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Concise Review

Preclinical Evidence for the Use of Oral Mesenchymal Stem Cell-Derived Extracellular Vesicles in Bone Regenerative Therapy: A Systematic Review

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Abstract

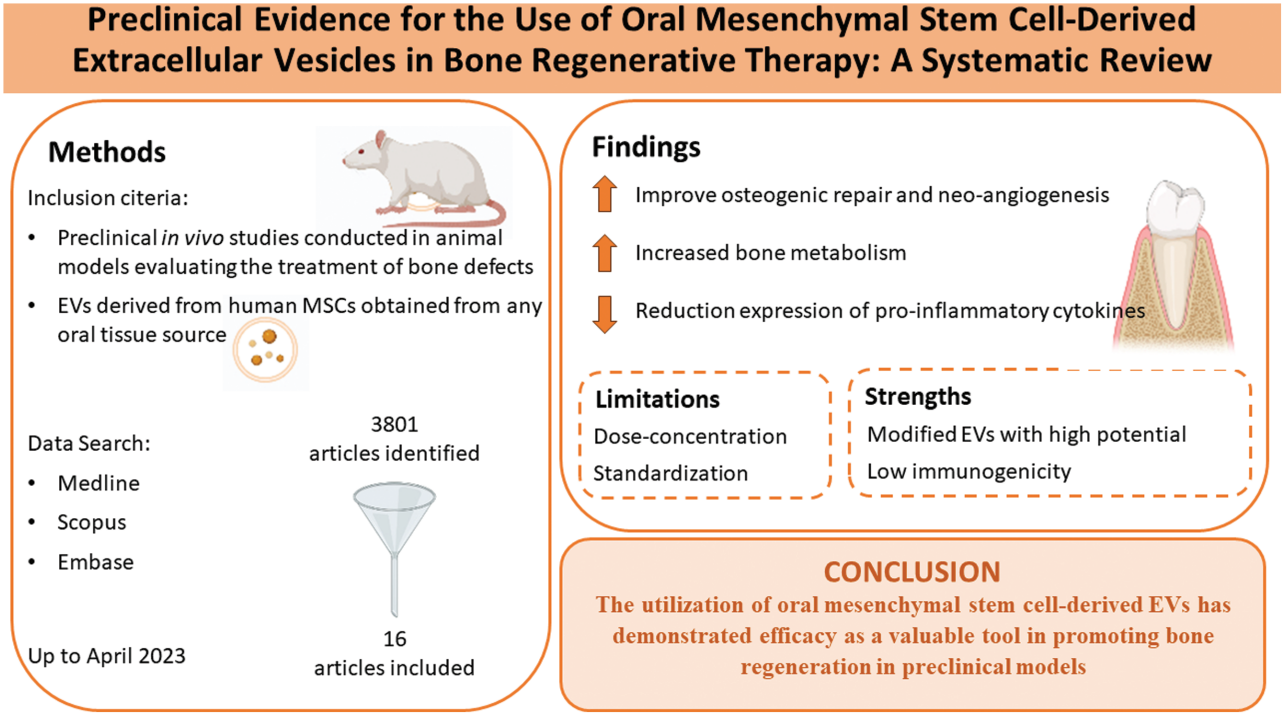
The development of extracellular vesicles (EVs) therapies has revolutionized personalized medicine, opening up new possibilities for treatment. EVs have emerged as a promising therapeutic tool within this field due to their crucial role in intercellular communication across various cell types and organisms. This systematic review aims to evaluate the therapeutic potential of oral mesenchymal stem cell (MSC)-derived EVs for bone regeneration, specifically focusing on findings from preclinical models. Sixteen articles meeting the inclusion criteria were selected following document analysis. The biological effects of oral MSC-derived EVs predominantly involve the upregulation of proteins associated with angiogenesis, and inflammation resolution, alongside the downregulation of proinflammatory cytokines. Moreover, these therapeutic agents have been found to contain a significant quantity of different molecules (proteins, lipids, DNA, microRNAs, etc) further contributing to their modulatory potential. The findings from this systematic review underscore that oral MSC-derived EVs, irrespective of their specific population, have the ability to enhance the osteogenic repair response in maxillary bone or periodontal defects. In summary, this systematic review highlights the promising potential of oral MSC-derived EVs for bone regeneration based on evidence from preclinical models. The comprehensive assessment of their biological effects and the presence of microRNAs underscores their therapeutic significance. These findings support the utilization of oral MSC-derived EVs in enhancing the osteogenic repair response in various maxillary bone or periodontal defects, providing insights into the mechanisms involved and potential therapeutic applications in the field of personalized medicine.

Key words: cellular therapy; extracellular vesicles; oral mesenchymal stem cells; bone regeneration; preclinical models; angiogenesis; osteogenesis; proinflammatory cytokines.

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Graphical Abstract



Significance Statement

This systematic review highlights the therapeutic potential of oral mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) for bone regeneration. The review demonstrates that these EVs exhibit biological effects associated with angiogenesis promotion, inflammation resolution, and modulation of proinflammatory cytokines. Furthermore, the presence of microRNAs in these EVs enhances their modulatory potential. The findings emphasize that oral MSC-derived EVs have the ability to enhance osteogenic repair response in maxillary bone or periodontal defects. This supports their utilization in personalized medicine for various bone regeneration applications.

Introduction

Reconstructing large bone defects poses a significant challenge in the field of regenerative medicine.[1] Current approaches involve surgical procedures using various biomaterials, such as grafts and scaffolds, with standardized or customized shapes. While these biomaterials provide osteoconduction, there is a growing emphasis on enhancing the osteogenic properties of grafts through individualized medicine. Tissue engineering strategies utilizing mesenchymal stem cells (MSCs) have emerged as promising therapies, garnering considerable attention due to their regenerative potential and associated benefits.[2-4]

In the field of dentistry, the utilization of oral MSCs has garnered significant interest due to their availability from various sources. The oral cavity, including teeth and supporting tissues, harbors abundant populations of MSCs that possess remarkable self-renewal capacity and the ability to differentiate into multiple mesenchymal lineages, including myofibroblasts, osteoblasts, adipocytes, and chondroblasts.[5,6] Importantly, oral MSCs are relatively accessible[7] and offer advantages in terms of reduced ethical concerns associated with their acquisition and use.[8]

To date, researchers have successfully isolated and characterized 5 distinct types of human dental stem/progenitor cells. These include human exfoliated deciduous teeth (SHEDs), gingival MSCs (GMSCs), dental apical papilla (SCAPs), dental follicle stem cells (DFSCs), tooth germ stem cells (TGSCs), dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs).[5,6,9,10] Furthermore, oral MSCs have also been successfully extracted from other sources, such as the maxillary alveolar bone and grafted bone areas, during socket preservation or maxillary sinus floor augmentation procedures.[11,12]

Recent studies have highlighted the crucial role of stem cells and their secretomes in tissue repair. In addition to their ability to expand and differentiate to directly replace damaged tissues, stem cells have been found to release a secretome enriched with bioactive molecules.[13] These secretomes consist of a diverse range of paracrine factors, including extracellular vesicles (EVs), and other biologically active molecules.[14] EVs are well-defined nano-sized secreted vesicles that includes exosomes, microvesicles, and apoptotic bodies and are originated from various cell types, including oral MSCs.[15] Especially EVs derived from oral tissue MSCs show more advantages than EVs from other MSCs sources in tissue repair and regeneration. This is due to their lower invasiveness and easier accessibility for sample collection.[9]

The EVs encapsulate a variety of molecules, such as mRNA, miRNA, and proteins, which play critical roles in intercellular communication, antigen presentation, and RNA transfer.[16] Recent research highlights that EVs released by cells act as signaling factors, interacting with recipient cells by migrating

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to distant sites, and reaching the target cells. Communication between EVs and cells can occur in various ways, such as by fusing with the recipient cell's membrane, being taken up by the cell through endocytosis or binding to the cell's surface through specific ligand-receptor interactions without releasing their internal contents.[9] Because EVs can naturally transport materials between cells, they have been extensively investigated as possible carriers for drug delivery. Moreover, aside from their capacity to transport external therapeutic substances, specific EVs also have inherent therapeutic properties, particularly beneficial in the field of regenerative medicine.[17]

Emerging evidence suggests that EVs derived from MSCs possess therapeutic potential comparable to that of the MSCs themselves, particularly in the context of bone regeneration and healing.[18,19] This highlights the significant role of EVs as potent mediators of regenerative processes. The therapeutic potential of EVs derived from oral MSCs has garnered considerable attention in the field of regenerative medicine. These EVs have the ability to induce and support endogenous regeneration while also modulating the immune response, making them an attractive option for cell-free regenerative therapies.[20] In fact, it has been proposed that EVs derived from oral MSCs may play a crucial role in osteogenic differentiation and periodontal regeneration,[21,22] by regulating bone formation, promoting osteoclast formation, and regulating bone resorption.[15] This offers a promising avenue for the development of cell-free therapies for the treatment of periodontitis and other osteolytic diseases.[23]

However, it is important to note that the use of these therapeutic tools in humans has yet to be extensively documented. Therefore, the objective of this systematic review is to provide a comprehensive analysis of the current preclinical animal model data on the use of EVs derived from oral MSCs in bone regenerative therapy.

Methods

Protocol and Registration

This systematic review (SR) follows the guidelines outlined by the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE), as described by de Vries et al.[24]

The protocol for this SR has been registered in the International Prospective Register of Systematic Reviews (PROSPERO) under the registration number CRD 42022316045.

PICO Question

1. Population: Animal models with bone defects.
2. Intervention: Administration of EVs derived from human MSCs (hMSCs) sourced from any oral tissue for the treatment of various types of bone defects.
3. Comparison: Animal models with bone defects that received different modalities of regeneration treatment.
4. Outcomes: Comparison of the following outcomes between different modalities of regeneration treatment in animal models with bone defects:

   - Primary: Histomorphometric and histological assessment of bone regeneration.
   - Secondary: Evaluation of bone protein expression.

Focused Questions

1. Do oral MSC-derived EVs induce a bone regenerative effect in animal models?
2. Is there a difference in the bone regenerative potential between EVs derived from MSCs sourced from different oral tissues?

Eligibility Criteria

Only studies that met all the following criteria were included in this study:

1. Preclinical and in vivo studies conducted in animal models of both sexes, all ages, and species specifically evaluating the treatment of bone defects using EVs derived from oral MSCs.
2. Utilization of preclinical animal models, including calvaria defects, fractures, distraction osteogenesis, osteonecrosis, osteoporosis, and irradiation-induced bone loss.
3. EVs derived from human MSCs obtained from any oral tissue source: bone marrow (BMSCs) including bone jaw, dental pulp (DPSCs), apical papilla (SCAPs), gingival tissue (GMSCs), dental follicle (DFSCs), oral adipose tissue (ADSCs), periodontal ligament (PDLSCs), deciduous teeth (SHEDs), and grafted bone (GBSCs).
4. Utilization of any delivery routes, including implementation with a scaffold, injection into the defect, intravenous injection, and intramuscular injection.
5. Animal studies conducted on subjects with no systemic conditions or genetic modifications.

Exclusion Criteria

In vitro and clinical studies, editorials, literature reviews, and letters to the editor were excluded from consideration for inclusion in this study.

Search Strategy

Two independent reviewers (A.O. and A.V.B.) performed a comprehensive search of the MEDLINE (PubMed), Scopus, and Embase databases up until April 2023. The objective of the search was to identify preclinical animal model studies examining the effects of MSC on bone regenerative therapy. The search was conducted without any limitations on publication date or language. The complete search strategies for each database are provided in Supplementary Table S1. Furthermore, a manual search was performed, which involved reviewing the references of identified articles, including full-text evaluation and related reviews, following the recommendations by Greenhalgh and Peacock.[25]

Data Collection, Extraction, and Management

Screening and Selection of Papers

Two experienced authors (J.M.M. and A.V.B.), who were calibrated using the Cohen's kappa test,[26] independently assessed the titles and abstracts of the identified studies for potential inclusion in the review. Rayyan Systems Inc (https://www.rayyan.ai/) was utilized to facilitate the screening process. Once potentially relevant studies were identified, full-text articles were obtained for further evaluation. In case

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of any disagreements, a third reviewer (A.O.) was consulted to reach a consensus through discussion.

Search Results and Evaluation

The studies that met the eligibility criteria underwent data extraction, which was conducted independently by 2 researchers (A.O. and A.V.B.), utilizing predefined spreadsheets. In the event of any discrepancies, a discussion involving a third reviewer (J.M.M.) was conducted to achieve a consensus. If any data were found to be missing, inquiries were made to the corresponding authors for clarification. For each selected study, the following variables were collected: author(s)'name, year of publication, animal model, source, size of EVs in nanometers, isolation methods, study design, characterization methods, administration route, and results.

Risk of Bias in Individual Studies

The risk of bias (RoB) in each study was assessed using the SYRCLE's risk of bias tool.[27]

Two independent investigators (E.A.C. and A.O.) evaluated the RoB of all included studies separately.

Results

Study Selection

The initial search strategy yielded a total of 3801 articles, with 1310 from Scopus, 1673 from Embase, and 818 from Medline. After removing duplicates, 2 independent reviewers (Cohen's kappa test = 0.85) identified 2175 abstracts for title and abstract screening. Following the analysis of these documents, a total of 25 articles were selected for full-text assessment. The selection process is visually represented in the PRISMA flowchart (Fig. 1), and the reasons for exclusion are summarized in Supplementary Table S2. Ultimately, only 16 articles met the eligibility criteria and were included in this systematic review.

Studies Characteristics

All the studies included in this systematic review were conducted between 2018 and April 2023, and they utilized rodents as the research model. Specifically, 4 studies were conducted in Italy,[28-31] 9 studies were conducted in China,[21-23,32-37] and 3 studies were conducted in the US.[38-40]

Regarding the animal models used, all the included studies utilized rodents, either rats or mice. Among them, 6 studies used Wistar rats,[21,28-31,33] 8 studies used Sprague-Dawley rats,[22,32,34-37,39,40] and 2 studies used mice[23,38] (Supplementary Table S3).

Characteristics of Experimental Bone Defects and Techniques Used for Induction

1. Seven studies employed a rotary instrument, such as a dental bur or trephine, to induce critical defects of various sizes in the parietal calvarial bone. Among them, 4 studies reported the use of a 5 mm diameter and 0.25 mm,[28,29,31] or 1-mm height bone defect.[32] One study created smaller defects measuring 4 mm in diameter and 0.25 mm in height,[30] while 2 studies mentioned the use of 2.7 mm and 5 mm diameter defects,[35,38] without specifying the height.
2. Five studies focused on periodontal bone defects. Among them, only 3 studies provided information about the defect sizes: $3 \times 1.5 \times 2$ mm[22]; $4 \times 2 \times 1.5$ mm,[33] and $2 \times 1 \times 0.8$ mm.[36]
3. Two studies did not specify the sizes of the periodontal bone defect.[23,39] Instead, they described the induction of periodontitis by utilizing a silk ligature model to create a periodontal defect in the first molar of the upper jaw.
4. Four studies specifically targeted the maxillae. Among them, 2 studies utilized an alveolar defect model with a defect of $4 \times 3 \times 2$ mm[34] and $2 \times 1 \times 0.8$ mm.[36] The remaining 2 studies employed a mandibular defect model, creating a 2 mm diameter defect in the mandibular angle of the rat[21]; the specific size of the defect model was not reported in the study by Lee et al[40]
5. Finally, one study used as a model defect on the femoral condyle.[37]

Scaffold Materials Used for Tissue Regeneration in Bone Defects

Studies employed various types of scaffolds for regeneration purposes. These included a collagen (COL) in studies conducted by Giuliani et al,[29] Yu et al,[34] and Lee et al.[40] Wang et al[37] utilized collagen I and nanohydroxyapatite scaffolds. Pizzicannella et al[31] utilized a 3D COL scaffold. Diomede et al,[28] Giuliani et al,[29] and Pizzicannella et al[30] used 3D-printed polylactide (PLA) scaffolds with Diomede et al[28] additionally employing a polyethyleneimine (PEI) coating on the PLA scaffold. Jin et al[21] employed a 3D-peptide hydrogel (Puramatrix), while Zhao et al[36] utilized gelatin sodium alginate hydrogels. Lei et al[22] and Zhao et al[35] used a Corning Matrigel matrix (Matrigel).

Other studies utilized tissue engineered scaffolds made from poly(L-lactic acid) (PLLA),[38] or polydopamine (PDA)-modified poly(lactic-co-glycolic acid) microspheres (PMS-PDA).[32] Finally, ß-tricalcium phosphate (ß-TCP) was used as a scaffold in 2 studies,[22,33] while the remaining 2 studies[23,39] did not mention the use of any scaffold.

Oral Source of EVs

EVs derived from human MSCs were isolated from various oral tissues, including human periodontal ligament (hPDLSCs),[22,29,31,34-36] human gingiva (hGMSCs),[28-30,37,39] human exfoliated deciduous teeth (hSHEDs),[20,29,30] and human dental pulp (hDPSCs).[21,38,40] In some of these studies, EVs were cocultured with rat bone marrow MSCs (rBMMSCs),[23,34,35,37-39] human adipose-derived stem cells (hADSCs),[21] bone marrow mesenchymal stromal cells (BMMSCc),[32] and human umbilical vein endothelial cells (HUVECs)[33,37] (Supplementary Table S3).

Results From Periodontal Ligament Mesenchymal Stem Cells-Derived Extracellular Vesicles (PDLSCs-EVs)

The majority of studies demonstrated favorable outcomes with the use of hPDLSCs/EVs. A quantitative analysis of bone volume/tissue volume revealed increased bone repair in rat calvarial defects treated with hPDLSC-derived EVs/Matrigel.[35] Histological evaluations demonstrated enhanced regenerative capacity in the 3D-COL scaffold enriched with hPDLSCs-derived EVs coated with polyethylenimine (PEI-EVs) (3D-COL/hPDLSCs/PEI-EVs).[31] The 3D-COL/hPDLSCs/PEI-EVs group exhibited improved integration and

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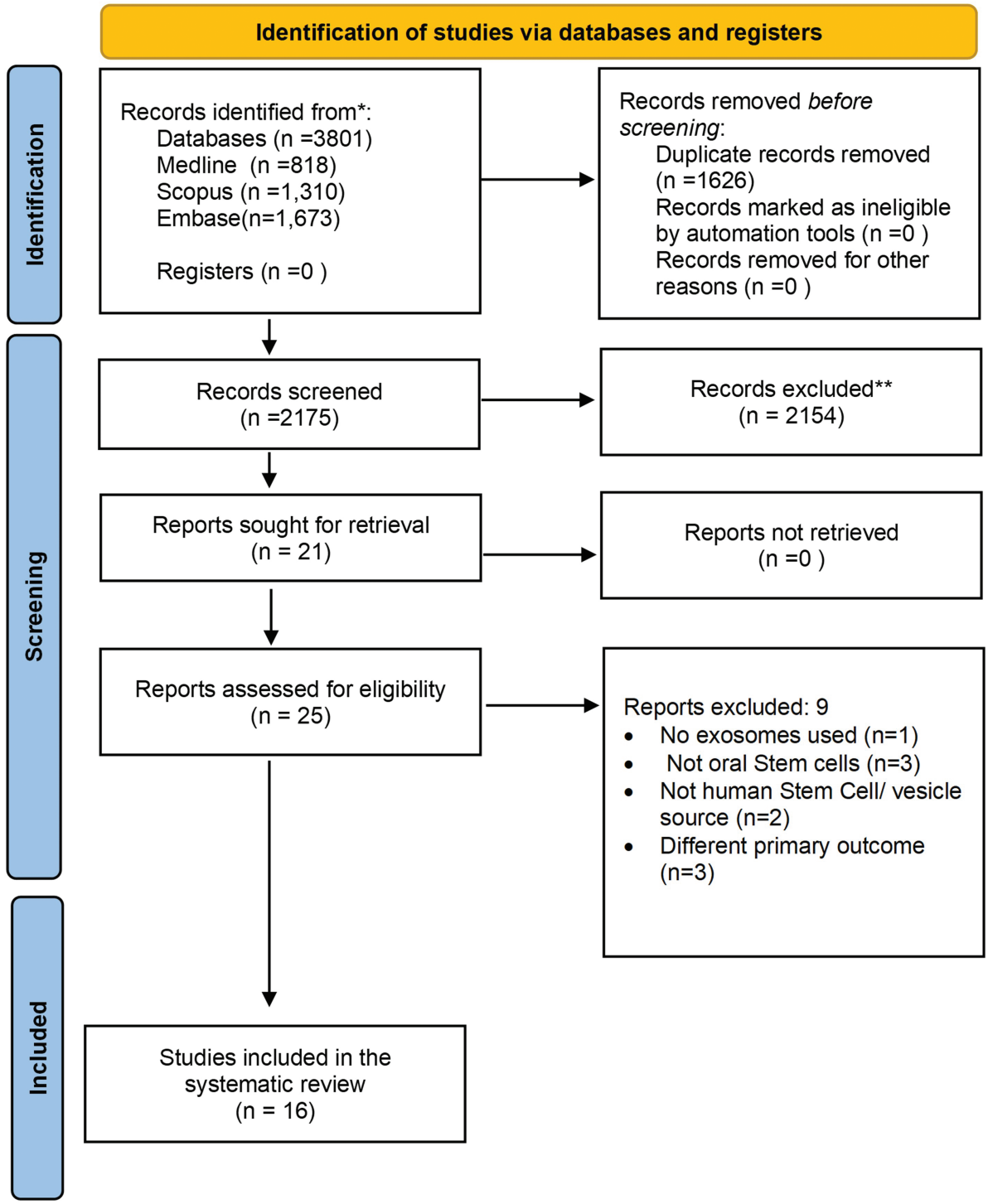


Figure 1. PRISMA 2020 flow diagram for new systematic reviews which included searches of databases.

regenerative potential, with visible osteoclasts and osteoblasts in the native bone after 6 weeks of grafting. Furthermore, the deposition of osteoid and calcification, leading to bone formation, was observed in the center of the bone defect with embedded osteocyte-like cells.[35]

Bone volume/trabecular volume analysis revealed increased new bone formation, improved healing, enhanced osteogenesis, and angiogenesis in the hydrogel + EVs group.[36]

In a rat model of periodontitis, Lei et al found that the alveolar bone mass was significantly higher in the group treated with EVs-loaded Matrigel compared to the group without EVs.[22] In addition, Yu et al demonstrated that the group receiving the 3D microenvironment (SM-Exo) with Matrigel exhibited the highest osteogenesis efficiency and a greater amount of newly regenerated bone tissue.[34] Conversely, Giuliani et al evaluated the distribution of relative bone mineral density (rBMD) using EVs from MSCs in combination with a collagen membrane (COL/hPDLSCs/EVs) and (PLA/hGMSCs/EVs).[29] Their findings indicated that the use of EVs resulted in lower mineralization levels and a wider density distribution.

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Results From Gingival Stem Cell-Derived EVs (GMSCs-EVs)

The utilization of EVs in combination with PLA scaffolds (PLA/hGMSCs/EVs) demonstrated an expanded range of bone density distribution,[29] enhanced growth of bone tissue at the defect site,[28] and a notable rate of bone regeneration.[30] The incorporation of engineered EVs into the samples resulted in new bone deposition, blood vessel formation, and the presence of osteoblast-like cells within the grafted bone defects, leading to complete repair of the calvarial defect.[28] However, histomorphometric analysis revealed that these samples exhibited a range of newly formed bone between 1.73% and 9.71%, with a total surface area (including extracellular matrix and residual graft biomaterial) of 90.2% to 98%.[28]

Furthermore, Wang et al demonstrated a positive therapeutic outcome in bone regeneration by utilizing EVs that promote osteogenesis and angiogenesis properties, particularly in relation to vascularization.[37]

Results With Exfoliated Deciduous Teeth Stem Cells-Derived Extracellular Vesicles (SHEDs-EVs)

It has been found that EVs derived from human exfoliated deciduous teeth stem cells (hSHED-EVs) could promote proliferation, migration, and osteogenesis of BMSCs.[33] In rats with ligature-induced periodontitis, bone loss height was significantly decreased in hSHED and SHED-EVs groups, suggesting that they both mitigate periodontitis bone loss.[23] Similarly, the use of hSHED-Exo/ß-TCP was shown to promote alveolar bone regeneration by enhancing osteogenesis and angiogenesis.[33]

The calvaria defect with porous microspheres + bioinspired polydopamine with hypoxic EVs condition (PMS-PDA + H-EVs) showed abundant newly formed bone tissues at 8 weeks. The bone volume fraction (BV/TV) was measured to be 58.8 ± 5.9%, and the bone mineral density (BMD) was 529.5 ± 59.3 mg/cm^3, which were significantly higher than the values observed in other groups.[32]

Results With Dental Pulp Stem Cells-Derived EVs (DPSCs-EVs)

The localized delivery of osteogenic EVs (OS-EVs) using EVs-releasing spheres (EVs-MS) resulted in the most effective regenerative outcome. Furthermore, the OS-EVs-MS-functionalized scaffold group exhibited a significant increase in mineralized bone volume within the bone defect. However, the BMD in the mineralized areas was found to be similar across the groups.[38]

Micro-CT images demonstrated that both the low dose (40 μg/mL) and high dose (4000 μg/mL) hDPSC-EVs groups exhibited greater quantities of new bone and BV/TV compared to the control group. However, there were no significant differences between the 2 experimental groups at weeks 3 or 5 (21). Swanson and colleagues suggested that OS-EVs could enhance bone healing in vivo in a dose-dependent manner.[38] Lee and collaborators reported that DPSCs + EVs displayed osteoinductive and osteogenic properties, although no statistically significant differences were observed in bone mineral density among the different groups.[40]

In summary, the majority of studies have shown that MSCs/EVs, irrespective of their specific origin, possess osteogenesis and angiogenesis properties in bone regeneration.

EVs MicroRNA Expression

Yu et al discovered notable differences in the expression of 25 microRNAs (miRNAs) between EVs secreted by PDLSCs in the 3D strain microenvironment (SM-Exo) and those obtained from the 3D culture microenvironment (Exo).[34] Among these 15 miRNAs (miR-10b-5p, miR-10a-5p, miR-200a-3p, miR-196b-5p, miR-200c-3p, miR-200b-3p, miR-125a-5p, miR-202a-5p, miR-449a, miR-619-5p, miR-335-3p, miR-10a-3p, miR-142-3p, miR-122-5p, let-7a-3p) were upregulated, while and 9 miRNAs (miR-574-5p, miR-382-3p, miR-210-3p, miR-212-3p, miR-363-3p, miR-665-3p, miR-7-1-3p, miR-6511b-5p, miR-532-3p) were downregulated in SM-Exo compared to Exo.[34]

Finally, Pizzicannella and coworkers described upregulation of miR-2861 and miR-210,[30] while Wu et al reported overexpression of miR-196a, miR-21, and miR-10b.[33]

Osteogenic Differentiation

Osteogenic differentiation in the studies was assessed using various techniques. Alkaline phosphatase (ALP) staining[21,23,32,36-38,40] and alizarin red staining (ARS)[21-23,28,30,32-34,36-38,40] were commonly utilized to evaluate osteogenic differentiation. In addition, BMD micro-CT analysis[29,36,37,40] was performed in some studies.

Different markers were employed to analyze the osteogenic differentiation capacity. These markers included RUNX Family Transcription Factor 2 (RUNX2),[21,28,30,32,36,37,39,40] bone morphogenetic protein 2 (BMP2),[21,28,32,39] bone morphogenetic protein 4 (BMP4),[28] osteocalcin (OCN),[32,36,37,39,40] alkaline phosphatase (ALP),[35,37,40] vascular endothelial growth factor A (VEGFA), osteopontin (OPN), collagen 1A1 (COL1A1).[30]

RoB Assessment Results

The assessment of RoB using SYRCLE's tool[27] resulted in a total of 416 entries (Supplementary Table S4). Among these entries, 41.11% were categorized as Unclear, 37.02% as Yes, and the remaining 21.87% as No. The majority of the SYRCLE's items (#3, #5, #6, #7, and #8) were marked as Unclear, indicating uncertainty regarding the blinding and randomization of outcome assessments. None of the studies assessing the use of EVs scored Yes in Items #3, #5, #6, #7, and #8, mostly due to a lack of reporting. The included studies did not provide data on animals' allocation concealment, blinding of investigators or outcome assessors, and management of incomplete outcome data. As a result, Item #9 was often answered as No, due to the impossibility to assure the absence of selective outcome reporting. On the other hand, Items #1, #2, #4, and #10 were mostly scored Yes. The authors described a random component for allocating animals to intervention groups, ensured a similar number of animals in each group, minimized the influence of housing factors by using syngeneic animals, and reported all the outcomes as stated.

Discussion

Despite being a novel approach, stem cell-based therapies have not yet reached an advanced stage of development. This is primarily due to the disadvantages associated with their use, including the unpredictable behavior of stem cells and the undefined duration of their activity. However, the utilization of EVs derived from stem cells represents a promising frontier in personalized therapeutics. These vesicles exert a paracrine

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effect within a specific time frame, acting on the patient's own cells. They have the ability to activate target cells, prevent apoptosis and stimulate the differentiation of intrinsic progenitor cells in the patient's tissue. These effects are mediated by the functional proteins, mRNA, or microRNAs present in these vesicles.[41]

Our objective was to investigate the in vivo effects of using EVs derived from oral MSCs in inducing bone regeneration. Oral MSCs are an effective source of EVs for bone regeneration because are easily accessible. Their EVs have lower immunoreactivity and greater biological stability than MSCs. Moreover, EVs have no potential to differentiate, making them more controllable and possessing higher biosecurity.[9,42] The findings of this systematic review conclude that EVs derived from various populations of intraoral mesenchymal cells have the ability to improve the osteogenic repair response in maxillary bone or periodontal defects through the following mechanisms:

1. Promotion of neo-angiogenesis, as demonstrated in studies.[30,31,37]
2. Enhancement of proliferation and differentiation of intrinsic stem cells toward the osteoblastic lineage, accompanied by increased bone metabolism.[32]
3. Reduction in the expression of certain proinflammatory cytokines.[39]

Use of Scaffolds

The application of such therapies necessitates the use of scaffolds to deliver both EVs and the associated stem cells or the conditioned media produced in these cells and their EVs. These scaffolds, often custom-made through 3D bioprinting, can be made from various biomaterials such as hydroxyapatite, PLGA, collagen membranes, or polylactide. These biomaterials can have different biological effects on the bone substrate, potentially influencing the host response.[43]

However, it is important to note that the scaffold alone, regardless of its composition, does not facilitate complete regeneration of the bone defect. This applies not only to the volumetric or morphometric aspects but also to the distribution of mineralization density when compared to a natural in vivo control.

No scaffolds have shown clear superiority over others in terms of bone regeneration and mineralization when used in combination with biological agents or conditioned culture media. However, when scaffolds are supplemented with stem cells, an increase in the mineralization of the constructs can be observed. Nevertheless, the mineralization density distribution in these cases tends to fall below the desired levels. When a combination of a matrix with stem cells and EVs (whether engineered or not) is utilized, it is possible to create a condition that closely resembles a healthy state.

Metabolic Pathways

MicroRNAs, mRNAs, and proteins enclosed within EVs exert a paracrine effect on recipient cells, triggering various signaling pathways that regulate metabolic activities within the bone environment. Through distinct molecular mechanisms, these components can modulate the expression of over 2500 genes involved in bone metabolism. In a study by Gao et al, a Kyoto Encyclopedia of Genes and Genomic (KEGG) signaling pathway enrichment analysis was conducted, revealing that modified EVs have the potential to enhance bone regeneration by targeting endogenous cells, inducing angiogenesis, and regulating bone metabolism.[32] Notably, these effects were mediated through the VEGF signaling pathway, thyroid hormone synthesis, and the upregulation of specific circRNAs associated with focal adhesion, a process that influences proliferation, cellular migration, and functional differentiation. In their study, Jin et al conducted a KEGG pathway analysis and identified the top 30 enriched pathways.[21] Their experimental model using hDPSC-EVs revealed enrichment in pathways associated with glycolysis, cytoskeletal GTPases, and signal recognition, which are characteristic of EVs. Among the identified pathways, the insulin signaling pathway exhibited the highest number of enriched genes. Traditionally, this metabolic pathway was primarily associated with glucose metabolism. However, recent studies have increasingly highlighted its involvement in osteogenesis and angiogenesis.[44] Furthermore, proteins associated with the MAPK pathway, such as MAPK1 (ERK2), MAPK3 (ERK1), MAPK8 (JNK1), MAPK9 (JNK2), and MAPK10 (JNK3), were also found to be enriched in the insulin signaling pathway.[21]

Wu et al reported similar findings, indicating that the phosphorylation of MAPK was enhanced in various types of stem cells upon stimulation with EVs.[33] They also suggested that this signaling pathway played a significant role in the angiogenesis and osteogenesis promoted by EVs derived from stem cells obtained from human deciduous teeth. Concretely, the Erk proteins are known to be involved in cell-EVs interactions, as well as the activation and stabilization of Runx2. Swanson and colleagues demonstrated that specific miRNAs expressed in EVs were capable of upregulating Runx2 expression, coinciding with Erk phosphorylation.[38] Importantly, this effect was independent of the BMP-related Smad-1 pathway.

Zhao and colleagues demonstrated that treatment with EVs significantly enhanced phosphorylation of AKT and ERK1/2 within a short timeframe of 15 minutes.[35] This suggests that the activation of AKT and ERK1/2 by EVs is partially mediated through the adenosine receptor signaling pathway. The role of the transforming growth factor-beta (TGF-ß) signaling pathway in osteogenic differentiation and bone metabolism through the use of EVs has also been described. Wei et al demonstrated that the phosphorylated Smad 5 (p-Smad 5) pathway was activated during the differentiation of mesenchymal cells derived from osseous tissue when treated with EVs derived from stem cells from human deciduous teeth.[23]

Other important metabolic pathways involved in bone remodeling could be downregulated. In this sense, Lei et al demonstrated that treatment with EVs suppressed the protein expression of active ß-catenin (nonphosphorylated ß-catenin),[22] an essential molecule that activates downstream signaling in the Wnt pathway.[15] According to their findings, the application of EVs decreased the mRNA levels of Wnt1, Wnt3a, Wnt10a, and ß-catenin in PDLSCs derived from patients with periodontal pathology to levels comparable to PDLSCs isolated from healthy subjects.[22]

MicroRNAs and Gene Expression

In addition to multiple bioactive molecules involved in angiogenesis, bone formation, and metabolism, including proteins such as PDGF (platelet-derived growth factor), EGF (epidermal growth factor), or FGFs (fibroblast growth factors), another biologically important component in the content of EVs is mRNA and microRNA. MicroRNAs carried in EVs

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can affect various cellular functions. The expression profile of miRNAs varies depending on the specific cell type and the microenvironment that regulates it.[45]

Therefore, it is plausible that each study on miRNAs in stem cell-derived EVs from different intraoral sources may show different miRNA upregulation or downregulation patterns. Thus, in the existing literature on miRNAs expressed in EVs derived from stem cells of oral origin, there is not any expressed miRNA from EVs that has been described at least in 2 separate different researches. However, although the miRNAs found are different, it seems that all of them are implicated in the modulation of osteogenesis signaling pathways. Nevertheless, their effects on the mechanisms involved in the progression of bone regeneration remain unclear and require further in-depth studied.

From the animal models utilized in the studies included in this systematic review, significant insights can be obtained regarding the genes that exhibit overexpression following the administration of EVs derived from oral stem cells, as well as the proteins encoded by these genes. The extent of detail in this data varies depending on the analysis technique employed, which encompasses a range of methods such as next-generation sequencing (NGS), immunohistochemistry, reverse transcription polymerase chain reaction (rt-PCR), or western blotting. By meticulously analyzing this data, several key findings can be derived:

1. The overexpression of angiogenesis-related proteins, such as VEGF-A, KDR, SDF-1, and FGF2, has been reported in studies conducted by Wu et al,[33] Wei et al,[23] Wang et al,[37] and Lee et al.[40] In addition, several osteogenesis-related proteins, including COL1A1, BMP2, RUNX2, OSX, ALP, OCN, and OPN, have shown increased expression in these studies. Moreover, EVs derived from oral stem cells have been associated with the upregulation of immune-modulatory-related proteins, such as IL-10 and FOXP3, as well as the downregulation of proinflammatory cytokines. This has been observed in studies conducted by Zarubova et al[39] and Wei et al[23]
2. The effect of EVs on target cells can be dose-dependent. In a study by Wei et al, it was demonstrated that EVs derived from deciduous teeth cells had a suppressive effect on the expression of certain proinflammatory cytokines, specifically reducing the expression of IL-6 and TNF-α.[23] However, at a high concentration of EVs (10 μg/mL), the opposite effect was observed, resulting in an increase in IL-6 and TNF-α expression. This higher concentration of EVs may exacerbate inflammation instead of suppressing it.[23] Similarly, in the research conducted by Wang et al, the strongest osteogenic effect was observed with a medium dose (50 μg/mL) when rat bone marrow MSCs were treated with 25 μg/mL, 50 μg/mL, and 100 μg/mL of HGMSCs-EVs.[37] Regarding angiogenesis, it was found that EVs derived from human gingival MSCs (hGMSCs) significantly enhanced the angiogenic ability of HUVECs in vitro. Interestingly, this effect was also concentration-dependent, with higher concentrations of EVs showing a greater enhancement of angiogenesis.[37]
3. Studies have shown higher expression of genes in models that used scaffolds grafted with both common stem cells and their derived EVs, compared to models where only stem cell EVs were administered.[28,29] NGS technology and Gene Ontology (GO) analysis have revealed that the combination of MSCs and EVs incorporated into a matrix leads to the upregulation of 31 identified genes involved in the regulation of ossification processes. These genes include FHL2, BMP2, TWSG1, CCDC47, FAM20C, ERCC2, LEP, TOB2, IMPAD1, CHRDL1, MINPP1, HIRA, MYBBP1A, JAG1, MEF2C, SUCO, SFRP1, SOX9, SIX2, RHOA, PDLIM7, IFT80, SMAD1, HDAC7, ASF1A, ID3, SNAI1, PEX7, RPL38, BMP2K, and BCAP29. Among these genes, 19 are involved in the "regulation of osteoblast differentiation" and "osteoblast differentiation" (FHL2, BMP2, TWSG1, CCDC47, FAM20C, HIRA, MYBBP1A, JAG1, MEF2C, SUCO, SFRP1, PDLIM7, SMAD1, IFT80, HDAC7, ASF1A, ID3, SNAI1, and BCAP29).

In this study, the combination of scaffolds, MSCs and EVs resulted in the upregulation of 9 genes associated with osteogenesis and genes involved in osteoblast differentiation through TGF-ß signaling. The upregulated genes included integrin (ITGA6), basement membrane laminins (LAMB3, LAMA1, and LAMC1), membrane proteins involved in cell-to-cell and cell-to-matrix interactions (CTNNA1, VCAN, CD44, and THBS2), and matrix metalloproteinase inhibitors (TIMP3).[28] In addition, the analysis showed the downregulation of genes associated with cell-cell adhesion (ITGA3, ITGB5, ITGAV, ACTB, CTNNB1, and CTGF) and genes encoding extracellular matrix (ECM) constituents of the basement membrane (LAMA3, TNC, GAPDH, and COL4A2). These findings indicate that the therapeutic combination enhances the regulation of adhesion molecules, ECM, and osteogenic genes.[28]

Limitations and Strengths

An upcoming development in this emerging therapeutic approach involves modifying MSCs to enhance the composition and quantity of their EVs. By utilizing these modified EVs as targeted therapeutic tools, it is anticipated that specific biological actions can be further amplified. There are several possibilities to enhance or modify the secretion of EVs from MSCs and thus improve their therapeutic potential. MSCs culture parameters, such as providing a 3D environment and adjusting the cell seeding density, can influence the yield of EVs. Moreover, MSCs can be genetically modified to enhance the quantity and effectiveness of the EVs they produce, thereby increasing the therapeutic potency of the secreted EVs. Researchers are currently working on developing MSCs that are therapeutically optimized by overexpressing specific proteins and miRNAs.[46]

However, despite the promising results, there are still limitations in the use of EVs as therapeutic agents. One of the most important drawbacks is determining the appropriate dose-concentration required to achieve the desired biological effect. As mentioned, it has been observed that different concentrations of the same EVs can lead to opposing effects[23] or different biological responses, such as the promotion of osteogenesis versus neoangiogenesis.[37] This limitation becomes even more significant when considering that the biological effect of EVs can be influenced by their origin from specific oral locations.

It is crucial to acknowledge that the studies discussed in this review utilize EVs derived from various oral MSCs. While

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the vehicle itself, EVs, is well understood, there is still limited comprehensive knowledge regarding the specific content of these particles, even when engineered with specific molecules. Another significant limitation inferred from this study is the lack of standardization in the utilization of these EVs. This includes variations in their cellular origin, isolation methods for application, and carriers employed for different types of bone defects and biological substrates. As a result, these variations inevitably lead to different biological actions, making it challenging to establish consistent, and standardized protocols for their use.

Conclusions

In conclusion, the utilization of oral MSC-derived EVs has demonstrated efficacy as a valuable tool in promoting bone regeneration in preclinical models. These EVs have shown notable improvements in osteogenic repair and neoangiogenesis, leading to enhanced bone metabolism and reduced inflammatory mediators. These findings highlight the potential of EVs as a promising therapeutic approach for bone regeneration in clinical settings.

However, further studies are necessary to elucidate the underlying biological mechanisms responsible for these regenerative effects. A deeper understanding of these mechanisms is crucial for optimizing the clinical outcomes associated with the application of oral MSC-derived EVs in bone regeneration. In addition, it is vital to address potential risks that may arise from the utilization of these novel biological systems.

Therefore, additional studies are needed to further explore and enhance the therapeutic potential of EVs, ensuring their safe and effective clinical implementation in the field of bone regeneration.

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Conflict of Interest

The authors do not have any financial interest in the companies whose materials may be mentioned in this article.

Author Contributions

A.O., K.B., P.G.-M.: conception; A.O., A.V.B., J.M.M., E.C.C.: design of the manuscript, critical review for relevant intellectual content; A.O., P.G.-M.: original draft; A.O., A.V.B., J.M.M., E.C.C., M.P.-M., P.G.-M., S.G.M.: writing review and editing; All authors: final approval of the version to be published.

Data Availability

No new data were generated or analyzed in support of this research.

Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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