

Review Article

Dental Mesenchymal Stem Cell Secretome: A Cell-free Therapeutic Approach for Tissue Regeneration

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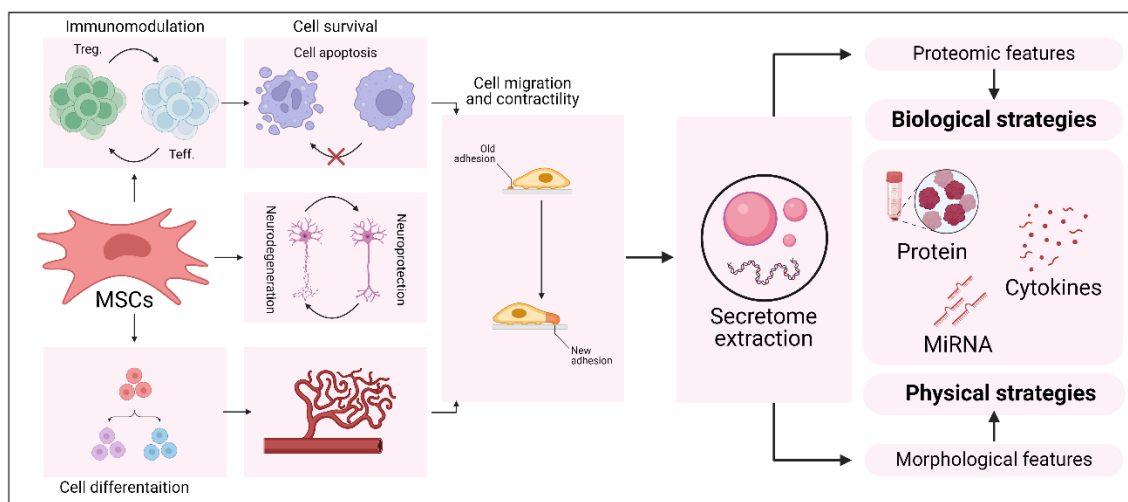
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Abstract

Dental mesenchymal stem cells (MSCs) constitute a promising candidate for tissue regeneration in regenerative medicine. While cellular transplantation of MSCs has shown potential for achieving functional tissue regeneration, several clinical challenges hinder its implementation. An alternative approach, harnessing the secretome of MSCs, has gained attention as a cell-free therapeutic strategy. The secretome encompasses the bioactive molecules released by MSCs, which have been shown to exert profound effects on tissue regeneration. This review comprehensively analyzes the dental MSCs secretome, understanding the potential of the MSCs secretome, addressing the obstacles associated with its utilization, and its potential as a valuable resource for regenerative therapies. In summary, the secretome of dental MSCs holds great promise as a cell-free therapeutic strategy for tissue regeneration. This review highlights the importance of further research and standardization efforts to fully exploit the therapeutic potential of the MSCs secretome and realize its clinical translation in regenerative medicine.

Keywords: Dental mesenchymal stem cells, Secretome, cell-free therapy, tissue regeneration



Graphical abstract. MSCs: Mesenchymal stem cells, Treg: Regulatory T-cells, and Teff: Effector T-cells.

Introduction

Mesenchymal stem cells (MSCs), including dental-derived stem cells, have emerged as a promising therapeutic option, being the focus of many studies in regenerative medicine ¹. Although cellular transplantation of several types of MSCs is a viable approach for achieving functional tissue regeneration, implementing this paradigm in clinical settings is still hindered by several significant clinical challenges ². It was hypothesized that the therapeutic effects of transplanted MSCs were attributed to their migration and differentiation into specialized cells. However, only a small percentage of cells were successfully engrafted within the compromised host tissue ³. Research has focused on characterizing the secretory capacity of MSCs in their surrounding environment. The chemicals released and referred to as secretome can be easily extracted and have been shown to impact tissue regeneration significantly ^{4,5}.

Compared to cell-based therapy, one of the advantages attributed to the MSCs secretome is its convenience in terms of preservation, sterilization, packaging, and long-term storage without compromising its qualities⁶. In addition to correctly determining appropriate dosages, efficiently producing large amounts of a substance, and avoiding invasive extraction methods. This approach offers significant time and cost savings ^{7,8}. Nevertheless, certain obstacles must be addressed to fully utilize the MSCs secretome as a cell-free therapy and ensure its therapeutic efficacy.

1. Mesenchymal stem cells

MSCs are multipotent cells residing in numerous adult body tissues, hallmarked by their self-renewal abilities and differentiation potential upon proper

stimulation ^{9,10}. In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy established minimal criteria to identify and characterize human MSCs. They include the ability of the cells to adhere to plastic under standard culture conditions, the expression of CD105, CD73, and CD90, while lacking expression of CD45, CD34, CD14, CD11b, CD79a, CD19, and human leukocyte antigen-DR (HLA-DR) surface molecules as well as their *in vitro* trilineage differentiation capacity ⁹.

MSCs have a crucial role in organogenesis and the postnatal repair of organs ^{11,12}. Their multilineage differentiation potential, in addition to their potent immunomodulatory properties and ability to secrete a broad range of trophic factors and cytokines demonstrated promising cell-based therapies for various diseases ¹³. Although bone marrow-derived mesenchymal stem cells (BM-MSCs) are frequently utilized in clinical treatments ^{14,15}, their harvesting from bone marrow is a highly invasive, time-consuming, and insufficient process ^{16,17}. Therefore, in recent years, researchers have identified stem cells derived from different dental tissues as innovative and potential candidates for cell-based therapy and regenerative medicine ^{18,19}.

2. Dental mesenchymal stem cells

Dental MSCs are unique adult MSCs derived from the ectomesenchyme's neural cells ²⁰. Various subpopulations of dental-derived stem cells have been discovered, such as dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAP), stem cells from human exfoliated deciduous teeth (SHED), dental follicle stem cells (DFSCs), and gingival mesenchymal stem cells (GMSCs) ²¹⁻²⁶. Dental MSCs possess notable benefits, including their widespread accessibility, ease of

acquisition, and minimally invasive nature^{27,28}. Consequently, they have emerged as a promising therapeutic avenue for tissue regeneration and repair²⁹⁻³². Detailed characteristics of human dental MSCs are shown in Table 1.

3. Mesenchymal stem cell-based therapy and its limitation

Despite the favorable therapeutic abilities of MSCs, the progress of MSCs-based cell therapies has been hindered due to the absence of a standardized protocol for the isolation process. The field lacks a standardized protocol for *ex vivo* expansion, clonal populations, and culture conditions^{41,42}. Generating good manufacturing practice (GMP) grade human MSCs necessitates the presence of an officially recognized stem cell laboratory and adherence to national regulatory frameworks. The ability to achieve a significant level of cell production and banking continues to present challenges, particularly due to the associated high costs. Additionally, there is a lack of consensus regarding the most suitable source of MSCs for various indications⁴³.

Moreover, there is a lack of clarity regarding the effective delivery route, optimal dosage of infusion, and administration frequency^{41,42}. The effective route of delivery represents a significant challenge. The percentage of engrafted cells after transplantation is usually negligible, which may affect the survival rate and long-term therapeutic potential of MSCs⁴⁴. Although the intravenous route for human MSCs administration is considerably safe, it is essential to acknowledge the potential occurrence of more severe adverse effects, such as thrombosis or unfavorable inflammatory reactions⁴⁵.

Additionally, stem cell transplantation carries the risk of tumorigenesis⁴⁶. Several factors can influence the probability of tumor formation following the transplantation of MSCs. These factors include the donor's age, the characteristics of the host tissue, the presence of growth regulators in the recipient tissue, and the mechanisms that govern the activity of MSCs at the specific location where they are transplanted^{47,48}. Furthermore, the genetic stability and chromosomal aberrations of MSCs might be

Table 1: Characteristics of human dental MSCs³³.

Stem cells	CD antigen expression		Other representative markers	<i>In vitro</i> differentiation capacity	<i>In vivo</i> tissue formation capacity
	Positive	Negative			
DPSCs <small>34-37</small>	CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105, CD106, CD146, CD166	CD14, CD31, CD34, CD45, CD117, CD133	STRO-1, Nestin	dent (od), mes (os, ad, cho, myo), ect (neu)	dent (dentin, pulp), mes (adipose, muscle)
SHED <small>35,38</small>	CD13, CD44, CD73, CD90, CD105, CD146	CD14, CD19, CD34, CD43, CD45	STRO-1, Oct-4, Nanog, Nestin, SSEA-3, SSEA-4	dent (od), mes (os, ad, cho, myo, endo), ect (neu)	dent (dentin), mes (bone, microvessel)

PDLSCs 37	CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105 CD106, CD146, CD166	CD31, CD34, CD45	STRO-1, Scleraxis	dent (cem), mes (os, ad, cho), ect (neu)	dent (cementum, PDL), mes (alveolar bone)
DFSCs 39	CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105, CD106, CD166	CD31, CD34, CD45, CD133	STRO-1, HLA class 1	dent (cem), mes (os, ad, cho), ect (neu)	dent (cementum, PDL), mes (alveolar bone)
SCAP 36	CD49d, CD51/61, CD56, CD73, CD90, CD105, CD106, CD146, CD166	CD14, CD18, CD34, CD45, CD117, CD150	STRO-1, Nestin, Survivin	mes (ad), ect (neu)	dent (dentin, pulp)
GMSCs 40	CD29, CD44, CD73, CD90, CD105, CD106, CD146, CD166	CD34, CD45, CD117	STRO-1, Oct-4, Nanog, Nestin, SSEA-4, HLA- ABC, Sox-2, Tra2-49, Tra2-54	mes (os, ad, cho), ect (neu, glia cell), end (definitive endoderm cell)	mes (bone, cartilage, fat, muscle), ect (epithelia, neural tissue)

Ad (adipocyte), cem (cementoblast), cho (chondrocyte), dent (dentinogenic lineage), ect (ectodermal lineage), end (endodermal lineage), endo (endothelial cell), hep (hepatocyte), PDL (periodontal ligament), mes (mesodermal lineage), od (odontoblast), os (osteoblast), , myo (myoblast), neu (neuronal cell).

compromised due to modifications and prolonged in vitro culturing ⁴⁸. The complex, underrecognized legal framework for cell-based therapies presents an additional barrier to clinical implementation ⁷.

4. The era of cell-free therapy based on MSCs secretome

The basic concept underlying MSCs therapy is their capacity to migrate to injury sites and differentiate into various cell types to promote tissue regeneration. However, it has been demonstrated to be insufficient to produce a significant therapeutic effect ^{49,50}. Even though different studies suggest that MSCs exert various biological effects by promoting cellular proliferation and cell-to-cell interactions ^{51,52}, the accumulated experience indicates that their beneficial effects are primarily attributable to the secretion of paracrine factors ⁵³. Therefore,

the research era shifted toward cell-free therapy, introducing MSCs secretome as a promising candidate for application in the field of novel medical biotechnology ⁵⁴.

Secretome effectively addresses the challenges associated with the utilization of stem cells themselves. The safety concerns related to the transplantation of proliferating living cells, such as immunological incompatibility, tumorigenicity, emboli formation, transmissible infections, and the potential for MSCs to enter senescence, have been resolved. Additionally, secretome possesses advantages in dosage determination and potency evaluation compared to conventional therapeutic approaches. The secretome can be preserved without applying potentially toxic cryopreservative agents ^{7,54}.

Utilizing secretome-derived products presents a more cost-effective and feasible

option for clinical applications. The usage of secretome obviates the need for the time-consuming and expensive processes involved in expanding and maintaining cell lines. This phenomenon can be attributed to the ability to pre-prepare secretome for therapeutic purposes in significant amounts, ensuring its availability for treatment when needed^{7,54}. Considering the aforementioned factors, asserting that secretome-based cell-free therapies provide a novel pathway toward safer, more standardized, scalable, and manageable treatments⁵⁵.

5. Secretome as a novel approach for cell-free therapy

MSCs possess the capacity to generate a diverse array of chemokines, cytokines, growth factors, and extracellular matrix components. The secretome/ conditioned medium (CM) refers to a collection of molecular components that are actively secreted into the extracellular area. The secretome/CM is classified into two main categories: soluble proteins and a vesicular fraction composed of extracellular vesicles (EVs)⁵⁶. Bioactive factors encompass a diverse array of molecules, such as cytokines, chemokines, growth factors, interleukins, proteins, messenger RNAs (mRNAs) in the form of free nucleic acids, microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and lipids including ceramide, cholesterol, and sphingolipids^{6,57}.

5.1. Soluble factors

An array of growth/differentiation factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), platelet-derived endothelial cell growth factor, hepatocyte growth factor (HGF), epidermal growth factor, insulin-like growth factor I and II (IGF-I, IGF-II), fibroblast growth factor-2 (FGF-

2), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF) fibroblast growth factor-7 (FGF-7), heparin-binding epidermal growth factor, neural growth factor (NGF), and brain-derived neurotrophic factor (BDNF)⁵⁸ were demonstrated in MSCs secretome. Furthermore, anti-inflammatory cytokines, including transforming growth factor-(TGF)- β 1 and interleukins (IL), including IL-6, IL-10, IL-27, IL-17, and IL-13, and pro-inflammatory cytokines including IL-8, IL-9, and IL-1 β were identified. Moreover, granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and prostaglandin E2 (PGE2) were present⁵⁸. The key benefit of bioactive molecule therapy is its capacity to enhance safety. Nevertheless, the effectiveness of bioactive substances as a therapeutic therapy is hindered by their limited stability in extracellular environments, as they are prone to rapid hydrolysis⁵⁹.

5.2. Extracellular vesicles

EVs are membrane-packed vesicles secreted by various cell types that act as a system of intercellular communication. They consist of a lipid bilayer that envelops bioactive molecules⁶⁰. EVs are distinguished by a distinct cargo consisting of mRNAs, miRNAs, proteins, or DNA. The genetic material remains protected throughout the oxidative extracellular environment and can be sent to remote cells to regulate the healing of injured tissue⁶¹. Based on size and biological origin, EVs are classified into exosomes (EXOs), microvesicles (MVs), and apoptotic bodies.

5.2.1. Exosomes (EXOs): EXOs are homogenous and small, spanning in diameter from 40 to 100 nanometers. They are liberated from the cell via exocytosis due to fusion with the cell membrane; they originate in multivesicular bodies^{62,63}.

Annexins, tetraspanins (CD63, CD81, and CD9), and heat-shock proteins (Hsp60, Hsp70, and Hsp90) are abundant in EXOs which are usually used for their identification⁶⁴.

5.2.2. Microvesicles (MVs): MVs, also called ectosomes, exhibit a wide range of sizes, reaching from 100 to 1000 nm in diameter. Their surface markers originate from the cells that produce them and are generated via direct branching from cellular plasma membrane^{65,66}. MVs comprise lipids and proteins in addition to mRNA and miRNA⁶⁷.

5.2.3. Apoptotic bodies: The largest type of EVs discharged by cells during the process of apoptosis, commonly exhibiting a size range of 1000-5000 nm. The process of synthesis of apoptotic bodies is a dynamic phenomenon governed by the morphological stages of apoptotic cell disassembly, which are regulated by protein kinases⁶⁸. Apoptotic bodies contain a variety of active biomolecules and intact organelles derived from apoptotic dead cells⁶⁹.

5.3. Mechanism of EVs in intercellular communication

EVs secreted by cells serve as paracrine factors, engaging in interactions with recipient cells by migration to remote sites and subsequent targeting of specific cells⁷⁰. Functional communication between EVs and cells can involve different types of interactions, including fusion of the EVs-plasma membrane, endocytosis uptake, and binding of EVs to the cell surface via the ligand-receptor without delivery of the contents⁷¹. These interactions exemplify the diverse mechanisms through which EVs can engage with cells. Intercellular communication via EVs offers the advantages of transporting hydrophobic materials, protecting the content against

deterioration by external enzymes, and their negligible *in vivo* immunogenicity⁷².

6. Approaches to produce MSCs secretome and its products

The preparation of MSCs-CM consists of leaving the cells in culture for a certain period before using centrifugation to collect their components. The supernatant must be centrifuged to remove detached and apoptotic cells, waste tissue, and cell debris. The resulting product can be utilized directly or further processed through filtration, fractionation, and/or concentration⁷³. Using ultrafiltration technology, various filtration modules with several molecular weight cut-offs (MWCOs) can be selected, allowing a complete or partial retention of the secretome components⁷³.

Centrifugal ultra-filter units with MWCOs of <3 KD are used to retain and concentrate the whole CM^{74,75}. By fractionating CM, it is possible to correlate a particular molecular subset or CM fractions with a specific measured effect¹.

Several methods were established to isolate and purify EVs. Although each method has pros and cons, ultracentrifugation-based procedures are the gold standard for generating high-quality EVs. Secretome (or EVs) can be sterilized using filtration without apparent loss of efficacy⁷⁶.

7. Characterization of MSCs secretome

Various approaches can be employed to identify the secreted proteins of the secretome [Figure 1]. The most employed techniques for identifying bioactive components in the MSCs-CM are liquid chromatography with tandem mass spectrometry (LC-MS/MS), in addition to proteomic and transcriptomic analyses⁷⁷⁻⁷⁹. Targeted detection techniques such as

antibody array, enzyme-linked immunosorbent assay (ELISA), flow cytometry, and western blotting are typically employed to quantify known proteins. These methods are used to isolate the proteins in the secretome. These techniques are usually followed by mass

spectroscopy, serial analysis of gene expression, DNA microarray, and RNA sequencing. The techniques mentioned above may be integrated with bioinformatics instruments (software and databases) to assess and analyze the secretome-proteome outcomes^{80,81}.

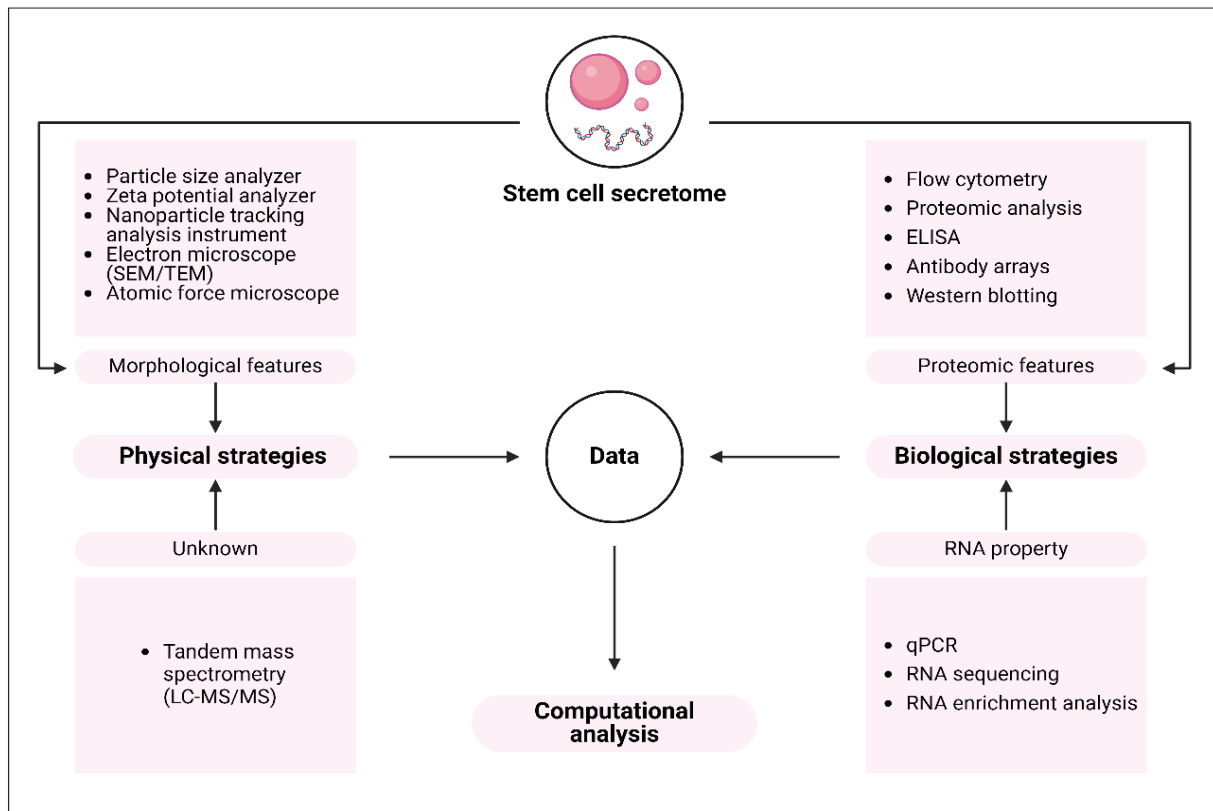


Figure 1: Characterization of stem cell secretome.

Real-time quantitative PCR detecting system (qPCR) is utilized to assess the enrichment and composition of RNA in the secretome of stem cells. Other physical characteristics of EVs, such as surface charge, particle number, size distribution, and morphology, can be assessed through the utilization of instruments such as electron microscopes and particle size analyzers^{82,83}.

8. MSCs secretome storage

The majority of studies stored dental MSCs-CM at -80°C ⁸⁴⁻⁸⁷, while some at -20°C ^{88,89}. These various forms of storage did not affect CM's capabilities. Previous research has shown that freeze-thaw does not affect the size of exosomes or compromise the integrity of EVs membranes; however, the size of exosomes decreases by 60% after two days at a physiological temperature of 37°C ⁹⁰. Exosomes are stable at -20°C for up to six months without experiencing a loss of

metabolic activity ⁹¹. Although Zhou et al. reported that exosomes frozen at -20°C experienced substantial depletion, freezing at -80°C enabled nearly complete recovery even after a storage period of up to seven months ⁹².

9. Dental MSCs secretome biological effects

9.1. Anti-apoptotic effect

Pro-apoptotic proteins, like Bcl-2-associated x protein (Bax), tumor protein 53 (p53), and cleaved caspase-3, were significantly reduced following dental MSCs-CM-based therapy in addition to significant increase in the expression of anti-apoptotic B-cell lymphoma 2 (Bcl-2) in parenchymal cells, preventing their loss during inflammation ^{6,93-95} [Figure 2].

9.2. Angiogenic effect

Dental MSCs-CM promoted angiogenesis via secretion of pro-angiogenic factors, including VEGF-A, FGF-2, PDGF, IGF-1, angiopoietin-2, metalloproteinase (MMP)-3, TGF- β , GM-CSF, GCSF, and IL-8 ^{6,96} [Figure 2].

9.3. Neuroprotective and neurotrophic effects

Several factors responsible for reduction of neurotoxicity such as VEGF, fractalkine, and the A β -degrading enzyme neprilysin were detectable in dental MSCs-CM ⁹⁷. Additionally, dental MSCs-CM contains growth factors that support neural growth

and differentiation, such as BDNF, NGF ⁹⁸⁻¹⁰⁰, nestin, Sox-1, β -tubulin III ¹⁰⁰, HGF ⁹⁸ and IGF ⁹⁹, besides Neurofilament 200 and S100 ¹⁰¹⁻¹⁰³ [Figure 2].

9.4. Immunomodulatory and anti-inflammatory effects

The immunoregulatory effects of dental MSCs-CM are achieved through the upregulation of M2 macrophages and the modification of pro-inflammatory conditions ¹⁰⁴⁻¹⁰⁶. In addition, dental MSCs-CM inhibited the production of pro-inflammatory cytokines, including NF- κ B, interferon (IFN)- γ , tumor necrosis factor (TNF)- α and interleukins (IL-1 β , IL-18, IL-17, IL-13, IL-6, IL-4) ^{44,82,83,107} and enhanced the anti-inflammatory cytokines (TGF- β 1, IL-10) ¹⁰⁸. The balance between these anti-inflammatories and pro-inflammatory cytokines may determine the final effect [Figure 2].

9.5. Osteogenesis

Dental MSCs-CM promotes osteogenesis by upregulating osteoblastic and chondrogenic marker expression (Osterix, SOX-5) ¹⁰⁹. TGF- β - bone morphogenetic proteins (BMP) signaling pathway plays a pivotal role in osseous regeneration induced by dental MSCs-CM through upregulating the expression of TGF- β 1, TGF- β 2, BMP2, BMP4, MMP8, runt-related transcription factor 2 (Runx2)g, and SOX-9 was detected ¹¹⁰, as well as VEGF, and collagen type I alpha 1 chain (COL1A1) ¹¹¹.

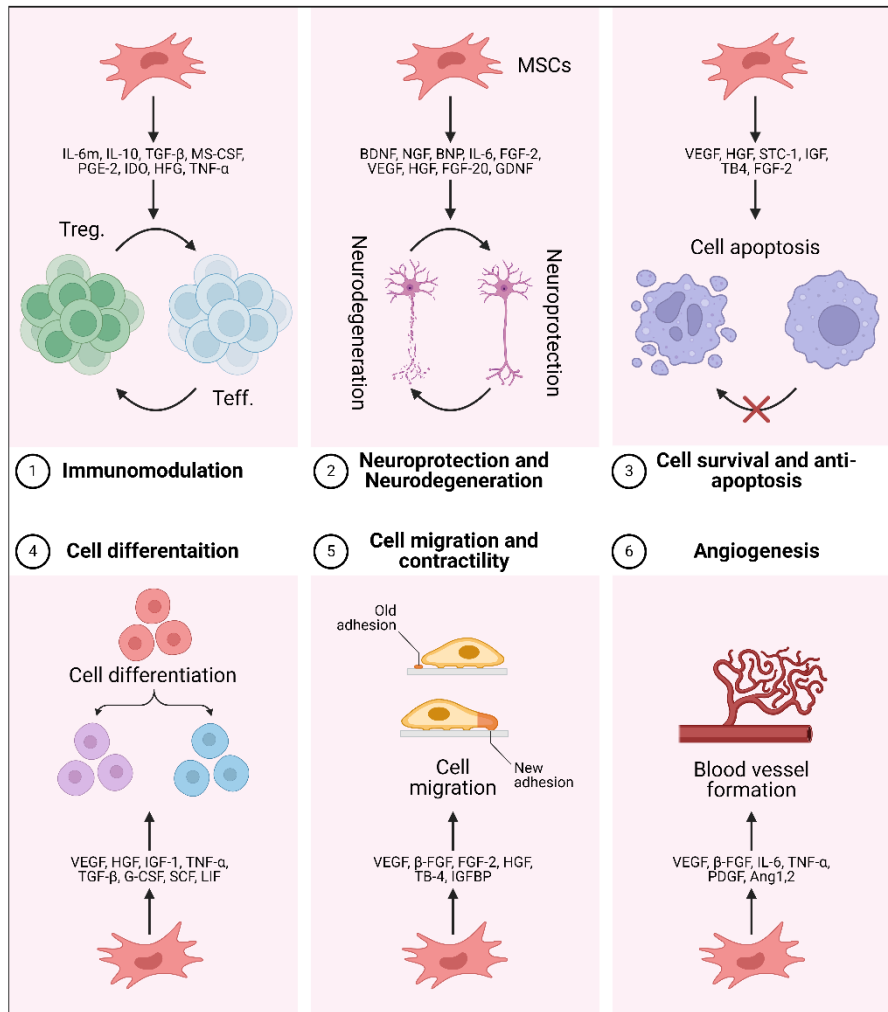


Figure 2: Biological effects of MSCs-CM.

9.6. Modulation of oxidative stress

In an *in vitro* model of multiple sclerosis, it has been documented that PDLSCs-CM¹¹² and GMSCs-CM⁹³ reduced oxidative stress markers such as superoxide dismutase (SOD)-1, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2, significantly. Furthermore, the immunomodulatory and anti-apoptotic properties of these MSCs-CM significantly inhibited the death of neural cells. Similarly, DPSCs-CM and SHED-CM reduced levels of reactive oxygen species (ROS) in neural cells¹¹³, a murine

model of multiple sclerosis¹¹⁴, and Alzheimer's disease⁹⁹.

10. The superiority of dental MSCs secretome and its derivatives

In terms of composition, the secretome profile of dental MSCs differs from that of other MSCs. SCAP-CM demonstrated elevated expression of proteins linked to metabolic processes and transcription, as well as chemokines and neurotrophins compared to BM-MSCs-CM¹¹⁵. A total of 124 cytokines exhibited shared expression among DPSCs-CM, SCAP-CM, and DFSCs-CM. It is worth mentioning that DPSCs-CM exhibited a considerably

elevated expression level of 23 cytokines that are associated with odontoblast differentiation, as well as pro-inflammatory

and anti-inflammatory cytokines ¹¹⁶. All dental secreted factors are listed in Table 2.

Table 2: The main factors found in the dental MSCs-secretome ¹¹⁷.

MSCs	Secretome	Contained Factors
DPSCs ¹¹⁸	CM	EGF, Endoglin, Endothelin-1, Eotaxin-1, Follistatin, G-CSF, GM-CSF, HGF, Leptin, IFN α 2, IFN γ , IL-12, IL-13, IL-15, IL-1 β , IL-5, IL-8, IL-9, PDGF-AA, TGF- α , TGF- β 1, TGF- β 2, TGF- β 3, TNF α , TNF β , VEGF-A, VEGF-C, VEGF-D
PDLSCs ^{119,120}	CM	99 proteins, including matrix proteins, enzymes, growth factors, cytokines, and angiogenic factors
	EVs	Non-coding RNAs: antisense RNAs, long non-coding RNAs, miRNAs (MIR24-2, MIR142, MIR335, MIR490, and MIR296)
SCAP ^{115,121}	CM	2046 proteins, included chemokines, angiogenic, immunomodulatory, anti-apoptotic, neuroprotective factors and extracellular matrix proteins
	EXOs	593 PIWI-interacting RNAs (piRNAs)
SHED ¹²²	CM	FGF-2, IL-10, PDGF, TGF- β 3, HGF, INF- γ , VEGF, IL-6
GMSCs ¹²³	EVs	Transcripts for growth factors such as TGF- β , FGF, VEGF, neurotrophins, such as NGF, BDNF and members of the Wingless/Integrated family
DFSCs ¹²⁴	CM	Osteogenic lineage related proteins

In comparison to BM-MSCs-CM, DPSCs-CM demonstrated enhanced angiogenic, anti-apoptotic, and neurite outgrowth capabilities, migration activity, immunomodulation in vitro ^{125,126}, and vasculogenesis in vivo ¹²⁶. DMSCs-CM derived from DPSCs, SCAP, and DFSCs exhibited a greater capacity for nerve regeneration than BM-MSCs-CM, as indicated by significantly increased colony formation and neurite extension. This indicates that DMSCs-CM are associated with enhanced neural differentiation and maturation, which could be attributed to significantly higher expressions of

BDNF, neurotrophin-3 (NT-3), and BDNF in DMSCs-CM.

Owing to their neural crest origin, Dental MSCs-EVs exhibit superior therapeutic potential in models of neurological disorders, dental diseases, and wound injury compared to other MSCs-EVs ¹²⁷. Compared to BM-MSCs-EXOs, DPSCs-EXOs have promoted differentiation in Tregs by increasing the release of anti-inflammatory cytokines IL-10 and TGF- β ¹²⁸. Five crucial miRNAs involved in microtubule cytoskeleton organization were identified in the EVs of PDLSCs ¹¹⁹. Additionally, SCAP-EXOs comprised RNAs that primarily function in binding

and catalytic activities, metabolic processes, cellular behavior, and biological control ¹²¹.

11. Novel strategies to increase therapeutic efficacy and production of MSCs secretome

To attain the intended therapeutic efficacy, it appears impractical to increase the dosage and frequency of administration of MSCs-CM. Therefore, boosting the potency of MSCs-CM, with subsequent enhancement of therapeutic benefits is a top priority. Various ways have been proposed to enhance the optimization of MSCs-CM components, which can be broadly categorized into genetic modification group and non-genetic modification group (pre-activation/priming) ¹²⁹ [Figure 3].

11.1. Modifications in culture conditions (priming)

An area of intense research has emerged around priming MSCs to enhance their immunomodulatory properties, migratory potential, and/or hypo-immunogenicity. The most widely used priming techniques involve treatment with:

11.1.1. Three-Dimensional (3D) culture

3D platforms are constructed to mimic the naive cellular niches in vivo. Three-dimensional cultures have been shown to increase cell yield and stimulate the secretion of trophic factors in comparison to conventional monolayer cultures ¹³⁰. Characterization of 3D-MSCs-CM revealed enrichment of the obtained CM profile on anti-inflammatory actors such as IL-10 and lower levels of pro-inflammatory cytokines such as IL-6 or IL-2 compared with 2D-MSCs-CM ¹³¹.

11.1.2. Culture medium

Sagaradze et al. identified variations in factor concentrations among MSCs-CM cultured in two distinct growth media ¹³². the utilization of MSCs-CM in vivo may elicit immunologic reactions due to the variability and uncertain composition of fetal bovine serum (FBS) across different batches, which includes impurities ^{133,134}. Serum-free or chemically defined medium is the most acceptable alternative ¹³⁵. Chemically defined medium is not only serum-free but also devoid of hydrolysates or supplements whose composition is unknown. Notably, cultures of MSCs lacking serum secreted a greater quantity of angiogenic factors ^{136,137}. Additionally, researchers noted that MSCs-CM obtained in the presence of serum exhibited cytotoxicity when used at full concentration (100%). However, its beneficial effects diminished upon dilution, unlike MSCs-CM obtained under serum-deprived conditions ¹³⁸.

11.1.3. Hypoxia

Under hypoxic conditions, there was an observed upregulation in the total protein content of the secretome. Notably, the intermittent hypoxic state significantly increased the expression of NGF and GCSF ¹³⁹. miRNAs concerned with the proliferative, differentiative, and inflammatory phases are overexpressed under hypoxia ¹⁴⁰. Hypoxia has been documented to stimulate the release of hypoxia-inducible factor (HIF-1), a protein that exerts regulatory effects on cellular processes, including metabolism, differentiation, proliferation, migration, and survival ¹⁴¹.

11.1.4. Biochemical stimuli

Biochemical stimuli have become a widely used strategy in therapies based on the MSCs-CM. Stimulated MSCs secrete molecules and EVs to hinder biological

signals and, thus, produce more significant amounts of miRNAs, metabolites, lipids,

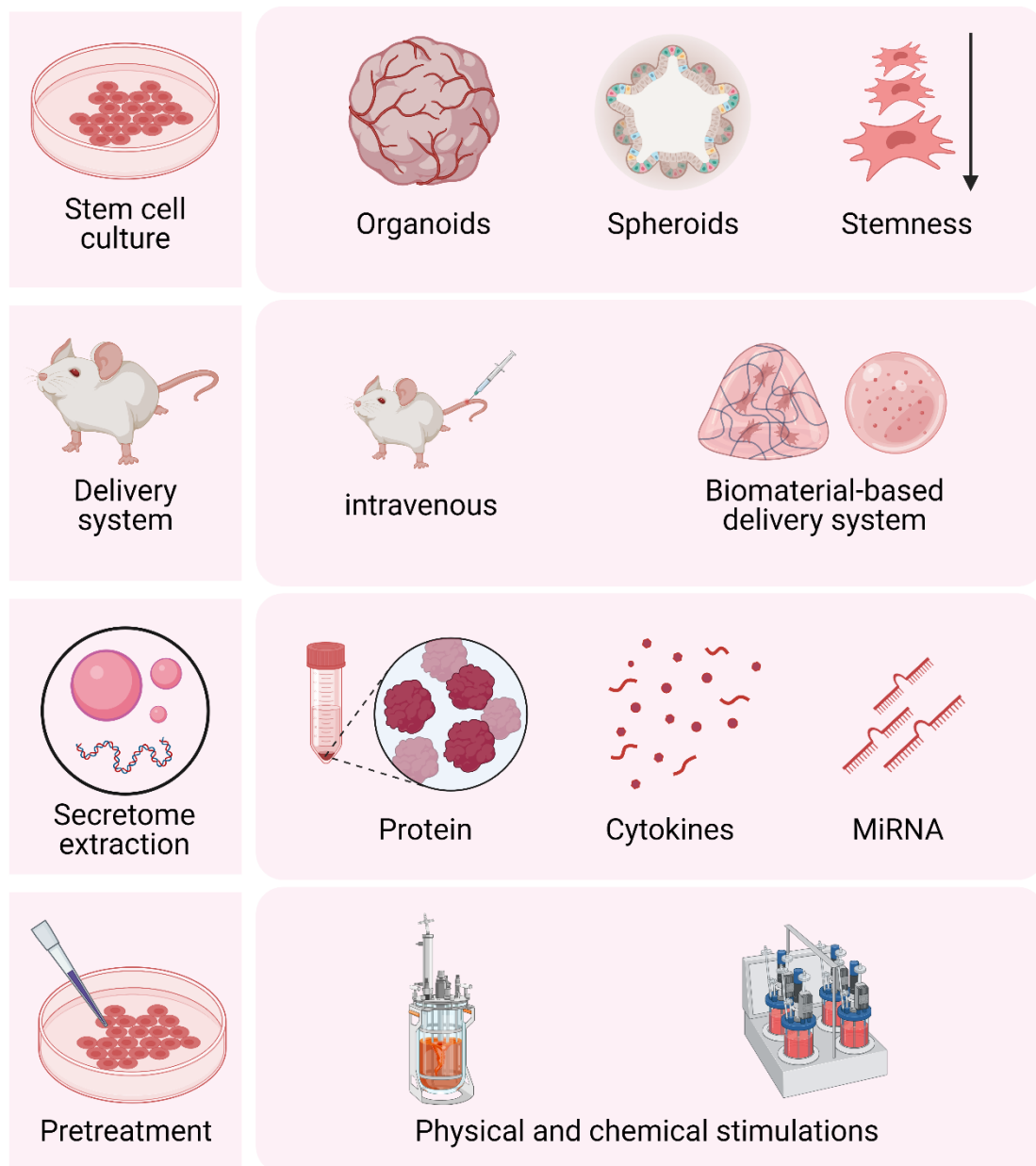


Figure 3: Schematic illustration of the up-to-date approaches for stem cell culture, cell pretreatment, secretome extraction, and secretome delivery.

and proteins ¹⁴². IFN- γ and TNF- α were utilized to prime MSCs. The exosomes obtained from these cells exhibited noteworthy enrichment of therapeutic and bioactive molecules with pro-angiogenic, proliferative, anti-inflammatory, and antifibrotic properties ¹⁴³. Moreover, exposure to IL-1 β and TNF- α enriches the CM in proteins such as GM-CSF, monocyte

chemoattractant protein-1 (MCP-1) and MMP 8 & 9 ¹⁴⁴.

11.1.5. Mechanical stimuli

Mechanical stimulation of MSCs is another method for manipulating their behavior and secretome profile. MSCs-CM are responsive to their substrate's rigidity and other mechanical properties ¹⁴⁵. Surface

topographies can modify the morphology of stromal cells and exert a quantitative impact on their cytokine secretion profile¹⁴⁶.

11.2. Genetic modifications

The application of genetic modification techniques to manipulate the expression of specific genes in MSCs through transfection or transduction methods has garnered growing interest. Transfection is the nonviral introduction of nucleic acids into cells via microinjection, electroporation, and nanocarriers (lipids, polysaccharides, peptides, inorganic materials, and polymers)¹⁴⁷. Transduction includes the introduction of nucleic acids into cells via viral vectors, such as lentivirus and adenovirus. Transfection is less effective in comparison. Nonetheless, concerns regarding the immunogenicity and mutagenicity of the viral vectors pose safety risks that prevent the clinical application of this method¹⁴⁸.

12. Delivery routes of MSCs secretome

MSCs-CM can elicit their therapeutic effects via various routes of administration. However, the immediate challenge in this field is still to find a safe, effective, and controlled manner for its delivery¹⁴⁹. There are systemic and local delivery routes.

Systemic delivery is the most prevalent method for delivering MSCs-CM. Nevertheless, intravenous administration frequently leads to an overabundance of MSCs-CM accumulation in the liver and spleen; therefore, in this instance, multiple injections at a higher dosage are necessary to achieve the desired therapeutic effect^{150,151}. Local delivery, with the assistance of various biomaterials, can enhance specific tissue delivery and retention with lower working dosage, increase stability, and advance the functioning of MSCs-CM.

Local administration could be either device-assisted technology or biomaterial-based approaches¹⁵².

Device-assisted technology includes inhalation, which is non-invasive and easy to use with good patient compliance¹⁵³, and needle-free injection drives CM through the skin with shock waves, gas pressure, or electrophoresis¹⁵⁴. Biomaterial-based approaches include (i) Successfully developed nanoparticles to overcome biological barriers, avoid nonspecific biodistribution, and evade immune clearance, leading to efficient and effective delivery in vivo^{155,156}. (ii) Injectable hydrogels can function as a barrier to prevent rapid clearance of encapsulated MSCs-CM, increasing their retention for sustained release in situ^{157,158}. (iii) Microneedle is a minimally invasive technique that can be used for the controllable and sustained release of different drugs. (iv) Scaffold patches fabricated using synthetic polymers, collagen, fibrin, and decellularized matrix with biocompatibility and biodegradability properties can be excellent carriers for various cells and growth factors^{159,160}.

13. The therapeutic role of dental MSCs-CM in the non-dental tissue regeneration

13.1. Neural tissue regeneration

Dental-CM and its EVs demonstrated the ability to promote neuro-regeneration in the central and peripheral nervous systems¹⁶¹. In a mutant superoxide dismutase mouse model of amyotrophic lateral sclerosis, systemic administration of DPSCs-CM improved motor neuron survival and neuromuscular junction innervation¹⁶². Additionally, as a potential treatment for Parkinson's disease, SHED-EXOs grown on three-dimensional laminin-coated alginate microcarriers have been discovered to prevent the apoptosis of

dopaminergic neurons, thereby protecting nerves ¹⁶³.

13.2. Immunological diseases

SHED-CM intravenous injection significantly ameliorated arthritis symptoms and reduced joint degradation in a rat model of rheumatoid arthritis owing to the anti-inflammatory properties of SHED-CM, which were linked to the promotion of M2 macrophage polarization and the suppression of osteoclastogenesis ¹⁶⁴. By reducing the polarization of M1 macrophages and inhibiting the production of ROS via the ROS-MAPK-NF κ B-P65 signaling pathway, DPSCs-EXOs can be utilized as a therapeutic intervention for spinal cord injury ¹⁶⁵. PDLSCs-CM and EVs demonstrated immunosuppressive effects in a mouse model of experimental autoimmune encephalomyelitis via reduction of pro-inflammatory cytokines IL-17, IFN- γ , IL-1 β , IL-6, TNF- α , and induce anti-inflammatory IL-10. In addition to attenuating apoptosis-related p53, Caspase 3 and Bax expressions ¹⁶⁶.

13.3. Cardiovascular injuries

SHED-CM intravenous administration significantly reduced the size of myocardial infarction in addition to significant reduction in levels of cytokines including TNF- α , IL-6, and IL- β , thereby protecting the mouse heart from hypoxic injury, as evidenced by enhanced cardiac function. This disparity could be attributed to the elevated hepatocyte growth factor (HGF) and VEGF concentrations ¹⁶⁷.

13.4. Solid-organ regeneration

DPSCs-CM demonstrated the existence of a variety of liver lineage proteins, including hepatocyte nuclear factor and hepatocyte growth factor receptor *in vitro*, thereby promoting liver repair and regeneration ¹⁶⁸.

Moreover, SHED-CM decreased lung fibrosis and improved survival rates in a mouse model of acute lung injury ¹⁶⁹.

13.5. Tumor progression

One notable characteristic of dental MSCs-EVs is their favorable drug-loading capacity and ability to target tumors specifically. The utilization of DPSCs-EXOs cultured with gemcitabine demonstrated a notable suppression of cellular proliferation in pancreatic cancer cell lines ¹⁷⁰.

13.6. Soft tissue regeneration

Local administration of GMSCs-EVs exhibiting upregulation of IL-1 receptor antagonist facilitates the cutaneous wound healing process, hence providing more evidence of the advantageous impact of GMSCs-EVs on the restoration of soft tissue damage ¹⁷¹. In addition, GMSCs-EXO combined with hydrogel promoted healing of diabetic rats' skin wounds through upregulation of collagen deposition, and remodeling in addition to promotion of re-epithelialization, ¹⁷². In addition, GMSCs-EXO enhanced angiogenesis which was confirmed through increased density of microvessels in the wound bed suggests that the skin defects in diabetic rats are receiving an adequate supply of oxygen and nutrients. These factors can facilitate the healing process ¹⁷².

14. The therapeutic role of dental MSCs-CM in the dental and paradental tissue regeneration

14.1. Periodontal diseases

Healthy PDLSCs-EXOs can suppress the excessive activation of the classical Wnt signaling pathway within an inflammatory milieu, which preserves the osteogenic potential of endogenous stem cells and

facilitates the process of periodontal bone repair ¹⁷³. Periodontal bone loss was inhibited in a mouse model of periodontal maxillary bone loss by EVs derived from TNF- α preconditioned GMSCs. This effect could be ascribed to the increased expression of miR-1260b in GMSCs-EVs ¹⁷⁴. Furthermore, SHED-EXOs inhibit lipogenesis and promote bone formation, as evidenced by the upregulation of Runx2 expression in mouse periodontitis model ¹⁷⁵.

14.2. Dentin-pulp complex regeneration

SCAP-EXOs introduced into the root fragment containing BM-MSCs and transplanted subcutaneously into immunodeficient mice promoted dentin-pulp complex regeneration. This effect could be attributed to activating the mitogen-activated protein kinase signaling pathway ¹⁷⁶. Furthermore, DPSCs-EXOs effectively stimulated odontoblastic differentiation in an ectopic tooth transplantation model; they induced the regeneration of dental pulp-like tissue ¹⁷⁷. DPSCs-EVs combined with a treated dentin matrix demonstrated promising potential for promoting dentinogenesis in a pulp exposure model employing miniature pigs ¹⁷⁸. Tertiary dentin development was stimulated in a rat molar pulpotomy model in response to the long-term release of DPSCs-EXOs and exosomes derived from an immortalized murine odontoblast cell line (MDPC) implanted in a microsphere synthetic polymeric carrier ¹⁷⁹.

14.3. Temporomandibular joint (TMJ) disorders

DPSCs-CM administration in a rat model of chemically induced TMJ osteoarthritis revealed reduced inflammation, enhanced ECM and subchondral bone repair and regeneration through downregulation of pro-inflammatory genes (MMP-13, MMP-

9, MMP-3, and MCP-1) ¹⁸⁰. By diminishing iNOS, MMP-13, and IL-1 β , SHED-CM significantly inhibited temporal inflammation and improved the integrity of the destroyed condylar cartilage ¹⁸¹.

14.4. Bone regeneration

The regenerative effects of DPSCs-EVs in the alveolar bone defect rat model suggested that alveolar bone MSCs are capable of absorbing DPSCs-EVs, which in turn significantly promoted the expression of Runx2, alkaline phosphatase, and osteocalcin, and the osteogenic differentiation capacity of alveolar bone MSCs 6 weeks post-operatively ¹⁸². When combined with materials, GMSCs and DPSCs derived EVs facilitated bone defects healing in rats and increased the expression of Runx2, VEGF-A, and osteopontin, which are osteogenic markers ^{57,183}. Additionally, the administration of GMSCs-CM resulted in a considerable upregulation of ossification-related genes in rat calvaria bone abnormalities ¹⁸⁴.

14.5. Salivary glands dysfunction

DPSCs-EXOs restored the activity of salivary gland epithelial cells during Sjogren's syndrome, suggesting that they may have therapeutic potential in the treatment of Sjogren's syndrome by inhibiting the mortality of salivary gland epithelial cells induced by IFN- γ ¹⁸⁵. Moreover, DPSCs-CM ameliorated Sjogren's syndrome by promoting regulatory T (Treg) cell differentiation and inhibiting T helper (Th17) cell differentiation by upregulating IL-10 and TGF- β 1 and downregulating IL-4 and 17a ¹⁸⁶.

15. Challenges and limitations surrounding the therapies based on secretome from dental MSCs

Despite the promising results of dental MSCs-CM and its derivatives as a cell-free approach, their role as sole therapy still requires further investigation. It is essential to match donor characteristics and tissue sources as an initial phase to develop and apply MSCs-CM-derived products precisely⁷. The lack of standardized MSCs isolation/expansion and secretome production protocols, continues to be among the main issues raised in the literature. This has led to unpredictability regarding the biological effects of secretome products, which appear to be significantly impacted by the preparation method¹⁸⁷. Determining the exact biochemical composition of MSCs secretome, in addition to the activity and half-life of its constituents, has proven to be an extremely challenging task⁷. Furthermore, the most significant obstacle to the therapeutic implementation of EVs is time-consuming and costly isolation processes. Moreover, the purified EVs yield is insufficient to permit extensive clinical application⁶⁴. Therefore, for MSCs-CM to be utilized in clinical practice, they must be expanded under well-controlled, scalable, and standardized GMP culture conditions⁷³.

Conclusions

Based on existing literature, dental MSCs-CM and its derivatives possess diverse capabilities relevant to regenerative medicine fields. Originating from dental tissues, these MSCs-CM demonstrate superior therapeutic potential compared to other MSCs-CM in tissue regeneration. Consequently, their utilization is increasingly recognized as a beneficial therapeutic approach.

The secretome of dental MSCs and its derivatives offer several advantages over cell-based therapies. These benefits encompass various aspects such as

streamlined manufacture, ease of storage, handling, packaging, extended product shelf life, and their potential as readily available biological therapeutic agents. Moreover, secretome-based therapies raise fewer safety concerns, positioning them as a prominent focus in regenerative medicine.

EVs play a crucial role in transferring bioactive molecules and protecting their cargo from hydrolysis. As a result, EVs are emerging as a promising cell-free therapy. Among all MSCs-EVs, dental MSCs-EVs hold excellent application prospects for both local and systemic diseases.

Despite the remarkable therapeutic potential of dental MSCs-CM and its derivatives, their clinical translation faces obstacles related to production and efficacy limitations. Factors such as culture conditions, separation techniques, characterization methods, and preconditioning strategies significantly impact the regenerative potential of dental MSCs secretome.

Further investigations are essential to assess MSCs-CM and its derivatives therapeutic efficacy, understand mechanisms of action, and address short and long-term complications. Complementary studies should focus on optimizing dental MSC populations, environmental cues, and secretome fractions for specific applications. Additionally, emphasizing the role of the microenvironment and exploring EVs' biological characteristics will enhance their therapeutic potential. Lastly, developing standardized methodologies and validation assays will facilitate clinical implementation.

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List of abbreviations

Ad	Adipocyte	MCP-1	Monocyte chemoattractant protein-1
Bax	Bcl-2-associated x protein	MDPC	Immortalized murine odontoblast cell line
Bcl-2	B-cell lymphoma 2	Mes	Mesodermal lineage
BDNF	Brain-derived neurotrophic factor	miRNAs	MicroRNAs
bFGF	Basic fibroblast growth factor	MMP-mRNAs	Matrix metalloproteinase-Messenger RNAs
BM-MSCs	Bone marrow derived-mesenchymal stem cells	MS	Microsphere
BMP	Bone morphogenetic protein	MSCs	Mesenchymal stem cells
Cem	Cementoblast	MVs	Microvesicles
Cho	Chondrocyte	MWCOs	Molecular weight cut-offs
CM	Conditioned medium	Myo	Myoblast
COL1A1	Collagen type I alpha 1 chain	Neu	Neuronal cell
COX-2	Cyclooxygenase-2	NF-κB	Nuclear factor kappa light chain enhancer of B cells
Dent	Dentinogenic lineage	NGF	Neural growth factor
DFSCs	Dental follicle stem cells	NT-3	Neurotrophin-3
DPSCs	Dental pulp stem cells	Oct-4	Octamer-binding transcription factor-4
Ect	Ectodermal lineage	Od	Odontoblast
ELISA	Enzyme-linked immunosorbent assay	Os	Osteoblast
End	Endodermal lineage	P53	Tumor protein 53
Endo	Endothelial cell	PDGF	Platelet-derived growth factor
EVs	Extracellular vesicles	PDL	Periodontal ligament
EXOs	Exosomes	PDLSCs	Periodontal ligament stem cells
FBS	Fetal bovine serum	PGE2	Prostaglandin E2
FGF	Fibroblast growth factor	ROS	Reactive oxygen species
GCSF	Granulocyte colony-stimulating factor	Runx2	Runt-related transcription factor 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor	SCAP	Stem cells from apical papilla
GMP	Good manufacturing practice	SHED	Stem cells from human exfoliated deciduous teeth
GMSCs	Gingival mesenchymal stem cells	SOD-1	Superoxide dismutase-1
Hep	Hepatocyte	Sox-	Sex determining region Y-box transcription factor-
HGF	Hepatocyte growth factor	TGF-	Transforming growth factor-
HLA-DR	Human leukocyte antigen-DR	Th-17	T helper-17
HIF-1	Hypoxia-inducible factor	TMJ	Temporomandibular joint
HSP-	Heat shock protein-	TNF-α	Tumor necrosis factor-α
IFN-α	Interferon-α	TNF-β	Tumor necrosis factor-β
IFN-γ	Interferon-γ	Treg	Regulatory T cell
IGF-	Insulin growth factor-	VEGF-	Vascular endothelial growth factor-
IL-	Interleukin-		
iNOS	Inducible nitric oxide synthase		
KGF	keratinocyte growth factor		
LncRNAs	Long non-coding RNAs		

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