) from a histopathological perspective (Lo & Rosenberg 2009). The NVU is a physiological unit with a specific structure composed of neurons, astrocytes,

oligodendrocytes, pericytes, vascular endothelial cells, and peripheral microglia (McConnell *et al.* 2017). A functioning NVU maintains normal brain homeostasis through dynamic intercellular interactions and signals between individual members of the NVU. After hypoxia and ischemia, each component of the NVU is damaged to a varying degree, causing a relative imbalance between each component that subsequently induces a neuroinflammatory cascade leading to brain damage (Zagrean *et al.* 2018). It was found that in a rat model of focal cerebral ischemia-reperfusion injury prepared by middle cerebral artery occlusion (MCAO), microvascular permeability initially increased at the early stage and further increased over time; subsequently, infiltration of molecules and inflammatory cells in peripheral blood caused excessive inflammation and brain edema, which led to more serious brain injury (Strbian *et al.* 2008). Therefore, the concept of NVU provides a new framework for basic and clinical research on ischemic brain injury. Further exploration of the pathogenesis of NVUs in ischemic brain injury is a prerequisite and breakthrough point for developing effective neuroprotective measures and significantly improving the prognosis of patients with ischemic stroke.

INNATE-IMMUNE INFLAMMATORY RESPONSES IN CEREBRAL ISCHEMIA/ REPERFUSION INJURY

The roles of Toll-like receptors in mediating ischemic brain injury

Although the mechanisms of ischemic brain injury remain unclear, inflammation has been demonstrated to be one of the main factors that mediates brain injury. Ischemic stroke leaves neurons in a pathological state of cellular oxygen/glucose deprivation (OGD), and toll-like receptor (TLR) signaling pathway is important innate-immune-response mechanism that promotes inflammatory responses after ischemic stroke (Famakin 2014).At present, a total of 10 TLRs have been found in humans, and TLRs are evolutionarily-conserved pattern-recognition receptors that recognize either pathogen-associated molecular patterns (PAMPs) related to infection or damage-associated molecular patterns (DAMPs) related to tissue damage. DAMPs include extracellular-matrix fibrinolytic proteins, hyaluronic acid, heparin sulfate, and even molecules found in intracellular compartments such as ATP, heat-shock proteins (HSPs), uric acid, and those derived from nucleus—such as high-mobility family protein B1 (HMGB1), DNA, double-stranded RNAs (dsRNAs), single-stranded RNAs (ssRNAs), and microRNAs (miRNAs) (Shichita *et al.* 2012). PAMPs recognized by TLRs are derived from bacteria, viruses, parasites, and fungi and include lipids, lipoproteins, and nucleic acids (C & microbiology 2013). Ischemic cell death leads to the formation of DAMPs, which activates TLRs. TLRs are widely expressed in glia—including microglia, astrocytes, and oligodendrocytes— and in neurons within both the central nervous system (CNS) and peripheral nervous system (PNS). Microglia secrete strong chemokines and cytokines under TLR2, TLR3, and/or TLR4 stimulation, while astrocytes secrete low levels of interleukin 6 (IL-6) under TLR3 stimulation (BM & neurobiology 2020; BJ *et al.* 2009). Among the many TLR families, TLR2 and TLR4 play the most important roles in the pathological process of cerebral ischemia-reperfusion injury (Wang *et al.* 2013).

TLR2 is mainly expressed in microglia, astrocytes, neurons, and endothelial cells in the CNS. TLR2 mainly transmits extracellular signals through a myeloid differentiation factor 88 (MyD88)-dependent pathway and activates cells to secrete inflammatory factors, pro-inflammatory factors, and pro-apoptotic factors. Studies have found that HMGB1 binds to TLR2 or TLR4 during the development of ischemic stroke, activating downstream signal-transduction pathways—such as phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), MyD88/nuclear factor κB (NF-κB), and mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (ERK) pathways—to stimulate the secretion of pro-inflammatory factors and the proliferation of different cell types (Marsh *et al.* 2009). In a study that used a rat model of ischemic stroke, it was found that brain edema and inflammatory reactions were significantly alleviated at 48 h after cerebral infarction in rats treated with a monoclonal anti-TLR2 antibody, indicating that TLR2 plays an important role in mediating secondary nerve injury and inflammation after ischemic stroke (Wang *et al.* 2011). In addition, TLR4, as a lipopolysaccharide (LPS) signal-transduction receptor, can activate signaling pathways including MyD88-dependent and MyD88-independent pathways; the former pathways recruit IL-1-receptor-associated kinase (IRAK), and phosphorylated IRAK then binds to TNF-receptor-associated factor 6 (TRAF6). Activated TRAF6 phosphorylates IκB kinase, which activates NF-κB and initiates transcription of inflammatory factors. In contrast, MyD88-independent pathways signal via IFN-β to induce expression of connexins (TRIF) and TRIF-related adaptor molecules (TRAM); subsequently, NF-κB and RIF are simultaneously activated to induce synthesis of related target genes (Zhu *et al.* 2016).

In summary, TLRs and their activated downstream inflammatory pathways play important roles in cerebral ischemic injury, and inhibiting TLRs and/or blocking their inflammatory signaling pathways can significantly improve infarct volume and nerve function in ischemic stroke (NA *et al.* 2019; Abdul *et al.* 2019). For example, osteoprotegerin (OPC) inhibits NF-κB receptor activator (RANK)/RANK ligand (RANKL) signal transduction. In $OPG^{-/-}$ mice, enhanced RANKL/RANK signaling helps to reduce infarct volume and brain edema by reducing post-ischemic inflammation. Furthermore,

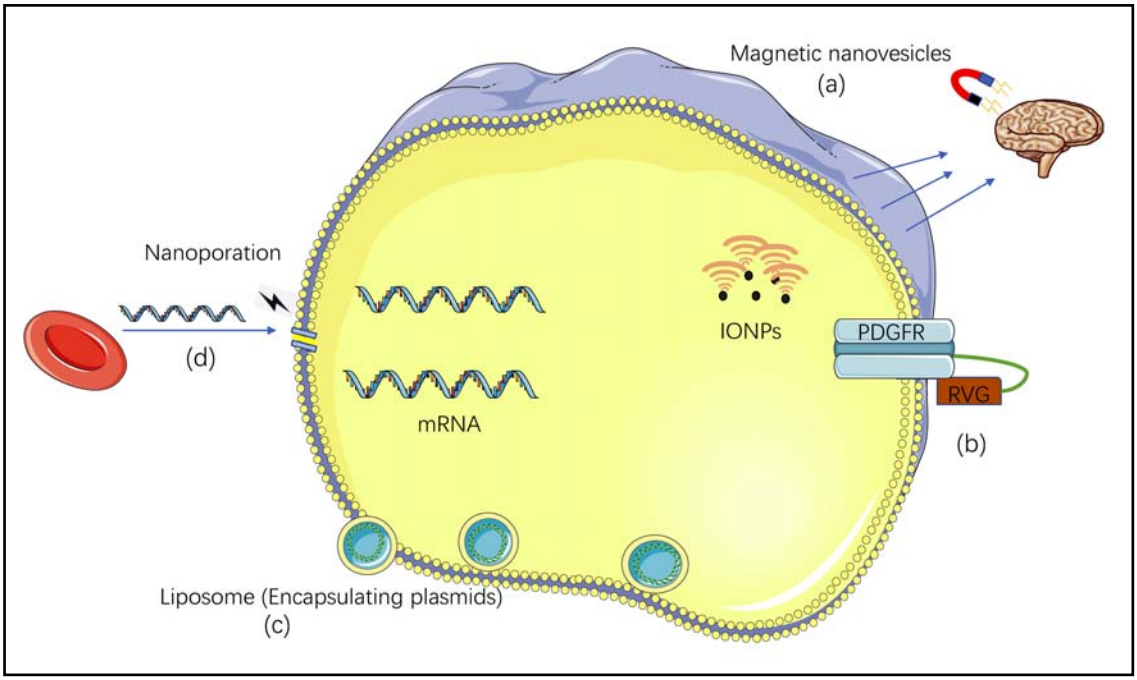

Fig. 1. Strategies to realize efficient encapsulation and targeting include: (a) IONPs could generate magnetism that allows MNV to target ischemic brains with the help of a magnetic helmet; (b) the fusion of RVG peptide and the transmembrane domain of PDGFR can enhance the targeting of EVs; (c) the fusion of the lipid bilayer of EVs with liposome forms EV-liposome hybrid; (d) nanoporation to deliver mRNA molecules into Evs.

OPG, RANKL, and RANK mRNA levels are increased during the acute phase of cerebral infarction and are expressed in activated microglia and macrophages. In LPS-stimulated neuron/glial co-cultures, RANKL/RANK signaling reduces inflammatory responses of microglia by inhibiting TLR4 signaling pathway (M *et al.* 2014; Kurinami *et al.* 2016). Neurosteroids such as estrogen, progesterone, and vitamin D3 can regulate TLR2 and TLR4 signal transduction and have potential neuroprotective effects (S *et al.* 2019). In addition, intravenous immunoglobulin (IVIg) protects neurons from HMGB1-induced neuronal cell death by regulating the activation of TLR2 and TLR4 signaling pathways (KZ *et al.* 2015).

Limitations of innate immune specific antagonists

The cascade of inflammatory responses induced by innate immunity plays a vital role in regulating activation of glia and release of pro-inflammatory cytokines and chemokines caused by cerebral ischemia. Inhibition of inflammasome signaling pathways can improve ischemic brain injury in cells and animal models (Yang *et al.* 2014; Zhang *et al.* 2014; Fann *et al.* 2013; Li *et al.* 2020). However, as cerebral ischemia injury involves complex cascade of pathophysiological processes, a single neuroprotective measure can only act on a certain cell type or a certain molecule in a specific signaling pathway; therefore, such a single-targeted approach cannot provide comprehensive neuroprotection from ischemic cascades. Some TLR/NLRP-specific antagonists may be hindered from passage across the blood-brain barrier (BBB) due to their large molecular weights. In addition, in order to study the effectiveness of innate-immune signaling ligands, rodents are widely used to simulate human PNS and CNS diseases. There are also differences in how mouse and human cells express innate-immune-response mechanisms. For example, in humans, both microglia and astrocytes express TLR4; in mice, only microglia express TLR4 (Zschaler *et al.* 2014). This may make it difficult to observe the same biological efficacies in animal models compared to those in patients with ischemic stroke. It is noteworthy that TLR antagonists may adversely affect CNS by inhibiting the phagocytosis of glia, reducing protein clearance and interfering with myelination (Leitner *et al.* 2019). Therefore, searching for neuroprotective measures that can reduce ischemia/reperfusion injury based on the realization of occlusion recanalization is urgent difficulty and heavily researched area in studies investigating ischemic stroke.

EXTRACELLULAR VESICLES (EVS) PROVIDE NEW DIRECTIONS FOR TREATMENT OF ISCHEMIC CEREBRAL INJURY

Extracellular vesicles (EVs), such as exosomes and microvesicles, are small membranous particles (40–1000 nm in diameter) secreted by cells in a constitutive or inducible manner. Almost all eukaryotic cells release EVs, so they are widely present in various body fluids such as blood, ascites, urine, and saliva. Endocytosis

leads to the formation of endocytic intraluminal vesicles (ILVs), each of which contains extracellular fluid and membrane-guide points to the outside of the cell. ILVs can fuse with each other to form an endosome. After the endosome matures, a multivesicular body (MVB) is formed. During the maturation process, the MVB envelope deforms inwardly, forming intraluminal vesicles, and each vesicle contains cytoplasmic content. In addition to being degraded by lysosomes, a MVB can fuse with the plasma membrane and be released extracellularly in the form of EVs. EVs benefit from their lipid-bilayer membranous structure, which protects intraluminal contents from degradation. A variety of active molecules—including transcription factors, cell-surface receptors, cytoplasmic/nuclear proteins, DNA, non-coding RNAs, and mRNAs—all use EVs as their carriers; hence, EVs play an important role in signal transduction between cells (Gao *et al.* 2019; Malm *et al.* 2016; Manek *et al.* 2018).

Damage to NVU is an important cause of ischemic brain injury. EVs can penetrate the BBB, have high stability in blood circulation, and can protect disease-related molecules (Olanrewaju & Hakami 2020). Previous studies have confirmed that EVs play an important role in neuroprotection and regeneration, such as the reconstruction of neurovascular units after ischemic stroke, and the potential role of EVs in the treatment of ischemic stroke (Zagrean *et al.* 2018). Here, we summarize the EV-mediated signaling networks among NVU components after cerebral ischemia, and discuss EVs as a novel target for the treatment of ischemic brain injury.

Neuronal EVs

Neurons are closely connected with cerebrovascular system and can meet their own metabolic needs by regulating blood flow in the brain. In addition, neurons, as a component of the NVU, change their coded information by changing the peaks and rates of action potentials to mediate neurotransmission and synapse generation (Azarfar *et al.* 2018). A previous study found that ischemia upregulated the expression of neuronal fossa protein-1 (CAV-1) to increase exosomal uptake in OGD-treated neurons, and human umbilical-vein endothelial cell-derived exosomal mirRNA-1290 reduced OGD-induced neuronal apoptosis (Yue *et al.* 2019). In addition, Xu *et al.* found that neurons secreted exosomal miRNA-132 and mediated the expression of the adhesion junction protein, VE-cadherin, through targeted regulation of eukaryotic elongation factor 2 kinase (EEF2K), as well as maintained integrity of cerebral blood vessels (Xu *et al.* 2017). Neural stem cells have the potential for self-renewal and multidirectional differentiation into neurons, astrocytes, and oligodendrocytes. A reduction in neural stem cells aggravates neuronal damage after cerebral ischemia (Jung *et al.* 2020). Ling *et al.* discovered that human urine-derived stem cell exosomes (USC-Exos) inhibited histone deacetylase 6 (HDAC6) activity by transferring exosomal miRNA-26a (miR-26a), and promoting OGD-treated neural stem-cell proliferation and neuronal differentiation (Ling *et al.* 2020). EVs can induce the differentiation of neural precursor cells, protect neurons, and serve as a communication carrier between neurons and other supporting cells. EVs also play an important role in resisting cerebral ischemic injury. Interestingly, TLR4 plays an important role in stroke-induced neurogenesis. In MCAO-induced stroke mice, although TLR4 attenuated the proliferation of subventricular region, TLR4 increased the number of neocortical neurons after stroke by promoting migration of neural stem cells (Moraga *et al.* 2014). EVs exhibit high targeting specificity and high stability, their roles in the mechanism of ischemic brain injury and neurogenesis mediated by TLR4 deserve more in-depth study.

Astrocytic EVs

Astrocytes play a key role in regulating the formation and function of neuronal synapses. They control blood-vessel tension and cerebral blood flow through their numerous fine protrusions. In addition, Astrocytes can also regulate the BBB and communicate with other cells by releasing various molecules including soluble nutrient factors (Maki *et al.* 2013). Researchers have found that exosomes derived from Astrocytes can affect the uptake, differentiation, and maturation of neurons and their changes under inflammatory conditions, providing a deeper molecular mechanism for the interaction between AST and neurons (You *et al.* 2020). Previous studies have confirmed that astrocyte-derived exosomes such as exosomal miR-92b-3q and prion protein (PrP) have protective effects on OGD-treated neurons (Huang *et al.* 2018; Xu *et al.* 2019; Holm *et al.* 2018). Recently, Pei *et al.* found that astrocyte-derived exosome, miR-190b, inhibited the expression levels of caspase-3 and Bax proteins in OGD-treated neurons, and also significantly reduced mRNA levels of TNFα, IL-6, and IL-1β, thereby inhibiting OGD-induced neuronal apoptosis and inflammation (Pei *et al.* 2020; Pei *et al.* 2019). In addition, in a rat model of traumatic brain injury (TBI), exosomal GJA1-20k (connexin-20k) derived from astrocytes inhibited TBI-induced neuronal apoptosis and up-regulated mitochondrial function (Chen *et al.* 2020). A variety of diseases in the CNS are related to demyelination. Researchers have found that patients with multiple sclerosis have a significantly increased risk of ischemic stroke (Hong *et al.* 2019). In addition to interacting with neurons, Zhang *et al.* found that when oligodendrocyte progenitor cells (OPC) were co-cultured with astrocytes, astrocytes up-regulated the expression of cell adhesion/integrin β4 (ITGB4) and promoted the secretion of OPC exosomes, thus mediating the differentiation of OPCs from G1 phase to S phase and subsequently promoting proliferation (Zhang *et al.* 2020). In addition, Astrocytes not only have neuroprotective effect, but also can destroy

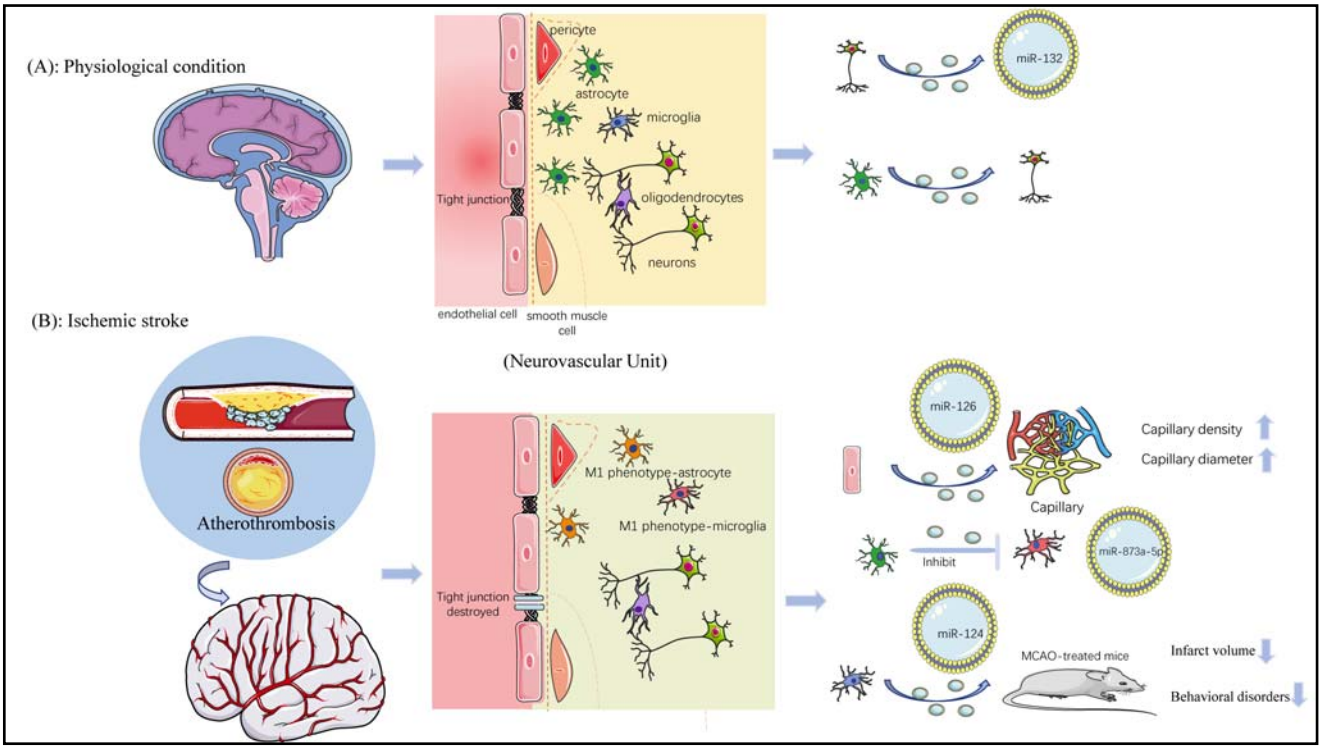

Fig. 2. Under both physiological and pathological conditions, exosomes mediate communication between cells in the central nervous system.

nerve cells and promote the occurrence of many kinds of neurodegenerative diseases. Astrocytes include two non-reactive types, type A1 and type A2. Under the stimulation of LPS, resting astrocytes transform into A1s, which can produce a large number of inflammatory factors. A2s are induced by cerebral hypoxia. During stroke, A2s secrete signals that support neuronal growth near the site of stroke. In human brain tissue with Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis, It can be observed that large number of A1s preferentially gather in the lesion area. A recent report suggests that, in spinal cord injury (SCI) rats, Intravenously-injected MSC-derived EVs could reduced A1 astrocytes via downregulation of phosphorylated p65 , Moreover, researcher observed decreased lesion area and expression of TNFα, Interleukin (IL)-1α and IL-1β, improved expression of Myelin Basic Protein (MBP), Synaptophysin (Syn) and Neuronal Nuclei (NeuN) compare to the contral group (Wang *et al.* 2018). Another report indicates that neuronal ephrin type-B receptor 1 (EphB1) can induce neuroprotective A2 astrocytes, partially linked to the STAT3 network, but in mouse models of amyotrophic lateral sclerosis (ALS), EphB1–STAT3-mediated anti-inflammatory pathways are inadequately activated (Tyzack *et al.* 2017), that may be due to the obstruction of the blood-brain barrier to macromolecular substances. Taken together, EVs are a key carrier of communication between astrocytes and NVU components, and play a key role in regulating inflammatory responses from brain injury.

EVs in endothelial cells

Vascular endothelial cells form the lining of cerebral blood vessels. Tight junctions (TJs), which are located between vascular endothelial cells, are composed of membranous junction proteins including occludin, claudins, and corresponding cytoplasmic proteins (ZO-1, ZO-2 and ZO-3). TJs represent the main structures for maintaining brain microvascular permeability and BBB integrity (Jo *et al.* 2013). After cerebral ischemic injury, these structures are destroyed, which increases the permeability of the BBB (Guo & Lo 2009; Jiao *et al.* 2011). Due to their location and functional characteristics, endothelial cells are the supporting cells that are first exposed to cellular damage and innate immune activation after cerebral ischemia. Therefore, endothelial cells are extremely important for maintaining NVU homeostasis. A number of studies have shown that in variety of disease phenotypes (e.g., tumor metastasis, extremity ischemia, wound healing), EVs carry related cargo proteins (or miRNAs) to regulate endothelial cell migration and proliferation, and promote vascular remodeling (Xu *et al.* 2020; Zhu *et al.* 2020; Masoumi-Dehghi *et al.* 2020). Various miRNAs are involved in the activation of TLR signals in ischemic brain injury (Paschon *et al.* 2016). Researchers have found via *in-vitro* experiments that monocytes co-stimulated with interferon alpha and LPS up-regulate exosomal miR-155, miR-146a/b, and miR125a, while also inhibiting exosomal miR-122 to activate cerebral-vascular-endothelial TLR4/Myd88/NF-κB signaling pathways to induce substantial release

of pro-inflammatory factors such as IL-1β and CCL-2 (Dalvi *et al.* 2017). In addition, Avenkat *et al.* found that in a mouse model of T2DM-stroke, endothelium-derived exosomal mir-126 significantly increased capillary density and artery diameter, and induced M2 macrophage polarization in the ischemic boundary region (Venkat *et al.* 2019). MiR-126 uses vascular cell adhesion protein 1 (VCAM-1) as the main target, promotes angiogenesis, and maintains vascular integrity, in addition to playing an important role in the development of ischemic stroke caused by small artery obstruction (Zhu *et al.* 2019). Endothelial progenitor cells (EPCs) are stem cells derived from bone marrow. They contribute to the homeostasis of the endothelial wall in acute and chronic ischemic diseases (Perrotta *et al.* 2020). EPCs are also early markers of stroke associated with acute microvascular disease. In patients with lacunar cerebral infarction, the number of EPCs on the first day of stroke is increased significantly (Wisniewski *et al.* 2020). Researchers have found that exosomal miR-21-5p derived from EPCs can inhibit the expression of thrombospondin (THBS1) and promote repair of endothelial cells in rats with carotid artery injury (Hu *et al.* 2019). Endothelial cells are activated during acute ischemic brain injury and release cytokines and chemokines, which have a positive effect on NVU reconstruction and brain protection. Zhou *et al.* found that in an MCAO rat model, exosomes derived from endothelial cells significantly reduced the volume of rat cerebral infarction and promoted proliferation and differentiation of neural precursor cells (Zhou *et al.* 2020). Therefore, cellular communication with EVs as carrier plays a bidirectional effect in the interaction between cerebral vascular endothelial cells and other NVU components.

Microglial EVs

Microglia are resident immune cells and comprise natural immune networks of the CNS. Another important feature of microglia is their phenotypic heterogeneity. When microglia are attacked by bacterial invasion, phagocytosis occurs with the release of inflammatory mediators (M1 phenotype). In contrast, when apoptotic cells or myelin fragments are removed, microglia release anti-inflammatory factors, which represent the M2-like phenotype. The heterogeneity of microglial phenotypes is closely related to cerebral ischemic injury (Galloway *et al.* 2019). A previous study has confirmed the influence of EVs on innate immune function of microglia and its interaction with CNS (Olanrewaju & Hakami 2020). Inhibiting the polarization of microglia to M1 phenotype and promoting their polarization to M2 phenotype are important targets for inhibiting ischemic brain injury. Researchers have discovered that miR-124, an exosome derived from M2 microglia, can significantly improve the infarct volume of MCAO-treated mice and reduce their behavioral disorders by inhibiting the activity of ubiquitin-specific protease 14(USP14) (Song *et al.* 2019). MiR-124 is a brain-specific miRNA that is highly expressed in microglia. Up-regulation of miR-124 expression contributes to the polarization of M2 microglia. Yang *et al.* found that in rats with TBI, exosomal miR-124 significantly reduced the protein levels of TLR4, MyD88, IRAK1, TRAF6, NF-κB p65, thereby promoting microglial M2 polarization (Yang *et al.* 2019). In addition, Jiang *et al.* found that miR-30d-5p-enriched exosomes derived from adipose stem cells can effectively inhibit autophagy-mediated conversion of microglia to M1 phenotype, reduce the area of cerebral infarction, and ameliorate ischemic brain injury (Jiang *et al.* 2018). In addition, NVU components are also involved in the regulation of microglial phenotypes. Researchers found that in LPS-induced microglia, astrocyte-derived exosomal miR-873a-5p significantly inhibited phenotypic transformation of microglia to M1 phenotype and reduced inflammatory reactions by reducing phosphorylation of ERK and NF-κ B p65, in addition to alleviating brain injury (Long *et al.* 2020). Yin *et al.* reported that neuronal cell-derived exosomal miRNA-21-5p can promote the M1 phenotype of microglia, and inhibiting the expression of miRNA-21-5P of exosomes is an important strategy to improve inflammatory responses after brain injury (Yin *et al.* 2020). In conclusion, EVs are important carrier for inducing the phenotyptic differentiation of microglia, and inhibiting the expression of inflammatory signaling.

Oligodendrocytic EVs

Oligodendrocytes are one of the main cell types in white matter. They can produce myelin sheaths to wrap around axons to effectively conduct electrical impulses and send signals to neurons. In addition, oligodendrocytes can provide a variety of nutritional factors to promote neuronal survival and axon growth *in vitro*. Oligodendrocytes are extremely sensitive to ischemic brain injury. Researchers found that in MCAO-treated rat model, oligodendrocytes colonized the ischemic injury area and strongly expressed 3R-Tau to myelinate damaged axons (Villa Gonzalez *et al.* 2020). Glutamate can stimulate NMDA and AMPA receptors on the surface of oligodendrocytes and mediate Ca^{2+} influx, thereby triggering oligodendrocytes to release exosomes containing proteins and RNAs, which are subsequently internalized by neurons (Fruhbeis *et al.* 2013). In addition, oligodendrocyte-derived exosomes can transport catalase and superoxide dismutase 1 (SOD1), as well as up-regulate the phosphorylation levels of Akt and ERk1/2 kinase, to promote survival of OGD-treated neurons and enhance their anti-stress ability (Frohlich *et al.* 2014). A previous study also showed that the firing frequency of neurons exposed to oligodendrocytic exosomes was increased significantly compared to that of controls (Venkat *et al.* 2020), indicate that the positive effects of oligodendrocytes on neurons are partly derived from exosomes they secrete . As a major iron

storage protein, heavy chain ferritin (FHC) plays an important role in maintaining the iron balance within cells. Furthermore, neuro-inflammation-mediated iron accumulation in the brain plays an important role in neuronal death (Pandur *et al.* 2019). Researchers have found that expression of FHC in the pyramidal layer of animals with transient cerebral ischemia is significantly increased (Yoo *et al.* 2016). Moreover, ferritin has antioxidant properties. Mukherjee *et al.* found that FHC is highly expressed in oligodendrocytes and acts on target cells through EVs as carrier. Hence, disrupting the release of EVs or FHC expression in oligodendrocytes may lead to neuronal loss and oxidative damage in ischemic mice (Mukherjee *et al.* 2020).

Pericytic EVs

Pericytes are located around the endothelial cell layer of the brain's capillary network. A large number of clinical and animal studies have confirmed that the biological functions of pericytes are related to cerebral blood flow, permeability of the BBB, maintenance of cerebrovascular formation, and neuroinflammation. In addition, studies have shown that under ischemic/hypoxic conditions, pericytes can obtain pluripotent stem cell activity and can differentiate into the main component of the BBB/NVU (Liu *et al.* 2020). Mayo *et al.* found via *in-vitro* experiments that the use of cobalt chloride (COCl2) to stimulate pericytes can activate the HIF pathway, regulate the release of exosomes, and promote angiogenesis (Mayo & Bearden 2015). Taken together, pericytes are an important component of the NVU, and research on EVs has provided a basis for further exploration of communication among cells within NVU.

Mesenchymal stem cells (MSCs) and EVs

MSCs are a type of self-renewing multipotent progenitor cell derived from human and mammalian bone marrow and the periosteum. MSCs can produce neurotrophic factors, such as nerve growth factor, brain-derived neurotrophic factor, or glial-derived neurotrophic factor. Previous studies have confirmed that MSCs can significantly inhibit the inflammatory response of ischemic brain injury and reduce neuronal apoptosis, as well as improve nerve function recovery (Bonsack *et al.* 2020; Sun *et al.* 2020; Paudyal *et al.* 2020). However, MSCs still have many limitations in clinical application, including low cell survival rate after transplantation, exhaustion of regenerative potential, and reduced cell differentiation ability (Gunawardena *et al.* 2019). In addition, systemic application of exogenous bone-marrow mesenchymal stem cells (BMSCs) in a rat model of stroke can cause pulmonary vascular obstruction, and transplanted cells can induce malignant transformation (Zhang *et al.* 2019). EVs are key factor in regulating the paracrine activity of MSCs. MSC-derived EVs provide the same therapeutic effects as cell-based therapies, but do not induce adverse reactions found in the latter. Various studies have confirmed that EVs derived from MSCs can significantly improve the neural function after ischemia injury, as well as promote synapse formation, axonal germination, and angiogenesis. The expression characteristics of MSC-derived EVs vary with differential states of cells. Recently, researchers have found that under standard culture conditions, exosomes released from human-cord-blood MSCs cannot improve neurological prognosis after stroke. However, exosomes obtained from co-culture of normal and OGD-treated MSCs can reduce the infarct area and swelling of ipsilateral cerebral hemisphere, protect neural function, and promote the rehabilitation of rats with ischemic brain injury (Nalamolu *et al.* 2019). In addition, Chen *et al.* found that using EP4 antagonists to block the prostaglandin E2/prostaglandin E2 receptor 4 (PGE2/EP4) signaling pathway of MSCs cells can increase the release of exosomes and induce exosomes to target specific cargo protein sorting (e.g., BBB-integrity support factors such as TIMP-1, IL-10, and brain-derived neurotrophic factor), thereby mitigating cognitive dysfunction of mice with TBI, reducing inflammation, and increasing the integrity of the BBB (Chen *et al.* 2019). The most serious disadvantage of using EVs to treat ischemic stroke is poor targeting of cerebral ischemic lesions after administration, resulting in poor therapeutic effects. The surface modification of EVs with Arg-Gly-Asp peptide promotes the accumulation of MSC-derived EVs in the ischemic regions (Tian *et al.* 2018). However, the binding of Arg-Gly-Asp peptides requires multiple chemical reactions, which may affect the function of EVs proteins. Recently, Kim *et al.* reported that MSC-derived magnetic extracellular nanovesicles (MNVs) containing iron oxide nanoparticles (IONPs) with the help of 3D-printed magnetic helmets significantly improved targeting and treatment effects of ischemic brain injury. IONPs are biocompatible, they can form ionization into iron ions and iron homeostasis, assimilated by the body (store excess iron ions in the form of ferritin). Moreover, IONP can phosphorylate c-jun N-terminal kinase (JNK) molecules and activate signal cascades associated with growth factor expression. After systemic injection of MNVs in MCAO-treated rats, magnetic navigation increased the location of MNV to the ischemic injury area by 5.1-fold, induced M1 macrophages around the infarct to polarize to M2, inhibited microglial activation, reduce inflammation, and promoted angiogenesis and anti-apoptosis, thereby significantly reducing infarct volume and improving neural function (Kim *et al.* 2020). EVs derived from MSCs can significantly inhibit the tissue inflammatory response mediated by innate immunity caused by ischemia, as well as alleviate inflammasome-mediated pyroptosis caused by inflammatory attacks. Researchers have found that combination of MSC-derived exosomes and rosuvastatin can significantly inhibit the gene expression of NLRP1 and NLRP3, and reduce the infarct volume of MCAO-treated rats (Safakheil &

Safakheil 2020). In addition, BMSC-derived exosomal miR-134 can be targeted to inhibit the expression and activity of Caspase-8, thereby significantly inhibiting OGD-induced oligodendrocytic apoptosis (Xiao *et al.* 2018). Recently, Liu *et al.* found that exosomes derived from BMSCs can inhibit the expression of TLR4, NLRP3, and Caspase-1 in OGD-treated cerebral vascular endothelial cells and increase p-p65/p65 ratio, thereby reducing the damage to endothelial cells caused by ischemia and hypoxia (Kong *et al.* 2020). Although their underlying mechanisms remain unclear, based on previous studies, recent studies on MSC-derived EVs have focused on regulating the expression of inflammatory signaling pathways in brain injury. In addition, elucidating the regulatory release of EVs from MSCs and the precise localization of EVs to damaged areas are also the focus of current and future studies.

Engineered EVs in Cerebral Protection

Another important feature of EVs is that they can be genetically engineered. Cell-or tissue-targeted peptides can be attached to the surface of EVs to achieve selective targeting of specific tissues and avoid unnecessary accumulation in other organs, thereby reducing systemic toxicity. For example, The fusion of lipid bilayer of EVs with liposome forms EV-liposome hybrid, which enables large DNA molecules (such as plasmids) to be encapsulated and transported, and alleviates the toxicity of liposomes (Lane *et al.* 2015). Moreover, although naturally secreted EVs contain a variety of RNAs, including miRNAs, and fragments, most fragments are tiny. MRNA molecules can be transferred from erythrocytes to EVs by electroporation, but this strategy is less efficient (Duan *et al.* 2020). Recently, Yang *et al.* reported use local and instantaneous electrical stimulation to stimulate plasmid DNA transfected cells from different sources,can produce EVs containing therapeutic mRNA and targeted peptides in large quantities (Yang *et al.* 2020). Similarly, engineered EVs can achieve targeting of brain tissue. The EVs produced by the fusion of rabies virus glycoprotein (RVG) peptide and the transmembrane domain of platelet-derived growth factor receptor(PDGFR) is more efficient than unlabeled EVs in transporting goods to the brain (Gyorgy *et al.* 2014). Furthermore, Yang *et al.* found that modified exosomes fused with rabies virus glycoprotein (RVG) and exosomal protein lysosomal-associated membrane glycoprotein 2b (Lamp2b) effectively transported miR-124 to the site of cerebral ischemic infarction. Promotion of neural precursor cells to acquire a neuronal phenotype at the infarct site mitigates brain injury (Yang *et al.* 2017). In addition, studies have confirmed that EVs have unique effects in ischemia/reperfusion injury of tissues and organs. In a rat model of ischemic acute kidney injury, EVs loaded with IL-10 (IL-10^{+}EV) made by engineered macrophages can improve renal tubular damage and inflammation caused by ischemia/reperfusion, and drive macrophages in the tubular interstitium to polarize to the M2 subtype. Delivery of IL-10 through EVs not only enhances the stability of IL-10, but also improves the targeting of cargo proteins to tissues (Tang *et al.* 2020). Therefore, we regard EVs as promising biomolecules, providing new strategies for reducing ischemia/reperfusion brain injury and improving the prognosis of patients with cerebral ischemia.

DISCUSSION

In this review, we introduced the mechanisms that induce inflammatory responses during ischemic brain injury and discussed potential therapeutic targets of innate immunity. Following synthesis of these previous findings, treatments targeting TLRs and inflammatory bodies may represent a promising strategy for mitigating secondary brain injury induced by innate immunity. Since MSCs have the ability to create a microenvironment that is conducive to repairing damaged tissues, they are a hot application in regenerative medicine. The excellent targeting-interaction properties of EVs and the their ability to protect cargo from immune recognition make them the most attractive research direction for elucidating brain-protection mechanisms during ischemic injury. Studies have confirmed that MSC-Exos can improve ischemic injury by suppressing innate immune inflammatory response. Interestingly, another study reported that in a mouse model of lower-extremity ischemia with femoral artery ligation, EVs (UMSC-Exo) derived from human-umbilical-cord mesenchymal stem cells (MSCs) inhibited pytoptosis from inflammasomes induced by local ischemia, including activation of NLRP3, cleavage of Caspase-1, and a subsequent increase in IL-1β and IL-18 levels, thus reducing cell death (Yan *et al.* 2020). In addition, MSC-EXOs can accumulate at tissue ischemic injury sites through integrins on their surface (Cao *et al.* 2021). EVs can connect various components of the NVU in series and play a bidirectional role in the event of cerebral ischemia injury. At present, there are still two main problems with EVs in affecting ischemic brain injury: First, due to the complexity of EVs and the diversity of their interactions with the surrounding environment, many unresolved issues and major knowledge gaps still exist. For example, their role in regulating the innate immune response is still relatively unclear; carefully designed experimental studies are needed to determine whether EVs have the same physiological efficacy and therapeutic potential as those observed in cellular and animal models. Second, as research interest in EVs increases, there will be a greater variety of ways to quantify EVs. However, there is no consensus on appropriate EV quantification, making it difficult for each study to be compared with another. Methods to overcome this heterogeneity require widely available standards with sufficient characteristics and multiple research comparisons across platforms. EVs

have opened up a promising path for the development of novel treatments. Their ability to freely cross the BBB makes them an effective carrier for drug delivery. Future research should focus on the role of EVs and their specific innate immunosuppressive molecules in influencing ischemic brain injury.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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AUTHOR CONTRIBUTIONS

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