Stem Cells International

Current Status and Perspectives of Human Mesenchymal Stem Cell Therapy 2021

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Research Article

Adipose and Bone Marrow Derived-Mesenchymal Stromal Cells Express Similar Tenogenic Expression Levels when Subjected to Mechanical Uniaxial Stretching *In Vitro*

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The present study was conducted to determine whether adipose derived mesenchymal stromal cells (AD-MSCs) or bone marrow derived-MSCs (BM-MSCs) would provide superior tenogenic expressions when subjected to cyclical tensile loading. The results for this would indicate the best choice of MSCs source to be used for cell-based tendon repair strategies. Both AD-MSCs and BM-MSCs were obtained from ten adult donors ($N = 10$) and cultured *in vitro*. At passaged-2, cells from both groups were subjected to cyclical stretching at 1 Hz and 8% of strain. Cellular morphology, orientation, proliferation rate, protein, and gene expression levels were compared at 0, 24, and 48 hours of stretching. In both groups, mechanical stretching results in similar morphological changes, and the redirection of cell alignment is perpendicular to the direction of stretching. Loading at 8% strain did not significantly increase proliferation rates but caused an increase in total collagen expression and tenogenic gene expression levels. In both groups, these levels demonstrated no significant differences suggesting that in a similar loading environment, both cell types possess similar tenogenic potential. In conclusion, AD-MSCs and BM-MSCs both demonstrate similar tenogenic phenotypic and gene expression levels when subjected to cyclic tensile loading at 1 Hz and 8% strain, thus, suggesting that the use of either cell source may be suitable for tendon repair.

1. Introduction

Mechanical forces are known to play a fundamental role in cell behaviour and their adaptation to their environment. These are particularly essential to maintain tissue homeostasis [1]. In tendons, it has been demonstrated that stretching produces superior repair outcomes [2–4], whereas disuse atrophy and tissue degeneration will occur when these tissues are not mechanically loaded for a long period of time [5, 6]. These have shown to result in the complete loss of tissue function [7]. It has become apparent that mechanical cues play a vital role in cellular signalling, through a series of molecular interactions, that results in genetic and protein expressions that maintain cellular function [8, 9]. Using this knowledge, a number of studies have attempted to exploit this process, known as mechanotransduction, to manipulate cell fate and outcomes [10–14]. One such attempt has included the use of mechanical stimuli in regulating the cellular differentiation of multipotent progenitor cells. The use of human mesenchymal stromal cells (MSCs) has garnered strong interest due to its ability to undergo self-renewable and multilineage differentiation. As such, MSCs have been sought after as a potential cell source to improve tissue repair and regeneration since these cells also exhibit proheal-ing and immunomodulatory effects [15–17]. Indeed, one method by which these cells can promote tissue repair is the differentiation of these cells that are implanted into damaged sites, as proposed by other researchers [18, 19]. Once MSCs are nested within the matrix of the target site, these cells will differentiate towards the native resident cells

thereby producing the necessary protein that would promote local tissue healing [20, 21]. This process can be regulated through several pathways such as local cytokine signalling, cell-surface interaction, and local tissue mechanical impulses [22]. From our own experience, intrainjection of autologous MSCs in joints with damaged cartilage results in moderate to good repair outcomes due to the presence of these cells within the damaged sites [23]. There has been a study indicating that 24% of injected MSCs were retained at the site of injury after 24 hours [24], and that these cells may undergo differentiation over time. The *in vivo* differentiation could be further enhanced when mechanical loading is then performed in the form of exercise regime [25]. In an attempt to further understand the underlying mechanisms that promote cellular differentiation, cells seeded in scaffolds and were subjected to mechanical loading *in vitro* [26], and the study shows that the loaded construct demonstrates differentiation expression, thus suggesting that mechanical loading may be the main contributor to the cell differentiation process.

In our previous studies, we were also able to demonstrate that by subjecting MSCs to cyclical stretching, these cells would undergo cellular differentiation and produce superior tendon protein expression [27, 28]. Such changes have also been observed in chondrocytes subjected to compressive loading in other studies [29, 30]. Together with our previous observation of cellular differentiation in damaged sites when cells are injected into these areas, we can therefore suggest that the mechanotransduction process that occurs in the transplanted MSCs may have been responsible for the observed positive outcomes [31]. Whilst the direct evidence of the direct role of mechanotransduction remains debatable, what cannot be refuted is the fact that MSCs do provide a significant positive musculoskeletal tissue repair outcome.

It is previously demonstrated that bone marrow has been a traditional source for MSCs harvest since bone marrow derived mesenchymal stromal cells (BM-MSCs) have demonstrated predictable results in musculoskeletal tissue engineering. There is a drawback in using BM-MSCs mainly due to invasiveness of the procedure involved in extracting these cells. It has been reported regularly that extraction from bone marrow results in minor donor site morbidity [32]. This, in addition to the low yield of cells from the bone marrow stroma [33], results in many studies describing alternative sources for MSCs. One such source is from adipose tissues. Adipose tissue derived mesenchymal stromal cells (AD-MSCs) have been shown to contain an abundance of MSCs, and since subcutaneous fat deposits are in large quantity in the human body, extracting these cells are easier and less painful for the donor [34, 35]. Despite its many promises, the potential use of AD-MSCs for the repair of damaged tendon does not appear to be explored with presently no previous works describing its potential when subjected to mechanical stimulation, i.e., stretching. Furthermore, while there has been some progress made in better understanding on how mechanical signals are sensed by MSCs [36, 37], the mechanotransduction processes that occur during tensile loading have not been well described.

Therefore, to demonstrate the potential efficacy of AD-MSCs as a source for tendon regeneration, a study was conducted to determine the tenogenic expression potential of these cells when subjected to cyclic tensile loading. This will be compared to BM-MSCs, which is presently being used as a therapeutic cell source for damaged tendons. It is hoped that the study will be able to demonstrate as to whether AD-MSCs can indeed be considered as a good candidate for tendon repair, and that further studies to develop this cell source should be conducted.

2. Materials and Methods

2.1. Bone Marrow and Adipose Tissue Harvesting from Patients. Five grams of adipose tissue sample and 5 cc of bone marrow were collected each from donors ($N = 10$; for MSCs isolation purpose) (mean age = 65.3; 6 females and 4 males) undergoing orthopaedic-related surgeries in University Malaya Medical Center with approval from the Medical Ethics Committee of University Malaya Medical Center (reference number: 20149-563). These samples were kept on ice throughout the transportation to the laboratory and processed for MSCs isolation within few hours after samples harvesting. From the 10 donors, 4 of the donors were used for quantitative experiments including cell proliferation assessment, total collagen assay, and gene expression assay, while the other 6 donors were used for qualitative/semiqualitative experiments including morphology assessment and MSCs characterization.

2.2. Isolation and Culture of Human Adipose Derived-MSCs (AD-MSCs). To isolate AD-MSCs, the harvested adipose tissue sample was rinsed using 1X phosphate-buffered (PBS) saline containing 1% penicillin-streptomycin until all visible blood and excessive fluid were eliminated. Small vessels and unwanted tissues were dissected away from the sample. Subsequently, the mixture was added with 0.1% (v/w) type I collagenase and incubated at 37°C for 1 h to allow the enzymatic digestion process to occur. After centrifugation, the pellet at the bottom of the tube containing the stromal vascular fraction (SVF) was collected (Figure 1) and transferred a cell culture flask containing complete cell growth medium (low-glucose DMEM supplemented with 10% fetal bovine serum, 1% of penicillin-streptomycin, and 1% GlutaMAX™-I) (Invitrogen-Gibco, USA) and incubated at 37°C in a humidified 5% CO_2 incubator. After 4 days of culturing, the digested tissue was then removed from the cell culture flask and discarded completely. Culture medium was changed every 3 days until reaching 80-85% of cell confluency. The AD-MSCs were subcultured up to passage 2 to be used in our experiments.

2.3. Isolation and Culture of Human Bone Marrow-Derived-MSCs (BM-MSCs). To isolate BM-MSCs, cell isolation was performed using our standard laboratory protocol as described in our previous publication [38, 39]. Briefly, bone marrow specimen was diluted with 1X PBS and gently layered onto the top of the density of 1.077 g/mL Ficoll-paque solution (Amersham Biosciences, Sweden). The

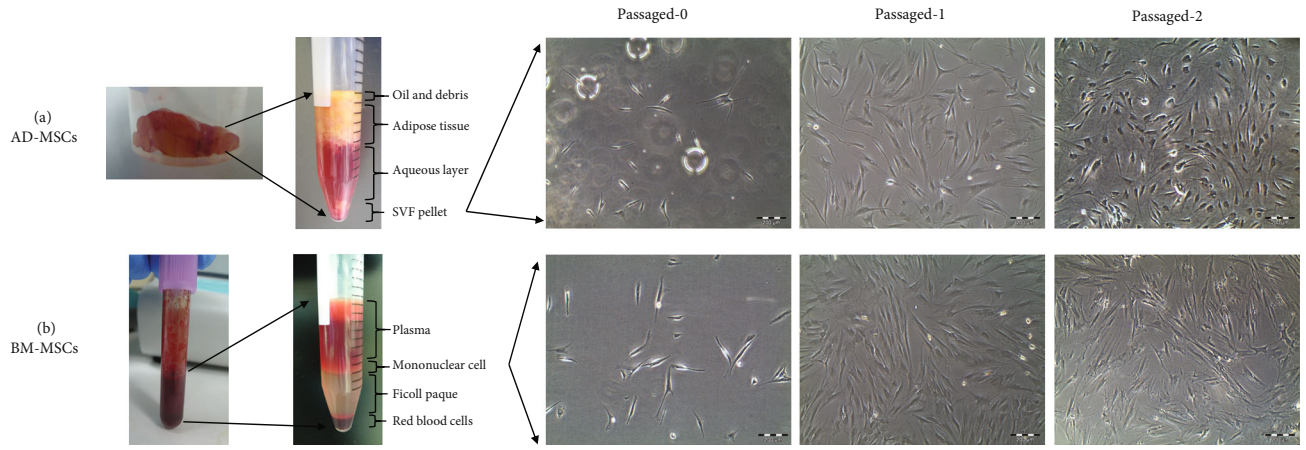

FIGURE 1: Morphology of isolated human AD-MSCs and BM-MSCs from adipose fat pad and bone marrow, respectively. (a) Enzymatic digestion of adipose tissue using collagenase type I, and the MSCs population isolated from the SVF. (b) MSCs isolated from bone marrow and the mononuclear cells isolated by Ficoll density centrifugation. At primary culture passage-0 (Day 6), fibroblastic as well as small clear cells can be observed. The number of clear cells was reduced during the passages and fibroblastic cells appeared to be the dominant cell type.

mononuclear cell layer was collected after undergoing gradient density centrifugation at 2,200 rpm for 25 min (Figure 1). The cell pellet was then extracted after second centrifugation and plated on a tissue culture flask containing complete cell growth medium. The cells were maintained in a humidified incubator at 37°C with 5% CO_2 on air. The subsequent medium changes were conducted at 3 day-intervals until 80%-85% confluence was reached. Cells were serially passaged until passage-2 prior to further experiments.

2.4. Characterization of Human AD-MSCs and BM-MSCs. To determine whether cells obtained were hMSCs, various tests including flow cytometry analysis for specific cell surface markers, cell morphological images, and the ability of the isolated cells to undergo trilineage differentiation were conducted (cells from 6 donors).

2.4.1. Cellular Morphology. Serial microscopic examinations were carried out at 3-day intervals in order to assess physical characteristics of the cells. The cellular morphology of cultured AD-MSCs and BM-MSCs at passage 0 (P0), passage 1 (P1), and passage 2 (P2) were captured using an inverted phase contrast microscope (Olympus CKX 41, Japan).

2.4.2. In Vitro Trilineage Differentiation. The multipotent capability of AD-MSCs and BM-MSCs were tested using specific StemPro® differentiation supplements (Invitrogen-Gibco, USA), inducing the cells into adipogenic, osteogenic, and chondrogenic lineages, with triplicates for each lineage, as described in our previous established protocol [28, 39]. The differentiation of these cells was confirmed through their phenotypic expression. The confluent passaged-3 cells were cultured with differentiation medium, respectively. The differentiation medium was changed every 3 days.

Briefly, for the adipogenic differentiation, 14 days after the culture initiation the cells were fixed with 4% paraformaldehyde for 30 min, rinsed with 60% isopropanol, and stained with Oil Red O (Sigma-Aldrich, USA) for 10 min. The slides were kept wet to keep the lipid vacuoles from disrupting. The slides were viewed and captured using light microscope (Nikon Eclipse TE2000-S, Japan). For osteogenic differentiation, 21 days after culture initiation, the filtered 2% Alizarin Red solution (pH 4.2) was added to the fixed cells for 3 min. Alizarin red staining was used to observe the matrix mineralization. To induce chondrogenic differentiation, pellet culture system was used. Twenty-eight days after the initiation of the culture, each chondrogenic cell pellet (1×10^6 passaged-3 cells) was fixed in 10% neutral buffered formalin for 1 hour and went through tissue processing (dehydrating in ascending concentrations of ethanol and clearing in xylene) overnight. The sample was then embedded in paraffin wax and sectioning at 4 μm using a microtome. The sections were then stained with 0.1% aqueous Safranin O for 5 min.

2.4.3. Evaluation of Cell Surface Markers by Flow Cytometry. Human AD-MSCs and BM-MSCs (1×10^6 cells/mL) were trypsinized, and the cells were washed with 1X PBS and resuspended in 100 μL of FASC stain buffer (BD Biosciences, CA, USA) before being transferred into polystyrene round-bottomed tube. Fluorescein isothiocyanate (FITC), or phycoerythrin (PE), or peridinin chlorophyll protein (PerCPCY5.5), or allophycocyanin-(APC-) conjugated anti-marker mAbs were used to stain the cells for 15 min in the dark. The tested markers including CD44, CD73, CD90, CD105, CD14, CD34, CD45, and HLA-DR were tested [38, 39]. After incubation, the cells were washed and then analysed using a flow cytometer (BD FACS Cantor II, BD Biosciences, CA, USA) with FACS DIVA software (BD, NJ, USA). Unstained and/or matched isotype controls were used to set background fluorescence levels.

2.5. Cell Seeding and Mechanical Straining System Set up. A total of 0.02% collagen type I (Sigma, St. Louis, USA) was used to coat the autoclaved transparent elastic silicone chambers (Strex, Japan). A total of 1×10^4 cells/cm^2 AD-

MSCs and BM-MSCs were seeded into each silicone chamber, respectively. After 48 h of culture, the medium was replaced with medium containing 1% FBS for 24 h. This step was conducted in order to synchronize the condition (by arresting the cells at the G0/G1 stage of their cell cycle progression) at the beginning of each experiment. Following 24 h of synchronization, the cell culture medium with a standard growth medium containing 10% FBS with no additional differentiation growth factors was replaced prior to mechanical stretching. The silicone chambers were mounted on a mechanical stretch device (ST-140-10, Strex, Cupertino, USA). A stretching rate of 1 Hz and a strain of 8% were applied to the AD-MSCs and BM-MSC seeded silicone flasks. The specimens were collected at 24 h and 48 h. Unstrained cells on silicone chambers in the same culture environment were used as control for this study.

2.6. Microscopic Evaluation. Microscopic images of the experimental cells at each time point were captured using an inverted tissue culture CCD camera-assisted microscope (Olympus CKX 41, Japan). Images from four visual fields of the cells were randomly captured. The morphology and alignment of the unstrained and strained cells on elastomeric substrate were then compared.

2.7. Cell Proliferation Assay. The alamarBlue® (AB) assay was used to assess cell proliferation. This assay utilizes the colorimetric quantitative analytical principle. At 0, 24, and 48 h, 10% AB reagent was added to the unstrained and strained cells in the culture medium. The samples were then incubated for 4 h at 37°C in a cell culture incubator, protected from direct light exposure. A total of 100 μL of the alamar-containing medium was collected and transferred to a 96-wells plate. The absorbance measurement was read on a microtiter plate reader at 570 nm wavelength while using 600 nm as a reference wavelength. Following the manufacturer's protocol, the percentage of AB reduction was calculated. For background values, medium without cells was used as the negative control group to correct the values of % AB reduction.

2.8. Total Collagen Biochemical Assay. A Sircol™ collagen assay kit (Biocolor, UK) was used to measure total extracellular soluble collagen. At 24 and 48 h of the experiment, the culture medium was collected from the experimental cells and mixed with 1 mL of Sircol dye reagent with vigorous agitation for 30 min. The mixtures were then centrifuged at 12,000 rpm for 10 min to collect the collagen dye complex. The unbound dye solutions were removed by draining the tubes carefully and washed the dye with ice-cold acid-salt wash reagent by centrifugation at 12,000 rpm for 10 min. The dye (which was bounded to the collagen pellet) was solubilized by adding 1 mL of alkali reagent. The absorbance of the samples was measured at 555 nm wavelength.

2.9. Gene Expression Assay. Total RNA from unstrained and strained cells was extracted using the RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. RNA concentration and purity were determined using spectrophotometer (Nano-Photometer, Germany) at the setting of A260/280. RNA integrity was verified by visualizing 18S and 28S rRNA bands on formaldehyde-agarose gels. Only samples with good quality were selected for RT^2 Profiler PCR arrays downstream analysis. An equal amount of RNA (500 ng) was used for reverse transcription using the RT^2 First Strand Kit (Qiagen, USA) using protocol steps that eliminated genomic DNA. qPCR experiments were performed using the customised RT^2 Profiler PCR array (Cat No./ID: CLAH22023) (SABioscience, USA) and RT^2 SYBR Green qPCR Mastermix (Qiagen, USA) on a real-time PCR instrument (CFX96, BioRad, USA). The temperature protocol included a start cycle for 10 min at 95°C, 40 cycles of amplification (15 s at 95°C and 15 s at 60°C), followed by a melt curve. The PCR array profiles the expression of selected nine genes (Table 1) involved in mesenchyme lineage. The housekeeping gene were *PGK1* (phosphoglycerate kinase 1) and *HPRT1* (hypoxanthine phosphoribosyltransferase 1). The housekeeping genes, RT controls, and PCR controls were included in each run. Relative expression of target genes was determined using the $\Delta\Delta$Cq method where the unstrained cell is the control group.

2.10. Statistical Analysis. The assays (cell proliferation, total collagen biochemical, and gene expression) were carried out in technical triplicates (n) per experimental run, using four independent samples from different donors (N) for each group. The data is presented as mean ± standard deviation (SD). Student's t-test was carried out to compare the differences in mean values. Statistical analyses were performed using SPSS software version 15.0 (SPSS Inc, USA), which took a probability value of $p < 0.05$ as statistically significant.

3. Results

3.1. Characterization of Human AD-MSCs and BM-MSCs. Results revealed that the morphology, surface antigen expression profiles, and the multidifferentiation capacity of human AD-MSCs and BM-MSCs were similar and conformed to the International Society for Cellular Therapy (ISCT) minimal characteristics of MSCs [40].

3.1.1. Plastic-Adherent Fibroblast-like Cells. After going through the culturing process in standard growth medium at 37°C incubation for 24 h, a proportion of the isolated cells from both adipose tissue and bone marrow demonstrated adherence to the plastic flask surface. Following medium change after 5 days of culture, they aggregated to form colony-forming-units. Rounded cells were observed to change into fibroblast-like morphology where the cells appeared spindle-shaped. Their appearance, however, varied, with heterogeneous shapes were observed, with features of elongated cells and multipolar projections. After 2 weeks in culture, the cells started reaching confluency and demonstrated fingerprint-like orientation. The cells appeared more homogeneous after cell passaging. Both AD-MSCs and BM-MSCs at passage-2 exhibited spindle-shaped morphology. However, AD-MSCs apparently grew at a relatively higher rapid rate compared with BM-MSCs, which could be observed under light microscopy (Figure 1).

Table 1: The genes of interest were determined in this study.

Related marker	Gene name	Abbreviation	Ref sequence	Catalog number
Tendon lineage	Collagen type I, α1	*COL1A1*	NM_000088	PPH01299F
	Collagen type III, α1	*COL3A1*	NM_000090	PPH00439F
	Decorin	*DCN*	NM_001920	PPH01900A
	Tenascin C	*TNC*	NM_002160	PPH02442A
	Biglycan	*BGN*	NM_001711	PPH01899A
Other mesenchyme lineage	Runt-related transcription factor 2	*RUNX2*	NM_001015051	PPH01897C
	SRY-(sex determining region Y-) box 9	*SOX9*	NM_000346	PPH02125A
	Peroxisome proliferative activated receptor, gamma	*PPARG*	NM_005037	PPH02291G
	Transgelin	*TAGLN*	NM_001001522	PPH19531F
Housekeeping gene	Phosphoglycerate kinase 1	*PGK1*	NM_000291	PPH02049A
	Hypoxanthine phosphoribosyltransferase 1	*HPRT1*	NM_000194	PPH01018C

3.1.2. In Vitro Trilineage Differentiation. Both AD-MSCs and BM-MSCs showed positive results for the differentiation experiments (Figure 2). This indicated that the MSCs isolated from the two cell types had multipotent ability, having the capacity to undergo trilineage differentiation which included osteogenic, adipogenic, and chondrogenic mesodermal lineages.

In adipogenic culture conditions, small lipid droplets appeared in both cell types on day 3, and they gradually spread homogenously. Not surprisingly, AD-MSCs displayed highly adipogenic cells with abundant Oil Red O positive lipid droplets as compared to BM-MSCs. This indicated that adipocyte formation was more extensive in AD-MSCs. Lipid vacuoles were not present in control cultures of either AD-MSCs or BM-MSCs. In osteogenic culture, nodule-like structures were observed in certain regions. By using Alizarin Red S, both cell types appeared red showing the presence of mineral deposition. In comparison, no accumulation of calcium oxalate crystals was observed in noninduced control MSC cultures that were stained. In the pellet culture system for chondrogenic differentiation, the size of the pelleted cells, i.e., AD-MSCs and BM-MSCs seemed to be increasing over time. Using Safranin O, the matrix of the both cell types demonstrated a pink-red colour, indicating sulphated proteoglycans or glycosaminoglycan deposition.

3.1.3. Immunophenotype Expression. Cells derived from adipose tissue and bone marrow expressed positive surface markers for CD73, CD44, CD90, and CD105 (Figure 3), which are markers for hMSCs (Table 2), particularly highly expressed were CD44 and CD90. They were negative for CD14, CD34, CD45, and HLA-DR marker expression. This indicated that the cells were not of hematopoietic or leucocytic in origin.

3.2. Effects of Cyclic Mechanical Stretch on Cell Morphology and Alignment. To determine the effects of mechanical stretch on cell morphology and organization, uniaxial cyclical tensile loading at 1 Hz of frequency and 8% of strain was applied on AD-MSCs and BM-MSCs. Mechanical stretch markedly altered the morphology and alignment of cells. The MSCs were randomly oriented before mechanical stimuli was applied (0 h), while both the AD-MSCs and BM-MSCs appeared to be perpendicular orientated to the direction of stretching after exposure to cyclic stretching. It also appears that the changes correspond to the duration of stretching (Figure 4). This may be due to the adaptation process of the cells, where cellular tensegrity tends to minimise the shape in order to reduce stresses in response to mechanical forces. In contrast, both unstrained AD-MSCs and BM-MSCs groups showed no specific cellular orientation, similar with 0 h group. The number of cells in all strained and unstrained groups appeared to increase with time. The increase in the number of cells was seen clearly especially for unstrained cells at 48 h group compared to the 0 h group. However, changes of cell number appeared small in strained cells compared to unstrained cells. Cells when strained exhibited a different morphology than unstrained cells, where the strained cells appeared elongated. There was no difference in morphology between the AD-MSCs and BM-MSCs strained group, where both cell types demonstrated similar appearance of spindle-shaped cells.

3.3. Effects of Cyclic Mechanical Stretch on Cellular Proliferation. The cellular proliferation rate of AD-MSCs and BM-MSCs was compared using alamarBlue absorbance reduction. Overall, alamarBlue® assay revealed that both unstrained and strained threads support the growth of human MSCs. Figure 5 shows that in unstrained conditions, AD-MSCs have higher cell proliferation compared with BM-MSCs, with statistically significant difference observed at 0 h and 24 h. Both cell types demonstrated gradual proliferation over time. However, when the MSCs were subjected to stretching, there was no difference between AD-MSCs and BM-MSCs either at the 24 h or 48 h time points. Mechanical stretching at 1 Hz and 8% demonstrated not to enhance cell proliferation in either cell group especially in AD-MSCs. In contrast, BM-MSCs were enhanced by mechanical stimulation after 24 hours of stretching, although it was not statistically significant. Nevertheless, a trend towards a decrease in cell proliferation was observed after 48 hours of stretching.

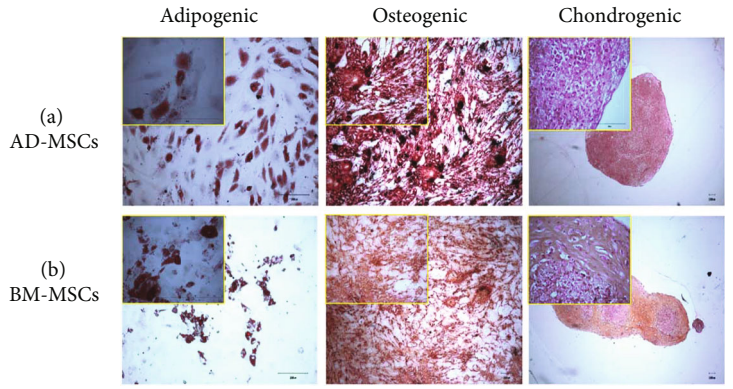

Figure 2: Trilineage differentiation potential of primary for (a) AD-MSCs and (b) BM-MSCs in adipogenic differentiation, osteogenic differentiation, and chondrogenic differentiation.

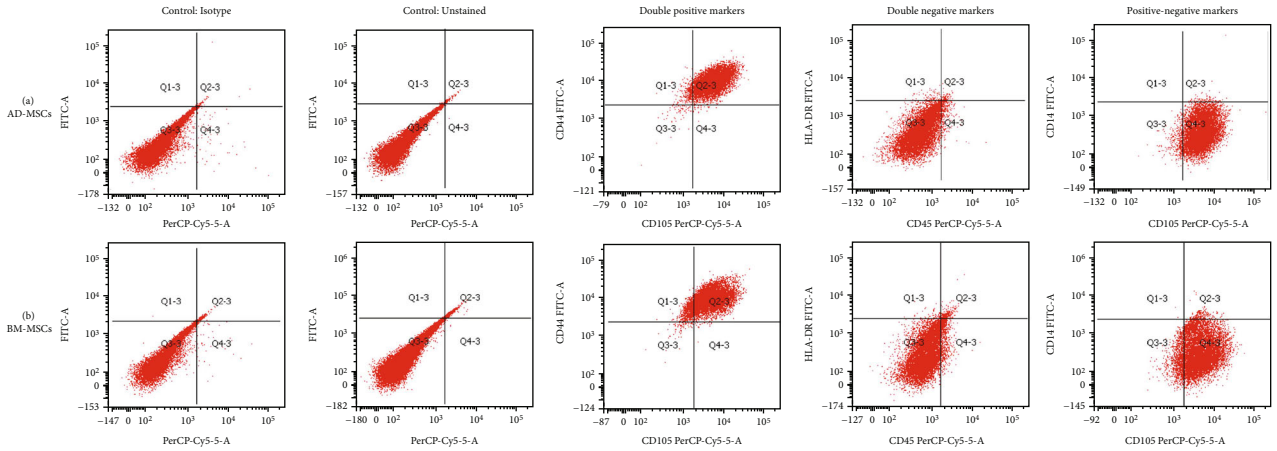

Figure 3: Immunophenotypic characterization of the surface of (a) AD-MSCs and (b) BM-MSCs using flow cytometry. The representative images showed the both type of MSCs expressed at least 85% of double-positive expression, double-negative, or coexpressed its positive and negative markers.

Table 2: Flow-cytometric analysis of expanded passaged-2 AD-MSCs and BM-MSCs.

Surface protein	% positive AD-MSCs	% positive BM-MSCs
Positive hMSCs markers		
CD73	92.5	94.8
CD44	99.1	98.8
CD90	100.0	100.0
CD105	96.9	97.0
Negative hMSCs markers		
CD14	0.3	1.2
CD34	5.5	2.2
CD45	0.3	0.7
HLA-DR	1.2	2.4

These results suggest that the proliferation rate of human MSCs is not influenced by stretching at 1 Hz and 8%, regardless whether MSCs derived from adipose tissue or bone marrow.

3.4. Effects of Mechanical Stretch on Total Collagen Expression of MSCs. The results showed that uniaxial stretching increased collagen production in cell cultures (Figure 6). At 1 Hz and 8% strain, when compared with unstrained groups with a normalized value 1, an increase in total collagen was noted in both AD-MSCs and BM-MSCs at both 24 h and 48 h. However, the increase of the collagen production was only statistically significant ($p < 0.05$) at 48 h. In terms of comparison between the AD-MSC and BM-MSC group, the BM-MSC group produced more collagen than the AD-MSC group at both time points, but only significantly different at 48 h.

3.5. Effects of Cyclic Mechanical Stretching on Mesenchyme Differentiation of MSCs. To investigate the regulatory genes during the tenogenic process when cells are subjected to cyclic tensile loading, the mRNA level of *COL1*, *COL3*, *DCN*, *TNC*, and *BGN* were determined. Both AD-MSCs and BM-MSCs (Figure 7) were triggered by mechanical simulation towards tenogenic lineage, but not to other mesenchyme lineages including osteogenic (*RUNX2*), chondrogenic (*SOX9*), adipogenic (*PPARG*), and smooth

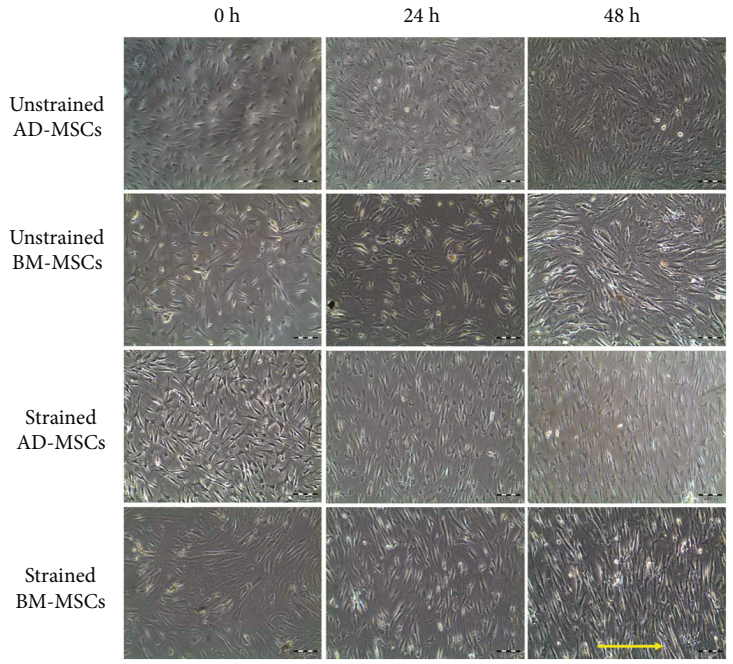

Figure 4: Effects of cyclic tensile loading on the morphology and orientation of both AD-MSCs and BM-MSCs. The strained cells presented an orientation perpendicular to the strain axis. The substrate was stretched in the direction of the yellow arrow.

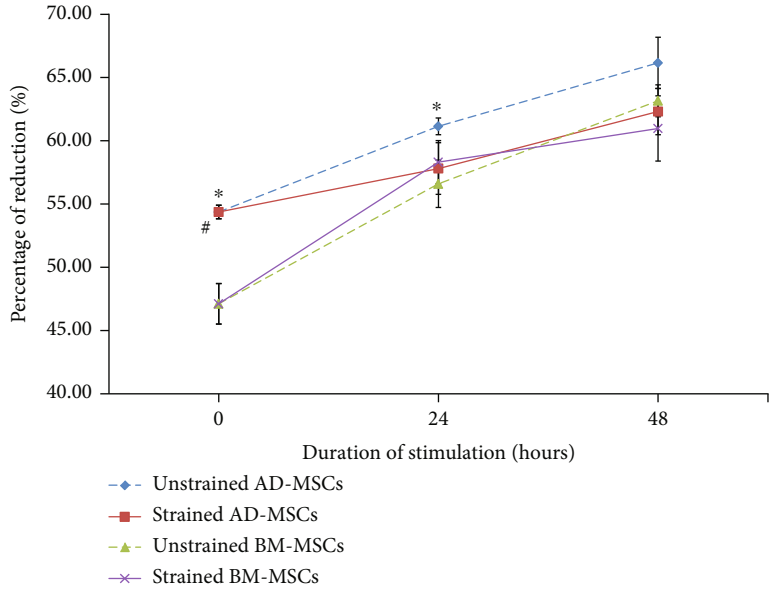

Figure 5: Comparison between the cellular proliferation of AD-MSCs and BM-MSCs with or without mechanical stimulation at different durations of cell culture. The cell proliferation rate higher in AD-MSCs as compared to BM-MSCs when left to grow on silicone chambers. This did not appear to be the case when cells were subjected to cyclical stretching at 8% and 1 Hz. There was no significant difference for both these types of cells and when compared to unstrained cells when mechanical stimulation was applied. Significance ($p < 0.05$) was indicated with an asterisk (∗) which compared unstrained AD-MSCs and unstrained BM-MSCs in different duration, while significance ($p < 0.05$) was indicated with a hash (#) which comparison between strained AD-MSCs and strained BM-MSCs in different duration. $N = 4$, $n = 3$. Error bar = ±SD.

muscle (*TAGLN*). Instead, our investigation demonstrated a downregulation of expression levels. Uniaxial strain regulated matrix remodeling by increasing *COL1* and *COL3* expression. The level of *COL3* expressed was higher in BM-MSCs than AD-MSCs. Compared to the collagen group, *DCN* also showed a similar pattern, where the expression was higher for a longer duration of stretching. The results showed *TNC* and *BGN* expression were upregulated in the

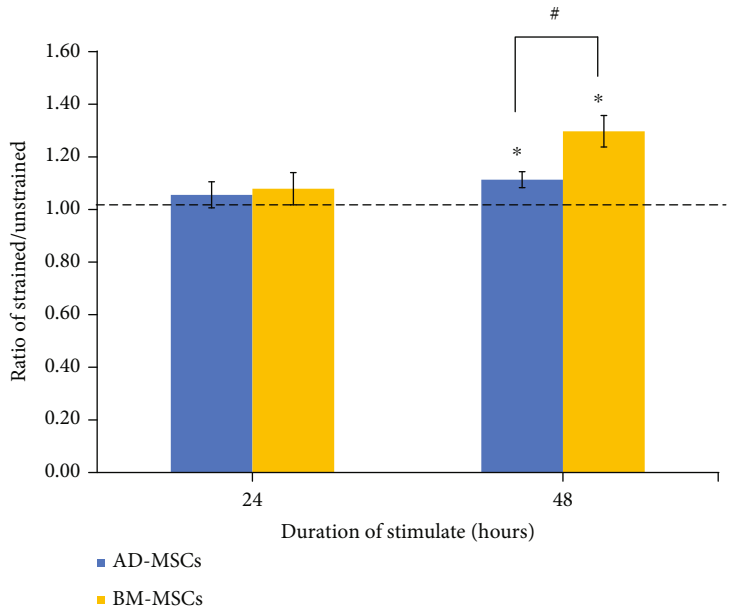

Figure 6: Extracellular collagen content analysis of AD-MSCs and BM-MSCs cultured at 1 Hz+8% at different duration of stretching. Statistical significance ($p < 0.05$) was represented by an asterisk (∗) which was compared to the unstrained cells represented by the Y axis (indicated as 1.00). Statistical significance ($p < 0.05$) was also represented by a hash (#) which is a comparison between AD-MSCs and BM-MSCs. $N = 4$, $n = 3$. Error bar = ±SD.

both cells types, more notably at 48 h. However, after stretching, the BM-MSCs showed higher and faster tenogenic gene expression than AD-MSCs although not significantly. These results suggest that both AD-MSCs and BM-MSCs have good potential to undergo tenogenic differentiation through mechanical stimulation.

4. Discussion

The present study demonstrated that from the same donor, no obvious differences can be observed between the morphology and response towards mechanical stretching in both AD-MSCs and BM-MSCs. These were also apparent in our flow cytometry, morphometric analysis, and characterization analysis. Although generally similar, there were some minor differences such as CD34, a surface marker of hematopoietic cells, which appeared to be slightly higher expressed in AD-MSCs, i.e., 5.5%. This, however, is not unexpected since such findings were also reported to be present up to 8.23% of the cell population [41]. It was demonstrated that the expression of CD34 in freshly isolated adipose stem cells will reduce over several passages but will retain some of its expression and not always completely absent [42]. When investigations were made to determine their proliferation and reorientation ability subjected to with or without stretching, both cells demonstrated contrasting outcomes. AD-MSCs proliferated better in static cultures. This finding is similar to a previously reported study where cell doubling time of AD-MSCs is 2 days as opposed to 7 days in BM-MSCs [41]. But when cyclic loading was applied, there were no differences observed. This observation is not unexpected, since we have demonstrated that lower strain values produced higher proliferation rates [38]. In this study, uniaxial cyclic strain modality is selected over other mechanical strain methods as it is thought to better mimic the type of mechanical strain experienced by MSCs in the human body [43]. What is worth noting is that cyclic loading results in the change in cellular proliferation rates to both MSCs types, demonstrating similar levels. This suggests that the internal mechanisms regulating cellular proliferation are stretch sensitive and may reset or overcome the natural cellular proliferation programming that exists in static culture conditions. This, however, would need to be proven in a more robust experiment.

Similar to *in vivo* conditions, both AD-MSCs and BM-MSCs are mechanosensitive and will realign in an arrangement perpendicular to the direction of loading. These changes were also time dependent and produced more prominent reorientation patterns over time. It has been suggested that when subjected to cyclic loading, actomyosin fibres undergo stretching that threatens cellular tensegrity [44]. The cell will strive to survive by elongating its shape and thus minimizing the energy required to maintain its integrity. The change in cell alignment and of adaptive processes through morphological changes are of natural physiological response and have resulted in the reorganization of cells' axes close to 100-110 degrees from the direction of loading [45, 46]. This in turn would avoid cell detachment or cell anoikis, which ultimately can lead to cell death [47, 48]. From our own experience, we were able to demonstrate that uniaxial tensile strain can significantly increase the Young's Modulus of the cell using cyclic loading modality, owing to the increased alignment of cytoskeleton components including F-actin fibres, thereby reducing the chance of cell failure [28].

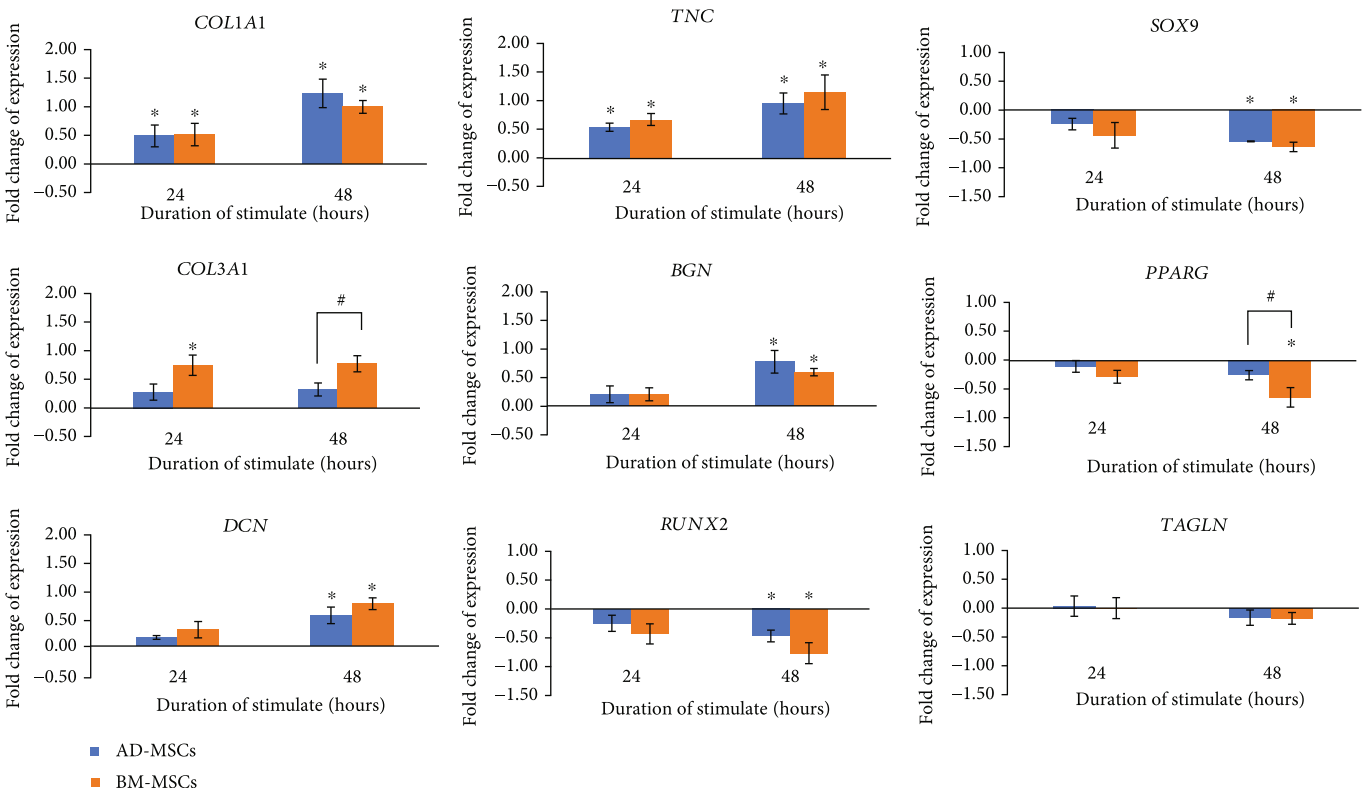

Figure 7: mRNA expression level of different genes in AD-MSCs and BM-MSCs subjected stretching at 24 h and 48 h. The expression level of each gene was normalised with the level of average housekeeping genes. The value of fold change was presented as ratio of strained group to the unstrained group. Statistical significance ($p < 0.05$) was represented by an asterisk (∗) in comparison to the unstrained cells represented by the Y axis (indicated as 0.00). Statistical significance ($p < 0.05$) was also represented by a hash (#) which is a comparison between AD-MSCs and BM-MSCs. $N = 4$, $n = 3$. Error bar = ±SD.

In terms of their differentiation potential, both the protein and gene expression analyses demonstrated a distinctive cellular differentiation towards tenogenic differentiation that of any other mesenchymal lineage. Indeed, between the two, BM-MSCs produced higher total collagen protein and *Col3* gene expression than AD-MSCs at 48 hours, signifying an increase of selected tenogenic expression rather than a global tenogenic expression. It has been previously suggested that appropriately managed mechanical loads at physiological levels would positively influence the expression of ECM and therefore stimulate the relevant mechanisms that will trigger tendon regeneration. On the contrary, aberrant mechanical loading alters the anabolic processes in tendons, resulting in the differentiation of tendon stem cells into non-tenocytes, such as osteocytes, which may lead to the development of degenerative tendinopathy [48–50]. Our previous studies demonstrated that the mechanical cyclic loading protocol used in this study, i.e., 8% strain at 1 Hz frequency produced the most optimal level of tenogenic differentiation for BM-MSCs [38]. The assumption made in this study was that AD-MSCs would respond in a similar manner to BM-MSCs when subjected to the same loading regimes. There may be a flaw in this assumption as the maximum tenogenic potential of AD-MSCs using a different regime was neither really explored nor revealed. Nevertheless, the present method did demonstrate a comparable potency to BM-MSCs, which was sufficient to answer our hypothesis.

In the present study, gene expression were the main indicators demonstrating the effects of stretching and that using *COL1*, *COL3*, *DCN*, *TNC*, and *BGN* as markers of tenogenic differentiation, and the experiments proved our hypothesis that stretching would indeed promote tenogenic expression. Such assumptions were in fact demonstrated previously in our prior report [38] and other studies such as those reported by Youngstrom et al. [51]. The choice of the panel of gene expression being investigated was reasonable having understood that these would lead to the protein expression which contributes to the matrix formation of tendon formation and repair. It is known that collagen type I is the primary matrix component of mature tendon/ligament, albeit not being exclusive to tendon tissue alone. Other matrix molecules including collagen types III, XII, and XIV; elastin; and proteoglycans may reflect the contents of tendon, albeit is in lower volume and is varied. Nevertheless, these are not specific to tendon as well. However, when these are considered holistically, these markers inherently provide sufficient indications of the ongoing repair process. In our experiments, we have demonstrated an increase in the synthesis of collagen types I and III at gene transcriptional levels, of which in tendons, this ratio has been used as an indicator of preferable tendon tissue repair outcomes [52, 53]. Such notion is supported further when several studies have indicated that collagen type III is increased during process of mechanotransduction process [54–56], which is apt

for the present experimental purposes. In general, the presence of collagen type I is important to resist mechanical loading whilst collagen type III is found to be involved in the early stages of tendon and ligament healing [53]. Therefore it is understandable that the ratio of collagen type III to collagen type I is being used in some studies, and that an increase in this ratio indicates tissues are undergoing the early stages of tendon healing [52, 57–59]. The use of other protein expressing gene markers, such as Decorin, provides further supporting evidence for the anticipated repair outcomes since this protein is the predominant proteoglycan component located in the tensile region of tendons which implicated in lateral fibril growth [60]. This fibre has been shown to correlate with size and density of collagen fibrils, and thus of the mechanical strength of tendon tissue itself [61]. Likewise, *BGN* is important for directing assembly of collagens [62], in addition to be essential in the maintenance of the putative tendon stem cell niche [63].

In considering the larger view of things, the relationship of *COL*, *DCN*, and *BGN* in our current studies appears overarching and provides a good and comprehensive indicator of the potential healing capacity of MSCs. In addition to expressing specific tendon related proteins, mechanical stimulation also triggers specific signalling pathways that lead to transcription of the regulatory genes of resident progenitor cells and in introducing MSCs into damaged tendon these cells, towards the activation of tenogenesis differentiation pathway. The mitogen-activated protein kinase (MAPK) pathway was found to be up-regulated in MSCs exposed to cyclic tensile strain, suggesting it as an important mechanotransductive pathway in MSCs differentiation [64, 65]. Another study of Kearney et al. found ERK and p38 to be involved in cyclic tension mechanotransduction, and stretch-activated cation channels are implicated to mediate collagen I gene expression [66]. Whilst there have been previous publications describing the effects of mechanical straining on MSCs differentiation, these mainly focuses on the differentiation of cells into smooth muscle cells, chondrocytes, and osteoblasts [26, 67, 68]. Like previous reports, our study demonstrated that AD-MSCs had good multilineage differentiation capacity and good cellular proliferation that was comparable to bone marrow [69, 70]. Nevertheless, it became apparent from our gene expression that uniaxial cyclical tensile loading on these two cells sources suppressed adipogenesis, chondrogenesis, and osteogenesis and instead strongly promoted tenogenesis. This appears to complement the results from our previous study involving human bone marrow MSCs [28]. Interestingly though, whilst prior report had mentioned that AD-MSCs produced superior mechanotransductive responses BM-MSCs when subjected to mechanical stimulation, our study was not able to establish this [41].

Whilst the study design was sufficiently robust, there were notable limitations to the present study. Firstly, inherent to any *in vitro* study design, biological variations that may occur due to the multiple donors for cell sources is unavoidable and may have influenced the study results. In order to reduce this, in most cases, we did our best to obtain both bone marrow and adipose derived-MSCs from similar individuals. This increased the likelihood to produce desirable observable related changes but does not address the variability of different individuals. To reduce the variation effect, a large sample size involving large population would be necessary, which in a laboratory experiment would be logistically and economically prohibitive. We accept this limitation in any *in vitro* experiment and interpreted the results accordingly to merely demonstrate the potential clinical efficacy prior to validating our results into a larger scale preclinical and/or clinical studies. Hence, we suggest a more robust and deeper investigation in the near future that would be necessary verify the findings of the present study before any further clinical implications can be made. Secondly, our preliminary study has shown that the size of cell culture chamber/device provide limited space for cellular expansion and results in maximal size expansion or confluency when experiments were conducted up to 48 to a maximum of 72 hours. In our previous study, we were able to demonstrate that the tenogenic differentiation of BM-MSCs could be triggered as earlier as 24 hours and enhanced phenotypic expression at 48 hours using the proposed mechanical strain and frequency. This would have been sufficient for the needs for the present experiments. To further extend the study beyond this would result in aberrant results as cells in culture would undergo proliferative contact inhibition. This was the rationale to limit the experiment to 48 hours. Moreover, considering that the continuous mechanical stimulation is a form of accelerated outcome stimuli, any results from this experiment would have been observed within this time period.

5. Conclusions

The present study demonstrates that mechanical stretching at 1 Hz and 8% strain did not promote cellular promotion but enhance tenogenic differentiation and protein expression for both AD-MSCs and BM-MSCs equally, suggesting that both cell sources are equally suitable in treating damaged tendon.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Hui Yin Nam and Mohd Rusdi Draman @ Yusof contributed equally to this work.

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Review Article

Cell-Based Transplantation versus Cell Homing Approaches for Pulp-Dentin Complex Regeneration

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Regenerative dentistry has paved the way for a new era for the replacement of damaged dental tissues. Whether the causative factor is dental caries, trauma, or chemical insult, the loss of the pulp vitality constitutes one of the major health problems worldwide. Two regenerative therapies were introduced for a fully functional pulp-dentin complex regeneration, namely, cell-based (cell transplantation) and cell homing (through revascularization or homing by injection of stem cells in situ or intravenously) therapies, with each demonstrating advantages as well as drawbacks, especially in clinical application. The present review is aimed at elaborating on these two techniques in the treatment of irreversibly inflamed or necrotic pulp, which is aimed at regenerating a fully functional pulp-dentin complex.

1. Introduction

Dental tissue regeneration requires the presence of specialized cells capable of the production of a tissue-specific extracellular matrix (ECM) [1, 2]. Stem/progenitor cells used in regenerative medicine are nonspecialized cells, demonstrating the ability of self-renewal and multilineage differentiation [3]. The potential of stem/progenitor cells, whether endogenous or exogenous, to adapt to various environmental niche could be exploited in regenerative endodontics and pulp-dentin tissue regeneration [4–6]. Therapeutic application of stem/progenitor cells is mainly dependent on the utilization of the transplanted cells, on suitable scaffolds and in combination with various growth factors to generate fully functional biological tissues [7]. Recently, the success demonstrated in animal models to repair/regenerate dental structures has paved the way for pulp-dentin organ regeneration approaches [8].

1.1. Cell-Based Transplantation for Pulp-Dentin Complex Regeneration (Table 1 and Figure 1). A suggested approach to address problems related to pulp-dentin tissue regeneration relied principally on the use of various sources of stem/-progenitor cells, combined with multiple scaffold systems and growth factors [9]. Human mesenchymal stem/progenitor cells (MSCs) have been extracted from many areas of the human body, including the bone marrow, the skin as well as the perivascular, the adipose, and the dental tissues [10–12]. Early trials and continuous animal studies were directed to investigate the effectiveness of cell-based transplantation on pulp healing and dentin regeneration [7, 13, 14]. Autologous transplanted constructs of dental pulp stem/progenitor cells (DPSCs) in combination with platelet-rich fibrin (PRF) proved to promote the regeneration of pulp-dentin-like tissue inside dogs' root canals [15]. A further animal study employing human DPSCs and platelet-derived growth factor

Table 1: Summary of cell-based transplantation studies for pulp-dentin complex regeneration.

Study	Study design				Outcomes		
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
Chen et al. 2015	Randomized controlled trial	Animal study; 3 dogs providing 60 root canals	Cell based	Group I: DPSC/PRF construct Group II: DPSCs only Group III: PRF granules only Group IV: blank controls without any exogenous transplanted grafts			DPSC/PRF construct led induced regeneration of dense pulp-like tissues with richly distributed blood capillaries. The deposition of regenerated dentin alongside the intracanal walls was evident.
Cai et al. 2016	Randomized controlled trial	Animal study; 6 rats, 12 incisors	Cell based	Group I: 6 untouched incisors Group II: 2 sham incisors Group III: 4 transplanted incisors			Immunohistochemistry revealed globular dentin and pulp-like tissue formation.
Jia et al. 2016	Randomized controlled trial	Animal study; 18 immature premolars from 2 dogs	Cell based	Group I: mineral trioxide aggregate Group II: absorbable gelatin sponge Group III: cDPSCs Group IV: Simvastatin group	Simvastatin stimulated cDPSC mineralization and induced DPSC pulp and dentin regeneration.	After 10 weeks, radiographic examination of pulpotomized teeth showed closure of the root apex and thickening of the root canal wall.	cDPSCs pretreated with SI, regenerated pulp that was filling most of the pulp cavity. Newly formed dentin deposits succeed to the primary dentin and odontoblasts were evident in the regenerated area. It was significantly higher than that in all other groups.
Iohara et al. 2016	Randomized controlled trial	Animal study; a total of 28 teeth from 5 dogs were randomly divided into 4 groups.	Cell based	Group I: pulpectomy only (no cells and no collagen) Group II: normal teeth Group III: transplantation of MDPSCs and 7.5 ng/mL G-CSF with an atelocollagen scaffold Group IV: collagen only	The signal intensity (SI) of MRI of the normal teeth was significantly higher than that of nonvital pulpectomized teeth and the controls of collagen transplanted teeth at 90 days. The stem cell transplanted teeth showed gradual decrease in the SI until 180 days which was similar to the normal teeth and significantly higher than that in the teeth transplanted with collagen alone without the stem cells.		One day after transplantation of collagen alone or MDPSCs and G-CSF with collagen, the root canal was filled with collagen like-fibers. Ninety days after the transplantation of MDPSCs and G-CSF with collagen, most of the root canal was filled with pulp-like tissue in which well-developed vasculature and dentin were formed along the dentinal wall. On day 180, the root canal was completely filled with pulp-like tissue and secondary dentin

Table 1: Continued.

Study	Study design			Outcomes			
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
Bakhtiar et al. 2017	Randomized controlled trial	Animal study; 32 premolars of 5 dogs	Cell based	Group A: MTA Group B: TDM Group C: TCP Group D: TDM scaffold impregnated with DPSCs+TDM Group E: TCP scaffold impregnated with DPSCs+TCP	The negative control group showed severe inflammation and granulation tissue formation. The positive control group was characterized by intact periodontal tissues and no inflammation.		was formed in the apical part and along the dentinal wall. Dentin bridge formation was absent in specimens of all groups. The SC+TDM group was associated with significantly more bone formation than other groups. Cementum was formed with a cellular and continuous pattern in all specimens.
Ito et al. 2017	Randomized controlled trial	Animal study; 48 female Wistar rats	Cell based	Group 1: RBMMSC/PLLA/Matrigel constructs Group 2: Matrigel constructs without RBMMSC Constructs were implanted into the cavity for 3, 7, or 14 days ($n = 8$ in each group).	Immunohistochemistry revealed that nestin-expressing odontoblast-like cells beneath the dentin at the border of implanted area increased until 14 days.		Considering RBMMSC constructs at 3 days, cells were located mainly along the implanted scaffolds. At 7 days, pulp tissue regeneration was created in almost the entire implanted region. At 14 days, pulp tissue regeneration continued throughout the implanted region.
Mangione et al. 2017	Randomized controlled study, split-mouth model	Animal study; 3 minipigs, of total 48 teeth	Cell based	Group 1: pDPCs were implanted in the left maxillary and mandibular teeth. Group 2: no pDPC scaffold was implanted in teeth of the right side.	Micro-CT examination of the treated teeth showed the formation of a reparative mineralized bridge in the remaining pulp of both groups. External root resorption was evident in all teeth.		With pDPSCs, reparative dentin bridge presented many abundant and joined nonmineralized areas.
Sueyama et al. 2017	Randomized controlled trial	Animal study; 40 female rats	Cell based	Group 1: PLLA implanted scaffolds with MSCs and Ecs Group 2: implanted scaffolds with MSCs Group 3: implanted acellular scaffolds Group 4: pulpotomy cavities were sealed with MTA only. Group 5: no pulpotomy	14 days after implantation; MSCs associating Ecs accelerated the pulp tissue regeneration and enhanced dentin bridge formation.		Teeth with MSC/EC constructs showed pulp healing and complete dentin bridge formation, but MSCs alone showed incomplete, thinner dentin bridges. Teeth implanted with acellular scaffolds were of poor tissue regeneration in the implanted area and incomplete hard tissue formation.

Table 1: Continued.

Study	Study design				Outcomes		
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
				(used as the normal control)			Teeth subjected to pulpotomy without implantation did not show pulp tissue regeneration.
El Ashiry et al. 2018	Randomized controlled trial, split-mouth design	Animal study; 12 dogs, 36 teeth	Cell based	Group A: tooth transplanted with a construct of autologous dental pulp stem cells with growth factors seeded in a chitosan hydrogel scaffold Group B: tooth received only growth factors with scaffold.	DPSC constructs resulted in complete root maturation., radicular thickening, root lengthening, and apical closure.		DPSC constructs showed regeneration of pulp-dentin-like tissue filling the emptied canals. The vascularized pulp-like tissue resembled the natural pulp. On the contrary, in the other group, no soft tissues were observed.
Cordero et al. 2020	Case report	Human mature molar with accidental root perforation	Cell based		Radiographic and cone-beam computed tomographic images indicated remission of the apical lesion. Clinically, normal responses to percussion and palpation tests	Tooth was responsive to the electric pulp test, and the vitality test indicated low blood perfusion units.	
Iohara et al. 2020	Randomized controlled trial	Animal study; aged dogs	Cell based	Group I: no treatment Group II: nanobubble treatment Group III: 0.05% trypsin for 10 min Group IV: 0.5% trypsin for 10 min Group V: 0.05% trypsin for 30 min Group VI: 0.05% trypsin for 10 min with nanobubbles			The amount of pulp-like regenerated tissues was three-times higher with 0.05 and 0.5% trypsin pretreatment for 10 min than that in the no treatment group. Moreover, the trypsin pretreatment induced higher pulp tissue vascularization compared with no pretreatment.

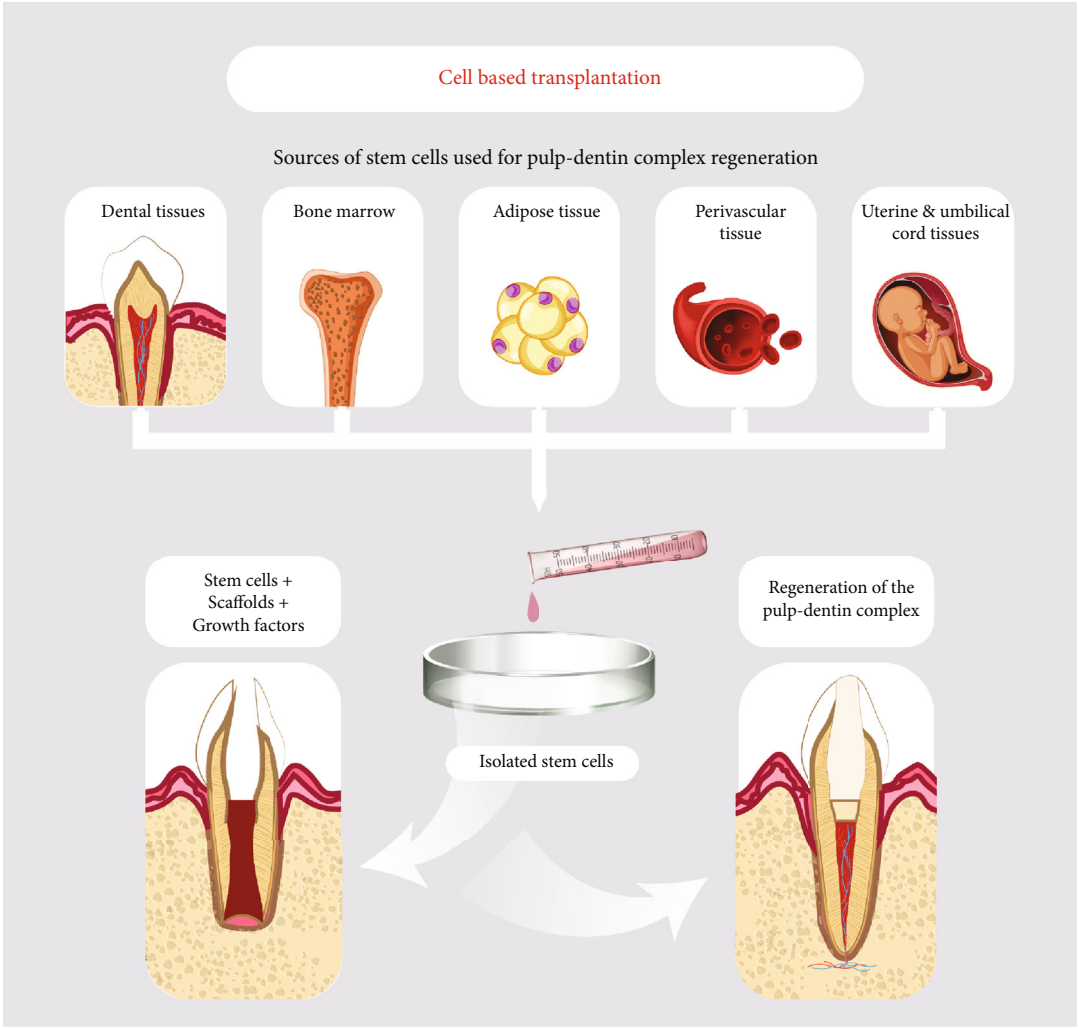

Figure 1: Cell-based transplantation method and sources of stem cells used for pulp-dentin complex regeneration.

(PDGF) constructs transplanted into the emptied root canals of rats induced the creation of globular dentin-like structure with odontoblastic cells and pulp-like tissues [16].

A trial to treat deliberately perforated pulp space of premolars of dogs using autogenous DPSCs, embedded in tricalcium phosphate (TCP) or treated dentin matrix (TDM) scaffolds, showed no dentin formation in all groups while cementum and vascular connective tissues were evident in all specimens [17]. A further study examined microvascular endothelial cells (ECs) coimplanted with rat bone marrow MSCs in pulpotomized rat models. Interestingly, after 14 days, immunohistochemical examination demonstrated healing of the pulp with complete dentin bridge formation in teeth implanted with MSCs and ECs, while those implanted with MSCs lacked the completion of the formed dentin bridge [18]. A further noninvasive regenerative pulpal approach was tested, using mobilized DPSCs freshly extracted from upper canine teeth of dogs, followed by autologous DPSCs transplantation in pulpectomized permanent teeth with apical closure. The study revealed that pulp tissue was completely regenerated 90 days following cell transplantation [19]. A novel trial on a rat model for dental pulp regeneration employed pulpotomized rat teeth, which were treated using buildups of rat bone marrow mesenchymal stem cells (BMMSCs). The tested buildups were implanted into the pulpotomized pulp chambers for 3, 7, or 14 days and then examined immunohistochemically. At 7 days, the pulp tissue was regenerated in almost the whole implantation area and regeneration continued to progress for 14 days with differentiation of odontoblast-like cells beneath the dentin at the margin of the implanted area evidenced by a detected nestin expression. Also, quantitative gene expression analysis disclosed the expression of sialophosphoprotein mRNA in the implanted area, suggesting the abundance of odontoblasts [20]. Chitosan hydrogel scaffold containing autologous DPSCs was further transplanted in the necrotic immature permanent teeth of dogs, regenerating pulp- and dentin-like tissues with complete root maturation radiographically and histologically [21]. However, not all the reported studies were successful. Implanting DPCs in TCP and TDM scaffolds, combined with transforming growth factor β, ascorbic acid 2-phosphate, and ascorbic acid 3-phosphate, did not promote the formation of a dentin bridge [17]. Also, porcine

DPCs failed to heal or regenerate partial pulpotomy defects of minipigs. Moreover, hyperemia in the residual pulp and external root resorptions were evident in the radicular area of all the treated teeth [22]. On the contrary, another investigation demonstrates that when combining collagen scaffold with granulocyte colony-stimulating factor (G-CSF), a total recovery of the pulp tissue was achievable in the pulpectomized teeth [19].

It was appealing to seek more uncommon supplementary derivatives to enhance stem/progenitor cells' activation and differentiation, dragging attention towards nondental medications. An animal study reported that a common drug used to treat hyperlipidemia, Simvastatin (SIM), succeeded in stimulating canine DPSCs, promoting pulp and dentin regeneration following pulpotomy [23]. Further animal studies suggested using glycogen synthase kinase (GSK-3) antagonists, a drug usually applied for the treatment of neurological disorders, which proved successful as a capping material of the pulpal exposure site, promoting dentin formation [24, 25]. Another animal study proved that pulp regeneration was enhanced in aged dogs' teeth by trypsin pretreatment of allogenically transplanted mobilized DPSCs [26].

A case report treating accidental root perforation of a mature permanent tooth, utilizing allogenic umbilical cord mesenchymal stem cells (UCMSCs) encapsulated in a platelet-poor plasma- (PPP-) based bio scaffold, demonstrated a clinically normal pulpal tissue in terms of vitality testing, palpation, and percussion testing at six-month and one-year follow-ups [27]. Moreover, two case reports showed a successful management of periapical lesions in permanent teeth treated with stem/progenitor cells from human exfoliated deciduous teeth (SHED), with the treated teeth responding normally to electric pulp testing and periapical tissue healing observed and maintained up to one year [28].

Collectively, cell-based therapeutic applications in the dental field and specifically dentin-pulp tissue regeneration still face a number of challenges. Future strategies should be directed towards overcoming these challenges and obstacles using an ideal combination of growth factors with properly matching scaffolds [17, 22]. Secure and controllable practice must be strictly followed to translate stem/progenitor cell research into human models, starting from protocols of stem/progenitor cells' tissue harvesting, the biocompatibility of the used scaffolds and biomaterials involved, and the safety of the technique itself and the predicted outcome [29, 30]. Finally, the endless mix and match trials between scaffolds of different origins, as well as electing the suitable growth factor/biological mediator, could govern the success or failure of regenerating a specialized tissue when employing the stem cell-based therapy [31].

1.2. Stem/Progenitor Cell Homing. As mentioned above for pulp-dentin complex regeneration, two strategies could be applied, namely, the cell-based transplantation therapy or the cell homing. In the latter, the regeneration is accomplished via chemotaxis of host endogenous cells to the injured tissue via biological signaling molecules. Stem/progenitor cell homing can be defined as the potential of stem/progenitor cells, whether endogenous or exogenous, to migrate into an environmental niche. MSCs can be delivered in situ or intravenously, or they can be recruited to sites of injury, through migration and homing [32]. Clinically, cell homing for pulp-dentin complex regeneration might be simpler and more economical to perform compared to the cell-based therapy and readily performed by clinicians without special training.

1.3. Stem/Progenitor Cell Homing Mechanisms (Figure 2). Homing approaches can be either systemic or nonsystemic. In nonsystemic homing, MSCs are locally transplanted at the selected tissue and are then directed to the region of injury through a chemokine gradient. Oppositely, in systemic homing, MSCs are delivered or recruited endogenously into the circulation and then undergo a series of processes, leaving the bloodstream and moving towards the site of injury. These complex processes involve tethering and rolling, activation, arrest, transmigration or diapedesis, and migration [33, 34]. Tethering is mediated by selectins on endothelial cells. MSCs exhibit CD44, which binds to the selectins and starts rolling along blood vessels [35]. This is followed by chemokine-mediated activation [36]. MSCs express the chemokine receptors CXCR4 [37] and CXCR7 [38, 39]. The stromal cell-derived factor (SDF-1) is the ligand to these receptors, where it binds to them to enhance homing to different tissues. Then, comes the process of arrest mediated by integrins, mainly by CD49d ($\alpha 4\beta 1$), which unites with VCAM-1 (CD106) present on endothelial cells [40]. In order to cut across the endothelial basement membrane, a process known as diapedesis or transmigration, MSCs produce matrix metalloproteinases (MMPs) mainly MMP-1, which plays a crucial role in tissue infiltration by MSCs [41]. Finally, MSCs migrate to injury sites. This step is regulated by chemotactic signals, produced as a reaction to tissue impairment. Numerous growth factors, such as insulin-like growth factor IGF-1 and platelet-derived growth factor (PDGF), can act as chemokines for MSCs [42]. Moreover, tumor necrosis factor (TNF-α) increases MSCs movement towards chemokines by increasing their expression of CCR3, CCR4, and CCR2 receptors [4, 43, 44]. In addition, the inflammatory cytokine interleukin- (IL-) 8 was proved to enhance migration of MSCs towards regions of injury [45, 46] and further promotes them to produce regenerative growth factors, such as vascular endothelial growth factor (VEGF) [47].

1.4. Routes of Administration and Delivery Methods. One important point in MSCs transplantation and their consequent therapeutic efficiency is the route of administration to provide the ultimate regenerative benefits with the least adverse effects. The most common delivery methods for MSCs are either by intravenous (IV) or intra-arterial infusion (IA) or by direct intratissue injection [48]. Several experimental studies proved the superiority of IA and IV delivery modes over other delivery routes [49, 50]. The IV route was proved to be the most convenient route for MSCs transplantation. It is less traumatic and reproducible and enhances widespread distribution in the affected regions, enhancing various biological effects [51]. However, this delivery method in nearly all cases causes entrapment of MSCs in the lungs, causing undesirable adverse effects, including embolisms. The reason for this lung entrapment relies probably on the amalgamation of physiological and mechanical factors, such as

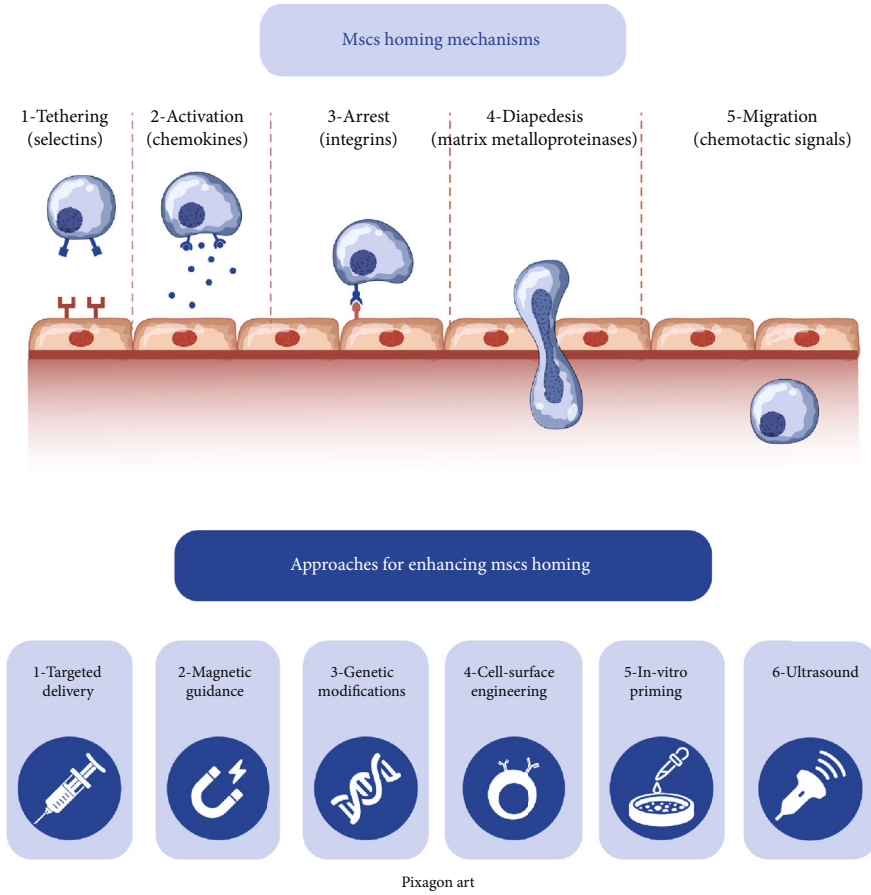

Figure 2: MSC homing mechanisms and different approaches for enhancing MSC homing.

the small size of blood capillaries, the vast network of capillaries, and the great adhesive characteristics of MSCs. It was also demonstrated that some cells could produce calcium deposits within the capillaries [52].

On the contrary, the IA route can be more efficient, as it provides a straightforward route to the injury site with an increased degree of cellular endurance and engraftment [53, 54]. Several studies proved the superiority of IA delivery route over the IV one. They demonstrated enhanced functional and histological results in IA delivery compared with IV injection of MSCs [49, 55]. IA transplantation of MSCs increases cellular migration, cellular density, and the number of homing MSCs to the target tissue, when compared to IV injection [56, 57]. Du et al. in a comparative study demonstrated greater angiogenesis and increased functional recovery with IA transplantation compared to IV injection utilizing human BM-MSCs in a rat model of ischemia [58]. Lundberg et al. confirmed these findings in a model of traumatic brain injury [59]. The main reason for the superiority of IA transport over IV mode is that the IA approach can bypass the pulmonary circulation and filtering organs, such as liver and spleen [60], thereby avoiding MSCs entrapment in lungs and liver [54], with a significant rise in number of cells with a more consistent cellular dissemination in target tissues [61, 62]. This will eventually lead to increased cell homing and improved therapeutic outcomes [58].

However, a probable limitation for the IA route is the possibility of vascular blockage in small arterioles and capillaries resulting in strokes. This may be attributed to the existence of large MSCs in the 20–50 μ size range [63, 64]. Several attempts have been performed to enhance the safety of IA transplantation via regulating infusion velocity and cell dosage [63, 65]. Moreover, real-time MRI could provide a useful tool in making the procedure more accurate and predictable, which is of ultimate importance for translation to clinical practice [66].

Direct injection delivery mode has the advantage of accurate localization of cells, despite being invasive. However, it has been proved that aside from the delivery route, only 1~5% of delivered cells disseminate within the target region for regeneration. The count of cells in the target region may thus be enhanced by maximizing the injection volume or enriching the cell concentration in the injectable volume [67–69]. In addition, the expression of adhesion molecules can promote homing of delivered MSCs [70, 71]. In this context, several approaches have been made to enhance MSC homing efficacy.

1.5. Enhancing MSC Homing (Figure 2). Cellular homing relies principally on specialized molecular interactions, not just passive diffusion. One of the main challenges facing MSCs therapeutic applications is enhancing their homing abilities [72].

Among the challenges is the fact that the expression of homing molecules, as CXCR4, is relatively low on MSCs [37, 73], and the in vitro expansion of MSCs further decreases the expression of their homing molecules [74, 75]. Thus, numerous approaches have been suggested to enhance MSC homing. Among these is targeted delivery, which relies on direct delivery of MSCs into the target region, employing nonsystemic rather than systemic homing [76]. In addition, magnetic guidance of MSCs to target tissues proved greater homing efficiency [77]. Furthermore, genetic modifications of MSCs via overexpression of homing factors such as VLA-4 and CXCR4 through viral transduction proved increased efficiency [78, 79]. Cell surface engineering approaches were suggested to modify the selectin ligand CD44, via transforming it into HCELL (the ligand for E- and L-selectin that MSCs utilize for homing), as MSCs normally express CD44, but not HCELL [80]. It was further demonstrated that coating MSCs with hyaluronic acid could upregulate CD44 expression [81]. Moreover, hypoxic conditions enhance hypoxia-inducible factor- (HIF-) 1a, which upregulates the expression of CXCR4 [82], CX3CR1 [83], and CXCR7 [84, 85].

A further strategy addressed modifying the target tissues, via overexpression of chemokines or via implantation of chemokine-coated scaffolds [86]. This allows tissues to be a more appealing target for homing MSCs. Moreover, irradiation of target tissues increases the expression of SDF-1, upregulating in MSC engraftment [87, 88] and homing [89]. Pulsed ultrasound applied to the target tissue may also enhance MSC homing [90], via altering gene expression of cytokines as bone morphogenic protein-2 (BMP-2), interleukins (IL-1α, IL-6, and IL-10), TNF-α, and growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), VEGF, and PDGF [91], causing disorganization of endothelial linings, enhancing vascular permeability, increasing secretion of SDF-1 on the tissue of interest, and upregulating CXCR4 expression [92].

1.6. Cell Homing for Pulp-Dentin Complex Regeneration (Revascularization) (Table 2 and Figure 3). Regenerative endodontics represents an alternative to root canal treatment, which is aimed at replacing the inflamed and necrotic pulp tissue with regenerated pulp-like tissue [93]. In this context, revascularization approaches of affected dental pulp were suggested as an innovative strategy to overcome the drawbacks associated with classical root canal treatment methods (e.g., fracture of the teeth and loss of vitality) [94]. A human study on mature necrotic teeth with large radiolucency concluded that the regenerative endodontic approaches have a success rate similar to nonsurgical endodontic treatment as a therapeutic alternative for mature necrotic teeth with radiolucency [95]. It could maintain the pulp vitality, leading to a reduction of apical periodontitis and enhance the periapical healing mechanism [96]. Basically, pulp revascularization is the reestablishment of angiogenesis inside the root canal but without the repopulation of odontoblasts, while the pulp regeneration means angiogenesis with presence of odontoblastic layer lining the dentinal surface, nociceptive as well as parasympathetic and sympathetic nerve fibers, interstitial fibroblasts, and stem/progenitor cells, which replenish the pulp cells in the newly regenerated pulp tissue [97]. According to American Association of Endodontists' (AAE) Clinical Considerations for a Regenerative Procedure, the primary goal should be the resolution of clinical symptoms/signs and elimination of apical periodontitis. The secondary goal should address the canal wall thickening and/or continued root maturation [98].

Pulp revascularization could be considered a type of cell homing strategy for pulp-dentin complex regeneration. This clinical procedure depends on the delivery of a blood clot (scaffold) inside the root canal, growth factors (mainly from platelets and dentin), and stem/progenitor cells. The stem/-progenitor cells of interest in revascularization are SCAP (stem cells of apical papilla) because of their anatomical positioning immediately adjacent to the termination of the root canal system, permitting easy cell delivery to the root canal [99, 100] and the greater superiority for dentin-like tissue formation [101, 102]. The root canal system is first disinfected with a combination of antibiotics or calcium hydroxide. In the second visit, the irrigation protocol during this clinical procedure is very critical as for the regeneration procedure to be successful; the irrigants should have bactericidal/bacteriostatic properties as well as an ability to promote survival and proliferative capacity of the patient's stem/progenitor cells. The irrigation protocols that include 17% EDTA promoted SCAP survival and attachment to the root canal dentinal wall [103].

Animal studies were performed to examine the tissues formed after revascularization, demonstrating ingrowth of cellular cementum-like tissues, formation of pulp-like tissue, thickening of the canal walls, closure of the root apex, and disappearance of periapical radiolucency [104, 105]. Histological sections were also performed in humans after fracture of a revascularized immature tooth (3.5 weeks after revascularization), showing that the canal was filled with loose connective tissue and a layer of flattened odontoblast-like cells lined along the predentin. Layers of epithelial-like cells, similar to the Hertwig's epithelial root sheath, further surrounded the root apex [106].

Alternative endodontic therapy is now possible, using the patient's own blood samples, where PRF and PRP are introduced inside the root canal. Easier and successful efforts for pulp revascularization and pulp tissue regeneration were reported by using evoked bleeding (EB), where the blood clot acts as a protein scaffold and interacts with endogenous stem cells and growth factors already abundant in the adjacent bone marrow tissues [107]. The highest reported cytokines and growth factors found in PRF are IL-1β, IL-6, IL-4, TNF-α, PDGF, VEGF, IGF-1, EGF, and transforming growth factor β1 (TGFβ1) [108], while PRP contains FGF, PDGF, VEGF, IGF-1, EGF, and TGFβ1 [109]. The superiority of PRP came from releasing an elevated number of proteins at early time intervals whereas PRF showed a sustained production of bioactive molecules throughout a duration of 10 days [110]. In the blood clot technique, the growth factors are released from the dentin matrix after conditioning of the dentin using EDTA (ethylene diamine tetra acetic acid) 17%-pH 7.2 during the revascularization technique. Thus, the dentin matrix acts as a reservoir of bioactive molecules, which provides a vital source of cell signaling molecules for initiating repair, including TGFβ1, bone

Table 2: Summary of cell homing studies for pulp-dentin complex regeneration.

Study	Study design				Outcomes		
					Primary outcomes	Secondary outcomes	
	Blind; random; design	Animal model/human	Type of study	Groups	Clinically and radiographically	Discoloration and sensibility test	Histology
Thibodeau et al. 2007	Randomized clinical study	Animal study; 60 immature teeth from 6 dogs	Cell homing	Group 1: no treatment (but disinfected) Group 2: blood clot Group 3: collagen solution Group 4: collagen solution +blood clot Group 5: negative control (left untouched)	Radiographic thickening of root canal walls, apical closure, and healing of periapical radiolucency in all the groups		Hard tissue deposition on radicular dentin in all groups except the negative control New vital tissues were formed in the root canals in all groups except in the negative group.
Shah et al. 2008	Pilot clinical study	14 cases of infected immature teeth	Cell homing	Blood clot revascularization	Radiographic resolution of periapical radiolucencies was judged to be good to excellent in 93% of the cases. The striking finding was complete resolution of clinical signs and symptoms and appreciable healing of periapical lesions in 78% of cases.		
Ding et al. 2009	Clinical study	12 patients, each with immature permanent tooth with chronic or acute apical periodontitis	Cell homing	Blood clot revascularization	Teeth ($n = 3$) were found to exhibit complete root development with a positive response to pulp testing.		
Lovelace et al. 2011	Clinical study	A total of 12 patients were included in this study.	Cell homing	This study consisted of 6 boys and 6 girls with immature permanent maxillary or mandibular single rooted immature tooth with open apices with diagnosis of pulp necrosis with apical periodontitis.	Molecular analyses of blood collected from the canal system indicated the significant accumulation of transcripts for stem cell markers CD73 and CD105 (up to 600-fold).		Histological analysis demonstrated that the delivered cells expressed both CD105 and STRO-1, markers for a subpopulation of mesenchymal stem cells.
Jadhav et al. 2012	Pilot clinical study	20 patients with nonvital, immature anterior teeth were randomly categorized into 2 groups; revascularization with or without PRP	Cell homing	Group I: blood clot Group II: using PRP	Clinically, all cases were asymptomatic with complete resolution of signs and symptoms. Radiographically, there was a marked difference in periapical healing, apical closure, and dentinal wall		

Table 2: Continued.

Study	Study design				Outcomes		
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
					thickening in group II in comparison with group I; however, root lengthening was comparable for both of the procedures.		
Shimizu et al. 2012	Case report	Human study	Cell homing	Revascularization/ regeneration procedure			At 3.5 weeks after revascularization, more than one half of the canal was filled with loose connective tissue similar to the pulp tissue. A layer of flattened odontoblast-like cells lined along the predentin. Layers of epithelial-like cells, similar to the Hertwig's epithelial root sheath, surrounded the root apex. No hard tissue was formed in the canal.
Mishra et al. 2013	Case report	An 11-year-old boy with the history of trauma was diagnosed with pulpal necrosis and symptomatic apical periodontitis in tooth #21.	Cell homing	Platelet-rich fibrin used	Clinical examination at 6 and 12 months revealed no sensitivity to percussion and palpation in tooth #21, and it responded positively to both electric pulp and cold tests. Radiographic examination showed resolution of periapical rarefaction, further root development and apical closure of the tooth #21 and its associated supernumerary tooth.		
Zhang et al. 2014	Randomized clinical study	Animal study; three 6-month-old beagles	Cell homing	Group 1: PRP Group 2: blood clot Group 3: negative control			Apical apex was closed. Pulp-like tissue (fibroblasts and blood

Table 2: Continued.

Study	Study design				Outcomes		
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
							vessels) was developed. Thickening of the canal wall with ingrowth of cellular cementum-like tissues (cementocyte-like cells) were present in the newly formed tissues. Large number of inflammatory cells were present in the PRP and blood clot groups.
Priya et al. 2015	Clinical case study	The present case evaluated PRP for pulpal regeneration in an avulsed mature incisor (>8-hour extraoral dry time) of an 11-year-old boy after delayed replantation.	Cell homing	The present case evaluated PRP for pulpal regeneration in an avulsed mature incisor (>8-hour extraoral dry time) of an 11-year-old boy after delayed replantation.	Nine- and 12-month radiographs revealed resolution of periapical radiolucency with no further progression of internal resorption. The tooth showed a positive response to thermal and electric pulp tests. The findings observed in this case warrant further research under controlled conditions to evaluate endodontic and periodontal regeneration in a tooth that would otherwise be expected to have an unfavourable prognosis.		
El Ashiry et al. 2016	Clinical study	20 patients with immature necrotic teeth with apical periodontitis	Cell homing	Blood clot group	Within 12-24 months, increase in dentinal wall thickness and root length and continued root development were observed.		
Shivashankar et al. 2017	Triple-blind randomized clinical trial	60 patients with necrotic immature tooth	Cell homing	Group A: PRF (scaffold) Group B: revascularization with conventional induced	At the end of 12 months, patients presented no pain and no signs of reinfection. No radiographic		

Table 2: Continued.

Study	Study design				Outcomes		
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
				bleeding Group C: PRP (biomaterial)	enlargement of the preexisting apical pathosis in all the three groups		
Song et al. 2017	Retrospective study	29 cases undergone revascularization between 2010 and 2014.	Cell homing	Revascularization group	Continued root development with apical closure in 79.35 of cases Revascularization associated intracanal calcification in 62.1% of the cases after 12-month follow-up		
Nageh et al. 2018	Clinical study	15 patients with necrotic pulp with symptomatic or asymptomatic apical periodontitis	Cell homing	PRF revascularization	All teeth survived after 12 months, no pain or swelling.	Pulp sensibility regained using electric pulp tester in 9 cases after 12-month follow-up.	
Neelamurthy et al. 2018	Clinical study	15 patients with immature and mature permanent teeth with pulpal necrosis and open apices	Cell homing	Bleeding group	After 10 months, 10 out of 13 patients showed root development and apical closure.	2 out of 13 patients showed a positive response to electric sensibility test.	
Arslan et al. 2019	Randomized clinical study	56 mature necrotic teeth with large periapical radiolucency	Cell homing	Group I: conventional root canal treatment (CRCT) Group II: regenerative endodontic procedures (REP)	No difference between the two groups regarding pain, palpation, swelling, sinus tract, and pain on percussion. Radiologically, absence and reduction of the radiolucency were 85% in the CRCT group and 92.4% in the REP group.	50% of REP-treated teeth responded positively to electrical vitality testing.	
Mittal et al. 2019	Clinical study	16 cases of necrotic immature permanent	Cell homing	Group I: PRF Group II: collagen	Clinically, patients were completely asymptomatic		

Table 2: Continued.

Study	Study design				Outcomes		
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
		teeth using PRF, collagen, Placentrex, and chitosan		Group III: Placentrex Group IV: chitosan	throughout the study period. Radiographically, all cases showed an improvement in terms of periapical healing, apical closure, root lengthening, and dentinal wall thickening. PRF and collagen gave better results than Placentrex and chitosan in terms of periapical healing, apical closure, and dentinal wall thickening.		
Ragab et al. 2019	Randomized controlled trial	22 patients suffering from immature necrotic permanent maxillary central incisors	Cell homing	Group A: blood clot Group B: using PRF revascularization	After a follow-up period of 12 months, most of the cases showed radiographic evidence of periapical healing and showed calcific bridges either cervical and/or apical.		
Arora et al. 2020	Case series	9 patients with infected immature molars	Cell homing	Bleeding group	After 60 months, all teeth showed continued root development and maintained functionality. All cases in both groups showed complete healing after 3 months.	None responded to vitality testing.	
Elsheshtawy et al. 2020	Randomized controlled trial	26 patients with immature permanent anterior teeth with necrotic pulps	Cell homing	Group 1: PRP (test) Group 2: blood clot (control)	One tooth in the PRP group had signs of reinfection after 6 months. In both groups, there was increase in root lengths and dentinal root widths and decrease in the apical foramen width and periapical area diameter.	No change in pulp sensibility using thermal and electrical pulp testing	
			Cell homing				

Table 2: Continued.

Study	Study design				Outcomes		
	Blind; random; design	Animal model/human	Type of study	Groups	Primary outcomes Clinically and radiographically	Secondary outcomes Discoloration and sensibility test	Histology
Rizk et al. 2020	Double-blinded randomized controlled trial	26 patients with maxillary permanent immature central incisors		Group I: PRP (scaffold) Group II: PRF (scaffold)	All teeth were survived after 12 months. Both groups showed marginal increase in radiographic root length and width. Increase in periapical bone density Decrease in apical diameter		
Rizk et al. 2020	Split-mouth double-blind randomized controlled trial	15 patients with bilateral necrotic upper permanent central incisors with open apex	Cell homing	Group I: blood clot Group II: PRF	Apical diameter in the PRF group is greater than of the blood clot group.		

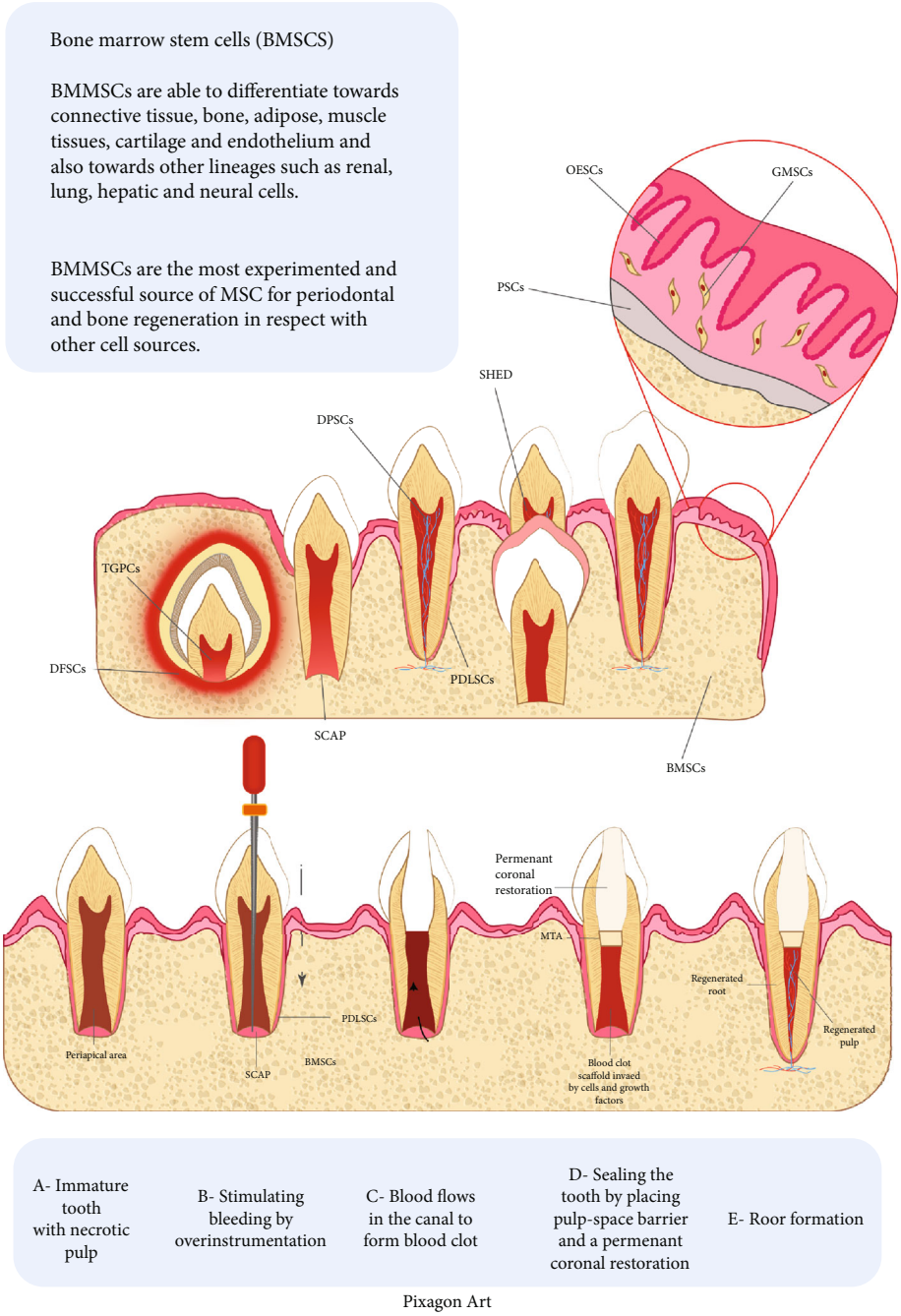

Figure 3: Different sources of stem/progenitor cells in the oral cavity and steps of revascularization.

morphogenetic proteins (BMPs), and VEGF [111]. PRF has proved to be an appropriate substitute to the blood clot technique, especially in cases where bleeding was very difficult to be obtained [107]. PRP and blood clotting technique used as scaffolds in immature traumatized permanent teeth with necrotic pulps also gave very good results [112]. In a clinical study on 30 patients with maxillary necrotic permanent immature central incisors, treating one group with PRP and the other with PRF scaffolds, teeth survived during the 12-month follow-up period. The teeth revealed marginal increase in radiographic root width and length, an increased periapical bone density, and a narrowing in apical diameter [113]. Other studies compared the effect of PRF, PRP, and the blood clot technique in the revascularization of necrotic teeth with open apex, demonstrating continued root development and maintenance of functionality, following different follow-up periods, yet with some teeth not responding to vital testing [2, 5, 6, 114–122]. A further investigation induced bleeding in root canals and used PRF in mature necrotic teeth, showing a regain in pulp sensibility [123]. In a further study, Kim et al. were able to regenerate tooth-like structure using cell homing approach [124].

Still, one of the drawbacks of the revascularization found among cases treated with this approach is the occasional intracanal calcification, which in some cases may progress to complete obliteration of root canals, affecting the normal function of the dental pulp tissues. This drawback could be attributed to multiple contributing factors such as the type of medicaments and the induction of intracanal bleeding [125, 126]. A recent review article evaluated the long-term outcomes of the apexification and the regenerative techniques in treating traumatized immature teeth with pulp necrosis and apical periodontitis, showing that the endodontic regenerative techniques appeared superior to apexification techniques in terms of root lengthening and root wall thickening [127].

1.7. Cell-Free Approach for Pulp-Dentin Complex Regeneration. Relying on "cell homing" concept, the cell-free approach is aimed at regeneration by enhancing proliferation, migration, and differentiation of intuitive stem/progenitor cells present near the root apex [128]. It was proposed that stem/progenitor cells' niches could initiate an appropriate microenvironment by releasing immunoregulatory molecules and enhancing paracrine effects to promote the differentiation of endogenous stem cells [129, 130]. Additionally, natural molecules and bioactive compounds have been proved to promote dentinogenesis [131, 132].

Conditioned medium (CM) can be described as the molecules released from living cells into the surrounding extracellular environment [133]. CM was found to stimulate cellular immunomodulation, proliferation, migration, and tissue regeneration [133–135] as it contains abundant amounts of proteins, lipids, nucleic acid, growth factors, cytokines, chemokines, and extracellular vesicles [136]. A recent study combined hDPSC conditioned medium with MTA for direct vital pulp therapy. It was assumed that the abundance of angiogenic growth factors such as PDGF, FGF, and VEGF [137] and immunomodulatory cytokines such as IL-6 and IL-8 [138] secreted by DPSCs and collected in hDPSCs' conditioned medium could modulate the inflammatory and regenerative processes in the dental pulp tissue, improve the orientation of the newly formed hard tissue, and enhance formation of dentin bridges [139].

Extracellular vesicles (EVs) derived from MSCs function as paracrine mediators in tissue regeneration and repair and resemble to a great extent the therapeutic efficacy of parental MSCs [140]. Extracellular vesicles (EVs) are defined by the MISEV2014 and the updated MISEV2018 as "particles naturally released from the cell that are delimited by a lipid bilayer membrane and are incapable of self-replication, i.e., do not contain a functional nucleus." EVs are a collective name including many subtypes of cell-released, membranous particles, known as microvesicles, microparticles, exosomes, oncosomes, ectosomes, and apoptotic bodies. EVs are characterized by the presence of luminal and transmembrane proteins and attenuation of extracellular or cellular non-EV proteins [141, 142]. The term "exosomes" usually refers to EVs that are formed by the endosomal system, opposite to ectosomes (microparticles and microvesicles) that bud from the plasma membrane. Particularly, intraluminal vesicles are unleashed into the extracellular environment as exosomes when the multivesicular body coalesces with the plasma membrane [143]. Exosomes are identified by their small diameter (40-100 nm) [144]. Moreover, they possess large amounts of tetraspanins (CD81, CD9, and CD63) and annexins, which are commonly used for their characterization [145].

Additionally, exosome vesicles were claimed to possess the ability to induce odontogenesis and augment dental pulp regeneration [146]. Accordingly, a study based on extracted exosome-like vesicles from rat Hertwig's epithelial root sheath (HERS) was tested. Dental pulp cells (DPCs) were united with HERS cell-derived exosome-like vesicles in an in vivo tooth root slice model, triggering the regeneration of hard reparative dentin-like tissue and soft tissue rich in blood vessels and neurons [147]. Moreover, in an interesting study, when SCAP-derived exosomes (SCAP-Exo) were put into a root slice containing BMMSCs and transplanted into immunocompromised mice, dentin and dental pulp-like tissues were formed in the root canal. Besides, when SCAP-Exo were evaluated in vitro, it was reported that dentin sialophosphoprotein expression and hard tissue deposition in BMMSCs treated with SCAP-Exo were significantly upregulated [148]. In another study, EVs were derived from DPSCs and EVs-fibrin gel constructs were manufactured as an in situ delivery system. Afterwards, DPSCs and endothelial cells were cocultured in the constructs. It was reported that EVs-fibrin gels promoted dental pulp regeneration by stimulating collagen deposition and enhancing angiogenesis through upregulating the expression of VEGF [149].

It is further well established that the usage of MSC-derived EVs possesses numerous advantages. First, it overcomes the ethical issues that limit the clinical translation of MSCs. Second, transplanting cells, which might have mutated DNA, can be avoided. Third, the dose of delivered MSCs rapidly declines posttransplant, in contrast to MSC-derived vesicles, which could attain a higher dose. Fourth, EVs are relatively small and can circulate easily, opposite to MSCs, which are too large to circulate smoothly via capillaries. However, the main disadvantage of utilizing MSC-derived vesicles is that they are static and cannot be produced in vivo. Moreover, the efficacy of EVs requires standard parameters to produce EVs of known content, develop storage techniques that preserve vesicle efficacy, and assess their therapeutic potential in well-controlled clinical trials [140].

2. Conclusion

Regenerative dentistry is no longer a dream, thanks to the current efforts to imply stem/progenitor cell-based techniques to enhance the regeneration of the pulp-dentin complex and to replace conventional endodontic pulp therapy. Yet, such novel therapies dictate careful testing first in vitro and in animal models, prior to human clinical translation [150]. Cell-based therapies still face many challenges, mainly economical and ethical concerns. Thus, efforts started to target cell homing for pulp-dentin complex regeneration as a simpler, safer, and reasonably priced approach compared to the cell-based transplantation therapy. However, the success and safety of MSCs administered via IV or IA routs, as well as directing such cells

towards the injured tissues, are not always guaranteed. Despite the great advancements in pulp-dentin complex regeneration through cell homing in the past years, they require further investigations and development. Cell homing techniques need to be examined in more realistic models, starting with animals then humans. Moreover, clinical trials are crucial to point out possible indications and contraindications. Thus, numerous aspects still need to be resolved to make it applicable and with predictable outcomes in clinical dental practice. The perspective of replacing conventional endodontic therapy, while retaining the tooth vitality in a practical and relatively safe way, provides hope for the clinical dental practice. Finally, any minor step towards the future is counted as an additional profit that must be preciously handled and searched thoroughly to be utilized later in the field of regenerative dentistry.

Abbreviations

AAE: American Association of Endodontists
BMMSCs: Bone marrow mesenchymal stem cells
BMPs: Bone morphogenetic proteins
BMP-2: Bone morphogenic protein-2
CCR2: C-C chemokine receptor type 2
CCR3: C-C chemokine receptor type 3
CCR4: C-C chemokine receptor type 4
CD105: Cluster of differentiation 105
CD44: Cluster of differentiation 44
CD49d ($\alpha4\beta1$): Integrin $\alpha4$
CD73: Cluster of differentiation 73
cDPSCs: Canine dental pulp stem cells
CRCT: Conventional root canal treatment
CX3CR1: CX3 chemokine receptor 1
CXCR4: C-X-C chemokine receptor type 4
CXCR7: C-X-C chemokine receptor type 7
DPCs: Dental pulp cells
DPSCs: Dental pulp stem cells
EB: Evoked bleeding
ECs: Endothelial cells
EDTA: Ethylenediaminetetraacetic acid
EGF: Epidermal growth factor
FGF: Fibroblast growth factor
G-CSF: Granulocyte colony-stimulating factor
GSK-3: Glycogen synthase kinase
HCELL: Hematopoietic cell E-/L-selectin ligand
hDPSCs: Human dental pulp stem cells
HERS: Hertwig's epithelial root sheath
HIF-1a: Hypoxia-inducible factor-1a
IA: Intra-arterial
IGF-1: Insulin-like growth factor-1
IL-1α: Interleukin-1 alpha
IL-1β: Interleukin-1 beta
IL-4: Interleukin-4
IL-6: Interleukin-6
IL-8: Interleukin-8
IL-10: Interleukin-10
IV: Intravenous
MDPSCs: Mobilized dental pulp stem cells
MMP-1: Matrix metalloproteinase-1
MMPs: Matrix metalloproteinases
MRI: Magnetic resonance imaging
MSCs: Mesenchymal stem/progenitor cells
MTA: Mineral trioxide aggregate
PDGF: Platelet-derived growth factor
pDPSCs: Pocrine dental pulp stem cells
PLLA: Poly L-lactic acid
PPP: Platelet-poor plasma
PRF: Platelet-rich fibrin
PRP: Platelet-rich plasma
RBMMSC: Rat bone marrow mesenchymal stem cells
REP: Regenerative endodontic procedures
SC: Stem cell
SCAP: Stem cells of apical papilla
SDF-1: Stromal cell-derived factor
SHED: Stem cells from human exfoliated deciduous teeth
SI: Signal intensity
SIM: Simvastatin
STRO-1: Stromal cell surface marker-1
TCP: Tricalcium phosphate
TDM: Treated dentin matrix
TGF$\beta1$: Transforming growth factor beta 1
TNF-α: Tumor necrosis factor
UCMSCs: Umbilical cord mesenchymal stem cells
VCAM-1 (CD106): Vascular cell adhesion molecule 1
VEGF: Vascular endothelial growth factor
VLA-4: Integrin VLA-4.

Data Availability

Data are available on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Clinical Efficacy and Safety of Human Mesenchymal Stem Cell Therapy for Degenerative Disc Disease: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Degenerative disc disease (DDD) can cause severe low back pain, which will have a serious negative impact on the ability to perform daily tasks or activities. For the past few years, mesenchymal stem cell (MSC) transplantation has emerged as a promising strategy for the treatment of DDD. However, the clinical efficacy of MSC in the treatment of DDD still lacks clinical evidence and is controversial. We conducted a meta-analysis with randomized controlled trials (RCTs) to evaluate the clinical efficacy and safety of MSC transplantation in patients with DDD. We searched major databases using terms from the database's inception through March 2021. The Cochrane bias risk assessment tool was used to assess quality. The analysis showed that MSC therapy could decrease visual analog scale (VAS) scores (SMD = −0.50, 95%CI = −0.68 ~ −0.33, $P < 0.00001$) and Oswestry Disability Index (ODI) scores (SMD = −0.27, 95%CI = −0.44 ~ −0.09, $P = 0.003$). The outcomes with subgroup analysis showed that MSC therapy could decrease VAS scores in 3 months ($P = 0.001$), 6 months ($P = 0.01$), 12 months ($P = 0.02$), and ≥24 months ($P = 0.002$) and ODI scores in ≥24 months ($P = 0.006$). Pooled analysis showed that MSC therapy has a higher ratio of patients at most thresholds but particularly at the MIC (minimally important change) ($P = 0.0002$) and CSC (clinically significant change) ($P = 0.0002$) in VAS and MIC ($P = 0.0005$) and CSC ($P = 0.001$) pain responders in ODI. Adverse events (AE) of treatment-emergent adverse events (TEAE), back pain, arthralgia, and muscle spasms were not statistically significant between the two groups. However, our further statistical analysis showed that MSC therapy may induce AE of TEAE related to study treatment (OR = 3.05, 95%CI = 1.11 ~ 8.40, $P = 0.03$). In conclusion, this study pooled the main outcomes and showed that MSC therapy could significantly decrease VAS and ODI scores in patients with DDD. Distinctly, the findings of this meta-analysis suggest a novel therapeutic strategy for patients with chronic low back pain (LBP) and lumbar dysfunction by DDD.

1. Introduction

Degenerative disc disease (DDD) is a multifaceted, progressive, and irreversible disease. It is an inescapable part of aging and may lead to a series of diseases or symptoms such as lumbar disc herniation, cervical spondylosis, discogenic pain, spinal stenosis, and spinal segment instability [1, 2]. DDD often results in severe LBP that would have a severe negative impact on the ability to perform daily tasks or activities [3]. This has developed into a public health problem that seriously affects the socioeconomic and quality of life of the people [4]. For patients who have failed conservative treatments (nonsteroidal anti-inflammatory drugs (NSAIDs), nonpharmacologic treatment with superficial heat, physical therapy, chiropractic, and/or acupuncture), it is particularly important to find new, safe, and effective treatment strategies [5]. Currently, the surgical treatment options for DDD mainly include laser, nucleus pulpoplasty, interbody fusion, and artificial disc replacement. Although these treatments have achieved good short-term results, long-term outcomes are affected by the high probability of recurrent pain. There is literature regarding the poor efficacy of spine fusion for treating LBP [6]. In addition, none of the previously mentioned treatment regimens could alleviate or reverse the course of disc

degeneration. Disc removal could also lead to further deterioration of the degeneration due to spinal instability [7, 8].

Therefore, the focus of current research on DDD is increasingly turning to regenerative methods to slow down continuous degenerative cascade. The degenerative disc microenvironment must stabilize in order to achieve this goal and eventually return to a normal physiological state. So, the potential therapeutic strategies include (a) reversing protein expression of proinflammatory cytokines/proteinases in intervertebral disc cells, (b) blocking proinflammatory cytokines/proteases in extracellular matrix, (c) producing new extracellular matrix in intervertebral disc cells by intervention, and (d) addition of additional cells to support regeneration in a degenerative intervertebral disc environment [9]. One promising therapeutic strategy for inducing intervertebral disc regeneration may be the use of progenitor cells and stem cells [10]. In recent years, the use of MSC for intervertebral disc regeneration has been most widely studied [10]. MSC has excellent immune privilege and immune evasion and suppresses ongoing immune response in a way that is not restricted by the human leukocyte antigen system [11, 12]. In the first place, we found that many animal studies of mesenchymal stem cell transplantation for DDD are particularly promising. Yim et al. [13] included 24 animal studies on MSC transplantation for DDD. All three types of mesenchymal stem cells have certain advantages in inhibiting intervertebral disc degeneration. To sum up, evidence suggested that MSC transplantation could increase disc space height in animal models. Bracingly, Orozco et al. [14] studied the injection of bone marrow MSC in 10 patients diagnosed with DDD and chronic back pain. After MSC transplantation treatment, 85% of patients had significantly reduced lumbar pain and disability in 3 months. After 6 and 12 months, a significant increase in the water content of the patient's intervertebral discs was observed, with moderate improvements [14]. Meanwhile, Noriega et al. [15] conducted a RCT in which 24 patients had been diagnosed with DDD. In the MSC group, bone marrow MSC were injected into each intervertebral disc. Results showed that the pain and disability of patients were significantly reduced at 3 months after MSC transplantation. Of greatest concern, the results of the study found no difference in VAS scores between the MSC transplantation treatment group and the control group at 12 months. Another RCT result also found that after MSC+allograft treatment, VAS score and ODI score decreased compared to baseline, but there was no statistical difference in 3, 6, and 12 months, compared to the control group with standard graft material [16]. The clinical efficacy of MSC transplantation in the treatment of DDD remains controversial. Recently, Noriega et al. [17] published long-term 42-month follow-up results of the RCT showing good differences after MSC treatment. Our team believes that for patients with DDD, choosing MSC transplantation is a positive effect on improving patients' VAS and ODI. However, there is currently no high-quality evidence-based medicine to support. In order to further explore the efficacy and safety of MSC transplantation in the treatment of DDD, we conducted this meta-analysis of published RCTs to study the therapeutic evidence of human MSC transplantation for DDD.

2. Materials and Methods

The detailed protocol for this study was designed in accordance with the Cochrane intervention review. The entire project has been registered on the PROSPERO website (CRD42021248707). The whole design and writing process of this meta-analysis were done one-to-one according to the of PRISMA.

2.1. Literature Search. We searched major databases including PubMed, Embase, and ClinicalTrials.gov using terms from the database's inception through March 2021. We screened the literature based on the participants, interventions, comparisons, outcomes, and study (PICOS) approach. The terms included the following: (1) degenerative disc disease, DDD, intervertebral disc repair, intervertebral disc degeneration, lumbar disc degeneration, and disc degeneration; (2) mesenchymal stem cell and allogeneic mesenchymal precursor cells; and (3) randomized controlled trials.

2.2. Extraction of Study Data. Two data extractors (He RR and Chen SC) screened the full text of MSC in the treatment of DDD and extracted the main observation indicators. The extraction of experimental data is mainly filled in the data extraction form table designed in advance. Disputable data were resolved through a third independent investigator (Xu YX). The main aspects of data extraction were registration, number of participants, age, treatment strategy, duration, and observed outcome.

2.3. Risk Assessment of Bias in Included Studies. To further address the risk of bias between included studies, we use the Cochrane risk of bias tool to assess the quality of the literature one by one for the included studies.

2.4. Outcome Indicators. (1) The efficacy outcomes are as follows: visual analog scale (VAS) of 3, 6, and 12 months or ≥24 months and Oswestry Disability Index (ODI) of 3, 6, and 12 months or ≥24 months. (2) The other efficacy outcomes are as follows: MIC and CSC of 6 and 12 months or ≥24 months. (3) The safety outcomes are as follows: adverse events (AE) of MSC therapy for DDD.

2.5. Inclusion and Exclusion Criteria. The inclusion criteria are as follows: (1) RCTs of studies; (2) participants with DDD; (3) the MSC group received MSC treatment and the control group received HA or rehabilitation treatment; and (4) follow-up time was longer than 3 months. Exclusion criteria are as follows: (1) nonrandomized trials; (2) ongoing RCTs without outcomes; (3) review, systematic review, or meta-analysis; and (4) case reports, prospective, or retrospective cohort studies.

2.6. Data Synthesis and Analysis. In this study, Review Manager 5.3 and Stata 12.0 were used to conduct statistics and analysis on the data of multiple outcomes, respectively. If the outcomes were dichotomous data, we analyzed the data by odds ratio (OR) and 95% confidence intervals (CIs). The contiguous data to be merged were represented by a standardized mean difference (SMD) and 95% CI. The χ^2

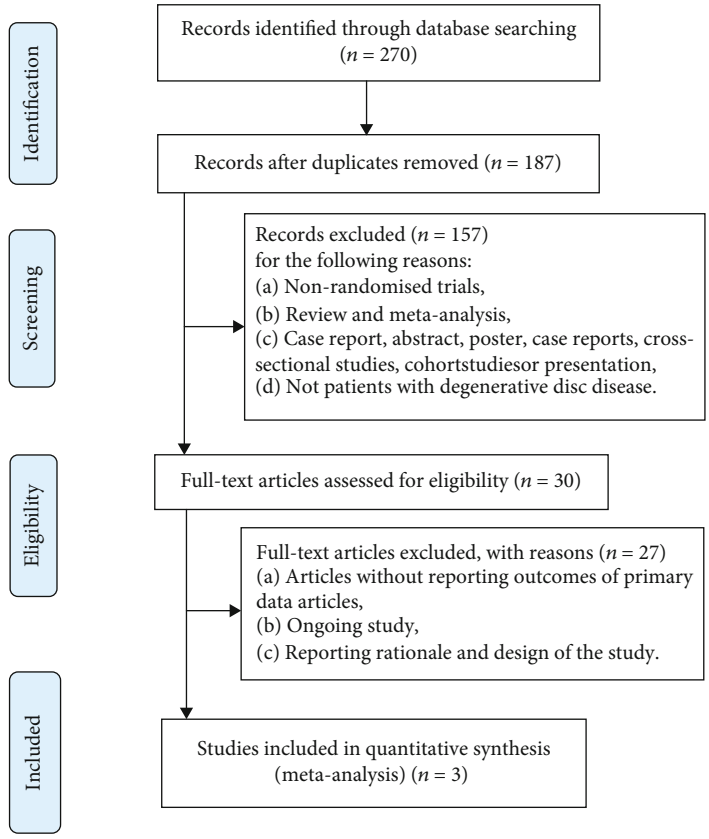

Figure 1: Flowchart of the retrieval strategy.

test and I^2 statistic were used to calculate the heterogeneity among the studies. The quantitative value of $I^2 < 25\%$ indicated mild inconsistency between the studies, and I^2 that ranged from 25% to 50% indicated moderate heterogeneity. If the $I^2 > 50\%$, this indicates that the study has serious heterogeneity. We would conservatively use the random-effect model to evaluate the statistical significance of the pooled outcomes, so as to reduce the influence of heterogeneity of this study. If $I^2 < 50\%$, the fixed-effect model was used for analysis. Subgroup analyses was used to study effect size of VAS of 3, 6, and 12 months or ≥24 months and ODI of 3, 6, and 12 months or ≥24 months.

3. Results

3.1. Screening of Studies. In this study, through the systematic retrieval of major databases, a total of 270 citations were retrieved from the establishment of the database to March 2021. 83 duplicate studies were excluded after importing the retrieved literature into NoteExpress. After reading the abstracts, 157 studies were excluded for several reasons: (a) nonrandomized trials; (b) systematic review or review; and (c) case reports, cross-sectional studies, and prospective or retrospective cohort studies. Next, after reading the full text, we excluded 27 ongoing studies and studies that were only basic principles and designs. In the end, we included three studies on MSC transplantation in the treatment of DDD for analysis (Figure 1).

3.2. Characteristics of Each Study. After screening of the inclusion criteria, 3 studies [15, 17, 18] with 104 participants were finally included in the analysis. The MSC group was treated with MSC, and the control group was treat with HA or mepivacaine. The registration numbers of two RCTs were NCT01290367 and NCT01860417. The MSC therapy was used in three RCTs that were allogeneic mesenchymal precursor cells and allogeneic mesenchymal stem cells. The amount of MSC transplants in the study of Amirdelfan et al. [18] was 6×10^6 and 1.8×10^7. The dosage of MSC transplants in the other RCT was 2.50×10^7. The efficacy outcomes were VAS of 3, 6, and 12 months or ≥24 months and ODI of 3, 6, and 12 months or ≥24 months. The other efficacy outcomes were MIC and CSC of 6 and 12 months or ≥24 months. The safety outcomes were AE of MSC therapy for DDD (Table 1).

3.3. Quality Assessment of Study. The clinical trials of Amirdelfan et al. [18] were divided into two groups according to a central randomization schedule and randomization list. In terms of selection bias, we assessed them as "low risk" studies. The study of Noriega et al. [15] did not explicitly address the randomized approach, which we assessed as "unclear" of selection bias. The clinical trial of Amirdelfan et al. [18] reported that the participants and radiographic reviewer were blinded to the assigned treatment but the investigator was not blinded. So, we assessed it as "unclear risk" in selection bias and performance bias. The trial of Noriega et al.

Table 1: Characteristics of each study.

Study	Registered number	Design	Participants	Intervention		Dosage	Follow-up	Outcomes
				MSC group	Control group			
Amirdelfan et al. [18] 2021	NCT01290367	RCT	80	MSC+HA	HA	6×10^6, 1.8×10^7	36 m	VAS,ODI, AE, CSC, MIC
Noriega et al. [17] 2021	NCT01860417	RCT	24	MSC	Mepivacaine	2.50×10^7	42 m	VAS, ODI, AE
Noriega et al. [15] 2017	NCT01860417	RCT	24	MSC	Mepivacaine	2.50×10^7	12 m	VAS, ODI, AE

Note: AE: adverse event; CSC: clinically significant change; HA: hyaluronic acid; ODI: Oswestry Disability Index; MIC: minimally important change; MSC: mesenchymal stem cell; RCT: randomized controlled trial; VAS: visual analog scale.

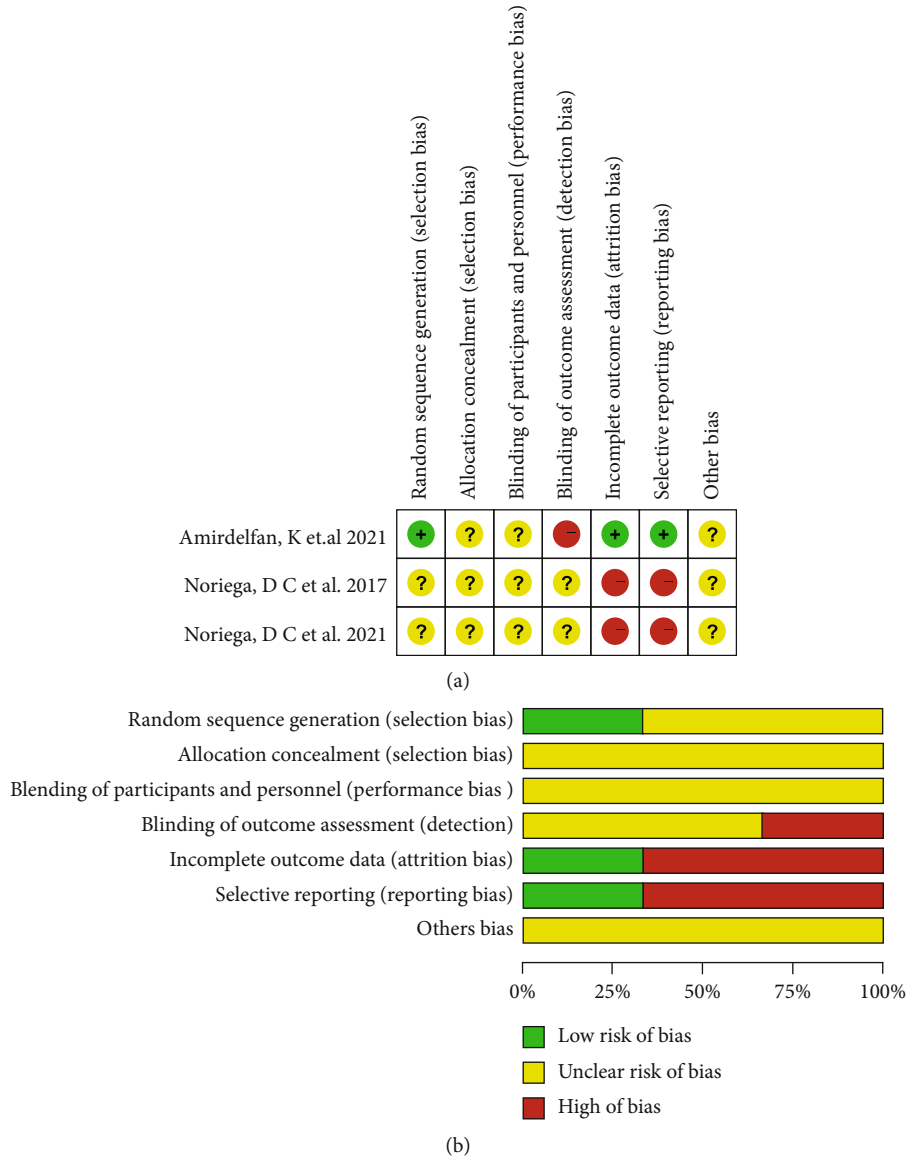

Figure 2: Quality evaluation of studies by the Cochrane collaboration manual. (a) Item-by-item detailed analysis of the summary of the risk of bias in studies. (b) The risk bias graph shows the quality summary of the study.

[15] reported that the participant, care provider, and outcome assessor were blinded, but we did not find the specific implementation plan for blinding, so we assessed it as "unclear risk" in detection bias, selection bias, and performance bias. Amirdelfan et al. [18] fully reported the outcome measures including the number of people lost to follow-up and the reasons for dropping out. We assessed it as "low risk of bias" in attrition bias and reporting bias.

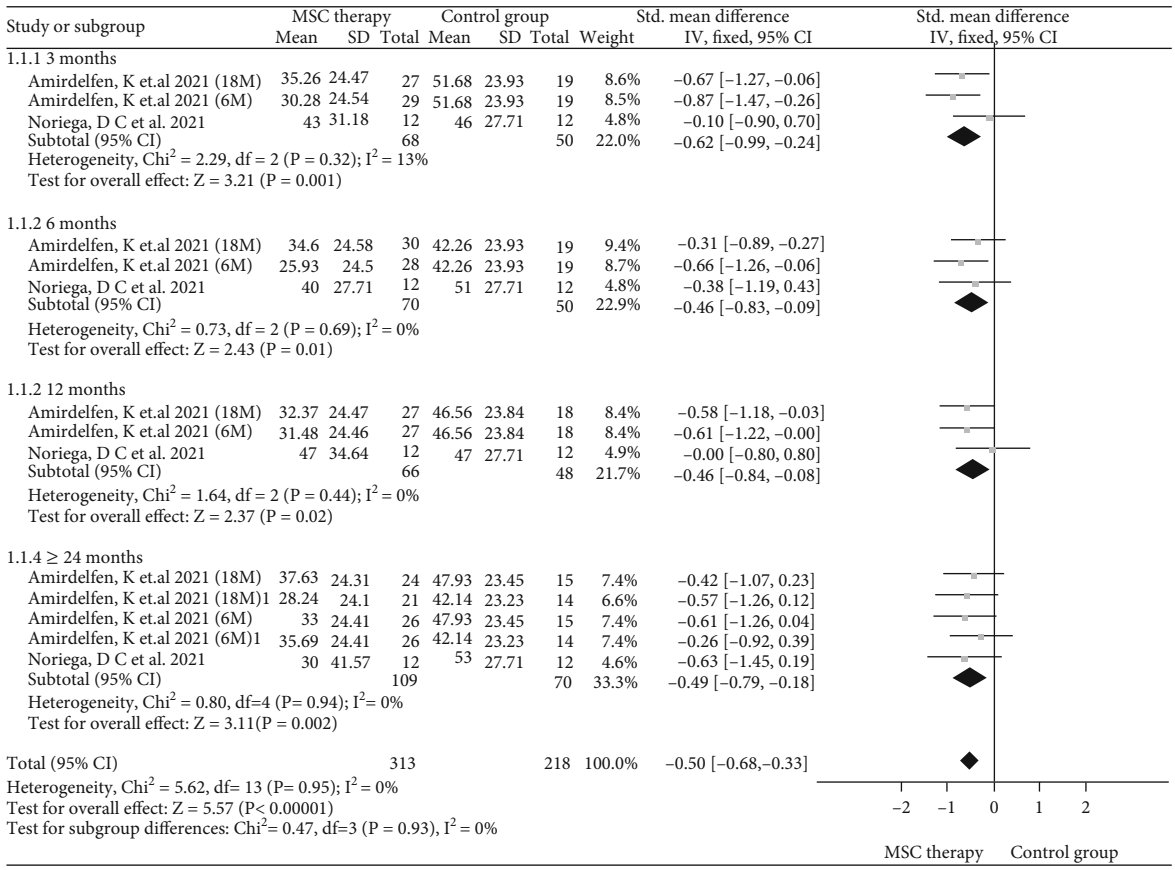

Figure 3: Forest plot of VAS scores between MSC therapy and control group.

The clinical trial of Noriega et al. [15] did not report the number of people lost to follow-up or dropped out, so we assessed it as "high risk of bias" in attrition bias and reporting bias (Figure 2).

3.4. Visual Analog Scale. VAS was reported in three studies [15, 17, 18] of MSC therapy and control group. We used a fixed-effect model to evaluate the statistical significance of the pooled analysis after testing for heterogeneity ($I^2 = 0 < 50\%$). The result of a meta-analysis showed that MSC therapy could significantly decrease VAS scores (SMD = −0.50, 95%CI = −0.68 ~ −0.33, $P < 0.00001$), compared with the control group. Subgroup analysis of VAS scores is as follows: the result with a fixed-effect model showed that MSC therapy could significantly decrease VAS scores in 3 months (SMD = −0.62, 95%CI = −0.99 ~ −0.24, $P = 0.001$), 6 months (SMD = −0.46, 95%CI = −0.83 ~ −0.09, $P = 0.01$), 12 months (SMD = −0.46, 95%CI = −0.84 ~ −0.08, $P = 0.02$), and ≥24 months (SMD = −0.49, 95%CI = −0.79 ~ −0.18, $P = 0.002$) (Figure 3).

3.5. MIC and CSC Responders in VAS. We used a fixed-effect model to evaluate the statistical significance of MIC and CSC responders in VAS of patients. Pooled analysis showed that MSC therapy has a high ratio of patients at most thresholds, especially in MIC (change ≥ 30% from baseline) (OR = 2.16, 95%CI = 1.43 ~ 3.25, $P = 0.0002$) and CSC (change ≥ 50% from baseline) (OR = 2.18, 95%CI = 1.44 ~ 3.31, $P = 0.0002$) thresholds. The pain responder rates of MIC and CSC for 2 groups in 6, 12, and ≥24 months are showed in Table 2.

3.6. Oswestry Disability Index. ODI scores were reported in three studies [15, 17, 18] of MSC therapy and control group. A fixed-effect model was used with heterogeneity analysis ($I^2 = 0\%$). The result of the meta-analysis showed that MSC therapy could decrease ODI scores (SMD = −0.27, 95%CI = −0.44 ~ −0.09, $P = 0.003$) (Figure 4). Subgroup analysis of ODI is as follows: the result with the fixed-effect model found that MSC therapy could significantly decrease ODI scores in ≥24 months (SMD = −0.43, 95%CI = −0.74 ~ −0.12, $P = 0.006$) in patients with DDD (Figure 4). However, no statistical differences were found in the subgroup analysis in 3, 6, and 12 months.

3.7. MIC and CSC Pain Responders in ODI. We used a fixed-effect model to evaluate the statistical significance of MIC and CSC pain responders in ODI of patients. Pooled analysis showed that MSC therapy has a high ratio of patients at most thresholds, especially in MIC (change ≥ 10 − point ODI from baseline) (OR = 2.06, 95%CI = 1.37 ~ 3.10, $P = 0.0005$) and CSC (change ≥ 15 − point ODI from baseline) (OR = 2.01, 95%CI = 1.33 ~ 3.05, $P = 0.001$) thresholds. The pain responder rates of MIC and CSC in 6, 12, and ≥24 months are showed in Table 2.

Table 2: Subgroup analysis.

Responder analyses	6 months	12 months	24 months	36 months	Overall effect
Pain responder					
MIC in VAS	OR = 2.53 [1.10, 5.84], $P = 0.03$	OR = 2.79 [1.22, 6.36], $P = 0.02$	OR = 2.12 [0.93, 4.48], $P = 0.07$	OR = 1.49 [0.67, 3.34], $P = 0.33$	OR = 2.16 [1.43, 3.25], $P = 0.0002$
CSC in VAS	OR = 2.59 [1.14, 5.90], $P = 0.02$	OR = 3.05 [1.31, 7.12], $P = 0.01$	OR = 1.78 [0.76, 4.16], $P = 0.18$	OR = 1.63 [0.71, 3.71], $P = 0.25$	OR = 2.18 [1.44, 3.31], $P = 0.0002$
ODI responder					
MIC in ODI	OR = 2.16 [0.95, 4.92], $P = 0.07$	OR = 1.86 [0.84, 4.15], $P = 0.13$	OR = 2.12 [0.93, 4.84], $P = 0.07$	OR = 2.12 [0.93, 4.84], $P = 0.07$	OR = 2.06 [1.37, 3.10], $P = 0.0005$
CSC in ODI	OR = 1.60 [0.71, 3.58], $P = 0.25$	OR = 1.86 [0.82, 4.23], $P = 0.14$	OR = 2.04 [0.88, 4.47], $P = 0.10$	OR = 2.81 [1.17, 6.74], $P = 0.02$	OR = 2.01 [1.33, 3.05], $P = 0.001$

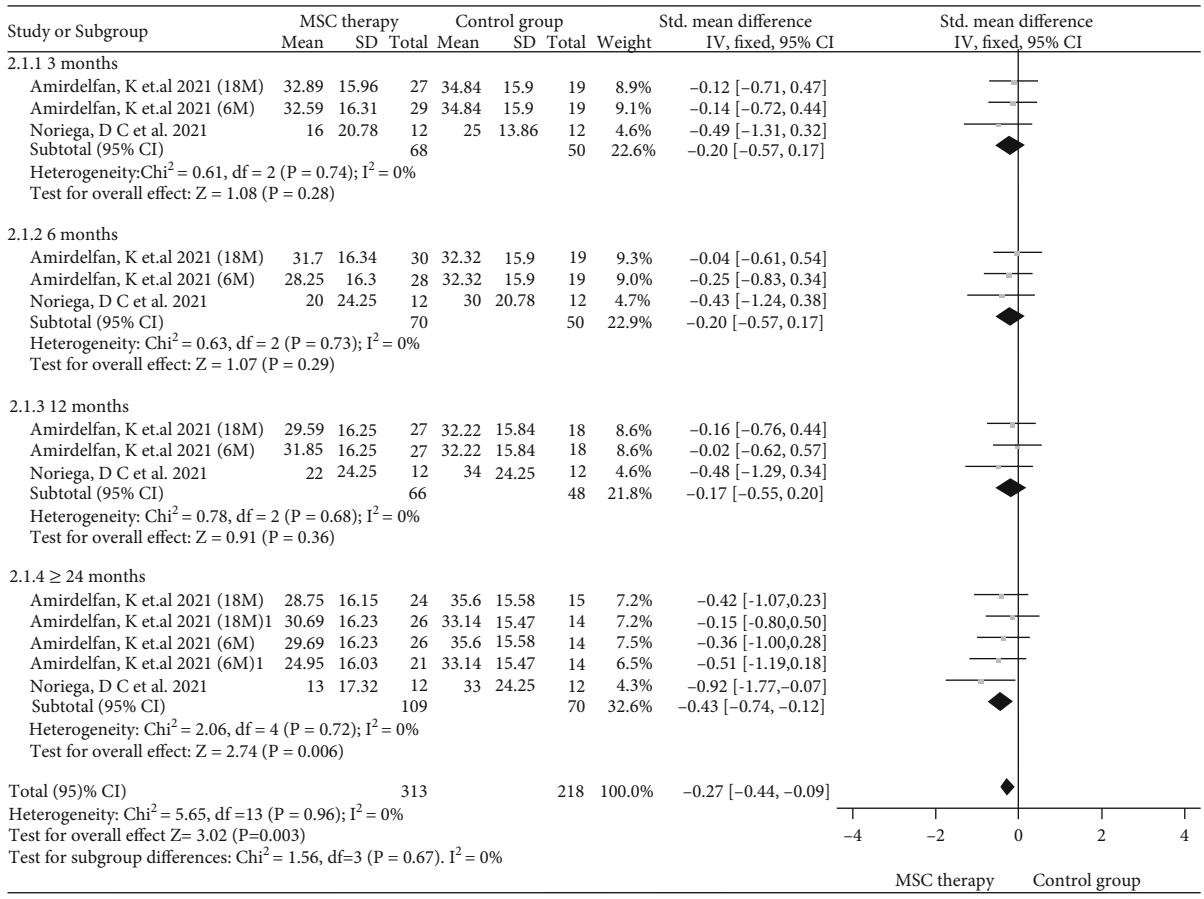

Figure 4: Forest plot of ODI scores between MSC therapy and control group.

3.8. Adverse Event. To further clarify the safety of MSC transplantation for DDD, meta-analysis was performed on the occurrence of AE. The result showed that AE of treatment-emergent adverse events (TEAE) (OR = 1.11, 95 %CI = 0.40 ~ 3.07, $P = 0.84$), back pain (OR = 1.23, 95%CI = 0.55 ~ 2.76, $P = 0.62$), arthralgia (OR = 0.63, 95%CI = 0.19 ~ 2.11, $P = 0.45$), and muscle spasms (OR = 2.11, 95% CI = 0.40 ~ 11.01, $P = 0.38$) were not statistically significant between two groups. However, our further statistical analysis showed that MSC therapy may induce AE of TEAE related to study treatment (OR = 3.05, 95%CI = 1.11 ~ 8.40, $P = 0.03$) (Table 3).

4. Discussion

4.1. Primary Efficacy Outcomes. Pain assessment is the prerequisite for pain treatment for chronic LBP by DDD. Accurate and timely assessment of pain can provide necessary guidance and assistance for clinical treatment and is the key to effective pain treatment [19]. The VAS is the most

Table 3: Adverse event analysis.

AE	OR and 95% CI	P
TEAE	OR (1.11), 95% CI (0.40, 3.07)	0.84
TEAE related to study treatment	OR (3.05), 95% CI (1.11, 8.40)	0.03
Back pain	OR (1.23), 95% CI (0.55, 2.76)	0.62
Arthralgia	OR (0.63), 95% CI (0.19, 2.11)	0.45
Muscle spasms	OR (2.11), 95% CI (0.40, 11.01)	0.38

commonly used scoring index in pain assessment [20]. The specific practice of VAS is to draw a horizontal line of 10 cm on the paper, and one end of the horizontal line is 0, indicating painless; the other end is 10, which means severe pain; and the middle part represents different levels of pain. The specific clinical assessment was as follows: (1)*Mild pain (1-3 points)*: the patient had pain but tolerable, lived a normal life, and had no disturbance in sleep. (2) *Moderate pain (4-6 points)*: the pain is obvious and intolerable, the patient is required to take analgesic drugs, and his sleep is disturbed. (3) *Severe pain (7-10 points)*: severe pain, unbearable pain, need to use analgesic drugs, severe sleep disturbance, autonomic nervous disorder, or passive posture. Kumar et al. [21] performed a trial of 10 eligible DDD patients. The result showed that primary efficacy outcomes of VAS for low back pain were significantly reduced in 1 month, 3 months, and 6 months after adipose tissue-derived MSC transplantation. The RCT of Noriega et al. [15] showed that VAS scores were significantly reduced at 3 months and 6 months after MSC transplantation. Of greatest concern, the study found that VAS scores increased at 12 months after MSC transplantation. Recently, Noriega et al. [17] published long-term 42-month follow-up results of the RCT. The result showed that MSC therapy could significantly decrease VAS scores at 42 months, compared with the control group in patients with DDD. In this study, we integrated the results of three RCTs, and the results showed that MSC therapy could significantly decrease VAS scores ($P < 0.00001$). A subgroup analysis of VAS scores was used for analysis. The result showed that MSC therapy could significantly decrease VAS scores in 3 months ($P = 0.001$), 6 months ($P = 0.01$), 12 months ($P = 0.02$), and ≥24 months ($P = 0.002$) in patients with DDD. Our study with a small sample size also found that MSC therapy has a high ratio of patients at most thresholds, especially in MIC ($P = 0.0002$) and CSC ($P = 0.0002$) thresholds. Our research results indicated that MSC therapy has shown excellent efficacy in reducing the VAS score of patients with DDD, whether it is short-term treatment or long-term follow-up.

ODI can accurately and reliably assess the treatment effect of patients with chronic LBP and the lumbar dysfunction by DDD [22]. ODI is composed of 10 questions, including the intensity of self-care, lifting objects, pain, sitting, standing, walking, disturbing sleep, social activities, sex life, and travel. There are 6 options for each question, and the maximum score for each question is 5 points. The higher the score, the more severe the patient's dysfunction [23]. Pang et al. [24] studied the feasibility and safety of MSC transplantation for patients with chronic discogenic LBP. After 2 years of follow-up, it was found that after MSC transplantation, the intervertebral disc pain was alleviated, and the ODI score was also significantly reduced. The RCTs of Noriega et al. [15, 17] showed that ODI scores were significantly reduced at 3, 6, 12, and 42 months after MSC transplantation. The results suggest that MSC transplantation is a potential alternative for the treatment of chronic discogenic LBP. In our study, ODI scores were reported in four studies of MSC therapy and control group. Pooled analysis indicated that MSC therapy could significantly decrease ODI scores ($P = 0.003$). Subgroup analysis found that MSC therapy could significantly decrease ODI scores in ≥24 months ($P = 0.006$) in patients with DDD. However, there was no statistically significant difference between 3, 6, and 12 months, but the ODI score had a tendency to decrease, which we believe may be caused by the insufficient sample size of the included studies. We evaluated the statistical significance of MIC and CSC pain responders in ODI of patients. Pooled analysis showed that MSC therapy has a high ratio of patients at most thresholds, especially in MIC ($P = 0.0005$) and CSC ($P = 0.001$) thresholds. To sum up, the results of this meta-analysis suggest that MSC transplantation could significantly alleviate pain and functional degradation in patients with DDD.

4.2. Primary Safety Outcomes. MSC has strong attractiveness and application prospects due to their low immunogenicity, easy access, and immunosuppressive potential [25–27], but the safety of MSC is still the first priority. Studies have shown that the quality of MSC depends more on the isolation conditions, cell culture technology, age of the donor, genetic characteristics, and medical history between different donors [28–30]. The quality difference of MSC is closely related to the AE in patients. To further clarify the safety of MSC transplantation for DDD, a meta-analysis was performed on the occurrence of AE. The result showed that AE of TEAE ($P = 0.84$), back pain ($P = 0.62$), arthralgia ($P = 0.45$), and muscle spasms ($P = 0.38$) was not statistically significant between two groups. However, our further statistical analysis showed that MSC therapy may induce AE of TEAE related to study treatment ($P = 0.03$). This study showed that there were no differences in serious AE of MSC transplantation for patient with DDD, compared with the control group. However, it is vital to pay close attention to whether the AE of patients is directly related to MSC therapy.

4.3. Limitations and Publication Bias. We found that there was mild inconsistency between the studies in VAS and

ODI scores. We analyzed the sensitivity of VAS and ODI scores. The analysis showed that the conclusion was credible, and there was no substantial change of the results with VAS and ODI scores. However, it should not be ignored that a few studies seriously limited the further analysis of publication bias and heterogeneity. To further address the risk of bias between included RCTs, we evaluated the quality of the literature using the Cochrane risk of bias tool. Our evaluation found that the included studies had a low risk of selection bias of randomization. The study describes the generation method of a random assignment sequence in detail. We believe that the results are reliable and stable to a certain extent, but we cannot rule out publication bias caused by a small sample size.

5. Conclusions

This meta-analysis pooled the main outcomes and showed that MSC therapy could significantly decrease VAS and ODI scores in patients with DDD. Distinctly, the findings of this meta-analysis suggest a novel therapeutic strategy for patients with chronic LBP and lumbar dysfunction by DDD. But, what is the optimal dose, frequency, time, and route of MSC transplantation at different stages of DDD? These crucial problems urgently require more randomized controlled trials to solve.

Conflicts of Interest

There is no conflict of interest in the publication of this paper.

Authors' Contributions

Baocheng Xie, Shichun Chen, and Yongxiang Xu contributed equally to this work. Weichao Han, Minyi Chen, and Runkai Hu reviewed the literatures. Yongxiang Xu and Ruirong He collected the data, and Shichun Chen analyzed the data. Shaobo Ding and Baocheng Xie designed this study. Ruirong He and Shaobo Ding are the cocorresponding authors.

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Review Article

Strategies to Improve the Quality and Freshness of Human Bone Marrow-Derived Mesenchymal Stem Cells for Neurological Diseases

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Human bone marrow-derived mesenchymal stem cells (hBM-MSCs) have been studied for their application to manage various neurological diseases, owing to their anti-inflammatory, immunomodulatory, paracrine, and antiapoptotic ability, as well as their homing capacity to specific regions of brain injury. Among mesenchymal stem cells, such as BM-MSCs, adipose-derived MSCs, and umbilical cord MSCs, BM-MSCs have many merits as cell therapeutic agents based on their widespread availability and relatively easy attainability and in vitro handling. For stem cell-based therapy with BM-MSCs, it is essential to perform ex vivo expansion as low numbers of MSCs are obtained in bone marrow aspirates. Depending on timing, before hBM-MSC transplantation into patients, after detaching them from the culture dish, cell viability, deformability, cell size, and membrane fluidity are decreased, whereas reactive oxygen species generation, lipid peroxidation, and cytosolic vacuoles are increased. Thus, the quality and freshness of hBM-MSCs decrease over time after detachment from the culture dish. Especially, for neurological disease cell therapy, the deformability of BM-MSCs is particularly important in the brain for the development of microvessels. As studies on the traditional characteristics of hBM-MSCs before transplantation into the brain are very limited, omics and machine learning approaches are needed to evaluate cell conditions with indepth and comprehensive analyses. Here, we provide an overview of hBM-MSCs, the application of these cells to various neurological diseases, and improvements in their quality and freshness based on integrated omics after detachment from the culture dish for successful cell therapy.

1. Introduction

Bone marrow-derived mesenchymal stem cells (BM-MSCs) have many merits as cell therapeutic agents, such as comparably easy in vitro handling, high plasticity, widespread availability, and immunosuppressive activity [1–3]. In addition, they have beneficial characteristics, such as anti-inflammatory, immunomodulatory, paracrine, and antiapoptotic ability, as well as homing capacity to the region of brain injury. Particularly, BM-MSCs can suppress inflammatory conditions in the central nervous system (CNS) and home to inflammatory brain injury [4–9]. To date, there have been many drugs developed to reduce the symptoms of CNS diseases because of irreversible neurological damage and limited regeneration in the brain, but these are associated with many adverse effects [10–12]. Therefore, BM-MSCs are a promising approach to treat neurological diseases, such as ischemia, traumatic brain injury, and neurodegenerative diseases, owing to their anti-inflammatory and immunomodulatory effects on such CNS neurological diseases [3, 13–16].

Most studies on BM-MSC-based therapies for neurological diseases have focused on the paracrine effects, immunomodulatory effects, and neuronal replacement through differentiation [17–19]. In addition, the MSC-based cell therapies have been applied to neurological diseases, which have no effective alternative treatments. Andrzejewska et al. summarized the application of MSC on the neurological diseases, including stroke, brain injury, Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and spinal cord injury, with experimental and clinical aspect [20]. Mukai et al. focused on clinical trial of the MSC transplantation in the neurological diseases with detailed condition of the clinical trials [21]. Moreover, Namestnikova et al. reported advantages of combinatorial methods, which are combination of coadministration of different stem/progenitor cell types, for the neurological diseases in animal and clinical study [22]. However, studies on the characteristics of human (hBM-MSCs) before transplantation are very limited. Depending on timing, before hBM-MSC transplantation into patients, after detaching these cells from the culture dish, cell viability, deformability, cell size, and membrane fluidity decrease, whereas reactive oxygen species (ROS) generation, lipid peroxidation, and cytosolic vacuoles increase, as shown in Figure 1 [9, 23].

hBM-MSC transplantation into patients is associated with an inevitable time-delay after cell detachment from the culture dish owing to various factors, including the injection formulation, transportation, and surgery preparation. Thus, an assessment of the quality and freshness of hBM-MSCs is important for successful hBM-MSC-based cytotherapy outcomes, and studies have tried to evaluate and preserve the quality and freshness of hBM-MSCs [23, 25]. However, conventional cell-based methods for evaluation, such as cell viability assays, fluorescence-activated cell sorting-based methods, and ultrastructural analysis, do not reveal the mechanism underlying changes in the quality and freshness of hBM-MSCs. Thus, omics, including genomics, transcriptomics, proteomics, and metabolomics, yield comprehensive information and can be interpreted using bioinformatic analysis [24, 26–31]. These technologies have been introduced to analyze the mechanism of changes in the hBM-MSCs status [9, 23]. Especially, the integration of transcriptomics and metabolomics with amino acid profiles is helpful to elucidate the quality and freshness of hBM-MSCs over time after trypsinizing cells [23]. Recently, advanced analysis has been used for the integration of omics, identification and in silico prediction of biological functions, and screening of upstream regulator molecules [32]. Moreover, discrimination methods, such as machine learning algorithms, have been used for investigations of correlations among each omics dataset, based on the large amount of data acquired from multiomic analysis. To evaluate and maintain the quality and freshness of hBM-MSCs, comprehensive multiomic analysis (big data) and proper machine learning algorithms for analyses of correlations within data are highly recommended rather than target approaches, according to the complexity of cellular changes after detachment from the culture dish. Here, we review three topics as follows: (i) hBM-MSCs, (ii) the application of hBM-MSCs to various neurological diseases, and (iii) the improvement of the quality and freshness of BM-MSCs after detachment from the culture dish for successful cell therapy.

2. hBM-MSCs

MSCs were first discovered in the bone marrow by Friedenstein in the 1970s [33, 34]. These cells are nonhematopoietic multipotent adult stem cells that are plastic-adherent with great capacity for proliferation, self-renewal, and differentiation [35, 36]. MSCs can be obtained not only from bone marrow but also from various tissues, such as adipose tissue, placenta, umbilical cord, and peripheral blood [37–40]. Although MSCs can be of different tissue origins, they must meet the minimal criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) as follows: (1) maintenance of plastic adherence; (2) ≥95% of the MSCs express surface molecules, such as CD73, CD90, and CD105, and do not express surface molecules, such as CD19 and HLA class I or CD11b, CD79a or CD45, CD34, and CD14; (3) capacity of trilineage differentiation in vitro into adipocytes (fat), osteoblasts (bone), and chondrocytes (cartilage); and (4) immunomodulatory activity [17, 41–46]. Additionally, MSCs can be differentiated into nonmesodermal-origin cells, including neurons, hepatocytes, cardiomyocytes, hepatocytes, and epithelial cells, which are of ectodermal and endodermal lineages [47–51].

Among MSCs, BM-MSC-based therapies have been promising strategy in preclinical and clinical trials based on tissue regeneration and wound healing attributed to the cell engraftment and differentiation properties of MSC [45, 52–55]. However, recent approaches for BM-MSC therapies have focused on paracrine effects in which MSC-derived vesicles are secreted containing various contents, such as soluble cytokines, growth factors, hormones, and miRNA, from immune cells and damaged tissues. This effect finally improves the efficacy of BM-MSC therapy [56–59]. As many studies have been reported regarding the efficacy of using exosomes derived from BM-MSCs on diverse diseases [59, 60], such BM-MSC-based therapies have been continuously suggesting to be promising strategies for clinical application to various neurological diseases [16, 20, 61, 62]. In addition, obtaining hBM-MSCs from adult tissue can avoid controversy regarding the ethical issues associated with the use of embryonic sources [63, 64]. Owing to these advantages, hBM-MSCs have strong potential in neurological diseases as a therapeutic tool.

3. Application of hBM-MSCs in Neurological Diseases

Neurological diseases, which cause neurological impairment, are characterized by irreversibility and progressive disorders, resulting in deterioration of the performance of regular activities because of the limited regenerative capacity for lost neurons and glial cells [16, 20, 65]. However, the landscape of treatment is limited, with restricted treatment options [16, 65]. Stem cell therapy, from preclinical to clinical trials based on the fundamental characteristics of stem cells, has shown promise as a potential treatment or to at least prevent progressive deterioration with neurological diseases, spinal

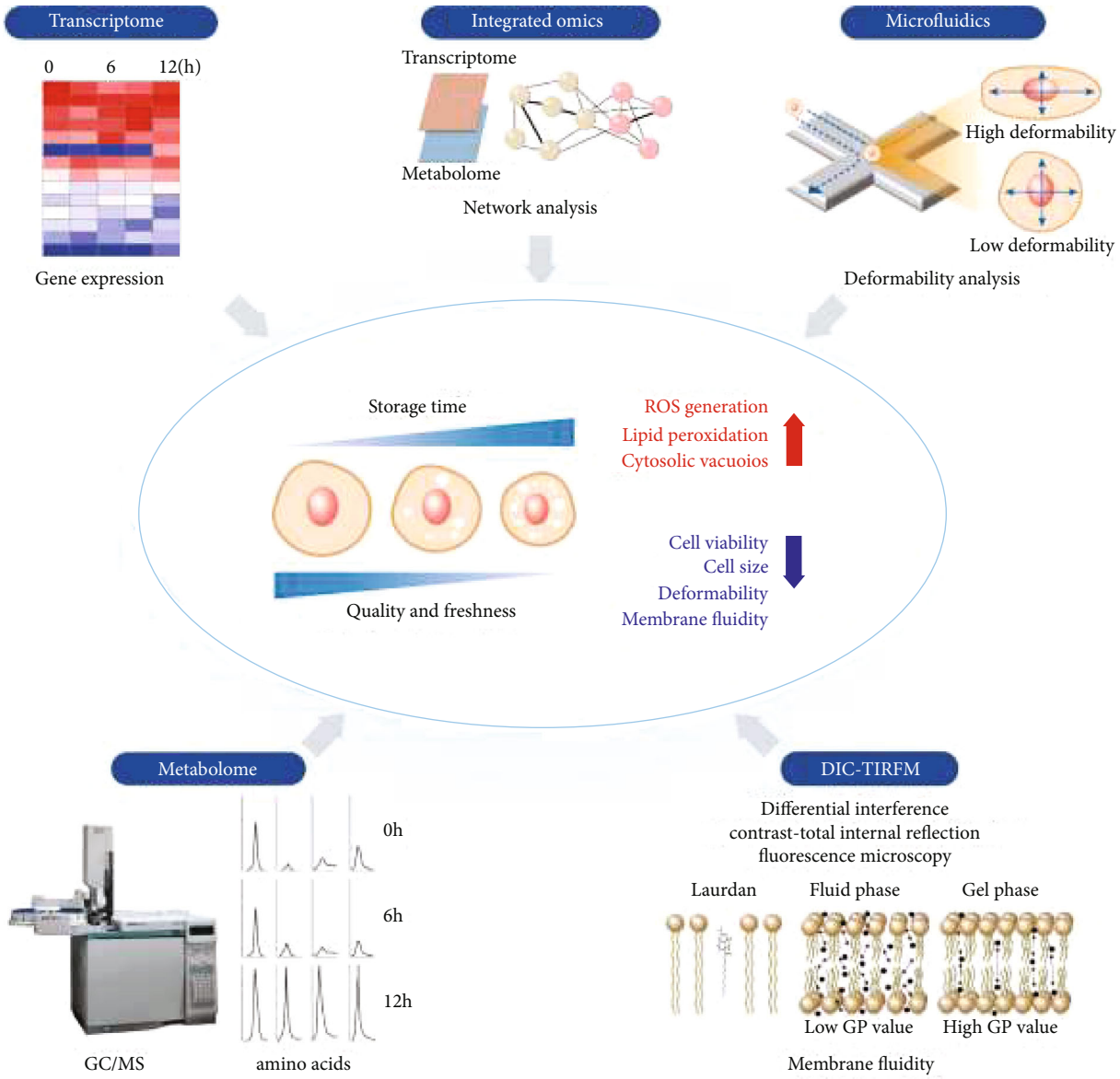

Figure 1: The quality and freshness of human bone marrow-derived mesenchymal stem cells (hBM-MSCs) are decreased over time after detachment from the culture dish. To evaluate the freshness and quality of hBM-MSCs, the metabolome is analyzed using gas chromatography–mass spectrometry (GC/MS), the transcriptome is analyzed using microarray, deformability is analyzed using microfluidics, and membrane fluidity is tested using differential interference contrast- (DIC-) total internal reflection fluorescence microscopy (TIRFM) in combination based on previous reports [9, 24].

cord injury, and myocardial infarction [65–67]. Several different sources, including neural stem cells, human umbilical cord blood cells, embryonic stem cells, hematopoietic stem cells, and MSCs, have been utilized in stem cell therapy [65]. Neural stem and embryonic stem cells have not been easy to apply in clinical fields or research because of the ethical issues (procured from aborted fetuses for allogenic transplantation), allograft rejection, or tumorigenic capacity [65, 68]. In recent years, more than half of registered stem cell trials have been conducted using MSCs because they are easy to acquire from the patients themselves, avoiding the ethical concerns and the possibility of harmful events [14, 65, 68]. In this section, we briefly review the neuroprotective and anti-inflammatory effects of hBM-MSCs via systemic transplantation, such as intravenous or intraarterial infusion, as shown in preclinical and clinical studies on ischemic stroke, traumatic brain hemorrhage, and neurodegenerative diseases, such as AD, HD, and PD.

3.1. Ischemic Stroke. The transplantation of hBM-MSCs could improve functional recovery and reduce the infarction size via neuroprotective and immunomodulatory effects after ischemic stroke in rats [69–74], a nonhuman primate model [75], and humans [14, 76–81]. Neuroprotection, nerve regeneration, and angiogenesis result from the paracrine effect of neurotrophic factors, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) [74, 82]. The local activation of

astrocytes and microglia/macrophages and the influx of leukocytes, including T cytotoxic cells, are significantly reduced [60]. Immunomodulatory effects as suppressors of inflammation were observed by decreasing the levels of proinflammatory cytokines, namely, interleukin (IL)-1α, IL-1?, IL-6, and tumor necrosis factor (TNF)-α and by increasing the levels of anti-inflammatory cytokines, including IL-4, IL-10, and interferon (INF)-? [60, 83, 84]. Moreover, rat BM-MSCs can suppress the inflammatory response by decreasing activated microglia, which are resident immune cells of the brain that produce proinflammatory cytokines at the cellular network level [85]. Interestingly, in an ischemic stroke animal model, hBM-MSCs were found to restore polyamine and free fatty acid compositions from metabolic disturbance to a near-normal state and maintain metabolic homeostasis [86, 87]. Migrated leucocytes aggravate neuroinflammation, enhancing cell death, blood–brain barrier (BBB) disruption, and vasogenic edema [71, 88, 89]. Leucocyte infiltration is facilitated by an increase in BBB permeability and endothelial cell adhesion molecule expression [89]. Leucocytes further enhance inflammation, increase cell death, and lead to BBB disruption and vasogenic edema [89]. Particularly, hBM-MSCs can decrease BBB permeability in the damaged neural tissue [71] and provide BBB integrity and maintenance through interactions with pericytes, astrocytes, and neurons [88].

An initial clinical trial of hBM-MSCs using autologous stem cells was conducted in 2005 for five patients with mid-cerebral artery occlusion, comparing results to those of twenty-five patients without stem cell therapy [14]. To date, phase I/II studies, including the first study, have reported the safety and feasibility of autologous or allogenic hBM-MSCs with long-lasting or transient neurological improvements [14, 76–81], functional improvements [80], and a short-term decrease in circulating T cells and inflammatory cytokines [90].

Although serious complications of hBM-MSCs have not been reported, there are some concerns about events such as pulmonary embolism with the intravenous injection of adipose MSCs [91] and allogeneic BM-MSCs [92], as well as the possibility of microembolism risk due to the closure of the lumens of small-diameter vessels related to the flow of cerebral blood, cell dose, infusion velocity [92], and innate procoagulant activity [67]. In addition, a previous study revealed that the deformability of hBM-MSCs decreased, and that the membranes of hBM-MSCs became stiffer via the peroxidation of plasma membrane lipids over time owing to the generation of ROS [23]. Cell dose and infusion velocity are important factors that trigger embolism; however, changing the decreased deformability of hBM-MSCs is an important contributing factor to these serious complications, considering cerebral infarction in patients with sickle-cell disease, which decreases the deformability of red blood cells [93]. Therefore, it is necessary to fully consider the quality and freshness of hBM-MSCs after dissociation from the cell culture dish.

3.2. hBM-MSCs in Traumatic Brain Injury. Traumatic brain injury is caused by primary injury facilitated by an initial insult and secondary injury occurring 1–3 days after the initial traumatic event. Primary injury includes a direct response to the initial insult, such as BBB disruption, cranial hemorrhage, brain swelling, and an acute reaction mediated by oxidative stress and excitotoxicity [94, 95]. Secondary injury is associated with the release of excitatory amino acids, ionic imbalances, intracellular calcium overload, mitochondrial dysfunction, and several immunological and inflammatory responses. This reaction induces ongoing neurodegeneration, diminished neurogenesis, axonal damage, and cell death [96, 97]. Since the complexity of injury-associated mechanisms has led to the need for multi-target treatment, several studies have been conducted using MSCs with various paracrine activities. In an animal model, subjects treated with rat BM-MSCs showed attenuated motor and cognitive deficits through the induction of trophic factors, such as BDNF and NGF, which promoted neurogenesis, neuroprotection, neural repair, immunomodulatory activity, and the secretion of bioactive factors, such as exosomes [15, 98, 99]. Some other studies using hBM-MSCs also showed functional improvements with immunomodulatory activity and the secretion of bioactive factors, such as exosomes [100, 101].

Several clinical studies have been conducted based on preclinical study results [102]. Cox et al. conducted intravenous injections of human BM-mononuclear cells in 25 patients after severe traumatic brain injury. Based on the results, there were no serious adverse events and the preservation of functionally critical regions, and the downregulation of inflammatory cytokines was observed [103]. Tian et al. injected autologous hBM-MSCs via lumbar puncture into 97 patients with subacute-stage traumatic brain injury and showed the safety and effectiveness of this therapy [104]. Zhang et al. conducted topical injection to the injured area using autologous hBM-MSCs and also showed the safety and feasibility of cell therapy [105].

3.3. hBM-MSCs in Neurodegenerative Disease. Neurodegenerative disease initially damages various types of neurons or glial cells but ultimately specifically causes the loss of function of certain cells, such as hippocampus and frontal lobe dysfunction in AD, striatal dopaminergic neurons in PD, or dysfunction of the striatal structure in HD. Although there are treatments to relieve symptoms for some neurodegenerative diseases, no treatments have been found that can modify the disease course [16, 20]. From this point of view, many studies have been conducted using BM-MSCs, which have the potential to replace lost cells and functional restoration through various paracrine activities [16, 20, 65].

3.4. AD. AD is a clinical dementia-presenting disease, and neuroinflammation mediated by the accumulation of amyloid beta plaques and neurofibrillary tangles is known as the main pathological mechanism [106, 107]. Based on this pathophysiology, several studies have been conducted using MSCs. In animal models, mouse or rat BM-MSC infusion improved cognitive impairment through various mechanisms, such as enhancing neurogenesis in the hippocampus [108, 109], increasing the level of acetylcholine [110], stabilizing and regenerating the synapse [111, 112], and modulating immunomodulatory activity [113]. Studies using hBM-MSCs have also shown reduced

amyloid beta deposition [114, 115] and increased amyloid beta clearance [116] and neurogenesis [117].

Based on animal research, several clinical trials are ongoing [20, 118]. Initial clinical trials using human umbilical cord or umbilical cord blood-derived MSCs (NCT01547689, NCT01696591, and NCT02054208) showed safety, but no positive results have been reported to improve the clinical status of AD patients. In addition, similar clinical trials are ongoing in several countries [20]. Although there have not been many clinical trials using hBM-MSCs, if the major pathophysiology of AD is associated with neuroinflammation, hBM-MSC therapy with paracrine effects might still be a promising treatment option [118, 119]. In the future, we expect that research using the replacement potential of BM-MSCs or that using BM-MSCs for early-stage AD will be performed.

3.5. PD. PD is a disease characterized by a gradual decrease in dopamine-producing neuronal cell in the *substantia nigra* and is accompanied by alpha synucleinopathy that results in the formation of Lewi bodies [120]. In PD, there are treatments to improve symptoms but no treatment options for the disease itself. For these reasons, therapy using MSCs in a PD animal model has been attempted. In experimental studies, rat BM-MSC administration has resulted in improvements in motor functions in PD animal models [121, 122], and other studies have shown that these results are associated with elevated dopamine levels in the striatum, enhanced neurogenesis, inhibited transmission of alpha-synuclein, and immunomodulatory effects [122–125]. A study has also shown that the preconditioning of BM-MSCs is more effective [126].

In clinical trials, the safety of hBM-MSC therapy was established in studies of transplantation through the stereotactic surgical method and intra-arterial administration using the cerebral artery, and improvements in motor function were observed in some patients [62, 127]. Currently, a phase II study is also being conducted for patients with idiopathic PD (NCT04506073). As results of previous experimental studies and preliminary data from clinical trials have shown that hBM-MSC treatment is safe and helpful in improving motor function, therapy using hBM-MSCs has the potential to comprise a disease-modifying treatment for PD patients.

3.6. HD. HD is a rare genetic disorder that causes cognitive impairment and movement abnormalities due to a mutation in the gene encoding the protein huntingtin, followed by damage to the striatal structure secreting gamma aminobutyric acid [128]. Effective treatment for HD has not been found. In an animal model, BM-MSC injection was mainly performed intracerebrally due to the selective damage to this area in HD. Transplanted rat or mouse BM-MSCs has been shown to activate endogenous neural stem cell proliferation and reduce apoptotic cell death through increases in BDNF or NGF levels in the striatal area, and as a result, the motor and memory function of the HD-model mice treated with MSCs were improved [129, 130]. Even with intranasal administration, an HD mouse model treated with mouse BM-MSCs showed an improved sleep cycle and survival time mediated by an increase in striatal expression of the factor associated with dopamine receptor protein and an immunomodulatory effect [131].

Based on these experimental studies, therapy with MSCs has been considered a potential disease-modifying treatment option for patients with HD, like that for other neurodegenerative diseases [132], but clinical trials have not yet been actively conducted. Zuccato et al. have reported low BDNF levels in HD patients, and that these low levels, considered one component of disease pathophysiology, are less useful as a biomarker of disease onset and progression in HD patients [133]. Owing to the complexity of symptomatology and pathophysiology, there have been observational clinical trials performed to clarify the clinical symptoms and detect potential therapeutic targets before cell therapy (NCT01937923). However, no positive results in humans have been found to date. Thus, BM-MSC treatment will be a meaningful potential treatment for HD patients, as previous experimental studies have shown that this approach improves functional activity and reduces brain atrophy.

3.7. Improvements in the Quality and Freshness of BM-MSCs after Detachment from the Culture Dish. Although hBM-MSCs are considered a potential therapeutic tool for various neurological diseases, a major bottleneck in the clinical application of hBM-MSCs is maintaining individual stem cell properties during ex vivo expansion, which is essential to achieve a therapeutically relevant number of cells. This is because only 0.001–0.01% of cells in the bone marrow are mononuclear cells [134]. After the expansion process, hBM-MSCs are detached from the culture dish and subjected to a serum-starved condition, which is largely different than their original environment, such as the MSC niche, and cells lose their useful properties [135–138]. Previous reports evaluated the freshness of hBM-MSCs kept in phosphate-buffered saline (PBS) over time after trypsinization, which can mimic ex vivo storage conditions [9, 23]. The cell viability was decreased through membrane peroxidation, and the number of cytosolic vacuoles was increased, depending on the PBS storage time, as shown in Figure 2 [9]. In addition, the expression levels of apoptosis and stress-related genes were significantly increased in hBM-MSCs after detachment from the culture dish over time [9, 23]. As hBM-MSCs are sensitive to microenvironmental conditions, stem cells stored in long holding induced cell aggregation and affected the differentiation potential of hBM-MSCs [23, 139, 140]. Therefore, hBM-MSCs should be transplanted as soon as possible after detachment from the culture dish. Even though the quality and freshness of hBM-MSCs is highly dependent on the preparation of cells and manufacturing practices, we have previously shown that the maximum storage time for optimal transplantation is within 6 h because profiles for transmission electron microscopy (TEM) imaging, gene expression, deformation index, storage time, cell viability, and metabolism are altered after storing cells for 6 h in holding conditions in the hBM-MSC group compared to the control group (0 holding stored hBM-MSC group) [9, 23].

The quality and freshness of hBM-MSCs after detachment from the culture dish were also previously analyzed with respect to viability, ultrastructure, deformability, cellular size, membrane fluidity, transcriptomics, and metabolomics [9, 23]. Cell

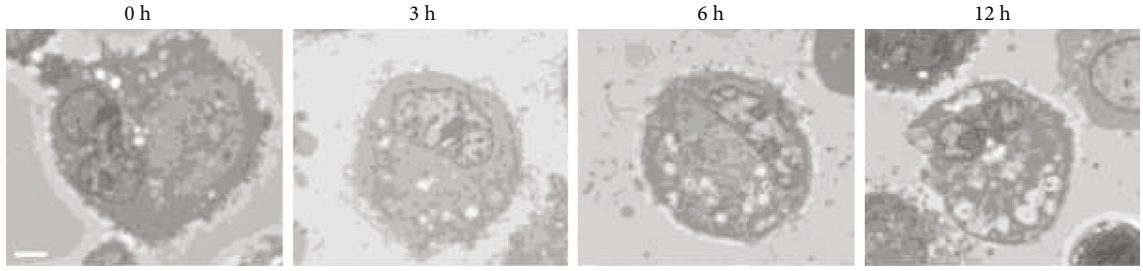

Figure 2: Representative images (×1,000) of starved human bone marrow-derived mesenchymal stem cells (hBM-MSCs). Cells were starved in phosphate-buffered saline at room temperature for 0–12 h in a previous report [9]. Scale bar = 2 μm.

deformability reflects the physicochemical changes in cellular components, such as nuclear organization, the cytoskeleton, and the membrane, in microfluidic devices [141]. For example, the deformability of red blood cells (RBCs) in diabetes, sickle-cell disease, and malaria is reduced, compared to that of healthy RBCs [142]. This is one reason as to why oxidative stress and lipid peroxidation reduce the deformability of RBCs [143]. It was reported that hBM-MSC deformability, used to analyze the quality and freshness of hBM-MSCs based on measurements using microfluidic devices, was slightly decreased after 12 h of storage [23] but was significantly reduced after 24 h of storage in PBS [144]. These results also suggested a decrease in cell deformability and membrane fluidity mediated by ROS generation and lipid peroxidation over storage time after cell detachment [23]. Therefore, these data suggest that for hBM-MSC-based cell therapy for neurological diseases, cell deformability in the brain with developing microvessels is one key point that should be considered.

An analysis of genes related to the quality and freshness of starved-hBM-MSCs for 6 and 12 h in PBS showed 27 genes that were changed, when compared to their levels in control hBM-MSCs (Table 1) based on previous reports [9, 23]. Compared to that after storage for 6 h, the gene expression was highly altered by storage for 12 h. Thus, we analyzed the transcriptomes of hBM-MSCs after 12 h based on three main functions, the generation of reactive oxygen, lipid peroxidation, and cell viability. The transcriptomic network related to each function in hBM-MSCs stored for 12 h was connected, and the functions were algorithmically predicted using Ingenuity Pathway Analysis. This in silico prediction indicated that ROS generation and lipid peroxidation were increased, and cell viability was decreased (Figure 3(a)). These data suggested that regulating redox homeostasis will be one key point to keep hBM-MSCs healthy and fresh in the pretransplantation stage.

Antioxidants can be used to eliminate ROS production. Accumulating studies have found that antioxidants can decrease the toxicity of ROS, including superoxide dismutase, glutathione (GSH), peroxidase, and vitamin E [145, 146]. To evaluate the effect of antioxidants and drug-targeting molecules, transcriptomic networks based on a combination of N-acetyl-L-cysteine (NAC) and glutathione were analyzed with predictions (Figures 3(b) and 3(c)). NAC targeted BCL2 apoptosis regulator (*BCL2*), fibroblast growth factor receptor 2 (*FGFR2*), and CD36 molecule (*CD36*), which were downregulated in the transcriptomic network (Figure 3(b)). Moreover, glutathione targeted BCL2 apoptosis regulator (*BCL2*), fibroblast growth factor receptor 2 (*FGFR2*), angiotensinogen (*AGT*), and albumin (*ALB*), which were downregulated in the transcriptomic network (Figure 3(c)). In silico prediction of the transcriptomic network indicated that NAC is more effective for the reduction of lipid peroxidation than glutathione. With NAC treatment, the lipid peroxidation level was suppressed, and the loss of cell viability was also slightly decreased. For GSH, the increase in the former function was less than that in the control, and the latter function showed a similar tendency to that of NAC-treated hBM-MSCs. Moreover, one study showed that antioxidants inhibit ROS production and help adipose tissue-derived mesenchymal stem cells maintain their stemness and ability to differentiate multidirectionally [145]. Taken together, it is highly possible that the quality and freshness of cells can be enhanced in the presence of antioxidants. Further studies require wet lab experiments to verify this in silico prediction.

There have been holistic advancements in the quantification of omics, including genomics, transcriptomics, small RNA-omics, proteomics, metagenomics, phenomics, and metabolomics [147]. Several layers of investigations, including those of the proteome, metabolome, transcriptome, genome, and epigenome, have resulted in the heterogeneity and high dimensionality of biological data. Hence, omics data could be combined in a sequential or simultaneous way to decipher the interplay of molecules. Recently, several studies have shown that the combined omics data lead to a better understanding of the biological system [148–151]. Shin et al. reported that NAC, a ROS scavenger, can protect hBM-MSCs from lipid peroxidation by integrating transcriptomics and metabolomics with amino acid profiling. Thus, they emphasized that multiomic analysis, such as the integration of transcriptomics and metabolomics (metabotranscriptomics), can be one strategy to overcome the limitations of conventional analyses of the condition of hBM-MSCs [23]. Moreover, studies on the application of miRNA to neurological disease have been reported based on post-transcriptional gene repression or the degradation properties of various miRNAs in multiple targets [152, 153]. Metabotranscriptomics integrated with small RNA-omic analysis might provide a clear rationale with respect to the importance of maintaining the quality and freshness of hBM-MSCs before clinical use.

Computational approaches, like machine learning, aid in handling vast amounts of generated data, such as omic big data. Machine learning can be classified into three types as follows:

Table 1: Genes related to the transcriptomic network of the quality and freshness of starved human bone marrow-derived mesenchymal stem cells (hBM-MSCs).

Entrez gene name	Symbol	Affymetrix ID	Location	Signal (fold change)[a]	
				6 h	12 h
Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	*PIK3CG*	206370_at	Cytoplasm	-11.70	-22.42
erbb2 interacting protein	*ERBIN*	232896_at	Cytoplasm	-1.10	-19.71
Solute carrier family 25 member 27	*SLC25A27*	230624_at	Cytoplasm	-9.20	-12.39
BCL2 apoptosis regulator	*BCL2*	207005_s_at	Cytoplasm	-7.73	-9.99
Peroxiredoxin 3	*PRDX3*	209766_at	Cytoplasm	1.85	-9.27
NADPH oxidase 5	*NOX5*	1553023_a_at	Cytoplasm	1.70	11.82
Neutrophil cytosolic factor 4	*NCF4*	205147_x_at	Cytoplasm	-1.19	12.5
Protein kinase AMP-activated catalytic subunit alpha 2	*PRKAA2*	238441_at	Cytoplasm	12.61	22.26
Phosphatase and tensin homolog	*PTEN*	242622_x_at	Cytoplasm	3.62	54.26
Angiotensinogen	*AGT*	202834_at	Extracellular Space	1.04	-27.13
TNF superfamily member 14	*TNFSF14*	233935_at	Extracellular Space	-1.19	-23.12
Albumin	*ALB*	211298_s_at	Extracellular Space	8.07	-20.19
Alpha-microglobulin/bikunin precursor	*AMBP*	214425_at	Extracellular Space	-4.55	-10.51
Adiponectin, C1Q, and collagen domain containing	*ADIPOQ*	207175_at	Extracellular Space	-3.15	-10.1
Serpin family B member 5	*SERPINB5*	1555551_at	Extracellular Space	6.58	10.19
Insulin-like growth factor 1	*IGF1*	209542_x_at	Extracellular Space	8.20	17.32
von Hippel-Lindau tumor suppressor	*VHL*	203844_at	Nucleus	-1.33	-37.9
MacroH2A.1 histone	*MACROH2A1*	1558779_at	Nucleus	1.76	12.75
Integrin subunit beta 1	*ITGB1*	215878_at	Plasma Membrane	12.40	-36.23
Fibroblast growth factor receptor 2	*FGFR2*	211400_at	Plasma Membrane	4.66	-15.37
Low-density lipoprotein receptor	*LDLR*	217103_at	Plasma Membrane	-12.50	-11.9
Glutamate metabotropic receptor 5	*GRM5*	207235_s_at	Plasma Membrane	-10.02	-10.17
CD36 molecule	*CD36*	242197_x_at	Plasma Membrane	3.27	-9.46
Presenilin 1	*PSEN1*	1559206_at	Plasma Membrane	-2.64	9.57
Fc fragment of IgG receptor IIa	*FCGR2A*	203561_at	Plasma Membrane	13.11	13.69
Toll-like receptor 7	*TLR7*	220146_at	Plasma Membrane	2.10	24.72
Prostaglandin E receptor 3	*PTGER3*	210375_at	Plasma Membrane	-17.22	27.73

[a]Normalized ratio of fold change of the signal at 6 and 12 h of storage, relative to the corresponding signal of the control group.

(i) supervised, (ii) unsupervised, and (iii) semisupervised or reinforcement. Among them, the unsupervised machine learning approach learns patterns from the unlabeled dataset and groups them based on data resemblance [154]. Especially, unsupervised methods of multivariate statistical analysis include principal component analysis, self-organizing maps, hierarchical clustering, and *K*-means. These methods reduce the dimensionality of data and can be used to visualize clusters (classifications) based on data similarity among samples. Particularly, *K*-means clustering is a traditional approach in unsupervised machine learning that can handle huge datasets to generate globular-shaped tight clusters using less computational time. Therefore, compared to other machine learning algorithms, *K*-means clustering is a very useful algorithm for the integration of omics data.

The integration of omics, advanced machine learning algorithms, and bioinformatic tools enable researchers to analyze feasible studies on the quality and freshness of hBM-MSCs based on the accurate discrimination of changes in the levels of omics data and the in silico prediction of phenomena using integrated transcriptomics and metabolomics. Therefore, to improve the efficacy of stem cell therapy with respect to the quality and freshness of hBM-MSCs, studies on comprehensive multiomic analysis (big data) and proper machine learning are required to analyze correlations within data. Moreover, in silico prediction is highly recommended, rather than a targeted approach, according to the complexity of dissociated hBM-MSCs.

In the review, we focused on describing strategies to improve the quality and freshness of hBM-MSCs for the treatment of neurological diseases. However, these factors are also affected by additional variables such as elevated temperature, high ionic strength, and nonoptimal substrate composition of the storage solution [139]. For example, storage temperature is an important factor affecting the quality of stored stem cells. Several temperature conditions were evaluated such as cold

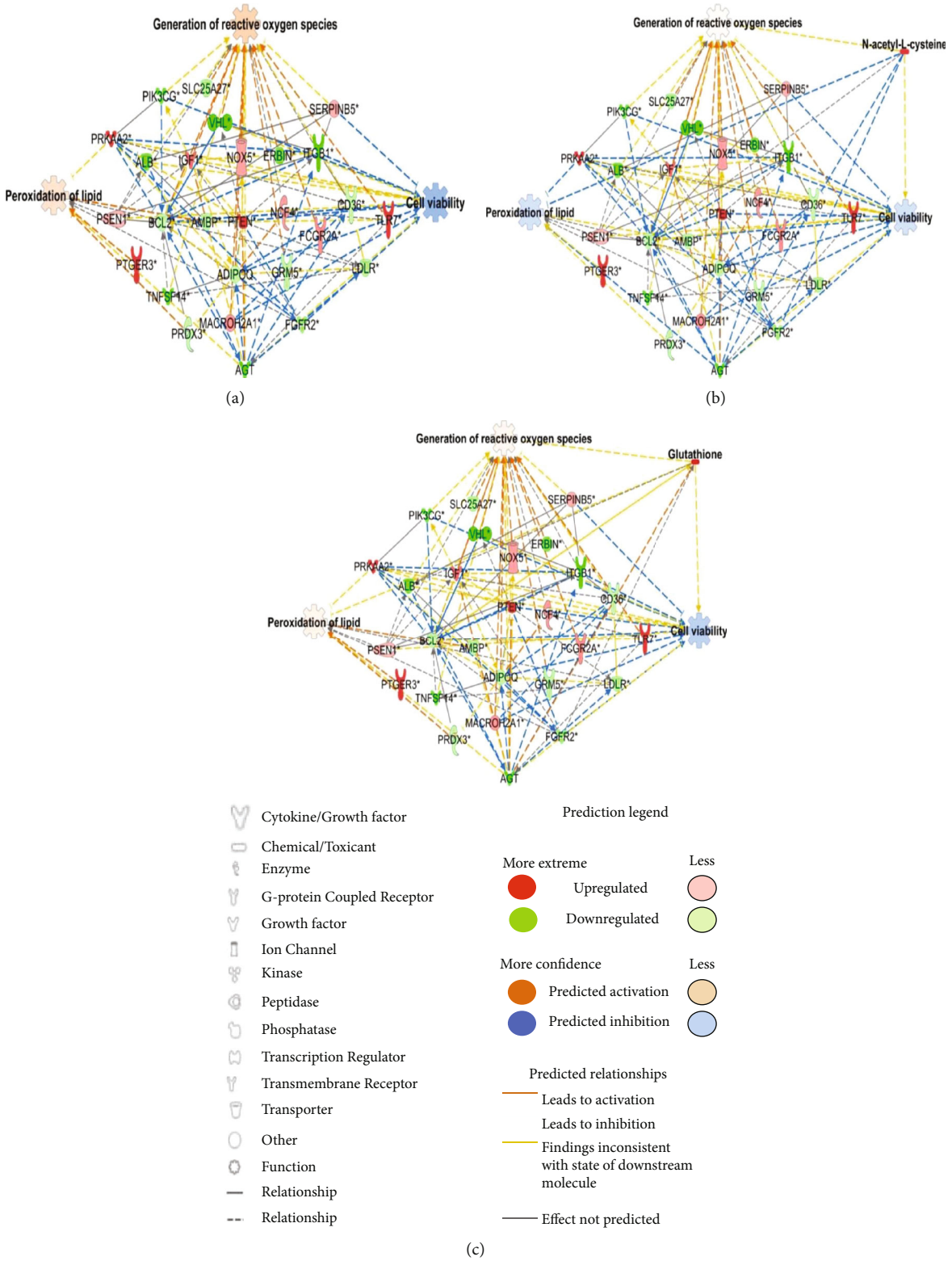

Figure 3: Transcriptomic network related to the quality and freshness of starved human bone marrow-derived mesenchymal stem cells (hBM-MSCs). (a) Analysis of the transcriptomic network with prediction using Ingenuity Pathway Analysis based on starved hBM-MSCs in phosphate-buffered saline for 12 h. Analysis of the transcriptomic network based on a combination of (b) N-acetyl-L-cysteine (NAC) and (c) glutathione (GSH) administered for 12 h. The analysis involved a fold change cut – off value ± 9. Green and red nodes indicate genes that were up and downregulated, respectively, compared to control levels. Orange and blue arrows indicate in silico prediction of function as activation and inhibition, respectively. Details of the shape and color, which were created with Ingenuity Systems (http://www.ingenuity.com), are described in the legends.

storage (4°C), low temperature (16-20°C), room temperature (25°C), physiological temperature (37°C), and cryopreservation [-20°C, -80°C, and -196°C (liquid nitrogen)] [139, 155]. There were advantages and disadvantages concerning the impact on storable time, differentiation capacity, viability, and protein secretion at the various temperatures [155].

Additionally, cryopreservation enables the storage of MSCs for a comparably longer period (over a month) than nonfreezing storage (one week). However, cryoprotective agents such as small (e.g., dimethyl sulfoxide, glycerol, ethylene glycol, and propylene glycol) and high molecular weight (e.g., sugars, polyvinylpyrrolidone, and hydroxyethyl starch) penetrating and nonpenetrating agents, respectively, are required to preserve the cellular functional and structural integrity [156]. Cryoprotective agents such as serum and serum alternatives have been used with dimethyl sulfoxide [156]. Moreover, the use of cell containers, impact of the freezing and thawing process, and the elution of cryoprotective agents should be considered during cryopreservation [156]. Free radical scavengers, ion chelators, protease inhibitors, and Rho-kinase inhibitors (Pinacidil, FDA-approved) have been used for the prevention of cryopreservation-induced cell death [157, 158]. However, the duration of storage time was the same in vitro, with improved therapeutic effects of hBM-MSCs observed using earlier passage (passage 2) than later passaged cells (passage 6) after *intravenous* administration of ex vivo cultured hBM-MSCs in a rat model for ischemic stroke [159]. Therefore, further studies are required to evaluate the quality and freshness of stored hBM-MSCs before use in human clinical trials.

4. Conclusion and Future Perspectives

Here, we reviewed hBM-MSCs, their application to neurological diseases, and improvements in the quality and freshness of these cells based on integrated omics after disassociation from the culture dish for stem cell therapy. As classical approaches are limited in terms of analyzing the quality and freshness of dissociated hBM-MSCs, the omics and machine learning approaches provide indepth and comprehensive information on the characteristics of the quality and freshness of dissociated hBM-MSCs. Therefore, further studies are needed regarding the integrated multiomic analysis, including genomics, transcriptomics, small RNA-omics, proteomics, phenomics, and metabolomics, in various hBM-MSCs conditions. Since multiomic is big data, application of machine learning algorithms for the multiomic analysis of hBM-MSCs will be one of the approaches for accurate discrimination and in silico prediction of the biological phenomena. Thus, these approaches will be helpful to analyze cellular changes of dissociated hBM-MSCs in the various conditions and improve their quality and freshness for successful stem cell therapy in neurological diseases.

Data Availability

The data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

D.Y.L., S.E.L., and G.L. conceived and designed the experiments. D.Y.L. S.N., D.H.K, M.H.L., J.S.H, S.B., and T.H.S. collected manuscript information, and J.H.A. helped with data analysis. D.Y.L., S.E.L., T.H.S., S.N., D.H.K., M.H.L., J.S.H., S.B., T.H.S., and G.L. wrote the paper. All authors read and approved the final manuscript. Da Yeon Lee and Sung Eun Lee contributed equally to this work.

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Review Article

Comparing the Therapeutic Potential of Stem Cells and their Secretory Products in Regenerative Medicine

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Cell therapy involves the transplantation of human cells to replace or repair the damaged tissues and modulate the mechanisms underlying disease initiation and progression in the body. Nowadays, many different types of cell-based therapy are developed and used to treat a variety of diseases. In the past decade, cell-free therapy has emerged as a novel approach in regenerative medicine after the discovery that the transplanted cells exerted their therapeutic effect mainly through the secretion of paracrine factors. More and more evidence showed that stem cell-derived secretome, i.e., growth factors, cytokines, and extracellular vesicles, can repair the injured tissues as effectively as the cells. This finding has spurred a new idea to employ secretome in regenerative medicine. Despite that, will cell-free therapy slowly replace cell therapy in the future? Or are these two modes of treatment still needed to address different diseases and conditions? This review provides an indepth discussion about the values of stem cells and secretome in regenerative medicine. In addition, the safety, efficacy, advantages, and disadvantages of using these two modes of treatment in regenerative medicine are also critically reviewed.

1. Introduction

Cellular therapy, also known as "cell-based therapy," involves the transplantation of human cells to stimulate the regeneration of damaged tissues and modulate the mechanisms underlying disease initiation and progression. Multiple types of human cells, including stem cells and progenitor cells, have been used to treat different diseases. Stem cell therapies using induced pluripotent stem cells (iPSCs) [1], embryonic stem cells (ESCs) [2], and adult stem cells such as mesenchymal stem cells (MSCs) [3] have been tested preclinically and clinically for years. Nowadays, MSC is widely used in the field of tissue engineering and regenerative medicine. In general, stem cell therapy has grown to become an attractive option to reduce the overall need for tissue transplantation and minimize the waiting time for patients [4]. Numerous clinical studies have indicated that stem cell administration is a safe and promising therapeutic approach. The transplanted cells

can differentiate to restore the structure and function of injured tissues [5, 6]. However, more and more evidence suggested that the transplanted cells promote tissue regeneration mainly through paracrine secretion.

Recent studies have shown that the transplanted cells secrete paracrine factors that directed the proliferation and differentiation of surrounding cells as well as produce chemoattractants that attracted the migration of effector cells to the injured sites. The "cell-free therapy" that utilizes the therapeutic molecules, i.e., secretome, secreted by stem cells has become more popular as it offers many advantages and avoids many limitations bothering the cell-based therapy. The composition of secretome is very dynamic, depending on the cell type and stimulus from their surrounding microenvironment [7]. Generally, stem cell-secreted secretome comprises (i) a complex mixture of soluble components such as growth factor and cytokines (obtained as the conditioned medium), (ii) a vesicular portion composed of extracellular vehicles (EVs), and (iii) cell organelles (e.g., mitochondria). It has been suggested that secretome can promote cell-cell communication, interact with other cells in their immediate environment, and transfer functional biomolecules to initiate tissue repair or regeneration. Generally, secretome has been found to possess proangiogenic, antiapoptotic, antifibrotic, anti-inflammatory, immunomodulatory, and proproliferative properties [8–10]. Nevertheless, extensive investigations are still required to better understand the therapeutic mechanism of secretome transplantation, its safety issues, and the clinical efficacy, mainly through clinical trials. In this review, the focus is on the values of stem cells and secretome in regenerative medicine, as well as discussing the latest insights on the safety, efficacy, advantages, and disadvantages of using these two modes of treatment.

2. Classification of Cell-Based Therapy

2.1. Stem Cell Therapy. Stem cell therapy can be categorized into autologous and allogeneic based on the tissue donor. To date, autologous stem cell transplantation has been performed for a broad range of purposes, such as to promote cardiac and cartilage regeneration, expedite wound healing, and improve aesthetic appearance. Autologous stem cells are used as they are readily available from many tissue sources and have a lower risk of life-threatening complications such as graft-versus-host disease (GVHD), free of ethical issues, and nonimmunogenic. The adverse events reported in the transplantation of stem cells are most likely unrelated to the treatment but to the underlying disease instead [11].

Allogeneic stem cell transplantation is gaining more attention in the past decade due to its advantages, such as reduction of functional variability through the pooling of cell products from multiple donors in a master bank, and it is readily available off-the-shelf for clinical applications. Allogeneic bone marrow-derived MSCs (BMSCs) mixed with autologous chondrocytes have been transplanted into the knee joint of patients with symptomatic cartilage defect, and the results showed the regeneration of hyaline cartilage with a high concentration of proteoglycans and type II collagen at 12 months [12]. A clinical trial on end-stage liver cirrhosis also revealed that allogeneic stem cell transplantation positively affects the patients' condition by improving the serum albumin levels and model for end-stage liver disease (MELD) scores after six months [13]. Paul et al. reported the immunomodulatory benefit of allogeneic MSC infusion by reducing the rejection of transplanted corneal during the immediate posttransplant period [14].

2.2. Stem Cell Derivatives/Secretome. In the field of regenerative medicine, the therapeutic effects of stem cells are not constricted to cell-cell interactions. A broad range of bioactive molecules is found in stem cell secretion, including growth factors, cytokines, chemokines, enzymes, extracellular matrix (ECM), and extracellular vesicles (EVs), collectively known as the secretome [15]. The secretome is a crucial component in exhibiting the therapeutic effect of stem cells (Figure 1).

3. Stem Cells

3.1. Source of Stem Cells. The collection of pluripotent stem cells (PSCs) such as ESCs is ethically controversial as it involves the destruction of possible human life. Furthermore, ESC is also considered an allogeneic source of cells which may cause immune incompatibility. However, there is an immediate solution to avoid ethical repercussions; the adult somatic cells can be reprogrammed into iPSCs which essentially functionally behaved as ESCs [16]. Genetically modified PSC is utilized in disease modeling to overcome the species-specific differences as observed in an animal model. It also serves as a potential cure to a currently permanent condition such as thyroid disease [17–19], cardiovascular disease [20], macular degeneration [21, 22], or Parkinson's disease that only can be managed with lifelong medications. Unfortunately, PSC has raised safety concerns as some research reported tumorigenicity [23] or epigenetic aberrations posttreatment [24–26]. A fail-safe suicide gene known as inducible caspase-9 (iCasp9) has been tested extensively in vitro and in vivo as a potentially viable solution to remove the residual pluripotent cell that may cause teratoma formation [27, 28].

On the other hand, multipotent cells have a narrower spectrum of differentiation than pluripotent stem cells and can differentiate into discrete cells of specific lineages. Examples of multipotent cells are hematopoietic stem cells (HSCs) and MSCs. HSCs can be isolated from peripheral blood, bone marrow, and umbilical cord blood, whereas MSCs can be found in bone marrow, umbilical cord, cord blood, placental, peripheral blood, adipose tissue, dental tissues, skin, salivary gland, and synovial fluid [29–32]. Although there are variations in molecular composition, surface antigen expression, differentiation capacity, and immunomodulatory property in MSCs isolated from different tissue sources [23, 33], however, functional analyses showed that all the secretome have similar functionality, i.e., to promote cell migration and inhibit cell apoptosis [34].

3.2. Mechanism of Therapeutics. MSCs were thought to promote tissue regeneration via transdifferentiation to replace

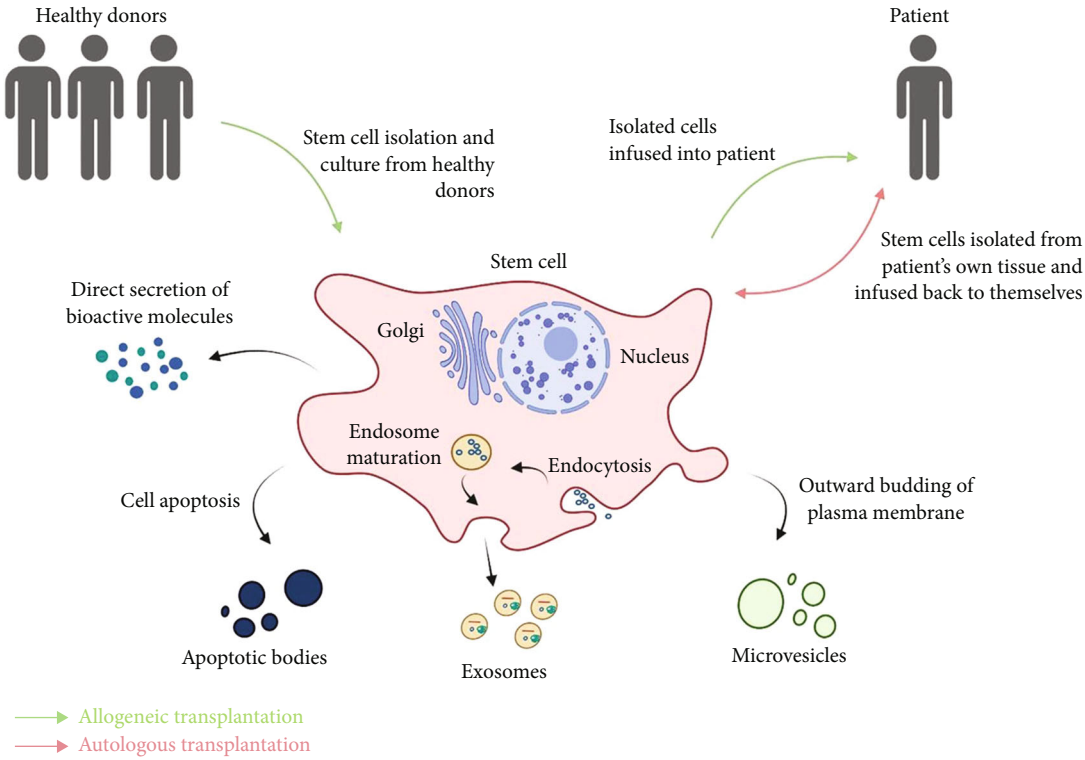

Figure 1: Classification of stem cell therapy and stem cell-derived secretome. Autologous stem cell transplantation involves the isolation of stem cells from the patient and infusion back to the same patient during treatment. Whereas in allogeneic stem cell transplantation, stem cells from single or multiple healthy donors are given to the patient. Stem cell secretome consists of bioactive molecules (including growth factors, cytokines, chemokines, enzymes, extracellular matrix) secreted directly out to the cell microenvironment or encapsulated within the extracellular vesicles that can be classified into three groups: apoptotic bodies which form during cell apoptosis, exosomes as the product of endosome maturation, and microvesicles by outward budding of the plasma membrane (created with BioRender.com).

the damaged cells and cell fusion to save the dying cells. However, many studies have found that these mechanisms are insufficient, and MSCs seem to secrete a myriad of paracrine factors, e.g., growth factors, chemokines, and cytokines, to promote tissue regeneration and modulate the immune response. This notion is supported by the low engraftment of the transplanted cells at the target site and rapid loss of the transplanted cells in vivo. The mechanisms employed by MSCs in tissue repair and immunomodulation have been excellently reviewed in previous publications [35–39]. In this section, we will only provide a glimpse at their mechanism of action in a short summary. In brief, MSCs secrete anti-inflammation, antiapoptosis, antioxidative, antifibrosis, proangiogenesis, promitosis, and chemotactic factors to stimulate tissue regeneration (Figure 2).

3.3. Multifactorial Crosstalk

3.3.1. Direct Signaling. Cell-cell signaling by direct contact allows stem cells to communicate and respond to other cells. It is not always necessary as stem cells have other soluble-dependent crosstalk as well. In a mixed immune cell culture such as peripheral blood mononuclear cells (PBMCs), cell contact is not required for MSCs to exert their anti-inflammatory effect. Contrastingly, when MSCs were introduced to a lymphocyte-only culture and cell-cell contact was prohibited, MSCs failed to induce FoxP3 and CD25 expressions in $CD4^+$ T cells [40]. The adhesion molecules ICAM-1 and VCAM-1 will not form in the lack of direct MSC-lymphocyte contact [41]. Moreover, MSCs require direct contact with immune cells to upregulate cell-surface proteins such as programmed death-ligand 1 (PD-L1) and Fas ligand to suppress inflammation [42, 43]. As a result, the immunoregulatory properties of MSC will not be exerted to their full potential. The modulation of dendritic cell maturation by MSCs also requires both direct cellular contact and the soluble factor, interleukin (IL)-6. Loibl et al. reported better results when endothelial progenitor cells were cocultured with MSCs as it significantly upregulated the mature endothelial cell marker, PECAM-1, relative to the transwell setup [44]. The immunosuppression of B cells was more efficient in direct cocultured with MSCs [45].

3.3.2. Secondary Crosstalk. Paracrine signaling is the main mechanism of MSC therapy. It was initially thought that MSC would migrate and engraft at the site of injury. Nonetheless, most of the MSC administrated intravenously are sequestered in the vasculature of the lungs, with only a few MSCs homed to the tissue of interest. Studies have also noted that exogenous MSCs unable to retain their population long enough to completely replace the affected tissue. Hence, the lasting reparative effect of MSCs is largely attributed to its

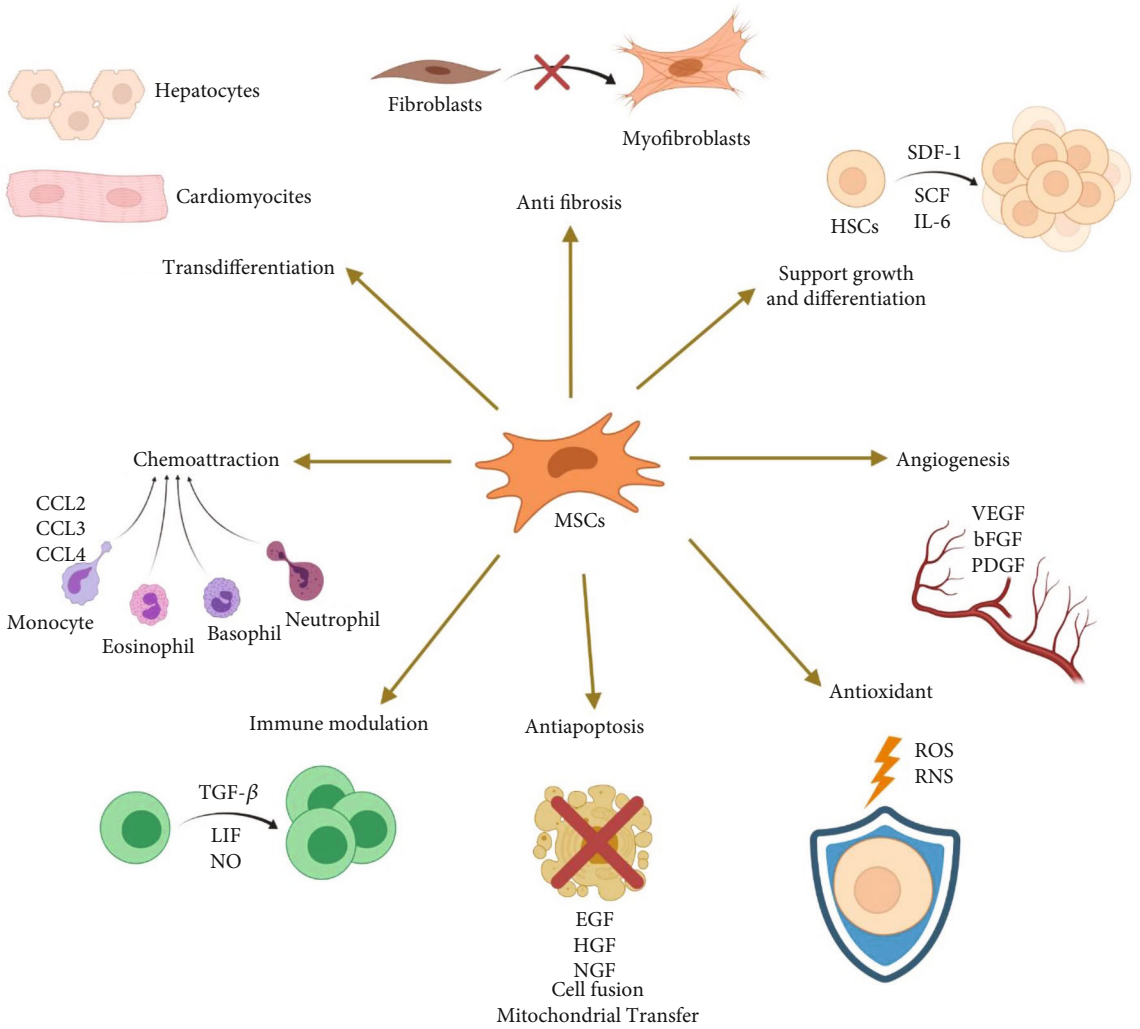

Figure 2: Mechanism of action of MSCs in tissue repair and immunomodulation. MSCs exert its therapeutic effects via various modulators. SDF-1: stromal cell-derived factor 1; SCF: stem cell factor; IL-6: interleukin-6; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; PDGF: platelet-derived growth factor; ROS: reactive oxygen species; RNS: reactive nitrogen species; EGF: epidermal growth factor; HGF: hepatocyte growth factor; NGF: nerve growth factor; TGF-β: transforming growth factor-beta; LIF: leukemia inhibitory factor; NO: nitric oxide; CCL: C-C motif chemokine ligand (created with BioRender.com).

ability to secrete trophic factors to ameliorate the inflammation in other parts of the body [35, 46–48]. Chin et al. claimed that the anti-inflammatory cytokine levels remained elevated from baseline up until six months post-MSC transfusion [46]. MSCs are known to secrete immunosuppressive factors such as transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), IL-10, prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), and galectin-1 into the circulatory system [47–51]. These molecules interact with the immune cells such as T and B cells to suppress their proliferation and differentiation, causing the polarization of macrophage to an anti-inflammatory phenotype and reduction of the pro-inflammatory milieu consists of cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), and IL-6 [46, 47, 49, 52–55].

3.3.3. Necrobiology. Necrobiology is a term used to describe the life processes associated with morphological, biochemical, and molecular changes related to cell death and the consequences and tissue response to cell death [56]. It encompasses four mechanisms by which derivatives of MSCs can retain significant clinical efficacy, including apoptosis, autophagy, mitochondrial transfer, and extracellular vesicle production [57]. The bioactive parts of dead or dying MSCs can trigger immunomodulatory properties in the host without the concern over cell survival and the formation of large aggregates [58–60].

(1) Apoptosis. Apoptosis of cultured MSCs can be induced via nutrient deprivation. In addition, some studies found that IFN-γ and TNF-α also can trigger MSC apoptosis through the nitric oxide (NO) [61] and Fas [62] pathways, respectively. One can consider inhibiting NO to prolong MSC survival, noting that it also will restrict the immunosuppression capacity of MSCs on the lymphocytes. Interestingly, Mancuso et al.'s study of knee osteoarthritis using an in vitro model revealed that apoptotic MSCs were more immunosuppressive than healthy MSCs [63]. Moreover, Chang et al.

found that apoptotic MSCs were more effective in attenuating organ damage in rat sepsis models compared to the healthy MSCs [64]. Cheung et al. found that monocytes that efferocytosed the apoptotic MSCs have higher expression of IDO, PD-L1, and cyclooxygenase 2 (COX2), and these cells secrete more PGE2 and IL-10 as well as lower TNF-α to subside the inflammation. Then, they proceeded to monitor the serum PGE2 levels of eight patients with severe steroid-resistant GVHD who received MSC therapy. It was found that the responders demonstrated increment in PGE2 levels while the nonresponders showed not changes in PGE2 levels. Albeit, in a small sample size of eight GVHD patients, this study teased the possibility of apoptotic MSCs in translational medicine [65].

(2) Autophagy. In terms of stem cells, autophagy plays a pivotal role in maintaining genomic stability and retain its potency and differentiation capacity [66]. Gao et al. discovered that autophagy may regulate MSC immunoregulation through TGF-β1 signaling. The proliferation of $CD4^+$ T helper cells was inhibited when cocultured with autophagic MSCs. However, when autophagy is inhibited, MSCs failed to suppress the proliferation of T cells [67]. Additionally, autophagy activation in MSC transplantation has protective effects on the damaged tissues. These protective effects of autophagy can be reversed using autophagy inhibitors such as 3-methyladenine and chloroquine. Autophagy can be induced by hypoxia and nutrient depletion, and it has been shown to protect MSCs in vitro [68]. Furthermore, Zhang et al. showed that hypoxic preconditioning on MSCs can enhance its functional survival to restore cardiac function in ischemia models [69]. Using rapamycin to induce the autophagy mechanism, Wang et al. showed that MSC-derived exosomes prevented acute kidney injury caused by cisplatin [70]. Similarly, Hou et al. induced autophagy by pretreating MSCs with starvation and rapamycin. In their study, it was shown that autophagy prevented the autophagic MSCs from irradiation injury and maintained the stemness after exposure to reactive oxygen species- (ROS-) induced damage [71]. Park et al. attributed the neuroprotective effects of MSCs to the higher levels of autophagy in a parkinsonian mice model and MPP^+ treated neuronal cell culture [72].

(3) Mitochondrial Transfer. MSCs are known to reprogram the host cells by the transfer of mitochondria. It is a process that requires direct cell-cell contact through tunneling nanotubes (TNT) or gap junctions [73]. Mitochondria also can be transferred via secreted EVs [56]. The mitochondrial transfer has a prominent role in protecting the recipient cells from oxidative stress, radiation injury, and hypoxic injury as well as recovering the mitochondrial membrane potential and aerobic respiration and modulating the host immune response [74, 75]. Upregulation of Miro 1, a mitochondrial Rho-GTPase, has been reported to enhance mitochondrial transfer, subsequently improve the MSC therapeutic efficacy [76].

(4) Extracellular Vesicles. According to MISEV 2018, EVs are nonreplicating particles of size 100-200 nm and encapsulated by a lipid bilayer [77]. MSC-derived EVs contain bioactive molecules including genetic materials, microRNAs, enzymes, signaling proteins, immunomodulatory factors, and growth factors [78]. EVs have the potential to be developed into cell-free therapy with the benefits of MSC immunomodulation but without the concerns of maintaining the cell viability or risk of immune rejection in allogeneic transplantation. Many studies showed that MSC-derived EVs are as effective as MSCs in treating diseases [79–81]. Apoptotic cells are known to produce different types of EVs and apoptotic bodies that can influence the surrounding cells. Apoptotic cell-derived EVs are rich in spliceosomes that alter the RNA splicing in recipient cells [82]. More data are showing that apoptotic cell-derived EVs play a significant role in immune modulation in autoimmunity, infection, and cancer, implicating that they are not just cell debris [83]. All these findings indicating that apoptotic cell-derived EVs could be an important medium of communication between the dead and living cells [84]. Nonetheless, to date, apoptotic cell-derived EVs are not well studies. Thus, what we know is still very limited.

4. Secretome

Secretome is often referred as a group of biologically active molecules or factors that are released by cells into their extracellular environment [85]. Although MSCs derived from various anatomical sites may exhibit similar morphological and immunophenotypic characteristics, numerous evidence showed that they secrete a distinct set of secretome that is normally associated with the host age and specific microenvironment that the cells were grown. The secretome may even fluctuate in response to various physiological changes and pathological circumstances. In general, MSC secretome is made up of a variety of growth factors, cytokines, and EVs that conferred its tissue repair and regenerative potential, mainly attributed to their capability to stimulate cell proliferation, formation of new blood vessels, and their immunomodulatory effects (Figure 3) [85, 86].

4.1. Growth Factor. Different investigations have shown that the growth factors present in MSC secretome may either work synergistically to exert their tissue regenerative potential or the presence of individual growth factors could be sufficient to achieve the desired therapeutic objective. For instance, brain injury such as stroke usually involves brain tissue damage due to a lack of blood supply. Hence, stroke therapy usually requires the promotion of new blood vessel formation and brain cell production, along with suppression of further cell death and inflammatory processes [85]. These have been successfully achieved via administration of BMSC and adipose tissue-derived MSC (AT-MSC) secretome that contain a mixture of hepatocyte growth factor (HGF), brain-derived neurotrophic factor (BDNF), fibroblast growth factors (FGF), and platelet-derived growth factor (PDGF) [7, 87]. Meanwhile, another study by Ding et al. has suggested a direct involvement of increased insulin-like growth factor-1 (IGF-1) levels that exhibited neuroprotective effect in a mouse model of brain stroke through regulating its ischemic and inflammatory condition to reduce the volume of brain infarct while improving the function of brain cells [88].

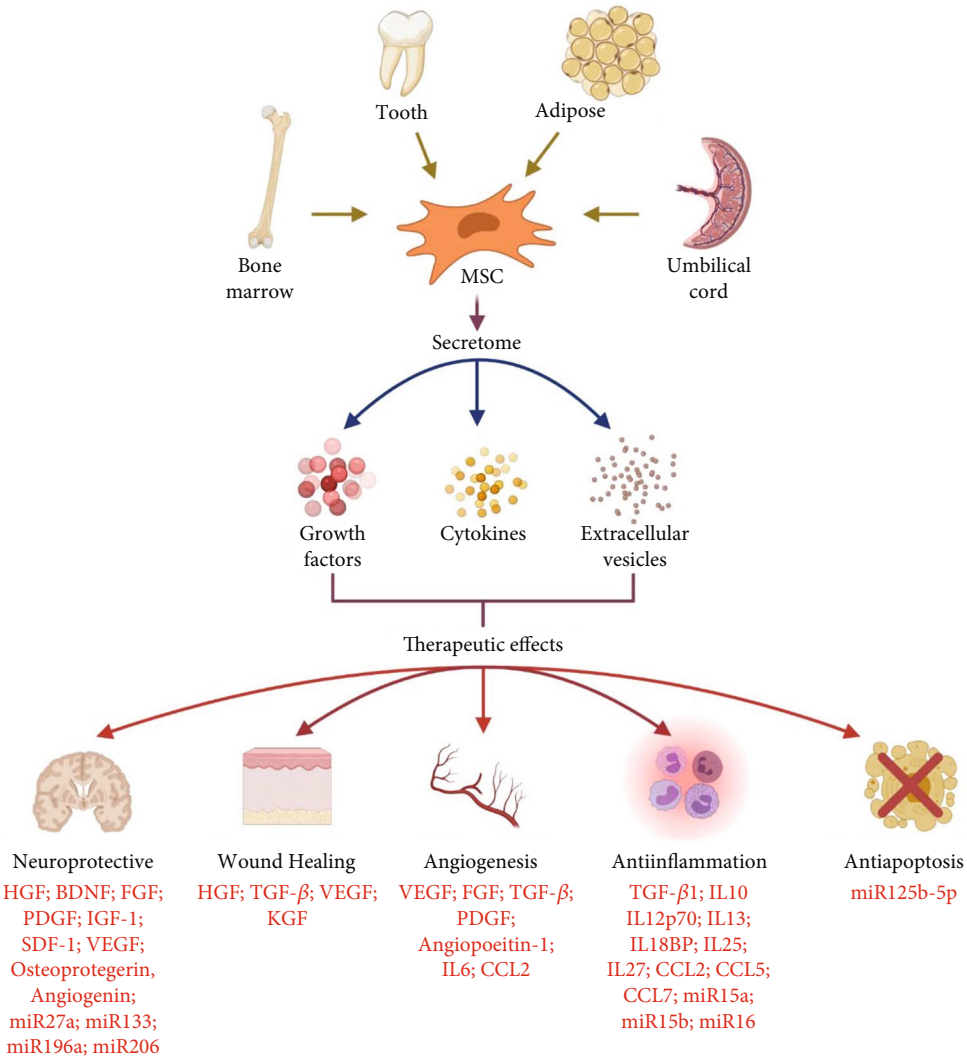

Figure 3: Mesenchymal stem cell sources, secretome content, and its therapeutic effects. MSCs can be derived from various sources, including bone marrow, teeth (deciduous vs. nondeciduous), adipose tissue, and umbilical cord. MSCs mainly rely on the secretome, which consists of various soluble factors (growth factors and cytokines) and extracellular vesicles to exert their therapeutic effects. Neuroprotection, acceleration of wound healing, induction of angiogenesis, suppressing of inflammation, and prevention of cell apoptosis are some of the reported therapeutic potentials of MSC secretome. HGF: hepatocyte growth factor; BDNF: brain-derived neurotrophic factor; FGF: fibroblast growth factor; PDGF: platelet-derived growth factor; IGF-1: insulin-like growth factor 1; SDF-1: stromal cell-derived factor 1; VEGF: vascular endothelial growth factor; TGF-β: transforming growth factor-beta; KGF: keratinocyte growth factor; IL: interleukin; miR: microRNA; CCL: C-C motif chemokine ligand (created with BioRender.com).

Similar observations also have been reported in Huntington's disease mouse model whereby transplantation of BMSCs led to elevated expression of stromal cell-derived factor-1 (SDF-1) to improve blood supply to the damaged brain striatum tissue via stimulation of angiogenesis [89]. The neuroprotective role of SDF-1 had also been verified in another rat model of Parkinson's disease whereby the grafted BMSCs inhibited apoptotic activities in the affected dopaminergic neuronal tissue, which significantly recovered the behavior of the diseased rats [90]. Besides brain injuries, therapeutic effects of growth factors present in MSCs have also been investigated for other pathologic conditions such as cutaneous injury, whereby the use of AT-MSC secretome that contains VEGF, HGF, transforming growth factor β (TGF-β), and keratinocyte growth factor (KGF) was able to induce greater cellular proliferation, trigger cell migration, and decrease the wound size at a faster rate [91–95]. The positive impact of these diverse growth factors in promoting angiogenesis [96], regenerating muscle tissue [97], and reducing incidences of premature infant diseases such as periventricular leukomalacia, retinopathy of prematurity, bronchopulmonary dysplasia, and necrotizing enterocolitis [98] also has been implicated.

4.2. Cytokines. Whilst growth factors are more frequently associated with induction of cellular proliferation or prevention of cell death for tissue regeneration, cytokines present in the MSC secretome play a more important role in regulating inflammatory activities in pathologic conditions to attain the therapeutic effect. In the MSC secretome, both anti-inflammatory cytokines (such as tumor necrosis factor β1

(TNF-β1), IL-10, IL-12 p70, IL-13, IL-18 binding protein, IL-25, and IL-27) and proinflammatory cytokines (such as TNF-α, interferon γ (IFN-γ), IL-1b, IL-6, IL-8, and IL-9) could be present. The impact of MSC secretome on the inflammatory process is usually governed by the balance of these anti- and proinflammatory cytokines [86]. For example, the destruction of pancreatic cells in autoimmune diabetes mellitus type 1 disease by proinflammatory cytokines, including TNF-α, IFN-γ, and IL-1b, could be reversed by treating the primary islet cells with MSC secretome containing significantly elevated levels of anti-inflammatory cytokines (IL-4 and IL-10), resulted in the prevention of cell apoptosis and improvement in insulin secretion [99]. The importance of anti-inflammatory cytokines produced by MSCs was further demonstrated by Hsu et al. who utilized MSCs to suppress inflammation-associated transplant arteriosclerosis through the secretion of IL-10 [100]. In another study by Ogata et al., MSC secretome was shown to stimulate bone healing in a rat bone defect model by increasing the migration of endogenous stem cells into the defect area. Subsequent analysis revealed the presence of various important cytokines in the MSC secretome which are essential to suppress inflammation as well as induce cell proliferation, angiogenesis, recruitment, and osteogenesis. These cytokines include chemokine ligand 2, chemokine ligand 5, chemokine ligand 7, and TNF-β [101].

4.3. Extracellular Vesicles. Other than growth factors and cytokines, EVs are another important subset of MSC secretome that play a crucial role in both normal and pathological processes through maintenance of homeostasis as well as regulation of immune function, tissue regeneration processes, and tumorigenesis. These EVs that carry therapeutic cargo, including nucleic acids, proteins, and lipids, are originally a method of communication between neighboring and distant cells. They can be divided into two types depending on their sizes, that is either exosomes (40-200 nm) or microvesicles (50-1000 nm) [4]. Example of the therapeutic proteins abundantly present in EVs secreted by MSCs includes osteoprotegerin and angiogenin that were found to be the key players for bone regeneration in a rat model of bone defect [101]. Besides proteins, microRNAs (miRNAs) inside the EVs secreted by BMSCs, such as miR27a, miR196a, and miR206, were also found to be crucial in triggering the expression of osteogenic genes for acceleration of bone regeneration in a rat model of calvarial bone defect [102]. On the other hand, miR133 is an important miRNA produced by MSCs to stimulate neuronal tissue remodeling in a rat model of stroke disease [103, 104]. Meanwhile, miR125b-5p is an example of miRNA that could exert antiapoptotic effect as demonstrated by its ability to suppress expression of proapoptotic BAK1 and p53 genes in a myocardial infarction model, hence preventing the death of cardiomyocytes and subsequently allowing the repair of the ischemic tissue [105]. Apart from modulation of tissue regeneration, some miRNAs are also able to regulate the immune system to suppress the extent of tissue injury. For instance, miR15a, miR15b, and miR16 could inhibit the expression of CX3CL1 to prevent recruitment of macrophages to the ischemic kidney, therefore reducing the inflammatory process in the injured kidney [106].

5. Delivery and Homing of Stem Cells and Secretome

Stem cells and secretome can be delivered via various routes of administration to elicit their therapeutic actions. Thus far, direct injection to the target site and intravenous injection is most widely used as they deliver the biologics to the target tissue more effective compared to other routes of administration.

Compared to exosomes, homing of stem cells to the target tissue is critical for the cells to exert their therapeutic effects. The efficacy of stem cell homing to the target tissue upon transplantation is very much dependent on the route of administration. There is an intrinsic relationship between different chemical factors and MSCs that influence its homing and reparative effects. Stromal-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) axis is imperative in the recruitment of MSCs to the injured tissue [107, 108] and inadvertently promotes neovascularization [109, 110]. The increased expression of SDF-1 after tissue injury stimulates the expression of CXCR4 on MSCs which improve stem cell homing and engraftment to the injured site [111]. Besides, Qin et al. described that SDF-1 regulates the MSC immunomodulatory effects through CXC chemokine receptor 7 (CXCR7). In low concentration, the proliferation of MSCs is induced, and the regulatory B cells produce various cytokines including (IL-6, IL-10, IL-4, IFN-γ, TNF-α) [112]. Zheng et al. suspected enhanced homing of CXCR4-overexpressing MSCs to the site of colitis resulted in the significant reduction of tumor formation when compared to the untreated group [113]. Similarly, Wang et al. observed improvement in cell migration using CXCR4-overexpressing MSCs, and the progression of diabetic retinopathy was hampered [47]. Without CXCR4 gene transfection, MSC is considerably less effective in repairing cardiovascular damage as the necessary vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) cannot be stimulated solely with SDF-1 [110, 114].

5.1. Direct Injection to the Target Tissue. Direct injection is a straightforward approach to deliver stem cells to the target tissue. For example, stem cells can be injected through the intraspinal and intrathecal route to treat spinal cord injury and intraarticularly to treat osteoarthritis [115, 116]. Direct injection can increase the homing of stem cells to the target tissue, and this is crucial as stem cells can self-renew and differentiate into the desired cells to repopulate and regenerate the injured or lost tissue. In addition, local delivery of stem cells to the target tissue is necessary as the secreted bioactive factors act in a paracrine manner and may be degraded in the bloodstream before reaching the target tissue when administered distantly. In the context of secretome such as exosomes, direct injection is applicable as well. MSC exosomes were effective in repairing critical size osteochondral defects in immunocompetent rats, as evidenced by the increased cellular proliferation and infiltration, enhanced matrix synthesis,

Table 1: Recent studies applied secretome and its components via intravenous (IV) route.

Cell type	Disease model	Application method	References
Cardiac stem cells	Cardiac myopathy	IV injection of 30×10^9 exosomes	[122]
BMSCs	Pulmonary hypertension	IV injection of culture media (30 μg/100 μL)	[123]
BMSCs	Asthma	IV injection of culture media (500 μg/mL)	[124]
BMSCs	Lung fibrosis	IV injection of 10 μg EVs	[125]
MSCs	Myocardial inflammation	IV injection of 200 μL of 300 μg exosomes	[126]
iPSCs	Limb ischemia	IV injection of 200 μg exosomes	[127]
hMSC544 cells	Ovarian cancer	IV injection of 100 μL exosomes	[128]
Schwann cells	Peripheral neuropathy caused by type 2 diabetic	IV injection of 200 μL exosomes	[129]
BMSCs	COVID-19	IV injection of 15 mL exosomes	[130]

and presence of regenerative immune phenotype [117]. These results are achieved via intraarticular injection of exosomes.

Even though many researchers have analyzed the therapeutic efficacy of different routes of delivery for stem cell therapy, however, there is still no congruous consensus on the optimal delivery method among the different reports [118]. Although direct injection of cell-based treatment (either stem cell or secretome) to the affected tissue is appealing and has long been documented, this approach may accompany problematic complications if it is not carefully planned, performed, or managed. The migration of stem cell or lymphatic drainage is the physiological process that would reduce the number of injected cells or quantity of secretome initially present in the target tissue vicinity. In addition, the hostile wound environment with intense inflammation is not ideal to support the survival of the transplanted cells [119].

5.2. Intravenous Administration. Delivery of cells through vein has been suggested in numerous preclinical studies and clinical trials [120]. Intravenous administration is advantageous because of its systemic distribution and ability to reach deeper tissues. However, intravenous administration also carries the risk of cell entrapment in the lung vasculature, and the retention time for the cells and their effects are short. The main concern of intravenous administration is to get enough cells to the target tissues. Harting et al. managed to infuse rats with MSCs intravenously to treat traumatic brain injury [121]. The group did not find cell homing to the target tissue. However, the rats still showed improvement in motor and cognitive functions. In terms of secretome, delivery via the intravenous route is safe due to the lower risk of embolism compared to the delivery of stem cells. In the case of neurological disorders, emboli of administered cells in the cerebral microvascular can exacerbate the disease and can be life-threatening. Intravenous administration of whole secretome or its components, i.e., exosomes, has been reported to be safe and capable to ameliorate several diseases (Table 1).

5.3. Scaffold. On top of that, new delivery strategies utilizing the biomaterials such as polymeric scaffolds and cell sheets can increase cell retention on top of providing a supporting matrix to enhance cell survival and functionality [131]. The polymeric scaffold stabilizes the stem cells and their soluble factors as well as permits sustained delivery of these bioactive factors. The structure also supports cell growth. The architecture of the scaffolds including stiffness and pore arrangement is an important regulator of stem cell differentiation. The microarchitecture of the scaffold has an impact on the differentiation of MSCs into cells of interest. Phadke et al. found the randomly oriented pores were better suited for osteogenic differentiation of MSCs when compared to the lamellar column-arranged pore network [132]. Multiple studies have focused on the development of insulin-producing cells to treat diabetes. Enderami et al. noted a significantly higher expression of glucose-regulating genes including Pdx1, insulin, glucagon, and Ngn3 genes in poly-L-lactic acid and polyvinyl alcohol (PLLA/PVA) 3D scaffold than in the regular 2D culture [133]. The 3D scaffold provides a supporting structure to maintain the cell-cell and cell-matrix interactions. The stem cells cultured in nanofibrous scaffolds generate pancreatic organoids which are morphologically and functionally similar to the mature pancreatic β-cells [134–136].

The main advantage of using cell sheets is the fabrication techniques that will not disrupt the cell-cell and cell-matrix contact [137]. Usage of cell sheets fabrication techniques such as temperature-responsive culture surface, photoresponsive polymer, and ultrasound irradiation enables the detached cells to maintain their cell surface proteins, cellular junctions, and extracellular matrix [138]. Cell sheets may be developed into an advanced cell delivery method for the treatment of many tissue injuries, including cardiovascular diseases, cutaneous wound healing, and tendon/ligament injuries. The combination of multiple cell sources in the fabrication of cell sheets may mimic the natural state of tissue to allow better grafting of cells and better tissue regeneration.

6. Stem Cells and Secretome Clinical Trials

6.1. Stem Cell Clinical Trials. Thus far, many clinical trials using MSCs have been completed, and some of the therapies, e.g., Cupistem®, Queencell®, Cartistem®, Cellgram®, Neurorata-R®, Prochymal®, Stempeucel®, and MesestroCell, have received market authorization in Korea, Canada, India, and Iran [139]. A list of worldwide clinical studies using stem cells in different phases can be found in National Institutes of

Health Clinical Trials.gov website (https://www.clinicaltrials.gov/) (Table 2).

6.1.1. Pluripotent Stem Cells. There are eight completed clinical trials on ESC transplantation, and seven of them are associated with eye disease and one for ischemic heart disease. The PSC clinical trials mainly focus on eye disease as the tissue is easily accessible for transplantation, and serious adverse events (SAEs) on the eye are less likely to be life-threatening. Furthermore, PSC transplantation is associated with higher risks of tumor formation. Since the eyeball is a confined space that has few vasculature connections with the rest of the body, the tumor is less likely to metastasize. However, none of the studies listed is presented with results. All these studies are either phase I or phase I/II, indicating that the translational research of PSC therapy is still in the early phase.

6.1.2. Multipotent Stem Cells. There are more completed MSC clinical trials compared to the PSC clinical trials as the cells are safer and have fewer ethical concerns. Many MSC clinical trials have published their results. Generally, MSC therapy is found to be safe and well-tolerated by the patients. In addition, some studies also reported the efficacy of MSC therapy to treat a battery of diseases. MSC therapy has received market authorization in several countries for the treatment of diseases such as Crohn's fistula, cartilage defects, osteoarthritis, major adverse cardiac events, amyotrophic lateral sclerosis, aGvHD, and critical limb ischemia [139]. Very recently, a parallel assigned controlled, nonrandomized phase I clinical trial has been conducted to evaluate the safety of human umbilical cord-derived mesenchymal stem cell (UC-MSC) infusion to treat patients with moderate and severe COVID-19 pulmonary disease [140]. Eighteen hospitalized COVID-19 patients were enrolled on the study, and nine of them received three cycles of intravenous infusion of UC-MSCs (3×10^7 cells/infusion). Twenty-eight days after the first infusion, no UC-MSC infusion associated SAEs were observed except for one patient in the treatment group that required mechanical ventilation compared to four patients in the control group. All patients recovered following the treatment and were discharged. These data showed that intravenous UC-MSC infusion is safe and well-tolerated in patients with moderate and severe COVID-19.

6.2. Secretome Clinical Trials. Clinical trials of cell-free therapy are taking the emerging field from basic science to clinical application. Numerous trials are/have been conducted for a huge variety of conditions. While there are reviews that have summarized previous clinical trials pertaining to the use of cell-free therapy, we intend to highlight several clinical studies that are recently published at the time of this writing (Table 3). Unfortunately, to date, the results from many of these clinical trials have yet to be published.

Overall, stem cell therapy has a longer history compared to cell-free therapy. A review on the stem cell clinical trials was published in the year 2011 [156]. One decade has passed since then, and a significant change in the current trend of stem cell clinical trials has been observed, most noticeably, the quantity (Figure 4). In 2011, 123 clinical trials using MSCs were recorded. Although some of the studies are in the combination of phase I/II, the majority are in phase II. The quantity of MSC clinical trials has grown tremendously, circa 25 times since the past decade. Notwithstanding, a total of 152 clinical trials using exosomes have also been recorded in the last 10 years. Although there is a huge surge in the number of clinical trials on MSCs and exosomes, the disease treated has not varied significantly and most of which are chronic diseases and disorders. While it is too hasty to draw a conclusion of the efficacy of cell-based therapy, the early observations of these trial results demonstrated that it is safe and feasible.

However, the clinical applications of MSCs or secretome are not without risks. Several pertaining concerns are promoting the growth of cancerous cells and nonspecific and undesirable differentiation of the transplanted cells at the target tissue. Perhaps, the most relevant risk of stem cell therapy is the malignant transformation of the administered cells. Many researchers have reported genomic instability in MSCs at higher passage [157, 158]. Thus, genotyping might be relevant to ensure the safety of the cells before transplantation.

7. Stem Cell vs. Secretome

7.1. Manufacturing. As a cellular product, the cell source poses the first major challenge to reproducibly manufacture clinically effective stem cells and secretome products. Stem cell manufacturing has been critically reviewed and discussed in the previous publication [159]. The production of stem cells is indeed a quite straightforward process. The stem cells can be grown on a large scale using bioreactors or large cell culture flasks under specific culture conditions [160]. Large-scale expansion is crucial to produce enough cells for downstream clinical application. Human platelet lysate (HPL) is often recommended as an alternative to fetal bovine serum (FBS) for good manufacturing practize- (GMP-) compliant stem cell expansion. Generally, stem cells cultured with HPL are smaller in size, display a tighter spindle-shaped morphology, and increased cell growth [161]. In addition, a chemically defined serum-free medium also can be used to replace the serum-based medium to avoid the batch-to-batch variation bothering the serum-based medium.

The manufacturing of clinically effective secretome is not an easy process. Notably, the quality and quantity of secretome are greatly influenced by the cell source and culture condition. Although secretome has been proven to work as effective as stem cells, nevertheless, it does not guarantee that the secretome harvested could work in the same way or as effective as the cultured cells. In vivo, transplanted stem cells produce secretome that could regenerate/repair the tissue or modulate the immune function in response to the signalling from the surrounding tissue. In contrast, this does not happen when the cells are grown in the laboratory. Therefore, it might be necessary to customize the culture condition that mimics the pathophysiological environment to produce clinically effective secretome [162].

When an appropriate cell source of clinically effective secretome is identified, the consistency of the cell source for

Table 2: Completed clinical trials that evaluated the safety, efficacy, and feasibility of stem cell therapy.

Type of stem cell	Treated disease	Trial design	Number of cells	Route of administration	Outcomes	References
Pluripotent stem cells	Ischemic heart disease	An open label, phase I study to assess both the feasibility and safety of epicardial delivery of a fibrin gel embedded with human embryonic stem cell- (ESC-) derived CD15+ IsI-1+ progenitors	NA	Epicardial transplantation of cells embedded in fibrin patch	Not yet available	NCT02057900
	Advanced Stargardt's macular dystrophy	A follow-up of a phase I/II, open-label, nonrandomized, 4-cohort, dose escalation, multicenter clinical trial to evaluate the long-term safety and tolerability of human ESC-derived retinal pigment epithelium (hESC-RPE) cellular therapy	0.05×10^6 to 0.2×10^6 hESC-RPE/kg	Subretinal injection	Not yet available	NCT02941991
	Stargardt's macular dystrophy	A phase I/II, open-label, nonrandomized, sequential, multicenter clinical trial to evaluate the safety and tolerability of hESC-RPE cellular therapy in patients with SMD and to evaluate potential efficacy endpoints to be used in future studies of hESC-RPE cellular therapy	0.05×10^6 to 0.2×10^6 hESC-RPE/kg	Subretinal injection	Not yet available	NCT01469832
	Outer retinal degenerations	A phase I/II, open label, nonrandomized, prospective study to determine the safety of hESC-RPE subretinal injection	0.1×10^6 hESC-RPE/kg	Subretinal injection	Not yet available	NCT02903576
	Stargardt's macular dystrophy	A phase I/II, open-label, nonrandomized clinical trial to evaluate the safety and tolerability of subretinal injection of hESC-RPE	0.05×10^6 to 0.2×10^6 hESC-RPE/kg	Subretinal injection	Not yet available	NCT01345006
	Age-related macular degeneration	A phase I/II, open-label, nonrandomized, sequential, multicenter clinical trial to evaluate the safety and tolerability of subretinal injection of hESC-RPE	0.05×10^6 to 0.2×10^6 hESC-RPE/kg	Subretinal injection	Not yet available	NCT01344993
	Age-related macular degeneration	A phase I/II trial, open-label, nonrandomized study to evaluate the long-term safety and tolerability of MA09-hRPE cellular therapy in patients with advanced dry age-related macular degeneration from one to five years following the surgical procedure to implant the MA09-hRPE cells	0.05×10^6 to 0.2×10^6 hESC-RPE/kg	Subretinal injection	Not yet available	NCT02463344
	Advanced Stargardt's macular dystrophy	A phase I/II trial, open-label, nonrandomized study to evaluate the long-term MA09-hRPE cellular therapy in patients with advanced Stargardt's macular dystrophy from one to five years following the surgical procedure to implant the MA09-hRPE cells	0.05×10^6 to 0.2×10^6 hESC-RPE/kg	Subretinal injection	Not yet available	NCT02445612

Table 2: Continued.

Type of stem cell	Treated disease	Trial design	Number of cells	Route of administration	Outcomes	References
Multipotent stem cells	Diabetic peripheral neuropathy	A phase II study to investigate the effects of mesenchymal stem cell (MSC) transfusion	NA	Intravenous infusion	Not yet available	NCT02387749
	Idiopathic pulmonary fibrosis	A phase 1b study to evaluate the safety and feasibility, particularly with respect to adverse acute hemodynamic effects and profibrosis of MSC treatment	1×10^6 or 2×10^6 hMSCs/kg	Intravenous infusion	Feasible and satisfactory short-term safety profile.	[141] NCT01385644
	Acute respiratory distress syndrome	A phase 1 clinical trial to test the safety of a single dose of allogeneic bone marrow-derived MSCs (BMSCs)	1×10^6, 5×10^6, or 10×10^6 of hMSCs/kg	Intravenous infusion	Safe and well tolerated	[142] NCT01775774
	Periapical periodontitis	A randomized, controlled phase I/II clinical trial to evaluate the safety and efficacy of human umbilical cord-derived mesenchymal stem cells (UC-MSCs) encapsulated in a plasma-derived biomaterial for regenerative endodontic procedures in mature permanent teeth with apical lesions	1×10^6 cells/biomaterial	Encapsulated in platelet poor plasma	Safe and effective	[143] NCT03102879
	Cleft lip and palate	A phase I trial to evaluate the feasibility and safety of deciduous dental pulp stem cells	1×10^6 cells/biomaterial	Embedded in hydroxyapatite-collagen sponge	Feasible and safe	[144] NCT01932164
	Knee osteoarthritis	A multicentre, phase I/II clinical trial to evaluate the clinical use of allogenic BMSCs	40×10^6 cells/kg	Intra-articular injection	Feasible and safe	[145] NCT01586312
	Knee osteoarthritis	A phase 2 study to determine the clinical response to autologous bone marrow aspirate concentrate and platelet-rich plasma injection	NA	Intra-articular injection	Not yet published	NCT02958267
	Frailty	A phase I/II randomized, double-blind, placebo-controlled clinical trial to evaluate the safety of MSC therapy	100×10^6 cells/kg	Intravenous infusion	Safe	[146] NCT02065245
	Retinal degeneration	A phase 1 study to report electroretinographic (ERG) findings in advanced glaucoma treated with a single intravitreal injection of BMSCs	1×10^6 cells/kg	Intravitreal injection	Not yet available	NCT02330978
	Spinal cord injury	A phase I/II study to evaluate the safety and efficacy of intrathecal administration of allogeneic UC-MSCs to patients with spinal cord injury	1×10^6 cells/kg	Intrathecal injection	Not yet available	NCT02481440
	Knee osteoarthritis	A phase I/II study to evaluate the feasibility and safety of the implantation of 40 million BMSCs in knees with osteoarthritis of grade II-IV (Kellgren and Lawrence)	40×10^6 cells/kg	Intra-articular injection	Not yet available	NCT01183728
	Chronic ischemic cardiomyopathy	A phase II trial to assess the feasibility, safety, and efficacy of *trans*-endocardial administration of autologous MSCs and cardiac progenitor cells (CPCs), alone, and in combination	150×10^6 cells/kg b.w.	*Trans*-endocardial injection	Feasible, safe, and effective	[147] NCT02501811
	Cardiomyopathy	A phase I study to examine the safety and feasibility of allogeneic human MSCs by *trans*-endocardial injection to cancer survivors	100×10^6 cells/kg	*Trans*-endocardial injection	Safe and feasible	[148] NCT02509156

Table 2: Continued.

Type of stem cell	Treated disease	Trial design	Number of cells	Route of administration	Outcomes	References
	Ischemic cardiomyopathy	A phase 1/2 randomized comparison study to test whether allogeneic MSCs are as safe and effective as autologous MSCs	20×10^6, 100×10^6, or 200×10^6 cells/kg	*Trans*-endocardial injection	Safe and effective	[149] NCT01087996
	Nonischemic dilated cardiomyopathy	A phase I/II study to assess the safety of autologous and allogeneic human MSC administration	100×10^6 cells/kg	Trans-endocardial injection	Safe	[150] NCT01392625
	Myocardial infarction	A phase II trial to compare the safety and efficacy of 2 doses of allogeneic BMSCs	20×10^6 or 100×10^6 cells/kg	*Trans*-endocardial injection	Safe	[151] NCT02013674
	Ischemic cardiomyopathy	A phase I/II study to demonstrate the safety of *trans*-endocardial injection of autologous MSCs and bone marrow mononuclear cells	100×10^6 or 200×10^6 cells/kg	*Trans*-endocardial injection	Safe	[152] NCT00768066
	End-stage liver disease	A phase I/II study to investigate the feasibility, safety, and efficacy of autologous MSC injection	$3 - 5 \times 10^6$ MSCs	Peripheral or portal vein injection	Feasible, safe, and effective	[153] NCT01440309
	Knee osteoarthritis	A randomized, phase 2b study to assess the efficacy and safety of a single intra-articular injection of adipose tissue-derived MSCs (AT-MSCs)	1×10^8 MSCs	Intra-articular injection	Safe and effective with satisfactory functional improvement and pain relief in patients	[154]
	Heart failure	A phase 1/2 randomized controlled trial to evaluate the safety and efficacy of the intravenous infusion of UC-MSCs in patients with chronic stable heart failure and reduced ejection fraction	1×10^6 MSCs/kg	NA	Safe and effective with improvement in quality of life	[155] NCT01739777
	COVID-19	A phase 1 clinical trial to evaluate the safety of human UC-MSC infusion in the treatment of patients with moderate and severe COVID-19 pulmonary disease	3×10^7 of UC-MSCs	NA	Safe and well tolerated without serious adverse events	[140]

NA: not available.

Table 3: Cell-free treatment in clinical trials for various diseases.

Target disease	Cell	Clinical trials identifier	Administration	Dosage	Results
COVID-19	MSC-derived exosomes	NCT04491240	Inhalation	$0.5 - 2 \times 10^{10}$ exosomes	No observable side effects in 30 days. Improvement in overall treatment is not insignificant compared to standard therapy.
Chronic ulcer	MSC conditioned media	NCT04134676	Topical	Unknown	Not available
Keloid	Umbilical cord-MSC conditioned medium	NCT04326959	Intralesional	1 mL/cm^3	Not available
Knee osteoarthritis	MSC conditioned medium	NCT04314661	Intra-articular injection	2 mL 2 weeks after 5×10^5 MSC cells	Not available
SARS-CoV-2-associated pneumonia	MSC-derived exosomes	NCT04276987	Inhalation	2.0×10^8 vesicles/3 mL for 5 days	Not available
Multiple organ failures after surgical repair of aortic dissection	MSC-derived exosomes	NCT04356300	Intravenous	150 mg exosomes for 2 weeks	Ongoing
Chronic low back pain	Platelet-rich plasma with exosomes	NCT04849429	Intrathecal	2 mL	Ongoing
Cerebrovascular disorders	MSC-derived exosomes	NCT03384433	Intravenous	Not available	Ongoing
COVID-19	MSC conditioned medium	NCT04753476	Intramuscular	0.5-1 mL (3 doses)	Ongoing

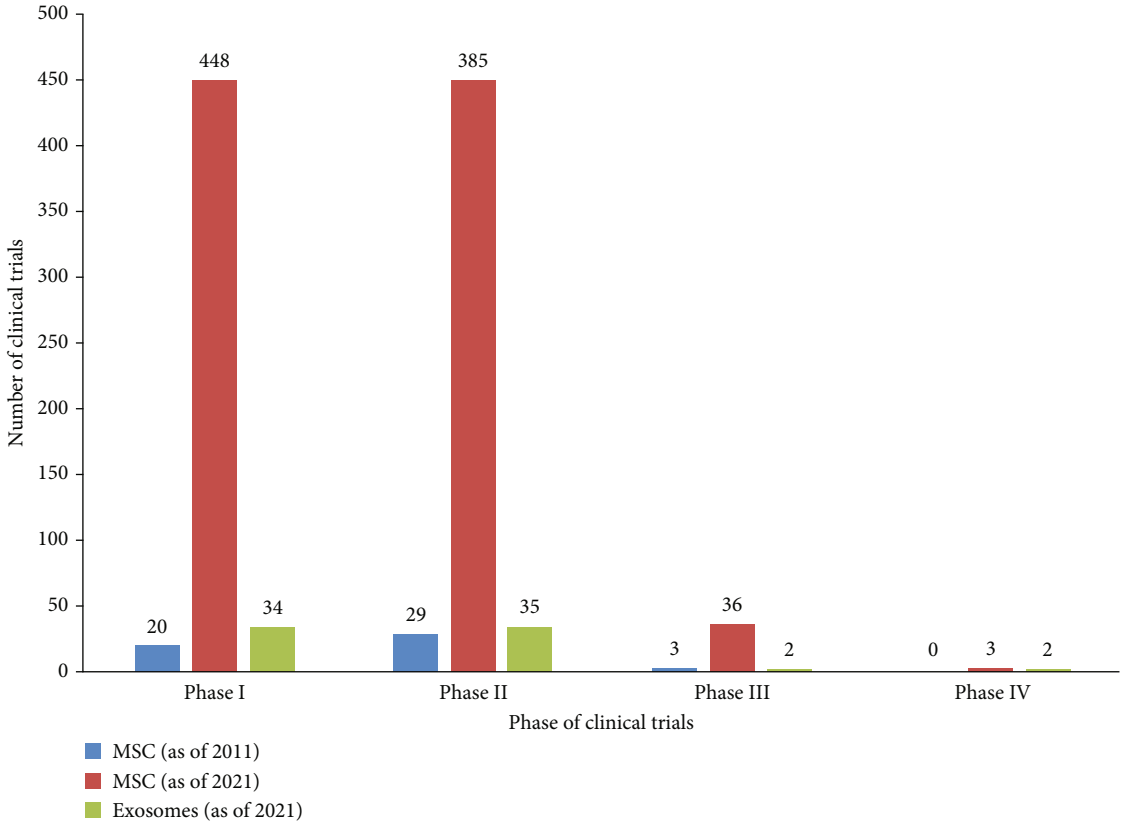

Figure 4: Number of mesenchymal stem cell (MSC) and exosome clinical trials between 2011 and 2021 by clinical phase (source: clinicaltrials.gov [accessed 5/5/2021]).

all subsequent batches of secretome production must be addressed. This could be achieved by pooling the cells from the same tissue source of different healthy donors to produce multiple batches of secretome, mitigating the challenges of biological variation in tissue sources and donors [163]. Due to the finite replication of the primary cells, a more practical approach is to use "immortalized" cell lines or PSC-derived stem cells. Although studies have reported that the regenerative properties of secretome [164] and sEVs [165] harvested from immortalized stem cells are not compromised. Nevertheless, all these must be carefully investigated to ensure that the immortalized cells are stable and continue to produce secretome products that are bioequivalent to those from non-immortalized parental cells. In addition, secretome enrichment protocols could also be employed to enhance the production of secretome in the laboratory [166]. It should be taken into consideration that the type of media used to harvest secretome may also affect the quality and efficacy of secretome. To avoid interference from HPL, basal media is often employed to harvest the secretome to determine the efficacy of stem cell secretome. Nevertheless, the sudden switch from nutrient-rich to basal media may change the cell's behavior and subsequently modifying the secretome profile. Would the basal media harvested secretome work as effective as the stem cells in the host and whether the secretome harvested from cells cultured in complete growth media might have better clinical efficacy compared to the secretome harvested from basal media are some of the questions that remain to be answered. Based on the abovementioned arguments, the quality of secretome preparation is dependent on the source, culture condition, and secretome enrichment protocol. Therefore, secretome manufactured using different protocols may have different disease-relevant potency.

7.2. Quality Control. Quality control is crucial to ensure the safety and efficacy of cell-based products. Adherence to the GMP regulations assures the identity, strength, quality, and purity of the products. Strict adherence to the quality management system helps to prevent contamination, mix-up, deviation, failure, and error during production [167]. It is important to note that a lot of efforts have been given to establish GMP facilities to produce cellular products for clinical applications [168]. Apart from the GMP facility, guidelines are in place to characterize the stem cells. For instance, MSCs should be characterized according to the guideline recommended by the International Society of Cellular Therapy (ISCT) [169].

The quality control for secretome is way more complex compared to the stem cells. As the secretome is a mixture of EVs and soluble proteins, it is challenging to identify the active components from this mixture and hence, more efforts are required to characterize the secretome. For instance, proteomic analysis is needed to identify the type of proteins and their concentration in the preparation [96, 170]. For EVs, particularly small EVs (sEVs), it needs to be characterized according to the guidelines published by the International Society of Extracellular Vesicles (ISEV). The identification of size and number would require either zetasizer or nanoparticle tracking analysis (NTA). Notably, quantifiable metrics defining the identity of sEV preparations should reflect the cellular origin of the sEVs in preparation, the presence of lipid membrane vesicles, and the degree of physical and biochemical integrity of the vesicles. The combination of these metrics could quantify the identity of sEVs and facilitate stratification and comparison of different secretome preparations [77, 171]. As EVs contain miRNAs, the molecular technique is also required to characterize the miRNA profile [172, 173].

7.3. Cost of Production and Treatment. There is an argument whether the cost of production and treatment is lower in secretome therapy in comparison to stem cell therapy. For stem cell therapy, the number of stem cells that could be isolated from patients/donors is low; therefore, the harvested cells are usually expanded in the laboratory to attain enough cells for clinical applications. The process can take weeks to months. During the cell expansion, media change is typically done every 3-4 days. The high volume of spent media means to be discarded is a potential source of secretome that can be used clinically after proper processing. The preparation of cell-free therapies from the spent media can greatly reduce the cost of production. However, need to bear in mind that the secretome or exosomes collected form the spent media could have different biological components when the cells are cultured in different conditions. Thus, it is imperative to determine safety and efficacy as well as to characterize the secretome or exosomes secreted by cells cultured in different conditions. In another words, not all spent media can be processed to produce safe and effective secretome and exosomes.

Furthermore, as the secretome cannot self-replicate and have a short half-life in vivo, thus, the secretome might need to be given more frequently to exert its therapeutic effect. In contrast, stem cells can self-renew and survive in the body for a longer period. Stem cells can respond to the signaling molecules released by the injured cells by secreting the appropriate paracrine factors to stimulate tissue regeneration. On the other hand, the contents of secretome are already defined in vitro. Thus, preconditioning of the cells at the culture condition that mimic the disease pathophysiological condition might be needed to produce clinically relevant secretome. The used of specific instruments or biochemicals to replicate the disease pathophysiological condition in vitro will incur extra costs. Finally, the cost of secretome production is still likely to be higher than the cells as it requires extra concentration and purification steps [174].

7.4. Advantages and Disadvantages. The use of stem cells as regenerative medicine for various diseases has been progressing well since the past decade. However, the type of stem cell suitable for different diseases is still under vigorous debate since each stem cell subtype possesses its advantages and limitations. For instance, ESCs can differentiate into various types of tissue but its limitations, i.e., ethical dilemma, genetic instability, and teratocarcinoma, might overweight the benefits [175]. MSCs show several superior properties for therapeutic use compared to other types of stem cells, including easy to harvest and expand, both autologous and allogeneic

cells can be used with minimal risk of rejection, free from ethical issues and have limited replicative lifespan, and hence have lower risks of malignant formation. However, MSCs are only capable to differentiate into certain lineages thus limiting their usage to only certain diseases [176]. Notably, stem cells could be differentiated into specific tissue as cell replacement therapy [177, 178]. This is the main advantage of stem cells over secretome. Another prominent property of stem cells is their ability to migrate to the site of injury (homing effect). Surprisingly, sEVs also possessed the homing ability. Studies showed that MSC-EVs were mainly accumulated in the inflamed kidneys [179] and injured brains [180, 181] in the acute kidney injury model and intracerebral haemorrhage models, respectively. The accumulation of systemically injected sEVs in the intracerebral hemorrhage model also showed that sEVs can cross the blood-brain barrier (BBB). Research also has indicated that stem cells could cross BBB. MSCs integrated into the endothelium through the adhesion molecules VCAM-1/VLA-4 and β1 integrin. After crossing the endothelial barrier, MSCs invade the host tissue via plasmic podia [182]. MSCs were also found to cross BBB through paracellular pathways that are normally inhibited by the presence of tight junctions [183]. These showed that both stem cells and secretome could be intravenously injected and reach the brain. Nevertheless, the bioavailability of these two subjects in the brain remains to be elucidated.

Accumulating evidence suggests that secretome possesses many advantages over stem cell transplantation. Cell degeneration or senescence in the host after transplantation is not a concern for secretome therapy. It was also reported that secretome has lower cell surface proteins compared to stem cells, which makes allogeneic secretome safer than allogeneic stem cells because of the lower risk of immunogenicity [184]. Irreplicable property and absence of DNAs in secretome greatly reduce the risk of DNA mutation and tumor formation in the host. The use of secretome also reduces the possibility of vascular obstruction compared to larger stem cells. The bioactive components of secretome can be easily modulated by culturing the cells in different conditions. Secretome is also easier to store compared to stem cells, i.e., stem cells need to be stored in liquid nitrogen to maintain their viability while secretome can be stored in -20°C. Finally, the requirement to evaluate the safety and dosage of secretome is less stringent in comparison to the stem cells, making the journey to the clinical setting smoother and faster. This is because stem cells are living cells, and the fate of the transplanted cells is more difficult to predict. Table 4 and Figure 5 summarize the comparison between stem cells and secretome from the perspective of manufacturing, quality control, cost of production, and treatment, as well as their advantages and disadvantages in clinical applications.

8. Future Perspective

Currently, cell-based therapies face two great challenges; how to anticipate decreased cell viability and biological functions during in vitro culture and how to prolong survival of transplanted cells. Consequently, several strategies can be envisaged to increase survival, immunomodulatory potential, and regenerative functions of cell-based therapy. Preconditioning, genetic modification, and tissue engineering are the dominant strategies. Furthermore, combinatorial approaches using nanotechnology could also improve the therapeutic performance of stem cells and secretome.

8.1. Stem Cells

8.1.1. Genetic Modification. The combination of stem cell biology and genetic engineering has great potential in regenerative medicine. Through genetic modification, the researcher could induce or determine the cell's specific differentiation pathway after injection or enhance the adhesion potential of the stem cell to specific target. After transplantation, the fate of MSCs would be stochastically determined based on the microenvironment and biochemical stimulation of the host body; therefore, not all transplanted cells would contribute to the regeneration of damaged organs. As recently demonstrated in mice, transplanted MSCs could differentiate into osteoblasts in the heart [185]. Although cell differentiation can be achieved using the biochemical or biophysical stimulus in vitro, however, reverse differentiation may occur after transplantation or withdrawal of stimulants [186]. Therefore, genetic modification of the transplanted stem cells would be the key to achieve a directed and irreversible differentiation into the desired lineage. Several studies have been conducted on the therapeutic applicability of genetically modified MSCs in animal models of diabetes, myeloma bone disease, GvHD, and myocardial infarction. Table 5 summarizes the modifiers, cell source, genetic engineering method, and applications from various studies.

In addition to the ability to differentiate, MSCs can be genetically engineered to home to the target tissue. For example, MSCs transduced with CXCR4 demonstrated higher homing in the mice model of myocardial infarction after intravenous administration [110, 235]. The overexpression of CXCR4 facilitated MSC aggregation and etching of collagenous tissue of the infarcted area [236]. Such strategies will help in the development of noninvasive cell therapies, since the route of administration is also important to avoid the formation of heterotopic tissue, especially in the case of genetically modified MSCs. On the other hand, poor cell survival after transplantation is a yet to resolved hurdle in MSC-based therapies. Studies show that genetic modification of MSCs with hypoxia-regulated heme oxygenase-1 (HO-1), Akt1, and Bcl-2 increased cell survival after transplantation in animal models by inhibiting cell apoptosis [236–238]. Thus, these strategies might be the possible solutions to increase the survival of MSCs after transplantation.

8.1.2. Tissue Engineering Using MSCs. Another area of regenerative medicine is to combine cells and scaffolds to create a 3D implant. Tissue engineering seeks to recreate the in vivo environment to promote the development of tissues needed for transplantation. Various approaches have been studied, including protein-impregnated scaffolds [239], gene vector-incorporated templates [240], and cell-scaffold combinations (Table 6). Scaffolding alone has been shown to help repair certain types of damage [239]. However, incorporating MSCs

Table 4: Comparison between stem cell and secretome.

Aspect	Stem cell	Secretome
Manufacturing	(i) General culture condition is normally used but special culture condition might be needed to produce specific cells (e.g., chondrogenic media to produce chondrogenic-differentiated MSCs) (ii) The consistency of the cell source has to be maintained for allogeneic stem cells (iii) May contain elements of external sources (FBS, HPL) (iv) Require a large number of cells for clinical applications	(i) General culture condition can be used but special culture condition mimicking the pathophysiological condition of the target diseases might be needed to produce the "bioequivalent" secretome (ii) The consistency of the cell source has to be maintained (iii) Enrichment protocol might be needed to enhance the production of secretome (i) Secretome may contain elements of external sources (FBS, HPL) (ii) High volume of media is collected, and it needs to be processed and concentrated for clinical applications
Quality control	(i) Stem cell markers are well established (ii) The characterization techniques are well established (iii) Specific functionality assay is needed to determine the efficacy	(i) The characterization is complex since secretome contain many elements such as growth factors, cytokines, and extracellular vesicles (ii) Specific functionality assay is needed to determine its efficacy
Cost of production and treatment	(i) Cost can be reduced via large-scale expansion of allogeneic stem cells (ii) Treatment dose is easier to be justified by number of cells	(i) Repetitive collection of secretome from spent culture media can greatly reduce the cost of production (ii) Extra cost is needed for downstream processing of secretome (concentration and purification) (iii) Treatment dose is vague (protein amount vs number of particles) (iv) It is unsure which component(s) of the secretome are exerting therapeutic effects
Advantages	(i) Stem cells can be differentiated into specific lineages to improve the therapeutic efficacy and treat different diseases (ii) Mesenchymal stem cells are easy to isolate and expand, have low immunogenicity (both autologous and allogeneic cells can be used clinically), free from ethical issues, and have limited replicative lifespan, hence safe from malignant formation (iii) Can be reprogrammed into pluripotent stem cells (PSCs) (iv) Can cross blood-brain barrier (BBB) (v) Can migrate and home to the target tissue in response to the signal release by the injured cells (vi) Living cells can exert the therapeutic effects for a longer period. Thus, less frequency of administration is needed (e.g., once in every 6 months)	(i) The therapeutic components of the secretome could be customized by modifying the culture condition (preconditioning) (ii) Can cross BBB (iii) Can circulate and home to the target tissue (iv) Low risk of mutation, carcinogenesis, and immunogenic as they are not living cells (v) Lower risk of vascular obstruction as they are smaller in size compared to stem cells (vi) Easier to store (vii) Cell degeneration or senescence in the host after transplantation is not a concern
Disadvantages	(i) Higher risk of mutation and carcinogenesis (especially the PSCs) (ii) Ethical issue (embryonic stem cells) (iii) Might illicit host immune response to reject the transplanted cells (especially the allogeneic stem cells) (iv) Cell degeneration or senescence in the host after transplantation (v) Potential vascular obstruction (vi) More stringent storage condition to maintain the cell viability (vii) More optimization is needed to improve the safety and efficacy (e.g., optimum dosage and route of administration)	(i) Cannot be used as cell-replacement therapy and relying on the proliferation of host cells for tissue regeneration (ii) Lack of understanding on its mechanism of action (iii) Lack of long-term safety data (iv) Lack of standardization (v) Short half-life in the body. Thus, might need more frequent administration (vi) Difficult to purify the specific therapeutic components (e.g., exosomes). Thus, the secretome products are highly heterogeneous

into the scaffold improves the in situ repair process by acting as the precursors and stimulators [241].

Over the decades, much effort has been devoted to study the physical and chemical properties of various biomaterials, as these properties affect the differentiation pathway and adhesion capacity of MSCs. For example, the elasticity of a polyacrylamide matrix seeded with MSCs determines their differentiation pathway into neuronal, muscle, or bone lineage based on crosslink density [247]. Furthermore, studies indicated that the presence of carboxyl or hydroxyl groups

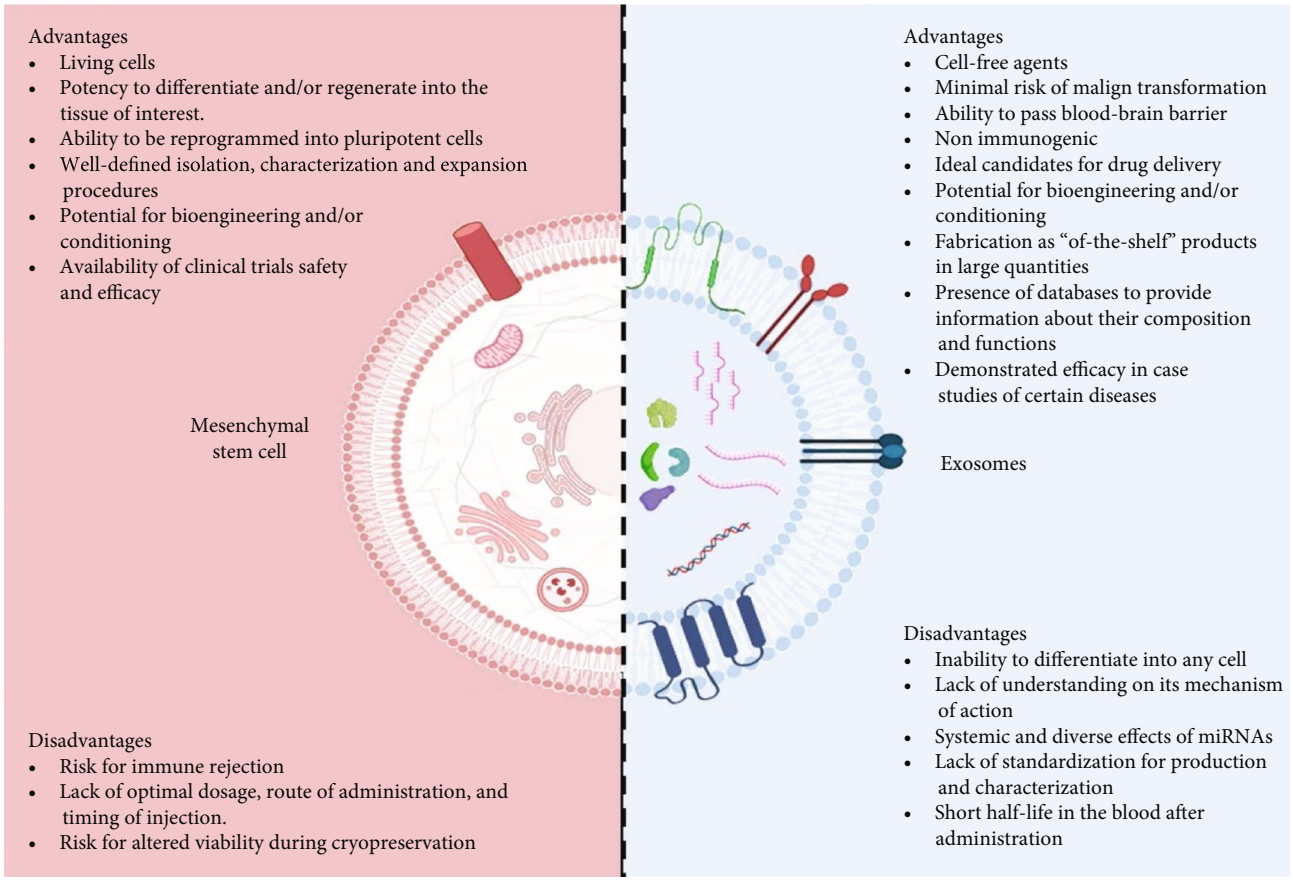

Figure 5: Advantages and disadvantages of MSCs and MSC-derived exosomes (created with BioRender.com).

on the scaffold surface prioritizes chondrogenic differentiation, while amino and sulfhydryl groups promote bone formation [248]. In addition to biometric properties, graft angiogenesis is another important factor in ensuring cell survival and therapeutic efficiency. The host's blood vessels can invade the transplant, but the process is very slow, and it takes weeks to vascularize just a few millimeters. Therefore, researchers incorporate angiogenesis-promoting factors such as endothelial progenitor cells (EPCs) and VEGF to hasten graft angiogenesis [249]. Unfortunately, no perfusion was observed upon implantation [250]. Currently, there are no established angiogenesis strategies available to support transplantation of large tissue due to delayed angiogenesis which resulted in cell apoptosis and necrosis. The approaches mentioned above only could increase the likelihood of angiogenesis.

8.2. Exosomes

8.2.1. Preconditioning. Both 3D culturing and pretreatment of MSCs with cytokines, hypoxia, or chemicals are reliable methods to increase exosome secretion (Figure 6) [251]. In addition, MSC gene and cell surface modifications may be used to improve the therapeutic effect of exosomes.

(1) Increasing Exosome Production. Increasing the secretion of exosomes is an important but unmet process. Studies have shown that 3D culturing methods such as bioreactors and microcarriers could significantly increase the production of exosomes by cultured MSCs [252]. Generally, MSCs are processed on 2D surfaces in plastic dishes that do not reflect the physiological niches of MSCs. Therefore, the use of a 3D porous scaffold structure, such as beads, microfiber, or any other type of carrier is an attractive method to increase exosome production. One study showed that antifungal agents, i.e., imidazole and nitrefazole, significantly increase the production of exosomes in prostate cancer cells [253]. In this case, nitreprazole increased the level of the protein Rab27a, which regulates MVB exocytosis. Other chemicals, such as azole and pentetrazole, have also been shown to activate exosome biogenesis-related molecules, i.e., Alix and NSmas2. The techniques may be employed to increase the production of exosomes from MSCs by modulating the biogenesis and release of exosomes [253]. On the other hand, gene editing is another effective way to increase the production of exosomes. There are several important genes, such as phospholipase D2, that are important for the biosynthesis and secretion of exosomes, and the overexpression or dysfunction of these genes promotes exosome secretion. For example, the overexpression of phospholipase D2 led to a twofold increase in the number of secreted exosomes [254].

(2) Hypoxia Preconditioning. Hypoxia culture is commonly used to prime MSCs. Several studies found that exosomes

Table 5: Genetic modifications in human MSCs and the disease models tested.

Factor overexpressed	MSC source	Method	Disease	Reference
Akt	Human umbilical cord	Adenovirus	Acute myocardial infarction	[187]
Angiotensin II type 2 receptor	Human bone marrow	Lentivirus	LPS-induced acute lung injury	[188]
Arginine decarboxylase	Human adipose tissue	Retrovirus	Spinal cord injury	[189]
Basic fibroblast growth factor (bFGF)	Human bone marrow	Lentivirus	Angiogenesis	[190]
Brain-derived neurotrophic factor (BDNF)	Human umbilical cord blood	Plasmid transfection	Neurological injury and disease	[191]
	Human bone marrow	Lentivirus	Neuronal degeneration	[192]
C-C chemokine receptor type 2 (CCR2)	Human bone marrow	Lentivirus	Ischemic stroke	[193]
CXC chemokine receptor 4 (CXCR4)	Human umbilical cord	Lentivirus	Radiation- induced lung injury	[194]
Cytosine deaminase (CD) and herpes simplex virus thymidine kinase (HSV-tk)	Human umbilical cord blood	Lentivirus	Ovarian cancer	[195]
Ephrin-B2	Human bone marrow	Plasmid transfection	Ischemic tissues	[196]
Forkhead box protein (Foxa2)	Human adipose tissue	Plasmid transfection	Acute liver injury	[197]
Glial-derived neurotrophic factor (GDNF)	Human adipose	Lentivirus	Renal interstitial fibrosis	[198]
	Human bone marrow	Adenovirus	Nephrotoxic serum nephritis	[199]
Glucocorticoid-induced tumour necrosis factor-related receptor (GITR)	Human bone marrow	Plasmid transfection	Small cell lung cancer	[200]
Granulocyte chemotactic protein-2 (GCP-2)	Human adipose tissue	Lentivirus	Myocardial infarction	[201]
Heme oxygenase-1 (HO-1)	Human embryonic stem cell	Lentivirus	Myocardial infarction	[202]
Hepatocyte growth factor (HGF)	Human bone marrow	Retrovirus	Bladder outlet obstruction	[203]
	Human umbilical cord	Lentivirus	Myocardial infarction	[204]
	Human bone marrow	Adenovirus	Liver fibrosis	[205]
	Human umbilical cord	Adenovirus	Injured sinonasal mucosa	[206]
	Human umbilical cord	Adenovirus	Parkinson's disease	[207]
	Human umbilical cord blood	Plasmid transfection	Liver fibrosis	[208]
	Human bone marrow	Lentivirus	Spinal cord injury	[209]
Hepatocyte nuclear factor 4a (HNF 4a)	Human umbilical cord	Lentivirus	Hepatocellular carcinogenesis	[210]
Human N-cadherin	Human umbilical cord blood	Lentivirus	Myocardial infarction	[211]
Hypoxia inducible factor-1a (HIF-1a)	Human bone marrow	Lentivirus	Angiogenesis	[212]
IL-4	Human adipose tissue	Lentivirus	Multiple sclerosis	[213]
IL-10	Human amniotic fluid	Human amniotic fluid	Liver fibrosis	[214]
	Human bone marrow	AAV	Acute ischemic stroke	[215]
Leptin	Human bone marrow	Lentivirus	Myocardial infarction	[216]
LIM-homeobox transcription factor islet-1 (ISL1)	Human bone marrow	Lentivirus	Myocardial infarction	[217]
miR-101-3p	Human bone marrow	Lentivirus	Oral cancer	[217]
miR-16-5p	Human bone marrow	Plasmid transfection	Colorectal cancer	[218]
miR-199a	Human bone marrow	Plasmid transfection	Glioma	[218]
miR-199a-3p	Human bone marrow	miRNA transfection	Renal ischemia/reperfusion injury	[219]
miR-let-7d or miR-154	Human bone marrow	Lentivirus	Lung injury	[220]
miRNA-181	Human umbilical cord blood	Lentivirus	Myocardial ischemia-reperfusion injury	[221]

Table 5: Continued.

Factor overexpressed	MSC source	Method	Disease	Reference
Neuregulin 1 (NRG1)	Human adipose tissue	Adenovirus	Cerebral ischemia	[222]
Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	Human amniotic fluid	Lentivirus	Acute lung injury	[194]
Oct4 and Sox2	Human adipose tissue	Plasmid transfection	Liver injury	[223]
PARKIN	Human Wharton's jelly	Plasmid transfection	Parkinson's disease	[224]
Pigment epithelial-derived factor (PEDF)	Human bone marrow	Lentivirus	Hepatocellular carcinoma	[225]
SRC3-specific short hairpin RNA (sh-SRC3)	Human bone marrow	Lentivirus	Multiple myeloma	[226]
sST2	Human adipose tissue	Lentivirus	Occupational asthma	[227]
	Human adipose tissue	Lentivirus	Endotoxin-induced acute lung injury	[228]
Thioredoxin-1 (Trx-1)	Human umbilical cord	Adenovirus	Acute radiation injury	[229]
Tissue matrix metalloproteinase inhibitor 2 (TIMP2)	Human umbilical cord	Lentivirus	Myocardial infarction	[230]
TNF-related apoptosis-inducing ligand (TRAIL)	Human bone marrow	Lentivirus	Non-small-cell lung cancer	[231]
	Human adipose tissue	Plasmid transfection	Non-small-cell lung cancer	[232]
	Human adipose tissue	Lentivirus	Glioblastoma multiforme	[233]
Transforming growth factor b1 (TGF-b1)	Human bone marrow	Lentivirus	Angiogenesis	[190]
Vascular endothelial growth factor (VEGF)	Human bone marrow	Lentivirus	Peripheral nerve injury	[234]

Table 6: Tissue engineering therapies using MSCs.

Disease	Study organism	Cell	Scaffold	Outcome	Reference
Osteochondral defect	Rabbit	Autologous MSCs	Injectable synthetic ECM	Cartilage filled the full-thickness defect	[242]
Spinal cord injury	Rat	Autologous MSCs	Hydrogels	Enhanced ingrowth of axons in the lesion and improvement in function	[243]
Critical size bone defect	Mouse	OSX-modified murine MSCs	Collagen sponge	Enhanced bone formation	[244]
Tendon defect	Rat	C3H10T1/2 cells stably transfected with BMP-2 and active Smad8 variant	Collagen sponge	Tendon regeneration	[245]
Articular cartilage defect	Rabbit	Autologous MSCs modified with TGF-1	Chitosan scaffold	Enhanced repair; defect filled with hyaline cartilage	[246]

derived from MSCs cultured in hypoxic condition showed greater angiogenic potential compared to exosomes secreted by MSCs cultured in normoxic condition [255]. The exosomes secreted by hypoxia primed MSCs were uptaken more effectively by the target cells compared to exosomes derived from MSCs cultured in normoxic condition. The uptaken exosomes promoted the VEGF expression and protein kinase A signaling pathway activation in the target cells, which resulted in improved angiogenesis [256, 257]. However, the reason for these phenomena is still unclear, and how different culture conditions influence the uptake of exosomes needs to be further investigated.

(3) Chemical Preconditioning. In contrast to hypoxic priming, the effects and mechanisms of biomolecule priming in exosomes are better studied. Various studies have compared the therapeutic effect between lipopolysaccharides (LPS) preconditioned and unconditioned exosomes. LPS conditioned exosome showed higher regeneration potential for liver disease preclinically by reducing the expression of IL-6 and TNF-β [258] and upregulated the expression of THP-1, which in turn stimulate the synthesis of more anti-inflammatory cytokines and contributed to the polarization of M2 macrophages [259]. A recent study has also shown that macrophages cultivated with exosomes from LPS-primed MSCs expressed higher levels of STAT3 gene, secretion of cytokines (IL-10 and IL-15), and growth factors (FLT-3 L) which play vital roles in cell regeneration and anti-apoptosis [260]. Several other molecules have been tested as preconditioners, including thrombin to improve fibroblast proliferation, enhance anti-inflammatory effects, accelerate wound healing [176] and melatonin to increase BCL2,

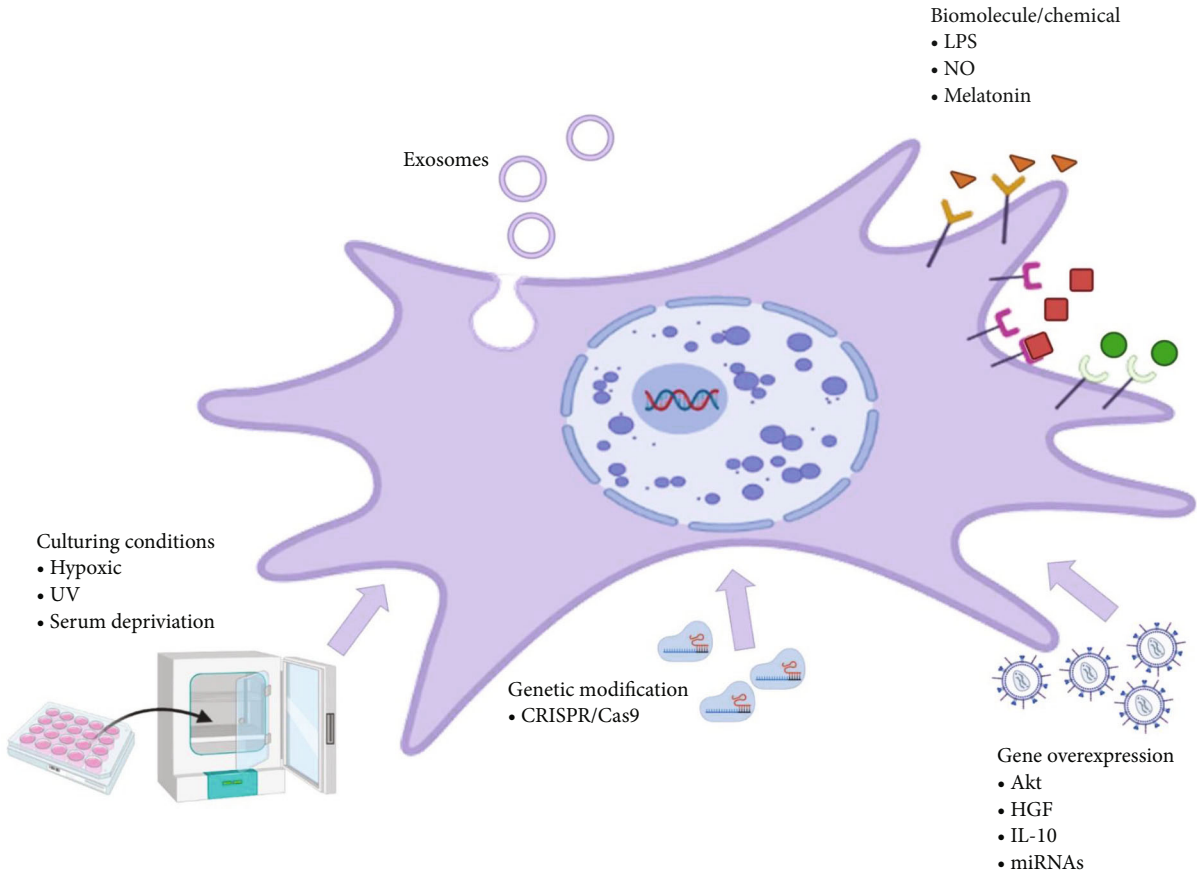

Figure 6: Preconditioning approaches to enhance the secretion and therapeutic efficacy of exosomes. The yield of secretome can be increased by preconditioning strategies such as introducing hypoxic and serum deprivation culture conditions and genetic modification using CRISPR technology as well as overexpression of certain genes. LPS: lipopolysaccharides; NO: nitric oxide; IL: interleukin; miRNAs: micro RNAs; UV: ultraviolet (created with BioRender.com).

HO1, IL-10, and VEGF expression, and suppress the expression of various apoptosis-related genes such as ICAM1, HIF1, NFkB, and IL-1β in a rat model [261]. Exosomes derived from deferoxamine-primed MSCs contained higher levels of miR-126a that support angiogenesis [262].

8.2.2. Genetic Modification. In 2010, a study reported that the paracrine factor secreted by MSC-overexpressed GATA-4 increased blood vessel formation and cell survival [263]. Next, in a mouse model of myocardial infarction, exosomes secreted by the genetically modified stem cells with GATA-4 were more effective in increasing angiogenesis and reducing the number of apoptotic cardiac cells compared to the exosomes secreted by native stem cells [264]. MSC-derived exosomes that overexpress GATA-4 and CXCR4 have been shown to contain cardioprotective antiapoptotic miR-19a that activates Akt and ERK signaling pathways [265, 266]. Similarly, exosomes from MSCs that overexpress SDF-1 have been shown to prevent apoptosis of cardiomyocytes and exhibit improved cardiac regeneration properties [267]. Genetic modification methods have also been investigated to improve the therapeutic potential of exosomes for musculoskeletal disorders, liver and lung disorders, and inflammation-related disorders.

8.2.3. Combining Nanoparticles, Exosomes, and Stem Cells. Nanotechnology is the term used to cover the design, construction, and utilization of functional structures with at least one characteristic dimension measured in nanometers. In recent years, the application of nanotechnology in stem cells has made great advances. Currently, nanotechnology is utilized to control the proliferation and differentiation of the transplanted stem cells.

Carbon nanotubes (CNTs) are widely used in various fields, including medicinal chemistry, molecular electronics, and tissue engineering, due to their unique mechanical, physical, and chemical properties. CNTs can be designed and filled with DNA or peptide molecules to improve their properties and functions, such as biocompatibility and recognition capability in the molecular treatment of diseases [268–270]. In a study that examined the effect of CNTs on the proliferation and differentiation of human stem cells, the result showed that CNTs inhibit the proliferation of cells of the embryonic kidney cell line HK293 and reduce the adhesion efficiency of cells in a dose- and time-dependent manner, but similar CNTs can stimulate the formation of bumps on the surface of human osteoblasts and fibroblasts, which are one of the active cells in the immune response [271]. Nanomaterials such as CNTs have enormous potential in the field

of regenerative medicine in several areas, including (1) the development of nanovehicles to deliver biomolecule-based products to MSCs and (2) the creation of new biomedical applications for electroactive CNTs in combination with MSCs. However, despite the immense potential of nanoparticles, the method of delivering nanoparticles to the target cells was still a major problem. The maximum size of particles entering cells is 25 nm to 700 nm; so, it is difficult for nano-sized particles to penetrate cells due to the tension and adhesion strength of the cell surface. As an alternative, the nanoparticles can be bonded to the external cell membrane.

9. Conclusion

Regenerative medicine holds an immense potential for a variety of diseases in which there is a high unmet clinical need. Regenerative medicine covered a wide range of subbranches including cell and gene therapies and tissue engineering applications. Stem cells have been the focus for years because of their biological potential, and paracrine effect is the pivotal mechanism in stem cell-based tissue regeneration. Thus, cell secretome has attracted great attention as therapeutics in recent years and has been suggested as alternative to stem cell therapy as cell-free agents. The high degree of confidence in cell-based therapy is vividly indicated by the significant increase in the number of ongoing and planned clinical trials worldwide. Despite the relatively slow rate of translational success from laboratory to clinics, expectations, optimism, and excitement surrounding this field remain great.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Review Article

Progress in Mesenchymal Stem Cell Therapy for Ischemic Stroke

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Ischemic stroke (IS) is a serious cerebrovascular disease with high morbidity and disability worldwide. Despite the great efforts that have been made, the prognosis of patients with IS remains unsatisfactory. Notably, recent studies indicated that mesenchymal stem cell (MSCs) therapy is becoming a novel research hotspot with large potential in treating multiple human diseases including IS. The current article is aimed at reviewing the progress of MSC treatment on IS. The mechanism of MSCs in the treatment of IS involved with immune regulation, neuroprotection, angiogenesis, and neural circuit reconstruction. In addition, nutritional cytokines, mitochondria, and extracellular vesicles (EVs) may be the main mediators of the therapeutic effect of MSCs. Transplantation of MSCs-derived EVs (MSCs-EVs) affords a better neuroprotective against IS when compared with transplantation of MSCs alone. MSC therapy can prolong the treatment time window of ischemic stroke, and early administration within 7 days after stroke may be the best treatment opportunity. The deliver routine consists of intraventricular, intravascular, intranasal, and intraperitoneal. Furthermore, several methods such as hypoxic preconditioning and gene technology could increase the homing and survival ability of MSCs after transplantation. In addition, MSCs combined with some drugs or physical therapy measures also show better neurological improvement. These data supported the notion that MSC therapy might be a promising therapeutic strategy for IS. And the application of new technology will promote MSC therapy of IS.

1. Introduction

Stroke is the second leading cause of death in the world after ischemic heart disease [1]. Ischemic stroke (IS) accounts for 87% of all stroke patients, and its incidence rate is still rising [2]. Due to acute neurological deficits caused by focal cerebral ischemia, it has brought different degrees of disability burden to a large number of patients. Currently, there are few treatment options for ischemic stroke. Intravenous injection of tissue plasminogen activator (t-PA) can recanalize the blocked vessels. However, this treatment is limited by a short time window (≤ 4.5 hours) and the risk of secondary cerebral hemorrhage [3]. Mechanical thrombectomy (MT) can extend the treatment time window to 24 hours, but this kind of special operation can only be performed in a few qualified hospitals and needs to go through strict screening of indications and contraindications; only a few patients can accept MT treatment [4]. In addition, rehabilitation treatment can only bring limited functional improvement; there are still a large number of patients with permanent disabilities [5]. Therefore, it is imperative to develop a new treatment for ischemic stroke.

Stem cell therapy has been widely studied in different central nervous system diseases (such as autoimmune encephalomyelitis, spinal cord injury, and stroke) in recent 20 years [6–8]. There are many types of stem cells, including embryonic stem cells, neural stem cells, hematopoietic stem cells, and mesenchymal stem cells [9]. As early as 1970, Friedenstein et al. cultured fibroblast precursor from the cell suspension of guinea pig spleen and bone marrow, which is now called mesenchymal stem cell [10]. Later studies found that these cells have the potential of multidirectional differentiation. They can not only differentiate into mesoderm lineage cells such as osteoblasts, chondrocytes, adipocytes, and muscle cells [11–13] but also differentiate into endoderm and neuroectoderm cells, including endothelial cells [14], hepatocytes [15], neurons [16], and glial cells [17]. MSCs lack HLA-II molecules and rarely express costimulatory molecules, so they are nonimonogenic or hypoimmunogenic [18]. Due to its pluripotent differentiation and immune tolerance, MSCs

have become one of the most promising candidate cells in stem cell therapy.

In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed a set of minimum standards for the definition of bone marrow MSCs: First, MSCs must show the property of plastic adherence in standard culture conditions. Second, MSCs must express endothelial surface markers (CD73, CD90, and CD105) and be negative for hematopoietic markers (CD11b, CD14, CD19, CD34, CD45, CD79α, and HLA-DR). Third, MSCs must be capable of differentiating to osteoblasts, adipocytes, and chondroblasts in vitro [19].

In recent years, a large number of studies have proved that the application of MSCs can reduce the area of cerebral infarction after ischemia and promote the recovery of neural function. The therapeutic mechanism of MSCs in IS has not been fully understood, which may be related to its neuron replacement, neurogenesis, angiogenesis, and anti-inflammatory effects. The extracellular vesicles (EVs) produced by MSCs may also play an important role in this process. A large number of preclinical studies have proved its safety. However, there is no reliable and authoritative scheme for MSCs in the treatment of IS. The best choice of MSCs in source selection, treatment dose, treatment time window, administration method, and treatment strategy needs to be explored scientifically. This article will make a comprehensive review on the progress of MSCs in the treatment of IS.

2. Method

Review the literature and summarize the effects, strategies, related mechanisms, safety, and clinical application of MSCs in IS treatment through searching the PubMed database with key words: ((ischemic stroke[Title/Abstract]) OR (cerebral ischemia[Title/Abstract]) OR (cerebral infarction[Title/Abstract]) OR (middle cerebral artery occlusion[Title/Abstract]) OR (ischemic brain injury[Title/Abstract])) AND ((Mesenchymal Stem Cells[Title/Abstract]) OR (Bone Marrow Stromal Cells[Title/Abstract])). And a total of 296 results were presented over the past five years. We excluded 120 reviews, systematic review, comments, and irrelevant articles and finally got 176 research articles.

3. Mechanism of MSCs in the Treatment of IS

3.1. Immunomodulatory. Inflammation and immune response play an important role in the pathogenesis of stroke. After focal cerebral ischemia, reactive oxygen species, proinflammatory cytokines, and chemokines are released from damaged brain tissue, causing the activation of resident inflammatory cells such as microglia and astrocytes [20]. At the same time, a large number of inflammatory factors lead to the destruction of the blood-brain barrier, and blood-derived inflammatory cells enter the ischemic brain tissue, which expands the inflammatory response and activates the adaptive immune response [21, 22]. Although some studies have shown that inflammation after ischemia can promote the repair of brain tissue and the recovery of neural function in the chronic phase [23], more evidence supports the adverse effects of inflammatory response. The continuous infiltration of immune cells and the continuous expansion of the inflammatory response can cause neuronal necrosis, brain edema, and aggravate secondary brain injury [24, 25].

As mentioned above, inflammatory factors lead to the destruction of blood-brain barrier after ischemia, and then chemokines such as monocyte chemoattractant protein-1 (MCP-1) and stromal cell-derived factor-1 (SDF-1) can attract large-scale invasion of peripheral immune cells [21]. Researchers found that transplanted MSCs can maintain the integrity of blood-brain barrier and reduce the leakage of inflammatory cells in brain parenchyma by downregulating the expression of aquaporin-4 and reducing the release of neutrophil matrix metalloproteinase-9 (MMP-9) [26, 27]. In addition, MSCs can reduce the production of MCP-1 by secreting anti-inflammatory cytokine TGF-β, thus blocking the migration of CD68 + immune cells to the ischemic areas [28].

Studies have shown that the immunomodulatory effect of MSCs is related to the regulation of proinflammatory and anti-inflammatory cytokines. By coculturing MSCs with oxygen- and glucose-deprived (OGD) neurons, Huang et al. found that MSCs may play an anti-inflammatory role by secreting IL-6 and reducing the expression of proinflammatory cytokine TNF-α [29]. The potential signaling pathway may be related to the inhibition of NF-κB activity by MSCs [30]. Similarly, in the animal model of IS, researchers also confirmed that MSCs can reduce the levels of proinflammatory cytokines TNF-α and IL-1β and reduce the infarct volume after focal cerebral ischemia [31]. Studies have shown that intra-arterial application of MSCs can inhibit the activation of acid sensing ion channels (ASICs) and then decrease the expression of the inflammasome, which leads to the inhibition of the activation of IL-1β [32]. Liu et al. reported that transplanted MSCs could alleviate nerve injury after focal cerebral ischemia by upregulating the expression of anti-inflammatory cytokine IL-10 and downregulating the expression of proinflammatory cytokine TNF-α [33]. A recent study showed that the above immunoregulation effect is achieved by MSCs enhancing Wnt/β-catenin signaling pathway, which is also related to MSCs mediated reduction of apoptotic cells after IS [34]. The cytokines involved in the immunoregulation of MSCs also include IL-23 and IL-17. In ischemic stroke, the inflammatory IL-23/IL-17 axis has been proved to be related to ischemia-reperfusion injury [35]. The experimental results of Ma et al. showed that MSCs injected via the caudal vein could reduce the infarct volume and promote the recovery of neurological function by downregulating the IL-23/IL-17 axis [36]. The above evidence suggests that MSCs can regulate the balance of proinflammatory and anti-inflammatory factors in the ischemic areas, and the same phenomenon is also observed in peripheral blood [37].

The activation of resident immune cells has a great impact on the inflammatory response after stroke. Previous in vitro experiments showed that MSCs could promote microglia to transform from a harmful neurotoxic phenotype, mainly releasing proinflammatory molecules to a beneficial neuroprotective phenotype producing anti-

inflammatory molecules through CX3CL1 release [38]. Recently, Tobin et al. found that microglia in ischemic lesions of MSC-treated rats showed the same morphology of small cell bodies and a large number of branchings as that of inactive ones [39]. Similarly, the experimental results of Oh et al. showed that intravenous injection of MSCs could induce proinflammatory M1 microglia to differentiate into anti-inflammatory M2 microglia after IS and exert anti-inflammatory effects by increasing the expression of IL-1ra (an anti-inflammatory cytokine) [40]. Further studies show that the activation of cAMP-response element binding protein (CREB) induced by MSCs may be related to it [40]. In vivo and in vitro studies by McGuckin and colleagues have shown that MSCs can cause low expression of microglia activation markers (ED1 and Iba) and astrocyte proliferation markers (GFAP) [41]. It is suggested that the immunomodulatory effect of MSCs may be related to the inhibition of these two kinds of brain resident inflammatory cells, which may be related to a noncanonical JAK-STAT signaling of unphosphorylated STAT3 [41].

The proinflammatory effect of MSCs has also been reported. The experimental results of Li et al. showed that the release of inflammatory factors in the infarct lesions of MSC transplantation group increased on the second day after the establishment of the middle cerebral artery occlusion (MCAO) model but decreased on the seventh day [42]. Guan et al. reported that MSC transplantation increased the proportion of TNF-1α and IL-1β positive immune cells in the infarcted cortex of MCAO rats, which supported the immune-promoting effect of MSCs in the early stage of infarction [43]. However, MSC transplantation is still beneficial to the functional recovery of MCAO rats on the second day [42]. Therefore, more effort is required to further explore the exact mechanism of MSC-mediated immunoregulation in the pathological process following IS in the further.

3.2. Neuroprotection. After the occurrence of IS, the ischemic focus of brain tissue was formed, which was divided into the central ischemic core and the surrounding ischemic penumbra. Most cells died in the ischemic core, and the structure of the ischemic penumbra changed, but the neurons still survived [20]. With the prolongation of infarct time, hypoxia and hypoglycemia lead to the decrease of ATP production and cell death in ischemic penumbra neurons. Moreover, high levels of glutamate from the ischemic core can induce the production of apoptosis mediators such as nitric oxide and oxygen free radicals in ischemic penumbra and cause neuronal apoptosis [20]. Therefore, the protection of neurons in the ischemic penumbra is the key to the treatment of IS.

By inducing MCAO in rats, the researchers found that compared with the control group, the expression of antiapoptotic factor Bcl-2 protein in MSC group was significantly increased, and the expression of p53 protein was significantly decreased (the induction of p53 is related to neuronal apoptosis) [44]. In addition, the density of neurons around the ischemic area increased after MSC transplantation [44]. These evidences suggest that administration of MSCs can reduce neuronal apoptosis.

After transplantation, MSCs directly release or increase the release of endogenous neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [45, 46], nerve growth factor (NGF) [47], glial cell line-derived neurotrophic factor (GDNF) [48], and basic fibroblast growth factor (bFGF) [49] to achieve neuroprotective effect. Chen et al. found that MSC treatment significantly increased bFGF in the ischemic border area of rats with middle cerebral artery occlusion, accompanied by a significant decrease of apoptotic cells in the ischemic border area [49]. In addition, the researchers found that transplantation of BDNF gene-modified MSCs could further increase the level of BDNF in the lesion area and further reduce neuronal apoptosis [45, 46]. The same enhancement effect was also observed in GDNF gene modified MSCs [48].

Studies have shown that fibronectin plays a neuroprotective role after IS. Fibronectin gene knockout mice show increased neuronal apoptosis and infarct size after transient focal cerebral ischemia [50]. The researchers found that six weeks after transplantation, transplanted MSCs still retained their fibronectin producing properties, suggesting that fibronectin may be involved in the neuroprotective effect induced by MSCs [51]. The imbalance of calcium ions in ischemic brain tissue after stroke can trigger the over activation of calcineurin (CaN) and cause neuronal apoptosis. Researchers found that MSC transplantation reduced the expression of CaN in the lesion, resulting in decreased neuronal apoptosis after IS [52].

In addition to apoptosis, MSCs can also play a neuroprotective role by alleviating parthanatos and necroptosis. By coculturing MSCs with OGD neurons, Kong et al. found that MSCs could protect neurons from parthanatos by reducing the nuclear translocation of apoptosis inducing factor (AIF) [53]. Moreover, the decrease of neuronal necrosis kinase RIP1 and RIP3 induced by MSCs was highly correlated with the decrease of neuronal necroptosis [53]. In addition, the experimental results of Nazarinia et al. showed that transplanted MSCs could reduce neuronal autophagy by increasing the expression of mTOR, thus playing a neuroprotective role after cerebral ischemia [54].

3.3. Angiogenesis. After IS, capillaries were destroyed, blood-brain barrier permeability increased, aggravating the inflammatory reaction, neuronal necrosis, and brain edema. Neovascularization after stroke helps to restore the blood and oxygen supply of the affected brain tissue, thus promoting nerve recovery, which may be a key factor in the survival of ischemic neurons [55]. The researchers found that patients with higher microvessel density at the edge of the ischemic area had a longer survival time, suggesting that poststroke angiogenesis plays an indispensable role in the prognosis of stroke patients [55].

Through three-dimensional analysis of the capillaries in the lesions, the researchers found that the number of new capillaries at the edge of the lesions in MCAO mice transplanted with MSCs increased significantly, which proved the role of MSCs in promoting angiogenesis [56, 57]. Studies have shown that neovascularization is mainly composed of endogenous endothelial cells but rarely differentiated from

transplanted MSCs [57]. Moreover, there was no significant correlation between the microvessel density and the number of mesenchymal stem cells in the peri-infarct area [58]. Therefore, the replacement of vascular endothelial cells may not be the main mechanism of MSCs. The current view is that MSCs transplanted into the infarcted areas can promote angiogenesis by directly releasing or increasing endogenous nutrients such as vascular endothelial growth factor (VEGF) [29], angiopoietin-1 (Ang-1) [59], placental growth factor (PlGF) [60], and basic fibroblast growth factor (bFGF) 45-47 [61]. Both Ang-1 and VEGF have strong angiogenic effects, but their effects are not exactly the same. VEGF can promote the formation of immature vascular trunk, and Ang-1 participates in the maturation and stability of vessels [62, 63]. It has been reported that MSCs can reduce infarct size by releasing Ang-1 and VEGF to promote angiogenesis. Interleukin-1β may play an important role in this process [64]. Toyama et al. investigated the effects of Ang-1 gene-modified MCSs (Ang-MCS), VEGF gene-modified MCSs (VEGF-MCSs), and Ang-1 gene combined with VEGF gene-modified HMCS (Ang-VEGF-MCSs) on angiogenesis in infarcted area of MCAO rats and compared their therapeutic effects [56]. Both Ang-MCS group and Ang-VEGF-MCS group showed an increase in capillary volume and a decrease in infarct size, of which the Ang-VEGF-MCS group achieved the greatest benefit [56]. Surprisingly, transplantation of VEGF overexpressed MSCs can lead to increased infarct size and neurological deficits, which suggests that angiogenesis may require the coexpression of vascular endothelial growth factor and angiotensin-1 [56].

The signaling pathway of angiogenesis induced by transplanted MSCs remains to be explored. After induction of MCAO model, Guo et al. first confirmed the increase of neovascularization in the infarcted area of MSC-treated rats [65]. In addition, by Western blotting and double immunofluorescence staining, they found that the level of Notch 1 protein and Notch 1 positive microvessels in the lesion area increased significantly, suggesting that MSCs promote angiogenesis by activating the Notch signaling pathway in the endothelial cell of ischemic brain tissue after stroke [65]. Further study by Zhu et al. showed that the activation of Notch signal may be related to the secretion of VEGF-A by endothelial cells [66]. The administration of DAPT (a gamma secretase inhibitor, which can inhibit the activation of Notch signal) led to the decrease of vascular endothelial growth factor-A and the inhibition of angiogenesis after MSCs transplantation [66]. By coculturing the supernatant of MSC culture with human aortic endothelial cells, Hong et al. found that the former could inhibit hypoxia-induced endothelial cell apoptosis and promote angiogenesis [67]. This beneficial effect may be related to the activation of PI3K Akt signaling pathway, which may be one of the potential signaling pathways for MSCs to promote angiogenesis after IS [67].

3.4. Neural Circuit Reconstruction. Under suitable conditions, MSCs can differentiate into neurons and glial cells [68]. The original idea is that bone marrow mesenchymal stem cells can differentiate and replace damaged nerve cells after transplantation. However, although MSCs transplanted into the cortex around the infarcted area can express neuron-specific markers, the differentiated neurons are immature, with round shape and few fiber processes [51]. More importantly, they lack the voltage-gated ion channels needed to generate action potentials [69]. Therefore, the neural replacement mechanism may not be one of the mechanisms of MSCs in the treatment of IS.

After cerebral ischemia, endogenous neurogenesis occurs in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampus. The newly formed neural progenitor cells can migrate to the infarcted area and further differentiate into neurons [70]. However, due to the unfavorable microenvironment full of inflammatory mediators and lack of nutrients after ischemia, most of these neural progenitor cells are facing the fate of rapid apoptosis, which limits the reconstruction of the neural network in the damaged area [70]. It is reported that MSCs can increase the number of neural progenitor cells and promote endogenous neurogenesis after IS [71, 72]. Through the electrophysiological recording of evoked field potentials, Song et al. found that the activity of neuronal circuits in the peri-infarct cortex of mice treated with MSCs was significantly increased [72]. Further studies have shown that MSCs can promote the migration and survival of neuroblasts to the ischemic penumbra and increase the number of neurons in the ischemic penumbra [72]. The increased expression of SDF-1 and polysialization enzyme induced by MSCs mediates the increased migration of neuroblasts to the injured site [73]. In addition, it is speculated that BDNF secreted by MSCs can promote the proliferation of neural stem cells in SVZ, increase the number of neural progenitor cells, and play a nutritional role in the process of proliferation, differentiation, and migration of neural progenitor cells, so as to prevent premature apoptosis [71, 74].

After IS, axonal sprouting and synaptic connection reconstruction of intact neurons promote the repair of neural function. However, the formation of glial scars in the ischemic area and the production of axon inhibitory proteins limit the reconstruction of the neural network [75, 76]. Liu et al. confirmed that the interhemispheric and intracortical axonal connections in the motor cortex around the infarction increased after stroke, and the application of MSCs significantly enhanced this effect [77]. Shen et al. demonstrated that MSC treatment significantly reduced the axonal loss and increased the expression of synaptophysin [78]. Further studies showed that MSCs could promote the reorganization of neural connections by reducing the thickness of the glial scars and the expression of Nogo-A (an inhibitor of axon growth) [78]. In addition, MSCs transplanted into the lesion may also promote axonal growth by downregulating the expression of neurocan (an axon elongation inhibitory molecule) and upregulating the expression of tPA in reactive astrocytes in the glial scars [75, 79, 80]. The above evidence indicates that MSC treatment can weaken the physical and chemical barrier effect of glial scars on axonal regeneration after infarction.

The transplanted MSCs may also promote axonal growth after cerebral ischemia by releasing nutrients. Song et al. showed that the expression of axon growth associated

protein-43 (GAP-43) increased, and the expression of axon growth inhibitory proteins rock II and NG2 decreased in the cortex around the infarction in the MSC-treated mice, accompanied by the increase of axon density in this area. Further experiments showed that the increased expression of GAP-43 may be related to bFGF secreted by MSCs. In addition, in view of the role of BDNF in promoting and maintaining axonal branching, the effect of MSCs on axonal growth may also be related to the release of BDNF [81, 82].

After cerebral ischemia, the neural circuit reconstruction is affected by myelin reformation. It has been reported that transplanted MSCs can increase the number of oligodendrocyte progenitor cells in the peri-infarct area, corpus callosum, and SVZ [83, 84]. Recent reports by Tobin et al. also confirmed the above views. By measuring myelin basic protein in ipsilateral hemispheric tissue lysates of MCAO rats, the authors found that the total amount of myelin basic protein increased significantly after MSC treatment, suggesting that the role of MSCs in promoting neural circuit reconstruction also includes promoting myelin formation [39]. In conclusion, MSCs contribute to the reconstruction of neural circuits by inducing endogenous neurogenesis, promoting axonal budding and myelin regeneration, and specific signaling pathways remain to be investigated.

3.5. Mitochondrial Transfer. Transferring healthy mitochondria to damaged cells may be one of the mechanisms of MSCs in the treatment of ischemic stroke. Tunnel nanotubes (TNTs) are nanoscale tubular structures that connect adjacent cells. As a new intercellular communication mechanism, they can promote the exchange of components between adjacent cells [85]. By coculturing MSCs with human umbilical vein endothelial cells subjected to oxygen glucose deprivation and reoxygenation (OGD/RO), Liu et al. found that TNTs could be formed between MSCs and endothelial cells. Moreover, under the induction of OGD/RO, the functional mitochondria in MSCs transport to endothelial cells in a single direction, thus protecting endothelial cells from hypoxia injury [86]. In a paper published in 2019, the same author demonstrated that MSC transplantation after IS can also protect cerebral vascular endothelial cells through this intercellular connection. Their experimental results show that MSCs transplanted into the peri-infarct area can transfer their active mitochondria to the damaged microvascular endothelial cells, thus promoting angiogenesis, reducing infarct size, and improving neurological function [58]. Furthermore, the application of TNT inhibitors significantly reversed this effect, suggesting that TNTs play an important role in the mitochondrial transfer of this activity [58].

Besides vascular endothelial cells, MSCs can also transfer mitochondria to astrocytes and neurons damaged by oxidative stress, promoting their survival and proliferation [87, 88]. This beneficial effect also depends on the direct contact between cells, because the survival rate of neurons decreased after MSCs and neurons were separated by porous transmembrane [88]. In addition, Miro1, a mitochondrial Rho-GTPase 1, was upregulated in oxidative injured neurons and promoted the transfer of mitochondria from MSCs to neurons [87]. By coculturing Miro1 overexpressing MSCs with damaged neurons, the researchers found that more neurons survived, while Miro1 inhibited MSCs caused the opposite result [88]. Further in vivo experiments showed that Miro1 overexpressing MSCs could significantly improve neurological function compared with normal MSCs after transplanted into cerebral infarction animals [88]. In conclusion, the increased expression of Miro1 in neurons after IS can cause transplanted MSCs to transfer their healthy mitochondria to damaged neurons, thus increasing the metabolic activity or survival of neurons. The direct contact between MSCs and neurons and the establishment of TNT connections play an important role in this process.

3.6. EV Transfer. Mesenchymal stem cell-derived EVs (MSC-EVs) are spherical cytoplasmic components secreted by mesenchymal stem cells, which contain a large number of soluble bioactive components such as lipids, proteins, mRNAs, and microRNAs [89]. It can regulate the activity and function of target cells by combining with target cells and transferring the above cell components and genetic genes into target cells [89]. As a key messenger between MSCs and injured cells, MSC-EVs play an important role in the treatment of IS with MSCs.

By coculturing MSCs with OGD neurons and brain microvascular endothelial cells (BMEC), the researchers found that the former could reduce the apoptosis of damaged neurons and restore the tube formation of BMEC [90]. The addition of GW4869 (an inhibitor of EVs secret) can reverse this beneficial effect, which suggests that MSC-EVs may be the main mediator of the neuroprotective and angiogenic effects of MSCs [90]. Xin et al. injected rats with MSCs-EVs via the tail vein 24 hours after the induction of IS [91]. Compared with the control group, the density of axons and synaptophysin immunoreactive areas increased in the treatment group [91]. Immunofluorescence staining showed that the number of doublecortin (marker of neuroblasts) positive and von Willebrand factor (marker of endothelial cells) positive cells increased [91]. These evidences suggest that MSC-EVs can induce angiogenesis, neurogenesis, and neural circuit reconstruction after IS. Zhao et al. explored the anti-inflammatory effect of exosomes, the main components of MSC-EVs, in ischemic cerebral infarction. They confirmed that intravenous injection of MSC-derived exosomes 2 hours after IS resulted in a significant decrease in neurological severity score (NSS) and a significant improvement in motor function 7 days later [92]. In vitro, OGD microglia were cocultured with MSC-derived exosomes. It was found that the latter could inhibit the activation of M1 microglia, increase the number of M2 microglia, downregulate the levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-12), and upregulate the levels of anti-inflammatory cytokines (TGF-β and IL-10) [92]. These data suggest that MSC-EVs are involved in immunomodulation, neuroprotection, angiogenesis, and neural circuit remodeling after transplantation of MSCs into the ischemic brain.

MSC-EVs may play a role by mediating the transfer of microRNA. First, Moon et al. demonstrated that 24 hours after MCAO induction, intravenous injection of MSC-EVs produced angiogenesis and neurogenesis, and this effect

was positively correlated with the dose of MSC-EVs [93]. The contents of miR-184 and miR-210 in MSC-EVs were more abundant than those in fibro EVs [93]. Transfection of miR-184 and miR-210 into neural stem cells and human umbilical vein endothelial cells could increase the proliferation of these two cells, suggesting that MSC-EVs may induce the proliferation of vascular endothelial cells and neural stem cells after IS through miR-184 and miR-210 [93]. Secondly, MSC-EVs containing miR-29b-3p inhibitor could increase the apoptosis of oxygen-glucose-deprived neurons and decrease the angiogenesis of BMEC, while MSC-EVs overexpressing miR-29b-3p had the opposite effect [90]. This suggests that miR-29b-3p may mediate the neuroprotective and angiogenic effects of MSC-EVs. Mir-29b-3p may play a role by inhibiting PTEN and then activating Akt signaling pathway [90]. Furthermore, the experimental results of Geng et al. showed that MSC-EVs overexpressing miR-126 significantly increased the number of doublecortin positive and von Willebrand factor positive cells compared with normal exosomes [94], which suggests that miR-126 may be involved in EV-mediated angiogenesis and neurogenesis. Finally, MSC-EVs may play an indirect role in nerve repair after IS. In vitro experiments by Xin et al. showed that MSC-EVs overexpressing miR-133b could increase the secretion of exosomes by astrocytes, while the latter could significantly increase the number and length of axons [95].

In conclusion, immunoregulation, neuroprotection, angiogenesis, and neural circuit reconstruction may be the main mechanisms of MSCs in the treatment of IS, while the secretion of nutritional cytokines, the transfer of mitochondria, and the transfer of extracellular vesicles may be the main ways of MSCs acting (Figure 1).

4. Selection of MSCs

4.1. Different Sources of MSCs. Bone marrow is the first tissue to isolate MSCs. However, the production of MSCs in bone marrow is low. The proliferation and differentiation potential of bone marrow-derived MSCs (BM-MSCs) decrease with age, and invasive bone marrow puncture is needed to obtain them [96], which makes bone marrow may not be the best source of MSCs. Besides, bone marrow, MSCs were also isolated from other tissues, including adipose tissue, placenta, umbilical cord, and dental pulp.

There are a large number of functional mesenchymal stem cells in adult adipose tissue. Adipose-derived MSCs (AD-MSCs) can be obtained by collagenase digestion of adipose tissue. Compared with BM-MCS, AD-MSCs are easier to obtain and cultivate enough autologous grafts [97]. Immunogenicity of human allogeneic AD-MSCs is lower than that of allogeneic BM-MCS [98], while autologous AD-MSCs show lower immunogenicity [99]. Due to its considerable clinical transformation potential, AD-MSCs are the most studied MSCs in IS besides BM-MSCs. Studies have shown that after the establishment of MCAO model, intravenous injection of AD-MSCs has the same curative effect as injection of BM-MSCs, and AD-MSC has even more advantages in reducing infarct size and improving neurological function [100]. Many other animal experiments have also proved that AD-MSC transplantation after IS has immunomodulatory effects [101–103], neuroprotective effects [102], angiogenesis effects [104], and neural circuit reconstruction effects [104]. It is worth noting that the experiment of Mangin et al. showed that intravenous injection of AD-MSCs could not improve the infarct size and neurological function after IS in diabetic or hypertensive mice [105]. Frutos et al. reported that intravenous transplantation of AD-MSCs can improve the function of hyperglycemic rats after cerebral infarction but has no effect on hypertensive rats [106, 107].

Dental pulp provides an accessible, noninvasive, high proliferation potential source of mesenchymal stem cells [108]. The extracted wisdom teeth are similar to the adipose tissue on the operating table. If they are not used for stem cell extraction or other purposes, these tissues will be discarded as clinical waste. Similar to BM-MSCs, the beneficial effect of dental pulp-derived MSCs (DP-MSCs) may be mediated by paracrine mechanisms rather than substitution [108, 109]. Song et al. compared the therapeutic effects of intravenous injection of human DP-MSCs and human BM-MSCs in MCAO model rats. There was no significant difference in the improvement of neurological function between the two groups, but the DP-MSC group showed smaller infarct volume [110]. In addition, the experimental results of Wu et al. showed that periodontal ligament stem cells (PDLSCs) were more effective than DP-MSCs in promoting the recovery of neural function after cerebral ischemia [111]. It is worth noting that a recent study reported that human DP-MSCs have the ability to produce action potentials after differentiation into neurons in vitro [112]. Whether they can be converted into functional neurons in animal models of IS remains to be explored.

Umbilical cord-derived mesenchymal stem cells (UC-MSCs) were extracted from umbilical cord perivascular tissue and Wharton's jelly (mucoid connective substance surrounding umbilical cord vessels). As the same as placenta, umbilical cord is easy to obtain as the waste after delivery, and there is no ethical problem. A number of studies have compared MSCs derived from the umbilical cord, dental pulp, bone marrow, and adipose tissue and found that MSCs derived from the umbilical cord have stronger proliferation activity [113, 114]. Studies have shown that UC-MSCs and placenta-derived MSCs (PL-MSCs) can alleviate neurological deficits after cerebral ischemia in rats, and their potential mechanisms are similar to those described above [96, 115–117]. It is worth noting that the data of Liao et al. showed that after transplantation of human UC-MSCs, more than 90% of the blood vessels around the cerebral ischemic area contained transplanted mesenchymal stem cells, which were integrated into the blood vessels and partially differentiated into endothelial cells [115]. The authors suggest that UC-MSCs can play the role of vascular remodeling by directly differentiating into vascular cells, which is not common in the experiment of using BM-MSCs.

Most of the BM-MSCs were extracted from long bone or iliac bone. Abiko et al. extracted BM-MSCs from rat skull and used them in MCAO model rats [118]. Their experimental results showed that compared with normal BM-MSCs, rats transplanted with skull-derived MSCs showed better

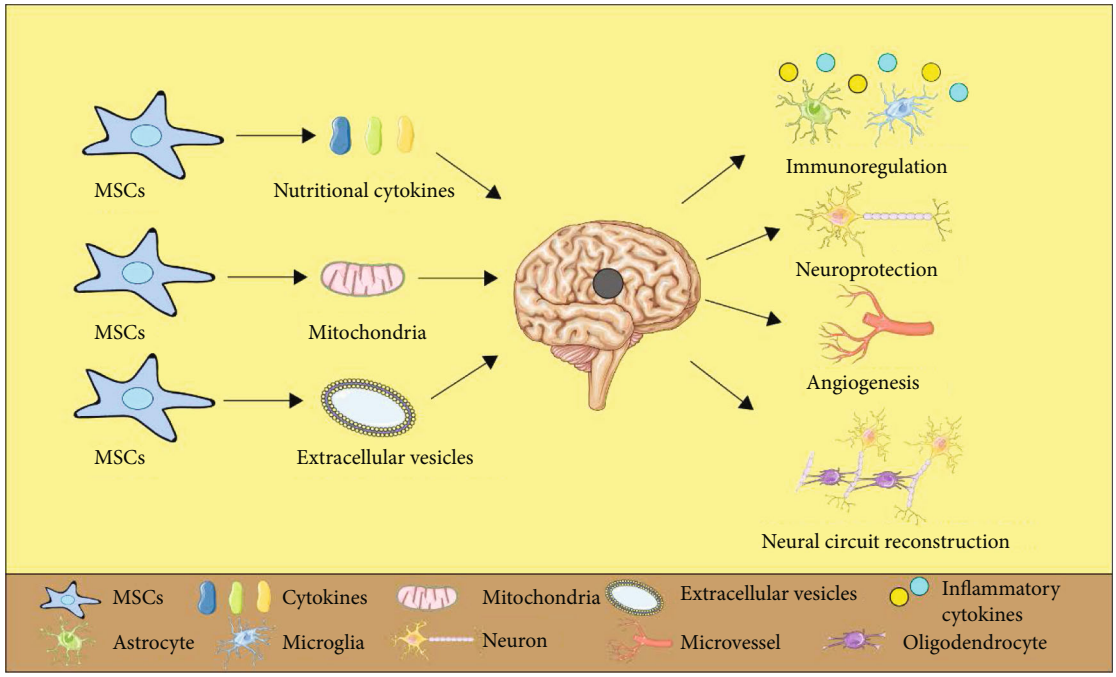

Figure 1: Mechanisms of MSCs in the treatment of IS. This figure contains elements available at Servier Medical Art repository, which is licensed under a Creative Commons Attribution 3.0 Unported License.

neurological recovery, which may be related to the latter's ability to secrete more BDNF and VEGF [118, 119]. Interestingly, MSCs were also extracted from human turbinate. Lim et al. found that MSCs derived from human turbinate can promote neurogenesis after cerebral ischemia in rats, and the effect of improving neural function after IS is similar to that of AD-MSCs transplantation [120]. However, it is not easy to obtain MSCs from human skull and turbinate, and its clinical application potential is limited.

In addition to BM-MSCs, MSCs from adipose, dental pulp, umbilical cord, and placenta are the most promising types of MSCs for clinical treatment due to their easy availability and strong expansion ability. Future research also needs to clarify the consistency and difference of the mechanism of MSCs from different sources, and which kind of MSCs can obtain the maximum efficacy and the minimum adverse reactions in IS, so as to determine the most suitable source of MSCs for clinical application.

4.2. Autologous or Allogenic? Although autologous MSCs are the safest, allogeneic MSCs have more advantages. First of all, autologous MSCs need a long time to culture and expand, which limits its application in the acute stage of IS, while allogeneic MSCs can be obtained and expanded from the freezer more quickly, thus avoiding the delay of time window. Second, patients with IS usually take antiplatelet or anticoagulant drugs, and the application of autologous MSCs may lead to secondary hemorrhage. Allogeneic MSCs from healthy donors have no such concerns. Third, age is a factor that affects the physiological characteristics of MSCs. Studies have shown that MSCs from elderly donors have decreased proliferation and differentiation ability, and the ability to secrete nutrients such as BDNF, VEGF, and insulin-like growth factor (IGF) is also affected [121–123]. Animal experiments have proved that transplantation of BM-MSCs from old people can improve the neurological function of rats after cerebral infarction and less than transplantation of MSCs from young people, and the effects of BM-MSCs from young people on anti-inflammation, angiogenesis, and secretion of nutritional factors are more significant [123]. However, IS patients are generally older, so allogeneic MSCs obtained from young healthy donors may be more effective.

5. Routes of Transplantation

5.1. Intraparenchymal Delivery. Intracerebral transplantation is effective in the treatment of experimental ischemic stroke [27, 124]. Direct injection of MSCs into the brain parenchyma can lead to the largest number of MSCs in the lesion area [27], with less side effects outside the brain, and may bring the best neurological improvement [124]. A meta-analysis of 46 articles showed that different routes of administration affected the therapeutic effect of MSCs in the treatment of IS [125]. Intracerebral administration was the best, followed by intra-arterial administration, and finally intravenous administration. However, neurosurgery is not acceptable to all patients [125]. Although the application of stereotactic technology can avoid craniotomy, it may still persecute the local brain parenchyma and blood-brain barrier, lead to additional neuronal damage and inflammatory reactions, and even cause complications such as hemorrhage and epilepsy. Therefore, whether the intracerebral delivery pathway is suitable for clinic application remains to be further studied and discussed.

5.2. Intraventricular/Intrathecal Delivery. Intraventricular or intrathecal injection of MSCs can spread to various parts of the central nervous system through cerebrospinal fluid. Lim et al. injected MSCs into the lumbar spinal cord sheath of rats with IS and found that compared with intravenous injection, MSCs injected intrathecally could effectively migrate to the infarcted area and could differentiate neurons and astrocytes, which promoted the improvement of motor function of rats with cerebral infarction [126]. A recent study reported that MSCs injected into the corpus callosum could migrate not only to the infarcted areas but also to the choroid plexus (CP) [127]. In vitro, the coculture of CP and MSCs showed that they could promote each other's proliferation, and this interaction may be related to neurogenesis [127]. Therefore, the authors suggest that MSC injected into the lateral ventricle containing CP is an appropriate way of transplantation. Although intraventricular or intrathecal administration is less likely to cause secondary injury than intraparenchymal administration, it is still more invasive than intravascular administration, and its inconvenient operation limits its clinical application.

5.3. Intravenous Delivery. The advantage of intravenous injection is that it avoids intracranial invasion, less trauma, and is simple and easy to operate. Many preclinical trials of MSCs in the treatment of IS have used intravenous injection and achieved good results, including the reduction of infarct volume and improvement of neurological function. Although different studies have reported that the therapeutic effect of the intravenous injection route on ischemic cerebral infarction may be the smallest, the benefits are still considerable [124, 125]. The limitation of intravenous injection is that it needs to reach the artery through the systemic venous circulation and then enter the brain through blood-brain barrier. The result of long-distance migration is that only a small part (4%) of MSCs injected intravenously can be located in ischemic brain tissue, and most of them stay in peripheral organs such as the lung, liver, spleen and kidney, and especially the lungs [47, 128]. Due to its large volume (18 μm diameter), AD-MSCs have a higher pass clearance rate through the pulmonary circulation. The risk of intravascular injection lies in the possibility of pulmonary embolism. Cases of pulmonary embolism caused by intravascular injection of AD-MSCs have been reported in a journal, although it is not used for the treatment of IS [129].

5.4. Intra-arterial Delivery. Intra-arterial injection is another method of intravascular administration; most of the studies take internal carotid artery injection. Intra-arterial injection not only retains the advantages of minimally invasive intravascular administration but also can bypass the pulmonary circulation and improve the concentration of MSCs in the lesion. Li et al. injected 2×10^6 MSCs into rats through the internal carotid artery and found that 21% of MSCs entered the brain and promoted the improvement of function after cerebral ischemia [130]. Zhang et al. compared the effects of intra-arterial, intravenous, and intracerebral transplantation in MCAO model rats and found that the intra-arterial pathway showed the greatest degree of neural function recovery [131]. The disadvantage of intra-arterial injection is that it may lead to the formation of intra-arterial emboli, decrease of cerebral blood flow, and cerebral microvascular embolism, and it is related to the dose of injected cells [132]. Clinical trials have proved the effectiveness and safety of intra-arterial injection of MSCs in patients with IS [133], so intra-arterial injection may be another suitable intravascular route.

5.5. Intraperitoneal Delivery. Intraperitoneal injection is a less used way of MSC administration. This way of administration can cause a large number of MSCs to accumulate in the abdominal cavity, while the number of MSCs migrating to other organs is very small [134]. An experiment in rats with hypoxic ischemic encephalopathy (HIE) model showed that the number of MSCs injected intraperitoneally homed to the ischemic area was less than that injected intravenously [135]. However, Barzegar et al. showed that intraperitoneal injection of MSCs showed a high survival rate [116]. These authors further demonstrated that intraperitoneal injection of MSCs into MCAO mice also showed an effective neuroprotective effect, which may be related to the significant recovery of cerebral blood flow after administration [116].

5.6. Intranasal Delivery. Intranasal route is a new way of stem cell transplantation. MSCs transplanted intranasally can bypass the blood-brain barrier at the nasal mucosa and then enter the brain through the olfactory sensory nerves or further transfer to other intracranial regions through cerebrospinal fluid [136]. Chau et al. reported that intranasal administration of MSCs reached the peri-infarct area 6 hours after delivery, and MSCs transplanted intranasally could reduce the infarct volume and promote the recovery of neurological function [137]. Zhao et al. showed that intranasal administration of MSCs can improve the permeability of blood-brain barrier and promote the recovery of neurological function after IS, which may be related to the promotion of revascularization in the infarcted area [138]. Researchers found that intranasal administration of MSCs in neonatal hypoxic-ischemic injury model mice can show the recovery of cognitive, sensory, and motor functions [139]. It is worth noting that intranasal administration of less MSCs can achieve the same effect as intracranial administration [139]. The advantages of intranasal injection lie in its noninvasive, simple operation, and repeated administration. However, compared with rodents, the human olfactory bulb is smaller. Whether intranasal administration of MSCs to IS patients can achieve the same effect as experimental animals remains to be proved by clinical trials.

In conclusion, in the current research on MSCs in the treatment of IS, intraparenchymal, intravenous, and intra-arterial administration are the three most widely used methods, and different administration methods have their own advantages and disadvantages (Table 1). Intravascular injection may be an appropriate route for MSC administration. Intranasal administration, as a new way of stem cell transplantation, has great potential in clinical application. Future studies need to evaluate and compare the safety and effectiveness of different ways of administration, so as to select the most suitable way of administration.

Table 1: Advantages and disadvantages of different MSC transplant approaches.

Routes	Advantages	Disadvantages	Reference
Intraparenchymal	Highest homing rate; low off-brain side effect	Highly invasiveness; additional brain tissue damage	[27, 124]
Intraventricular/intrathecal	Allow for migration to different parts of CNS; high homing rate	Invasiveness	[126]
Intravenous	Low invasiveness; easy operation	Stagnation in peripheral tissue; pulmonary embolism	[128, 129]
Intra-arterial	Low invasiveness; considerable homing rate	Microvascular occlusion	[130, 132]
Intraperitoneal	Low invasiveness; high survival rate	Low homing rate	[116, 134]
Intranasal	Noninvasiveness; easy operation; repeated administration	Lack of clinical trial evidence	[136–138]

6. Timing of Transplantation

Different laboratories have studied the efficacy of MSCs transplanted at different time points after IS. Omori et al. divided the rats into two groups; one group was injected with 3×10^6 MSCs at 6 hours after stroke induction, and the other group was injected with 1×10^6 MSCs at 6 hours, 24 hours, and 48 hours, respectively [140]. The researchers found that the two groups achieved the same improvement in neurological function, suggesting that the time window of MSCs for the treatment of IS can be extended to 48 hours after cerebral infarction [140]. Hess et al. reported in a clinical trial that early administration of MSCs within 24-48 hours of the onset of symptoms of acute ischemic cerebral infarction may lead to a better one-year prognosis. Ishizaka et al. injected 1×10^6 MSCs into the internal carotid artery of rats on days 1, 4, and 7 after MCAO induction (D1, D4, and D7). They found that the D1 group showed the earliest improvement of motor function, followed by the D4 group, but the D7 group did not recover. There was no significant difference in the degree of recovery between the D1 and the D4 groups [141]. This study expanded the treatment time window of MSCs to 4 days after IS and suggested that the early application of MSCs may get faster recovery. After intranasal administration of 1×10^6 MSCs on the 3rd, 4th, 5th, and 6th day after IS, Chau et al. found that the motor function of mice was significantly improved on the 14th day, which suggested that the administration of MSCs in the delayed phase of IS was still beneficial [137]. In addition, it has been reported that MSCs can still significantly improve the neurological function of rats after 1 month of infarction, although it is used in a relatively large dose (3×10^6) [142].

Due to the different ways of administration, dosage, and evaluation of neurological function in different studies, the best administration time cannot be obtained objectively. A meta-analysis involving 141 articles divided the administration time of these studies into four groups: 0-6 hours, 12-24 hours, 2-7 days, and > 7 days [143]. It was found that compared with the 12-24 hours group and > 7 days group, the score of comprehensive neurological function was significantly improved in the 2-7 days groups [143]. Moreover, there was no significant difference between 0-6 hours and 2-7 days, which suggests that 0-6 hours and 2-7 days after IS may be the best time for administration [143]. On one hand, in the early stage after IS, local brain tissue lacks oxygen and energy, and the inflammatory reaction is strong, which leads to the low survival rate of MSCs. Therefore, administration between 2-7 days after IS may increase the survival rate of MSCs, so that MSCs can play a greater role. On the other hand, the significant improvement of neural function induced by administration of MSCs at 0-6 hours may be related to the timely rescue of neurons in the ischemic penumbra, early intervention of inflammatory cells, and activation of the inflammatory cascade. In addition, the study also found that, compared with other groups, 0-6 hours administration caused the most significant improvement in sensorimotor outcomes [143]. Therefore, early administration within 7 days after stroke may be the best time for treatment.

Although the best time for MSCs to treat IS is still controversial, it is undeniable that MSCs expand the treatment time window of ischemic stroke, so that those patients who cannot receive tPA thrombolytic therapy for more than 4.5 hours can benefit from MSCs.

7. Doses of Transplantation

Studies have shown that intravenous transplantation of MSCs between 5×10^5 and 3×10^6 is effective in rodent stroke models [144]. In a preclinical study, MSCs were given at doses of 1×10^5, 5×105, and 1×10^6 24 hours after stroke induction. It was found that compared with the control group, only rats in the 1×10^6 group showed improved neurological function [40]. There are also studies using a relatively large dose (3×10^6) of MSCS, which found that compared with the 1×10^6 group, the infarct size of rats in the high dose group decreased by 20% and showed better neurological recovery. A phase I clinical trial has proved that intravenous infusion of 0.5×10^6, 1.0×10^6, and 1.5×10^6 MSCs/kg allogeneic MSCs is safe and effective in patients with chronic ischemic stroke.

Is the greater the dose of MSCs, the greater the benefit? Lin et al. injected 1×10^6 and 4×10^6 human UC-MSCs intravenously 24 hours after MCAO in mice and found that a high dose of human UC-MSCs did not cause a more significant reduction of infarct size. The authors suggest that this may be due to the fact that most of MSCs remain in peripheral tissues after intravenous injection of a large doses of MSCs, so that the number of MSCs eventually reaching the central nervous system is not as much as expected [145]. A meta-analysis showed that the benefit of neurological function may not be directly proportional to the dose of MSCs but may be inverse U-shaped, as the benefit of behavioral function decreases at the highest dose of MSCs [125], which may be related to the disadvantages of intravascular administration. When MSCs are given a large dose via an artery or veins, it may cause microvascular obstruction or embolus formation and then decrease the perfusion of the brain or other organs. Therefore, it is necessary to grasp the relationship between effectiveness and safety to obtain the optimal dosage. More clinical and preclinical studies are needed to get the dose value corresponding to the apex of the inverse U-shape.

8. Therapeutic Strategy

Although MSCs have great potential in the treatment of ischemic stroke, due to the low homing rate, survival rate, and poor differentiation ability after transplantation, the effect of MSCs in the recovery of neurological function after cerebral infarction is still unsatisfactory, so researchers developed a variety of strategies to increase the efficacy of MSCs in ischemic infarction from different perspectives. The main strategies include pretreatment, gene transformation or overexpression, combination therapy, and MSC-EV transplantation.

8.1. Pretreatment of MSCs. Pretreatment is a strategy to change the culture environment of MSCs in different ways before transplantation to enhance their functional characteristics. Hypoxic culture is one of the most commonly used pretreatment methods for MSCs. Adapting to the hypoxic environment in advance may make MSCs play a better role in the face of barren environment in vivo. An appropriate hypoxia environment can increase the proliferation rate of MSCs and promote their differentiation into different mesenchymal cell lines in vitro [146]. By transplant of normoxic and hypoxic cultured MSCs into MCAO model animals, Hu et al. found that compared with the normoxic group, hypoxic preconditioning resulted in increased migration to the ischemic penumbra and improved survivability in adverse environments [147]. These benefits may be related to the increased expression of C-X-C chemokine receptor type 4 (CXCR4) in MSCs after hypoxia. As a ligand of SDF-1, the increased expression of CXCR4 promotes MSCs homing to the infarcted area [147]. Chen et al. showed that the enhanced migration and survival of MSCs after hypoxic preconditioning may be related to the inhibition of caspase-3 activation and the increased expression of hypoxia inducible factor-1α (HIF-1α) [148]. In addition, the expression of BDNF and VEGF in MSCs pretreated by hypoxia increased more significantly and promoted angiogenesis and nerve regeneration more significantly [148]. Moreover, the authors think that 8 hours is the best time for hypoxic preconditioning [148]. Kong et al. reported that MSCs cultured in a hypoxic environment expressed higher levels of CD200, which may be related to the decreased activation of microglia and the increased expression of anti-inflammatory cytokines such as IL-10 and TGF-β after MSC transplantation, suggesting that hypoxic preconditioning may enhance the immunomodulatory ability of MSCs after IS [117]. It should be noted that the transplantation of conditioned medium and exosomes derived from hypoxic preconditioning MSCs into MCAO model animals also showed a greater reduction of infarct size and improvement of neurological function [149, 150]. Moreover, hypoxic preconditioning can also improve the protective effect of conditioned medium derived from aged BM-MSCs on ischemic neurons, thus partially offsetting the adverse effect of age on the transplantation of autologous bone marrow stem cells [151].

In addition to culture in a hypoxic environment, there are many ways to pretreat MSCs. In vitro studies suggest that IL-1-treated MSCs can secrete more granulocyte colony stimulating factor (G-CSF) and reduce the secretion of inflammatory mediators in activated microglia [152]. The same authors injected medium derived from IL-1-treated MSCs into MCAO model animals and found that the conditioned medium could reduce the infarct volume by 30% 48 hours after stroke and improve the neurological function score [153]. The above evidence suggests that IL-1 pretreatment could induce MSCs to transform into anti-inflammatory and pronutritional phenotypes and play a beneficial role in cerebral infarction. Tobin et al. compared the efficacy of interferon-γ pretreated MSCs and normal MSCs in MCAO model animals and found that although there was no significant difference in functional improvement between the two, interferon-γ pretreated MSCs may have more advantages, which reflected in the fact that interferon-γ pretreated MSCs can induce activated microglia to secrete less proinflammatory cytokines and induce oligodendrocyte differentiation and myelination more effectively [39]. VX-765 is a selective caspase-1 inhibitor. Sun et al. found that transplantation of VX-765 pretreated MSCs resulted in more anti-inflammatory cytokines and less proinflammatory cytokines and apoptotic cells than nonpretreated MSCs. The enhanced anti-inflammatory and antiapoptotic effects of VX-765 may be related to the activation of autophagy by regulating AMPK/mTOR signaling pathway [154]. Different from the mechanism mentioned above, the authors believe that the increase of autophagy is beneficial to the treatment of IS by MSCs. Therefore, the role of autophagy in the treatment of is by MSCs remains to be further studied and explored.

MSCs-EV is one of the important mechanisms of MSCs in the treatment of IS. Cholesterol, as an important component of EVs, participates in the production, secretion, and functional regulation of EVs [155]. Barzegar et al. cultured human PL-MSCs with cholesterol lipid supplemented media and found that human PL-MSCs treated with cholesterol lipid could release more EVs, and the survival rate of vein

transplantation was also significantly improved [116]. When 1×10^5 human PL-MSCs treated with cholesterol lipid were intravenously injected into mice after MCAO induction for 1 hour, the researchers found that they could reduce the infarct size and restore neurological function, while the same low dose of common human PL-MSCs did not show any protective effect [116]. These evidences suggest that pretreatment of MSCs with cholesterol lipids can improve the efficacy of MSCs in the treatment of IS by enhancing the release and survival ability of EVs.

Three-dimensional (3D) aggregation is a new method of MSC culture. MSCs cultured in this way form a spheroid composed of 500-10000 tightly packed cells. Compared with 2D adherent MSCs, their migration ability and survival ability in hypoxia environment were enhanced, and the release of anti-inflammatory and nutritional factors was increased [156]. Yuan et al. verified the above characteristics of 3D aggregate-derived MSCs in MCAO model animals and showed smaller infarct size after transplantation of 3D aggregate-derived MSCs, suggesting that 3D aggregation is an effective pretreatment measure to enhance the efficacy of MSCs after IS [157]. Most stem cell studies use fetal bovine serum (FBS) to culture MSCs. Moon et al. compared the efficacy of MSCs cultured in fetal bone serum, normal health control serum, and stroke patient serum in MCAO model animals and found that rats transplanted with MSCs cultured in stroke patient serum showed more significant angiogenesis and neurogenesis, which is an inspiration for the way of autologous MSC transplantation [158].

8.2. Gene Transfection or Overexpression. It is a potential therapeutic strategy to enhance the therapeutic effect of MSCs in the treatment of IS by transfecting specific genes with viral vectors or plasmids to make MSCs overexpress certain molecules or proteins. After IS, the homing of transplanted MSCs is mainly mediated by the interaction between chemokine receptors on the surface of MSCs and high levels of chemokines in ischemic lesions. C-C motif chemokine ligand 2 (CCL2) is one of the most expressed chemokines in the ipsilateral cerebral hemisphere after IS. It mediates the transfer of a variety of cells to the brain by interacting with C-C motif receptor 2 (CCR2). Huang et al. transplanted CCR2 transgenic MSCs in MCAO model animal and found that this kind of CCR2 overexpression MSCs can more effectively migrate to ischemic lesions and mediate the protection of blood-brain barrier and the more significant improvement of neural function [159]. In addition, Lee et al. found that transplantation of CCL2 overexpressed MSCs resulted in a more significant increase in angiogenesis and neurogenesis and a more significant reduction in inflammatory response [160]. Moreover, the researchers also reported that overexpression of the neurogenic transcription factor neurogenin-1 can upregulate the expression of chemokine receptors CCR1, CCR2, and CXCR4 in MSCs, thus increasing the homing of MSCs to ischemic regions and promoting the further improvement of neural function [161]. The above evidence suggests that the more MSCs migrate to ischemic lesions, the more beneficial it will be. Promoting the homing of MSCs by genetic means is a potential therapeutic strategy.

Immunomodulation is an important mechanism of MSCs in the treatment of IS. As mentioned above, transplanted MSCs can regulate the inflammatory response after IS by upregulating the level of anti-inflammatory cytokines and downregulating the level of proinflammatory cytokines. Nakajima et al. reported that intravenous injection of IL-10 overexpressing MSCs resulted in a more significant reduction of infarct size and a more significant improvement of neurological function than normal MSCs [162]. Specifically, this kind of transgenic MSCs can lead to higher levels of anti-inflammatory cytokine IL-10 in lesions, which can inhibit the activation of microglia and the secretion of proinflammatory cytokines more effectively [162].

The repair of neural circuits is another important mechanism of MSCs in the treatment of IS. Noggin is an extracellular bone morphogenetic protein (BMP) antagonist, which promotes neurogenesis by inhibiting BMP signaling [163]. Chen et al. transplanted Noggin gene transfected MSCs intravenously 6 hours after induction of MCAO model and found that compared with the normal MSC group, this kind of MSCs could significantly increase noggin level in rat brain and more significant neurogenesis in ipsilateral SVZ [164]. Interestingly, Lu et al. transplanted MSCs cotransfected with BDNF gene and Noggin gene and found that these MSCs did not show additional antiapoptotic effects but showed additional anti-inflammatory effects [165]. Specifically, compared with MSCs transfected with BDNF gene or Noggin gene alone, CO transfected MSCs more effectively inhibited the activation of TLR4/MyD88 pathway and the expression of MMP-9 and reactive oxygen species (ROS) [165].

Since MSCs may play a therapeutic role mainly through paracrine, many researchers choose to overexpress some cytokines or nutritional factors to increase the therapeutic effect. Fibroblast growth factor 1 (FGF1), as a member of the paracrine FGF family, is abundant in neurons and can mediate neuroprotection [166, 167]. Ghazavi et al. investigated the effect of AD-MSCs transfected with FGF1 and found that compared with normal AD-MSCs, the former could increase the level of FGF1 in the ischemic lesions, reduce apoptotic cells, and infarct size more significantly [61]. In addition, Linares et al. found that FGF21 transfected MSCs have strong antiapoptotic ability in vitro in the face of oxidative stress and inflammatory environment, which suggests that FGF21 transfected MSCs may have stronger survival ability and neuroprotective ability in infarcted lesions [168]. LV et al. found that transplanted MSCs overexpressing HIF-1α had increased viability in ischemic lesions and showed a more significant reduction of infarct size and recovery of neurobehavior, which was related to the further decrease of proinflammatory cytokines and increase of neurotrophic factors [169]. HIF-1α is a protective regulatory factor produced by cells in the face of hypoxic environment. The therapeutic effect of HIF-1α overexpressing MSCs coincides with that of hypoxic preconditioning MSCs mentioned above. As mentioned in the mechanism section above, transplantation of VEGF expressing transgenic MSCs is not conducive to the treatment of IS. However, a recent study

reported the opposite results. In this study, transplantation of VEGF expressing transgenic MSCs resulted in smaller infarct size, more significant angiogenesis, and neurological improvement [170]. The reasons for the two results are unknown, which may be related to different administration methods, administration timing, and measurement time points. Other studies transfected with different cytokines, such as BDNF, GDNF, PLGF, and hepatocyte growth factor (HGF), are shown in Table 2.

8.3. Combination Therapy. In recent years, the research focus of MSC treatment strategy is to enhance the efficacy of MSCs in the treatment of IS by combining with other drugs or treatment measures. Minocycline is a kind of tetracycline antibiotic. Due to its anti-inflammatory and antiapoptotic effects and good blood-brain barrier penetration, many studies have reported its beneficial effects in the central nervous system [171, 172]. Cho et al. showed that compared with transplanting MSCs alone, the combination therapy showed smaller infarct size and more significant improvement in neurological function, which may be related to minocycline enhancing the neurogenesis and angiogenesis of MSCs [173]. Simvastatin, as a class of HMG-CoA reductase inhibitors, was initially used to reduce cholesterol, and its application alone has also been proved to improve the prognosis after IS. Cui et al. found that Simvastatin can significantly increase the expression of the chemokine CXCR4 in MSCs, promote the homing of MSCs, and further promote angiogenesis and neural function recovery [174]. Combined therapy can also be achieved by intranasal administration. Shen et al. administered MSCs combined with IGF-1 into the nose and found that this strategy increased the ability of MSCs to promote angiogenesis and neurogenesis and further increased the cerebral blood flow in the ischemic area [175].

Because the repair of blood-brain barrier leads to the decrease of the passing rate of peripheral drugs, the drug treatment in the chronic stage of stroke often cannot achieve the desired effect. Although it has been reported that intravenous injection of MSCs at 1 month after stroke can improve motor function, this beneficial result may be attributed to the relatively large drug dose (3.0×10^6) [142]. It has been reported that mannitol combined with temozolomide can inhibit the increase of blood-brain barrier permeability caused by endothelial tight junction proteins [176]. Choi et al. applied this strategy to MSCs in the treatment of IS and found that although MSCs were not detected in the brain parenchyma; these two drugs combined with MSCs could improve the behavior defect by increasing the brain parenchyma metastasis of MSC-derived microvesicles (a type of MSC-EVs), which was not observed in the MSCs only treatment group [177]. This combined strategy provides a new method for the treatment of chronic stroke.

Many traditional Chinese medicines have been proved to enhance the efficacy of MSCs in the treatment of IS. Radix Angelica Sinensis is a kind of Chinese herbal medicine with neuroprotective effects. Sodium ferulate (SF) and n-butylidenephthalide (BP) are the two main active components of Radix Angelica Sinensis. Study has shown that BP can enhance the interaction of SDF-1α/CXCR-4, promote MSCs to move to an ischemic focus, and promote MSCs to differentiate into astrocytes more effectively [178]. Zhang et al. injected SF, BP, and MSCs intravenously into MCAO rats and found that this combination therapy can further increase the levels of VEGF and BDNF in ischemic lesions and more effectively promote angiogenesis and neural function recovery [179]. Other research groups have also confirmed the enhancement effect of angelica extract on the efficacy of MSCs [180]. Tetramethylpyrazine (TMP), an active component extracted from Chinese herb Rhizoma Chuanxiong, has also been shown to increase the expression of CXCR4 in MSCs, thus promoting the homing of MSCs to infarcted brain tissue [181]. In the MCAO model animal, the combined application of TMP and MSCs further promoted the expression of VEGF and BDNF, resulting in a more significant improvement of neurological function score [182]. Other laboratory studies have shown that this enhancement effect may be related to the anti-inflammatory and neurogenesis effects of TMP on MSCs [183]. Other herbs with enhanced effects on MSCs in the treatment of IS include Salvia miltiorrhiza [184], Icariin [185], and Borneol [186].

In addition to drugs, some physical therapies combined with MSCs have also been proved to be beneficial. Morimoto implanted the electrical stimulator into the inner and outer sides of the cranial cavity of rats [187]. It was found that electrical stimulation could increase the movement of MSCs injected into the corpus callosum toward the ischemic focus, which was related to the increased level of SDF-1α. The rats in the combined group showed a smaller infarct size [187]. Electroacupuncture (EA) is a physical therapy that combines traditional acupuncture and electrical stimulation. Studies have shown that EA can increase the expression of BDNF and VEGF mRNA in a cerebral ischemia animal models and promote functional recovery [188]. Kim et al. treated mice with EA once a day from day 5 to day 16 after MCAO and found that EA combined with MSC transplantation could significantly improve the motor function of mice after cerebral infarction, which may be related to the promotion of neurotrophic factor secretion and neurogenesis [189]. Another report from the same laboratory showed that EA could increase the differentiation of TrkB gene transfected MSCs into mature neurons and increase the levels of BDNF and neurotrophin-4/5 more significantly [190]. Bi et al. placed the head on an ice bag for 3 hours immediately after MCAO induction and injected 1×10^6 MSCs into the ventricle 24 hours later [191]. It was found that this mild hypothermia treatment increased the homing efficiency and angiogenesis ability of MSCs and significantly reduced the neurological function score [191]. In addition, MSC treatment combined with exercise or rehabilitation also showed a beneficial effect on the efficacy of MSCs [192, 193].

The rise of nanotechnology is of great help to regenerative medicine. Some new nanomaterials are used in the research of MSCs in the treatment of ischemic stroke. In vitro experiments showed that nitrogen-doped carbon nanocages (NCNCs) could enhance the inhibitory effect of MSCs on microglia activation [194]. Compared with transplanted MSCs alone, intravenous injection of MSCs combined with

Table 2: Gene transfection in the treatment of IS with MSCs.

Transfected gene	Transfected vector	Cell type	Dose	Transplantation route	Administration time	Outcome (compared with normal MSCs)	Reference
BDNF	Adenovirus	hBM-MSCs	5×10^5	Intracerebral injection	24 hours after MCAO	Fewer apoptotic cells; smaller infarcted volume; improvement of neurological function	[45]
GDNF	Adenovirus	hBM-MSCs	1.0×10^7	Intravenous injection	3 hours after MCAO	Smaller infarcted volume; higher function recovery	[48]
HGF	Herpes simplex virus type-1	BM-MSCs	1.0×10^6	Intracerebral injection	2 or 24 hours after MCAO	Fewer apoptotic cells; smaller infarcted volume; higher function recovery	[208]
PIGF	Adenovirus	hBM-MSCs	1.0×10^7	Intravenous injection	3 hours after MCAO	More angiogenesis; smaller infarcted volume; higher function recovery	[60]
FGF1	pCMV6-entry vector	AD-MSCs	2.0×10^6	Intravenous injection	0.5 hour after MCAO	Fewer apoptotic cells; smaller infarcted volume; higher function recovery	[61]
Ang; VEGF; Ang+ VEGF	Adenovirus	hBM-MSCs	1.0×10^6	Intravenous injection	6 hours after MCAO	Ang/Ang+ VEGF: more angiogenesis; smaller infarcted volume; higher function recovery. VEGF: infarct size increased; function deteriorated	[56]
VEGF	Adenovirus	BM-MSCs	1.0×10^6	Intracerebral injection	24 hours after MCAO	More angiogenesis; smaller infarcted volume; higher function recovery	[170]
Hif-1α	Lentivirus	BM-MSCs	1.0×10^6	Intracerebral injection	24 hours after MCAO	Lower level of proinflammatory cytokines; higher level of neurotrophins; smaller infarcted volume; higher function recovery	[169]
TSP4	Lentivirus	BM-MSCs	2.0×10^6	Intravenous injection	3 hours after MCAO	Higher levels of Ang-1 and vWF; more angiogenesis; higher function recovery	[209]
IL-10	Adeno-associated virus	hBM-MSCs	1.0×10^6	Intravenous injection	0 or 3 hours after MCAO	Lower level of proinflammatory cytokines and microglial activation; smaller infarcted volume; higher function recovery	[162]
CCR2	Lentivirus	BM-MSCs	2.0×10^6	Intravenous injection	24 hours after MCAO	More homing; less BBB leakage; higher function recovery	[159]
CCL2	None	hUC-MSCs	1.0×10^6	Intravenous injection	1 and 4 days after MCAO	More homing; more angiogenesis and neurogenesis; less neuroinflammation; smaller infarcted volume; higher function recovery	[160]
Ngn1	Retrovirus	hBM-MSCs	1.0×10^6	Intra-arterial injection	2 hours after MCAO	More homing; fewer apoptotic cells; less neuroinflammation; higher function recovery	[161]
Noggin	Adenovirus	BM-MSCs	5.0×10^6	Intravenous injection	6 hours after MCAO	More neurogenesis; smaller infarcted volume; higher function recovery	[164]

NCNCs in mice with cerebral infarction showed higher levels of IL-10, lower levels of TNF-α, and smaller infarct volume [194]. Nazarian et al. found that modafinil-coated gold nanoparticles (AuNPs) can promote the antiapoptotic ability of MSCs and further reduce the area of cerebral infarction, which is accompanied by a significant increase in the levels of BDNF and GDNF [195]. Zuo et al. reported that the combination of cerium oxide nanoparticles with human UC-MSCs could obtain the antioxidant effect of the former and enhance the anti-inflammatory effect of the latter. Specifically, the levels of ROS and inflammatory factors (TNF-α, IL-6, and IFN-γ) in the brain tissue of rats with cerebral infarction after transplantation of human UC-MSCs labeled with nanoceria were significantly decreased [196]. Yao et al. proposed a new nanoplatform to load MSCs. This method allows us to quantitatively detect cell migration by SPECT imaging after transplantation, and it can continuously release cobalt protoporphyrin IX to protect cells from oxidative stress, thus increasing the survival of MSCs in ischemic lesions [197]. The MCAO model mice transplanted with MSCs through this method showed better neurological recovery [197].

8.4. MSC-EV Transplantation. MSC-EVs may be one of the main mechanisms of MSCs in the treatment of IS. In recent years, many studies have used isolated MSC-EVs alone in the treatment of IS, showing that the curative effect is no worse than that of MSCs alone. Some studies even reported that injection of MSC-EVs alone showed more significant improvement in neurological function than MSC

transplantation [93]. Therefore, transplantation of MSC-EVs alone seems to be a good alternative strategy, which has many advantages compared with MSCs. First of all, MSC-EVs injected intravenously can reach the infarct lesion more effectively [93]. Because of its smaller volume and lipid double-layer vesicle structure, MSC-EVs do not stay in peripheral organs like MSCs after intravenous injection and are easier to cross the blood-brain barrier. Secondly, the risk of vascular occlusion and microvascular thrombosis is reduced after transplantation, and MSC-EVs do not have the potential of tumor transformation because they cannot self-replicate. Third, MSC-EV has a robust structure and can be stored at -80° for a long time without loss of biological activity [198]. Finally, intravenous injection of MSC-EVs can reduce the peripheral immunosuppression (i.e., the decrease of B cells, NK cells, and T cells) after IS [95]. In addition, MSC-EVs can also be enhanced by genetic engineering. As mentioned above, MSC-EVs overexpressing certain miRNAs have stronger efficacy. Combination therapy also appears to be feasible, as AD-MSCs combined with AD-MSC derived exosomes intravenously administered 3 hours after IS resulted in smaller infarct size and better improvement in neurological function than either alone [199]. Therefore, MSC-EVs may be an effective alternative to MSCs, which has great potential in the treatment of ischemic stroke. At present, there are few clinical trials on MSC-EVs, and its efficacy and safety in stroke patients need to be further evaluated.

9. Clinical Trial

A large number of preclinical data have proved the feasibility of MSCs in the treatment of IS, and the clinical administration of stem cell therapy is also expected. A number of clinical trials have proved the effectiveness and safety of MSCs in the treatment of IS. The earliest clinical trial included only 30 subjects, five of whom received 1×10^8 MSCs at 5-7 weeks after acute stroke. During the 1-year observation period, no adverse events were reported in these five patients. And the Basel index (BI) of these five patients was significantly improved, suggesting a certain improvement in neurological function [200]. Lee et al. enrolled 85 patients with severe IS. During the five-year follow-up, MSC treatment group had higher cumulative survival rate, more patients with low modified Rankin Scale (mRS) score (0-3), and no adverse reactions [201]. Additionally, a randomized controlled trial conducted by Jaillard et al. showed that although intravenous injection of autologous mesenchymal stem cells did not improve BI, mRS, and National Institutes of Health Stroke Scale (NIHSS) 2 years later, it promoted the improvement of motor function score [202].

In recent years, more and more different clinical trials have shown the possibility of diversified clinical transformation. A phase I/II clinical trial by Levy et al. demonstrated for the first time that a single intravenous injection of allogeneic BM-MSCs is safe and effective. None of the 15 serious adverse reactions during follow-up may be related to stem cell therapy. In addition, intravenous injection of 1.5 million cells/kg allogeneic MSCs in phase 2 showed significant improvement in BI score and NIHSS score [203]. Deng et al. conducted a phase II clinical trial to evaluate the safety and efficacy of intrathecal infusion of allogeneic BM-MSCs in the treatment of IS for the first time. 59 subjects received intrathecal infusion of allogeneic BM-MSCs four times a week (1×10^6 cells/kg body weight), mainly to evaluate the mRS score and the occurrence of adverse events after 90 days of treatment [204]. The project is still in progress. Some literatures have also reported the clinical trials of modified MSCs in the treatment of IS. Steinberg et al. transplanted SB623 cells, BM-MSCs transfected with Notch-1 gene, into the brain of 18 patients with chronic stroke; results showed significant improvements in the European Stroke Scale (ESS) score, NIHSS score, Fugl-Meyer (F-M) total score, and F-M Exercise Scale score after 24 months of treatment [205]. There is a table of clinical trials of MSCs in the treatment of patients with IS (Table 3).

10. Discussion

In this study, we mainly focused on the mechanism, application parameters, and treatment strategies of MSCs in the treatment of ischemic stroke IS. On the one hand, the mechanism of MSCs in the treatment of IS was the focus of previous reviews [206, 207], but most of the previous articles in this area were not detailed enough. Here, we make a relatively comprehensive review and summary of the mechanism of action of MSCs and reviewed the research hotspots of the mechanism of action in recent years, namely, mitochondrial transfer and extracellular vesicles. We believe that this will provide a significant reference for the follow-up study of the mechanism of MSCs. On the other hand, application parameters and treatment strategies are the key to the clinical transformation of MSCs in the treatment of IS, but little attention of previous review was paid to these two aspects. This manuscript also makes a comprehensive review and summary of these two aspects, including the summary of the best application parameters of MSCs (i.e., the optimal dose and the optimal time window) and the display of the latest optimized treatment measures (i.e., pretreatment and combined treatment). We believe that this is of great significance to the development of follow-up clinical trials. In a word, we hope that both basic research and clinical trials can obtain useful information from this manuscript, so as to promote the progress of MSCs in the treatment of IS.

Immunomodulation, neuroprotection, angiogenesis, and neural circuit reconstruction are the main mechanisms of MSCs in the treatment of IS. Except for paracrine, mitochondrial transfer or extracellular vesicle transfer may also be the main pathway through which MSCs act, and MSC-EVs may be an effective alternative strategy for MSCs in the treatment of IS. MSC therapy extends the time window for treatment of ischemic stroke, and early administration within 7 days after stroke may be the best time for treatment. Intravascular injection of MSCs may be an appropriate way for clinical application, but we should pay attention to their adverse reactions. Intranasal administration is also a promising way of MSC transplantation. The optimal dose for treatment with MSCs is uncertain, but there is no positive linear correlation between dose and efficiency. There are a lot of researches on

Table 3: Clinical trials of MSCs in the treatment of patients with IS.

Type of trial	Stroke type	Sample sizes	Cell type	Dose/single (*S*) or multiple (*M*)	Route	Time of adm. from stroke onset	Follow-up	Result	Reference
RCT	Acute IS	30	BM-MSCs/autologous	$5 \times 10^7/M$	IV	4-5 weeks 7-9 weeks	1 year	Significant improvement in BI. No significant difference in NIHSS and MRI scan	[200]
RCT	Acute IS	85	BM-MSCs/autologous	$5 \times 10^7/M$	IV	5 weeks 7 weeks	5 years	No significant side effects. Patients with mRS 0–3 significant increased	[201]
OL-PT	Chronic IS	12	BM-MSCs/autologous	$0.6 - 1.6 \times 10^8/S$	IV	36–133 days	1 year	No side effects. Decreasing of infarct volume by>20% at 1 week	[210]
OL-PT	Subacute IS	11	BM-MSCs/autologous	$85 \times 10^6/S$	IV	7–30 days	6 months	No side effects. Improvement in NIHSS, BI, and mRS	[211]
SB-CT	Acute IS	20	BM-MNCs/autologous	$1.59 \times 10^8/S$	IA	5–9 days	180 days	No side effects. No significant differences in neurological function	[212]
OL-PT	Chronic IS	36	BM-MSCs/allogeneic	$1.5 \times 10^6/S$	IV	>60 days	12 months	No side effects. Significant improvement in BI and NIHSS	[213]
OL-PT	Chronic IS	18	SB623 cells/allogeneic	$2.5 \times 10^6/S$ $5.0 \times 10^6/S$ $10 \times 10^6/S$	IC	>60 days	24 months	All experienced at least 1 treatment-emergent adverse event. Significant improvements in NIHSS F-M and ESS	[205]
RCT	Subacute IS	31	BM-MNCs/autologous	$1.0 \times 10^6/S$ $3.0 \times 10^6/S$	IV	<2 weeks	2 years	No significant improvements in NIHSS, BI, and mRs. Significant improvements in motor function	[202]
OL-PT	Chronic IS	12	BM-MNCs/autologous	Not provided	IV	3-24 months	4 years	No side effects. Significant improvements in mBI at 156 and 208 weeks	[214]

RCT: random control trial; OL-PT: open label prospective trial; SB-CT: simple blinded control trial; IV: intravenous; IA: intra-arterial; IC: intracerebral; adm: administration.

enhancing the therapeutic strategies of MSCs, but whether it is pretreatment or gene modification, combination therapy, or MSC-EVs, they are mainly based on the mechanism of MSCs in the treatment of IS. Clarifying the mechanism of action, molecular regulation, and signal pathways of MSCs will promote the discovery of more beneficial therapeutic strategies.

10.1. Limitation. Several limitations of the current review should not be ignored. First, we only reviewed the relevant literatures in PubMed database. Some articles not included in this database may be omitted, resulting in incomplete review. Secondly, in this study, there is no in-depth investigation and summary on MSC-EVs and micro-RNA which is the research hotpot of MSCs recently. Thirdly, the safety of MSCs in the treatment of IS, which is crucial to clinical transformation, has not been investigated in this study.

10.2. Future Directions. MSC therapy extends the time window for treatment of ischemic stroke, and early administration within 7 days after stroke may be the best time for treatment. Although the application of intravascular injection of MSCs may be an appropriate treatment for IS, more efforts might be required to determine the potential adverse reactions. And delivery MSCs through the intranasal route could also be a promising way of MSC transplantation. Furthermore, the optimal dose for treatment with MSCs needs to be investigated. Additionally, there are many different treatment strategies to optimize the efficacy of MSCs; researchers should carry out clinical trials in this area to achieve better clinical transformation in the future.

11. Conclusion

In summary, MSC transplantation provides hope for the treatment of IS. Further study of its mechanism and optimization of its treatment strategy will lay a solid foundation for the clinical transformation of MSC therapy.

Abbreviations

IS: Ischemic stroke
MSCs: Mesenchymal stem cells
EVs: Extracellular vesicles
MSCs-EVs: MSC-derived EVs
t-PA: Tissue plasminogen activator
MT: Mechanical thrombectomy
MCP-1: Monocyte chemoattractant protein-1

SDF-1: Stromal cell-derived factor-1
MMP-9: Matrix metalloproteinase-9
OGD: Oxygen and glucose deprived
ASICs: Acid sensing ion channels
CREB: cAMP-response element binding protein
MCAO: Middle cerebral artery occlusion
BDNF: Brain-derived neurotrophic factor
NGF: Nerve growth factor
GDNF: Glial cell line-derived neurotrophic factor
bFGF: Basic fibroblast growth factor
AIF: Apoptosis inducing factor
VEGF: Vascular endothelial growth factor
Ang-1: Angiopoietin-1
PlGF: Placental growth factor
bFGF: Basic fibroblast growth factor
Ang-MCS: Ang-1 gene modified MCSs
VEGF-MCSs: VEGF gene modified MCSs
Ang-VEGF-MCSs: Ang-1 gene combined with VEGF gene modified HMCS
SVZ: Subventricular zone
SGZ: Subgranular zone
GAP-43: Axon growth associated protein-43
TNTs: Tunnel nanotubes
OGD/RO: Oxygen glucose deprivation and reoxygenation
MSC-EVs: Mesenchymal stem cell-derived EVs
BMEC: Brain microvascular endothelial cell
BM-MSCs: Bone marrow-derived MSCs
AD-MSCs: Adipose-derived MSCs
DP-MSCs: Dental pulp-derived MSCs
PDLSCs: Periodontal ligament stem cells
UC-MSCs: Umbilical cord-derived mesenchymal stem cells
PL-MSCs: Placenta-derived MSCs
IGF: Insulin-like growth factor
HIE: Hypoxic-ischemic encephalopathy
CXCR4: C-X-C chemokine receptor type 4
HIF-1α: Hypoxia inducible factor-1α
3D: Three-dimensional
FBS: Fetal bovine serum
CCL2: C-C motif chemokine ligand 2
CCR2: C-C motif receptor 2
FGF1: Fibroblast growth factor 1
HGF: Hepatocyte growth factor
BMP: Bone morphogenetic protein
ROS: Reactive oxygen species
TSP4: Thrombospondin-4
SF: Sodium ferulate
BP: n-Butylidenephthalide
TMP: Tetramethylpyrazine
EA: Electroacupuncture
NCNCs: Nitrogen-doped carbon nanocages
AuNPs: Modafinil-coated gold nanoparticles
BI: Basel index
mRS: Modified Rankin Scale
NIHSS: National Institutes of Health Stroke Scale
ESS: European Stroke Scale
F-M: Fugl-Meyer total score.

Conflicts of Interest

The authors report no conflicts of interest.

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