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*Review*

# Cell Secretome from Mesenchymal Stem Cells for Periodontal Regeneration

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**Abstract:** Periodontitis affects approximately 50% of the global adult population and results in varying degrees of periodontal destruction. The regeneration of periodontal tissue is in great demand but is currently difficult to achieve. The cell secretome from mesenchymal stem cells ($CS^{msc}$) has shown promise in promoting periodontal regeneration and is a translational alternative for mesenchymal stem cell (MSC)-based therapy. The practical components of $CS^{msc}$ are soluble secretions and extracellular vesicles. The mechanisms of $CS^{msc}$-induced tissue regeneration may lie in its regulation of the local microenvironment, modulating immune cells such as macrophages and stimulating local host MSCs, a cell population with a direct effect on tissue regeneration. Therefore, $CS^{msc}$ has been suggested as a promising cell-based product for future periodontal regenerative therapy. Hence, more studies should be conducted to analyse the effective components of the MSC secretome, explore the underlying mechanisms, and obtain functional $CS^{msc}$ for clinical translation in periodontal regeneration.

**Keywords**: mesenchymal stem cells; periodontal regeneration; secretome; extracellular vesicles; cell-free therapy

## 1. Background

Periodontitis, characterised by the destruction of periodontal tissue (Figure 1), is a chronic inflammatory disease that occurs in periodontal tissue (including gingiva, periodontal ligament, alveolar bone, and cementum). Periodontitis affects half of the adult population globally, making it the leading cause of tooth loss. Furthermore, its close link to systemic disorders further underscores its significance, imposing a heavy burden on overall health [1]. Given the significant demand for periodontal regeneration and the inherent difficulties in achieving it, there is a pressing need to develop efficacious methods for periodontal regeneration, despite the present challenges.

Mesenchymal stem cell (MSC)-based therapy is an effective method for periodontal regeneration. According to a meta-analysis conducted in 2017, MSC-based therapy showed promising effects in the treatment of periodontal defects in animals [2]. Furthermore, the clinical application of MSCs has shown positive results in periodontal regeneration [3–6]. However, another meta-analysis published in 2020 showed that MSC-based therapy has a negligible impact on treating intrabony periodontal defects, suggesting low-quality evidence in clinical practice [7]. Notably, studies that investigated MSC-base therapy for other diseases also found that the curative effect of MSCs was unstable [8]. Therefore, it is necessary to further clarify the effective components and mechanisms of MSC therapy to improve its clinical efficacy.

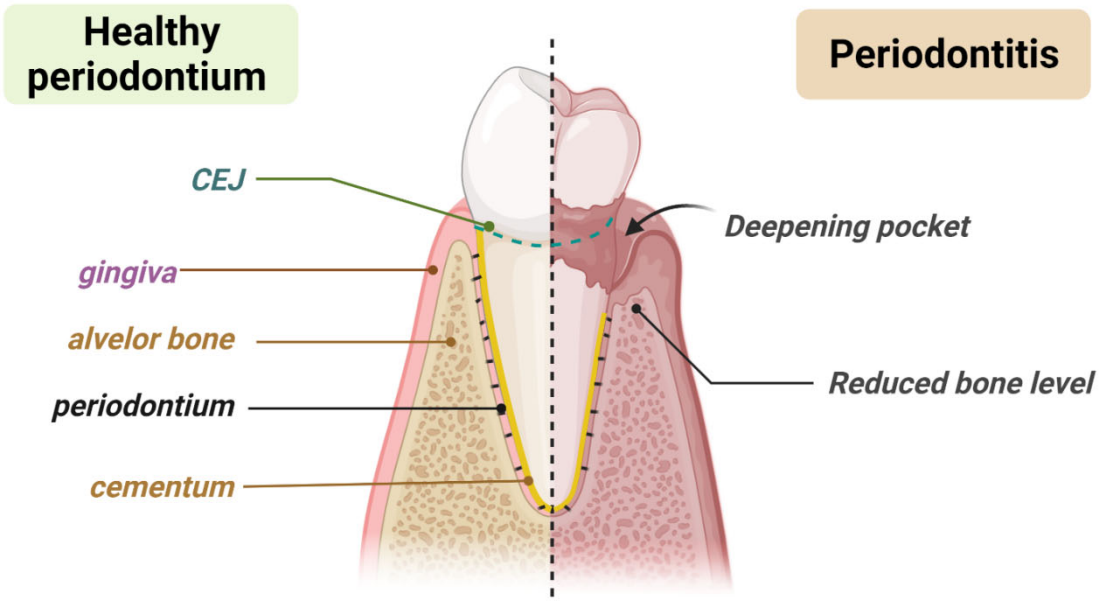


**Figure 1.** Healthy periodontal tissue and periodontitis.

### *1.1. The Clinical Efficacy of MSCs-Based Approaches for Periodontal Regeneration*

The results of randomised controlled trials on the clinical applications of MSCs in periodontal regeneration are summarised in Table 1 [3,4,6,9–17]. In these studies, the effect of MSCs in repairing periodontal soft tissue defects (gingival recession) was highly favourable [4,9,10], however, there are great differences in the effect of MSCs on periodontal defects [3,6,11–17]. We hypothesize that the controversial efficacy of MSCs therapy in periodontal regeneration may be attributed to the multitude of factors influencing the healing of periodontal bone defects. These factors include, but are not limited to, the following aspects: (1) the healing capacity of specific individuals [18], (2) the tooth type [18], (3) the morphology of osseous defect [19], (4) surgical planning and surgical proficiency [18], (5) the source of MSCs [5,20], (6) the carrier [21], the time of stem cells co-culture with scaffold materials before MSCs transplantation, and the inoculation density of cells; (7) the preparation standard, storage, and transplantation of MSCs may also greatly impact the therapeutic potential and state of cells, which exacerbates the instability of the results. Therefore, a study with a small sample size may be insufficient to identify the real impact of MSC therapy.

**Table 1.** Summary of randomized controlled clinical trials evaluating the efficacy of MSCs in periodontal regeneration.

| References | Participants | Stem Cell Origin | Defect Type | Treatment | Follow-Up Period | Results |
|---|---|---|---|---|---|---|
| Köseoğlu et al. (2012) [10] | 22 sites from 11 patients (split-mouth design) | autologous gingival fibroblasts | gingival recession defects (Miller I) | I: cells + collagen membrane C: collagen membrane | 12 months | Both treatments resulted in a significant gain in root coverage when compared with the baseline. A statistically significant increase was detected in the percentage of root coverage in the I group when compared with the C group. |
| Zanwar et al. (2014) [9] | I: n = 12 patients C: n = 12 patients | human umbilical stem cells | gingival recession defects | I: cells + bioresorbable PLA/PGA membrane, C: SCTG | 6 months | Stem cells in combination with bioresorbable PLA/PGA membrane resulted in significantly higher clinical attachment level (CAL) gain than SCTG. |
| Zanwar et al. (2017) [4] | I: n = 7 patients C: n = 7 patients | human umbilical stem cells | gingival recession defects | I: cells + PLA/PGA membrane C: PLA/PGA membrane | 6 months | Stem cell with PLA/PGA membrane showed significantly higher mean root coverage compared to PLA/PGA membrane only. |

**Table 1.** *Cont.*

| References | Participants | Stem Cell Origin | Defect Type | Treatment | Follow-Up Period | Results |
|---|---|---|---|---|---|---|
| d'Aquino et al. (2009) [3] | 34 sites from 17 patients (split-mouth design) | autologous dental pulp-derived MSCs | distal defects to the second molar | I: cells + collagen sponge scaffold C: collagen sponge scaffold | 12 months | An increase of clinical attachment that was quantitatively higher at the I site than at the C site. Additionally, I site samples consisted of well-organized and well vascularised bone; bone from control sites was immature, with fibrous bone entrapped among new lamellae, incomplete and large Haversian channels. |
| Cubuk et al. (2023) [17] | 26 sites from 13 patients (split-mouth design) | autologous dental pulp stem cells (DPSCs) | distal defects to the second molar | I: DPSCs seeded onto Leukocyte and platelet-rich fbrin (L-PRF) C: L-PRF alone | 6 months | The L-PRF and L-PRF+ DPSC groups showed a signifcant reduction in PPD (1.65 ± 1.01 mm and 1.54 ± 0.78 mm) and CAL (2.23 ± 1.45 mm and 2.12 ± 0.74 mm), respectively. There was no diference between the groups for any periodontal parameters. No signifcant diferences were found between the groups regarding the radiographic vertical bone loss (VD) or relative bone density (rBD). |
| Chen et al. (2016) [11] | I: n = 20 defects C: n = 21 defects | autologous PDL-MSCs (PDLSCs) | intrabony defects | I: cell sheets + Bio-Oss® C: Bio-Oss® | 12 months | No clinical safety problems that could be attributed to the investigational PDLSCs were identified. Each group showed a significant increase in the alveolar bone height (decrease in the bone-defect depth) over time. However, no statistically significant differences were detected between the cell group and the control group. |
| Ferrarotti et al. (2018) [12] | I: n = 15 defects C: n = 14 defects | autologous dental pulp stem cells (DPSCs) | intrabony defects | I: Cells + collagen sponge C: collagen sponge | 12 months | I sites exhibited significantly more probing depth (PD) reduction (4.9 mm versus 3.4 mm), CAL gain (4.5 versus 2.9 mm) and bone defect fill (3.9 versus 1.6 mm) than controls. Moreover, residual PD < 5 mm (93% versus 50%) and CAL gain ≥ 4 mm (73% versus 29%) were significantly more frequent in the I group. |
| Shalini et al. (2018) [13] | I: n = 14 defects C: n = 14 defects | PDL-MSCs (PDLSCs) niche | intrabony defects | I: A-PDLSC Ni + open flap debridement (OFD) C: OFD | 12 months | Treatment of intrabony defect by direct transplantation of autologous PDLSCs niche in comparison with OFD showed a significant reduction in probing pocket depth and gain in CAL. Radiographically, there was alveolar crest improvement, decrease in defect area, and increase in defect density in A-PDLSC Ni group. |
| Abdal-Wahab et al. (2020) [14] | I: n = 10 defects C: n = 10 defects | gingival fibroblasts | intrabony defects | I: Cells + β-TCP scaffold + collagen membrane C: β-TCP + collagen membrane | 6 months | The intervention group reported a significantly greater reduction in vertical pocket depth, greater CAL gain and higher radiographic bone gain compared with control. |
| Sánchez et al. (2020) [15] | I: n = 9 patients C: n = 10 patients | autologous PDL-MSCs (PDLSCs) | intrabony defects (1-2 walls) | I: (Bio-Oss Collagen® + MSCs) C: (Bio-Oss Collagen®) | 12 months | No serious adverse events were reported. Patients in the test group (n = 9) showed greater CAL gain (1.44, standard deviation [SD] = 1.87) and probing pocket depth (PPD) reduction (2.33, SD = 1.32) than the control group (n = 10; CAL gain = 0.88, SD = 1.68, and PPD reduction = 2.10, SD = 2.46), without statistically significant differences. |
| Apatzidou et al. (2021) [16] | Group-A: n = 9 Group-B: n = 10 Group-C: n = 8 | autologous bone marrow MSCs | intrabony defects | Group-A: cells+ collagen scaffolds + fibrin/platelet lysate. Group-B: collagen scaffolds + fibrin/platelet lysate. Group-C: minimal access flap surgery | 12 months | Radiographic evidence of bone fill was less pronounced in Group-B, although clinical improvements were similar across groups. |
| Sreeparvathy et al. (2024) [6] | 34 sites from 17 patients (split-mouth design) | autologous peripheral blood mesenchymal stem cells (PBMSCs) | intrabony defects | I: PRFM + PBMSCs + open flap debridement (OFD) C: PRFM alone + OFD | 6 months | At 6 months, radiographic parameters revealed significant reduction in defect depth (DD) and significant defect fill percentage (DFP) values in the test group compared with the control group. The I group showed significant improvement in PPD and CAL at the end of 6 months |

**Table 1.** *Cont.*

| References | Participants | Stem Cell Origin | Defect Type | Treatment | Follow-Up Period | Results |
|---|---|---|---|---|---|---|
| Sreeparvathy et al. (2024) [6] | 34 sites from 17 patients (split-mouth design) | autologous peripheral blood mesenchymal stem cells (PBMSCs) | intrabony defects | I: PRFM + PBMSCs + open flap debridement (OFD) C: PRFM alone + OFD | 6 months | At 6 months, radiographic parameters revealed significant reduction in defect depth (DD) and significant defect fill percentage (DFP) values in the test group compared with the control group. The I group showed significant improvement in PPD and CAL at the end of 6 months |

Abbreviations: I, intervention group. C, control group. PLA/PGA, polylactic acid (PLA)/ acid/polyglycolic acid (PGA). SCTG: subepithelial connective tissue graft. CAL: clinical attachment level. PDL, periodontal ligament. PDLSCs, periodontal ligament stem cells. PD, probing depth. OFD, open flap debridement. β-TCP and β-calcium triphosphate.

To clarify this problem, the following suggestions may need to be considered: (1) The interference induced by different patients' healing abilities can be reduced by increasing the sample size or adopting a split-mouth design. (2) The interference induced by different defect types can be reduced by increasing the sample size or by using a paired design. (3) For cell therapy, more studies are needed to determine detailed treatment conditions; for example, optimal scaffold material of MSCs, cell inoculation density of MSCs, and co-incubation time of MSCs and scaffold material before treatment. (4) Periodontal regenerative surgery possesses a high degree of technical sensitivity [18]; therefore, experienced surgeons are also needed to ensure the reliability of experimental results.

### *1.2. The Challenges of MSCs Therapy for Periodontal Therapy*

MSCs therapy remains challenging owing to the following limitations: (1) a limited number of cells; (2) likelihood of tumour formation; (3) difficulty of administration to the target tissue; (4) requirements for storage in liquid nitrogen and the associated infrastructure for revival; and (5) lack of uniformity in MSCs, which limits the clinical application of MSCs [8,21]. However, these limitations may be variable in different stem cell application scenarios; therefore, we will analyse the relevance of these limitations to periodontal regenerative therapy.

**A limited number of cells from periodontal tissues.** Periodontal ligament stem cells (PDLSCs) may be the best donor cells for periodontal repair compared to MSCs derived from other tissues [5,21]. Therefore, a major limitation of the application of MSCs in periodontal therapy is the shortage of cell sources. At present, PDLSCs are isolated from the donor periodontal ligament after tooth extraction, which limits the possibility of obtaining donor cells both in animal and clinical studies. Even when considering the use of allogeneic PDLSCs without considering possible immune rejection, the source of PDLSCs remains limited.

**Likelihood of tumour formation.** It is very rare for MSCs to form tumours after clinical application. Additionally, tumors originating from the periodontal ligament have never been reported. Considering that the periodontal ligament is the most important component of periodontal tissues, and it is the very donor tissue of PDLSCs, the possibility of tumour formation by MSCs, especially PDLSCs, in periodontal regeneration will be even lower.

**Difficulty of administration to target tissue.** In periodontal therapy, there is no concern of targeted administration difficulty. Reaching the target site is a common problem of stem cell application, while the special anatomical structure of periodontal tissue makes it simple to directly transplant stem cells into periodontal pockets and periodontal defects, especially intrabony defects (Figure 1).

**Requirement for storage in liquid nitrogen and the associated infrastructure for revival.** Poor homogeneity of stem cells and difficulties in their storage, transportation, and recovery are common problems in all stem cell therapies. Using the effective components of stem cells to replace living cells can greatly improve the uniformity of stem cell products and the convenience of storage and transportation. More importantly, this approach will greatly reduce the risk brought by cell recovery. Risk control in stem cell therapy will greatly improve its efficacy and stability, which is of great significance for the clinical application of stem cell-based therapy. Here, we summarise the challenges of MSC therapy in periodontal therapy in Table 2.

**Table 2.** Challenges of MSCs therapy for periodontal therapy.

| Challenges | For General Use | For Periodontal Use | Reasons | Comments |
|---|---|---|---|---|
| limited cell donors | limited | very limited | PDLSCs isolation needs tooth extraction. | It is the major limitation of the application of MSCs in periodontal therapy. |
| likelihood of tumour formation | rare | improbable | Periodontal ligament tumours have never been reported. | It is safer for MSCs application in periodontal tissue. |
| difficulty in administration to the target tissue | hard except superficial sites such as skin | easy | The special anatomical structure of periodontal tissue makes it easier for MSCs administration. | It is easy to administrate MSCs to periodontal pockets and periodontal defects, especially intrabony defects. |
| infrastructure for cell revival | yes | yes | Many clinical centers lack the necessary experimental conditions for cell revival, and improper cell revival procedures can lead to changes in cell state and function. Furthermore, some clinical centers do not have the corresponding experimental equipment, which limits the implementation of MSC therapies. | Using the effective components of stem cells to replace the living cells themselves can greatly improve the convenience of storage, transportation and clinical application. |

### *1.3. MSCs-Based Cell-Free-Therapy May Be an Effective Way for Periodontal Regeneration*

Two studies from different research groups reported that MSC-based cell-free therapy can effectively promote periodontal regeneration [22,23]. In the study conducted by Nagata et al., the authors prepared three cell conditional media (CM) from periodontal ligament stem cells (PDLSC-CM) at different concentrations (original: PDLSC; low: 17- to 29-fold concentration: PDLSC-moderate; and 450-fold concentration: PDLSC-high) and transplanted them into rat periodontal defects. PDLSC-CM transplantation enhances periodontal regeneration in a concentration-dependent manner. However, this study only reported the best concentration of PDLSC-CM within a certain range, and there was no inflection point in the concentration-dependent effect curve. We speculated that the authors may not have used a higher concentration owing to the fact that it is difficult to obtain a higher concentration using ultrafiltration with their method. Therefore, further research may obtain higher PDLSC-CM concentrations using the freeze-thawing method to find the optimal PDLSC-CM concentration for periodontal regeneration. In addition, lyophilised powder is easier to store and transport, which is beneficial for industrial applications.

### *1.4. Why Could MSC-Based Cell-Free Therapy Be an Effective Alternative to MSCs for Periodontal Regeneration?*

The fundamental reason why MSC-based cell-free therapy could replace MSCs is that the therapeutic role of MSCs depends more on their immunomodulatory function than the direct proliferation and differentiation of MSCs. Previous studies have shown that the role of MSCs in tissue regeneration stems from their direct proliferation and differentiation to form the target tissue. However, an increasing number of studies have shown that this is not true. Here, we summarise three reasons why MSC-based cell-free therapy could be an effective alternative to MSCs for periodontal regeneration as follows: (1) The low MSCs engraftment rate documented in injured areas disproves the hypothesis that MSCs repair tissue damage by replacing cell loss with newly differentiated cells [24]. Therefore, the functional benefits observed after MSCs transplantation in experimental models of tissue injury may be related to the secretion of soluble factors acting in a paracrine manner. (2) The majority of studies have shown that MSCs are immunocompromised with immunomodulatory and/or anti-inflammatory properties, which are responsible for their therapeutic effects. MSC-derived factors also have immunomodulatory functions similar to MSCs, as they suppress both innate and adaptive immunity by attenuating maturation [25]. (3) Pre-clinical studies have demonstrated equal or even improved organ function upon infusion of MSC-based cell-free therapy compared with MSC transplantation [24]. A human clinical study of alveolar bone regeneration also indicated that MSC-based cell-free therapy was used safely and with fewer inflammatory signs and appeared to have great osteogenic potential for regenerative medicine of alveolar bone [26], which is a major part of periodontal tissue. Therefore, MSC-based cell-free therapy could be an effective alternative to MSCs.

These cell-free therapies have several advantages: (1) they are safer owing to lower amounts of membrane-bound proteins (such as major histocompatibility complex molecules) and do not present concerns about tumour development; (2) the protein, peptide, RNA, and lipid mediators secreted by MSCs could be concentrated, frozen, or lyophilised without loss of activity, which makes them easier to store, transport, and commercialise; and (3) they can infiltrate target organs and have better uniformity compared to MSCs (although this point is not very important for MSCs in periodontal regeneration). Therefore, mesenchymal stem cell-derived products may present new methods of tissue regeneration with great potential and competitiveness.

### *1.5. Mesenchymal Stem Cell Secretome ($CS^{msc}$): The Definition*

As has been noted, MSC-based cell-free therapy is a very promising new model of periodontal treatment. Therefore, it is necessary to clearly define its meaning to facilitate future research. We use "cell secretome from mesenchymal stem cells ($CS^{msc}$)" to define it. $CS^{msc}$ mainly includes cytokines, extracellular vesicles (EVs), and other MSC products. Compared to the definition of cell-free therapy, $CS^{msc}$ can better reflect the product characteristics derived from MSCs, including cytokines and extracellular vesicles (EVs).

Methods to isolate $CS^{msc}$ mainly include four parts:

(1) Preparation of MSCs. Expand MSCs in suitable growth medium (e.g., DMEM, α-MEM) supplemented with 10% fetal bovine serum (FBS) until they reach 70–80% confluence. Verify the MSCs' viability, morphology, and identity markers (e.g., CD73, CD90, CD105 positive; CD14, CD34, CD45 negative).

(2) Transition to Serum-Free or Low-Serum Medium. Gently wash the MSCs 1–2 times with phosphate-buffered saline (PBS) or serum-free medium to remove residual serum and metabolites. Replace the growth medium with serum-free medium (e.g., DMEM/F12) or low-serum medium (e.g., 1% FBS). Serum-free media are preferred to avoid contamination of CM with serum proteins.

(3) Conditioned Medium Collection. Incubate MSCs in serum-free or low-serum medium for 24–48 h. Avoid prolonged incubation to prevent cell stress or death that may alter CM composition. Carefully aspirate the medium without disturbing the cell monolayer. Ensure MSCs are viable after CM collection (e.g., using Trypan Blue exclusion).

(4) Processing of Conditioned Medium. Centrifuge the collected medium at 1000–2000× *g* for 5–10 min to remove cellular debris and suspended particles. Filter the supernatant through a 0.22 μm sterile filter to remove residual debris and ensure sterility. However, it is worth noting that different parameters may need to be adjusted to obtain $CS^{msc}$when the desired components vary.

$CS^{msc}$ contains a diverse array of bioactive components secreted by MSCs. The key components typically found in $CS^{msc}$ include growth factors (e.g., VEGF, FGF, HGF, and EGF) that promote cell proliferation, migration, and differentiation, cytokines (e.g., IL-10, TGF-β, and TSG-6) that regulate immunity and inflammation, and extracellular vesicles carrying miRNAs, proteins, and lipids. It also contains enzymes involved in extracellular matrix remodeling (e.g., MMPs and TIMPs), immunomodulatory molecules (e.g., PGE2, IDO, and HLA-G), and metabolites such as lactate, kynurenine, and short-chain fatty acids [27]. These components collectively contribute to its therapeutic potential in tissue repair, immune regulation, and anti-inflammatory effects. The composition of $CS^{msc}$ is influenced by several factors, including the source of MSCs (e.g., bone marrow, adipose tissue, or umbilical cord) [28], which determines their distinct secretory profiles, and culture conditions, such as serum-free media, hypoxia, or 3D culture systems [29], which can modulate the types and amounts of secreted factors. Additionally, preconditioning MSCs with specific stimuli [30], such as inflammatory cytokines or hypoxic environments, can enhance the secretion of targeted bioactive molecules, further tailoring the CM composition to specific therapeutic needs.

As one of the main components of $CS^{msc}$, MSC cytokines mainly include interleukins (IL) and growth factors, among which IL-6, IL-8, TIMP, and VEGF are the most abundant components [31,32]. As another main component of $CS^{msc}$, MSC-EVs include exosomes, microvesicles (MVs), and apoptotic bodies, which are nano- and micron-sized heterogeneous vesicles. Both cytokines and extracellular vesicles have the capacity to regulate the local immune response, thereby influencing inflammation and tissue regeneration. This review mainly introduces the role of $CS^{msc}$, summarises the possible mechanisms, and highlights the problems to be solved, providing a reference for the application of $CS^{msc}$ in periodontal regeneration.

## 2. $CS^{msc}$ in Periodontal Regeneration: Roles of MSC Cytokines

### *2.1. The Components of MSC Cytokines*

Initially, due to the limitations of detection technology, only a small number of targeted biomarkers have been detected through low-throughput detection methods (mainly ELISA). Subsequently, cytokine protein array is used to analyse cytokine expression in human cord blood-derived mesenchymal stem cells (CB-MSCs). Several cytokines, interleukins (IL), and growth factors are secreted by CB-MSCs, among which IL-6, IL-8, TIMP-1, and TIMP-2 are the most abundant components. Later, a cytokine secretion profile of human bone marrow (BM)-derived MSCs is reported using antibody arrays, identifying 120 cytokines and chemokines, which features a predominant hybridisation signal for IL-6 and moderately elevated signals for IL-8, TIMP-2, MCP-1, VEGF, and OPG [32]. With the development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology, more MSC secretions have been found. It was reported that 258 proteins specifically expressed by murine MSCs

were isolated, and 54 of which were classified as secreted proteins [31]. A more systematic integrated approach to human MSC secretome analysis was used by Sze et al., which included LC-MS/MS detection, antibody arrays, microarrays, bioinformatics and 201 unique proteins were identified [33]. To date, only a few studies have analysed the specific soluble factors secreted by PDLSCs through antibody arrays and reported that various proangiogenic factors (such as tissue inhibitor of metalloproteinase 1, urokinase-type plasminogen activator, and VEGF) and growth factors (such as insulin-like growth factor binding protein 6 (IGFBP6), IGFBP2, and platelet-derived growth factor receptor) were detected in PDLSC-CM [22]. Moreover, Suh. et al. combined proteome and transcriptome analysis, where not only 187 significant proteins were identified in PDLSC-CM, but also a panel of differentially expressed genes were revealed [34]. PDLSC may be the best donor cells for periodontal regeneration [5,21]; therefore, a more comprehensive component analysis of PDLSC cytokines is necessary in the future.

### *2.2. Efficacy of MSC Cytokines for Periodontal Regeneration*

**Characteristics of periodontal regeneration microenvironment.** Periodontitis is a chronic inflammatory disease. Although controlling periodontal inflammation and removing inflammatory granulation tissue and dental plaque, which are unfavourable for periodontal regeneration during periodontal surgery, are essential in periodontal regenerative treatment, it is still a challenge to modulate inflammatory cells in periodontal tissues during periodontal regenerative surgery. The periodontal repair process is not only the healing process after surgical trauma; the process is also influenced by the chronic inflammatory microenvironment. Correspondingly, MSCs secrete many soluble factors in response to both local injury and infection, which helps build a favourable microenvironment for periodontal regeneration.

**A single MSC cytokine is not enough for periodontal regeneration.** Although both single and combined use of MSC cytokines have been shown to promote periodontal regeneration [35,36], the latter may be better at meeting the needs of periodontal regeneration. Tissue healing is a highly coordinated, dynamic process that requires different cytokines at different stages. Moreover, periodontal tissue includes a variety of tissue components (soft tissues, including gingiva and periodontal ligament, as well as hard tissues, including alveolar bone and cementum). The tissue characteristics and healing speed are different, and periodontal regeneration requires these tissues to be arranged in order to restore the function of periodontal tissue. Therefore, the use of a single cytokine to promote periodontal regeneration is inadequate. For example, BMP-2, a powerful cytokine for bone regeneration, was under the spotlight a while ago. Research on BMP-2 has achieved very good results in animal studies and has even been promoted for clinical applications in periodontal therapy. Unfortunately, researchers have later found that the application of BMP-2 can effectively promote bone tissue regeneration but usually leads to problems such as ankylosis [37] and increased cancer risk [38]. Therefore, a single MSC cytokine is insufficient for periodontal regeneration.

Multi-cytokine combination mimicking the effective components of MSC cytokines may represent a new promising method for periodontal regeneration. As the process of tissue repair involves the participation of a variety of cytokines and better tissue regeneration may depend more on the joint action of a variety of cytokine networks than a single cytokine, we assume that the combined application of multiple cytokines may also be better for periodontal regeneration. A study has shown that a variety of cytokine combinations simulating $CS^{msc}$ can achieve good periodontal regeneration [36]. Due to the biosafety and better regeneration-promoting effects of MSC cytokines, the multi-cytokine combination mimicking the effective components of MSC cytokines may represent a new promising method for periodontal regeneration.

### *2.3. Potential of MSC Cytokines for Periodontal Therapy*

Although MSC cytokines can promote periodontal regeneration, in terms of source, these factors are not extracted from MSC-CM in studies using these factors to promote periodontal regeneration, owing to their very low concentration in MSC-CM. If MSC cytokines with specific components are extracted from MSC-CM, it means that a large number of MSCs need to be cultured; this not only bring in technical difficulties but also results in high cost. Therefore, on the basis of effective MSC cytokine analysis, using a cytokine cocktail to mimick the effective components and proportion of MSC cytokines may be an effective way to solve this problem. To achieve effective bionics, we need to fully understand the composition of the cytokines secreted by MSCs, especially those secreted by PDLSCs.

In addition, similar to the disadvantages of cytokines therapy, MSC cytokines and their biomimetic mixture are degraded rapidly *in vivo*. Therefore, after determining the effective components, it is also necessary to develop appropriate carriers so that these effective components can be released into the tissue at an appropriate dose at an

appropriate healing stage. An in-depth study of these issues will not only develop new periodontal regenerative methods, but also provide valuable references for the regeneration of other tissues. Third, the formation of blood clots is the first step in natural healing. Many studies have suggested the importance of platelets in tissue healing. In view of the wide application of platelet products (such as PRF, FGF, and CGF) in periodontal clinics, the combined application of MSC cytokines and platelet products may also be a promising new method for periodontal regeneration.

## 3. CS$^{msc}$ in Periodontal Regeneration: Roles of MSC-EVs

### *3.1. Some Basic Knowledge about MSC-EVs*

**A brief introduction of MSC-EVs.** Currently, EVs can generally be classified into two major categories: ectosomes and exosomes [39]. Ectosomes encompass a range of vesicles, including microvesicles(MVs), microparticles, and larger vesicles, with diameters spanning from approximately 50 nm~1 μm. In contrast, exosomes have a size range of about 40~160 nm (average 100 nm) [39,40]. Due to the diverse range of characteristics exhibited by EVs in terms of their biogenesis, size, content, and structure, as well as technological limitations and various complexities, we are still hindered from effectively separating EVs into distinct subsets that accurately reflect their unique attributes [41,42]. Some studies do not separate them on purpose and consider them as EVs, as recommended by the International Society for Extracellular Vesicles [40,43]. Figure 2 shows BMMSC and its EVs.

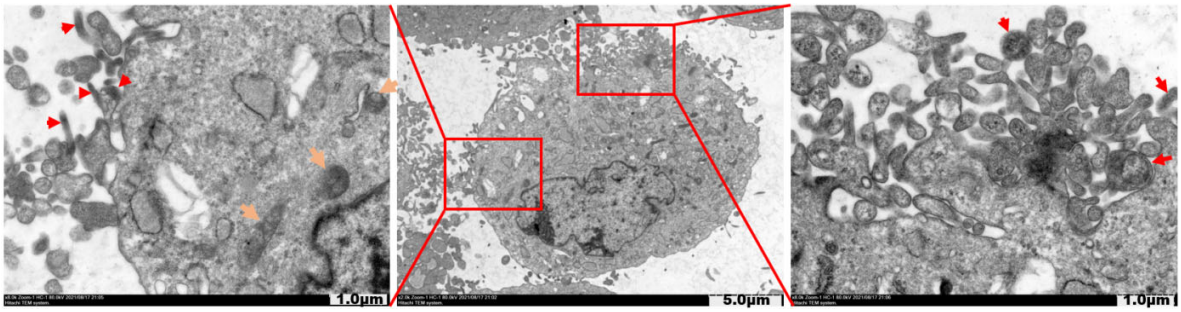


**Figure 2. MSC and its extracellular vesicles (TEM).** Extracellular vesicles of MSCs are composed of a series of vesicles with different sizes wrapped by a membrane structure. The vesicles contain materials with different electron density. It should be noted that the electron density of some components in extracellular vesicles (red arrow) is similar to that of intracellular mitochondrial vesicles (orange arrow).

**Biogenesis and release of EVs.** A long-held assumption is that EVs form mainly at two subcellular sites: ectosomes emerge through budding at the plasma membrane (PM), while exosomes are generated via the endocytic pathway, where the budding of late endosomes leads to the creation of intraluminal vesicles within multivesicular bodies (MVBs) [44,45]. Genetic or epigenetic manipulation has implicated several families of proteins involved in EV biogenesis and release, including Rab GTPases, ARRDC1, and ESCRT complexes [46,47]. In addition to membrane proteins, lipids are also believed to influence EV formation [46]. The packaging of certain RNAs into EV subsets may occur. The larger the EV, the more likely it is to incorporate a cytoplasmic entity [48,49]. This concept is supported by some data, such as the finding that large EVs and their parent cells have highly correlated RNA expression profiles, while the RNA expression of small EVs differs significantly from that of the source cell [46]. These findings suggests that the components of large EVs are more likely to be affected by the state of their parent cells. Although the biogenesis of EVs has not yet been fully elucidated to date, it is assumed that EV biogenesis and secretion are driven by constitutive secretory pathways or that they are secreted as a response to growth conditions or treatments [45,50]. Moreover, the content of vesicles varies with respect to the mode of biogenesis, cell type, and physiological conditions [51], suggesting that EVs can be obtained for specific purposes by changing their parent cells.

**The EV-cell interaction.** As a way of communication between cells, the interaction between EVs (from the parent cell) and recipient cells is the premise of their biological function. EVs might exert effects on cells through contact, uptake, fusion, degradation, or a combination of these modalities[46]. The EV-cell interaction is likely a handshake; factors on the surface of both membranes contribute [46], which means that independently of the change in EVs or the change in recipient cells, it will affect the therapeutic effect of EVs. In addition, it should be noted that although there is nonspecific EV uptake, specific EV uptake certainly exists. For example, endogenous

exosomes present in the circulation and interstitial fluid of zebrafish are taken up only by endothelial cells and macrophages but not muscle cells (despite being bathed in EVs) [52]. Figure 3 shows the EVs around the surface of the macrophages treated with BMMSC-CM.

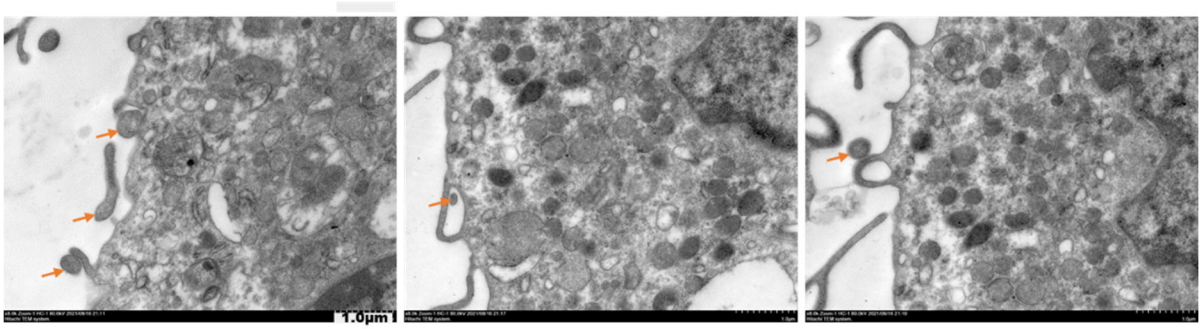


**Figure 3. EVs around the macrophage surface (TEM).** After bone marrow derived macrophages were treated with bone marrow derived mesenchymal stem cells conditioned medium for 24 h, the macrophages were collected for TEM examination. Extracellular vesicles around the cells are marked by orange arrow. Scale bar: 1 μm.

**How EVs exert their functions?** EVs contain many biologically active materials from their parental cells such as RNAs, DNAs, proteins, and lipids, which can be transported to their recipient cells to mediate intercellular communication and signalling [51]. According to the ISEV membrane and EV workshop [46], responders believe that EVs primarily interact with target cells by signalling through proteins displayed on the target cell surface or endosomal lumen. Transferring functional RNA, proteins, and lipids are seen as a secondary effect, even though this effect is widely known (Figure 4). Most researchers believe that EVs are indirectly a form of nutrition or molecular recycling for recipient cells. In recent years, the transfer of mitochondria and their components by EVs has also received attention. Mitochondria or their components (such as mtDNA and mtRNA) are mainly transferred through large EVs, which serve as reservoirs for mitochondrial components and protect them against degradative enzymes [53–55]. Therefore, the material and information from the mitochondria of donor cells are retained, meeting the requirement to reprogram effector cells to accomplish complex adjustments. It has been reported that mtDNA is present within and/or on the surface of EVs [56], and it is worth noting that in some cases, the amount of mtDNA in EVs can be up to twice that of nDNA [55].

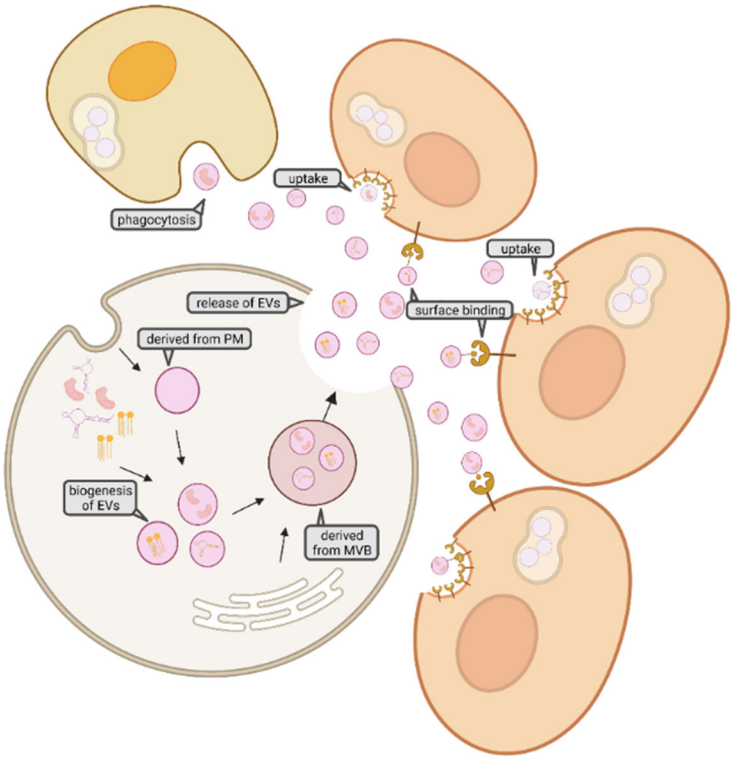


**Figure 4.** Biogenesis, release, and function mode of EVs.

### *3.2. MSC-EVs Are Effective in Periodontal Regeneration*

In addition to MSC cytokines, MSC-EVs are the most important components of $CS^{msc}$. Previous studies have shown that MSC-derived small EVs promoted periodontal regeneration [57]. Moreover, small EVs from both lipopolysaccharide-preconditioned MSCs and 3D-cultured MSCs improved periodontitis status in an inflammatory microenvironment [58–60]. An important restriction factor of periodontal regeneration is the chronic inflammatory microenvironment; therefore, these studies provide valuable support for the application of EVs in periodontal repair. In addition, the components of EVs are favourable for periodontal regeneration. Exosomes from MSCs have been reported to promote periodontal regeneration in rats [61] and, more importantly, improve the clinical outcomes of nonsurgical periodontal treatment in humans [62].

According to previous consensus, the components of MVs are closer to their parent cells than those of exosomes [46], Therefore, we believe that the role of MSC-MVs in periodontal regeneration deserves more attention, despite no direct evidence that MSC-MVs can promote periodontal regeneration. Although there is no direct evidence of the role of apoptotic bodies in periodontal healing, it has been reported that apoptotic bodies are related to tissue healing and can promote M2 polarisation [59,63,64]. This indicates that they may have a positive effect on periodontal repair, because good periodontal healing is closely related to M2 polarisation of macrophages [65].

### *3.3. How to Obtain Modified MSC-EVs for Periodontal Regeneration*

The physical conditions that produce EVs may be a more noteworthy way to regulate EVs components. For example, cardiac regeneration and angiogenesis were significantly enhanced in animals treated with exosomes derived from Akt-modified MSCs compared to those treated with normal MSCs [66]. Additionally, atorvastatin enhanced the therapeutic efficacy of MSC-derived exosomes in acute myocardial infarction [67]. The previously mentioned studies suggested that appropriate pre-treatment of MSCs could change the therapeutic effect of MSC-EVs, which is a promising method for future EV-based therapy. Considering the inflammatory nature of the periodontal regenerative microenvironment, MSC pre-treatment by simulating the inflammatory microenvironment may be an effective way to promote periodontal regeneration. Studies have shown the benefits of lipopolysaccharide-preconditioned MSC-EV-based periodontal regenerative therapy [60].

In addition, the in vivo clearance of unmodified EVs following their administration is rapid. Thus, these engineered surface modifications extend the biodistribution, stability, and pharmacokinetic profiles of EVs, thereby facilitating drug delivery [68]. EVs can be bioengineered through modifications such as the loading of drugs or attachment of molecules to their surface; EV bioengineering includes engineered EVs and EV mimetics [68]. Therefore, using EV bioengineering to make MSC-EVs expressing specific surface markers conducive to periodontal regeneration or using EV mimetics technology to produce artificial EV imitating the active components of PDLSC-EVs may provide a new method for periodontal treatment.

## 4. Possible Mechanisms of $CS^{msc}$ Functions in Periodontal Regeneration

$CS^{msc}$ influences periodontal regeneration through several interconnected mechanisms. The bioactive molecules secreted by mesenchymal stem cells (MSCs) into the conditioned medium play a key role in enhancing cellular behaviors critical to periodontal tissue repair and regeneration. As shown by Figure 5, the primary mechanisms mainly include:

$CS^{msc}$ contains chemoattractants such as SDF-1α activate the CXCR4/SDF-1α signaling axis, attracting endogenous stem cells to the injury site and promoting their involvement in tissue repair. Extracellular vesicles and microRNAs, including miR-126 and miR-210, target key signaling pathways like PI3K/AKT and VEGF signaling, which stimulate angiogenesis, endothelial cell proliferation, and vascularization, further improving the microenvironment [69].

$CS^{msc}$ contributes to extracellular matrix (ECM) remodeling by regulating matrix metalloproteinase activity and the TIMP/MMP balance, ensuring appropriate ECM degradation and rebuilding [70]. It also activates the TGF-β/SMAD pathway to promote collagen synthesis and ECM production, creating a supportive environment for cell adhesion and tissue regeneration.

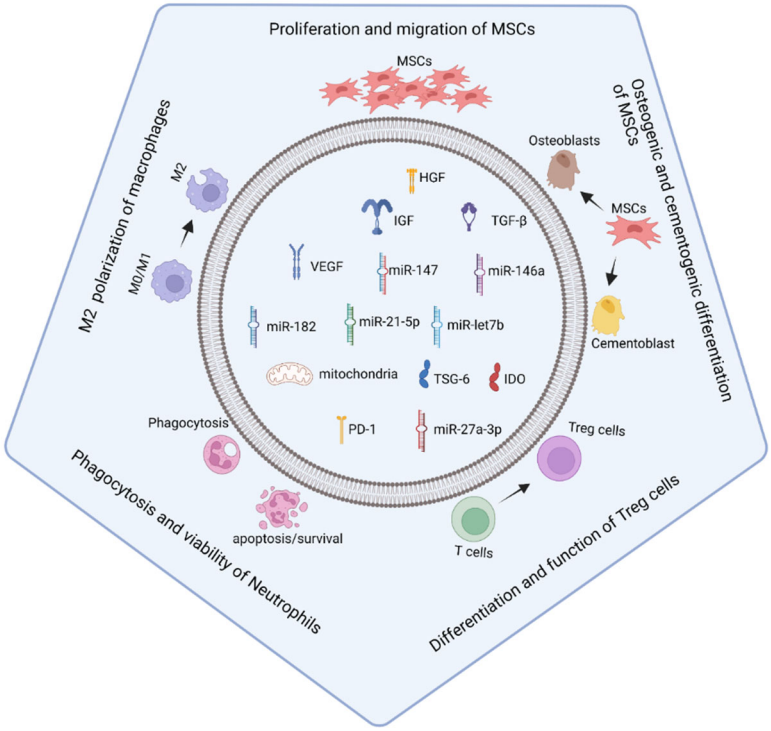


**Figure 5. Possible mechanism of CS$^{msc}$ functions in periodontal regeneration.** Periodontal regeneration is a dynamic process that involves multiple cells that are highly coordinated in time and space. Immune cells and MSCs are the two most important cell types in this process. The former provides a microenvironment for tissue healing, while the latter participates in tissue repair. Therefore, we considered these two cell types as effector cells to discuss the mechanism by which CS$^{msc}$ promotes periodontal regeneration. **Cell Recruitment, Microenvironment Enhancement and Extracellular Matrix Remodeling.**

### (1) Osteogenic Differentiation, Bone Regeneration, Cementum Formation and Promote angiogenesis.

CS$^{msc}$ contains growth factors such as BMP-2 and BMP-7 that activate the SMAD signaling pathway, promoting osteoblast differentiation and mineralization [71]. The Wnt/β-catenin signaling pathway is also activated by factors within CS$^{msc}$, enhancing bone matrix synthesis and regulating osteogenic differentiation [72]. Cementum formation is also supported by CS$^{msc}$. CS$^{msc}$ stimulates cementoblast activation and enhances the deposition of cementum proteins like cementum attachment protein (CAP) [36]. This process is essential for re-establishing the functional attachment of periodontal tissues to the tooth root and is further supported by activation of the integrin/FAK pathway [73], which enhances cellular adhesion and ECM interaction.

CS$^{msc}$ has a significant impact on angiogenesis due to its rich composition of growth factors, cytokines, and extracellular vesicles [28]. Key angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietins, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) within CS$^{msc}$ play pivotal roles in promoting endothelial cell proliferation, migration, and tube formation. VEGF in CS$^{msc}$ binds to its receptor VEGFR-2 on endothelial cells, activating downstream pathways like PI3K/Akt and MAPK/ERK, which enhance endothelial cell survival and stimulate angiogenesis [74]. Additionally, CS$^{msc}$ contains exosomes that carry pro-angiogenic miRNAs, such as miR-126 and miR-210 [75], which further modulate angiogenic signaling by regulating gene expression in recipient endothelial cells. Moreover, HGF in CS$^{msc}$ contributes to vessel remodeling and stabilization by interacting with endothelial and smooth muscle cells, while angiopoietins enhance vascular maturation. Through these combined effects, CS$^{msc}$ creates a pro-angiogenic microenvironment, which is particularly beneficial in tissue regeneration and wound healing.

### (2) Immunomodulatory Effects.

CS$^{msc}$ contains anti-inflammatory factors such as IL-10 and TGF-β, which inhibit pro-inflammatory pathways, including the NF-κB signaling cascade, and suppress oxidative stress. CS$^{msc}$ also modulates the JAK/STAT3 pathway, enhancing the polarization of macrophages from the pro-inflammatory M1 phenotype to the reparative M2 phenotype. This creates a favorable environment for tissue healing and limits immune-mediated tissue

damage [27,76]. Additionally, $CS^{msc}$-derived exosomes carry miRNAs like miR-146a, which further inhibit NF-κB signaling, reducing the secretion of pro-inflammatory cytokines such as TNF-α and IL-6.

The immunomodulatory effects of $CS^{msc}$ further support tissue regeneration by regulating immune cell behaviour, as describes below.

### *4.1. $CS^{msc}$ May Improve the Regenerative Microenvironment by Immune Regulation*

**$CS^{msc}$ may promote periodontal regeneration through M2 polarization.** Our previous studies showed that $CS^{msc}$ promotes the polarisation of M0 macrophages to M2 macrophages [65,77]. Other studies have reported similar results [78]. In addition, both our research and previous studies have shown that M2 polarisation of macrophages is closely related to improved periodontal regeneration [52,57]. The important mechanism by which $CS^{msc}$ promote periodontal regeneration could be promoting M2 polarization of macrophages. It is necessary to explore the components of MSC that may promote M2 polarisation during periodontal healing. The mechanism could be associated with the secretion of the following MSCs: (1) Cytokines: MSC-secreted TGF-β regulates lipopolysaccharide-stimulated macrophage M2-like polarisation via the Akt/FoxO1 pathway [79]. Specifically, TGF-β transduce signals via binding to type I (TGFRI) and typeII (TGFRII) transmembrane heteromeric serine/threonine kinase receptors, which are expressed on monocytes or macrophages. Then, Smads are activated and form a heteromeric complex, inducing specific genes transcription [80]. In addition, VEGF, one of the main cytokines secreted by a variety of MSCs, including PDLSCs [22] and BMMSCs [32], can significantly induce M1 macrophages to shift to an M2 phenotype. By binding to VEGF receptors on macrophage, VEGF promoted immunosuppressive genes expression, reinforcing M2 phenotype. Moreover, the conditioned media from human endometrial stromal cells induces changes in macrophage polarisation similar to that induced by VEGF treatment [81]. (2) EVs: Several studies have indicated that MSC-derived EVs alter disease outcomes via support of macrophage polarisation [78]. The mechanism of EV-induced-M2 polarization of macrophages may be as follows: (a) miRNAs in EVs, such as miR-147 [82], miR-146a [83], miR-182 [84], miR-21-5p [85], miR-let7 [86], and miR-27a-3p [87] may promote M2 polarisation of macrophages; (b) mitochondria in EVs also contribute to EV-induced M2 polarisation of macrophages [88]; and (c) other molecules such as TSG-6 [89,90] could also induce macrophage M2 polarisation. Particularly, the surface of EVs expresses adhesion molecules, phosphatidylserine, milk fat globule membrane proteins, and other molecules, which interact specifically with molecules on the surface of recipient cells [91]. It can directly fuse with the plasma membrane of the receptor cell and release its contents into the cytoplasm of the receptor cell. Alternatively, endocytosed vesicles can fuse with the membrane of endosomes, leading to the release of their contents into the cytoplasm.

Among the above mechanisms by which $CS^{msc}$ regulates macrophage polarisation, mitochondrial transfer has attracted increasing attention in recent years [65,88,92]. Jackson et al. found that MSCs can promote macrophage phagocytosis through mitochondrial transfer, suggesting that mitochondrial transfer may be an important mechanism by which MSCs regulate macrophage function, consistent with the results of Morrison et al. [88,92]. A study reported that mitochondrial transfer from MSCs to macrophages restricts inflammation and alleviates kidney injury in mice with diabetic nephropathy [93]. Interestingly, macrophages treated with $CS^{msc}$ achieve a higher number of mitochondria transfer than MSCs [92], indicating that mitochondria in $CS^{msc}$ may be more easily taken up by macrophages, which supports the potential application of $CS^{msc}$ in periodontal regeneration other than MSC.

**Effects of $CS^{msc}$ on neutrophils.** Neutrophils are immune cells that appear during the early stages of periodontal healing. Wang et al. summarized the roles of neutrophils in tissue repair as three possible strategies: (1) neutrophils can clear necrotic cellular debris, (2) neutrophils release effectors that promote angiogenesis and regeneration, and (3) phagocytosis of apoptotic neutrophils results in the release of anti-inflammatory and reparative cytokines [94]. Other studies have reported that macrophage function in tissue repair and remodelling requires IL-4 or IL-13 along with apoptotic neutrophils [95]. Based on the above-mentioned biological role of neutrophils in tissue regeneration, we speculate that phagocytosis, survival, and apoptosis of neutrophils are critical biological features during tissue healing.

Previous studies have shown that MSC exosomes and MSC-CM can enhance the phagocytic capacity of neutrophils [96–98], thus promoting the clearance of necrotic cellular debris. MSC exosomes and $CS^{msc}$ can also regulate the viability of neutrophils. However, studies have shown inconsistent results regarding the impact of $CS^{msc}$ on neutrophil viability. Some studies have reported that $CS^{msc}$ can induce neutrophil apoptosis. For instance, Vincent et al. reported that $CS^{msc}$ attenuated LPS-induced acute lung injury by inducing neutrophil apoptosis [99]. Other studies have reported that MSC-exosomes can improve neutrophil viability [96,100]. We speculate that the effects of $CS^{msc}$ on neutrophil viability may depend on the stage of inflammation and tissue repair.

Mohammad et al. [98] compared the effects of MSC exosomes with MSC-CM on neutrophil function and apoptosis. They concluded that MSC exosomes improved the viability more than phagocytosis of neutrophils, whereas MSC-CM increased phagocytosis more than the survival of neutrophils. Although there is a lack of clarity on how MSC secretion affects neutrophils, these results illustrate the different functions of MSC-CM and MSC-exosomes and further suggest the necessity of comprehensively analysing the components of MSC-CM and clarifying the functions of each component.

**Effects of $CS^{msc}$ on T cells.** Regulatory T (Treg) cells are also important for tissue regeneration [101,102]. Both MSC cytokines and MSC EVs can modulate T cells. For example, not only MSC-secreted IDO induces Tregs [103] and inhibits allogeneic T cell responses through regulating Th1 and Th2 activities [104], but also MSCs secreted PD-1 ligands also exert immunosuppressive effects directly on T-cells by suppressing the activation of CD4+ T cells [105]. Upon PD-1 ligands binding to PD-1, HP-2 within the PD-1 complex were activated, leading TCR proximal signaling molecules dephosphorylation and immune activation is impaired [106]. Additionally, MSC-derived exosomes suppress the immune response by enhancing Treg function [107]. It has also been reported that mitochondrial transfer from MSCs to T cells induces Treg differentiation and restricts inflammation [108], and the alleviation of the inflammatory response may be conducive to periodontal regeneration.

**Macrophages may be the primary effector cells in the immunomodulatory function of $CS^{msc}$.** Macrophages may serve as more crucial vital effector cells in the immunomodulatory function of $CS^{msc}$. The study conducted by Németh et al. showed that macrophages may be the primary effector cells for MSC-based treatment of sepsis induced by caecal ligation and puncture (CLP) [109]. To test this hypothesis, they examined the effects of BMSCs in mice that genetically lack mature T and B cells (*Rag2*$^{-/-}$) or are depleted of natural killer (NK) cells with an antibody. The effect of BMSC injections on the survival of the mice was present in both these models, suggesting that lymphocyte populations of T, B, and NK cells do not mediate the effect of BMSCs in the CLP model [109]. However, BMSCs were no longer effective in mice lacking monocytes or macrophages [109]. In addition, when $PKH26^{pos}$ MSCs, either resting or inflammatory-primed, were co-cultured with unfractionated peripheral blood mononuclear cells, EVs were mostly internalised by monocytes and scarcely by lymphocytes after 24 h up to day 4. In fact, at the end of co-culture, the percentage of $PKH26^{pos}$ monocytes was 75.11 ± 3.24 in the presence of resting $PKH26^{pos}$ MSCs and 61.27 ± 8.11 in the presence of inflammatory-primed $PKH26^{pos}$ MSCs. Among lymphocyte subsets, $CD19^{pos}$ B cells displayed the highest EV uptake (6.86 ± 10.26%) compared to $CD56^{pos}$ NK cells (1.35 ± 0.46%) and $CD3^{pos}$ T cells (0.702 ± 0.30%) in the presence of resting MSCs. Although EV do not necessarily enter the cells [46], the above studies suggest that macrophages may be the main target cells of MSC-EVs.

### *4.2. $CS^{msc}$ May Regulate the Function of Tissue MSCs*

**$CS^{msc}$ may promote the proliferation and migration of MSCs in periodontal tissue.** Previous studies have shown that the therapeutic effect of MSCs in tissue regeneration is mainly associated with the secretion of cytokines and growth factors, which are critical for tissue regeneration [110–112]. Multiple growth factors have been found in $CS^{msc}$, such as VEGF, IGF, HGF, and TGF-β, and growth factors contained in $CS^{msc}$ promoted the migration and proliferation of PDLSCs [113]. In addition, these growth factors also enhanced tube formation by vascular endothelial cells and accelerated angiogenesis [113,114]. This may help address the prevalent issues in periodontal regeneration: insufficient functional cells and shortage of blood supply. Therefore, $CS^{msc}$ may promote periodontal regeneration by enhancing cell proliferation and accelerating angiogenesis in periodontal tissue.

**$CS^{msc}$ may induce stem cells to differentiate into bone-forming and cementum-forming cells.** Multiple growth factors have been found in $CS^{msc}$, such as VEGF, IGF, HGF, and TGF-β. These growth factors have been shown to promote osteogenic differentiation of MSCs [115]. It has been reported that $CS^{msc}$ triggers osteogenesis in PDLSCs, suggesting important implications for periodontal engineering [116]. On the other hand, apical tooth germ-conditioned medium could provide a cementogenic microenvironment and promote the differentiation of both GMSCs and PDLSCs along the cementoblastic lineage [117,118]. MSCs, particularly GMSCs, are difficult to transform into cementoblasts under normal culture conditions in vitro. The above results suggest that bone marrow-derived $CS^{msc}$ can provide a microenvironment for stem cells to differentiate into osteoblasts, whereas dental papilla-derived $CS^{msc}$ can provide a microenvironment for stem cells to differentiate into cementoblasts. The microenvironment is critical during periodontal regeneration; therefore, one of the mechanisms of $CS^{msc}$ promoting periodontal regeneration may be the enhancement of the osteogenic or cementogenic ability of cells in periodontal tissue.

## 5. CS$^{msc}$ for Periodontal Regeneration: Summary and Perspectives

### *5.1. MSC-Secreted Soluble Factors versus MSC-EVs for Periodontal Regeneration*

As mentioned earlier, the concentration of cytokines in CS$^{msc}$ is very low. At present, there are no reports on the extraction of cytokines from CS$^{msc}$ for periodontal regeneration. However, there have been many studies on EVs derived from MSCs to promote periodontal regeneration. In this regard, EVs seems to be a more promising component of CS$^{msc}$ for promoting periodontal regeneration. However, it is worth noting that EVs have recently been recognized as an alternative secretory mechanism. Interestingly, cytokines/chemokines exploit these vesicles to be released into the extracellular milieu and appear to modulate their release, trafficking, and/or content [119]. Therefore, there is still much work to be done to draw definitive conclusions. In summary, CS$^{msc}$ contains a large number of proteins, peptides, hormones, and other secretions, as well as a series of EVs of different sizes ranging from tens to thousands of nanometres, as summarised in Figure 6. Additional research should be dedicated to determining whether EVs or cytokines primarily mediate the effects of CS$^{msc}$, and exploring whether there is a synergistic interaction between these two entities.

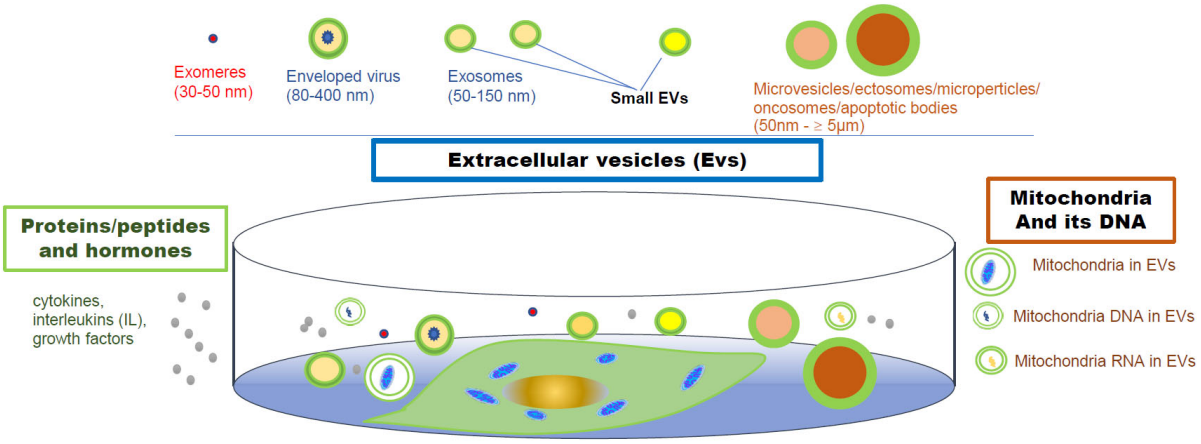


**Figure 6. Schematic diagram of CS$^{msc}$.** CS$^{msc}$ contains mainly soluble secretion (green rectangle) and EVs (blue rectangle). In recent years, the role of mitochondrial and their related components (orange rectangle) in CS$^{msc}$ has also attracted extensive attention.

### *5.2. Perspectives*

**Finding the best donor cells for periodontal repair and exploring the reasons.** MSCs from different tissues exhibit different cytokine induction and signal transduction properties. The secretome has been found to be similar among BMMSCs derived from three different donors, and similar but not identical to that of umbilical cord blood (CB)-derived cells, suggesting that the trophic nature of MSCs might depend on the cell origin, but not on the donors [32]. Hwang et al. [120] compared cytokine gene expression in MSCs from the human placenta, CB, and BM and found that most types of MSCs had a similar expression pattern. However, MSCs from the BM, placenta (amnion and decidua), and CB express cytokines differently. A meta-analysis summarising several in vivo studies showed that PDLSCs may be the best donor cells for periodontal tissue repair compared to MSCs derived from other tissues [5]. Unfortunately, there is a lack of comprehensive comparisons between the secretomes of PDLSCs and MSCs from other tissues. By analysing the secretory characteristics of periodontal ligament-derived MSCs, it is possible to determine why periodontal ligament-derived MSCs have a stronger ability to promote periodontal repair than other sources of MSCs, which may also find new target molecules for periodontal regeneration.

**Finding the methods for sufficient harvest, accurate separation, and functional analysis of different EVs.** EV isolation is of primary importance for obtaining EVs intended for therapeutic purposes. Currently, a method that can simultaneously incorporate multiple factors such as purity, yield, specificity, quality, and cost is not available. The development of three-dimensional cultures will provide important technical support for obtaining a large number of EVs.

Another technical limitation of EV research is the markers. At present, specific markers of different EVs have not been identified, and current technology cannot effectively separate different EVs. Therefore, it is difficult to determine their function. By developing more selective isolation techniques, it should be possible to distinguish between the different subpopulations of vesicles and accurately define their biogenesis, cargo, and function, which

is useful not only for understanding the 'language' of EVs, but also for the development of novel therapeutic strategies [121].

In addition, it is important to identify the key signalling pathways and molecules that control EVs secretion. For example, it has been reported that the PI3K/Akt signalling pathway can increase the formation of EVs [85]; therefore, activating PI3K/Akt and its downstream Akt and mTOR may help MSCs form more EVs, thus promoting tissue regeneration. Many research results support this inference. For example, human amniotic MSCs and $CS^{msc}$ can promote the healing of skin trauma, and their mechanism depends on the activation of the PI3K/Akt signalling pathway [122]; activation of PI3K/Akt signalling pathway is also conducive to the repair of cardiac tissue [66] and bone tissue [123].

**Steps towards a comprehensive analysis of components in conditioned medium of MSCs, particularly odontogenic MSCs.** As mentioned above, the components of $CS^{msc}$ from different cell sources are different. As PDLSCs are the best stem cells for periodontal regeneration, it is necessary to comprehensively analyse their secretory components. Once we understand the effective cytokine components in $CS^{msc}$, the components of artificial $CS^{msc}$ will provide the possibility for the development of more effective, stable, and low-cost treatments, as shown by Sakaguchi [36].

**Exploring the effects of $CS^{msc}$ on the effector cells during periodontal healing.** Different cells have different ways of ingesting EVs, such as clathrin-dependent endocytosis or phagocytosis in neurones, macropinocytosis by microglia, phagocytosis or receptor-mediated endocytosis by dendritic cells, caveolin-mediated endocytosis in epithelial cells, and cholesterol and lipid raft-dependent endocytosis in tumour cells [121]. On the other hand, a single cell can use more than one uptake mechanism for different EVs [124]. Further research is required to understand which cells mainly receive EVs, and what happens when specific cells receive these EVs. Regarding periodontal regeneration, it is necessary to explore the effect of exogenous administration of $CS^{msc}$ on periodontal tissue cells, which directly repair the tissue and immune cells, especially macrophages, which are very important for periodontal regeneration [51,65,125].

**Attaining pre-treatment MSCs or gene-modified MSCs for periodontal regeneration.** Mounting evidence shows the therapeutic potential of MSC pre-treatment or gene-modified [126,127], and it has been reported that lipopolysaccharide (LPS) pre-treatment can enhance the therapeutic effect of dental follicle stem cell-derived small extracellular vesicles for periodontitis [59]; therefore, MSC pre-treatment or gene modification may represent an important direction of $CS^{msc}$ related research in the future. Considering the inflammatory nature of the periodontal regeneration microenvironment, achieving a stronger ability for concurrent immune regulation and stem cell recruitment in the $CS^{msc}$ and promoting the osteogenic and cementoblastic differentiation ability of stem cells in periodontal tissue may be directions worthwhile.

**Following interdisciplinary research.** The developments in other disciplines will also help in the future application of $CS^{msc}$. For example, it has been reported that cysteine–arginine–glutamic acid–lysine–alanine functionalized small EVs, which can target fibrin to accumulate and retain in bone defects, can enhance bone repair [128]. There are also many reviews summarizing the potential regenerative roles of engineered EVs [129–131], which further support the notion that engineered EVs and EV mimetics may offer a novel approach for periodontal repair. The author believes that exploring EVs containing mitochondria with diverse functions could be an intriguing avenue of research, particularly given a recent article that provides a functional classification of mitochondria, which further fuels our optimism [132].

## 6. Conclusions

$CS^{msc}$ is a promising cell-based product for periodontal regenerative therapy. Both soluble molecules and active ingredients in EVs promote periodontal regeneration. Future research should focus on obtaining sufficient, component-specific, effective, stable, and safe $CS^{msc}$ to promote periodontal regeneration. This not only requires further analysis of the effective components of $CS^{msc}$ and clarification of their function, but also an in-depth understanding of how these active ingredients function to improve $CS^{msc}$-based periodontal regeneration. This could even lead to the development of new approaches that are designed from a therapeutic concept originating from $CS^{msc}$ but are not limited to MSCs. In addition, finding pre-treatment or gene-modified MSCs that generate $CS^{msc}$ with the desired function, exploring feasible carriers for $CS^{msc}$, modifying the engineered EVs, and producing biomimetic components of $CS^{msc}$ could be important research directions in the future. Additionally, investigating its role in immune modulation, particularly the polarization of macrophages toward a regenerative phenotype, could provide insights into its anti-inflammatory properties. Long-term animal studies are needed to assess the durability of regenerated tissues, while advanced models like organ-on-a-chip could offer more accurate simulations of human periodontal conditions. Combining $CS^{msc}$ with existing therapies, personalizing treatments

for patients with comorbidities, and decoding the functionality of exosomes will further refine its clinical potential and pave the way for innovative strategies in periodontal regeneration. We firmly believe that $CS^{msc}$ will bring revolutionary changes to periodontal regeneration as well as other wound healing.

While $CS^{msc}$ holds significant promise for therapeutic applications, several potential drawbacks must be addressed to ensure its efficacy and safety. The variability in its composition, influenced by the source of MSCs and culture conditions, can lead to inconsistent therapeutic outcomes. Additionally, the short half-life of bioactive molecules in $CS^{msc}$ may reduce its effectiveness, necessitating repeated administrations or advanced delivery systems. Unlike live MSCs, $CS^{msc}$ cannot dynamically adapt to changing microenvironments, potentially limiting its regenerative potential. Moreover, its lack of target specificity could result in off-target effects, such as fibrosis or abnormal tissue growth, and there is a risk of immunogenic responses, particularly with allogeneic sources. Production scalability and costs pose further challenges, along with regulatory hurdles due to the complex nature of $CS^{msc}$. Safety concerns also exist regarding its potential to promote tumorigenesis through factors like VEGF. Addressing these challenges through standardized production, improved delivery systems, and rigorous safety evaluations will be essential to optimize $CS^{msc}$ for clinical use.

## Author Contributions

B.C. and L.L. contributed to the conception and drafted the manuscript. X.Z. and Q.L. contributed to critically revising the manuscript. F.Y. and Y.X. contributed to the conception and critical revision of the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work. All authors have read and agreed to the published version of the manuscript.

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## Institutional Review Board Statement

Not applicable.

## Informed Consent Statement

Not applicable.

## Data Availability Statement

Not applicable.

## Conflicts of Interest

The authors declare no conflict of interest.

## List of Abbreviations

| | |
|---|---|
| BM | Bone marrow |
| BMMSCs | Bone marrow-derived mesenchymal stem cells |
| CB | Cord blood |
| CBMSC | Cord blood-derived mesenchymal stem cell |
| EV | Extracellular vesicle |
| GMSC | Gingival-derived mesenchymal stem cell |
| IL | Interleukins |
| ILV | Intraluminal vesicles |
| LC-MS/MS | liquid chromatography tandem mass spectrometry |
| MSC | Mesenchymal stem cell |
| $CS^{msc}$ | Cell secretome from mesenchymal stem cells |
| MSC-CM | Mesenchymal stem cell conditioned medium |
| MV | Microvesicles |
| PDLSCs | Periodontal ligament stem cells |

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