Chapter 7

Advanced Regenerative Techniques Based on Dental Pulp Stem Cells for the Treatment of Periodontal Disease

Ana-Maria Seciu, Oana Craciunescu and Otilia Zarnescu

Additional information is available at the end of the chapter

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Abstract

Recent progress in periodontology intended to reduce the risk represented by periodontal disease for systemic disorders and general human health condition. In this chapter, we overview the advantages and limitations of current techniques based on occlusive membranes for periodontal regeneration. Special emphasis is paid to advanced techniques using stem cells from dental pulp for the regeneration of bone defects caused by the chronic periodontal disease. Stem cells isolation, *in vitro* expansion and characterization techniques are presented. Therapeutic strategies of stem cells delivery using natural polymeric carriers are discussed. Stem cell-scaffold constructs application in bone tissue engineering is proposed, taking into account the marked decline of healing, and regenerative processes in elderly individuals. Future researchers envisage multiple effects of engineered constructs with antimicrobial, anti-inflammatory, and regenerative activity for periodontal treatment.

Keywords: cell carrier, collagen, dental pulp, periodontitis, regenerative medicine, scaffold, stem cells, tissue engineering

1. Introduction

Periodontium is a structural and functional tissue, which facilitates the anchoring of teeth in the maxillary and mandibular bones. It consists of two hard tissues, the cementum and the alveolar bone, and two soft tissues, the gingival connective tissue, and the periodontal ligament [1]. The periodontal tissues provide structural support at the tooth-jaw interface, a resilient attachment of the teeth during mastication and protection against the pathogenic

microbial flora from the oral cavity [2]. Periodontal remodeling is a necessary process in response to occlusal changes and dental abrasion, while the gingiva can initiate an immune response against pathogenic bacterial flora [3]. At the molecular level, tissue remodeling is accomplished by resorption and deposition of extracellular matrix components [4].

Periodontitis is the second most common oral infectious disease after dental caries, which affects the periodontium. It is a result of the inflammatory response caused by the accumulation of bacterial plaque at the gingival edge of the tooth. In its first phase, gingivitis, an inflammatory process occurs in the gingival tissue causing oral discomfort, but the following phases of periodontal disease are characterized by progressive destruction of the supporting tissues of the tooth, increased dental mobility, impairment of dental function, and, finally, tooth loss [5]. Periodontitis involves a cascade of temporally and spatially coordinated molecular processes, which degrade the host tissues in a similar way to other tissue remodeling actions. Researchers indicated the involvement of main components of the periodontal ligament extracellular matrix in the initiation and progression of periodontitis [1, 2], but the molecular mechanisms have not been identified. The presence of chronic inflammation in association with bacterial plaque leaded to enzymatic degradation of the extracellular matrix components and an increase of soluble glycosaminoglycans fractions [6], carboxy-terminal telopeptides of type I collagen (COL) molecule [7], and the 40 kDa pro-apoptotic fragment of fibronectin (FN) [8]. In addition, increased levels of pro-inflammatory cytokines (tumor necrosis factor TNF-α, interleukin IL-1β), matrix metalloproteases (MMP-8, MMP-9) and apoptosis events called anoikis, which induce detachment of cells from the matrix, were observed [9]. The main mechanism of tissue damage encountered in periodontitis is extracellular matrix degradation, in particular, COL catabolism, by matrix metalloproteinases (MMP-1, MMP-3, MMP-8, MMP-9, and MMP-13). These enzymes are synthesized and activated by resident fibroblasts and macrophages during the inflammatory process, under the action of proinflammatory cytokines [10]. Their activity is regulated at the transcriptional level, post-translational level and through tissue inhibitors of metalloproteinases [11]. Other mechanisms coordinated by external bacterial virulence factors or proinflammatory cytokines secreted by host T-lymphocytes, at the inflammatory situs, induce the expression of receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL is expressed by numerous cell types, including osteoblasts and lymphocytes, and is found in soluble form or associated with the cell membrane [12]. RANKL binds to its monocyte-expressed receptor RANK, inducing the formation of multinucleated osteoclasts. In the same time, osteoprotegerin (OPG) is a secreted receptor that acts as a competitive inhibitor of RANKL. Unlike healthy periodontal tissues, there are very high levels of RANKL and low OPG concentrations found in the gingival crevicular fluid of damaged periodontal tissues [13]. The balance within RANKL/RANK/OPG system controls the physiological processes involved in bone turnover and loss. Besides tissue damage, the cytokine network promotes fibroblast activation and proliferation, which can lead to fibrosis [5].

Periodontal disease presents a high risk for the general health, leading to systemic diseases, such as rheumatoid arthritis, chronic bronchitis, and pulmonary fibrosis [14]. Periodontal therapy targets the structural and functional regeneration of the complex structure of the periodontium, aiming the restoration of cementum lining the tooth root and the periodontal ligament attached to the cementum, together with the formation of new alveolar bone and gingiva. In the same time, the subgingival space needs protection against the pathogenic bacterial flora [2]. Stem

cell biology has enabled the development of innovative cellular therapies that stimulate the endogenous regenerative process of the periodontal tissue [15]. Thus, two-dimensional (2D) cell sheets or three-dimensional (3D) cell aggregates served as grafting materials, but only for small periodontal defects due to their low stability [16]. There are numerous stem cell sources with potential application in periodontal regeneration involving both oral and extra-oral tissues. A major problem of stem cells clinical application in periodontal regeneration strategies is their low survival rate after transplantation [17]. In order to improve the therapeutic properties of stem cells, they are implanted in biomaterials that must meet the requirements of the biological environment. A detailed investigation of stem cells-biomaterial interaction, in correlation with the role of stem cells secretome represented by cytokines, growth factors and chemokines is needed. Tissue engineering is a strategic approach for periodontal regeneration in controlled conditions. Clinically meaningful results were obtained using complex biomaterials capable of spatial and temporal guiding of the periodontal regeneration [18]. The use of composite biomaterials can be complementary to existing clinical procedures, such as guided tissue regeneration (GTR) or combined with cellular therapies and/or bioactive molecules. These emerging technologies of regenerative medicine were tested *in vitro*, in preclinical studies, and clinical trials for periodontal tissue engineering [1, 19]. They facilitate the integration of stem cells into the surrounding milieu and functional reconstruction of the periodontal complex [16].

This chapter describes the advantages and limitations of current techniques and proposes advanced techniques based on stem cells and cell carriers for the treatment of periodontal disease. Special emphasis is paid to dental pulp stem cells (DPSCs) isolation, expansion conditions *in vitro,* and their use for the regeneration of bone defects caused by the chronic periodontal disease. Their delivery after injection within natural polymeric carriers is discussed as a new therapeutic strategy for periodontal lesions.

2. Current techniques of periodontal regeneration

2.1. Guided tissue regeneration

Many clinical and biological factors can impair the process of periodontal tissue regeneration. The tissue is in permanent contact with the external environment of the oral cavity and presents a risk of infection during the regeneration process. There are several types of mechanical stress, caused by occlusal forces or dental gum stretching, which affects the mucous membranes and reduces bone resorption. Surgical techniques of periodontal tissue regeneration include root surface modification, bone grafting, and GTR. The most common regenerative treatment GTR is based on the application of barrier membranes, in order to restrict epithelial and gingival connective cells in periodontal defects and to increase the number of cementoblasts and osteoblasts that synthesize extracellular matrix and, finally, new functional tissue [20]. On GTR market, several products are intended for the clinical treatment of intraosseous defects or class II fissure defects. They consist of non-resorbable membranes of synthetic materials (expanded polytetrafluoroethylene, nylon on silicone) or resorbable membranes made of organic materials, presenting different composition, and structure. In case of using non-resorbable membranes, bone regeneration is low, and a second

surgery is needed to remove them. The resorbable membranes composed of COL types I or III extracted from bovine tendon or porcine skin are enzymatically degraded and their resorption time varies with the cross-linking degree. Polymeric membranes of polylactic acid or copolymeric materials of polylactic acid/polyglycolic acid are biocompatible, but their degradation depends on composition, pH, and presence of enzymes and bacterial infection [21]. Resorbable membranes, such as Avitene® are obtained from bovine pericardium crosslinked with diphenylphosphorylazide, Collistat® is a semi-occlusive membrane (pore size 0.004 μm) prepared from bovine dermis, BioMend® is made of type I COL from bovine tendon and exhibit variable efficacy, depending on the shape and size of the defect [22], Paroguide® from COL, and chondroitin sulfate (CS) helps to repair the periodontal ligament, cementum, and alveolar bone, without signs of inflammation [23]. Resorbable membranes have many beneficial properties as medical products, being hemostatic, chemotactic, and biocompatible, but they are not stable enough to allow the formation of new tissue. These membrane barriers are used during GTR to stimulate selective cell repopulation of the periodontal defects. Therefore, biocompatibility is the most important feature of a barrier membrane and any sign of cytotoxicity can lead to irreversible destruction of the periodontal tissue. In addition, the membranes must exhibit structural and mechanical properties, in order to maintain space and to resist against external forces. They should initiate tissue integration and be easily used in order to reduce intervention time and patients discomfort [24].

2.2. Limitations

Clinical trials have showed that periodontal treatment is effective in achieving the primary goal of preventing disease progression and loss of affected tooth. Long-term studies on periodontal disease noted that 95% of treated teeth were not lost for 10 years [25]. Although there are encouraging results, existing treatment methods have numerous limitations, and indicate the lack of complete periodontal tissue regeneration. Thus, most treatments of periodontal pocket lead to gingival retraction, which can progress over time in poor esthetics, increases tooth mobility, and affects dental functions [5]. In intraosseous defects, minor regeneration of periodontal tissue may occur in the apical region of the defect [26]. GTR can induce partial remodeling and restoration of the cortical bone, but total regeneration of bone or periodontal ligament does not occur [27]. It was noted that the biological process of epithelium growth and differentiation needed a layer of connective tissue to mediate the passage of signaling molecules [28]. Studies suggested that, in contrast to superficial connective tissue of the gingiva, deep connective tissue prevented epithelial migration and leaded to formation of a simple epithelium, phenotypically similar to the junctional epithelium [28]. Besides epithelium ingrowth, specific signaling pathway involved in the periodontal repair process is not known.

3. Stem cell therapy for periodontal regeneration

Stem cell research is one of the most promising areas of biology due to its therapeutic implications [29]. Stem cells are defined as immature, undifferentiated cells with self-renewal capacity, clonogenicity (the ability to form cell colonies), and cellular differentiation capacity [30]. The regenerative capacity of adult tissues depends on their own stem cell populations that

have the ability to self-renew and form progenitor cells capable of differentiating into specialized cells. Mesenchymal stem cells (MSCs) are pluripotent cells that can differentiate into any cell type from all three embryonic layers, including periodontal tissue associated cells. Due to their differentiation capacity, widespread tissue distribution and promising results obtained in both preclinical and clinical models of tissue repair, MSC are increasingly used in tissue engineering. Ideal stem cells must be non-immunogenic, easy to obtain, highly proliferative, and have the ability to differentiate into desired cell type. Extraoral stem cells from adipose tissue and bone marrow, as well as intraoral stem cells from periodontal ligament, dental pulp, papilla, and follicle cells were used in periodontal tissue engineering, to repair damaged parodontium [24, 31, 32]. Dental tissues are easily accessible sources of stem cells, which can be used in cell therapy of periodontal disease and maxillofacial reconstruction due to their ability to form bone, dentin and pulp tissue [15, 33, 34].

3.1. Dental pulp stem cells (DPSC) role in periodontal regeneration

The dental pulp is the vital organ of the tooth, presenting very good repair and regenerative capacity. DPSC with high proliferative capacity and multipotency were used in orthopedics and maxillofacial reconstruction for regeneration of bone and dental tissues, respectively [35]. DPSC transplantation into the human alveolar bone initiated dental pulp regeneration [36]. In addition, a graft of DPSC from the child to the parent differentiated into osteoblasts and regenerated the mineralized tissue [37] or into odontoblasts responsible for reparative dentin secretion [38, 39]. Cultivation of a large number of DPSC in medium without xenogeneic serum granted these cells with major impact in regenerative medicine and clinic applications [40, 41]. Moreover, DPSC were reprogrammed into induced pluripotent stem cells at a higher rate than other cell types [42], confirming their periodontal tissue regenerative capacity [43, 44].

MSC was first isolated from the adult dental pulp by Gronthos et al. [45] and, then, from primary dentition extracted during periodontal and oral surgery [46]. DPSC from primary dentition presented higher proliferative capacity and differentiation potential, compared to DPSC from permanent teeth [47]. Still, scientific interest has lately turned to adult DPSC because the processing and storage of stem cells from primary dentition is not possible for the majority of the population [29]. DPSC were also isolated from inflamed pulp tissue and their markers profile was similar to that of cells from normal tissue [48]. DPSC is easily accessible and available in a larger amount than MSC from bone marrow due to their high proliferation rate [49, 50].

The regenerative quality of DPSC can be influenced by the isolation process and biological parameters, such as the age of the donor, its general health status and oral health, long-term storage conditions and post-freeze viability [29, 51, 52]. Dental pulp aging results in changes that are difficult to distinguish from physiological and pathological processes. An obvious change caused by aging is pulp size decrease due to continuous secretion of dentinal matrix (secondary dentinogenesis). As a result, the number of constituent cells (odontoblasts, fibroblasts, MSC) decreases, COL and crosslinked fibers increase, lipid infiltration and calcification take place [53]. These changes suggest a decline of DPSC characteristic functions with increasing age. In conclusion, isolation of DPSC is recommended from healthy young adult donors, in order to present clinical applicability in stem cell therapy.

3.2. DPSC isolation

Both explant culture and enzymatic digestion protocols are efficient for DPSC isolation from adult teeth, considering a pulp weight of at least 0.2 g for establishing a viable primary culture [54]. Third molars (wisdom tooth) are usually used after their extraction during mandatory surgical or orthodontic treatment of healthy adults (21–34 years), with patient's agreement, according to the bioethics rules in force. Immediately after extraction, the molars are placed in 10 mM sterile phosphate buffered saline (PBS) supplemented with a mixture of antibiotics (200 U/ml penicillin, 500 μg/ml streptomycin, 400 μg/ml neomycin, and 2.5 μg/ml amphotericin) and transported to the lab, on ice, for processing or storage at 4°C, for up to 24 h (**Figure 1**). Dental pulp is extracted from the pulp chamber of molars and subjected to enzymatic digestion in a solution of 3 mg/ml type I collagenase and 4 mg/ml dispase, at 37°C, for 1 h (**Figure 1**). The obtained cell suspension is filtered through 70 μm cell strainer and cultured in minimum essential medium eagle-alpha modification (α-MEM) supplemented with 20% fetal bovine serum, 100 μM L-ascorbic acid, 2 mM L-glutamine and 1% antibiotics mixture, in a humid atmosphere with 5% CO_2, and at 37°C. The culture medium is changed every 3 days.

3.3. DPSC properties

DPSC present typical characteristics of MSC isolated from other sources, like specific phenotype, *in vitro* renewal capacity, distinctive cell surface antigens, and clonogenicity [38]. Periodic observation of DPSC seeded in a culture plate is usually performed using an inverted microscope. At 24 h of *in vitro* cultivation, DPSC adhered to the plastic surface, and in the first week of cultivation, they started to form colonies, similar to mesenchymal-type stem cells. At 12 days of cultivation, the colonies reached confluence, and the first passage was performed. Hematoxylin-eosin staining of confluent DPSC revealed the fibroblast-like morphology, characteristic for the mesenchymal phenotype (**Figure 2A**). DPSC culture processed for transmission electron microscopy [55] exhibited typical ultrastructure of MSC with spherical or irregular shaped nucleus (N) containing a large amount of euchromatin and mitochondria-rich cytoplasm (**Figure 2B**). Some cells presented rough endoplasmic reticulum (ER) with dilated cisterns (**Figure 2B**).

DPSC cultures cultivated in standard conditions were investigated for self-renewal by colony-forming units (CFU-F) analysis. At 14 days of cultivation, cell clusters of different sizes and densities were observed (**Figure 2C**). This demonstrated that subpopulations of cells were able to generate new colonies from a single cell.

MSC immunophenotyping consists in cell surface antigens analysis using flow cytometry. DPSC at passage 3 (2×10^5 cells) were washed in PBS and incubated with primary antibodies directed to specific antigens, at 4°C, for 30 min. After centrifugation at 1200 rpm, for 10 min, cells were resuspended in PBS and analyzed at a flow cytometer. Unlabelled cells were used as a control. Data are processed as histograms using provided software and the results are expressed as percentages. Flow cytometry of DPSC cultures showed similar profile to that of mesenchymal-type stem cells. Specific markers, such as CD29, CD44, CD73, and CD90 were expressed at high levels, ranging between 96 and 100%. DPSC were negative for hematopoietic markers, CD34, CD45, and CD133, in accordance with previous studies [56]. STRO-1 is a specific marker present in stromal precursors from bone marrow with multiple differentiation

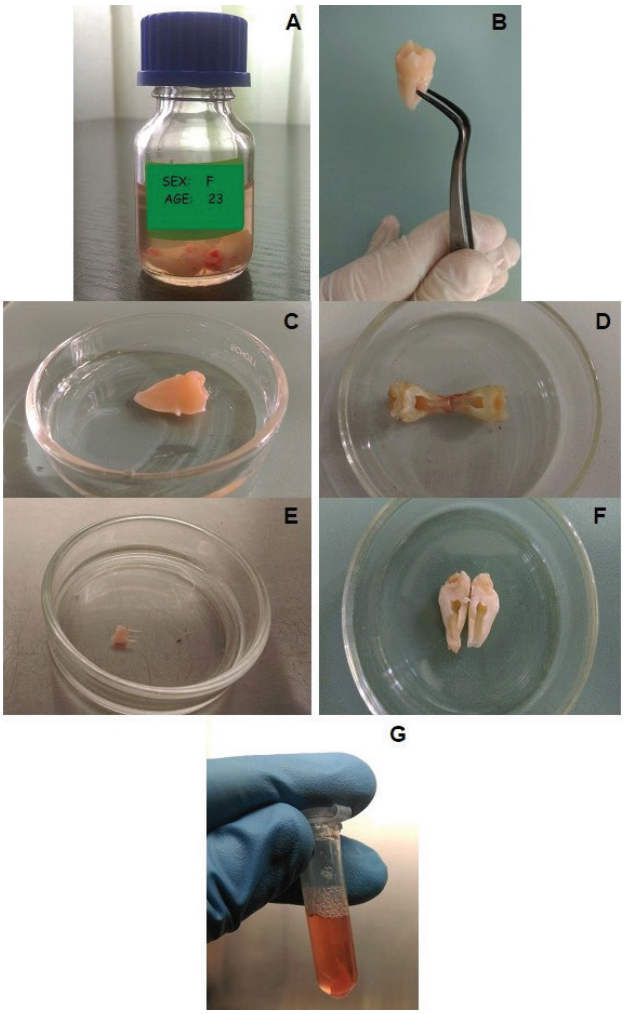

Figure 1. Isolation of stem cells from the third molar dental pulp by enzymatic digestion. The sample was transported to the lab on ice (A). Third molar (B) was thoroughly washed in 70% ethanol and PBS, pH 7.4 (C). The pulp chamber was mechanically exposed (D), the dental pulp was extracted (E) and the tooth remained empty (F). The dental pulp was digested in enzymatic solution (G).

potentials and, in particular, osteo/odontogenic. To date, DPSC populations expressing this marker [57, 58], as well as populations characterized by STRO-1 negative immunophenotype [56] were reported.

3.4. DPSC multidifferentiation capacity

DPSC exhibit plasticity and can be differentiated into osteoblasts, odontoblasts, osteocytes, chondrocytes, adipocytes, myocytes, cardiomyocytes, neural, and hepatocyte cells [29, 35, 59].

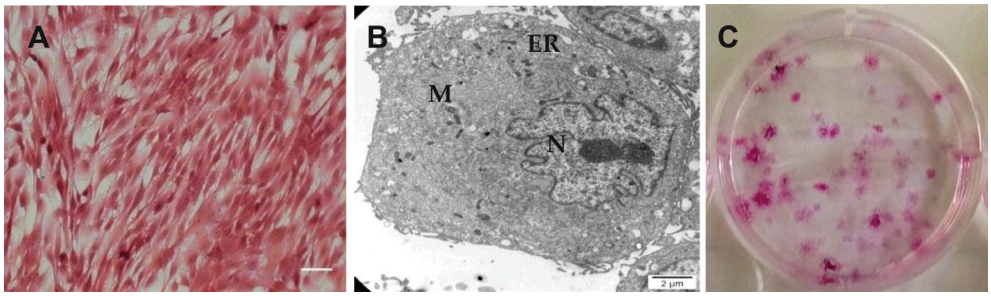

Figure 2. Light micrograph of DPSC at confluence showed characteristic spindle-shaped cell morphology (A, hematoxylin-eosin staining, bar = 10 μm). Transmission electron micrograph of DPSC revealed the N, mitochondria (M), and rough ER (B, bar = 2 μm). Colony-forming unit analysis of DPSC detected cell clusters after 14 days of *in vitro* cultivation (C, hematoxylin-eosin staining).

For DPSC differentiation, isolated cells are expanded *in vitro* by trypsinization and, at passage 5, 6, are seeded in 6-wells culture plates, at a density of 5×10^4 cells/ml in α-MEM until reaching confluence. Then, the culture medium is replaced with osteogenic differentiation medium based on 1 μM dexamethasone, 20 mM sodium β-glycerophosphate, and 45 mM L-ascorbic acid-2-phosphate [60]. The plates are incubated in a humid atmosphere with 5% CO_2, for 21 days and the medium is changed every 3 days. For adipogenic differentiation of DPSC, the monolayers are cultivated in specific induction media based on 5 μg/ml insulin, 10^{-6} M dexamethasone, 0.5 mM isobutylmethylxanthine, and 60 μM indomethacin [60]. Chondrogenic differentiation is performed in pellet mass culture (2×10^5 cells/pellet) placed in conical polypropylene tubes, centrifuged at 500 × *g*, for 5 min and cultivated in specific induction medium based on 5 μg/ml linoleic acid, 1× insulin-transferrin-selenium concentrate, 10 ng/ml transforming growth factor β1, 14 μg/ml ascorbic acid, and 10^{-7} M dexamethasone [60]. Specific staining protocols are used to analyze the morphology of differentiated stem cells.

Osteogenic differentiated cells fixed in 4% paraformaldehyde solution in PBS were stained with 2% alizarin red S, for 30 min [61], and images acquired at an inverted light microscope. DPSC cultivated in osteogenic differentiation medium, for 21 days presented characteristic calcium mineral deposits, visualized as strong red color (**Figure 3A**). Adipogenic differentiated cells stained with 0.5% Oil Red O solution in isopropanol, for 30 min [61] showed morphological changes, intracellular lipid droplets accumulation and reduced proliferation rate (**Figure 3B**). The spheroid of chondrogenic differentiated cells stained with 1% Alcian blue solution, pH 2.5, for 30 min [61] presented specific proteoglycans storage within the extracellular matrix (**Figure 3C**).

Morphological observations can be confirmed by immunofluorescence [55] or microarray investigations [62] that evidentiate specific markers expression at protein and gene level, respectively. In osteogenic differentiated DPSC, analysis of osteocalcin, osteopontin, and type I COL is performed. Adipogenic differentiation is marked by secretion of adiponectin and lipoprotein lipase, while DPSC differentiated toward chondrocytes express aggrecan, SOX-9 and type II COL [60, 62]. In each experimental model, control DPSC grown as undifferentiated cells in normal culture medium have no reaction for these specific markers.

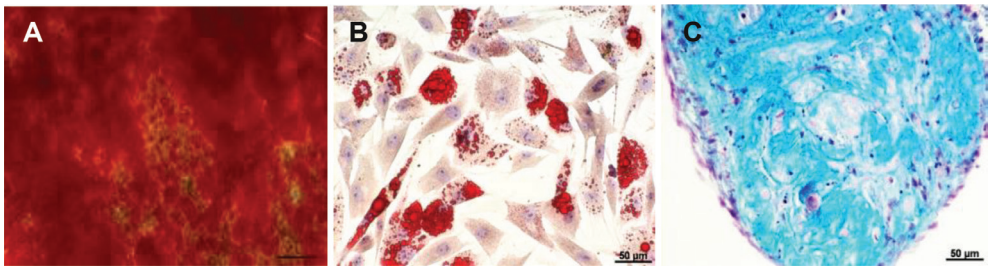

Figure 3. *In vitro* multilineage differentiation of DPSC after three weeks of cultivation in specific culture media. Osteogenesis was evidentiated by calcium deposits (A, Alizarin red S staining, bar = 100 μm). Adipogenesis was observed as intracellular lipid droplets accumulation (B, Oil Red O staining, bar = 50 μm). Chondrogenesis was showed by proteoglycans deposits in the extracellular matrix (C, Alcian blue staining, bar = 50 μm).

4. Advanced techniques for periodontal regeneration

Gene therapy and cell therapy are two advanced techniques that represent an effective solution to maximize the delivery of molecules involved in periodontal regeneration and to reduce limitations of currently used regenerative treatments. Gene therapy is a strategy for modulating host immune response triggered by dental microbiota. It consists of the direct insertion of certain genes into patient cells or indirect gene delivery using a carrier [24]. Their cellular transport is considered a more secure approach because it requires isolation of target cells and insertion of therapeutic genes under controlled conditions [32].

Cell therapy envisages induction of complete periodontal regeneration using triple complexes of cells injected in scaffolds containing bioactive molecules. The scaffold helps maintain the space of lost tissue and to protect the regenerated tissues of infection and mechanical stress. In addition, a tailored scaffold in terms of viscosity can promote high percentage of cells retention in injectable transplantation therapies [63]. Bioactive molecules, including bone morphogenetic proteins (BMP), enamel matrix protein derivatives and growth factors, such as platelet-derived growth factor, were also used to stimulate periodontal regeneration. BMP are multifunctional polypeptides that belong to the transforming growth factor-β superfamily, their main feature being the ability to induce ectopic bone formation. Bone regrowth in periodontal defects was achieved in various animal models by BMP application together with carrier systems [24]. Growth factors have regulatory effects on immune function, proliferation, and differentiation of periodontal tissue cells. Both *in vitro* and *in vivo* studies demonstrated the efficiency of platelet-derived growth factor in increasing osteoblast cells population for bone regeneration, endothelial cell multiplication for the capillary formation and fibroblasts proliferation for collagen synthesis and connective tissue regeneration [24].

4.1. Cell-scaffold constructs for periodontal regeneration

Solid biomaterials in which the cells of interest are seeded represent the most used cell transport method [64]. Their great advantages are the ease of application and the ability to encapsulate and concentrate cell suspensions at the target site. After implantation, these

cells-biomaterial constructs stimulate cell proliferation and differentiation, resulting in tissue development [15]. The structure and porosity of biomaterials are important to allow nutrients absorption and to avoid cell apoptosis in the central region of the construct. Also, the structural integrity and mechanical properties of solid biomaterials should be adjusted for specific applications of bone and periodontal regeneration. Analysis of 1-year clinical trial on 7 adult patients (24–40 years old) demonstrated that a construct of autologous DPSC in 3D COL sponge could restore the mandible bone defects occurring after molar extraction, with final regeneration scores of 70–100% [40]. Other polymeric composites, hybrid materials or biomaterials incorporating biological active factors, such as platelet-rich plasma and fibrin-rich plasma, have been developed to transport DPSC for human periodontal tissue formation [65–68]. Implantation in animal models demonstrated repair of affected cementum, periodontal ligament, and alveolar bone. *In vitro* and *in vivo* studies demonstrated that DPSC in 3D systems have high osteogenic potential and increased the bone tissue production [38, 69]. Further tailoring is needed in order to be used as dental implants for bone tissue engineering.

4.2. DPSC-scaffold constructs fabrication

Natural proteic, polysaccharidic, and glycoproteic polymers having high molecular weight, such as COL types I, III, and V, CS contained by decorin and biglycan proteoglycans and FN were reviewed as main components of the oral extracellular matrix [4]. They were mixed in different weight ratios, at room temperature, for 2 h, to obtain biocompatible variants of composite material with regenerative properties. The mixtures were conditioned as 3D porous scaffolds by lyophilization [70]. Sterile scaffolds (~0.25 cm^3) were placed in 24-wells culture plate and 200 μl α-MEM culture medium supplemented with fetal bovine serum, containing 5×10^5 DPSC were injected into the scaffold. Cell suspension was absorbed during 30 min of incubation in a humid atmosphere with 5% CO_2, at 37°C. Cell-scaffold constructs were covered with 500 μl culture medium and incubation continued in standard conditions. The culture medium was changed every 48 h.

4.3. Biologic activity of DPSC-scaffold constructs

Assessment of cell viability, membrane integrity, cell adhesion, and proliferation are useful tools, in order to select optimal ratios between scaffold's polymers and to establish DPSC cultivation conditions.

A method for assessing DPSC viability within cell-scaffold constructs cultivated in standard conditions, for different periods of time is live/dead assay based on cellular esterase activity. Calcein AM penetrates living cells membrane and is transformed into fluorescent calcein under the action of esterases. Ethidium homodimer-1 penetrates only cells with the damaged membrane, intercalates DNA double helix, and emits red fluorescence. Live/dead assay allows simultaneous staining of live and apoptotic cells, measuring both cell viability and plasma membrane integrity. For the experiment, DPSC-scaffold constructs incubated in standard cultivation conditions, for 72 h are washed in PBS and, then, 20 μM calcein-AM and 5 μM ethidium homodimer-1 in PBS are added. The plates are incubated in the dark, at room temperature, for 20 min. The images of cell-scaffold constructs are acquired at 490 nm using an inverted fluorescence microscope equipped with a photo camera. A large population of

viable cells colored in green was observed in COL-CS-FN composite material after 72 h of cultivation (**Figure 4A**). Adhered cells were distributed throughout the entire scaffold. Cells in different stages of apoptosis, colored in red, were present in very low number, indicating a very good biocompatibility of the natural composite scaffold toward stem cells from dental pulp tissue.

For observations on DPSC infiltration capacity and the degree of cell colonization within composite materials, scanning electron microscopy is a useful technique. Cross sections of cell-scaffold constructs cultivated in standard conditions, for 48 h are fixed in 2.5% glutaraldehyde in cacodylated buffer, at room temperature, for 20 min. Then, samples are visualized using an environmental scanning electron microscope, operated at 15 kV, in an inert nitrogen atmosphere. Scanning micrographs showed that DPSC infiltrated the 3D composite scaffold and adhered to pore walls as isolated cells (**Figure 4B**) or group of cells (**Figure 4C**).

For cell proliferation assessment in cell-scaffold constructs, MTS test is applicable after PBS washing to remove unattached cells. Cells are incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate, at 37°C, for 3 h. The tetrazolium salt is reduced in the presence of mitochondrial dehydrogenases from the viable cells and generates a colored product. Thus, its quantity measured as optical density at 490 nm is directly proportional to the number of metabolically active cells. The degree of DPSC proliferation in COL-CS-FN composite scaffold was reported to a control of DPSC on the 2D plastic surface. The results showed that, at 48 h of cultivation, cell proliferation was 1.46 times higher than in control and, at 96 h of cultivation, DPSC proliferated 1.62 times more than in control. These data indicated that 3D composite scaffold stimulated DPSC proliferation.

The influence of FN on cell adhesion within DPSC-scaffold constructs can be analyzed using DNA fluorometric assay. The constructs are minced and mixed with a lysis solution of 30 mM saline sodium and 0.2 mg/ml sodium dodecyl sulfate, at 37°C, for 1 h. The mixture is then centrifuged at 13,000 × *g*, for 15 min and the supernatant used to determine the DNA content.

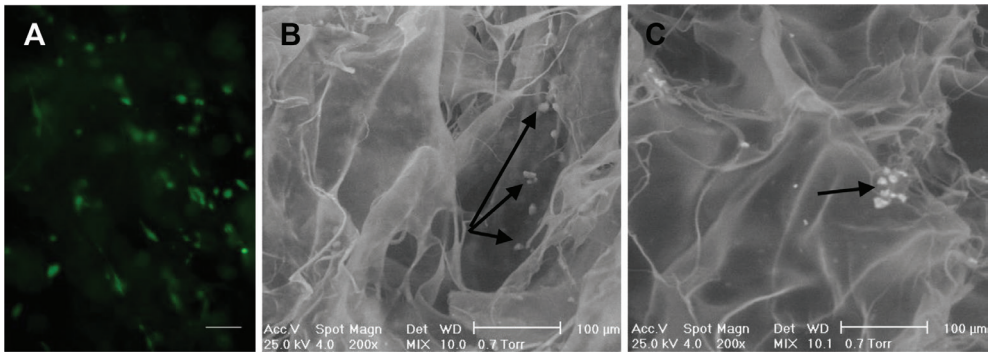

Figure 4. Constructs of DPSC in 3D porous composite scaffold based on collagen, CS and fibronectin in $10{:}1{:}10^{-8}$ (w/w/w) ratio. Fluorescence microscopy showed viable DPSC in biocompatible constructs after 72 h of cultivation in α-MEM (A, bar = 50 μm). Scanning electron micrographs revealed isolated DPSC (B, bar = 100 μm) or cell groups (C, bar = 100 μm) within the composite scaffold.

Briefly, over 10 μl of supernatant, 190 μl of working solution is added, followed by a vortexing step, incubation at room temperature, for 2 min, and the optical density is recorded at a fluorometer for nanoquantities. Taking into account a content of 8 μg DNA/cell [71], the results can be expressed as cell count/scaffold. Statistical analysis using Student's test on control-sample pairs of interest show differences considered significant at $p < 0.05$. The results obtained for DPSC cultivated in COL-CS-FN composite scaffold in standard conditions, for 96 h showed the significantly higher number of DPSC (786,675 ± 23,600 cells/scaffold) than in COL-CS scaffold (718,335 ± 25,100 cells/scaffold). This could be due to FN presence in materials composition and indicated its usefulness for improvement of cell adhesion. FN supports cell adhesion through synergistic action of both integrin-binging regions and N-glycans [72]. Variation of FN concentration and distribution is a topic of interest for cell-scaffold construct fabrication.

Further incubation of DPSC-scaffold constructs in standard conditions indicated that cell adhesion and proliferation decreased, probably as a result of cell migration outside the porous scaffold. Tailoring the pore size of 3D materials by polymer concentration, lyophilization temperature or cross-linking should adjust the adhesion and proliferation of DPSC for longer periods of cultivation.

4.4. Osteogenic properties of DPSC-scaffold construct

Osteogenic differentiation of DPSC is of interest in repairing bone defects, including in dentistry for elderly people with a deficit of stem cells. DPSC-scaffold constructs could serve as biomimetic experimental models, in order to improve the fabrication of dental materials with modified surfaces, and enhanced bioactivity. Individual components of ECM, like type I COL and FN, promote osteogenic differentiation of stem cells, but the process is influenced by environmental culture conditions [73]. COL-CS-FN composite scaffolds injected with DPSC were cultivated in specific osteogenic differentiation medium, for 21 days. Conditioned medium was harvested at 3, 6, 9, and 12 days of cultivation and analyzed for secretion of specific markers, such as alkaline phosphatase, calcium, and type I COL, using specific and sensitive techniques.

MSC are characterized by the low activity of alkaline phosphatase, while its increased activity is an index of cell differentiation into fully functioning osteoblasts. The analysis of alkaline phosphatase activity uses 50 μl supernatant incubated with 7.34 mM p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8, containing 2.58 mM $MgCl_2$, at 37°C, and for 30 min. The reaction is stopped with 1 M NaOH solution and the optical density is recorded at 410 nm using a microplate reader. The alkaline phosphatase activity is calculated using a standard curve of p-nitrophenol and expressed as mM p-nitrophenol/min. For comparable results, the protein concentration of cell supernatants is determined using Bradford method. The results obtained for DPSC in COL-CS-FN scaffolds indicated that alkaline phosphatase activity increased by 4 times in the first 10 days of cultivation in osteogenic medium (**Figure 5**). After another 10 days of cultivation, the enzymatic activity reached 10 times higher values than at 3 days of cultivation. The steep slope of alkaline phosphatase activity profile demonstrated the differentiation of DPSC into osteoblasts within 3D composite scaffolds of COL-CS-FN cultivated in osteogenic differentiation medium.

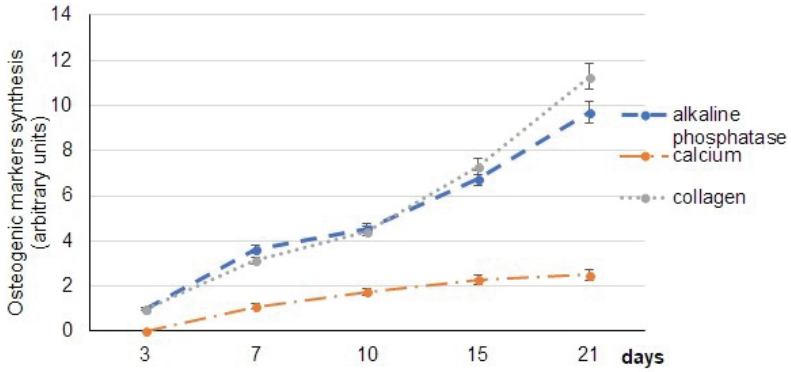

Figure 5. DPSC cultivated within 3D porous composite scaffold based on collagen, CS, and fibronectin in $10:1:10^{-8}$ (w/w/w) ratios, in the presence of osteogenic induction medium, for 21 days. Increasing quantities of alkaline phosphatase, calcium, and type I collagen showed DPSC differentiation into osteoblasts.

Determination of calcium amount deposited by cells can be performed using a quantification assay kit based on o-cresolphthalein dye reagent, which forms purple stable complexes with calcium ions. Supernatant of culture medium (50 μl) is incubated with 90 μl chromogenic reagent and 60 μl buffer, at room temperature, for 10 min. The optical density is recorded at 575 nm using a microplate reader and final results are expressed as nm calcium/μl. In case of DPSC in COL-CS-FN composite scaffold, the results indicated a rapid increase of calcium secretion in the first 10 days of osteogenic differentiation and, then, the values reached a plateau (**Figure 5**).

An important marker of stem cells differentiation into osteoblasts is type I COL synthesis. To avoid the interference of COL present in composite materials or degraded during incubation in the culture medium, it is recommended to use an antibody that specifically detects the propeptides from the C-terminal end of type I procollagen molecule. These are enzymatically cleaved and released into the medium only during the synthesis of triple helical COL molecule. The amount of type I procollagen secreted is quantified by incubation of 50 μl culture supernatant in 96-wells culture plate pre-coated with a specific monoclonal antibody, at 37°C, for 1 h. After subsequent washing steps, as provided by ELISA protocol, the incubation with peroxidase conjugate polyclonal antibody is performed, followed by substrate addition. Optical density is read at 450 nm using a microplate reader and the results are calculated as ng type I procollagen/ml. The results obtained for DPSC in COL-CS-FN scaffold presented similar variation to that of alkaline phosphatase activity (**Figure 5**). The curve profile of procollagen synthesis increased throughout the entire cultivation period, but higher concentrations of type I procollagen were registered after 10 days of cultivation in osteogenic medium (**Figure 5**).

In conclusion, specific markers of osteoblastic differentiation, alkaline phosphatase, calcium, and type I COL were secreted by DPSC cultivated in direct contact with COL-CS-FN composite materials. This indicates the possible use of these constructs not only as experimental models *in vitro* but as materials with osteogenic effect in periodontal tissue engineering. In addition, the values of all markers were significantly higher ($p < 0.05$) than those obtained in 2D DPSC cultures. The values demonstrated that 3D porous composite materials had a

positive effect on calcium secretion and mineralization process useful in bone repair, as well as the capacity of cell-scaffold constructs to improve new extracellular matrix formation.

5. Conclusions

Periodontitis is a very common condition that poses a challenge to oral tissue engineering. Periodontal disease affects all four tissues of the periodontium, namely the gingival tissue, periodontal ligament, cement and alveolar bone and can be partially treated by classic periodontal surgical methods. Specialized therapeutic methods, such as GTR, stem cell therapies, and innovative biomaterials including bioactive molecules, envisage the functional reconstruction of the periodontal complex, and reduction of periodontal disease risk for general health. Stem cells from numerous sources have potential application in periodontal regeneration because they are non-immunogenic, have high proliferation rate and ability to differentiate into the desired cell type. Stem cells from dental pulp are easy to obtain and currently studied in combination with 3D porous biomaterials of certain porosity, mechanical and regenerative properties. Cell culture data demonstrated that COL-CS-FN composite material had the capacity to improve the secretion of osteogenic markers and the synthesis of bone matrix. These studies are important and can provide progress in periodontology, especially for elderly individuals presenting marked decline of healing and regenerative processes. Future researchers envisage multifunctional engineered constructs with antimicrobial, anti-inflammatory, and regenerative activity for use in periodontitis treatment.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Ana-Maria Seciu[1], Oana Craciunescu[2]* and Otilia Zarnescu[1]

*Address all correspondence to: oana_craciunescu2009@yahoo.com

1 Faculty of Biology, University of Bucharest, Romania

2 National Institute of Research and Development for Biological Sciences, Bucharest, Romania

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