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Review

Mesenchymal stem cell-derived extracellular vesicles/exosome: A promising therapeutic strategy for intracerebral hemorrhage

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ABSTRACT

Intracerebral hemorrhage (ICH) is the second largest type of stroke with high mortality and morbidity. The vast majority of survivors suffer from serious neurological defects. Despite the well-established etiology and diagnose, there is still some controversy over the ideal treatment strategy. MSC-based therapy has become an attractive and promising strategy for the treatment of ICH through immune regulation and tissue regeneration. However, accumulating studies have revealed that MSC-based therapeutic effects are mainly attributed to the paracrine properties of MSC, especially small extracellular vesicles/exosome (EVs/exo) which are considered to be the key mediators of the protective efficacy from MSCs. Moreover, some papers reported that MSC-EVs/exo have better therapeutic effects than MSCs. Therefore, EVs/exo has become a new choice for the treatment of ICH stroke in recent years. In this review, we mainly concentrate on the current research progress on the use of MSC-EVs/exo in the treatment of ICH and the existing challenges in their transplation from lab to clinical practice.

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Abbreviations: ICH, Intracerebral hemorrhage; EVs, extracellular vesicles; Exo, exosome; SAH, subarachnoid hemorrhage; MSCs, Mesenchymal stromal cells; db-ICH, Intracerebral hemorrhage (ICH) induced by diabetes; CAA, Cerebral amyloid angiopathy.

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1. Introduction

Intracerebral hemorrhage (ICH) refers to non-traumatic intracranial parenchymal hemorrhage. According to the statistics of the World Health Organization, the total annual incidence of ICH is 24.6 per million individuals [1,2], and the incidence is positively correlated with age [3] . According to the available data, there are differences among different races, with Asians appearing to have the highest incidence. It has become a significant global concern due to its high mortality and morbidity, with the vast majority of survivors have legacy paralysis, aphasia and other severe comorbidities after ICH [4]. Although it has become a global public health problem, progress has been made in the treatment of ICH and related complications, effective clinical treatment which can significantly improve ICH prognosis is still unavailable. Therefore, ICH has no effective treatment option currently.

Mesenchymal stem cells (MSCs) are considered as promising therapies for the treatment of ICH. A large number of studies have confirmed the improvement of ICH (in-vitro/in-vivo) models by MSCs. However, it also carries risks of tumorigenicity and immune rejection [5]. Further studies have found that MSCs mainly act through paracrine mechanisms. Extracellular Vesicles, especially exosomes are the key mediators of their effect [6,7]. Recently, researchers have begun to explore the role of mesenchymal stem cell-derived Extracellular Vesicles/exosomes (MSC-EVs/exo) and found that MSC-EVs/exo have the advantages of crossing the biological barrier, convenient storage and transportation, satisfied biosafety, low immunogenicity and small immune rejection compared with MSCs. Therefore, EVs/exo, an important paracrine factor of MSCs, have been examined as potential MSC-based therapies. Multiple groups have reported the therapeutic potential in the treatment of ICH by MSC-EVs/exo.

This review focused on summarizing the efficacy, mechanism and current challenges on the use of MSC-EVs/exo in the treatment of ICH, providing guidance for the translation of MSC-EVs/exo from lab to clinical practice.

2. Pathologic mechanisms of injury in ICH

The site of ICH is usually located in the (lentiform nucleus) shell, pons, brain parenchyma, etc., mostly with sudden (or acute) symptoms of headache, nausea, vomiting, varying degrees of disturbance of consciousness and limb paralysis [8].

The onset of ICH is closely related to hypertension, cerebral amyloid angiopathy and anticoagulation therapy, which are demonstrated to have the risk of cerebral hemorrhage [9]. The concept of hypertensive intracerebral hemorrhage was established by vascular pathological examination in patients with progressive hypertension and ICH. In patients with progressive hypertension, intimal hyperplasia with hyalinization occurs in the vessel wall of perforating branch vessels, called 'pseudoaneurysms' [10,11], which predisposes them to focal necrosis and vessel wall rupture. These microscopic 'pseudoaneurysms' are associated with small amounts of extramural bleeding which is usually subclinical and imperceptible. However, when the coagulation system cannot compensate the destruction of blood vessel wall, heavy bleeding may occur. Cerebral amyloid angiopathy (CAA), usually asymptomatic, is an important cause of primary lobar ICH in older adults. CAA is characterized by deposition of Congo red in small and medium-sized vessels in the brain and leptomeninges. This weakens the vessel wall structure, making it prone to bleeding. CAA usually presents as spontaneous lobar hemorrhage. This lesion site helps to distinguish CAA-related ICH from hypertensive ICH, the latter of which occurs more frequently in the putamen, thalamus and pons [12].

The pathological process of hemorrhagic stroke is complex, mainly including the formation and enlargement of hematoma, increased intracranial pressure, damage of blood-brain barrier, cerebral edema, neuronal apoptosis and neurological dysfunction. The initial hemorrhage or continued hemorrhage and hematoma expansion after ICH causes immediate primary brain injury due to both global increased intracranial pressure (ICP) and mechanical compression of local structures [13]. Secondary brain injury is caused by the physiologic response to the hematoma (primarily edema and inflammation) as well as the clot-originated toxic components. Edema contributes to the disruptions of ion balance, including K^+, Cl^- and Na^+ [14]. Diapedesis of neutrophils occurs within days and subsequently, microglia are activated after ICH [15]. Free radical generation may contribute to the iron imbalance-induced brain injury [16]. Furthermore, the disruption of blood-brain barrier by the toxic effects of red blood cells and/or hemoglobin breakdown products leads to increased brain water content in the hemorrhagic area, resulting in edema and exaggerated brain damage [17]. We summarized the mechanism of brain injury after ICH in Fig. 1. At present, the main treatments for ICH include anti-hypertensive, surgery to remove hematoma and improving coagulation dysfunction. However, they are usually accompanied by the risks of recurred bleeding, infection, nerve injury and effective perfusion pressure deficiency caused by surgery.

As a hotspot of regenerative medicine, MSC-EVs/exos have been widely used to treat many diseases. Similarly, MSC-EVs/exos have also been adopted to treat ICH-induced brain injury with extraordinary efficacy. However, the complicated pathogenic mechanism in ICH and complex repair mechanism of brain injury makes it difficult to achieve comprehensive and in-depth studies. In view of the limitation of the current research, we searched articles mainly come from PubMed, Embase, Elsevier, web of science and other databases using the following keywords: (ICH) OR (Intracerebral hemorrhage) AND (exosomes) OR (extracellular vesicles) from

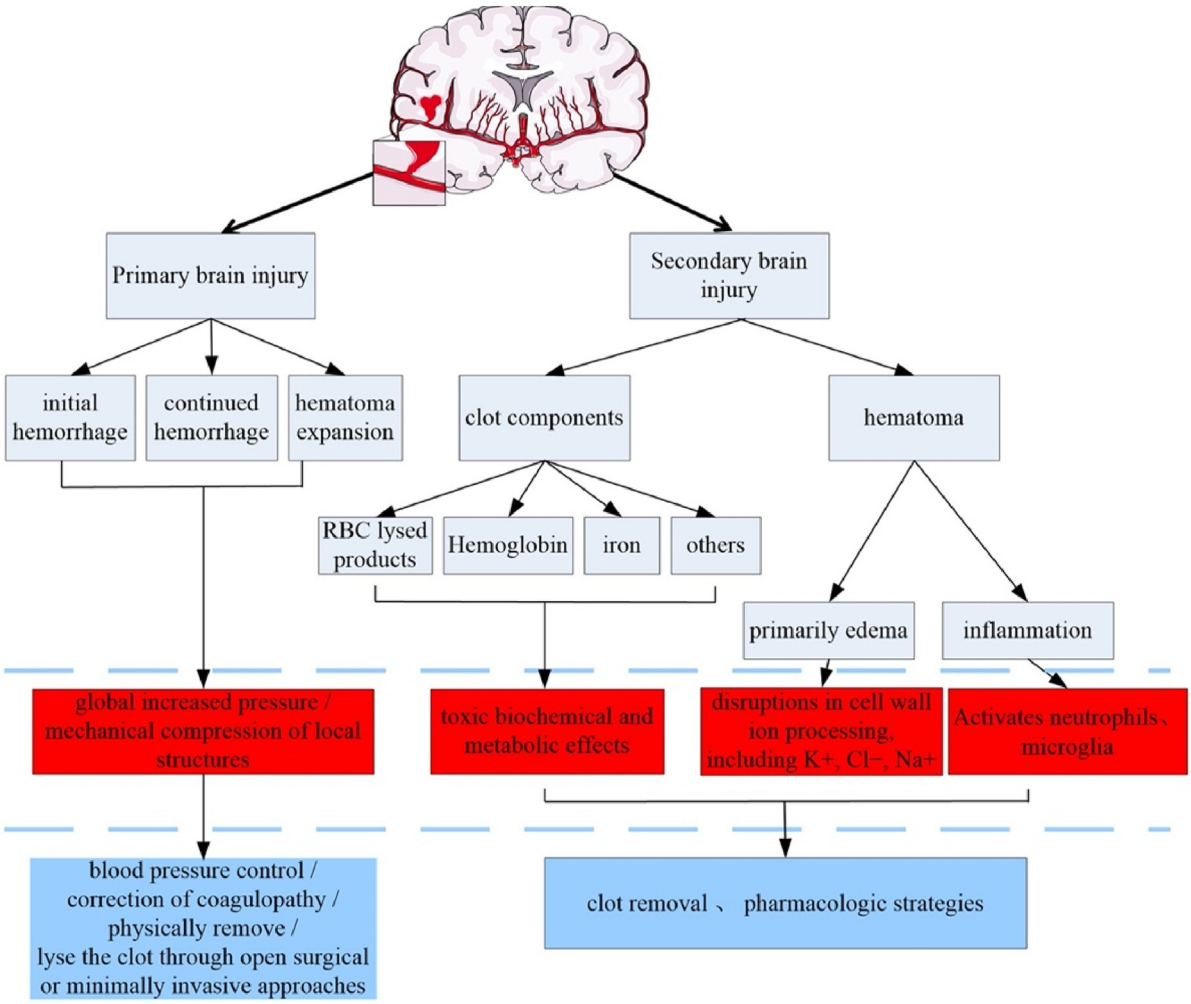

Fig. 1. Mechanisms of brain injury.

January 2016 to September 10th, 2022. A total of 324 publications were searched. 270 duplicates or not related to exosomes and intracerebral hemorrhage. 42 review articles were ruled out. Ultimately, 12 studies were included (Fig. 2 Searching strategy) [18–29]. We summarized the functions and mechanisms of MSC-EVs/Exo in repairing brain injury, which can give us a deeper understanding to draw up therapeutic strategies in theory.

3. MSC-derived EVs/exo: Definition and biological functions

Extracellular vesicles (EVs) are membranous vesicles released by cells, which are divided into three types according to their diameter: exosomes (30–150 nm), microvesicles (100 nm-1μm), and apoptotic bodies (1–5 μm) [30] . The detailed characters of different types of EVs/exo are summarized in Table 1.

Exosomes (exo) are 30-150 nm in diameter membrane vesicles of endocytic origin secreted by almost all cell types in vitro which mediate intercellular communication in physiological and pathological conditions. It can be actively secreted and produced by a variety of cells in the body such as immune cells, stem cells, cardiovascular cells, reticulocytes, platelets, nerve cells, and tumor cells and are widely distributed in cell supernatants, tissues and many types of body fluids (including peripheral blood, urine, saliva, milk, ascites, amniotic fluid, etc) [31–33].

Exosomes are formed by invagination of the cell membrane to generate multivesicular bodies, which are fused with the cell membrane and secreted into the extracellular environment. Exosomes carry a large number of specific proteins (such as integrin proteins, immunomodulatory proteins, transmembrane proteins, exosome-producing proteins, etc.), RNA (such as mRNAs and non-coding RNAs (miRNAs, IncRNAs, circRNAs, etc)), DNA, lipids, etc. Vesiclepedia database is a manually curated compendium of molecular data (lipid, RNA and protein) identified in different classes of EVs. All of the EV-DNA gene data derived from human samples are included on the site www.evdnadatabase com. When exosomes were first discovered in 1980, they were originally regarded as waste products excreted by the cell [34]. Rothman et al. discovered the transport regulatory mechanism of extracellular vesicles and obtained the Nobel prize in physiology or medicine in 2013. Since then, researchers have conducted a lot of researches on their biological source, material composition and transportation, cell-to-cell signaling and distribution in body fluids, and found that exosomes have a variety of functions. The function of exosomes depends on the cell type from which they originate and can be involved with immune response, antigen presentation, cell migration, cell differentiation, tumor invasion, etc [35,36].

In recent years, with the broad application of various MSCs obtained from bone marrow, fat tissue, umbilical cord, placenta, urine, etc., MSCs-derived EVs/exos have also been widely studied in

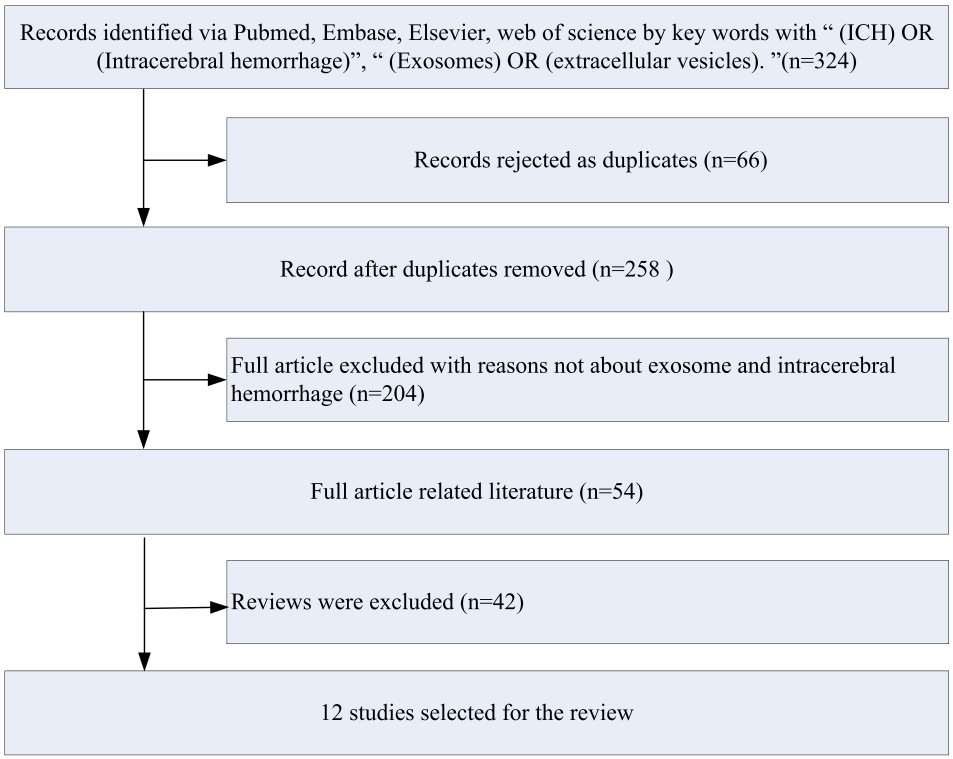

Fig. 2. Searching strategy.

a variety of diseases. Compared with MSCs, MSC-EVs/exos have the following advantages: i: paracrine function, paracrine effect of exosomes as stem cells [37]; ii: Strong biological effect. Exosomes can cross the biological barrier and directly fuse with the target cells to exert a strong biological effect [38]; iii: Easy to store and transport. It can be stored and transported at -80°C for a long time with the active ingredients wrapped by the cell membrane and not easily destroyed; iv: Exosomes can be further modified to load specific cargo like drug; v: High biosafety because of its low immunogenicity as a cell-free therapy [39–42]; vi: Concentration, dosage, delivery route and time are easy to control, the yield is easy to increase. The study of MSC-EVs/exo in ICH also prospers for their amazing therapeutic effects. Multiple sources of MSC-EVs/exo can improve ICH and related complications by inhibiting neuron apoptosis, reducing neuroinflammation, and suppressing cell differentiation. We summarized the advanced knowledge in respect of the source, function and related mechanism of MSC-EVs/exo in the treatment of ICH in Table 2.

4. Mechanism of MSC-EVs/exo in the treatment of ICH

The early treatment of ICH mainly lies in removing the hematoma and protecting the surrounding tissue of the hematoma. The later stage is mainly to repair damaged nerves [43]. As an emerging biological therapy, EVs/exo represents a multi-field breakthrough in neural repair. Although many studies have explored the relationship between MSC-EVs/exo and ICH, the repair mechanism is controversial. Nerve repair is inseparable from a series of complex reactions and interactions between cells and EVs/exos. EVs/exo delivers bioactive substances to the target cells through diffusion, endocytosis and receptor-mediated transport. A large number of studies have shown that EVs/exos play a role in the treatment of ICH by following two ways: (1) direct action: exosome surface proteins recognize target cell receptors to induce signal transduction and transmit intercellular information; (2) Indirect effect, exosomes carry substances for the treatment of ICH: Exosomes can fuse with target cells to deliver bioactive factors to act on receptors

Table 1
Characteristic of different types of extracellular vesicles.

Characteristic	Extracellular vesicles		
	Exosomes	Microvesicles	Apoptotic bodies
Size (nm)	30 ~ 150	100–1000	1000–5000
Morphology	Cup/round shaped	Various shaped	Heterogenous
Contents	DNA, mRNA, retrotransposons, small interfering RNAs, and other non-coding RNA, cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules		Nuclear fractions, cell organelles
Markers	Tetraspanins (such as TSPAN29 and TSPAN30), ESCRT components, PDCD6IP, TSG101, flotillin, MFGE8	Integrins, selectins, CD40 ligand	Extensive amounts of phosphatidylserine
Origins/mechanisms	Endosomes/Intraluminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	Plasma membrane/Outward budding of cell membrane	Plasma membrane/Outward blebbing of apoptotic cell membrane

on the surface of target cells to achieve information transport. Nerve repair usually involves reducing inflammation, angiogenesis and nerve remodeling. At present, the role of MSC-EVs/exo in nerve repair mainly focuses on the above three facets. The therapeutic mechanism of MSC-EVs/exo may be related to cell proliferation, angiogenesis, inhibition of apoptosis, release of active factors and promotion of nerve regeneration.

4.1. Anti-apoptosis

Apoptosis is a kind of autonomous programmed cell death controlled by genes. Physiological apoptosis is essential for maintaining the stability of the internal environment [44]. A number of factors trigger cell death in perihematoma and remote brain regions [45], including programmed cell death (apoptosis, autophagy, and pyroptosis) and unprogrammed cell death (necrosis) (Fig. 3). This ICH-induced cell death leading to neuronal cell loss is a significant cause of high mortality and morbidity after ICH. Therefore, anti-apoptosis may play an important role in alleviating and reversing the adverse outcomes of ICH.

The exosomes derived from miR-133b modified MSCs can reduce neuronal apoptosis after ICH by inhibiting RhoA expression and activating ERK1/2/CREB in vitro [22]. Previous researches suggested that EGb761 ameliorates neuronal apoptosis and promotes angiogenesis in experimental intracerebral hemorrhage via rSK1/GSK3beta pathway [46]. Moreover, a prior study revealed that EVs derived from bone marrow MSCs were transferred to neurons of a subarachnoid hemorrhage mouse model via miR-21 to promote neuronal survival and alleviate cognitive impairment after subarachnoid hemorrhage [21]. Meanwhile, a study found that knockdown of exo/miR-206 derived from umbilical cord MSCs, by targeting BDNF, mediates the TrkB/CREB signaling pathway, inhibits apoptosis, and significantly improves neurological deficits and brain edema, so as to prevent early brain damage caused by subarachnoid hemorrhage [28]. Another study observed that MiR-26b-5p-modified hUB-MSCs-derived exosomes inhibited apoptosis and the expression of inflammatory mediators during subarachnoid hemorrhage through MAT2A-mediated p38 MAPK/STAT3 signaling pathway in early brain injury [29].

4.2. Reduce neuroinflammation

Neuroinflammation is inflammation of the nervous tissue, resulting from brain injury, strokes, intracerebral hemorrhages and other traumatic events [47,48] . Neuroinflammation is a crucial factor in the pathogenesis of ICH. During the early activation of ICH microglia, both cell death and breakdown products trigger an inflammatory cascade leading to increased neuronal death. ICH induces an extensive neuroinflammatory response, which seems to be responsible for the exaggeration of brain damage [49–52]. Therefore, reducing neuroinflammation is regarded as a potential target for therapeutic intervention.

MSC-EVs/exo can deliver microRNAs, proteins or other contents to endow the anti-inflammatory effects [53]. Recently, a study from reported that BMSC-exos reduce neuroinflammation and have a neuroprotective effect in the brain tissues after SAH by inhibiting NF-κB and activating AMPK pathways. BMSC-exos also regulate the polarization of microglia toward the M2 phenotype by downregulating IL-1β, CD16, CD11b, INOS, and upregulating the expression of CD 206 [19]. Another study found that BMSC-EVs carry miR-183-5p into the ICH site of rat brain tissues and repress the NLRP3 pathway by targeting PDCD4, thus alleviating neuroinflammation after diabetic ICH [25]. Interestingly, systemic exosomal/miR-193b-3p treatment attenuates the inflammatory response by acetylation of NF-κB p65 via suppressing the expression and activity of HDAC3. These effects alleviated neurobehavioral impairments and neuroinflammation following SAH [26]. Therefore, MSC-EVs/exo may play an anti-inflammatory effect in the pathological process of cerebral hemorrhage by inhibiting inflammatory factors and inhibiting the activation of M2 microglia.

4.3. Angiogenesis

Angiogenesis, the formation of capillaries from preexisting blood vessels, plays a central role in a variety of physiological and pathological conditions. With the occurrence of cerebral hemorrhage, the vascular endothelium is destroyed. The adjacent tissues and blood vessels are also subject to the ischemic stress after cerebral hemorrhage, which further aggravates brain damage. A recent finding indicate that HIF-1α plays a critical role in ICH-induced angiogenesis [54]. Promoting cerebral angiogenesis and neurogenesis is also an important strategy to ameliorate brain functional damage after ICH.

BMSC-EVs were also shown to play a role in angiogenesis [55]. BMSC-exo transplanted in mice with ischemic stroke showed marked improvement in angiogenesis and neurogenesis, thereby promoting the recovery of neurological function [56]. A study found that exosomes from MSCs pretreated with ischemic rat heart

Table 2
The functions and related signaling pathways of MSC-EVs/exos from different sources in the treatment of ICH.

Source of Exos	Function(s)	EVs or Exo/Involved miRNA or contents	Pathway(s)	Reference	Model
BMSCs (rat)	Alleviate neuroinflammation	EVs/miR-183-5p	PDCD4/NLRP3p	Ding et al. (2021)	ICH
	Reduce inflammation/Promote M2 polarization	EVs/–	AMPK/NF-κB	Han et al. (2021)	SAH
	Inhibiting microglial activation/Reduce neuroinflammation	Exo/–	CX3CL1/CX3CR1	Chen et al. (2020)	SAH
	Neuroprotection/anti-inflammation/ neurological recovery	Exo/miR-193b (HDAC3)	–	Lai et al. (2020)	SAH
	reduce neuronal apoptosis	EVs/miR-21-5p	PTEN/Akt	Gao et al. (2020)	SAH
	anti-apoptotic	Exo/miR-133b modified	mediating RhoA and ERK1/2/CREB	Shen et al. (2018)	ICH
	Reducing neuronal apoptosis, Inhibiting microglial M1 polarization	Exo/MiR-146a-5p	IRAK1 and NFAT5	Duan et al. (2020)	SAH
	Reduce neuronal apoptosis	Exo/miR-21 overexpressing	NF-κB	Zhang et al. (2018)	SAH
	Inhibiting M1 microglia activation	EVs/miR-140-5p	ALK5/NOX2	Qian et al. (2022)	SAH
AD-MSCs (rat)	Neuroprotection/neurological recovery/restore the white matter integrity	EVs/–	–	Otero-Orteg et al. (2017)	ICH
HucMSCs (rat)	suppress neuronal apoptosis	miR-206-Knockdown Exosomes	BDNF/TrkB/CREB pathway	Zhao et al. (2019)	SAH
	inhibited cell apoptosis and inflammatory mediator expression	Exo/MiR-26b-5p-modified	MAT2A-mediated the p38 MAPK/STAT3	Liu et al. (2021)	SAH

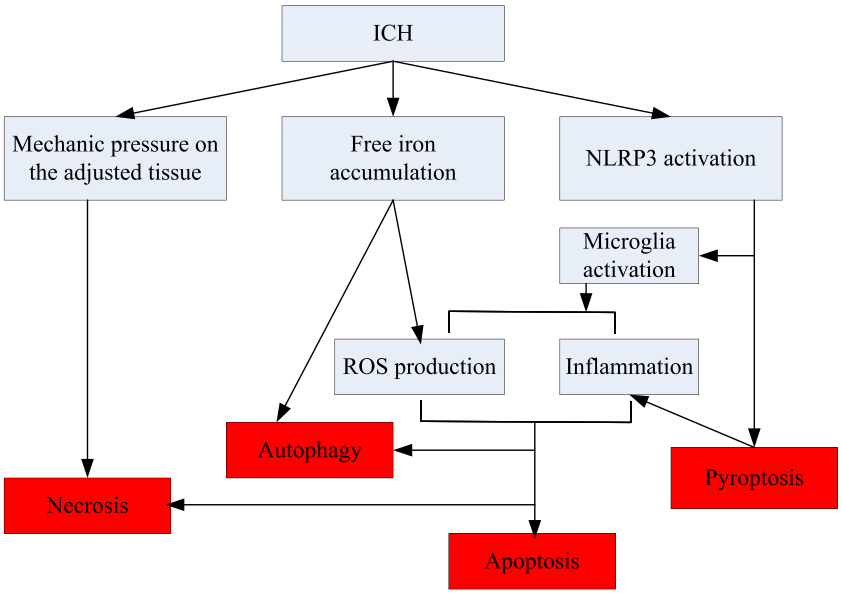

Fig. 3. The main pathway of ICH-induced cell death.

extract promote angiogenesis by delivering DMBT1 to repair brain injury [57]. Interestingly, it is reported that exosomes from mouse brain endothelial cells (EC-Exo) could significantly increase primary cortical neuron axonal growth and promote endothelial capillary formation resulting in increased axonal density, myelin density, blood vessel density, arterial diameter and neurological cognitive function [58].

4.4. Immunomodulation

In recent years, numerous research groups have disclosed the immunomodulatory effects of EVs in various disease models [59,60]. In general, EVs can play an immunosuppressive or immunostimulatory role dependent on the active ingredients within EVs and disease type engaged [61]. Several studies suggest that attenuation and modulation of immune responses may be a promising approach for ICH treatment [62,63]. In acute stroke, the products of microglial activation and cell death trigger an inflammatory cascade that damages vessels and parenchyma within minutes to hours after ischemia or hemorrhage. Immune interventions that limit brain inflammation, vascular permeability, and tissue edema must be rapidly implemented to reduce acute immune reaction-mediated destruction and avoid subsequent immunosuppression. Several current findings suggest that these goals may be achieved in ischemic and hemorrhagic stroke using multiple sclerosis disease-modifying drugs, suggesting that successful immune interventions can slow and reverse brain injury after ICH. Therefore, exosomes may improve the outcome of ICH through immune intervention.

4.5. Carriers for drug delivery

As we mentioned earlier, exosomes are the most widely characterized class of secreted membrane vesicles that carry proteins RNAs and serve as a cell-to-cell communication tool. Like liposomes, they can transport their cargo to the plasma membrane and provide a barrier against premature transformation and elimination, thus are increasingly seen as an alternative to liposomes as drug delivery vehicles. EVs/exo are naturally secretory vesicles isolated from the patient's own cells, so they may have low toxicity. They are ubiquitous in the body. Almost all cell types, various body fluids (eg. blood, saliva, urine, milk, etc.) secrete exosomes, so it can be inferred that they are more biocompatible in vivo. MSCs transplantation has been extensively tested in a variety of disease indications and has been shown to be safe in numerous clinical trials. For example, studies have found that Infusion of human-MSC exosomes into immunocompetent mouse model of acute myocardial ischemia have been shown to be therapeutic and without obvious adverse effects [64,65]. Stem cells can produce exosomes on a large scale [66,67]. Thus, MSC- EVs/exo may be ideal carriers for drug delivery [68,69].

The blood-brain barrier in the brain prevents the flow of endogenous molecules, exogenous biological agents and immune-monitoring cells (e.g., macrophages), thus maintaining central system homeostasis [70]. When neuropathy occurs, many therapeutic agents cannot reach the corresponding target cells due to the protective effect of the blood-brain barrier, limiting the treatment of neurological diseases. MSC-EVs/exo promotes functional recovery, synaptic remodeling, neurogenesis and angiogenesis in animal experimental stroke models [71]. Currently, MSC-EVs/exo can be used as a drug delivery vehicle for the treatment of cardiovascular diseases [72,73], neurological diseases. MSC-EVs/exo loaded with peroxidase was found to successfully cross the blood brain barrier and ameliorate the disease state of Parkinson's disease [74]. At present, studies have confirmed the effectiveness of MSC-EVs/exo acting directly in the treatment of ICH (Table 2), but the use of MSC- EVs/exo as a drug delivery vehicle for ICH looks like a promising treatment method that still requires extensive research.

5. Challenges in the treatment of hemorrhagic stroke by MSC-EVs/exo

Exciting results have been achieved in the treatment of hemorrhagic stroke by MSC-EVs/exo in animal models, but the following problems still need to be considered and solved.

5.1. The standards for the purity and quality control of isolated MSC-EVs/exo

As we all know, EVs/exo mediate cell-to-cell communication by transporting macromolecules such as proteins and RNA. In order to identify cargo molecules in exosomes, it is necessary to isolate exosomes in advance. However, due to the small size and low density of exosomes, they coexist with a variety of particles with similar properties (microvesicles, apoptotic bodies, protein aggregates, etc.), resulting in difficult isolation. Several biochemical methods such as ultracentrifugation, density gradient centrifugation, size exclusion chromatography and polymer-mediated precipitation have been used to isolate exosomes [31,33,75–87] (Table 3). However, the different viscosity of biological fluids, the adhesion of exosomes surface and the variable macromolecular composition of exosomes may comprise the exosome preparation of consistent quality. On the other hand, the preparation of exosomes by these methods usually requires a long processing time and special equipment. It is important to note that these difficulties associated with existing exosome isolation methods may also complicate the identification of exosome markers and may not adequately distinguish exosomes from other types of extracellular vesicles.

Recently, researchers from Augusta University in United States reported a new in situ labeling method for exosome marker recognition such as CD63 that bypasses the exosome separation step. The study showed that the engineering of ascorbic acid peroxidase APEX variants and secrete fusion protein (CD63), aspect markers in intracellular vesicles or conditioned medium of secreted outside secrete specific expression in the body, the induced protein of biotin and excitation signal, and through the protein mass spectrometry proved that oxidative stress caused by the accumulation of protein markers [88]. Another research team constructed a strategy for the separation and detection of small extracellular vesicles based on controllable self-assembly DNAzyme by integrating DNAzyme technology and aptamer technology. The EVs/exo clusters formed can be effectively enriched by ordinary centrifuge without the help of ultra-high-speed centrifuge. Later, the EVs/exo cluster was cut and dispersed again. Synchronized quantitative detection was achieved under the controlled rapid shearing of the self-assembled DNAzyme. The whole procedure was completed within 1.5 hours. This system can quickly and effectively enrich, separate and detect EVs/exo [89]. Anyway, new methods are still being tried. An exosome isolation method of time-saving, cost-effective and high purity is the consistent pursuit of the researchers.

5.2. The storage of MSC-EVs/exo

MISEV 2018 defined the essential requirements for identifying EVs [19]. MSC-sEV preparations must first meet the International Society for Cellular Therapy (ISCT) minimal criteria: (1) At least one protein of each category 1 to 3 must be evaluated in any EV preparation; (2) At least one negative protein marker should be evaluated; (3) Electric or atomic force microscoping should be performed; and (4) Single particle feature should be analyzed. Moreover, MSC-sEV-specific antigens need to be identified. According to the ISCT minimal criteria, MSC surface antigens, such as CD73, CD90, and CD105, have been found in many published MSC-EV proteomics datasets. In contrast, three non-MSC surface antigens (CD14, CD34, and CD11b) from the ISCT minimal criteria were not found in these datasets.

Compared with the standards for identification, the storage criteria of EVs/exo are also very important. Although EVs/exo is a vesicle structure with a double lipid membrane, which has good stability and protects the biomolecules in the body from various enzymes in the body fluids, thus maintaining their integrity and biological activity. However, the integrity and bioactivity of EVs/exo extraction are also affected by storage media, storage temperature and storage time. There is no universal consensus on storage standards. Therefore, it is necessary to study exosome preservation techniques in order to protect their biological activity for transport and clinical application.

At present, the main protection techniques used are freezing, freeze drying and spray drying [90] . After the EVs/exo are extracted, they are generally suspended in phosphate buffer [7]. At present, the most commonly used storage method is cryopreservation, but it may lead to changes in the shape and physical properties of EVs/exo, the formation and aggregation of multiple layers of vesicles. Repeated freeze-thaw will lead to changes in the biological characteristics, content and marker composition of EVs/exo surface molecules. Conventional EVs/exo storage conditions in the laboratory are generally 4°C, -20°C, -80°C. In one study, researchers assessed the stability of EVs/exo stored at 4°C, -20°C and -80°C for up to 28 days and compared them to fresh EVs/exo. They found that different storage temperatures and storage periods affected the stability, size and number of EVs/exo compared to freshly isolated EVs/exo, also affected their cell absorption and biological distribution [91] . The researchers immediately analyzed the freshly isolated EVs/exo or used them after short-term storage at 4°C or -20 °C. The results showed that the EVs/exo stored at -20°C and -70°C were relatively intact, while the expression of CD63 is lost at 4°C and room temperature, and the expression of HSP70 was also reduced under room temperature storage conditions. Compared with 4°C, -70°C and fresh samples, the concentration of EVs/exo protein and RNA components in EVs/exo stored at room temperature was significantly reduced. Studies have shown that a temperature lower than -20°C is preferable for the long-term storage of EVs/exo. However, -80°C storage is still recommended for long-term preservation of EVs/exo for therapeutic applications.

It is worth noting that EVs/exo from different sources may have very different stability due to their heterogeneity when compared in the same storage environment. It has been found that the extracellular vesicle DNA in serum is stable in different storage environments, but its RNA will be significantly degraded when the plasma is stored at 4°C or long-term storage at−20°C. Most miRNAs are very stable in this context, indicating the potential of EVs/exo miRNAs as biomarkers, except for one study that found miR-122 and miR-145 to be very unstable in serum [92]. After freezing of semen for up to 30 years at −80°C, there were no significant changes in the shape, physical properties, nucleic acid protein content and type of EVs/exo. In contrast, the cryopreserved samples of EVs/exo in bronchoalveolar lavage fluid became larger and the abundance of more than half protein molecules changed or even disappeared compared with the fresh sample. Therefore, the optimal storage conditions for EVs/exo from different kinds of sources still need to be explored. Although 4°C preservation is easy to lead to the loss of proteins and nucleic acids in EVs/exo, it can avoid the destruction of vesicles caused by the freeze-thaw process. EVs/exo are recommended to be stored at −80°C, but it is sometimes difficult to maintain such low temperature conditions during handling or transportation.

Interestingly, studies have found a lyophilization method to preserve exosomes, using trehalose as a protective agent during freezing. Trehalose can provide bioprotective effects to stabilize proteins, cell membranes and liposomes; prevent the aggregation of proteins as well as EVs/exo; reduce the formation of ice during freezing and decline the loss of extracellular vesicles during isolation and preservation, etc [91]. Still, more research is needed to confirm its reliability in the treatment of ICH.

Table 3
Major isolation technology of EVs/exo.

Isolation technique	Advantage	Disadvantage
Differential ultracentrifugation	The gold standard isolation approach suitable for large-volume specimens, especially cell culture supernatant, urine, etc. low cost	lengthy timescales; the requirement of a large number of cells or biological fluids; non-vesicular macromolecule contamination
Density-gradient centrifugation	High purity of EVs; preservation of EV activity	Time and labor consuming; expensive equipment requirements; large amount of pre-work and complicated steps; low EV yield
Ultrafiltration	Short operation time; low equipment cost; suitable for large-volume specimens	Labor consuming; moderate purity; possible loss due to clogging membrane; potential physical changes induced by shear stress; low EV yield
Immunoaffinity capture	High specificity and purity; easy operation; no potential mechanical damage	High-cost; low efficiency; environment-susceptive ligands activity susceptible to processing environment; low EV processing volume and yields
Size-exclusion chromatography	High purity; easy operation; good reproducibility; preservation of the native state of EVs; suitable for most of downstream analysis	Time consuming; relatively high preparation cost
Polymer Precipitation	Short operation time; preservation of the native state of EVs; high yield; easy accessibility	Contaminations of non-EVs; affection on downstream analysis and quantification of EV samples
Microfluidic techniques	Low sample consumption; fast processing time; high sensitivity; suitable for quantitative detection of scarce samples	Low sample capacity; nonspecific binding

5.3. The appropriate delivery route of MSC-EVs/exo for the treatment of ICH need to be clarified

EVs/exo drug delivery systems have a variety of routes, among which common routes include intravenous, subcutaneous, intraperitoneal, intratumoral, intranasal and oral. Recently, an exosome-based room-temperature stable respirable lyophilized powder mRNA vaccine was reported. The lyophilized powder of Lung-Exo-based mRNA vaccine was prepared by loading the mRNA encoding novel coronavirus spike protein (Spike) into Lung-Exo by electroporation followed by lyophilization. Therefore, the mode of inhalation administration was increased [93]. Therefore, the inhalation method of administration was increased. For intracerebral hemorrhage (in vivo/in vitro) experiment, there are currently intravenous injection, intraventricular injection, drug encapsulation as a drug carrier and other methods. In essence, the routes of administration are closely related to the therapeutic effect of various diseases, meaning that the different routes of administration affect the biological distribution and rapid clearance rate of drugs in the body. Therefore, it is necessary to study the influence of exosome drug delivery system on the outcome of cerebral hemorrhage.

6. Prospectives

To sum up, the treatment of MSC-derived EVs/exo has achieved good results in animal experiments. The future of MSC-derived EVs/exo therapy has great potential. However, there are some challenges from lab to clinical practice that need to be resolved. The clinical application of BMSC-EVs/exo to ICH remains in their fancy. More experimental studies and repeated evaluations are needed to demonstrate the safety and efficacy. Nevertheless, it is expected that exosome-based cell-free therapy will open up a new approach for the treatment of ICH.

Author contributions

ND and LW: conceptualization, project administration, Science and Technology Project of Sichuan Province, National Natural Science Foundation of China, and funding acquisition.

YZ and LL: writing—original draft preparation. JD and MM: writing—review and editing. YZ, HW and GY: visualization. All authors contributed to the article and approved the submitted version.

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Declaration of competing interest

The authors declare that they have no competing interests.

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