SVN | Stroke and Vascular Neurology

Therapeutic application of exosomes in ischaemic stroke

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ABSTRACT

Ischaemic stroke is a leading cause of long-term disability in the world, with limited effective treatments. Increasing evidence demonstrates that exosomes are involved in ischaemic pathology and exhibit restorative therapeutic effects by mediating cell–cell communication. The potential of exosome therapy for ischaemic stroke has been actively investigated in the past decade. In this review, we mainly discuss the current knowledge of therapeutic applications of exosomes from different cell types, different exosomal administration routes, and current advances of exosome tracking and targeting in ischaemic stroke. We also briefly summarised the pathology of ischaemic stroke, exosome biogenesis, exosome profile changes after stroke as well as registered clinical trials of exosome-based therapy.

INTRODUCTION

Ischaemic stroke, accounting for more than 80% of stroke population, is a severe worldwide disease with high disability and heavy economic burdens.[1 2] However, the clinical treatments are limited with only recombinant tissue plasminogen activator and thrombectomy in the very acute phase.[3] Therefore, it is urgent and crucial to develop feasible therapeutic treatments in stroke.

Recently, exosomes have gained increasing interest in stroke due to their diagnostic and paramount therapeutic applications.[4–6] Exosomes are a subset of extracellular vesicles with a diameter ranging from 30 to 150 nm,[7] and their regenerative roles in stroke have been well documented.[4 6 8] Compared with cell-based therapy, exosome-based therapy shows similar substantial protective effects while reduces potential tumourigenic and immunogenic side effects.[4] The underlying mechanisms of exosome therapeutic effects are primarily via transferring their enriched cargo, especially microRNAs (miRNAs). Mature miRNAs are single-stranded nucleic acid consisting of 20–25 nucleotides and can target multiple genes via binding to the 3'-untranslated region and repressing mRNA transcription, which can be specifically enriched in exosomes and regulate the proliferation, apoptosis, survival and differentiation of target cells.[9 10] These mechanisms have been carefully discussed in previous reviews.[4 6 8]

Herein, we mainly focus on current advances in the therapeutic application of different cell derived exosomes, exosome administration routes, exosome tracking and targeting in ischaemic stroke. We also briefly discuss the pathology of ischaemic stroke, biogenesis of exosomes, exosome profile changes after stroke and registered exosome-based clinical trials for stroke.

ISCHAEMIC STROKE PATHOLOGY

Ischaemic stroke could immediately result in ion balance disruption, metabolism failure and reduce general protein synthesis in brain cells, then induce peri-infarct depolarisation, excitotoxicity, oxidative stress, inflammatory response and blood-brain barrier (BBB) disruption, which altogether cause the death of neurons, astrocytes, microglia, oligodendrocytes and endothelia cells.[11 12] Noteworthily, cells in the peri-infarct area are still alive in the early phase of ischaemia; however, most of them are difficult to survive due to the unfriendly microenvironment. It is important to note that with these progressions, stroke also initiates the repair and remodelling processes soon after, including angiogenesis, neurogenesis, synaptogenesis and oligodendrogenesis.[11 13] Finally, newly formed neurovascular unit could partially replace the injured tissue and improve outcomes.[14]

Inflammation response after ischaemic stroke

Inflammatory response possesses both the detrimental and beneficial influences in the progression of cerebral ischaemia.[15–17] In the acute phase of stroke, inflammation could over-activate immune system. Resident microglia and infiltrating peripheral immune cells are activated by inflammatory factors or cellular components released from dying cells, then producing various inflammatory factors, chemokines, adhesion molecules and tissue-destructive enzymes, and activating complement system.[18–20] These altogether accelerate BBB damage and exacerbate brain injury in a vicious circle.[19] On the other hand, immune cells could also release anti-inflammatory factors and neurotrophic factors, clear cell

debris and toxic substances and promote neurovascular unit repair, especially in the late phase.[16 21] The detailed inflammatory and immune responses after stroke are discussed in these reviews.[16–19 21 22]

BBB disruption and recovery after ischaemic stroke

BBB is structurally formed by endothelia cells, pericytes, base membrane, astrocyte endfeet, the linking tight and gap junctions and extracellular matrix components.[23] BBB dynamically mediates substance exchange and cellular crosstalk between brain and peripheral blood, and maintains cerebral microenvironment homeostasis.[24] Following ischaemic stroke, dysfunctions of endothelia cells, pericytes and astrocytes, disruptions of tight junctions and basement membrane, oxidative stress and inflammatory response altogether lead to increasing BBB permeability, and even cause its breakdown.[23 25–27] BBB disruption could induce vasogenic oedema, extravasation of toxic blood components and infiltration of immune cells in brain parenchyma. Consequently, it causes secondary brain injury.[28] The BBB disruption usually lasts for 7 days after ischaemic stroke. After then, BBB permeability recovers and gradually returns to normal state due to the decrease of inflammatory factors, increase of growth factors, proregenerative effects of immune cells and integration of progenitor cells.[28]

Angiogenesis, neurogenesis and oligodendrogenesis after ischaemic stroke

Following ischaemia onset, endothelial cells, neurons, astrocytes, microglia and oligodendrocytes mainly undergo necrosis in the ischaemic core region.[11 29] Though the border zone surrounding the core, known as ischaemic penumbra, is less affected and has collateral blood supply, cells in this area are challenged with autophagy and apoptotic cell death.[30 31] For the detailed mechanisms of cell death and oxidative stress after stroke, readers can refer to these reviews.[32–38] Meanwhile, brain ischaemia also triggers local angiogenesis, neurogenesis and oligodendrogenesis.

Angiogenesis involves the coordination of basal lamina matrix detachment and remodelling, endothelial cell proliferation, migration and elongation, along with the participation of pericytes to form new microvessels.[39] Stroke induces the overexpression of matrix metalloproteases, which are important for basal lamina matrix detachment.[40 41] Moreover, ischaemia upregulates integrins and angiogenic factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor, fibroblast growth factor (FGF), angiopoietin-1/-2 and Tie-2, which could activate endothelial cells and promote angiogenesis.[39] In addition, endothelial progenitor cells are motivated and participated in angiogenesis.[42 43] Adult neural stem cells (NSCs) are found to located in the subventricular zone of lateral ventricle, the subgranular zone of dentate gyrus and the posterior periventricular area.[44 45] Ischaemia could induce the expression of neurogenic factors such as epidermal growth factor receptor

(EGFR), transforming growth factor α (TGF-α), FGF, chemokine such as stromal cell-derived factor 1 and transcription factors, which accompanies with the repair of injured neuronal axon, dendrites and synapses.[44 46] These bioactive molecules can activate NSC proliferation, migration along white matter fibre tracts or blood vessels[47] and differentiation. Recently, reactive astrocytes were found to trans-differentiate into neurons after ischaemia,[48] and they had great potentials to be reprogrammed into neuron via overexpression of neurogenic transcription factors including NeuroD1, Sox2, neurogenin-2.[49 50] The proliferation, migration and maturation of oligodendrocyte progenitor cells contribute to oligodendrogenesis and remyelination after cerebral ischaemia.[51] However, the differentiation of oligodendrocyte progenitor cells into myelinating oligodendrocytes is limited and impaired in the injured brain.[52] Therefore, therapeutic strategies aiming at modulating and enhancing endogenous repair process exert promising potential in ischaemic stroke treatment.

BIOGENESIS OF EXOSOMES

Exosomes are special extracellular vesicles originated from endosomes with a size of 30–150 nm in diameter.[53 54] They were first found in investigating the transferrin receptor fate during the maturation of sheep reticulocytes into red blood cells in 1983,[55 56] and named as 'exosomes' in 1987.[54] Since then, successive studies demonstrated that various types of cells were able to release exosomes, from immune cells, tumour cells, epithelial cells to neurons and glia cells. The presence of exosomes were also discovered in all kinds of biofluids including blood, urine, saliva, cerebrospinal fluid, bile, semen, ascites, amniotic fluid and breastmilk.[53 57] In late 1990s, exosomes were found to function as intercellular communication mediators.[58 59] Excitingly, exosomes were first found to carry nucleic acids such as miRNA and mRNA in 2007.[60] Now, it is well accepted that exosomes contain certain enriched proteins, lipids, DNAs, mRNAs and miRNAs derived from their original cells, and can be released by cells with active biological functions.[57 61]

The biogenesis of exosomes involves the formation of multivesicular bodies (MVBs) from early endosomes, the produce of intraluminal vesicles (ILVs) in MVBs as well as the transport and fusion with plasm membrane to release ILVs from MVBs.[57] Inward budding of early endosome membrane forms MVBs and then further ILVs. The endosomal sorting complexes required for transport with their associated proteins (ALIX, VPS4, etc), tetraspanin proteins (CD63, CD81, etc) and chaperone proteins (HSP70) are involved in cargo sorting.[57 61] Then, the Rab proteins and other unknown intracellular effectors modulate the transportation and secretion of MVBs. For detailed descriptions of exosome formation and secretion, please referred to these reviews.[53 57 61]

Exosomes are small and non-toxic extracellular vesicles, which can easily cross the BBB without provoking immune

responses as a novel shuttle.[62] These properties make exosomes an ideal and adjunct therapeutic approach for post-injury regeneration, such as engineering exosomes as exquisite delivery platforms for drugs, bioactive molecules and specific immune-modulators.[4 63 64]

EXOSOME PROFILE CHANGES AFTER STROKE

After stroke, the profile of exosomes synthesised and released from brain cells alters and these exosomes can pass through BBB to cerebrospinal fluid or peripheral blood, which can possibly serve as potential biomarkers for stroke diagnosis and prognosis.[63 65 66] Additionally, exosomes are also released to blood from endothelial and blood cells in response to stroke. Several studies have examined the changes of circulating exosomal contents including proteins and nucleic acids in stroke. In a proteome screening of microvesicles from five lacunar infarction patients' plasma and follow-up study of 5 years' outcomes, the upregulation of myelin basic protein, coagulation cascade proteins and focal adhesion, and the downregulation of albumin was associated with adverse outcomes.[67] Another study examined inflammasome proteins form serum and serum exosomes in patients who had a stroke, results suggested that apoptosis-associated speck-like protein was a potential biomarker for stroke.[68] Besides, exosomal proteins cystatin C and CD14 were related with cerebral white matter lesions and progressing brain atrophy.[69]

As for exosomal miRNA pattern changes, serum exosomal miR-126 was found to decrease at 3 hours and back to normal at 24 hours after rat transient and permanent ischaemia, while serum miR-126 showed no significant changes in transient ischaemia, suggesting that exosomal miR-126 may be more sensitive and specific to ischaemia.[70] In patients with acute stroke, serum exosomal miR-9 and miR-124 were in significantly higher levels and positively associated with clinical scores.[71] Similarly, analysing circulating exosomal from patients who had an ischaemic stroke within 24 hours or 72 hours onset found that miR-134 and miR-233, respectively were upregulated and positively correlated with severe clinical scores, poor outcomes and inflammatory responses.[72 73] Moreover, plasma exosomal miR-422a and miR-125b-2-3 p were found to decrease in subacute phase of ischaemic stroke while miR-422a increased in the acute phase, and both could serve as biomarkers for ischaemic monitor and diagnosis.[74] Detecting plasma exosomal miRNA-30a-5p and miR-21-5 p from 143 ischaemic patients divided into hyperacute, acute, subacute and recovery phases showed that combination of these two miRNAs can be used for diagnosing and distinguishing different phases of ischaemic stroke.[75]

Importantly, some treatments for stroke could also induce the change of exosomes. In mice transient ischaemic stroke, mice received moderate exercise for 4 weeks before ischaemia showed elevated miR-126 levels in endothelial progenitor cell exosomes isolated form the total brain tissue and periphery circulation exosomes. This miRNA was associated with neuron protection against apoptosis, axon growth, angiogenesis and neurogenesis.[76] Similarly, in rat cardiac ischaemia, long term exercise elevated circulating exosomal miR-342-5 p levels and prevented cardiomyocyte from apoptosis via targeting caspase 9 and Jnk2. Further analysis of the origin of exosomal miR-342-5 p showed that exercise directly promoted endothelial cell to synthesis miR-342-5p.[77] In semaphorin 3A (Sema-3A, a molecule that inhibits axonal outgrowth) inhibitor treatment of rat ischaemic stroke, Sema-3A inhibitor induced the upregulation of several genes including ptgds in astrocyte-derived exosomes.[78]

EXOSOME-BASED THERAPY AND APPLICATION
Mesenchymal stem cell derived exosomes

Mesenchymal stem cell (MSC) transplantation has been shown to promote post-stroke recovery in animal models and clinical trials (table 1).[79] Growing evidence suggests that stem cells exert their therapeutic effects mainly via paracrine mechanisms, among which exosome releasing is intensively investigated.[4 6] In ischaemia, MSC derived exosomes have been well shown to promote stroke recovery.

In rat transient middle cerebral artery occlusion (tMCAO) model, intravenously delivered MSC exosomes enhanced function recovery via promoting neurogenesis, neurite remodelling and angiogenesis.[80] In order to compare the therapeutic effects of MSC exosomes and MSCs in ischaemia, MSC exosomes were intravenously administrated 1, 3 and 5 days after tMCAO, while MSCs were given 1 day after tMCAO. Results showed that MSC exosomes exhibited closely the same effects as MSCs in terms of neuroprotection, angiogenesis and immunomodulation.[81 82] Interestingly, the effects of MSC exosomes partly depend on MSC culturing conditions. For instance, exosomes derived from brain extract treated or oxygen glucose deprivation (OGD) treated MSCs showed better therapeutic effects, which could be due to the enrichment of some certain functional proteins in exosomes.[83 84] Excitingly, MSC exosomes were also proved to be effective in primate stroke model. In macaca mulatta cortical hand motor cerebral injury model, intravenous injection of MSC exosomes 24 hours and 14 days post-injury promoted fine hand motor function recovery.[66] Further mechanism studies reveal that MSC exosomes not only reduced neuroinflammation and shift microglia into restorative functions in aged macaca mulatta,[85] but also hampered injury related hyperexcitability and restored excitatory–inhibitory balance.[86]

These effects could be ascribed to functional miRNAs or proteins transferring and further downstream signalling pathway activation. Intranasal delivery of MSC exosomes before ischaemia reduced neuronal death, promoted oligodendroglia maturation and inhibited microglia-mediated neuro-inflammation possibly via Toll-like receptor 4/CD14/NF-kB signalling pathway.[87 88]

Table 1 Published studies of mesenchymal stem cell derived exosomes in ischaemic stroke

Animals	Stroke model	Time of treatment	Routes of exosome delivery	Exosome modifications	Proposed mechanisms	Ref
Rat	2 hours tMCAO	24 hours after ischaemia	Tail vein	No	Promote neurite remodelling, neurogenesis and angiogenesis	80
Mouse	30 min tMCAO	1, 3 and 5 days after ischaemia	Femoral vein	No	Immunomodulation	82
Rat	30 min tMCAO	1, 3 and 5 days after ischaemia	Femoral vein	No	Immunomodulation	81
Rat	pMCAO	48 hours after ischaemia	Common carotid artery	No	Promote angiogenesis, neurogenesis, anti-inflammation	83
Ovine fetuses	25 min global hypoxia-ischaemia	1 hour and 4 days after ischaemia	Intravenously	No	Preserve baroreceptor reflex sensitivity and reduce white matter injury	152
Rat	2 hours tMCAO	24 hours after ischaemia	Intravenously	Enriched in miR-17-92 cluster	Enhance oligodendrogenesis, neurogenesis and neurite remodelling	97
Mouse	30 min tMCAO	Once every other day for 14 days after ischaemia	Tail vein	RGD-exosomes loaded with miR-210	Promote VEGF expression and angiogenesis	147
Mouse	Photothrombosis	24 hours after ischaemia	Tail vein	RVG-exosomes loaded with miR-124	Promote neurogenesis	98
Rat	Endothelin-1 subcortical stroke	24 hours after ischaemia	Tail vein	No	Promote white matter repair	90
Mouse	5 min bilateral common carotid arteries occlusion	Right before ischaemia	Intracerebroventricular injection	No	Inhibition of Cox-2 expression	89
Rat	2 hours tMCAO	Immediately after ischaemia	Tail vein	No	Reduce oedema	84
Rat	Perinatal brain injury	Before ischaemia	Intranasally	No	Neuroprotection, anti-inflammation	88
Rat	2 hours tMCAO	24 hours after ischaemia	Intra-arterially	Enriched with miR-134b	Promote neurite remodelling	153
Macaca mulatta	Cortical hand cerebral injury	24 hours and 14 days post injury	Intravenously	No	Not studied	154
Aged macaca mulatta	Cortical hand cerebral injiry	24 hours and 14 days post injury	Intravenously	No	Reduce inflammation, shift microglia towards restorative functions	85
Macaca mulatta	Cortical hand cerebral injury	24 hours and 14 days post injury	Intravenously	No	Dampen injury-related hyperexcitability restores excitatory–inhibitory balance	86

RVG, rabies virus glycoprotein; tMCAO, transient middle cerebral artery occlusion; VEGF, vascular endothelial growth factor.

In global ischaemia, MSC exosomes ameliorated hippocampal spatial learning and memory deficits potentially through inhibition of cyclo-oxygenase-2 expression.[89] In subcortical ischaemia, MSC exosomes promoted axonal sprouting and white matter repair.[90] The promotion of axonal growth was mediated by exosomal transferring miR-17-92 cluster and activating the PTEN/mTOR signal pathway in vitro.[91] The protection of oligodendrocyte from apoptosis was through targeting caspase-8 by exosomal miR-134.[92] Another study using lentiviruses to knock-in and knock-down miR-133b in MSCs showed that the effects of MSCs was partially mediated by exosomal transferring miR-133b to the astrocytes and neurons.[93] In OGD, MSC exosomes protected neurons from apoptosis via transferring let-7-5p and subsequently inhibiting caspase-3 expression.[94]

Recently, strategies of engineering exosomes to enhance their therapeutic effects are emerging as hotspots.[95][96] For instance, MSC exosomes enriched with the miR-17-92 cluster showed greater improvements on oligodendrogenesis, neurogenesis and neurite remodelling than control.[97] In a mouse photothrombosis model, researchers modified MSC exosomes with rabies virus glycoprotein (RVG, a neuron targeting peptide) by fusing the peptide with exosomal protein lysosome-associated membrane glycoprotein 2b (Lamp2b), then loaded with miR-124-mimics via electroporation. These engineered exosomes were shown to efficiently carry miRNA-124 to the ischaemic region and ameliorate brain injury by promoting neural progenitor differentiation.[98]

NSC derived exosomes

The investigations of NSC derived exosomes in stroke have been attracting interest since 2018 (table 2). A study compared the effect of exosomes from NSCs and MSCs, which were derived from the same pluripotent stem cell line in thromboembolic stroke model. NSC exosomes were proved to exert greater function improvement and infarct volume reduce than MSC exosomes, which were associated with more potent effect in polarising macrophage toward M2 phenotype and inhibiting inflammation.[99] Additionally, the effect of NSC exosomes on reducing infarct volume may through preservation of astrocyte function.[100] Moreover, NSC exosomes also showed promising therapeutic effects in aged stroke mice.[99] Importantly, in porcine stroke model, NSC exosome treatment also reduced infarct volume and brain oedema, promoted white matter integrity and function recovery.[101]

Adipose-derived stem cells derived exosomes

Adipose-derived stem cells (ADSCs) can be easily obtained from surgical adipose waste tissue and have been proven to be a promising candidate for ischaemic treatment. Recently, exosomes from ADSCs are emerging as a substitutive therapy (table 3).[102] In vitro, ADSC exosomes promoted brain microvessel endothelial cell migration and tube formation, while tailored miR-181b-5p overexpressing ADSC exosomes further enhanced angiogenesis via downregulating its target transient receptor potential melastatin 7 and upregulating hypoxia-inducible factor-1α and VEGF.[103] Systemic administration of miRNA-126 overexpressed ADSC exosomes could effectively inhibit neuro-inflammation, reduce neuron death, promote neurogenesis and functional recovery than normal and miRNA-126 knockdown ADSC exosomes in rat ischaemia.[104] Similarly, ADSC exosomes enriched with miR-30d-5p further prevented cerebral injury via promoting M2 microglia polarisation than normal and miR-30d-5p knock-down group. Mechanism study showed that miR-30d-5p suppressed autophagy-mediated M1 microglia polarisation by targeting autophagy-related genes beclin-1 and autophagy-related genes 5.[105][109] In rat ischaemia, intraventricular administration of ADSC exosomes engineered with a multifunction protein pigment epithelium-derived factor more potentially repressed neuron apoptosis via promoting autophagy-associated protein little computer 3 expression.[106]

Other cell derived exosomes

Apart from stem cells, other cells derived exosomes also exhibited therapeutic effects in stroke (table 4). Here, we discussed the applications of exosomes from astrocytes, endothelial cells and microglia.

Astrocyte derived exosomes were showed to reduce infarct volume and inhibit neuron apoptosis via

Table 2 Published studies of neural stem cell derived exosomes in ischaemic stroke

Animals	Stroke model	Time of treatment	Routes of exosome delivery	Exosome modifications	Proposed mechanisms	Ref
Mouse	TE-MCAO	2 hours, 14 hours, 38 hours/6 hours, 24 hours, 48 hours (in aged mice) after ischaemia	Tail vein	No	Immunomodulation, inhibit inflammation	99
Mouse	1 hour tMCAO	2 hours after ischaemia	Internal jugular vein	No	Preserve astrocyte function	100
Pig	pMCAO	2 hours, 14 hours, 24 hours after ischaemia	Intravenously	No	Protect the integrity of BBB and WM	101

BBB, blood-brain barrier; pMCAO, permanent middle cerebral artery occlusion; TE-MCAO, thromboembolic middle cerebral artery occlusion; WM, white matter.

Table 3 Published studies of adipose-derived stem cell derived exosome in ischaemic stroke

Animals	Stroke model	Time of treatment	Routes of exosome delivery	Exosome modifications	Proposed mechanisms	Ref
Rat	50 min tMCAO	3 hours after ischaemia	Intravenously	No	Anti-inflammation, anti-apoptosis	155
Rat	tMCAO	Immediately after ischaemia	Tail vein	Enriched with miR-30d-5p	Reduce autophagy and inflammation, and promote microglia M2 polarisation	105
Rat	tMCAO	Not mentioned	Intravenously	Enriched with miR-126	Promote neurogenesis, angiogenesis, anti-inflammation	104
Rat	1 hour tMCAO	3 days before ischaemia	Lateral cerebral ventricle injection	Loaded with pigment epithelium-derived factor	Promote autophagy	156

tMCAO, transient middle cerebral artery occlusion.

regulating autophagy.[107] Exosomes from OGD preconditioned astrocytes attenuated OGD induced neuron death via shedding miR-92b-3p.[108] In rat permanent ischaemia, Sema-3A inhibitor could reduce astrocyte activation and promote axonal elongation via GTPase-1/R-Ras/Akt/GSK-3β signalling pathway. They also confirmed that exosomes from OGD Sema-3A inhibitor treated astrocyte further promoted neuron axonal outgrowth than exosomes from normal and OGD astrocyte.[78]

However, these effects were adverse in normal and OGD endothelial cells exosomes. Exosomes from normal microvascular endothelial cells inhibited astrocyte apoptosis in vitro, ameliorated BBB disruption, reduced infarct volume and promoted neurological function recovery in vivo, while OGD endothelial cell exosomes all presented reversed effects.[109] Differences in exosomal protein and miRNA contents isolated from the same cell under different cultures may explain for these discrepancies.[110] Interestingly, remote ischaemic postconditioning increased exosomes in serum and reduced ischaemia-reperfusion injury partially via exosomes released from endothelial cells in femoral arteries.[111 112] Treating SH-SY5Y cells with exosomes from OGD human umbilical vein endothelial cells (HUVEC) inhibited cell apoptosis, promoted cell migration and invasion under OGD.[112] Moreover, endothelial cell exosomes suppressed macrophage activation and inflammation via transferring miR-10a and inhibiting NF-kB signalling pathway.[113] Intravenously injecting brain endothelial cell exosomes 3 days after ischaemia in diabetes mice improved neurological outcomes and cognitive function, enhanced axon outgrowth and myelin density, promoted angiogenesis and M2 macrophage polarisation. However, miR-126 knockdown endothelial cell exosomes largely abolished these beneficial effects.[114]

Exosomes from IL-4 stimulated BV2 microglia cells promoted HUVEC tube formation.[115] Systemic administration of M2 microglial exosomes reduced infarct volume, promoted neurological function recovery and inhibited neuron apoptosis possibly via transferring miR-124 and modulating ubiquitin-specific protease 14

in neurons.[116] These studies collectively suggested that exosomes play a critical role in intercellular communication and could be used for therapeutic applications.

ROUTES OF EXOSOME ADMINISTRATION

There are different exosome administration routes in stroke, which could be divided into two broad categories, respectively, systemic and local administration. Systemic administration includes intravenous injection via tail vein, femoral vein or internal jugular vein, intra-arterial injection via common carotid artery, intraperitoneal and intranasal delivery. Exosomes delivered via tail vein is the most used route in rodent models of ischaemic stroke.[80 90 98 99 105 109 116] Visualisation of indium-111-labelled exosomes injected 1-hour post ischaemia by single photon emission CT (SPECT) showed that exosomes appeared at the infarcted area 1-hour post injection, and were largely cleared from the brain by 24 hours.[99] Another study revealed that the clear time of exosomes from blood rapidly began 1 hour and gradually lasted from 1.5 to 6 hours post injection.[117] Similarly, 3-^{125}I-iodobenzoyl norbiotinamide labelled exosomes displayed a distribution phase with a half-life of 1.5 min then an elimination phase with a longer half-life of 6 hours via measuring the radioactivity in the blood.[118] Notably, the majority of exosomes were trapped in the liver, lungs, spleen, kidney, stomach and intestines.[119 120] In contrast to intravenous injection, intraperitoneal injection displayed decreased accumulation in the liver, increased distribution in the pancreas and slightly higher whole body accumulation.[119] Intraperitoneal administration allows larger amount of exosome than other systemic administration routes, but exosomes rapidly diffused in the cavity of peritoneal.[121 122] In a rat model of perinatal brain injury, dynamic examination of intranasal administrated exosomes prior to ischaemia revealed that exosomes appeared in the frontal brain as early as 30 min post administration and evenly distributed in the whole brain 3 hours post administration.[88] Biodistribution analysing intravenous and intranasal delivery of

Table 4 Published studies of other cell derived exosomes in ischaemic stroke

Animals	Stroke model	Time of treatment	Source of exosomes	Routes of exosome delivery	Exosome modifications	Proposed mechanisms	Ref
Mouse	pMCAO	1 hour after ischaemia	Astrocyte	Tail vein	No	Anti-apoptosis	107
Mouse	90 min tMCAO	30 min after ischaemia	Brain microvascular ECs	Tail vein	No	Protect BBB integrity, inhibit astrocyte activation	109
Mouse	Photothrombosis	3 days after ischaemia	Brain endothelial cells	Intravenously	Enriched with miR-126	Promote angiogenesis, neurogenesis and M2 macrophage polarisation	114
Rat	2 hours tMCAO	Immediately after ischaemia	Macrophage cell line	Tail vein	No	Promote M2 microglial polarisation	157
Mouse	90 min tMCAO	1, 2, 3 days after ischaemia	Microglia cell line	Tail vein	No	Promote neuronal survival	116

pMCAO, permanent middle cerebral artery occlusion; tMCAO, transient middle cerebral artery occlusion.

exosomes labelled with gold nanoparticles after cerebral ischaemia showed that the amount of intranasal delivered exosomes in the brain was more than twofold higher than intravenous delivery 1-hour post administration. There still were substantial amount of exosomes retaining in the brain after intranasal delivery, while nearly negligible amount after intravenous delivery 24 hours post administration. Importantly, a fair amount of intranasal delivered exosomes also accumulated in the lungs, spleen and kidney.[123] These results suggested that intranasal delivery was a more efficient and promising non-invasive therapeutic strategy for exosome administration in ischaemic stroke.

Reported local administration is usually the cerebral ventricle injection.[89 106] In global ischaemia, intracerebroventricular injected MSC exosomes rescued hippocampal synaptic transmission deficits, inhibited cyclo-oxygenase-2 expression and promoted spatial learning and memory.[89] Additionally, ADSC exosomes delivered via lateral cerebral ventricle for constitutive 3 days before ischaemia ameliorated infarct volume and cell apoptosis 3 days after tMCAO.[106]

EXOSOME LABELLING AND TRACKING
Fluorescence microscopy
Exosomes can be visualised under fluorescence microscopy through directly labelling exosomes with fluorescent dye or indirectly overexpressing fluorescent proteins conjugated to exosomal enriched proteins or miRNAs in parental cells. Lipophilic fluorescent dye such as PKH-26 and Dil-Red can label exosome membrane through inserting aliphatic chains into the lipid bilayer.[124] A study compared the bio-distribution between liposomes and tumour-derived exosomes labelled with Dil-Red, results revealed that exosomes showed the similar bio-distribution profiles with liposomes in vivo, mostly accumulated in liver and spleen when intravenous administrated and retained in tumour when intratumour injected.[125] This method is relatively easy to operate. However, the lipophilic fluorescent dye could incorporate with other cellular lipid membranes for long periods and may not reflect the true half-life of exosomes.[117 126] In order to circumvent this disadvantage, a study labelled exosomes with fluorescent dye using clickchemistry by cross-linking the exosomal membrane proteins with alkyne group.[127]

Another approach is to fuse exosomal membrane protein or exosomal RNA cargos with fluorescent protein reporter gene or peptide, such as enhanced green/red fluorescent protein or Cy3.[77 126] These can dynamically visualise exosomal spatiotemporal release, uptake and cargo exchange between cells.[126]

Bioluminescent imaging
Bioluminescent imaging (BLI) is based on light emitting-enzyme catalysed reactions to report cellular activity at molecular level.[128 129] Compared with traditional imaging methods, BLI is a powerful imaging technology

for molecular imaging in living cells and small animals without the need to sacrifice animals.[128] This enables the investigations of ongoing biological processes in vitro and vivo. One of the most used light emitted enzyme is gaussia luciferase, which can react with its substrate coelenterazine and then emit strong chemiluminescent signal.[129 130] In order to dynamically detect exosome uptake and mRNA translation in recipient cells, researchers fused exosome mRNA cargos with gaussia luciferase reporter gene. The gaussia luciferase mRNA translation occurred within 1 hour, peaked at 12 hours, declined between 12 and 24 hours and continued up to 72 hours post exosomes delivered to recipient cells.[126] Systemic administration of exosomes labelled with lactadherin (a membrane-associated protein mainly presented in exosomes) conjugated gaussia luciferase demonstrated similar biodistribution pattern as PKH-26 labelled exosomes, but displayed shorter half-life.[120] Exosomes displayed rapid distribution and then followed by longer clearance, and the clearance time was much longer in macrophage-depleted mice, suggesting that macrophages might be involved in the clearance of exosomes from circulation system.[131]

MRI

MRI can provide high-resolution imaging for monitoring stem cells and exosome tracking and distribution in a non-invasive and radiation-free manner.[132–135] In the first study to track exosome by MRI, exosomes were loaded with 5 nm superparamagnetic iron oxide nanoparticles through electroporation. Dynamic tracking exosome homing from food pad to local lymph nodes showed that exosomes retained the iron cargo through the whole process for at least 48 hours.[136] But electroporation induces a temporal disruption of exosomal membrane, which can alter exosomal contents and influence their function. Therefore, another study innovatively incubated ADSCs with ultrasmall superparamagnetic iron oxide nanoparticles then isolated the exosomes.[132 133] However, the detection limitation of MRI tracking was 3 µg and 5 µg of exosomes in vitro and in vivo, respectively.[133]

CT

CT produces images based on the computer processing of X-ray from different angles and is widely used in clinic. A study labelled MSC exosomes with glucose-coated gold nanoparticles and detected the biodistribution of exosome after intranasal administration in the normal and ischaemic brain by CT. In vivo CT imaging showed that exosomes migrated and accumulated in the ischaemic region 3 hours and still within the ischaemic site 24 hours post intranasal administration, while displayed no region specific accumulation and were cleared from the brain after 24 hours in the normal mice.[123] They further used CT to track the migration and accumulating of intranasal administrated MSC exosomes in stroke, autism, Parkinson's disease and Alzheimer's disease. Results revealed that exosomes specifically targeted and accumulated in the associated pathological brain region up to 96 hours

post-delivery, while showed diffused distribution and elimination by 24 hours. These accumulation patterns were related to neuro-inflammation signal.[137]

SPECT and positron emission computed tomography

SPECT and positron emission CT (PET) are two main clinical nuclear imaging tools, and acquire images by detecting gamma rays generated directly from gamma-emitting radioisotopes and indirectly from positron-emitting radionuclide, respectively.[138] Different from MRI and CT emphasising on anatomy imaging, nuclear imaging is a molecular and functional imaging that can visualise and measure bimolecular metabolism and cellular activity throughout the whole-body.[139 140] Compared with SPECT, PET imaging has higher resolution and sensitivity.[140] The major advantages of nuclear imaging are the unlimited tissue penetration and quantitative analysis with the shortcoming of low tissue anatomical information.[139] Therefore, they are usually combined with CT or MRI. Dynamic visualising intravenously injected ^{99}mTc-HMPAO labelled exosomes in normal mice by SPECT/CT showed that the uptake of exosomes were mainly in liver and spleen 30 min post-injection, and in salivary glands 3 hours post-injection, but very few in the brain at any time point.[141] Up to date, the SPECT/CT and PET imaging of exosomes labelled with radiotracers are mainly focused on tumour and no reports in cerebral ischaemia.[142–144] For example, tumour cell derived exosomes were labelled with iodine-131 for SPECT/CT imaging and ex vivo gamma counter could quantitatively evaluate the amount of exosomes delivered to each organ.[144] Another study labelled breast cancer cell derived exosomes with copper-64 for PET imaging and polyethylene glycol for enhanced in vivo properties. The accuracy of PET imaging of intravenous delivered exosome tissue biodistribution was confirmed by ex vivo gamma counting.[143] These suggested than SPECT and PET are potentially imaging methods for exosome tracking in vivo and can pave the way for the development of future mechanism researches, therapeutic and diagnostic applications.

EXOSOME TARGETING

Exosome targeting has emerged as an innovative and important engineering strategy for enhancing and optimising therapeutic effects by targeting specific tissue and cell type. The basic principle of exosome targeting is to fuse specific tissue homing or cell targeting peptide to exosome membrane proteins, such as Lamp2b. A study fused neuron-specific RVG peptide (specifically binds to the acetylcholine receptor) to Lamp2b and isolated exosomes form dendritic cells overexpressing fused Lamp2b. Then these modified exosomes were loaded with exogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) short interfering RNA (siRNA) by electroporation. Intravenously injected GAPDH siRNA loaded plus RVG-fused exosomes specifically knockdown

GAPDH expression in striatum, midbrain and cortex, but not in the liver, muscle, heart and other organs. Further examination of the colocalisation of siRNA and brain cell specific marker revealed that RVG-conjugated exosomes targeted primarily to neurons, microglia, oligodendrocytes and their precursors.[145] Therefore, intravenous administration of RVG modified MSC exosomes 1-day post cerebral ischaemia exerted greater therapeutic effects on promoting neurogenesis and reducing brain injury.[98] To specifically target brain endothelial cells, MSC exosomes were conjugated with the cycle (Arg-Gly-Asp-D-Tyr-Lys) peptide (c(RGDyK)) using clickchemistry, which had high affinity to integrin $\alpha_v\beta_3$ in reactive cerebral endothelial cells after ischaemia. Analysing the fluorescence intensity of intravenous injected exosomes in different organs showed that compared with unmodified exosomes, c(RGDyK) modification increased the amount of exosomes in the brain, liver and lung, and the amount in the brain was higher than that in the liver and lung, suggesting that c(RGDyK) conjugation enhanced exosomal tropism to ischaemic brain region. Further administration of curcumin loaded c(RGDyK) exosomes to ischaemic mice effectively inhibited inflammation and cellular apoptosis in ischaemic region compared with control groups.[146] In mice ischaemia, MSC exosomes were modified with c(RGDyK) and loaded with miR-210 (RGD-exo:miR-210). Intravenously injected RGD modified exosomes mainly accumulated in the ipsilateral brain and liver, RGD-exo:miR-210 more potently upregulated VEGF, integrin β3 expression and promoted angiogenesis compared with RGD modified exosomes.[147] EGFR could also serve as a specific target for tumour and ischaemic region with angiogenesis. GE11 peptide (amino-acid sequence YHWYGYTPQNVI) specifically binds to EGFR, but less mitogenic than EGF. The uptake of EGF and GE11 peptide modified exosomes showed an EGFR concentration-dependent manner, but EGF modified exosomes stimulated cellular EGFR signalling.[148]

Recently, new methods have been developed to further enhance exosomes targeting. In a study, researchers engineered exosome membrane to create a membrane-anchoring platform termed 'cloaking', which could provide anchoring sites to embed any biotinylated tissue-specific antibodies or homing peptides onto exosomes. Examination the uptake of the engineered exosomes with cardiac fibroblasts target bio-DR, muscle targeting peptide MTP and ischaemic targeting peptide sequence CSTSMLKAC showed specific target cell and tissue accumulation and homing than control group.[149] Another way to load functional proteins into exosomes was to fuse the target protein with a WW tag, which could be recognise by L-domain-containing protein Ndfip1 and induce ubiquitination and protein loading into exosomes.[150] Collectively, these researches enabled the application of modulating exosome tropism and targeting to improve exosome treatment efficiency.

EXOSOME CLINICAL TRIALS IN STROKE

Many preliminary works have proven that exosomes were sufficient to promote neurovascular remodelling and functional recovery in ischaemic stroke. However, the clinical investigations of exosome-based therapy in stroke have just begun to explore, and well-designed clinical trials are warranted to promote exosome translation to clinic. Up to now, only one registered clinical trial NCT03384433 was reported in exosome treatment of stroke. Researchers are aiming to evaluate the administration of allogenic MSC exosomes enriched with miR-124 on improvement of disability in patients with acute ischaemic stroke. This clinical trial is still under recruiting, and patients will receive 200 mg exosomes from MSC transfected by miR-124 via stereotaxic administration 1 month after ischaemia onset. Measurement of modified ranking scale and adverse treatment related events will be conducted 12 months post exosome administration. Due to the therapeutic mechanisms of exosomes are largely mediated by miRNAs, we also reviewed miRNA clinical trials in stroke. Noteworthily, most of miRNA clinical trials are focused on the prediction, diagnosis and prognosis roles of miRNAs in stroke research. For example, in order to identify serum miRNAs for the stroke risk prediction, researchers finally examined and analysed the serum miRNAs from 1523 controls aged 40–69 years and 173 cerebrovascular disorder cases aged over 69 years. Using three step strategy, they identified 10 miRNAs correlated with a predicted risk of stroke, 7 of which were significantly associated with cerebrovascular disorder. Finally, they constructed a 3-miRNA (miR-1268b, miR-4433b-3p and miR-6803-5p) combination model for risk predicting before stroke onset.[151] Clinical trial NCT04175691 and NCT04230785 will analyse the expression pattern of circular RNA, miRNAs and long non-coding RNA using next-generation sequencing in patients to verify related biomarkers for detection and prognosis of acute ischaemic stroke, and for progression and prognosis of acute ischaemic stroke with endovascular treatment, respectively. Importantly, one on recruiting study NCT03577093 tries to investigate the molecular mechanisms of microRNA-494 mediating cell cycle regulation after cerebral ischaemia. They designed to recruit patients who had a stroke within 6 hours onset aged 18–80 years and examine peripheral blood DNA samples.

CONCLUSIONS AND FUTURE PERSPECTIVES

Exosomes have gained increasing interest in cerebral ischaemia and have been considered to be potential therapeutic strategy. Numerous evidence demonstrates that exosomes derived from either stem cells or other cells exert protective and restorative effects in stroke. Furthermore, engineered exosomes exhibit greater therapeutic benefits. Therefore, exosomes could be tailed specifically with restorative genes, proteins, drugs and molecules to obtain better efficacy. However, the dose, frequency and routes of exosome delivery still do not have concise

agreement and need further investigation to accelerate exosome-based therapy from bench to bedside. The development of exosome tracking and targeting will advance our understanding of exogenous exosome bio-distribution, pharmacokinetics and further promoting therapeutic effects and clinical translation of exosome-based therapy in stroke.

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