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Review Article

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The Preclinical and Clinical Applications of Mesenchymal Stem Cells in Stroke

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ABSTRACT

Stroke is a kind of sudden onset of cerebral circulatory disorders with transient or permanent symptoms and signs of brain dysfunction clinically, it has a high incidence rate and a high mortality rate and is one of the severe cerebrovascular diseases that threaten human life and disability seriously and bring the heavy economic burden to patients and their families, there was no ideal treatment for it. Mesenchymal stem cells (MSCs) are a type of primitive undifferentiated cells with multi-directional differentiation potential and self-replication ability, they have the characteristic of convenient source and can differentiate into various types of somatic cells including nerve cells, cardiomyocytes, and osteoblasts and secrete various growth factors including brain derived neurotrophic factor, nerve growth factor, and epidermal growth factor, demonstrating the enormous application potential in many kinds of diseases including stroke. In this review, we summarized the basic characteristics and sources of MSCs, recent research progression of preclinical application and clinical application of MSCs in stroke, and the application limitations and the prospect were also discussed, hope our review may provide some useful clues for the related researchers.

Key words: *MSCs, Stroke, Preclinic application, Clinic application*

INTRODUCTION

It is reported that stroke has been becoming the second leading cause of death and the third leading cause of disability in the world [1]. The number of stroke patients has been increasing significantly year by year in recent ten years, because of the characteristics of high incidence rate, high recurrence rate, high disability rate, and high mortality, almost 70-85% of stroke patients experience varying degrees of loss of life and work abilities, bringing significant economic burden for families and society [2-4]. Many kinds of risk factors could result in stroke including hypertension [5, 6], diabetes mellitus [7], high fasting blood glucose [8, 9], smoking [10, 11], and drinking [12, 13], these risk factors pose significant obstacles to the prevention and treatment of stroke. How to effectively treat and alleviate the symptoms of stroke patients is becoming a global problem in the world.
Stem cells are a kind of special cells with self-renewal ability and multi-directional differentiation potential [14], they can be divided into three types including totipotent stem cells, pluripotent stem cells, and unipotent stem cells according to the developmental potential of stem cells [15, 16]. Multiple studies have demonstrated that MSCs could differentiate into different cell types including osteoblasts [17, 18], chondrocytes [19, 20], and nerve cells [21, 22] under specific conditions; MSCs could also express, synthesize, and secrete many kinds of bioactive molecules including growth factors, cytokines, signal peptides and exosomes which regulating play the important role in cell metabolism, immunity, differentiation, and proliferation in the body [23, 34]; and they also have the

characteristic of advantageous distribution towards the site of injury [25, 26], those advantages of MSCs exhibit the enormous application potential in regenerative medicine. Moreover, many studies have demonstrated that MSCs also exhibit significant advantages in alleviating and treating stroke. For example, Smith *et al*. reported that hematopoietic stem cells could reduce infarct volume, mortality rate, and microglial activation by regulating metallothionein-1 in ischemia-reperfusion injury mice model [27]; Jiao-Jiao Peng *et al*. demonstrated that repetitive transcranial magnetic stimulation could promote the embryonic stem cells-derived human neural stem cells to differentiate into neuron-like cells and to accelerate functional recovery in middle cerebral artery occlusion rat model [28]; and Wang *et al*. found that ZL006 could improve the homing ability of neural stem cells into the ischemia-injured site and accelerate the neuronal differentiation in focal cerebral ischemic male rat [29]. In this review, we will introduce the characteristics and sources of MSCs, summarize the recent research progression of MSCs application in stroke, and the application limitations and the prospect of MSCs were also discussed, hope our review may provide some clues for the related researchers **(Figure 1)**.

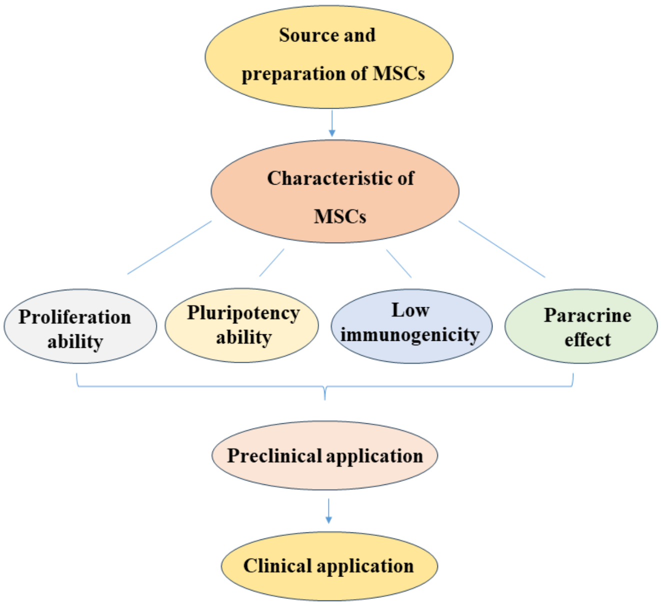

Figure 1. Graphical abstract of the review article

Characteristics and source of MSCs

Source and preparation of MSCs

MSCs are a kind of pluripotent adult stem cells with self-renewal ability derived from the mesoderm, which could differentiate into various cell types; MSCs distributed in connective tissue and interstitium of organs including adipose tissue, bone marrow, umbilical cord, dental pulp, placenta, amniotic fluid, muscle tissue, and thymus tissue [30-32]. The preparation of MSCs is relatively simple and diverse, for example, the isolation methods of bone marrow MSCs include a conventional tissue-adherent method, bone tissue digestion method, density gradient centrifugation method, immunomagnetic bead sorting method, and flow cytometry separation method [33]; the isolation methods of umbilical cord MSCs and adipose-derived stem cells include tissue block adhesion method [34], enzyme digestion method [35], adsorption column method [36] and direct centrifugation method [37]. Those different isolation methods of different kinds of MSCs have their advantages and disadvantages and could be selected according to practical requirements. We have summarized the advantages and disadvantages of the common isolation methods in **Table 1**.

Table 1. The advantages and disadvantages of the common isolation methods

Isolation method	Advantages	Disadvantages
Adherent screening method	simple operation, time-saving, less pollution	low cell purity

Density gradient centrifugation method	high quality and higher purity	strict and complex operations, low cell activity
Tissue digestion method	simple operation, low cost	collagenase could affect the cell activity and proliferation ability
The immunomagnetic bead sorting method	simple and efficient, and high purity	magnetic separator required, high cost, complex operations
Flow cytometry separation method	high purity	complex operations, high cost and time-consuming

Proliferation ability of MSCs

MSCs have the advantage of being capable of large-scale amplification and a strong proliferation ability in vitro. In theory, MSCs have high physiological activity and could proliferate infinitely. Some evidence suggested that MSCs could still maintain pluripotency and immunogenicity after many passages. Qinjun Zhao *et al*. compared the biological properties of human umbilical cord MSCs at P3, P6 and P15, found that human umbilical cord MSCs at P3, P6 and P15 had the higher consistency in morphology, expression of biomarkers and cytokines [38]; Urszula Kozlowska *et al*. compared the biological activity of bone marrow MSCs, adipose tissue-derived MSCs, skeletal muscles-derived MSCs and skin MSCs which cultured for ten weeks, found that four different types of long term cultured MSCs exhibited the basic relatively consistent phenotypes of MSCs, especially, long term cultured bone marrow MSCs and long term cultured adipose tissue-derived MSCs could keep the expression of Sox2 and Oct4 and multilineage differentiation ability [39]; Shannon S Connard *et al*. compared the immunomodulatory properties changes of P3 equine bone marrow MSCs, P6 equine bone marrow MSCs, and P9 equine bone marrow MSCs, found that there were no significant differences in promoting cytokine synthesis and expression of nterleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) in three different passage of bone marrow MSCs [40]; Mengbo Yang *et al*. demonstrated that P2 human umbilical cord MSCs and P8 umbilical cord MSCs had the same cell morphology and same expression of surface markers [41]; Mahnaz Babaahmadi *et al.* provided the evidences that bone marrow MSCs with long-term passages and the early passages of WJ-MSCs and BM-hMSCs had the similar therapeutic effects in collagen-induced arthritis rat [42], reflecting the same application advantages of long-term cultured MSCs and short-term cultured MSCs with a large amount of amplification.

Pluripotency ability of MSCs

Evidence demonstrated that MSCs could differentiate into three different germinal layers including neural cells [43], cardiomyocytes [44], kidney cells [45], and skin cells [46]; MSCs could also express the pluripotency markers NANOG, OCT-4, and SSEA-4; MSCs exhibit the excellent pluripotency ability. Aleksandra Musiał-Wysocka *et al*. isolated the MSCs from Wharton's jelly of the umbilical cord and confirmed that expression of NANOG, OCT-4, and SSEA-4 in Wharton's jelly of umbilical cord-derived MSCs was lower than in iPSCs, and hypoxia could improve the expression of NANOG, OCT-4, and SSEA-4 and increase its pluripotency [47]. Interestingly, the pluripotency ability of MSCs could be enhanced through various physical and chemical pathways. For example, Ana Borojević *et al*. reported that Vitamin D3 could increase the expression of pluripotency markers in human Bone Marrow MSCs through SIRT1 signaling [48]; Mekhemar *et al*. found that Thymoquinone from Nigella sativa could improve the expression of TLR3 and NANOG in Gingival MSCs [49]; Gitika Thakur *et al*. demonstrated that the special 3D culture could provide the cell microenvironments to enhance the expression of pluripotency markers in Wharton's jelly MSCs [50]. Therefore, MSCs exhibit enormous clinical application potential including neurological diseases and kidney diseases because of the pluripotent characteristics of MSCs.

Low immunogenicity of MSCs

Low immunogenicity is another important characteristic of MSCs for clinical application. It is reported that MSCs do not express major histocompatibility complex class II (MHC-II) molecules, MSCs could exhibit low immunogenicity in their immune characteristics and do not actively release their identity information when entering the host; and MSCs could evade the host's immune response through immune privilege during their participation in the immune process [51-53].

Paracrine effect of MSCs

MSCs could express, synthesize, and secrete many kinds of bioactive molecules including growth factors, cytokines, transcription factors, and signal peptides to regulate metabolism, cell differentiation, proliferation, migration, and apoptosis to balance the internal homeostasis of the body, the paracrine effect of MSCs plays the important role in tissue regeneration and organ repair [54, 55].

The preclinical application of MSCs in stroke

Due to the rapid development of stem cell technology, MSCs have been widely used and proven to alleviate and treat stroke in many kinds of animal models. The preclinical application of MSCs is mainly based on the different characteristics of MSCs including proliferation and pluripotency ability, low immunogenicity, and paracrine effect acting on animal models to explore their therapeutic effects and underlying mechanisms. For example, Fenjun Jiang *et al*. obtained a kind of neurotrophic factor-secreting MSCs from the bone marrow MSCs, found that this kind of neurotrophic factor-secreting MSCs could secrete highly level of neurotrophic factors including glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) compared with the bone marrow MSCs, and could reduce the infarct volume and increase the functional recovery in ischemic stroke rats [56]; Oh Young Bang *et al*. compared the effects of normal MSCs and CXCR4 overexpression of MSCs on rat stroke model, found that the migration of CXCR4 overexpression of MSCs was better than normal MSCs, and CXCR4 overexpression of MSCs could promote the behavioral recovery significantly compared with normal MSCs [57]; and Sanghun Lee *et al*. demonstrated that CCL2-overexpressing human umbilical cord MSCs could improve the functional recovery and decrease the stroke volume in middle cerebral arterial occlusion rats compared with normal human umbilical cord MSCs [58]. We summarized the recent preclinical application of MSCs in stroke in **Table 1**. In summary, cell resources mainly include humans, mice, and rats; stem cell type includes neural stem cells, adipose-derived MSCs, bone marrow MSCs, umbilical cord blood MSCs, and hair follicle stem cells; the main stroke modeling method is middle cerebral artery occlusion; the administration dosage focus on 1.0×10^5 cells, the details have been shown in **Table 2**.

Table 2. The recent preclinical application of MSCs in stroke

Cell resource	**Stem cell type**	**Model**	**Dosage**	**Administration method**	**Therapeutic effect**	**Mechanism**	**References**
Human	NSCs	Middle cerebral artery occlusion rat	5×10^5/20uL	Stereotactic injection	reduced brain tissue atrophy and memory functional loss	neuroprotective and pro-angiogenic paracrine activities by JAK2/STAT3	[59]
Mouse	ASCs	middle cerebral artery occlusion mice	2×10^6/20uL	intraperitoneal injection	infarct size was reduced, and neurological recovery was increased	EVs containing miR-25-3p regulate autophagy	[60]
Human	BMMSCs	distal middle cerebral artery occlusion mice	1×10^6	intraperitoneal injection	increased peri-infarct blood flow and vascular density and reduced infarct volume	upregulation of Rabep2	[61]
Mouse	NSCs	transient middle cerebral artery occlusion mice	/	intraperitoneal injection	reduction in neurological deficit along with reduced infarct area	Jak2/Stat3 pathway	[62]
Rat	BMMSCs	middle cerebral artery occlusion rat	5×10^6 cells/kg	intraperitoneal injection	reductions in infarct size and inhibition of microglial activation	upregulated neuron-glia antigen 2	[63]
Rat	MSCs	middle cerebral artery occlusion rat	/	intraperitoneal injection	improving the mitochondrial activity and functional recovery	mitochondria transfer	[64]

Human	BMMSCs	transient middle cerebral artery occlusion mice	1×10^6/50uL	intraperitoneal injection	improving functional recovery	activation of PI3K/Akt pathway	[65]
Human	NSCs	middle cerebral artery occlusion (MCAO) and oxygen-glucose deprivation rat	/	intraperitoneal injection	improving the spatial memory performance	promoting β-catenin nuclear translocation	[66]
Mouse	MSCs	permanent focal cerebral ischemia mice	5×10^5	intraperitoneal injections	improving functional recovery	gap junction-mediated cell-cell interaction	[67]
Human	BMMSCs	1 hour middle cerebral artery occlusion rat	3×10^5 cell/9μL	intraperitoneal injections	brain-to-periphery migration	lymphatic and inflammation pathways	[68]
Mouse	NSCs	middle cerebral artery occlusion mouse	1×10^5cell /μL	intraperitoneal injection	neurological function improvement	inhibited p53-mediated Proapoptotic Pathway	[69]
Mouse	NSCs	injecting endothelin in the right pons	0.5×10^6 /μL	intraperitoneal injection	improving neurological function	overexpressed BDNF and Dlx2	[70]
Human	UCBMSCs	Middle cerebral Artery Occlusion rat	5×10^5	intraperitoneal injection	improve neuroprotection, decrease inflammation, and increase angiogenesis	release cytokines and decrease inflammation	[71]
Human	UCBMSCs	Middle cerebral Artery Occlusion rat	2.5×10^5	intraperitoneal injection	improved the recovery of sensory and motor function	/	[72]
Mouse	BMMSCs	bilateral common carotid artery occlusion mouse	2×10^6	intraperitoneal injection	improved neuroprotective property	attenuating the host cell response	[73]
Rat	HFSCs	Middle cerebral Artery Occlusion rat	1×10^6 cells/mL	intraperitoneal injection	reduced the infarct volume and promoted neurological recovery	/	[74]

Abbreviations: MSCs: Mesenchymal stem cells; NSCs: Neural stem cells; ASCs : Adipose-derived MSCs; BMMSCs: Bone marrow mesenchymal stem cells; UCB-MSCs: Umbilical cord blood mesenchymal stem cells; HFSCs: Hair follicle stem cells.

The clinical application of MSCs in stroke

It is essential to conduct systematic studies on patients or healthy volunteers to confirm the efficacy and safety of the investigational drug for final validation. Similarly, to verify the therapeutic effect of MSCs in stroke, numerous clinical trials have been conducted around the world in the past ten years. The results demonstrated that there were 16 clinic trials (phase I stage or phase II stage) of MSCs application in stroke had been reported from 2014-2023; stem cell type mainly included neural stem cells (5 clinic trials), bone marrow MSCs (4 clinic trials) and adipose MSCs (3 clinic trials); the dosage of MSCs were variant from 0.5×10^6 to 3.0×10^8 cells; intraperitoneal injection was the main administration; injection period was from 3 months to 30 months; there were 6 clinic trials reported that intraperitoneal injection of MSCs had the adverse effect in patients, the ratio was 37.5% (6/16); the main countries for conducting clinical trials were China (5 clinic trials), Japan (2 clinic trials) and Spain (2 clinic trials). The details are summarized in **Table 3**.

Table 3. The clinical application of MSCs in stroke from 2014-2023

Clinic trial type	Stem cell type	Stroke type	Dosage	Administration method	Period	Adverse effect	Country	References
open-label intervention study	BMMSCs	perinatal arterial ischaemic stroke	45-50×10^6	intraperitoneal injection	3 months	a mild transient fever of 38°C without the need for clinical intervention	Netherlands	[75]
prospective randomized controlled trial	MSCs	chronic major stroke	5×10^6 cells/mL	infused via the antecubital vein	3 months	unknown	South Korea	[76]
a prospective, multicentre, single-arm, open-label study	NSCs	ischaemic stroke	2×10^6 cells	stereotaxic injection	6-12 months	no cell-related adverse events	United Kingdom	[77]
single-center, open-label, randomized controlled trials	BMMSCs	moderate-severe ischemic carotid stroke	unknown	intravenous injection	6-24 months	yes	France	[78]
randomized, double-blind, placebo-controlled, multicentre, phase 1/2 clinical trial	DPSCs s	acute ischaemic stroke	1-3×10^8	intravenous administration	30 months	unknown	Japan	[79]
Phase I/II Study	BMMSCs	chronic stroke	3.6-12.4×10^6 cells/kg	Intravenous transfusion	12 months	15 serious adverse events including infections, vascular disorders, and pain syndromes	USA	[80]
single-centre, randomized, double-blinded, parallel-controlled trial	NSCs	ischaemic stroke	2.5×10^6 cells/100uL	intranasal administration	12 months	unknown	China	[81]
a phase IIb, multicentre, randomized, double-blind, placebo-controlled clinical trial	ASCs	ischaemic stroke	1.0×10^6 cells/mL	intranasal administration	24 months	unknown	Spain	[82]
phase I open-label clinical trial	ASCs	chronic ischemic stroke	1×10^8	intracerebral transplantation	6 months	no adverse events	China	[83]
an open-label, single-site, dose-escalation trial	NSCs	chronic ischaemic stroke	2.0-20×10^6 cells	stereotactic ipsilateral putamen injection	29 months	no adverse events	USA	[84]
a Phase I/II randomized, placebo-controlled trial	allogeneic MSCs	Chronic Stroke	0.5-1.5×10^6 cells/kg	intracerebral transplantation	12 months	15 serious adverse events	Canada	[85]
a single-site, phase I trial	NSCs	ischemic stroke	1.2-7.2×10^7 cells	intracerebral microinjection	24 months	unknown	China	[86]

a Phase I/II randomized, placebo-controlled trial	NSCs, MSCs	ischemic stroke	0.5-6.0×10^6 cells/kg	intravenous injection	24 months	low fever (<38.5°C)	China	[87]
a Phase II single-arm, open-label trial	autologous MSCs	stroke	0.5-2.0×10^8 cells	intravenous injection	6 months	unknown	Japan	[88]
A phase II, randomized, double-blind, placebo-controlled, single-center, pilot clinical trial	ASCs	acute ischemic stroke	1.0×10^6 cells/kg	intravenous injection	24 months	1 case of serious adverse event was found	Spain	[89]
a randomized controlled observer-blinded trial	bone marrow MSCs	severe ischemic stroke	1.0×10^6 cells/kg	intravenous injection	12 months	unknown	China	[90]

Abbreviations: MSCs: Mesenchymal stem cells; NSCs: Neural stem cells; ASCs : Adipose-derived MSCs; BMMSCs: Bone marrow mesenchymal stem cells; UCB-MSCs: Umbilical cord blood mesenchymal stem cells; HFSCs: Hair follicle stem cells; dental pulp stem cells: DPSCs.

Conclusion and perspectives

Stem cell therapy is becoming one of the most promising treatment methods for difficult diseases including stroke because of its convenient preparation of MSCs, excellent differentiation, renewal, and repair capabilities, and low immunogenicity. While stem cell technology is becoming increasingly mature and has good development prospects, there are still many urgent problems that need to be solved for applications of MSCs. (1) Heterogeneity of MSCs. The main manifestation is heterogeneous cell populations with unclear definitions, varying sizes and shapes, and different epigenetic imprints of cells from different tissue sources [91, 92]; how to obtain a homogeneous population of MSCs with the same epigenetic imprints is an urgent issue to be solved. Isolation and seek for suitable subpopulations of MSCs seems to be an ideal method. For example, Hongwei Chen *et al*. identified the BAMBI^{high}MFGE8^{high} C1 subgroup by scRNA-seq technology, this kind of special umbilical cord MSCs had a unique phenotype and distinct transcriptomic profile, which exhibited excellent clinic application perspectives [93]; and Penghong Chen *et al*. identified four different types of subpopulations of Wharton's jelly MSCs including proliferative Wharton's jelly MSCs, niche-supporting Wharton's jelly MSCs, metabolism-related Wharton's jelly MSCs and biofunctional-type Wharton's jelly MSCs by single cell and spatial transcriptome sequencing technology; Subsequently, they isolated a special S100A9$^+$CD29$^+$ CD142$^+$ subpopulation from biofunctional-type MSCs, found that this kind of S100A9$^+$CD29$^+$CD142$^+$subpopulation had the better effect on wound healing that traditional Wharton's jelly MSCs [94]. This evidence may provide a new solution method for the heterogeneity of MSCs. (2) The standardization of methods for stem cell isolation, amplification, and differentiation. There are many kinds of MSCs including bone marrow MSCs, umbilical cord MSCs, adipose MSCs, and hematopoietic stem cells, and has been reported, that there were many kinds of isolation methods and culture methods for MSCs, the inconsistency and non-standardization of isolation methods and culture methods resulted in the huge barriers of MSCs applications. Establishing standardized quality control standards for MSCs may be one of the ways to solve the problem. (3) further verification for the safety and effectiveness of MSC therapy is still needed. At present, the application of MSCs is mostly limited to animal experiments, and there is a huge gap between animal experiments and clinical trials. How to obtain more reliable clinical trial data to support the MSCs therapy is also a big barrier. (4) Establishment and implementation of MSCs policies. The policies related to MSCs directly determine whether MSCs can enter the clinical stage from animal experiments to marketing approval, improving relevant regulations can provide a basis for the clinical application of MSCs and have a targeted approach.

Anyway, with the rapid development of MSC technology and solving the above problems step by step, MSC applications in stroke will make breakthrough progress in the future.

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