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Dental Stem Cells Characterization and Bone Regenerative Potential in Oral Medicine

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Abstract

The oral cavity has proven to be an important reservoir of stem cells with the advantage of being a non-invasive source. These cells potential for maxillo-facial bone regeneration has been well documented throughout the last five years. This article aims at reviewing and discussing the different types of dental stem cells available in the oral cavity, their lineage characterization and potential applications in oral medicine; cell collection, isolation and preservation; bone regenerative potential comparison between dental stem cells and bone marrow stem cells; and various factors affecting bone regeneration in the use of osteogenic cell groups.

Keywords

Dental stem cells, Osteoinduction, Gene induction, Osteointegration, Stem cells isolation, Mesenchymal stem cells, Bone regeneration, Scaffolds, Regulation factors

Abreviations

MSC: Mesenchymal Stem Cells, DSC: Dental Stem Cells, DPSCs - Dental Pulp Stem Cells, SHEDs: Stem Cells from Exfoliated Deciduous Teeth, SCAPs: Stem Cells from Apical Papilla, DLSCs: Periodontal Ligament Stem Cells, DFPCs: Dental Follicle Precursor Cells, BMMSCs: Bone Marrow Mesenchymal Stem Cells, BMP: Bone Morphogenic Protein, PLLA: Poly L-lactic Acid, ALP: Alkaline Phosphatase, BSP: Bone Sialoprotein, OSX: Ostenix, OPN: Osteopontin, PDGF: Plaquelet Derived Growth Factor, OC: Osteocalcin, Coll-1: Collagen Type I, CP23: Cementum Protein 23, FGF2: Fibroblastic Growth Factor 2, TNFα: Tumoral Necrosis Factor α, VEGF: Vascular Endothelial Growth Factor, NF-κB: Necrosis Factor κB, CEMP1: Cementum Protein 1, HIF1: Hypoxia Induction Factor 1, FGF9: Fibroblastic Growth Factor 9, HA/TCP–Hidroxiapatite + Tri-calcium phosphate

Introduction

Alveolar bone loss is a major concern, especially after tooth extraction. To overcome the alveolar bone loss and to increase support (in order to place dental implants, for example), many bone regenerative substitutes are available, such as: allografts, autografts, xenografts, synthetic biomaterials and osteoactive agents [1].

Calcium phosphate ceramics are one of the most used groups of synthetic bone substitutes [2], because of its similarity to the mineral phase of natural bone, absence of immunogenic reactions, excellent biocompatibility and osteoconductive potential [3].

Although calcium phosphate ceramics contribute to bone healing through osteoconduction, they generally lack osteoinductivity. Due to these limitations, surgeons and researchers have focused on developing alternative therapies, particularly studying the combination of osteoprogenitor cells, like mesenchymal stem cells (MSCs), with bone substitutes to improve their osteogenic properties [4].

Some studies indicate that increasing the number of grafted MSCs, associated with calcium phosphate ceramics biomaterials, result in better bone formation. This might be related to the crucial role of MSCs in osteoinduction [5].

There are many MSCs, such as: bone marrow-derived mesenchymal stem cells, umbilical cord blood-derived mesenchymal stem cells, adipose tissue-derived stem cells, muscle-derived stem cells and dental pulp stem cells [6]. Bone marrow was the most used source of MSCs. However, in recent years, dental stem cells have received extensive attention in the field of bone tissue engineering due to their easy access for dentists and oral surgeons, immunosuppressive properties, high proliferation, and the capacity to differentiate into odontoblasts, cementoblasts, osteoblasts, and other cells to be found in dental tissues [7,8].

The aim of the present review is to analyse the current state of the art about dental stem cells and their potential in oral medicine bone regeneration.

Material and Methods

A systematic search was made in the PubMed database.

The following combination of keywords was used: dental stem cells, osteoinduction, gene induction, osteointegration, stem cells isolation, mesenchymal stem cells, bone regeneration, scaffolds, and regulation factors.

Mainly review articles, clinical trials and case-reports were selected. Time frame of publication date was set from 2010 until

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Table 1: Therapeutic application of oral MSCs

DPSCs	These cells seem to be of great importance concerning periodontal regeneration as well as bone and dentin regeneration. However, further work on the subject needs to be developed [23,52,53]
	On the other hand, they have been able to generate a dentin pulp-like complex [11,16,53].
PDLSCs	These stem cells seem to be capable of generating bone, cementum, periodontal ligament-like structures and to increase periodontal regeneration [16,52]. When compared to other mesenchymal stem cells they appear to be more indicated when periodontal regeneration is intended [11,52].
SHEDs	This group of stem cells are believed to be a valuable alternative when periodontal regeneration is intended and also demonstrate the ability to reconstitute a dentin pulp-like complex [11,16,52,53].
	Moreover, SHED have shown bone regenerative capacity [16,23,53].
SCAPs	These stem cells demonstrated positive results in what concerns the formation of dentin pulp-like complex [11,16,23,54].
	Associated with PDLSCs, SCAPs were able to form a root-like structure when seeded onto hydroxyapatite-based scaffolds and implanted in pig jaws [52,54].
DFPCs	The micro-environments where this stem cells are implanted have great importance to determine which tissues they are capable of forming (e.g. DFPCs implanted in the non-mineralized omental pocket contributed to dentin regeneration; DFPCs implanted in the highly mineralized skull contributed to matrix formation; DFPCs implanted in the alveolar fossa contributed to the formation of root like tissues with a pulp-dentin complex and a periodontal ligament connecting a cementum-like layer to host alveolar bone). Another important information provided by these studies is that DFPCs demonstrated high potential towards tooth roots regeneration [52].
	Moreover, this group of stem cells is also capable of generating a bone/cementum-like tissue [16].

2015, and only papers available in English were included. The papers selection was performed by considering the presence of keywords in the title and abstract, and their relevancy to the research that has been made. Then, each paper was selected individually according to its content.

Results and Discussion

Types of dental stem cells

Dental tissues have been considered a potential source of MSC-like populations [9].

With the exception of enamel, which lacks ameloblasts or other cellular elements following tooth development, the periodontium and dentine continue to retain some regenerative or reparative capacities [10].

Dental MSCs are indicated for the regeneration of the dentin–pulp complex, bone, cartilage and neuronal tissues, among others [10,11]. Until recently, five different populations have been isolated and characterized in postnatal dental tissues and classified according to the tissue of origin: dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHEDs), stem cells from apical papilla (SCAPs), periodontal ligament stem cells (PDLSCs) and dental follicle precursor cells (DFPCs) [9,12] (Table 1).

Dental pulp stem cells (DPSCs): DPSCs are multipotent stem cells, with ectomesenchymal origin, which have advantages for clinical applications when compared to other mesenchymal stem cells derived from bone marrow, adipose tissue, peripheral blood, and umbilical cord blood. They are easily available from discarded teeth after extraction and they can be cryopreserved, retaining their multipotent differentiation ability [9,13].

The differentiation potential of DPSCs from natal teeth to adipogenic, osteogenic, chondrogenic, myogenic and neural-glial cells lines was also shown, and an intrinsic tendency of these cells to differentiate towards osteoblasts has been demonstrated [13].

Sometimes DPSCs are referred to as odontoblastoid cells, because they appear to synthesize and secrete dentin matrix like the odontoblast that they replace [14]. When transplanted *in vivo* (using immunocompromised mice as hosts), the cells derived from dental pulp generated functional dental tissue in the form of complexes like dentine/pulp. This might be due to the origin of their development as neural crest derived cells [12]. When cultured in ceramic substrates, such as hydroxyapatite or tricalcium phosphate, the cells are able to form bone, dentin and cementum-like tissues [9].

Stem cells from exfoliated deciduous teeth (SHEDs): SHED is a heterogeneous multicellular population of stem cells [15] identified as highly proliferative clonogenic cells capable of differentiating into a variety of cell types including neural cells, adipocytes and odontoblasts [12]. SHED cells represent a population of multipotent stem cells that are more immature than the DPSCs and, in vivo, they seem to be able to induce bone or dentin formation [13,14].

Also, SHED cells exhibit higher proliferation rates, increased population doublings, osteoinductive capacity and ability to form sphere-like clusters when compared to DPSCs [12].

Stem cells from apical papilla (SCAPs): The cells located in the apical papilla (root foramen area) represent another unique population of dental stem cells [12]. As long as the root develops, the dental papilla's location moves in an apical direction [13]. These cells show ability to differentiate into cells of the osteogenic, odontogenic, adipogenic, and neurogenic lineages [9]. It is thought that SCAPs may be responsible for the formation of primary odontoblasts that account for the formation of root dentin, whereas DPSCs seem to be the source of replacement odontoblasts that produce reparative dentine [9,12,13]. To conclude, SCAPs are derived from a developing tissue that may represent a population of early stem/progenitor cells, which may be a superior cell source for tissue regeneration [13], since they also showed other favourable characteristics, such as higher proliferative rate, telomerase activity and improved migration capacity [14-16].

Periodontal ligament stem cells (PDLSCs): The periodontal ligament provides nourishment to the teeth, regulates periodontal homeostasis and contains a population of progenitor cells – periodontal ligament stem cells [12]. PDLSCs are capable of differentiating into cells resembling cementoblasts, osteoblasts, adipocytes, chondrocytes, and fibroblasts [9]. This population seems to be more proliferative, have a faster cell growth rate and higher clonogenic capability than bone marrow mesenchymal stem cells (BMMSCs) [9,16]. These cells maintain their tissue regenerative potential even after recovery from frozen human tissue, which suggests the possibility of cryopreserved PDLSCs from extracted teeth being used for future therapeutic purposes [12].

Dental follicle precursor cells (DFPCs): Dental follicle stem cells exist in the dental follicle (loose connective tissue sac surrounding the enamel organ and the dental papilla of the developing tooth germ before eruption) [9,12]. These cells were first isolated from the follicle of human impacted third molars [14]. *In vitro*, following adequate induction, DFPCs have been demonstrating osteogenic, odontogenic and cementogenic differentiation capacity [9].

Gingiva as a source of stem cell: Progenitor cells and multipotent MSC subpopulation of cells have been isolated and characterised from gingival fibroblasts. These fibroblasts are easily accessible and have recently been used to derive induced pluripotent stem cell lines [12].

Collection, isolation and preservation of dental stem cells

Collection: The main source of dental stem cells is extracted teeth. A freshly extracted tooth is transferred into a vial containing transport solution and generally hypotonic phosphate buffered saline solution. The vial is carefully sealed and placed into a thermette and

then the carrier is placed into an insulated metal transport vessel. The thermette, along with the insulated transport vessel, maintains the sample in a hypothermic state during transportation. This procedure is described as sustentation. The time from harvesting to arrival at the processing storage facility should not exceed 40 hours [17].

DPSCs samples are obtained from dental pulp tissue from extracted third molars, exfoliating/extracted deciduous teeth and teeth extracted for orthodontic treatment, trauma or periodontal disease [18,19], while PDLSCs are obtained via scraping the mid third of the root in extracted teeth. SCAPs are isolated from the apical papilla tissue and can easily be removed from an extracted tooth with developing roots (very common in third molar extractions) [19-23].

Isolation: Almost every author uses a method of their own, which makes a detailed analysis of all experimental works impossible. Consequently, several studies need to be done in order to systematize laboratorial procedures [23].

The two methods most frequently used for DPSCs isolation are Enzyme-digestion and Explant outgrowth. Other methods like Magnetic activated cell sorting (MACS) and Fluorescence activated cell sorting (FACS) are also available [17,24-27].

Regardless of the chosen isolation method, common procedure dictates that the tooth surface is cleaned by washing it three times with phosphate buffered saline (PBSA) without Ca^{2+} and Mg^{2+}. The next step is disinfection followed by another washing with PBSA. Pulp tissue is isolated from the pulp chamber and placed into a sterile petri dish, washed at least three times with PBSA [17,25,26].

Enzyme-digestion method: Small pieces of pulp tissues are transferred into an enzyme solution, often collagenase/dispase, for 30-60min at 37°C. Afterwards, large cell aggregates are removed and single-cell suspensions are obtained by passing cells through a cell strainer. Single-cell suspensions are centrifuged for 5 min at room temperature. Suspensions are then seeded in culture dishes containing proliferation medium and incubated at 37°C in 5% CO_2. Culture medium can be substituted every three days until cell confluence is achieved [17,24-27].

Explant outgrowth method: Pulp tissue is placed in culture flasks with proliferation medium and then incubated at 37°C in 5% CO_2. Medium must be changed after outgrowth is observed [17,24-27].

A modified efficient method for dental pulp stem cell isolation: Recently, an additional method has been proposed. The main difference relies on the digestion of pulp tissues and fixing them under a cover slip in the medium. This new method provided a greater *in vitro* expansion compared to the two methods mentioned above. Moreover, the cells were less damaged and were, therefore, healthy enough to propagate longer *in vitro* than with other methods [24,26].

Preservation: Once the tissue samples are obtained, they are transferred under proper conditions to a tooth bank where they are stored. The approaches used for stem cell storage are cryopreservation and magnetic freezing [17].

Cryopreservation: Cryopreservation is the process of preserving cells or whole tissues by cooling them at sub-zero temperatures. Liquid nitrogen vapour is used to preserve cells at a temperature below -150°C. Cryopreservation of stem cells maintains the viability of these cells indefinitely [17].

Magnetic freezing: This technology is referred to as *cells alive system* (CAS), which works on the principle of applying a weak magnetic field to water or cell tissue, which will lower the freezing point of that body by up to 6–7°C. Using CAS, Hiroshima University (first proposed this technology) claims it can increase cell's survival rate in teeth up to 83%. CAS system is a lot less expensive and more reliable than cryogenics [17].

Comparison of human mesenchymal stem cells derived from dental pulp and bone marrow

BMMSCs are a population of multipotent, non-hematopoietic marrow-derived cells that are easily expanded in culture and differentiated into cells with an osteogenic phenotype [19].

Bone marrow was the first identified source of a cell referred as a MSC. These cells were the earliest discovered and well-known stem cell population used in bone tissue engineering [28].

Although they are widely studied and have presented favourable results, there was a need to search other alternative cell sources to overcome the disadvantages that these cells present, specially the morbidity associated to their collection. The main disadvantages of BMMSCs are the low stem cell yield from aspirated bone marrow, painful and highly invasive procedure of collection, as well as the decline, over time, of the number, proliferative capacity and maximal lifespan of MSCs derived from bone marrow [29].

Dental stem cells have emerged in the recent past as an alternative source to MSCs. They are considered multipotent cells with a high proliferation rate, opportunity to be safely cryopreserved, possessing immunosuppressive properties and expressing mesenchymal markers. They also have multi-differentiation ability, easy accessibility, high viability and ready ability to be induced into multiple cell lineages, such as: odontoblasts, adipocytes, neuronal-like cells, glial cells, osteoblasts, chondrocytes, melanocytes, myotubes, and endothelial cells [8,30,31].

PSCs share similar characteristics with BMMSCs. However, it is notable that DPSCs are superior to BMMSCs in certain aspects [32]. These cells have differences in expression of pluripotent stem cell genotype when compared to other types of MSCs. This might reflect their embryonic stem cell origin. Dental pulp is made of ectomesenchymal elements, containing neural crest-derived cells, which display plasticity and multipotential capabilities [5].

Ponnaiyan D et al. concluded that MSCs derived from dental pulp proliferate much faster than MSCs from bone marrow [7]. The authors demonstrated that DPSCs exhibit a significantly higher proliferation rate than BMMSCs and are, therefore, more appropriate for cell-based therapy in clinical application. This result may be due to the age related state of respective tissues, since DPSCs were isolated from third molars (the last permanent teeth to evolve and erupt at an earlier stage of development). In contrast to BMMSCs, DPSCs were more restricted in their adipocyte differentiation capacity, while exhibiting a stronger ability for osteoblast differentiation [30].

Many studies confirm the ability of DPMSCs to undergo osteogenic differentiation and their capacity of forming vascularized adult bone. Integration between the graft and the surrounding host blood supply was achieved [5,21,32,33]. Mori et al. also successfully induced DPSCs into osteoblasts [34].

Although the majority of studies support the advantages of DPSCs over other types of stem cells, there are some which claim opposite conclusions. Asatrian G et al. advocate that while dental pulp might pose as an alternative site for MSCs isolation, the use of DMSCs is limited by the small quantity of pulp in a tooth, by the proportionally smaller DMSC yield required for their expansion by long-term culture and the corresponding costs and risks that are incurred. Thus, according to this study, DPMSCs do not supplant therapies enlisting BMMSCs [35].

Bone tissue regeneration using DSCs - recent advances

In recent years, bone tissue regeneration has proven to be achievable using MSCs from the oral cavity. This is presented as an evolutionary step from common practiced clinical techniques such as the use of rich plasma alone or scaffolds carrying BMPs. Furthermore, bone regeneration is achieved in more significant capacity using stem cells than with previous methods [36]. Therefore, it is important to review the latest developments in the research of factors which may affect stem cells' potential as a biomaterial.

Scaffold architecture and composition

Scaffolds simulate an extracellular matrix in a three dimensional

model, as a carrier of signalling molecules aiming towards stimulation, differentiation and migration of cells, ultimately with the purpose of tissue regeneration [16].

Nanofibrous PLLA scaffolds are preferable to Solid Wall ones, as they exhibit higher adherence, proliferation and cellular differentiation values. Also, greater mineral deposition has been achieved in the matrix *in vitro*. Higher ALP activity, development of dense tissue (detected via sialoprotein concentration) and collagen levels have also been reported *in vivo* [37].

In recent times, Biocoral Scaffolds comprising DPSCs revealed elevated genomic expression of osteoblastic lines, along with high local values of BSP and osteocalcin. It was possible to observe DPSCs migration towards the inside of Biocoral pores, where the secretion of extracellular matrix and osteoblastic differentiation occurred [38].

Scaffolds HA/TCP carrying PDLSCs corrected furcation defects (Class II and III) combined with cementum and periodontal regeneration, after 12 weeks, in animal models. *In vitro* studies also verified positive results regarding osteogenesis and *in vivo* studies equally established favourable clinical and radiographic results. PDLSCs revealed the potential for the development of lamina-dura around titanium implants *in vivo* [16]. MSC Spheroids, in conjunction with osteoblastic induction agents, over the course of 7 days, have shown accelerated osteogenic properties, higher calcic deposition and bone regeneration *in vivo*, with significant increase in osteogenic markers concentration (OSX - 8.27x, RUNX2 - 1.57x, OPN – 1.94x, BSP – 1.33x) [39].

Genetic and nutritive regulation

Bone morphogenic proteins have long been utilized in scaffolds as stimulants for a hastened growth of bone matrix and cartilage [40]. Presently, the transfection of genetic factors to cellular populations in scaffolds, namely PDLSCs, resulted in an escalation of PDGF, with greater cementoblastic activity than reported in recombinant PDGF delivery [41].

A mutant variety of RUNX2 (a major transcription factor for bone development and osteologic differentiation) in which the C-terminal 5 amino-acid chain is missing, conveyed higher OC, OP, Coll-1 and CP23 concentrations in scaffolds prepared *in vitro*. Similar results have been achieved when the cells were stimulated with FGF2. Genetic regulation of cells was obtained through gene introduction via viral vectors and by incorporating the cell medium with genes in the form of recombinant proteins [42]. Bobby Sox has also been studied as a regulator of osteoblastic gene expression in DPSCs, with stem cell proliferation increasing 6.5 times over a period of 7 days when present. BBX variant 1 and 2 cDNA were previously cloned and, afterwards, overexpressed when electroporated in progenitor cells using a microporator. The knockdown of BBX using shRNA did not affect mineralization but a decrease in osteoblastic gene expression was observed. Neither procedure modulated cell proliferation [43].

A shift in miRNA expression in DSC populations undergoing osteoblastic differentiation has been detected with miRNA-218 being associated with mineralized tissue formation and downregulation of RUNX2. MiRNA-218 quantity analysis was conducted using a nanodrop spectrophotometer at baseline (day 0) when the peak expression of RUNX2 was detected [44].

Implant site determinants

Angiogenic and pro-inflammatory responses have been studied as means to regulate DPSCs' proliferation. Short-term exposure to TNFα induces cellular death through an increase in angiogenic signalling (VEGF) and NF-κB, which, once prematurely inhibited, promote mineralization. Long-term exposure prompts cellular proliferation through an increase in anti-apoptotic genes (BCL2) and Survivin [45].

Dexamethasone has been described as having an osteogenic impulse on PDLSC *in vitro*, vindicated by an upturn in intracellular calcium, ALP and mineralization. Two times more frequently calcified nodules were noted between days 21 and 28 of the experiment conducted, therefore leading to the question of a possible genetic induction being carried out by Dexamethasone [46].

Human serum has been studied as an option to substitute bovine serum as a support for osteomorphic inducting markers for DPSCs, solving possible disease transmission issues and xenogenic immune response, which both may occur when bovine serum is used. Human serum displayed higher cellular proliferation (13% more at each cellular cycle), greater surface expression of typical DPSC antigens, more significant mineral deposition, more ALP activity, and moreover, an increase in surrounding ossification in bone graft implanted *in vivo* [47].

CEMP1 has been reported as an inducer of cementoblastic phenotypes in PDLSCs. In relative hypoxic states, while the local oxygen tension is reduced, CEMP1 expression rises, leading to further mineral deposition. This mechanism may be regulated by means of HIF1 [48].

DFSCs showed maximum proliferation rates at heat-stress temperatures in the 39°C to 40°C interval, concomitant with higher expression of osteogenic markers, when compared to 37°C physiological temperature [49].

Other studies conducted

Led Nitrate (Pb^{2+}) posed a threat to adhesion of DPSCs and PDLSCs in scaffold matrixes, with changes to cell morphology, higher osteogenic deregulation and cells expressing local markers associated to stem lineages (Oct4, Rex1). Lower proliferation and mineralization rates were also observed [50].

FGF9 induces phosphorylation of ERK1/2, therefore it inhibits osteogenic induction of DPSCs. BMP in scaffold matrix may be able to compensate for this effect, especially since FGF9 also upregulates angiogenesis (VEGF) leading to an ostegenic effect [51].

Conclusion

MSCs are available for extraction from the oral cavity without performing invasive techniques. Various cell populations express stem cell markers, diverse differentiation and clinical application potentials, such as regenerative endodontics and periodontal regeneration among others.

The most deeply studied group of cells with maxilo-facial bone regeneration capacity are DPSCs, SHEDs and PDLSCs. All of these displayed promising both in *in-vitro* and *in-vivo* results, especially when compared to other widely utilized stem cell groups such as BMMSC. However, comparisons are still controversial among the scientific community. Other oral MSCs still lack extensive research and so far have not demonstrated much bone regeneration potential. Furthermore, many factors implied in scaffold architecture and composition, gene induction, cell nutrition and general medium exposures regulate cell proliferation, mineralized matrix deposition and osteogenic markers' expression on cellular surface.

Oral stem cell research exhibits a large potential for development, with a broad spectrum of clinical applications to be considered in the coming years.

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