Extracellular vesicles in mesenchymal stromal cells: A novel therapeutic strategy for stroke (Review)

YINGCHEN LI[1*], QILAI CHENG[2*], GUOHENG HU[3*], TIANHAO DENG[4], QIMEI WANG[4], JIANDA ZHOU[5] and XINPING SU[6]

[1]Post-doctoral Research Station of Clinical Medicine, The Third Xiangya Hospital, Central South University, Changsha, Hunan 410013; [2]College of Pharmacy, Gannan Medical University, Ganzhou, Jiangxi 341000; [3]Department of Neurology, The First Affiliated Hospital of Hunan University of Traditional Chinese Medicine, Changsha, Hunan 410007; [4]Department of Oncology, The Affiliated Hospital of Hunan Institute of Traditional Chinese Medicine, Changsha, Hunan 410006; [5]Department of Plastic Surgery, The Third Xiangya Hospital, Central South University, Changsha, Hunan 410013; [6]Department of Osteology, The Affiliated Hospital of Hunan Institute of Traditional Chinese Medicine, Changsha, Hunan 410006, P.R. China

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Abstract. A stroke is a focal cerebral insult that frequently causes severe neurological deficit and mortality. Recent studies have demonstrated that multipotent mesenchymal stromal cells (MSCs) hold great promise for neurovascular remodeling and neurological function recovery following a stroke. Rather than a direct replacement of parenchymal brain cells, the therapeutic mechanism of MSCs is suggested to be the secretion of soluble factors. Specifically, emerging data described MSCs as being able to release extracellular vesicles (EVs), which contain a variety of cargo including proteins, lipids, DNA and various RNA species. The released EVs can target neurocytes and vascular cells and modify the cell's functions by delivering the cargo, which are considered to mediate the neural restoration effects of MSCs. Therefore, EVs may be developed as a novel cell-free therapy for neurological disorders. In the present review, the current advances regarding the components, functions and therapeutic potential of EVs are summarized and the use of MSC-derived EVs as a promising approach in the treatment of stroke are highlighted.

Correspondence to: Professor Jianda Zhou, Department of Plastic Surgery, The Third Xiangya Hospital, Central South University, 138 Tongzhipo Road, Changsha, Hunan 410013, P.R. China
E-mail: zhoujianda@csu.edu.cn

Professor Xinping Su, Department of Osteology, The Affiliated Hospital of Hunan Institute of Traditional Chinese Medicine, 58 Lushan Road, Changsha, Hunan 410006, P.R. China
E-mail: 1824064377@qq.com

*Contributed equally

Abbreviations: AD-MSCs, adipose-derived mesenchymal stromal cells; ATPS, aqueous two phase system; BI, barthel index; BM-MSC, bone marrow mesenchymal stem cell; CM, conditioned media; CNS, central nervous system; DEX, dextran; ESC, embryonic stem cells; EVs, extracellular vesicles; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; F-M Score, Fugl-Meyer score; HPCs, hematopoietic progenitor cells; Hsp, heat-shock protein; IGF-1R, insulin-like growth factor 1 receptor; LDH, lactate dehydrogenase; MCA, middle cerebral artery; MSCs, mesenchymal stromal cells; MVs, microvesicles; MVBs, multivesicular bodies; NIHSS, National Institutes of Health Stroke Scale; PBS, phosphate buffer solution; PC12 cells, rat adrenal pheochromocytoma cells; PEG, polyethylene glycol; PS, phosphatidylserine; RT, room temperature; SN, supernatant; Tsg101, tumor susceptibility gene 101; UC, ultracentrifugation; WB, western-blotting

Key words: mesenchymal stromal cells, extracellular vesicles, microvesicles, exosomes, stroke

Contents

1. Introduction

Stroke is one of the leading causes of neurological dysfunction and mortality worldwide (1). Intravenous thrombolytic therapy has been demonstrated to effectively restore cerebral blood flow; however, the narrow therapeutic window (within 3-4.5 h following stroke onset) and serious hemorrhagic complications limit this treatment to only a small proportion of

stroke sufferers (2). Endovascular intracranial thrombectomy allows an extension of this therapeutic window and potentially diminishes the risk of intracranial bleeding; however, mechanical recanalization devices also exhibit limitations due to vessel tortuosity, arterial stenosis and inaccessibility of the thrombus (3). Therefore, there is an urgent need to investigate safer and more effective treatment to restore brain function. In recent years, multipotent mesenchymal stromal cells (MSCs) have emerged as promising candidates among the innovative treatment options, which may significantly promote the recovery of neural function following a stroke (4,5). However, rather than directly replacing parenchymal brain cells, the secretion of extracellular vesicles (EVs) is suggested to be the primary therapeutic mechanism of MSC therapy (6,7). EV-based therapy circumvents the disadvantages and limitations of MSC transplantation, including embolization and possible tumor differentiation, therefore it may be an alternative to MSCs in restoring neurological function (8). In the present review, recent advances regarding the components, functions and therapeutic potential of EVs have been summarized and the future of MSC-derived EVs as a promising cell-free therapeutic approach for stroke has been discussed.

2. Therapeutic potential of MSCs in the treatment of stroke

MSC are multipotent adult progenitor cells, which can be isolated from various sources, such as bone marrow, placenta, umbilical cord, umbilical cord blood and adipose tissues (9,10). MSCs have the unique abilities of self-renewal, proliferation and multipotent differentiation, and can differentiate into the different cell types of the three germ layers; neurons, adipocytes, osteoblasts, hepatocytes, endothelial cells and islet b cells (11-13). In recent years, MSCs have received attention from researchers worldwide and several features have identified them as promising candidates for the treatment of diseases of the central nervous system. For instance, MSCs may be derived, cultured and expanded with no serious ethical issues, and they can migrate into the brain parenchyma via the circulatory system (14). It has also been determined that MSCs exhibit immunosuppressive properties and immune privilege and therefore may be applied in allogeneic cellular transplantation (15,16). In addition, they release a number of anti-inflammatory factors, including indoleamine 2,3-dioxygenase, prostaglandin E2 and interleukin-10 following transplantation and reduce the inflammatory immune response following cerebral ischemia injury (17).

A number of previous studies have confirmed the remarkable neuroregenerative ability of MSCs. MSC infusion in animal models of stroke have led to reduced infarct volume, enhanced synaptogenesis, increased vessel density and improved neurological function (9). In clinical trials, transplantation of MSCs was demonstrated to be a safe and feasible therapeutic strategy for stroke patients (Table I) (18-24).

Following cell transplantation in a previous study, MSCs were hypothesized to integrate and differentiate into neural cells and replace damaged brain tissue, however, the majority of grafted cells became mechanically entrapped at the precapillary level and, ultimately, long-term survival was only observed in a limited number of delivered MSCs in the brain (25). Previous data have suggested that the therapeutic effects are attributed to the secretion of paracrine factors by MSCs rather than the differentiation of the administered cells (26-28). A previous study has identified a class of MSC-released EVs, which may be associated with the brain restoration and repair effects of MSC therapy (29). EVs serve an important role in intercellular communication by transferring protein and RNA cargo between parent and target cells. The EVs released by MSCs function in the brain and vessels and induce neurovascular remodeling, anti-apoptosis and anti-inflammatory effects, which are considered to be novel molecular mechanisms of MSC therapy (6,30-32).

3. Constitution and characteristics of extracellular vesicles

Aside from the biologically active cytokines or factors being important components of cell secretions, it seems that the majority of cells are able to release EVs constitutively or following stimulation (33). EVs are cell-derived membrane-bound entities that contain cytoplasmic components. Structural analyses have revealed that EVs are surrounded by phospholipid bilayers, which are predominantly composed of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine (PS) (34). In particular, the structure of the bi-lipid membrane provides an efficient platform for the interaction of EVs and target cells through vesicle-cell channels (35). With an endosomal origin, EVs contain various types of cellular components, such as cytoskeleton proteins, signaling proteins, lipids, RNA and DNA. The cargos of EVs are dependent on the original cell types and may be affected by the conditions of cell culture (36). Furthermore, EV proteins and RNAs typically participate in cell-cell communication to determine the biological effects of EVs on recipient cells (37,38). Present in all biological fluids in the body, such as serum, urine and cerebrospinal fluid, EVs may contain certain specific biomarkers of diseases, thus they are promising candidates in the diagnosis of different disorders (39,40). The abilities of transporting biomolecules and targeting specific cell populations further raise the possibility of EVs as therapeutics (41).

To date, the biochemical structure, composition, biological characteristics and physiological effects of EVs have only been partially elucidated. Nevertheless, the important role of EVs in intercellular communication has received much attention in the last 10 years, as these particles are capable of transferring biological molecules such as proteins, lipids, mRNAs and microRNAs to target cells (42). The timeline (1969-2016) of articles referring to extracellular vesicles, microvesicles and exosomes are presented in Fig. 1. The present study conducted a search for literature in the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/) and the following search terms were utilized: 'extracellular vesicles', 'microvesicles' and 'exosomes'. The original studies published in various languages were retrieved, while the reviews, editorials and articles investigating irrelevant objects were excluded.

Types of EVs. EVs can be categorized into three major types based on size and intracellular origin; exosomes, microvesicles (MVs) and apoptotic bodies. Exosomes originate from multivesicular bodies (MVBs) and are discharged via p53-regulated exocytosis, which is dependent on cytoskeleton reorganization but independent of intracellular calcium concentration (43).

Table I. Clinical trials of MSC therapy in patients with stroke.

Lead author, year	Study design	Brain infarct	Cells used	Route of application	Cell dose	Adverse effects	Outcome	(Refs.)
Bang, 2005	Control, n=25; treatment, n=5; 1-year follow up	Acute MCA infarct	Autologous BM-MSCs	IV	$5x10^7$ cells in two doses	None	Improved BI; decreased mRS	(18)
Lee, 2010	Control, n=36; treatment, n=16; 5-year follow up	Acute MCA infarct	Autologous BM-MSCs	IV	$5x10^7$ cells in two doses	None	Decreased mRS	(19)
Honmou, 2011	No control group; treatment, n=12; 1-year follow up	Chronic ischemic	Autologous BM-MSCs	IV	Single dose of $0.6\text{-}1.6x10^8$ cells	None	Decreased NIHSS, reduced lesion volume	(20)
Bhasin, 2011	Control, n=6; treatment, n=6; 6-month follow up	Chronic MCA infarct	Autologous BM-MSCs	IV	$5\text{-}6x10^7$ cells	None	Increased F-M score and mBI	(21)
Jiang, 2013	No control group; treatment, n=3; 6-month follow up	Acute MCA infarct	Allogeneic UC-MSCs	IA	Single dose of $2x10^7$ cells	None	Improved muscle strength and mRS	(22)
Bhasin, 2013	Control, n=20; treatment, n=6; 6-month follow up	Chronic ischemic	Autologous BM-MSCs	IV	$5\text{-}6x10^7$ cells	None	Increased mBI	(23)
Steinberg, 2016	No control group; treatment, n=16; 1-year follow up	Chronic Ischemic BM-MSCs	Modified allogeneic	IC	Single doses of $2.5x10^6$, $5.0x10^6$, or $10x10^6$ cells	None	Increased ESS, F-M score; decreased NIHSS	(24)

BI, Barthel index; BM-MSC, bone marrow mesenchymal stem cell; ESS, European Stroke Scale; F-M score, Fugl-Meyer score; IA, Intra-artery; IC, intracerebral; ICH, intracerebral hemorrhage; IV, intra venous; mBI, modified Barthel index; MCA, middle cerebral artery; mRS, modified Rankin Score; NIHSS, National Institutes of Health Stroke Scale; UC-MSCs, umbilical cord mesenchymal stem cells.

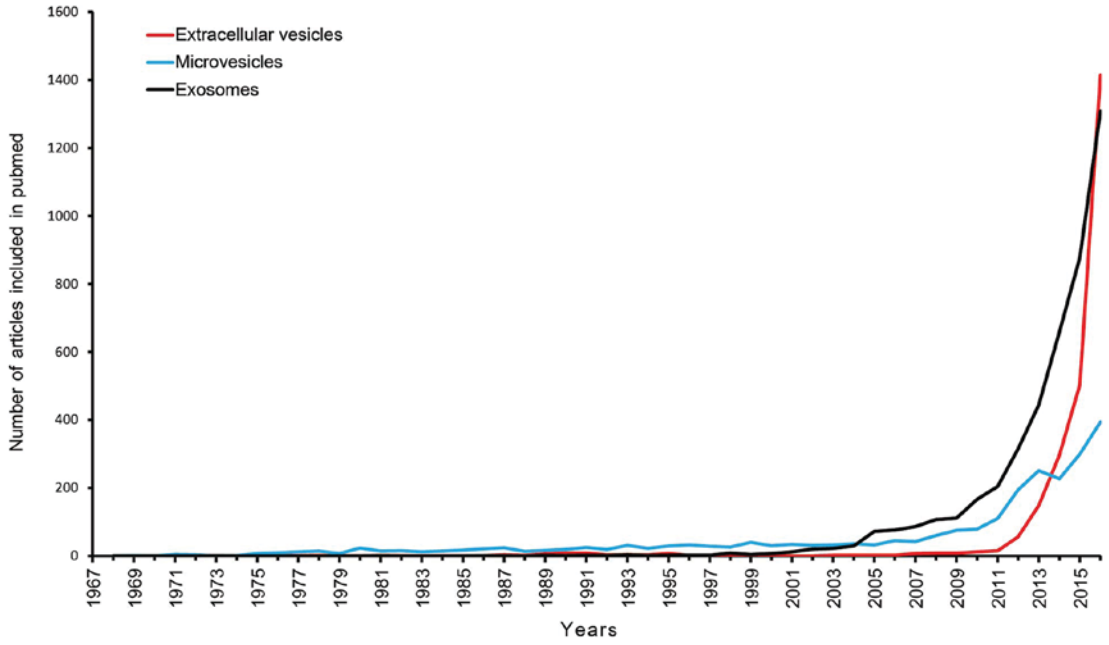

Figure 1. Timeline (1969-2016) of articles referring to extracellular vesicles, microvesicles and exosomes in PubMed (https://www.ncbi.nlm.nih.gov/pubmed/).

When MVBs fuse with cell membranes, the intraluminal particles are released from the cell and these particles are referred to as exosomes. Typically, exosomes are reported to be homogenous in size (30-120 nm) and have a density in sucrose of 1.13-1.19 g/ml (44). The most common markers of exosomes are tetraspanins [cluster of differentiation (CD)63, CD81 and CD9], heat-shock protein (Hsp)60, Hsp70, Hsp90, ALG-2-interacting protein X (Alix) and the origin-cell-specific markers (45). In addition, exosomes are rich in annexins, clathrin, lipid raft markers (flotillin-1 and flotillin-2), tumor susceptibility gene 101 (Tsg101), major histocompatibility complex (MHC) molecules and Rab family proteins, whereas PS is expressed at a low level in exosomes (45).

MVs, also known as microparticles, nanoparticles, ectosomes and shedding vesicles, are formed in response to specific stimuli (46). MVs have a diameter of 100-1,000 nm and a density in sucrose of 1.04-1.07 g/ml. They are released via direct budding and shedding from cytomembranes and this process is dependent on activation of calpain, calcium influx and the cytoskeleton (8,47). Large amounts of PS and lipid raft-associated molecules are characteristic markers of MVs and the membranes of MVs are rich in cholesterol, sphingomyelin and ceramide (43).

Apoptotic bodies, which are derived from cells undergoing apoptosis, represent the last kind of EVs. They are typically >1,000 nm and contain membrane contents, tightly packed organelles and fragmented DNA (48). Apoptotic bodies are exocytosed under the regulation of adenosine triphosphate-dependent enzymes from the cytomembrane, they express PS on the surface and have permeable membranes (45). Apoptotic bodies have been suggested to induce anti-inflammatory or tolerogenic responses when taken in by nearby cells; however, their specific role remains undefined (49).

The electron micrographs of exosomes and MVs released by human umbilical cord-MSCs are presented in Fig. 2. The ultrastructure of human umbilical cord-MSCs was visualized using transmission electron microscopy. The umbilical cord tissue was obtained in April 2017 from a patient at the Obstetrical department of the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine (Changsa, China). Briefly, MSCs were washed with PBS three times and digested with 0.25% trypsin-EDTA (cat. no. 25200-056; Invitrogen; Thermo Fisher scientific, Inc., Waltham, MA, USA) at 37˚C for 2 min. Following trypsinization with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), cells were pelleted via centrifugation at 200 x g for 5 min at 4˚C. The supernatant was removed and the cells were fixed with 1% glutaraldehyde solution at 4˚C overnight. Subsequently, the glutaraldehyde was discarded and 1% osmium tetroxide solution was added to the pellets and incubated for 24 h at 4˚C. The sample was dehydrated via graded ethanol washes and embedded in Durcupan resin for 6 h at room temperature. Sections (thickness, 60 nm) were cut using an ultramicrotome and placed on copper grids. A solution of 3% Uuranyl acetate and 2% lead citrate was used to counterstain the sections for 1 h at 37˚C. The sample was observed with a transmission electron microscope (Hitachi, Ltd., Tokyo, Japan, HT7800) at 80 kV. The key characteristics of the three types of EVs are summarized in Table II.

The strict separation of these vesicles by size or biogenesis has not yet been established. As all of these vesicle types are present *in vitro* and *in vivo*, the mixed vesicles cannot be dissected from each other once they have left the cell and in the present review, the term EVs is used to collectively describe the extracellular membrane vesicles.

Isolation of EVs. At present, there are a number of approaches used to isolate EVs, including ultracentrifugation (UC), polymeric precipitation, the use of size exclusions and aqueous two phase system (ATPS) (50).

UC is the most accepted method, which involves a series of centrifugation steps (8). Cell fragments and large vesicles are separated from EV-containing fluid by gradually raising

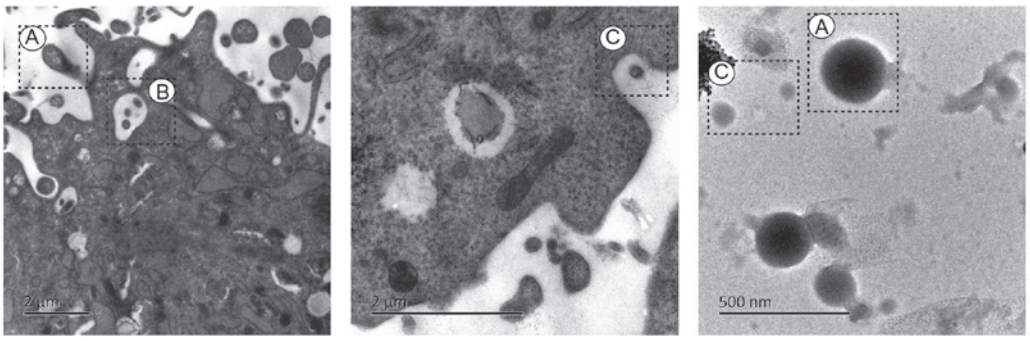

Figure 2. Transmission electron microscopy of human umbilical cord mesenchymal stromal cells. Massive blebbings of the membrane were observed in this image, the microvesicles, MVBs and exosomes are indicated in the dashed squares. (A) Microvesicles: Large (100-1,000 nm), derived from the plasma membrane. (B) MVBs: Contain exosomes. (C) Exosomes: Small (30-120 nm), derive from MVBs. MVBs, multivesicular bodies.

the centrifugal force (from 2,500-10,000 x g) and EVs are precipitated with two rounds of centrifugation at 100,000 x g for 2-3 h each time. This method is reliable, inexpensive and highly reproducible, but is lengthy and requires specialized laboratory apparatus (30).

Another isolation method is polymeric precipitation (e.g., ExoQuick-TC™; System Biosciences, LLC, Palo Alto, CA, USA), which can decrease the solubility of MVs and form precipitation (51,52). This approach has the advantages of a high yield and efficiency, but the poor purity obtained reduces the reliability of the results (53).

Size exclusions (e.g., filters) are applied by passing the sample through 0.8-μm pore filters to remove large particles and followed by centrifugation to concentrate the eluted EVs (30,54). It's a simple and low-cost technique, which can be used alone or in conjunction with UC, but this method has a low throughput as lots of EVs may adhere to the filters (55). Furthermore, the products may be contaminated by other vesicles of the same size (53).

Recently, Shin *et al* (55) demonstrated a simple and efficient method to isolate EVs from saliva using an ATPS. ATPS rapidly separated EVs from a mixture of vesicles and proteins by partitioning different types of vesicles into different phases; this method achieved a high yield and purity within a short time. However, the application of ATPS has not yet been tested extensively and a standard isolation method is still utilized in EV research. A schematic diagram for separation of EVs is represented in Fig. 3.

Characterization of EVs. Due to the ability of fluorescence activated cell sorting (FACS) to identify the same individual vesicles by different parameters, is the most commonly used method for the study of EV. The operational process of FACS is simple and the results can be quantified to a high level of quality (56). The main disadvantage is that a flow cytometer has poor discrimination under 500 nm and is only fit for detection of big vesicles. Previously, high-definition cytometers have appeared on the market and they are capable of detecting nanoparticles as small as 0.1-0.2 μm in diameter (57). Electron microscopy is typically combined with FACS to provide morphological information about the EVs (54). However, this technique has limitations in quantitative examination and the process is complicated and costly (58). Nanoparticle tracking analysis (NTA) is typically applied to analyze the mean size, modal value and size distribution of EVs (50). The vesicles with a diameter from 30-10,000 nm can be precisely and easily detected in only 5 min. The main limitation of this technique is that the quality of NTA is influenced by particle concentration and the samples need to be diluted properly to obtain reliable results (59). Additionally, enzyme linked ELISA and western blotting (WB) are used to analyze the proteins associated with EVs (60,61). Commonly used markers include tetraspanins, Tsg101, Alix, annexins, lipid raft-associated molecules and the origin-cell-specific surface proteins (62). However, both ELISAs and WB are unsuitable to quantify the protein expression level in EVs and can only be employed to demonstrate the presence of proteins (50). Commonly used methods for characterization of EVs are summarized in Table III.

Storage of isolated EVs. Reservoir vessels, buffers, storage temperature and programs have specific marked impacts on the results of EV experiments. It is advisable to store EVs in silicified vessels to prevent the adhesion of EVs to the vessel surfaces (50). PBS is considered to be the optimal choice for EV resuspension and the standard temperature for MV storage is suggested to be -80˚C (63). As for any bioactive substances, it is recommended to have the samples frozen and thawed quickly and to minimize the cycles of freezing and thawing, although it has previously been suggested that freeze/thaw cycles have no negative effect on EVs (58,64). In general, further study is required to determine the optimal storage conditions.

4. Biological functions of extracellular vesicles

EVs are released from cell membranes when the cells are stimulated by biological agonists (e.g. interleukin, endotoxin and apoptosis factors) or chemical stress (oxidative or hypoxic stress) (8,40). EVs were previously treated as only cell fragments or debris and their biological activities were ignored (35); however, an increasing number of studies have demonstrated that these tiny vesicles are important messengers in cell-to-cell communication as they can preserve and deliver cellular signals (65-67). EVs contain various types of proteins (cytoskeleton, surface molecules, receptors and enzymes), lipids similar to the cytomembrane and nucleic acids [mRNA, microRNA (miRNA), long non-coding RNA and DNA] (68-70). As multicomponent lipid vesicles, EVs have the ability to transfer cellular cargos to target cells and induce

Table II. Key characteristics of extracellular vesicles.

Characteristic	Exosomes	Microvesicles	Apoptotic bodies
Size	30-120 nm	100-1,000 nm	≥1,000 nm
Density in sucrose	1.13-1.19 g/ml	1.04-1.07 g/ml	1.16-1.28 g/ml
Origin	By exocytosis of MVB; process dependent on cytoskeleton reorganization but independent on Ca^{2+}	Outward budding of plasma membrane; process dependent on Ca^{2+}, calpain and cytoskeleton activation	Outward budding of apoptotic cell membrane
Markers	Tetraspanins, Tsg101, Alix, Hsp, annexins, Low exposure of PS, the origin-cell-specific markers	Lipid raft-associated molecules, high expression of PS	Expression of PS
Content	Proteins, lipids, mRNA and miRNA, rare DNA	Proteins, lipids, mRNA, miRNA, plasmid DNA	Intracellular fragments and cellular organelles
Isolation technique	Ultracentrifugation, electron microscopy	Ultracentrifugation, electron microscopy	Flow cytometry, electron microscopy
Storage	-80˚C	-80˚C	Not available

Hsp, heat-shock protein; MVB, multivesicular body; PS, phosphatidylserine; Tsg101, tumor susceptibility gene 101; Alix, ALG-2-interacting protein X; miRNA, microRNA.

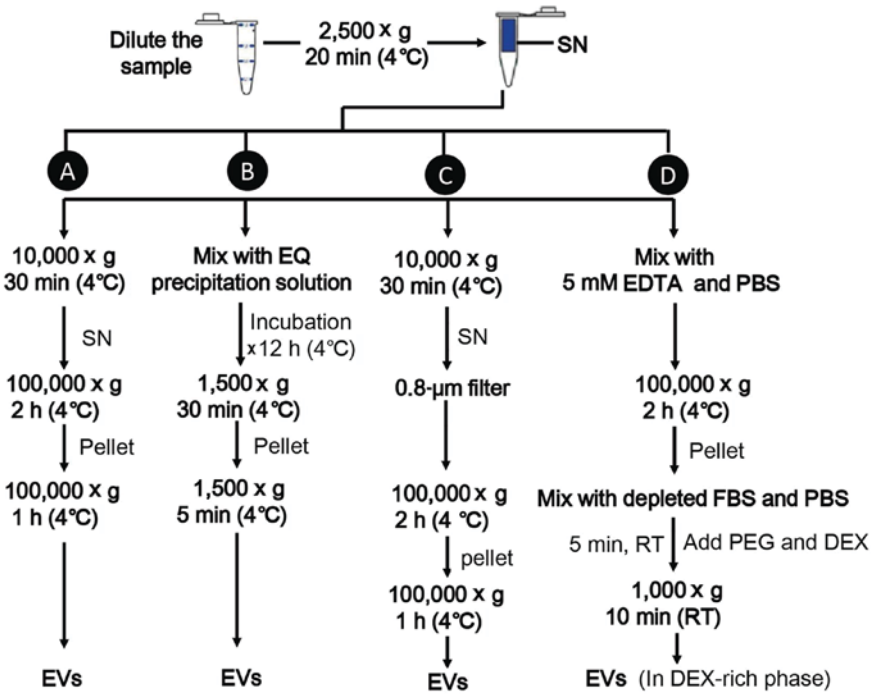

Figure 3. Isolation of extracellular vesicles. (A) Ultracentrifugation. (B) Polymeric precipitation. (C) Size exclusions, (D) ATPS. ATPS, aqueous two phase system; DEX, dextran; EDTA, ethylene diamine tetraacetic acid; EQ, ExoQuick; FBS, fetal bovine serum; EVs, extracellular vesicles; PEG, polyethylene glycol; RT, room temperature; SN, supernatant.

alterations of phenotype and behavior. Furthermore, they can protect the cytoplasmic components from damage or degradation by chemicals and enzymes (71,72). In general, EVs are able to mediate cellular interaction and exchange of information primarily through the pathways illustrated in Fig. 4.

Exosomes originate from MVBs and are discharged by membrane fusion, whereas MVs are released by direct budding from the cytomembrane. EVs may mediate cell-cell communication mechanisms including: i) Stimulation of recipient cells by functioning as signal complexes; ii) transfer of surface receptors or lipids into recipient cells; iii) delivery of cytoplasmic proteins and nucleic acids by the endocytic pathway; and iv) delivery of cytoplasmic proteins and nucleic acids by the membrane fusion.

EVs may directly stimulate the recipient cells by functioning as signal complexes. Previous studies have demonstrated that

Table III. Common methods for extracellular vesicle characterization.

Technique	Information acquired	Limitations
Electron microscopy	Morphology, size	Unquantifiable, complicated and costly
Fluorescence activated cell sorting	Phenotype, number	Limited working range
Nanoparticle tracking analysis	Size, concentration, size distribution	Dilution needed
ELISA and western blotting	Phenotype	Unquantifiable

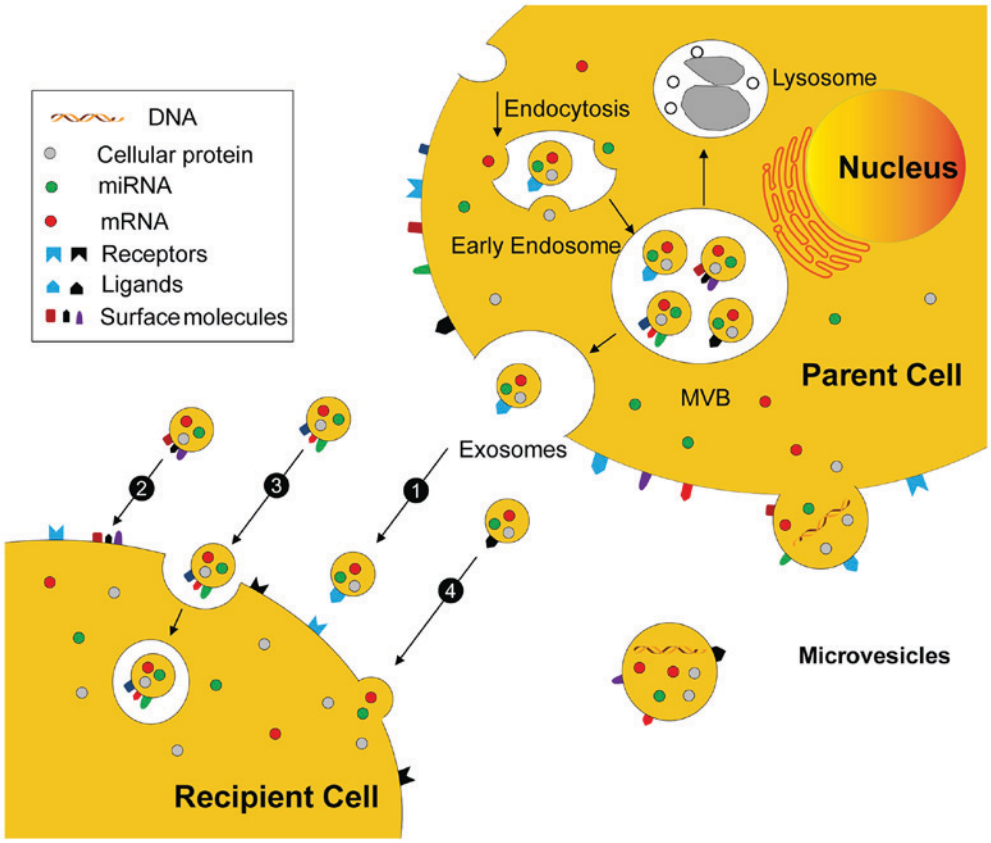

Figure 4. Biological functions of exosomes and microvesicles. (1) Stimulation of recipient cells by functioning as signal complexes; (2) Transfer of surface receptors or lipids into recipient cells; (3) Delivery of cytoplasmic proteins and nucleic acids via the endocytic pathway; (4) Delivery of cytoplasmic proteins and nucleic acids by membrane fusion. miRNA, micro RNA; MVB, multivesicular body.

EVs contain several types of surface molecules and receptor proteins including Fas-L protein, co-stimulator factors, adhesion molecules, MHC I and MHC II molecules (43,73,74). Therefore, cells with various receptors may be activated by EV ligands, e.g., exosomes expressing delta-like ligand 4, a membrane-spanning Notch ligand, which interacts with Notch receptors presented on endotheliocytes or neurocytes and causes angiogenesis and neurogenesis, respectively (75).

EVs may transfer surface receptors or lipids into recipient cells. For example, microparticles from platelets can deliver CD41 to endotheliocytes via membrane fusion, resulting in the proadhesive abilities of these cells (76). In addition, EVs are also able to act as vehicles for spreading infective agents between cells. The chemokine receptor 5 or C-X-C chemokine receptor type 4 from T lymphocytes can be diverted to non-lymphoid cells and this microparticle-mediated transfer may render these cells susceptible to human immunodeficiency virus infection (77,78).

Furthermore as part of the ligand and receptor interaction, EVs may also affect the function of target cells via the delivery of cytoplasmic proteins (79). EVs released by endotheliocytes can promote angiogenesis through the secretion of angiogenic stimulators, e.g., growth factors, proteases and their activators (80-82). In addition, Mitra *et al* (83) recently demonstrated that endotoxin-activated mononuclear cells induced cell apoptosis by transporting caspase-1 in microparticles. As a consequence, EV-mediated transfer of cellular factors and bioactive molecules may support the interaction effects between cell populations.

Finally, growing evidence indicates that EVs contribute to the transfer of nucleic acids and genetic information may be delivered to target cells, causing alterations of cellular activities and functions. Katsman *et al* (84) previously demonstrated that MVs released from embryonic stem cells (ESCs) contained a large amount of miRNAs, which may be transferred to human

Müller cells *in vitro*. In addition, it was demonstrated that ESC-released MVs increased the pluripotency and proliferation of hematopoietic progenitor cells (HPC) via delivery of ESC-derived mRNAs (85). The pretreatment of MVs with RNase may eliminate the biological effects on HPCs and this observation further confirmed the important role of mRNA transfer via MVs (85).

5. Extracellular vesicles as an alternative to MSCs

MSCs were demonstrated to release the highest amount of EVs out of a group of different cell lines, e.g., the human acute monocytic leukemia cell line, the primary human small airway epithelial cells and the mouse embryonic stem cell-derived insulin-producing cell line (86). There is no difference in morphological characteristics between the EVs released by MSCs and other cell types. In addition, aside from the common biomarkers, MSC-released EVs express certain specific surface antigens (including CD44, CD73, CD90 and CD105), which can be detected for their identification (30). As to the contents of the vesicles, the protein components of MSC-released EVs do not remain unchanged. In three independent batches of exosomes obtained from conditioned medium (CM) of MSCs, 379, 432 and 420 proteins were identified respectively, and only 154 unique proteins (~20%) were demonstrated to be in present in all (87). Functional clustering of those proteins was also performed in this study, which revealed that exosomes exhibit potential to initiate many biological processes, which is in accordance with the multiple therapeutic efficacies of MSCs (87).

Conversely, it has recently been demonstrated that MSCs produce many EVs that contain selected classes of mRNAs and miRNAs with specific functions (67,88,89). Based on gene ontology analysis, Eirin *et al* (66) demonstrated that EVs from adipose-derived MSC (AD-MSCs) were rich in different types of RNAs and their microRNA cargos (miRNA148a, miRNA 532-5p, miRNA 378) could target transcription factors or genes to induce angiogenesis, adipogenesis, apoptosis and proteolysis in recipient cells. In addition, it was reported that human bone marrow (BM)-MSCs are able to release exosomes containing mRNA for insulin-like growth factor 1 receptor (IGF-1R) and the transfer of IGF-1R mRNA may ameliorate cisplatin-induced renal dysfunction by increasing the proliferation of proximal tubular cells (90). Together, this suggested the possibility that MSCs may alter the expression of gene products and regulate the features and action of neighboring cells by EV-mediated delivery of nucleic acids, e.g., mRNA, small interfering RNA and miRNA.

As a consequence, EVs, by transferring specific types of biological molecules and genetic information, are suggested to be notable paracrine factors associated with signaling between MSCs and target cells and they may be an alternative to MSCs to restore organism function. A previous study demonstrated that the injured lung cells may deliver EVs containing lung-specific genes to BM cells, causing the expression of lung-specific proteins on MSCs (91). As a result, BM-MSCs were able to convert into cells with a pulmonary epithelial cell phenotype following transplantation into lethally X-ray-irradiated mice (91). Therefore, MSCs and injured cells may have bi-directional cell-cell communication during the process of tissue reparation. EVs secreted by injured cells may trigger the physiological activities of MSCs and MSC-derived EVs may induce the reparative and proliferative alterations of tissue cells (92,93).

6. Therapeutic effects of MSC-released extracellular vesicles on stroke

Although the application of MSC transplantation has attracted attention in neural regeneration research, the use of MSC-released EVs has also received intense scholarly interest. EVs may regulate neural survival, apoptosis, proliferation and regeneration following brain damage (Table IV) (32). MSC-secreted EVs have been demonstrated to promote neural repair and functional recovery in animal models of ischemic stroke. It was indicated that intravenous delivery of exosome-enriched EVs generated from BM-MSCs significantly improved axonal plasticity and neurite remodeling in the ischemic cortex of middle cerebral artery occlusion (MCAO) rats (94). In addition, a similar neurorestorative effect of MSC-derived EVs was exhibited by cortical neuron models of glutamate excitotoxicity (95). Following exposure to glutamate for 15 min, neurons were co-cultured with CM containing EVs of AD-MSC for 18 h. Compared with the control groups, MSC-CM reduced LDH release, inhibited neuron apoptosis and promoted neuronal regeneration and bioenergy restoration (95).

Furthermore, EVs from MSCs were reported to modulate signaling pathways to treat ischemic stroke. Lin *et al* noted that MVs released by BM-MSCs were able to protect PC12 cells from glutamate-induced damage (96). In that study, MVs enhanced protein kinase B (Akt) phosphorylation and B-cell lymphoma-2 (Bcl-2) expression and reduced Bcl-2-associated X protein and caspase-3 expression, and these effects were removed by inhibition of phosphoinositide 3-kinase (PI3K). This suggested that MSC-MVs acted as neural protective agents via activation of the PI3K/Akt pathway. In addition, Xin *et al* (97) previously observed that the expression of miRNA-133b in MSC-released exosomes increased when MSCs were cultured with ischemic brain extracts from rats subjected to MCAO. Further research demonstrated that EVs from BM-MSCs were able to transfer miRNA-133b to astrocytes and neurons in the ischemic boundary zone of rats, resulting in promotion of neural plasticity and functional recovery (94). These studies indicated that EV-mediated secretion of miRNAs contributed to the neuroprotective effects of MSCs on stroke.

With the exception of the beneficial effects on neurogenesis, EVs can improve angiogenesis following cerebral ischemia. It was demonstrated that intravenous administration of MSC-generated exosomes significantly increased the percentage of newly formed von Willebrand factor-positive cells in the ischemic zone (98). Compared with PBS-treated controls, rats receiving exosome treatment also demonstrated an improvement in neurovascular plasticity in the stroke affected hemisphere and promotion of neurological function recovery. Finally, it is important to emphasize that EVs may have immunosuppressive effects on cerebral inflammatory-related diseases. Doeppner *et al* (31) proposed that EVs from BM-derived MSC lineages were able to modify immune reactions and restore the reshaping ability of the injured brain

Table IV. Therapeutic Effects of MSC-released extracellular vesicles on stroke.

Model	Name/size (nm)	Isolation	Identify	Origin	Administration	Biological function	(Refs.)
Mice/MCAO	Extracellular vesicles/not shown	PEG plus UC	Micro BCA, WB	BM-MSCs	IV	Improved neurological impairment and angioneurogenesis, suppressed immune responses	(31)
Cortical neurons; glutamate excitetoxicity	MSC conditioned medium/-	- -	- -	Human AD-MSCs	*In vitro* co-culture	Inhibited neuronal cell apoptosis, promoted nerve regeneration and repair, restored bioenergy	(95)
PC12 cells; glutamate excitotoxicity	Microvesicles/ not shown	UC	Flow cytometry, Bradford method, TEM	BM-MSCs	*In vitro* co-culture	Activated the phosphoinositide 3-kinase/protein kinase B signaling pathway	(96)
Cortical neurons; not shown	Exosomes/ 40-100 nm	UC	TEM	BM-MSCs	*In vitro* co-culture	Increased neurite branch number, total neurite length and microRNA-133b levels	(97)
Rats/MCAO	Exosomes/ not shown	UC	Micro BCA assay	BM-MSCs	IV	Improved functional recovery, neurogenesis, neurite remodeling and angiogenesis	(98)
Rats; subcortical infarct model	Extracellular vesicles/50-100 nm	Exosome extraction kit	Electron microscopy, Nano Sight,	AD-MSCs	IV	Improved functional recovery, fiber tract integrity, axonal sprouting WB and immunofluorescence and white matter repair markers and restored white matter integrity	(109)
Rats; MCAO	Exosomes/not shown	UC	WB, TEM, micro BCA assay, qNano particle analysis	BM-MSCs	Intra-arterial injection	Increased functional improvement, neurite remodeling and brain plasticity	(110)

AD-MSCs, adipose derived mesenchymal stem cells; BCA, bicinchoninic acid; BM-MSC, bone marrow mesenchymal stem cell; IV, intravenous; MCAO, middle cerebral artery occlusion; PC12 cells, rat adrenal pheochromocytoma cells; UC, ultracentrifugation; PEG, polyethene glycol; Akt, protein kinase B; WB, western blotting; TEM, transmission electron microscopy.

Table V. Advantages and challenges of MSC based therapy and MSC-EV based therapy.

Type of therapy	Advantages	Challenges
MSC based therapy	Potential of proliferation and differentiation, release of MVs and other biological factors	Malignant transformation, tumor generation, microvascular obstruction
MSC-EV based therapy	No apparent adverse effects, capability to cross the blood brain barrier, no vascular obstructive effects, easy to be stored and engineered	Determine the specific benefits and mechanisms of MV administration, in-depth study of MV contents, potential side effects: tumor promotion

MSC, mesenchymal stem cells; EV, extracellular vesicles; MV, microvesicles.

following focal cerebral ischemia. Alongside the normalization of B lymphocytes, natural killer cells and T lymphocytes, a deactivation of dendritic cells was observed in the peripheral blood of MSC-EV-treated mice and the suppression of immune responses provided an appropriate extracellular environment for neurovascular remodeling and contributed to the functional recovery following a stroke.

7. Advantages and challenges of MSC-EV therapy

In spite of the indicated promised of MSC-based therapy in regenerative medicine, EVs demonstrate a number of advantages over MSCs in clinical applications. First, exogenous administration of MSCs may cause some serious side effects, e.g., malignant transformation, tumor generation or microvascular obstruction (99-101). In contrast, EVs, given their nanometer dimensions, have no vascular obstructive effects or apparent adverse effects following the in vivo allogeneic administration. Compared to the direct delivery of cells, EVs have a unique capability to easily cross the blood brain barrier, which is very important in the treatment of neurological disorders (102,103). Additionally, EVs can be stored at -80˚C for 6 months without degradation of their contents and this is important for the protection of soluble molecules including biological factors and nucleic acids (63). Notably, MSC-derived EVs can be modified to express a high level of biological factors, surface proteins, mRNAs and miRNAs that promote tissue repair and functional recovery and these engineered EVs may be utilized as a novel class of cell-based therapeutics (40,104).

Nevertheless, specific problems must be resolved if EVs are to be suitable for clinical applications. First, it must be determined to what extent EVs contribute to the therapeutic benefits of MSC-administration. Second, novel techniques are required to obtain large-scale production of EVs. Chen *et al* (105) previously suggested that transformation of the *Myc* gene may be a useful strategy to ensure an infinite supply of MSCs for the production of EVs and the high proliferative rate of MSCs may reduce the time and economic costs, and increase the output of EV-production. Third, further studies should look into the detailed mechanisms of the interaction between EVs and target cells and the contents of EVs, i.e., proteins, lipids and nucleic acids, must be intensively researched. Finally, it's imperative to investigate the potential side effects of EVs in therapy. Salido-Guadarrama *et al* (106) reported that tumor cells could achieve cell-cell communication by the release and transfer of miRNAs packed into tumor-secreted exosomes and, tumor-secreted exosomes served an important role in the establishment, maintenance and enhancement of tumor microenvironment, and they may act as mediators in cancer metastasis. Although EVs have no potential to directly form tumors, this does not mean that MSC-EV application has no risk of tumor promotion. Therefore, further studies are required to evaluate the long-term biological safety of EV administration. Table V exhibits the advantages and challenges of MSC based therapy and MSC-EV based therapy.

8. Conclusion

In recent years, the therapeutic potential of MSC-derived EVs has attracted a lot of attention. EVs should not be regarded as mere cell fragments or cellular waste and evidence has demonstrated that they are associated with the regulation of cell-cell communication and have significant biological effects on recipient cells. Remarkably, the idea of using EVs as a cell-free vaccine to eradicate tumors was conceived ~20 years ago (107) and clinical trials of EV-based therapy in cancer patients were initially conducted in the early 2000s (108). Currently, no clinical trials have been applied for an MSC-EV administration to treat strokes (http://clinicaltrials.gov) and it is too early to claim that MSC-derived EVs could be clinically used for functional recovery following cerebral ischemia. The specific mechanisms and the potential side effects of EV administration need to be fully investigated and novel techniques are required to obtain large-scale production of EVs. In addition, there is currently no internationally recognized standard for clinical level production and quality control of EV-based therapeutics. These unresolved problems are the remaining impediments to this therapy and, therefore, more animal experiments and clinical tests are required prior to MSC-EV application as a conventional treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Dr YL, Professor QC and Professor GH wrote the draft; Dr TD and Dr QW assisted with the literature search; and Professor JZ, and Professor XS revised and proofed the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol for analysis of human umbilical cord was approved by the ethics committee of the First Affiliated Hospital of Hunan University of Traditional Chinese Medicine (Changsa, China). Donors of human umbilical cord provided informed written consent prior to their inclusion in the present study.

Consent for publication

The patients have provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests and they have no financial relationships to disclose.

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