

Periodontal regeneration following implantation of cementum and periodontal ligament-derived cells

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Background and Objective: The periodontal regeneration of bone defects is often unsatisfactory and could be largely improved by cell therapy. Therefore, the purpose of this study was to evaluate the regenerative potential of implanting canine cementum-derived cells (CDCs) and canine periodontal ligament-derived cells (PDLDCs) in experimentally created periodontal intrabony defects in beagle dogs.

Material and Methods: Cells were obtained from premolars extracted from four beagle dogs. Three-wall intrabony periodontal defects, 3 mm wide and 4 mm deep, were surgically created in their second and fourth premolars and plaque was allowed to accumulate. Once the defects were surgically debrided, periodontal regeneration was attempted by random implantation of collagen sponges embedded with 750,000 CDCs, 750,000 PDLDCs or culture medium. After 3 mo of healing, specimens were obtained and periodontal regenerative outcomes were assessed histologically and histometrically.

Results: The histological analysis showed that a minimal amount of new cementum was formed in the control group (1.56 ± 0.39 mm), whereas in both test groups, significantly higher amounts of new cementum were formed (3.98 ± 0.59 mm in the CDC group and 4.07 ± 0.97 mm in the PDLDC group). The test groups also demonstrated a larger dimension of new connective tissue, resulting in a significantly more coronal level of histological attachment.

Conclusion: This proof-of-principle study suggests that cellular therapy, in combination with a collagen sponge, promoted periodontal regeneration in experimental intrabony periodontal defects.

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The periodontium is a complex organ comprising four mesenchymal tissue components (cementum, bone, gingiva and periodontal ligament) that act as a functional unit, providing the tooth with an attachment apparatus capable of withstanding masticatory forces (1). Amongst these components, the peri-

odontal ligament (PDL) has the important role of supporting the tooth and maintaining the homeostasis of the periodontium. This tissue is highly vascularized and contains many cell types, including fibroblasts and endothelial, epithelial, nerve and mesenchymal progenitor cells. These latter

cells are mature multipotent mesenchymal stem cells (MSC-PDL) (2) that are able to differentiate into multiple cell lineages, as well as to self-renew and maintain their multipotent capacity (3–5). MSC-PDL have demonstrated the potential to differentiate into osteogenic, adipogenic progenitor

cells (6) and precursors of the neural crest-like cells (7). It is not easy to identify MSC-PDL because there is no single antigenic marker specific for these cells (5); therefore, because MSC-PDL have many cell-surface molecules in common with mature hematopoietic cells, a combination of markers are used for their identification, such as CD13, CD29, CD44, CD59, CD90, CD10 (8) and STRO-1 (9). According to the International Society for Cellular Therapy (ISCT), three minimal criteria must be fulfilled for the *in vitro* identification of MSCs: adherence to plastic, a specific surface-antigen expression pattern (CD73⁺, CD90⁺, CD44⁺, CD105⁺, CD34⁻, CD45⁻, CD11b⁻, CD14⁻, CD19⁻, CD79a⁻ and HLA-DR⁻) and differentiation potential (osteogenic, chondrogenic and adipogenic lineages) (10). Canine MSCs derived from PDL have only demonstrated expression of CD146 and STRO-1 (11). MSCs have been isolated not only from the PDL, but also from the dental pulp (12,13), in exfoliated deciduous teeth (14) and in the apical dental papilla (15).

MSCs have seldom been used therapeutically and most cell therapy attempts in oral and maxillofacial surgery applications have employed bone marrow stromal mesenchymal stem cells (BMSMCs) because these cells favour osteogenic differentiation vs. other cell lineages (16). In canine models, BMSMCs vehicled in ceramic hydroxyapatite/tricalcium phosphate scaffolds (17,18) promoted bone regeneration in alveolar defects and enhanced bone-implant contact when compared with autogenous bone grafts plus platelet-rich plasma (19). In bone-regenerative applications the BMSMCs have been tested in combination with various scaffolds in sinus lift procedures (20) or for the treatment of cleft palate defects (21,22). These studies suggest that cell therapy may promote the bone-regenerative capacity of different bone grafts and hence reduce the donor site morbidity associated with autogenous bone grafting.

In periodontal regeneration the cell therapy approach has been rarely exploited. The periodontium destroyed during chronic periodontitis has lim-

ited capacity for self-regeneration. In fact, once the infection has been removed from the affected roots, the cells repopulating the wound will determine the nature of the tissue interface, mainly through the establishment of a long-junctional epithelium. This reparative process may be prevented when the epithelial and gingival connective tissue cells are excluded from the wound-healing process and cells from the PDL are allowed to colonize the wound, thus achieving periodontal regeneration (23). This periodontal-regenerative potential has been attributed to undifferentiated MSCs from the PDL that, together with appropriate growth and differentiation factors, may promote the formation of new cementum, connective tissue attachment and bone (1,24).

Even though the presence of MSCs in the PDL and the pulp is well established (9), it is not well known how these cells behave *in vivo* or the factors that influence their differentiation into the different cell lineages. A few investigations have attempted to isolate and culture these cells, as well as to study their potential to form periodontal tissue (25–28). Preisig & Schroeder (29) cultured PDL cells in root slices and developed an *in vitro* PDL-like tissue. In another experiment, demineralized roots with attached PDL cells were re-implanted *in vivo* in monkeys. Healing resulted in the formation of new collagen fibers perpendicularly oriented between the bone and the root. However, ankylosis and root resorption were also observed (30). These first attempts at regeneration demonstrated the need to isolate progenitor cells and to use suitable scaffolds to deliver the cells and growth factors to guide cell differentiation appropriately. Seo *et al.* (2) isolated human MSCs from the PDL and used hydroxylapatite/tricalcium phosphate scaffolds to implant these cells in artificially created periodontal defects in the mandibular molar of six immune-deficient rats. Eight weeks later the authors found a thin layer of cementum-like tissue with attached condensed collagen fibers, resembling Sharpey's fibers. Similarly, Zhao *et al.* (31) also implanted MSCs within a

polymer sponge in a fenestration-defect model in rats and reported differentiation of mice cementoblasts with the ability to form mineralized tissue after 6 wk.

Our research group has previously reported (32,33) a method for the isolation of MSCs derived from human PDL and their differentiation into cementoblasts and fibroblasts by immunocytochemistry and RT-PCR. These human cementum-derived cells (human CDCs) were positive for ameloblastin, amelogenin, cementum protein 1 (CEMP-1), cementum attachment protein (CAP) and osteocalcin. Once the methodology for the isolation and characterization of CDCs was established, we aimed to evaluate their capacity to promote periodontal regeneration in a preclinical experimental model. Accordingly, the goal of the present investigation was to evaluate the regenerative potential of implanted canine CDCs and canine periodontal ligament-derived cells (PDLDCs) within a collagen sponge (CS) used as scaffold in experimentally created, critical intrabony periodontal defects.

Material and methods

Cell isolation and culture

This experimental animal study was approved by the Ethical Research Committee of the 'Gomez Ulla' Central Military Hospital (Madrid, Spain). Four male, 1-year-old Beagle dogs weighing about 10 kg were selected and subjected to the prescribed quarantine period. Although the animals presented healthy periodontal tissues, full-mouth prophylaxis using ultrasonic scalers and manual curettes was carried out prior to the experimental phase. Canine CDCs and canine PDLDCs were isolated and characterized using the method previously reported by our research group (32). In brief, first and third mandibular premolars were carefully extracted under general anaesthesia (inhalatory anaesthesia – isoflurane – induced with intravenous propofol) and immediately placed in serum-free, alpha-modified Eagle's Medium (α -MEM/F12) nutrient mixture supplemented with 100 U/mL

of penicillin and 100 µg/mL of streptomycin. PDL was scraped from the roots using a 7/8 Gracey curette (Hu-Friedy, Chicago, IL, USA) and the tissue obtained was digested by incubation, at 37°C, in medium containing 3 mg/mL of collagenase type I and 4 mg/mL of dispase. After 40 min, digested tissue was centrifuged for 7 min at 200 g and washed four times with fresh medium. Cells were then passed through a 70-µm strainer onto Petri dishes containing α-MEM supplemented with 15% fetal bovine serum, 100 µM ascorbic acid 2-phosphate, 2 mM glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin, and the plates were placed inside an air incubator at 37°C with an atmosphere of 5% CO₂/95%. Under these conditions, a cluster of mesenchymal cells grew after 4 d *in vitro*, forming the so-called colony-forming unit. Every 2 d or so, when the cultures became semiconfluent, cells were detached and placed in a medium containing trypsin-EDTA. The number of PDLDCs doubled in a 2- to 3-d period and these cells were used for experiments within the fourth and fifth passages. The remaining root was left in the same medium, containing 3 mg/mL of collagenase type I and 4 mg/mL of dispase, for 40 min at 37°C. The root was then washed four times with fresh medium and thin root layers were scraped off in an apical-coronal direction using a 15c blade. These layers were washed and cut into smaller pieces using microsurgery scissors and then digested again by incubation, for 40 min at 37°C, in medium containing 3 mg/mL of collagenase type I and 4 mg/mL of dispase. Small tissue pieces were then plated in a Petri dish containing α-MEM supplemented with 15% fetal bovine serum, 100 µM ascorbic acid 2-phosphate, 2 mM glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin, and placed in a 5% CO₂/95% air incubator at 37°C. After 10 d of culture, a pool of canine CDCs grew, forming discrete cell colonies. Every 4 d or so, when the cultures became semiconfluent, the cells were detached using medium containing trypsin-EDTA. The number of CDCs

doubled in a 3- to 4-d period, and these cells were used for experiments within the fourth and fifth passages.

Immunocytochemistry

Canine CDCs and PDLDCs (at passages four to five) were fixed with 4% formaldehyde and analysed using immunocytochemistry. In brief, CDCs were incubated with primary antibodies against osteocalcin (1 : 100 dilution; Alexis Biochemicals, Lausanne, Switzerland), CEMP-1 (1 : 100 dilution) and STRO-1 (1 : 100 dilution; Santa Cruz Biotechnology, Heidelberg, Germany) in 10% goat serum for 90 min. PDLDCs were incubated with antibodies against osteocalcin (1 : 100 dilution), STRO-1 (1 : 100 dilution), CEMP-1 (1 : 100 dilution) and CD44 (1 : 100 dilution; AbCAM, Cambridge, UK) under the same experimental conditions. The samples were then incubated, for 60 min, with fluorescein isothiocyanate (FITC)-labelled secondary antibodies (Invitrogen, Barcelona, Spain). Stained cells were identified by fluorescence microscopy.

Flow cytometry analysis

PDLDCs (at passages four to five) were cultured in α-MEM supplemented with 15% fetal bovine serum, 2 mM glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Then, the cells were incubated with mouse anti-human serum to CD73, CD90, CD44, CD105, CD34, CD45, CD11b, CD80, CD19 and HLA-DR. Then, the cells were incubated with a 1 : 100 dilution of FITC-conjugated goat anti-mouse IgG and analyzed using an EPICS XL flow cytometer and the Expo 32 ACS software® (Coulter Electronics, Brookvale, Australia).

Mineralization and Von Kossa staining

Canine CDCs and PDLDCs (at passages four to five) were plated at a density of 10⁴ cells/well in sterile six-well plates and cultured in a mineralizing medium [Dulbecco's modified Eagle's minimal essential medium (DMEM)/F12] containing 15% fetal

bovine serum and 1% penicillin/streptomycin (diluted 100-fold from a stock solution containing 5000 U/mL of penicillin, 5000 U/mL of streptomycin, 50 mg/mL of ascorbic acid, 10 mM sodium β-glycerophosphate and 5 µM dexamethasone). The medium was changed every 2 d and mineralization was tested after 3 wk using Von Kossa staining and bright-field microscopy. For Von Kossa staining, the cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 10 min. After washing with distilled water, the cells were incubated in 5% silver nitrate at 4°C for 40 min. Subsequently, the cells were washed with water and 5% sodium thiosulfate solution was added at room temperature for 5 min. After removal of the sodium thiosulfate solution and washing, the cells were subjected to light microscopy for analysis.

Experimental study

The same animals that provided the extracted teeth for the isolation of CDCs and PDLDCs were used in the experimental study. Intrabony periodontal defects were created under general anaesthesia [inhalatory anaesthesia with isoflurane and then induced with intravenous (i.v.) propofol]. Buccal and lingual mucoperiosteal flaps were raised in contralateral jaw quadrants through intrasulcular incisions from the mesial aspect of the second premolar to the first molar. Using a hand-piece and fissure burs under copious irrigation, three-wall intrabony periodontal defects, measuring 3–4 mm in buccolingual, mesiodistal and apicocoronal directions, were surgically created at the mesial and distal aspects of second premolars and at the mesial aspect of the fourth premolars, thus generating six defects in each animal. Orthodontic wire ligatures were then placed and secured around the affected teeth and inserted into the defects before the flaps were repositioned and sutured with resorbable interrupted sutures (Ethicon, Summerville, NJ, USA). The dogs were then fed with a soft diet to allow plaque accumulation and thus mimic chronic periodontitis. After

1 mo of plaque accumulation, the dogs were sedated with 80 µg/kg of medetomidine, 20 µg/kg of butorfanol and 100 µg/kg of atropine sulfate and the ligatures were removed together with the accumulated plaque and calculus using ultrasonic scalers. Seven days later (5 wk after the creation of the defects), regenerative surgery was carried out using the same anesthetic protocol described earlier. After raising buccal and lingual mucoperiosteal flaps, the intrabony periodontal defects were carefully debrided and the roots were planed with curettes. The following intrabony measurements were recorded using a North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA): the distance between the alveolar bone crest and the cemento–enamel junction (ABC–CEJ), the distance from the ABC to the base of the defect (ABC–BD) and the bucco–lingual dimension of the defect. All measurements were rounded down to the nearest millimeter. With the use of a round bur, a reference notch was made at the base of the defect. The three modalities of treatment were always applied in the same sequence in order to avoid repeating the treatment in the defects. The randomization of treatments was performed by the tossing of a coin, selecting which treatment to start at the mesial intrabony periodontal defect of the second premolar (Table 1). The regenerative treatments tested consisted of the implantation of a CS (Xemax Surgical Products Inc., Napa, CA, USA) containing (a) > 750,000 CDCs (test), (b) > 750,000 PDLDCs (test) or (c) 50 µL of culture medium (control) into the periodontal defect. The flaps were then reposi-

tioned and sutured with resorbable interrupted sutures (Ethicon). To ensure the presence of viable cells in the scaffold (CS) we previously assessed the maintenance of living cells by incubating canine CDCs for 24 h at 37°C in a 5% CO₂/95% air atmosphere and then after 5 h in a refrigerator (4°C) to mimic the conditions to which cells are exposed during transit from the laboratory to the operating room. Cell viability was assessed by fluorescence microscopy after staining the scaffold with fluorescein diacetate (50 µg/mL, 3 min) and propidium iodide (20 µg/mL, 30 s) (Fig. 1).

After the experimental surgery, all animals received antibiotics (amoxicillin, 15 mg/kg intramuscularly, every 48 h for 7 d) and anti-inflammatory medication (meloxicam, 0.2 mg/kg subcutaneously, every 24 h for 3 d) and were fed with a soft diet for 2 wk to reduce potential mechanical trauma to the surgery area. A chlorhexidine gluconate (0.12%) spray was topically applied every third day for a 3-mo period.

Histological study

Three months after surgery, the dogs were killed by an overdose of sodium pentobarbital and the tissues were fixed by vascular perfusion through the carotid arteries with Karnovsky fixative solution (34). The lower jaw was then removed and immersed in Karnovsky solution for 1 wk. The experimental teeth were hemisected and tissue blocks containing the treated root and its surrounding soft and hard tissues were dissected and processed for ground sectioning according to the method described by Donath & Breuner (35). The tissue blocks were sectioned at the most central aspect of the defect using a dental radiograph to ensure proper orientation. One mesiodistal section was prepared from each tissue block and reduced to a thickness of approximately 20 µm using a microtome (Exakt®; Apparatebau, Norderstedt, Germany). The sections were stained using the Levai–Laczkó technique (36). All specimens were analyzed histologically and histometrically under a light microscope (Eclipse E800; Nikon Inc.,

Tokyo, Japan) equipped with a computerized image-analysis system (NIS Elements BR, Nikon DS-R1; Amstelveen, the Netherlands). A well-trained examiner (J.N.), blinded to the groups, carried out all histological evaluation and histometric analysis. To ensure reproducibility and calibration before the histometric analysis, a subset of 10 samples was measured in duplicate after 1 wk. This analysis resulted in 100% agreement between the duplicate measurements, within a range of ±0.3 mm. The following measurements were recorded in each section: the distance from the CEJ to the notch (CEJ–Notch); the distance between the gingival margin (GM) and the apical end of the junctional epithelium (JE) (GM–JE); the distance from the CEJ to the GM (CEJ–GM); the distance between the CEJ and the apical end of the JE (CEJ–JE); the distance between the apical extent of the JE and the most coronal cementum (JE–CC); the distance between the most coronal extent of newly formed cementum to the notch (CC–Notch); and the distance between the most coronal osseous crest and the notch (OC–Notch).

Statistical analysis

Means and standard deviations were calculated for each clinical and histological parameter in both experimental and control groups, with the beagle dog serving as the unit of analysis ($n = 4$). When only two means were compared, the Student's *t*-test was used. For more than two groups, statistical significance of the data was assessed using analysis of variance and compared using Bonferroni's multiple-comparison test. Differences were considered significant at $p < 0.05$.

Results

Culture and characterization of canine CDCs and PDLDCs

Isolation and culture of experimental cells obtained from the canine root-rendered cells with the distinct ability to form cell colonies and demonstrating the characteristic CDC and PDLDC phenotypes, was carried out.

Table 1. Distribution of the treatments used to treat intrabony periodontal defects

Area	Control	Test 1	Test 2
Mesial PII	4	2	2
Distal PII	2	3	3
Mesial PIV	2	3	3

The values given represent the number of treatments.

Control, culture medium; Test 1, canine cementum-derived cells (CDCs); Test 2, canine periodontal ligament-derived cells (PDLDCs).

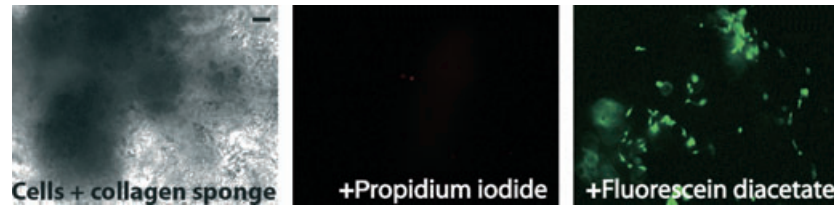


Fig. 1. Cell viability in the carrier [collagen sponge (CS)]. The CS was impregnated with canine cementum-derived cells (CDCs) in 50 μ L of culture medium and incubated for 24 h at 37°C in a 5% CO₂/95% air incubator and for 5 h in a refrigerator (4°C). The left image shows transmitted light of the cells in the CS. The center image shows dead cells stained with propidium iodide. The right image shows live cells stained with fluorescein diacetate. Bar = 5 μ m, the magnification was the same in all panels. The results shown are representative of three experiments.

PDLDCs started to spread out from the explants after 4 d *in vitro* (Fig. 2A), whereas CDCs did the same after 10 d *in vitro* (Fig. 2B). After 60 d *in vitro*, both cell types formed colonies with clear deposition of mineralized tissue

(Fig. 2C) and amorphous brown/black precipitates after Von Kossa staining (Fig. 2D). Immunocytochemical analysis was performed on fourth-passage (60 d *in vitro*) CDCs using anti(α)-CEMP-1, α -STRO-1 and α -osteocalcin

and on fourth-passage PDLDCs using α -STRO-1, α -CD44, α -CEMP-1 and α -osteocalcin. Figure 3A shows positive immunofluorescence staining in CDCs for α -CEMP-1 (78% of the CDC population) and α -osteocalcin (66%),

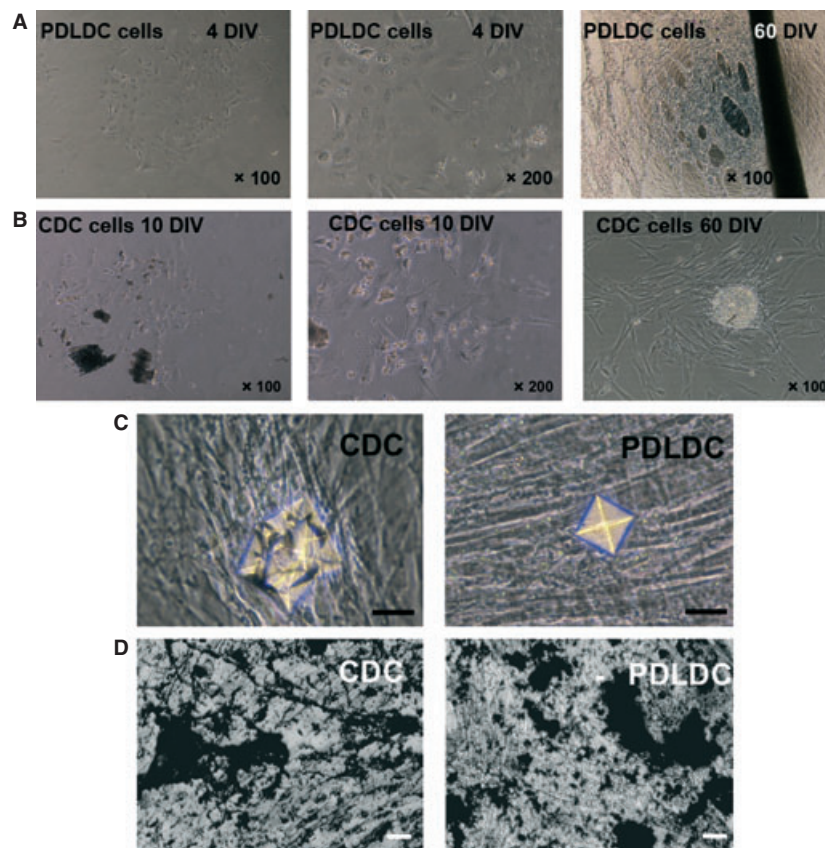


Fig. 2. Canine cementum-derived cells (CDCs) and canine periodontal ligament-derived cells (PDLDCs) form cell colonies and mineral. Teeth from young beagle dogs were used as a source of PDL. (A) Periodontal ligament was scraped from the tooth and digested using collagenase and dispase. After 4 d *in vitro*, the cells started to form colonies, termed fibroblast colony-forming units (CFU-F). At 60 d *in vitro*, the culture had completed four passages. (B) The external layers from root surfaces were removed and digested using collagenase and dispase. Cells from the cultured tissue explants started to spread out after 10 d *in vitro* and to form colonies and a mineralized tissue. A representative example at 60 d *in vitro* is shown. CDCs and PDLDCs cultured for 3 wk with dexamethasone, ascorbic acid and β -glycerophosphate were able to form mineralized tissue, as shown by light microscopy (C) and Von Kossa staining (D). Bar = 10 μ m in all panels. Data are representative of three independent experiments.

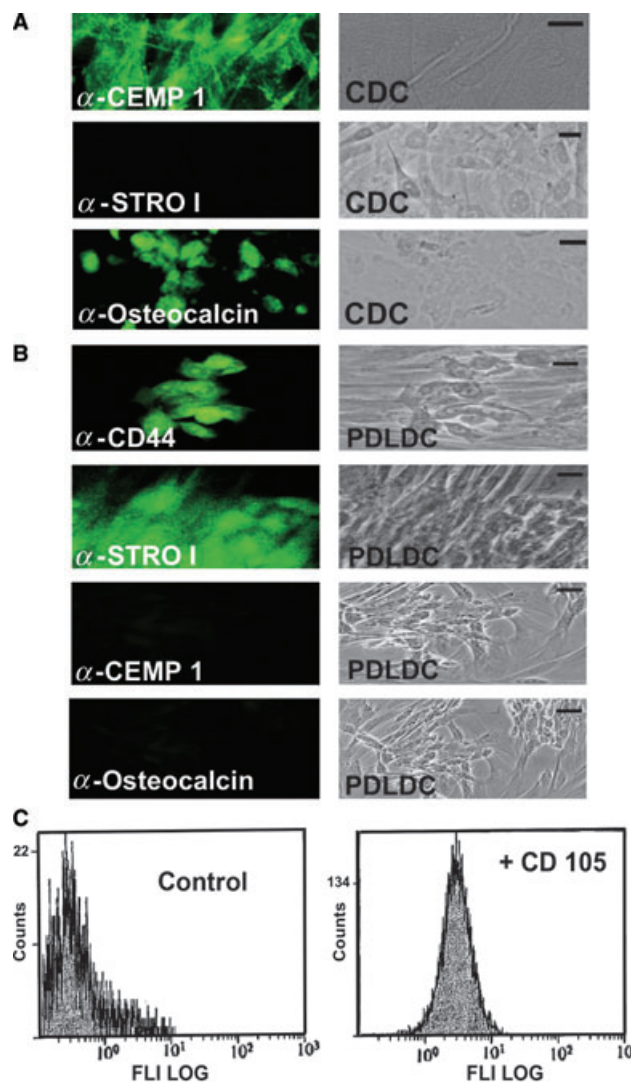


Fig. 3. Characterization of cell markers in canine cementum-derived cells (CDCs) and canine periodontal ligament-derived cells (PDLDCs). (A) CDCs were subjected to immunocytochemistry, using, as probes, anti-cementum protein 1 (α -CEMP-1), α -STRO-1 and α -osteocalcin, and positive cells were revealed with fluorescein-conjugated second antibody. Left, immunofluorescence images. Right, bright-field images. (B) PDLDCs were subjected to immunocytochemistry, using, as probes, α -CD44, α -STRO-1, α -osteocalcin and α -CEMP-1. Positive cells were revealed with fluorescein-labelled α -CD44 and α -STRO-1 as secondary antibodies. Left, immunofluorescence images. Right, bright-field images. The bar represents 10 μ m, the left panels have the same magnification as the corresponding right panels. (C) Flow cytometry analysis of expression of CD105 in PDLDCs. Cells were treated with antibodies against human CD105 and positive cells were tested by flux cytometry.

but not for α -STRO-1. Figure 3B shows positive immunofluorescence staining in PDLDCs for α -CD44 (6%) and α -STRO-1 (12%) but not for α -CEMP-1 and α -osteocalcin. We attempted to further characterize these cells by flow cytometry. We found that canine PDLDCs expressed CD105, a specific surface antigen of MSCs (Fig. 3). The canine-derived cells were

negative for a number of characteristic markers of MSCs when antibodies against human antigens were used (data not shown), implying either that the cells do not express these markers or, more likely, that the antibodies for human markers do not identify the antigens in canine cells. Unfortunately, canine-specific antibodies are not available at present.

Regenerative experimental study by application of a CS imbibed in CDCs, PDLDCs or culture medium

A critical step in experimental cell therapy is to test the efficiency of the cell carrier. We chose to test a CS as a cell carrier. Therefore, we tested the viability of CDCs or PDLDCs (at passages four to five) imbibed and cultured within the CS. The CS was able to maintain cell viability for 24 h of incubation and for 5 h in a cold environment (Fig. 1), two conditions required to fill the CS with cells and to transport them from the cell culture facility to the surgery room. CS-containing cells were applied to the defects, as described in the Material and methods, and the effects on different parameters were tested using histometry. Table 2 depicts the intra-surgical measurements recorded at the sites where the CS containing CDCs, PDLDCs or culture medium was applied. Postoperative healing occurred uneventfully in all animals and at 4 wk the periodontal tissues were healthy without any sign of infection. Specimens for histological analysis were obtained for all defects in the four experimental animals. Their gingival tissues had similar histological characteristics in the three study groups, depicting a parakeratinized epithelium covering the gingival margin with an underlying connective tissue rich in collagen fibers and fibroblasts, except for a band of inflammatory infiltrate confined to the sulcular and junctional epithelium (Fig. 4). The periodontal attachment apparatus, however, showed distinct histological characteristics when the groups were compared. The healing of the defects in both test groups demonstrated histological characteristics of periodontal regeneration, including formation of new cellular cementum and a significantly larger connective tissue attachment relative to the control group (Fig. 5). Conversely, in the control group, healing mostly occurred by repair, with limited formation of new cellular cementum coronal to the reference notch. No signs of root resorption or ankylosis were observed in either group. In the PDL between the newly

Table 2. Clinical measurements of periodontal defects

Parameter	Control (mm)	CDCs (mm)	PDLDCs (mm)
ABC-CEJ	3.25 \pm 0.32	2.62 \pm 0.31	3.50 \pm 0.50
ABC-BD	7.00 \pm 0.70	6.12 \pm 0.47	6.50 \pm 0.64
BOC-LOC	3.12 \pm 0.12	2.75 \pm 0.14	2.75 \pm 0.25

Data are shown as mean \pm standard deviation; $n = 4$.

Parameters include the distance between the alveolar bone crest and the cemento–enamel junction (ABC-CEJ), the distance between the alveolar bone crest and the base of the defect (ABC-BD) and the buccolingual width of the defect (BOC-LOC).

CDCs, cementum-derived cells; PDLDCs, periodontal ligament-derived cells.

formed bone and cementum, rich capillary vessels were observed (Fig. 5). Although new-bone formation was greater in the PDLDC group than in the CDC and control groups, the differences did not reach statistical significance.

The results from the histometrical analysis in the experimental (CDCs and PDLDCs) and control groups are shown in Fig. 6. The dimension of the epithelium (GM-JE) was not significantly different ($p > 0.05$) between the control group (2.45 \pm 0.20 mm) and the experimental groups (2.18 \pm 0.26 mm for the CDCs and 1.83 \pm 0.64 mm for the MSCDCs), representing 44, 39 and 33% of the defect, respectively. The dimension of the histological attachment (CEJ-JE), however, was significantly different ($p < 0.05$) between the control group (3.14 \pm 0.57 mm) and the experimental groups (1.64 \pm 0.28 mm for the CDCs and 1.68 \pm 0.50 mm for the PDLDCs), representing 56, 31 and 33% of the defect, respectively. The dimension of the gingival connective tissue adhesion to the root surface between the new cementum and the junctional epithelium (JE-CC) was 0.80 \pm 0.30 mm in the control, 0.30 \pm 0.10 mm in the CDCs and 0.15 \pm 0.08 mm in the PDLDCs; these differences were not statistically significant ($p > 0.05$).

The dimension of the newly formed cementum (CC-Notch) was also significantly different ($p < 0.05$) between the control group (1.56 \pm 0.39 mm) and the experimental groups (3.98 \pm 0.59 mm in the CDCs and 4.07 \pm 0.97 mm in the PDLDCs), representing 28, 77 and 74% of the defect, respectively. New-bone formation was similar in all groups, being 2.63 \pm 0.67 mm in

the control, 2.63 \pm 0.44 mm in the CDCs and 3.08 \pm 1.06 mm in the PDLDCs, representing 48, 54 and 57% of the defect, respectively (Fig. 6). Overall, there were no differences in any parameter evaluated between the CDCs and the PDLDCs, with similar results being obtained for both groups.

Discussion

The aim of this study was to evaluate whether a cell therapy based on the implantation during periodontal surgery of CS impregnated with either canine CDCs or canine PDLDCs in experimental intrabony periodontal defects would result in periodontal regeneration in the beagle dog. The histological findings obtained showed that this regenerative approach using cell therapy was able to promote significantly new attachment with formation of new cementum and new bone, and connective tissue attachment to a previously exposed root surface, when compared with the control group where the same carrier, but lacking the cells, was implanted in the defects.

The first phase of this investigation was to isolate canine CDCs and canine PDLDCs because there are few reports in the literature where these cells have been isolated in dogs. In a previous investigation from our research group (32) we reported a protocol for the isolation of human CDCs and PDLDCs, modifying the technique previously reported by Seo *et al.* (2). We followed this protocol in the present investigation. Once isolated, the cells obtained were able to grow and proliferate, forming colonies with the distinct capability of secreting mineralized matrix, as shown by

bright-field microscopy and Von Kossa staining. In order to further characterize the cells, we used antibodies against CEMP-1, a protein specifically present in human cementoblasts (37). Colonies from explants after 60 d of growth *in vitro* demonstrated positive immunostaining in a similar manner to previous investigations characterizing human cementoblasts (33,37). In contrast, PDLDCs were negative to α -CEMP-1, but positive to α -CD44 and α -STRO-1, antigens previously reported to be expressed in MSCs from human PDL (2,38). As there is controversy on the use of STRO-1 as a single marker for stem cells (7), the combination of several additional markers was used to positively identify PDLDCs. STRO-1 is also an early marker of pre-osteogenic cells, the expression of STRO-1 being progressively lost after cell proliferation and differentiation into mature osteoblasts (39). This pre-osteogenic population was further differentiated into an osteogenic cell population by growth in an osteogenic medium containing ascorbic acid 2-phosphate (39). We attempted to further characterize PDLDCs using flow cytometry. Using a large series of antibodies raised against a number of human stem cell markers, we found that canine PDLDCs stained positive for CD105 but negative for the remaining markers. Unfortunately, the lack of canine-specific antibodies does not allow us to conclude whether PDLDCs are MSCs or if they simply cannot be stained by human-specific antibodies. A further characterization of the phenotypic characteristics of these cells using canine-specific markers is warranted.

One of the key factors in cell therapy is the use of an appropriate carrier to ensure cell viability during delivery, as cells need to be maintained in a viable state and released during the healing process. In the present study we used a CS with a resorption time of 72 h. A similar carrier has been used to apply recombinant human bone morphogenetic protein-2 in the regenerative treatment of three-wall intrabony defects in dogs (40) and in the treatment of one-wall intrabony periodontal defects with the application of

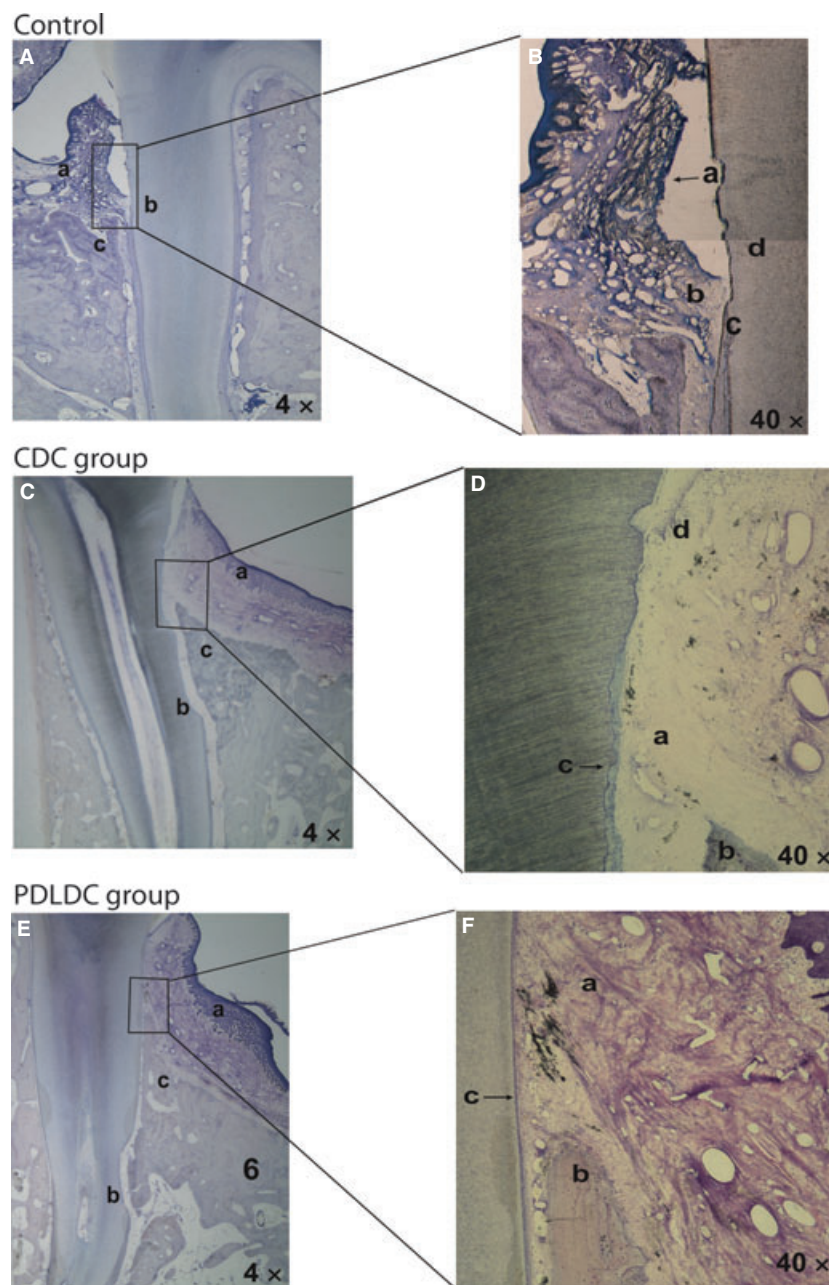


Fig. 4. Cellular therapy promotes connective tissue attachment. After 3 mo of healing in the control group, micrograph A shows a parakeratinized epithelium covering the gingiva (a), the root notch (b) and alveolar bone (c). (B) Higher magnification of the framed area shows the long junctional epithelium (a), connective tissue (b), cementum (c) and dentin (d) [Original magnification $\times 40$; samples were stained with Toluidine Blue (TB)]. In the cementum-derived cell (CDC) group, micrograph C also depicts parakeratinized epithelium covering the gingiva (a), the root notch (b) and alveolar bone (c). (D) Higher magnification of the framed area shows the presence of new connective tissue attachment in the CDC group. The connective tissue fibers (a) coronal to the osseous crest (b) are inserted perpendicularly to the newly formed cementum (c) and junctional epithelium (d) (original magnification $\times 40$; TB). In micrograph E, the periodontal ligament-derived cell (PDLDC) group shows parakeratinized epithelium covering the gingiva (a), the root notch (b) and alveolar bone (c). (F) Higher magnification of the framed area shows the presence of new connective tissue attachment. The connective tissue fibers (a) coronal to the osseous crest (b) are inserted perpendicularly to the newly formed cementum (c) (original magnification $\times 40$; TB).

growth/differentiation factor-5 (41). Hydroxyapatite/ β -tricalcium phosphate has also been used as a carrier for cell therapy in artificially created periodontal defects in mandibular molars

in rats (2) and in peri-implant defects in dogs (11) but the viability of cells in this scaffold is uncertain.

We have shown that canine CDCs or PDLDCs applied within a CS pro-

moted periodontal regeneration in an experimental three-wall intrabony periodontal defect. Significantly higher new-cementum formation and new periodontal tissue attachment was

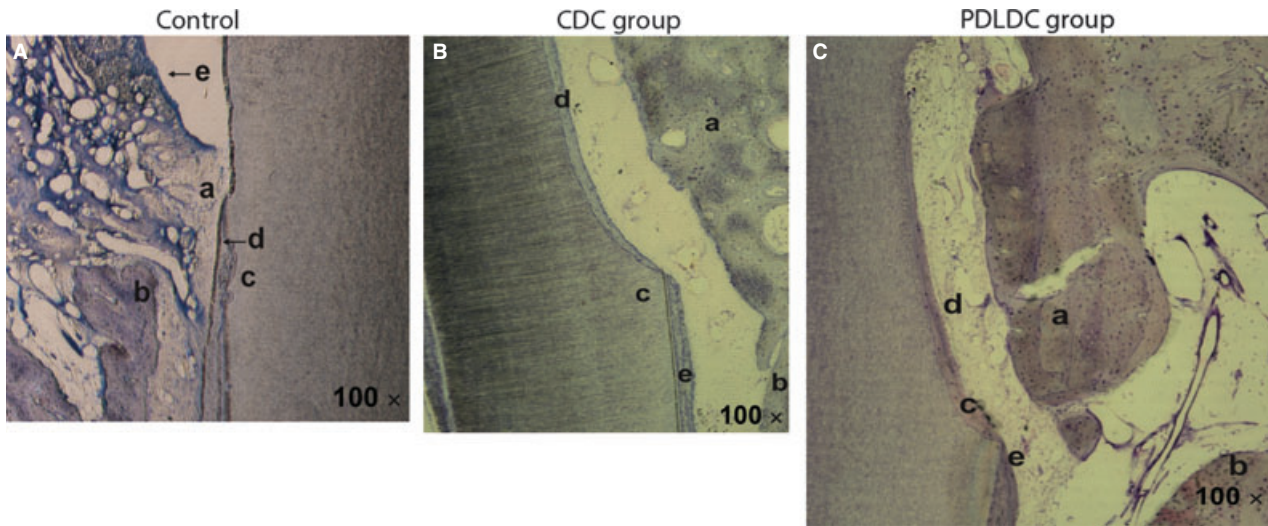


Fig. 5. Cell therapy with canine cementum-derived cells (CDCs) and canine periodontal ligament-derived cells (PDLDCs) promotes new bone formation, PDL and new cellular cementum. (A) Control group: connective tissue fibers (a) coronal to the osseous crest (b), the root notch (c), cementum (d) and the long junctional epithelium (e) [original magnification $\times 100$; Toluidine Blue (TB) stain]. (B) CDC group: new alveolar bone formation (a), old alveolar bone (b), root notch (c), new cementum formation (d) and old cementum (e) (original magnification $\times 100$; TB). (C) PDLDC group: new alveolar bone formation (a), old alveolar bone (b), root notch (c), new cementum formation (d) and old cementum (e) (original magnification $\times 100$; TB).

observed when compared with the application of CS without the cells. The PDLDC group also regenerated new cementum resembling a healthy periodontium, thus suggesting regeneration *ad integrum* of the periodontal tissues. Also, both CDCs and PDLDCs yielded similar results relative to periodontal regeneration. In other words, CDCs promoted as much periodontal regeneration as PDLDCs. This result is not unexpected because CDCs are able to produce specific growth factors and form new cementum, factors which are considered critical in the regeneration of periodontal tissue. Our results provide a proof of principle about the potential use of this mode of cell therapy for the treatment of periodontal intraosseous lesions. In a previous study using cell therapy with a seeding of fibroblast-like cells in artificial fenestration defects in dogs, the amount of new cementum formed was only 9–12% in most specimens (42). Better results were reported in similar defects with the use of hyaluronic acid as a carrier to the fibroblast-like cells, attaining 54% of new cementum in the experimental group vs. 40% in the control group (43). In this study, the amount of new cementum achieved was 77%

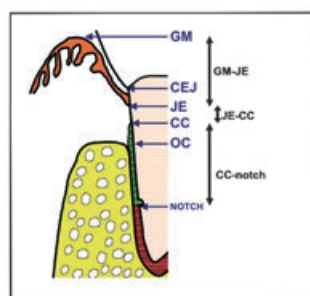
(3.98 mm) for the CDC group and 74% (4.07 mm) for the PDLDC group, both being significantly greater ($p < 0.05$) than the control group, which achieved 28% (1.56 mm) of new cementum. These values of new-cementum formation are similar to those reported in other studies when EMDs were used to treat buccal-dehiscence defects in monkeys (44) or two-wall intrabony defects in dogs (45). In contrast to the results obtained regarding new cementum, similar levels of new-bone formation occurred in the experimental and control groups, representing 54% in the CDC group, 57% in the PDLDC group and 48% in the control group. These results on new-bone formation may be explained by the experimental model used in this study, as the three-wall periodontal defect has a self-contained anatomy surrounded by bony walls that allows good stabilization of the blood clot and the promotion of new-bone formation. In spite of this, the PDLDC group showed a larger amount of new-bone formation, although the between-group differences were not statistically different.

One aspect difficult to avoid during healing after periodontal surgical procedures is the apical location of the

gingival margin relative to the CEJ (gingival recession). To avoid this outcome, minimally invasive surgical approaches have been recently described for the treatment of periodontal defects with the use of biologicals (EMD), and minimal gingival recession has been reported (46,47). In this study, both test groups failed to show any gingival recession, in contrast to the control group in which a gingival recession measurement of 0.72 mm was obtained. These results represent a gain of coronal level of histological attachment of 3.50 mm in the CDC group, 3.73 mm in the PDLDC group and 2.39 mm in the control group, the results in both test groups being significantly different ($p < 0.05$) from the result in the control group.

One of the shortcomings of this investigation was the lack of proof that the cells remained active during healing, because the transplanted cells were not labelled. It was therefore impossible to ascertain how these cells may have contributed to the tissue regeneration achieved. We demonstrated, however, that the cells were alive when they were implanted in the canine defects, and both groups using the cell therapy achieved significantly higher

A Schematic of histomorphometric sections



B Histometric results at 3 months post-surgery

Histometric section	Control (mm)	CDC (mm)	PDLDC (mm)
NOTCH-CEJ	5.53 ± 0.81	5.14 ± 0.17	5.41 ± 1.10
GM-JE	2.45 ± 0.20	2.18 ± 0.26	1.83 ± 0.64
CEJ-GM	-0.72 ± 0.38	0.51 ± 0.55	0.14 ± 0.68
CEJ-JE	3.14 ± 0.57	1.64 ± 0.28*	1.68 ± 0.50*
JE-CC	0.80 ± 0.30	0.30 ± 0.10	0.15 ± 0.08
CC-Notch	1.56 ± 0.39	3.98 ± 0.59*	4.07 ± 0.97*
New bone	2.63 ± 0.67	2.63 ± 0.44	3.08 ± 1.06

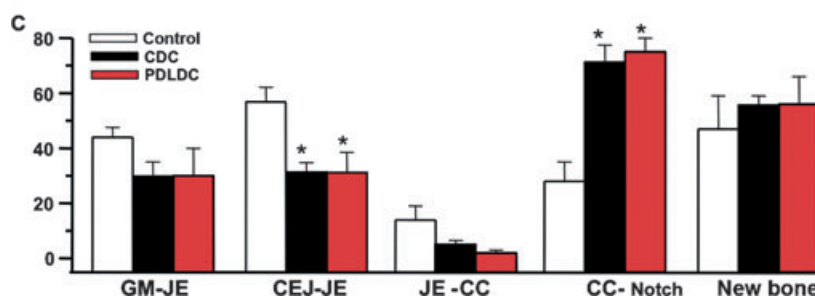
* $p < 0.05$ 

Fig. 6. Histometric results 3 mo after surgery. (A) Histometric landmarks used: gingival margin (GM), cemento–enamel junction (CEJ), apical extent of the junctional epithelium (JE), coronal level of newly formed cementum (CC) and coronal level of newly formed alveolar bone (OC). (B) Histometric distances (mm) between the notch and the CEJ, between the GM and the apical end of the junctional epithelium (GM-JE) and from the CEJ to the gingival margin (CEJ-GM). Length of the coronal level of histological attachment: the distance between the CEJ and the junctional epithelium (CEJ-JE). Length of the connective tissue adhesion: distance between the most apical extent of the junctional epithelium and the most coronal cementum (JE-CC), length of the newly formed cementum coronal to the notch (CC-notch) and length of the newly formed bone coronal to the notch (New bone). The length of the histological attachment level and the amount of new-cementum formation showed significant differences 3 mo after treatment. The unit of analysis is the number of dogs ($n = 4$), * $p < 0.05$. (C) Bars show the percentage distribution of the experimental values of the different parameters shown above (data are given as mean \pm standard error of the mean). * $p < 0.05$.

histological regenerative outcomes when compared with the control using the same carrier but lacking the cells. We may speculate that even if the cells died shortly after transplantation, they may have contributed positively to the healing process. Furthermore, if the cells remained viable, they could further differentiate into those cells responsible for the periodontal-regeneration process.

Although the number of dogs used in this investigation was relatively small, mainly for ethical reasons, the results obtained clearly suggest that the implantation of these cells has the potential to promote regeneration, thus paving the way for future cell-therapy studies in the treatment of periodontal disease. In conclusion, the cell therapy developed and tested in the present experimental study suggests

that the implantation of CDCs and PDLDCs in a CS in experimentally created periodontal defects in a canine model promotes the regeneration of periodontal tissues through the formation of new cellular cementum, new PDL and new bone. The results from this experimental investigation cannot, however, be extrapolated to the treatment of human patients. For this, first, a reliable and predictable noninvasive

technique for CDC and PDLDC extraction must be developed and tested. Second, these cells must be tested in the laboratory for consistent growth and differentiation. Third, their viability and release must be assessed in contact with different carriers. Finally, safety studies using the appropriate standard cell therapy protocols in humans must be carried out before this therapeutic approach can be implemented in human patients. Further research is warranted to clarify the specific biological activity in the tissue-regeneration process of cementoblasts and stem cells from PDL.

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