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Autologous periodontal ligament cells in the treatment of class II furcation defects: a study in dogs

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Abstract

Aim: The goal of this study was to histologically investigate the use of periodontal ligament cells (PDL cells) in tissue engineering to regenerate class II furcation defects.

Material and Methods: PDL cells were obtained from the mandibular tooth extracted from each dog (seven), cultured in vitro and phenotypically characterized with regard to their biological properties. Following, bilateral class II furcation lesions were created at maxillary 3rd premolars and were randomly assigned to the test group [PDL cells+guided tissue regeneration (GTR)] or the control group (GTR). After 3 months, the animals were euthanized to evaluate the histometric parameters.

Results: In vitro, PDL cells were able to promote mineral nodule formation and to express bone sialoprotein, type I collagen and alkaline phosphatase. Histometrically, data analysis demonstrated that the cell-treated group presented a superior length of new cementum (6.00 ± 1.50 and 8.08 ± 1.08 mm), a greater extension of periodontal regeneration (3.94 ± 1.20 and 7.28 ± 1.00 mm), a lower formation of connective tissue/epithelium (2.15 ± 1.92 and 0.60 ± 0.99 mm), a larger area of new bone (7.01 ± 0.61 and 9.02 ± 2.30 mm^2) and a smaller area of connective tissue/epithelium (5.90 ± 1.67 and 4.22 ± 0.95 mm^2), when compared with control group.

Conclusion: PDL cells in association with GTR may significantly promote periodontal regeneration in class II furcation defects in dog.

Key words: furcation defects; periodontal disease/surgery; periodontal regeneration; tissue engineering

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The ultimate goal of periodontal therapy is to regenerate tissues lost as a consequence of periodontal disease. The regeneration of damaged periodontal tissues is mediated by various periodontal cells and is regulated by a vast array of extracellular matrix informational molecules that induce both selective and non-selective responses in different cell lineages and their precursors (Bartold & Narayanan 2006). Guided tissue regeneration (GTR) is considered a valuable procedure for periodontal regenerative therapy and it is supported by clinical (Becker et al. 1988, Pontoriero et al. 1989, Pepelassi et al. 1991) and histological (Gottlow et al. 1984, Niederman et al. 1989) studies. However, the results obtained with this technique are not always predictable and frequently are subjected to complications (O'Neal et al. 1994, Park et al. 1995). In addition, although several procedures, such as grafting, root surface conditioning and application of growth factors, are performed for periodontal regeneration, the success of these therapies remains an unpredictable outcome (Sander & Karring 1995, Ripamonti & Reddi 1997, Venezia et al. 2004).

In this context, the use of tissue engineering has contributed to the growing interest in study regenerative techniques using autologous cells (Bartold & Raben 1996), especially mesenchymal stem. Recently, human periodontal ligament stem cells were isolated and characterized as a population of multipotent stem cells capable of forming cementum and periodontal ligament tissues upon in vivo transplantation (Seo et al. 2004). Animal studies have suggested that periodontal ligament cells (PDL cells), when cultured in scaffolds and then transplanted into periodontal

Conflict of interest and source of funding statement

The authors declare that there are no conflicts of interest in this study.

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defects, have multiple differentiation properties to regenerate periodontal tissues (Nakahara et al. 2004, Akizuki et al. 2005, Murano et al. 2006, Gault et al. 2010).

With regard to the cell-seeding technique, the type of the defect is very important to retain the implanted cell scaffold in the defect (Nakahara et al. 2004) and to avoid the epithelium migration (Gottlow et al. 1984). The class II furcation defect, one of the main indications for regenerative procedures (Jepsen et al., 2002; De Andrade et al., 2007; Keles et al., 2009) provides favourable anatomical characteristics for clot stabilization and protection. Therefore, the goal of this investigation was to evaluate, histometrically, the healing of class II furcation treated with transplanted autologous PDL cells associated with GTR.

Material and Methods

Animals

Seven adult beagle dogs, weighing approximately 15 kg (10–20 kg) and with a mean age of 1.46 ± 0.18 years were included in this blinded split-mouth study. All surgical procedures were performed under general anaesthesia with intravenous injection of sodium pentobarbital (30 mg/kg). The protocol of the study has been approved by the Institutional Committee on Animal Research of the State University of Campinas. One week before the surgery for teeth extractions, supra-gingival deposits were removed with an ultrasonic scaler and the teeth were wiped with gauze soaked in povidone-iodine. The same procedure was repeated 1 week before the surgery for the defect treatment.

PDL cell isolation and culture

Periodontal ligament explants (PDL explants) were obtained from the tooth root of bilateral mandibular second premolar and the first molar extracted from each dog. Briefly, teeth were extracted and placed in biopsy media composed by Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 250 μg/ml gentamicin sulphate, 5 μ/ml amphotericin B and 1% penicillin/streptomycin (Gibco BRL, Life technologies, Rockville, MD, USA). Then, the teeth were transferred to the laboratory facilities, washed with the biopsy media and the PDL explants were gently harvest from the middle third of the roots and pooled together. Following, PDL explants were enzymatically digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase (Gibco BRL, Life technologies) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 100 μm cell strainer (BD Falcon™, BD, Franklin Lakes, NJ, USA). Samples were expanded in 25 cm^2 culture flasks (BD Falcon™, BD), under a humidified atmosphere of 5% CO_2 at 37°C, in standard media composed by DMEM supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin (Gibco BRL, Life technologies). Then, samples were frozen for subsequent experiments and only cells in passages 2–3 were used.

Phenotypic characterization

Mineral nodule formation and gene expression analyses

In vitro, PDL cells were seeded (5×10^4 cells/cm^2) in standard media, in 60 mm dishes for gene expression analysis and in 24-well plates to assess the mineral nodule formation. To demonstrate the ability of PDL cells to initiate mineralized nodule, after 24 h, standard media was changed to osteogenic-inducing media (DMEM 10% FBS, 50 μg/ml ascorbic acid, 10 mM β-glycerolphosphate, 10^{-5} M dexamethasone), and in vitro mineral nodule formation was assessed on day 28 using the von Kossa assay. In parallel, for gene expression analysis, PDL cells were maintained in standard media until confluence was reached. Then, total RNA was obtained using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) for the expression of bone sialoprotein (BSP), type I collagen (COL I) and alkaline phosphatase (ALP). Total RNA was DNAse treated (Turbo DNA-free TM Kit, Applied Biosystems, Ambion, Austin, TX, USA), and 1 μg was used for cDNA synthesis following the manufacturer's recommendations (kit SuperScript® III First-Strand Synthesis System for RT-PCR, Invitrogen). Specific primers for dog glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (forward primer: 5′-CCAGAACATCATC CCTGCT-3′, reverse primer: 5′-ACTA CCTTCTTGATGTCGTCATATT-3′), ALP (forward primer: 5′-GGGCAACT CTATCTTTGGTCTG-3′, reverse primer: 5′-CTGGTAGTTGTTGTGAGCG-3′), BSP (forward primer: 5′-GGTACATA GGTCTAGCTGCAATC-3′, reverse primer: 5′-TGGTGCTGTTTATACCTTGC C-3′) and COL I (forward primer: 5′-G TGTCCGTGGTCTGACT-3′, reverse primer: 5′-TCACCTTTAGCACCAGG TTG-3′) were designed using software (Roche diagnosis GmbH, Manhein, BW, Germany). Reverse transcription-polymerase chain reactions (RT-PCR) were performed for one cycle of pre-incubation at 95°C for 10 min. and 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C and extension at 72°C for, respectively, 5/7 s (Gapdh), 3/6 s (ALP), 5/7 s (BSP), 2/8 s (COL I). Gapdh expression was used as an internal control of RNA integrity and efficiency of the reverse transcription process. Amplified samples were visualized on 2.0% agarose gels stained with ethidium bromide and photographed under UV light. Two independent experiments were performed.

Scanning electron microscopy (SEM) analysis

In order to exam cell morphology, spreading and adhesion to the scaffolds (BD 3D Collagen Composite Scaffold, BD Bioscence, Franklin Lakes, NJ, USA), a SEM analysis was performed. Cells were seeded into the scaffold in triplicates at a density of 3×10^5 cells/scaffold and incubated for 24 h in standard media. Media was then removed by aspiration, the scaffolds were gently washed with 2 ml of phosphate buffered saline (Gibco BRL, Life technologies), and fixed by immersion in Karnovsky solution. For stepwise dehydration, cell-seeded scaffolds were incubated in increasing concentrations of acetone (50%, 75%, 85%, 90%, 95% and 100%), and then dried by the critical point technique (Denton Vacuum DCP-1 – Critical Point, Denton Vacuum, Moorestown, NJ, USA). After sputter-coating with gold/palladium alloy, the scaffolds were examined in SEM (JEOL JSM-T330A, JEOL Ltd., Tokyo, Japan) with a magnification of 150–3500 times. The images acquired were used for descriptive analysis.

Surgical procedure and defect treatment

Six months after the extraction intervention, bilateral class II furcation lesions were surgically created at the buccal aspect of maxillary third premolars. To create the defects, a buccal mucoperiosteal

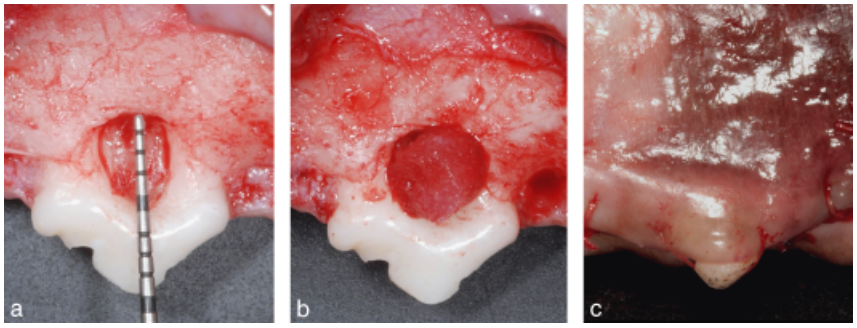

Fig. 1. Clinical illustration of treatment procedure: (A) aspect of the Class II furcation defect after the defect creation. (B) The collagen sponge scaffold, with (test) or without (control) the cell suspension was applied filling the defect. (C) Flaps coronally positioned and sutured after membrane positioning.

flap was raised to expose the alveolar buccal bone. Osteotomy was performed in the furcation area with high-speed diamond burs with constant irrigation and with #1 and 2 Ochsenbein chisels. The furcations lesions were standardized with a millimetre probe and measured 5.0 mm in the apico-occlusal direction and 2.0 mm in the bucco-lingual direction (Fig. 1A), following a technique described previously (Regazzini et al. 2004). On the root surface, the base of the defect was marked to establish a landmark for the histomorphometric analysis. Defects were randomly assigned to one of the following treatments:

A. Control group: a collagen sponge scaffold, without cells, was applied into the defect associated with an absorbable membrane (Resolut XT®, Regenerative Material, Gore-tex, Gore Associates, Flagstaff, AZ, USA) that was adapted to the defect The sponge was soaked with DMEM.
B. Test group: a cell-seeded collagen scaffold was applied into the defect associated with an absorbable membrane (Resolut XT®, Regenerative Material, Gore-tex, Gore Associates) that was adapted to the defect (Fig. 1B).

Cells were seeded at 3×10^5 cells and cultured in standard media for 24 h. To avoid immunological reaction, scaffolds seeded with cells were maintained in standard media without FBS during 12 h before transplantation (*serum starvation*).

In order to cover the membranes, the flaps were coronally positioned and sutured with interrupted sutures (Vicryl, Ethicon Inc, São José dos Campos, SP, Brazil) (Fig. 1C).

After surgical procedures, the animals were given an intra-muscular injection of penicillin (1:50,000 IU), and the same dose was repeated after 4 days. The post-operative plaque control was performed by daily topical application of 0.2% chlorhexidine gluconate, as well as by calculus removal and prophylaxis once a month, during the whole experimental phase.

Histomorphometric analysis

Three months after treatment, the animals were anaesthetized and the oral tissues were fixed by perfusion with 10% buffered formalin administered through the carotid arteries. The jaws were removed, dissected and the blocks containing the experimental specimens were obtained. Specimens were decalcified in a solution of equal parts of 50% formic acid and 20% sodium citrate for 4 months. The decalcified specimens were washed in running water, dehydrated and embedded in paraffin. Serial mesio-distal sections (7 μm thick) were obtained and stained with haematoxylin and eosin.

For histometric and descriptive histological analyses, the first and the last section where the landmarks on the root surface could be clearly identified were used to determine the number of sections necessary for each defect. The total number of sections was divided into three parts and the middle third of the defect was used to obtain sections for analysis. A total of six sections per site, which were selected in a way to show a constant distance among them, were used to obtain the mean for each parameter in each dog. The measurements were performed with an image analysis system (Image Pro Plus 3.0, Media Cybernetics, Silver Spring, MD, USA).

Linear measurements

The following linear measurements (mm) were obtained in the mesial and distal roots under the furcation:

1 *Total defect length*: total length of the root surface between notches on the mesial and distal roots;
2 *Tissue-free defect length:* portion of the total defect length with the absence of any new tissue formation;
3 *New cementum*: linear extension of the root surface covered by new cementum;
4 *Periodontal regeneration:* linear extension of the root surface covered by new cementum adjacent to newly formed bone, with functionally oriented inserted collagen fibers;
5 *Epithelium/connective tissue extension:* extension of the root surface covered by epithelium and connective tissue.

Area measurements

A line connecting both notches defined the apical limit of the defect and the following parameters (mm^2) was obtained by the point counting technique:

1 *Total defect area*: area limited by the apical line and the root surface in the furcation region;
2 *Non-filled area*: portion of the total defect area not filled with any tissue;
3 *Soft tissue area*: portion of the total defect area filled with epithelium and connective tissue;
4 *New bone area*: portion of the total defect area filled with new bone.

Statistical analysis

The data were averaged and the hypothesis that there was no difference between the groups regarding the evaluated parameters was tested by an intergroup analysis (e.g., control *versus* test) using the paired *t*-test ($\alpha = 0.05$). For the statistical analysis, the animal was used as the statistical unit ($n = 7$).

Results

In vitro characterization of PDL cells

In this study, to demonstrate the ability of PDL cells to promote mineral nodule formation, osteogenic differentiation was induced by the addition of culture

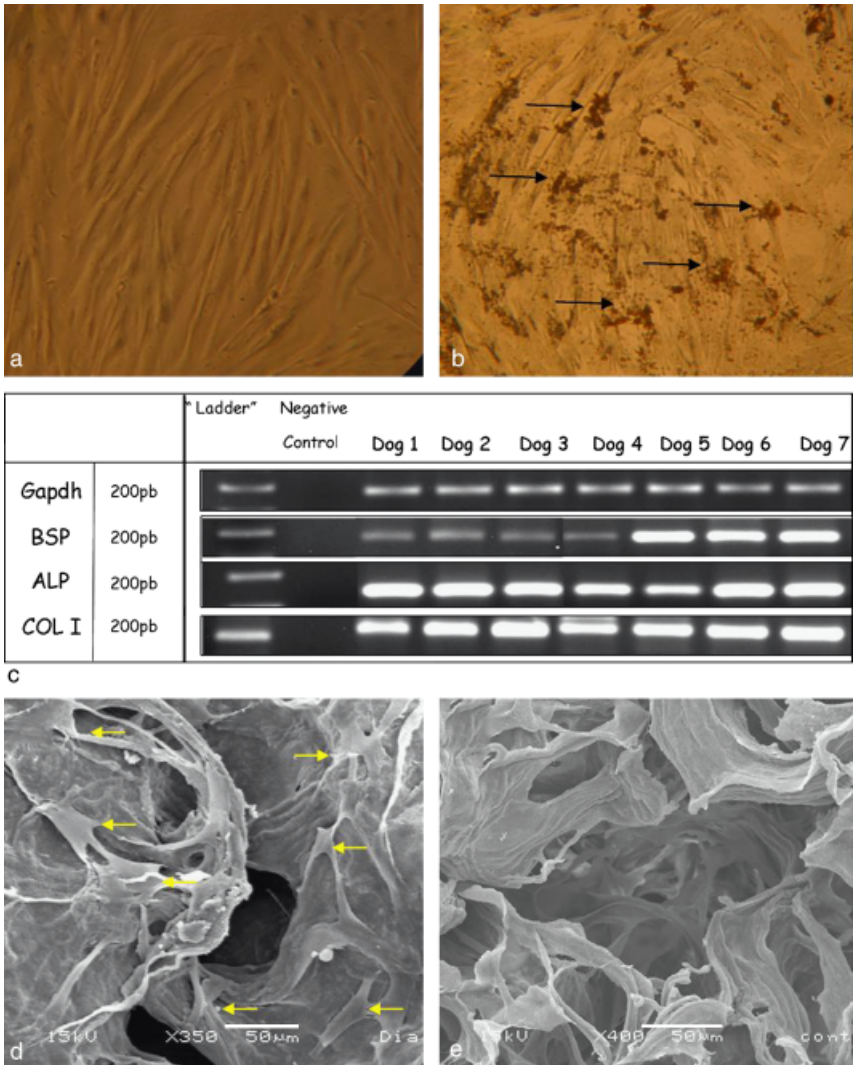

Fig. 2. Panel illustrating data for in vitro phenotypic characterization of cells used: (A–B) von Kossa assay: PDL cells cultured for 28 days in osteogenic conditions were able to produce mineral nodule deposition (A) as visualized by von Kossa staining (black colour indicates cell culture positive to von Kossa assay). PDL cells cultured in standard media were not capable of producing mineral nodule deposition. (C) Gene expression analyses: illustration of the photographic documentation of agarose gel 2% for genes Gapdh, BSP, ALP and COL 1. (D–E) Scanning electron microscopy analysis: after 3 days in culture, cells were attached and homogeneously dispersed on the carrier. PDL cells (D) seeded onto scaffolds (arrows) (450– × 550) and control scaffold (carrier without cells) (× 500) (E). PDL cells, periodontal ligament cells; ALP, alkaline phosphatase; BSP, bone sialoprotein; COL I, type I collagen.

media containing ascorbic acid-2-phosphate, dexamethasone and β-glycerol-phosphate. After 28 days, with no exception, PDL cells were able to produce mineral nodule deposition at variable levels as visualized by von Kossa staining (Fig. 2A and B). In addition, genes known to be expressed in PDL cells were qualitatively assessed by RT-PCR in order to confirm that the cells used in the current investigation were PDL cells. Data analysis demonstrated that, at variable levels, the cells obtained consistently expressed BSP, ALP and COL I (Fig. 2C). In order to exam cell morphology, spreading and adhesion to the scaffolds, an SEM analysis was performed. In general, after 24 h in culture, cells were attached and homogeneously dispersed on the carrier structure, presenting fibroblast-like morphologic appearance, confirming that the scaffold was suitable for the proposed ex vivo experiments, as demonstrated by the cell adhesion and spreading onto carrier structure (Fig. 2D and E).

Clinical observations

Clinically, the healing response was favourable for all treatments. No suppuration or abscess formation was observed during the 90 days and no exposure of membranes was observed in both groups during the healing period. At sacrifice, soft tissues had totally healed and exhibited no clinical signs of inflammation.

Histological observations

No inflammation and foreign body reactions were observed. Different stages of bone re-growth and periodontal ligament organization were observed in the healed furcation sites. Woven bone was the predominant type of bone formed, and in the control group, large bone marrow spaces were often present (Fig. 3A and B). The periodontal ligament was characterized by the presence of collagen fibres obliquely oriented to the root surface, extending between the new cementum and the newly formed bone. Down-growth of epithelium was found, restricted to the fornix of the furcation, in only four histological sections (different defects in the control group). The newly formed cementum was of a cellular, mixed intrinsic and extrinsic collagen fibre type. The presence of functionally oriented collagen fibres appeared to be closely related to the presence or absence of newly formed alveolar bone adjacent to the new cementum. Dentoalveolar ankylosis could be observed in one specimen of each group, corresponding to the same dog.

Histometrical analysis

Data analysis showed no significant differences between the groups regarding the initial defect area and extension. In addition, it was observed that cell-treated defects presented a superior length of new cementum and a lower formation of connective tissue/epithelium along the root surface.

The extension of periodontal regeneration, e.g., new cementum surface adjacent to new bone with inserting collagen fibres, was greater for test group (Table 1). The proportional distribution of the different healing patterns along the root surface is presented in Fig. 4.

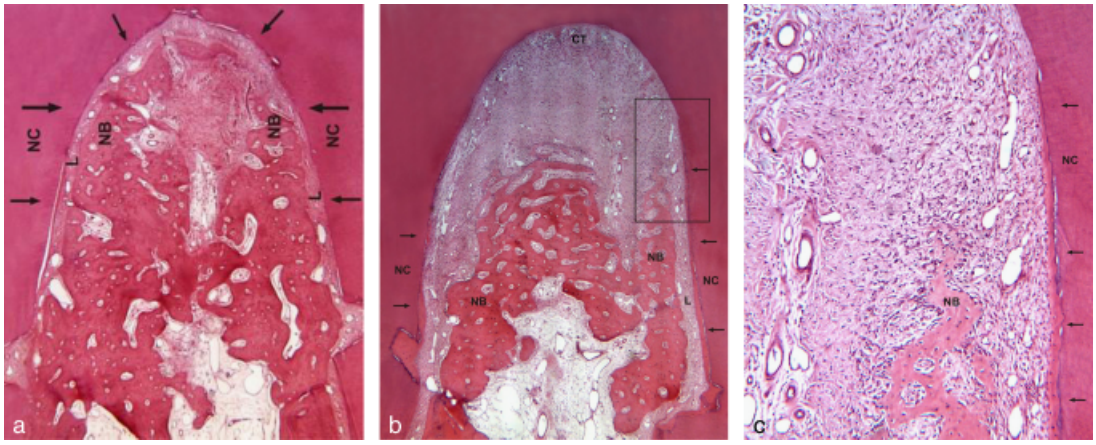

Fig. 3. Mesio-distal sections of the furcation defect. (A) Test group. The total defect area is filled in its majority with new bone (NB), which developed in continuity with the preexistent bone, and the whole extension of the defect (extension of the root surface between the two notches) is covered by the new cementum (→ NC). Note the periodontal ligament (L) extending between the new cementum and the newly formed bone (H&E staining; original magnification × 25). (B) Control group. In contrast to the scenario found in (A), connective tissue (CT) can be observed at the coronal part of the defect. New cementum formation (NC) and periodontal ligament (L) are restricted to the apical portion of the defect, with the connective tissue adaptation (CT) reaching its coronal part (H&E staining; original magnification × 25). (C) Higher power magnification of area delineated in (B) showing that the coronal part of the defect is not covered by NC (H&E staining; original magnification × 100).

Table 1. Extension (mean ± SD, mm) of linear parameters

	Control group ($n = 7$)	Test group ($n = 7$)	p value
Total defect length	8.39 ± 0.65	8.84 ± 0.31	0.18
Total new cementum	6.00 ± 1.50	8.08 ± 1.08	0.003*
Periodontal regeneration	3.94 ± 1.20	7.28 ± 1.00	0.001*
Epithelium/connective tissue extension	2.15 ± 1.92	0.60 ± 0.99	0.048*
Ankylosis	0.25 ± 0.53	0.16 ± 0.45	0.21

*Statistically significant ($p < 0.05$).

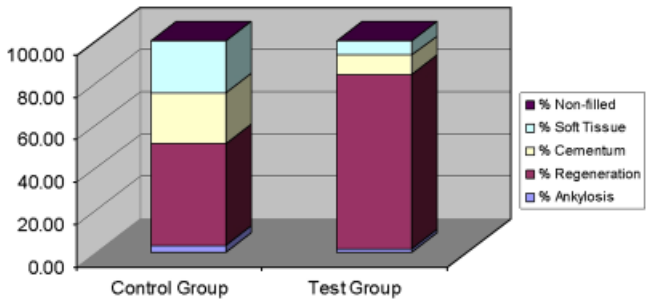

Fig. 4. Histometric linear parameters expressed as a percentage of the root surface.

Table 2. Area (mean ± SD, mm^2) of evaluated parameters

	Control group ($n = 7$)	Test group ($n = 7$)	p value
Defect area	12.93 ± 1.46	13.27 ± 2.17	0.718
Non-filled area	0.03 ± 0.06	0.02 ± 0.06	0.861
Soft tissue area	5.90 ± 1.67	4.22 ± 0.95	0.001*
New bone area	7.01 ± 0.61	9.02 ± 2.30	0.026*

*Statistically significant ($p < 0.05$).

The group in which the cells were applied showed a higher percentage of periodontal regeneration and lower proportion of soft tissue adaptation ($p < 0.05$) (Fig. 4). By comparing the two experimental groups with respect to the area of the defect, data analysis showed a larger area of new bone and a smaller area of connective tissue/epithelium for cell-treated defects (Table 2/Fig. 5).

Discussion

The present study evaluated, histometrically, the healing of Class II furcation defects treated by the application of autologous periodontal ligament cells (PDL cells) associated with GTR. In general, the findings of the present investigation demonstrated that the cell-treated defects presented a better final outcome, showing a greater extension of periodontal regeneration and a larger area of newly formed bone.

These findings are in accordance with previous studies, which demonstrated the potential of in situ tissue engineering using PDL cells for the periodontal regeneration (Nakahara et al. 2004, Akizuki et al. 2005, Murano et al. 2006, Gault et al. 2010). Nakahara et al. (2004), evaluating the effect of PDL cells seeded on periodontal fenestration defects, demonstrated that these cells induced cementum regeneration on the root surface. Similarly, Akizuki et al. (2005) using the PDL cells in dehiscence defects revealed that the formation of new cementum was significantly higher in the experimental group. Recently, Iwata et al. (2009) observed

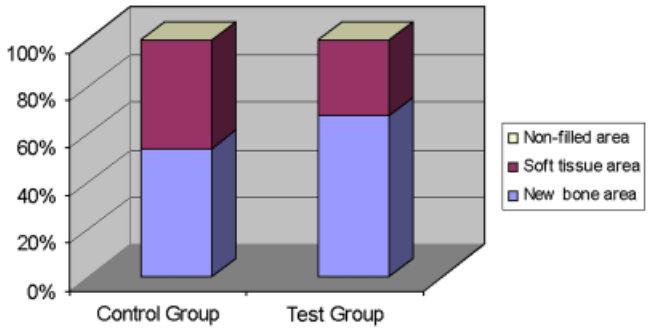

Fig. 5. Histometric area parameters expressed as a percentage of the defect filing. The proportion (%) of soft tissue (epithelium and connective tissue), new bone and non-filled area in the initial defect area.

complete periodontal regeneration with newly formed bone and cementum connecting with collagen fibres in all intrabony defects treated with cell sheets derived from PDL tissue.

The histometric results of the present study are in agreement with previous reports (Nakahara et al. 2004, Akizuki et al. 2005, Iwata et al. 2009) showing a superior length of new cementum in the cell-treated group. It has been suggested that, the direct contact of progenitor cells from the periodontal ligament with the root dentin surface may induce its differentiation into cementoblasts in vivo (Aukhil et al. 1986). In the group where the cells were applied the new cementum covered 91.40% (8.08 ± 1.08 mm) of the total defect length, and in the control group this value corresponded to 71.45% (6.00 ± 1.50 mm) ($p = 0.003$). In the present study, in order to reduce the risk of seeded cells leaking away from the defect, the cell treatment was associated with GTR. Therefore, both groups were treated with GTR membranes, which may explain the amount of cementum formation in the control group. The groups differed only by the application of the cells. Once new cementum covers the denuded root surface, epithelial/connective tissue cells may not be able to migrate on the surface. In the control group a higher formation of connective tissue/epithelium was observed along the root surface (2.15 ± 1.92 mm) when compared with the test group (0.60 ± 0.99 mm), $p = 0.048$. Moreover, studies have suggested that components of newly formed cementum matrix provide informational signals for development and insertion of collagen fibres in new bone and cementum (Grzesik & Narayanan 2002). The data of the present study showed that new cementum surface adjacent to new bone with inserting collagen fibres (periodontal regeneration) was greater for the test group (7.28 ± 1.00 mm) when compared with the control group (3.94 ± 1.20 mm), $p = 0.001$.

In contrast with previous studies (Nakahara et al. 2004, Akizuki et al. 2005), in the present investigation, a positive effect of transplanted cells in the amount of new bone formed was observed in the surgically created defects. Sites that received the scaffold with PDL cells showed a superior area of new bone (9.02 ± 2.30 mm) when compared with the control group (7.01 ± 0.61 mm) ($p = 0.026$). However, it should be noted that the type of defect used by Nakahara et al. (2004) and Akizuki et al. (2005) was different from the type of defect used in the present investigation. Fenestration and dehiscence defects, used in previous studies, have thin buccal alveolar plate and promote minimal space between the flap and the root surface contributing for the lack of effect in the bone regeneration. On the other hand, similar positive bone response was reported by Murano et al. (2006) that applied PDL cells in class III furcation in dogs and observed an accelerated bone formation (84.8%) when compared with the control group (12.2%).

Considering that statistically significant differences were observed in the histometric parameters between the groups in the present study (i.e., the test group showed a greater extension of periodontal regeneration and a larger area of newly formed bone than the control group), and that the inclusion of the cells was the only difference between the groups, it may be assumed that the cells may have favoured periodontal tissue formation. Hiraga et al. (2009) examined the bone-like matrix formation in vivo when PDL cells, isolated from green fluorescent protein-transgenic rats, were implanted into wild-type rats. The authors suggested that PDL cells have the ability to form periodontal tissues in vivo and could be a useful source for periodontal regenerative therapies.

In order to demonstrate that the cells used in the current study could be considered PDL cells, a series of assays were performed to evaluate known biological properties of these cells. As expected, PDL cells presented in vitro cementoblast/osteoblasts properties, which were characterized by the expression of COL I, ALP and BSP and by the ability of these cells to produce mineral nodule (Somerman et al. 1990, Arceo et al. 1991, Nohutcu et al. 1996, Iwata et al. 2010, Ribeiro et al. 2010). Within the context of the development of periodontal regeneration, COL I expression is associated with the formation of extracellular matrix and with the formation of periodontal fibers; ALP may be involved in the preparation of extracellular matrix for the ordered deposition of mineral, whereas the BSP expression is associated with matrix mineralization (Lian & Stein 1992).

With regard to the cell-seeding technique, a stable carrier scaffold is crucial for cell attachment. Experiments without a carrier scaffold could not be considered valid, because the applied cells would be washed away rapidly (Van Dijk et al. 1991, Lang et al. 1998, Dogan et al. 2002). The collagen sponge scaffold used in this study is a biodegradable material that is approved for clinical settings. An SEM analysis was performed confirming that the scaffold was suitable for the proposed ex vivo experiments, as demonstrated by the cell adhesion and spreading into carrier structure. Furthermore, the collagen sponge did not cause any significant harmful side effects such as remarkable inflammation during the healing period in any of the dogs.

It should be recognized that the type of defect used in the present study provides favourable anatomical characteristics for clot stabilization and protection. In addition, both groups were treated with GTR, that may justify the observation that more than a half of the defect area was occupied by new bone, even in the control group. Future studies are required to evaluate the use of PDL cells to treat more challenging defects, like class III furcation defects.

Within the limits of this animal study, it was concluded that PDL cells in association with GTR may be a viable approach to promote periodontal tissue regeneration (new cementum surface adjacent to new bone with inserting collagen fibres) in class II furcation defects. In light of these findings, the ready availability of periodontal ligament tissue from extracted teeth, such as third molars, may provide a supply of PDL cells that could be cultured in scaffolds and then transplanted into periodontal defects, when necessary. However, before clinical use, future studies are required to clarify the mechanism of action of transplanted PDL cells in the process of periodontal regeneration, to establish the ideal concentrations of cells in the scaffold and to evaluate the long-term outcome and safety of the procedure.

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References

Akizuki, T., Oda, S., Komaki, M., Tsuchioka, H., Kawakatsu, N., Kikuchi, A., Yamato, M., Okano, T. & Ishikawa, I. (2005) Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. *Journal of Periodontal Research* **40**, 245–251.

Arceo, N., Sauk, J. J., Moehring, J., Foster, R. A. & Somerman, M. J. (1991) Human periodontal cells initiate mineral-like nodules in vitro. *Journal of Periodontology* **62**, 99–503.

Aukhil, I., Simpson, D. M., Suggs, C. & Pettersson, E. (1986) In vivo differentiation of progenitor cells of the periodontal ligament. An experimental study using physical barriers. *Journal of Clinical Periodontology* **13**, 862–868.

Bartold, P. M. & Narayanan, A. S. (2006) Molecular and cell biology of healthy and diseased periodontal tissues. *Periodontology 2000* **40**, 29–49.

Bartold, P. M. & Raben, A. (1996) Growth factor modulation of fibroblasts in simulated wound healing. *Journal of Periodontal Research* **31**, 205–216.

Becker, W., Becker, B.E, Berg, L., Prichard, J., Caffesse, R. & Rosemberg, E. (1988) New attachment after treatment with root isolation procedures: report for treated Class III and Class II furcations and vertical osseous defects. *International Journal of Periodontics Restorative Dentistry* **8**, 8–23.

De Andrade, P. F., de Souza, S. L., de Oliveira Macedo, G., Novaes, A. B. Jr., de Moraes Grisi, M. F., Taba, M. Jr. & Palioto, D. B. (2007) Acellular dermal matrix as a membrane for guided tissue regeneration in the treatment of Class II furcation lesions: a histometric and clinical study in dogs. *Journal of Periodontology* **78**, 1288–99.

Dogan, A., Ozdemir, A., Kubar, A. & Oygur, T. (2002) Assessment of periodontal healing by seeding of fibroblast-like cells derived from regenerated periodontal ligament in artificial furcation defects in a dog: a pilot study. *Tissue Engineering* **8**, 273–282.

Gault, P., Black, A., Romette, J. L., Fuente, F., Schroeder, K., Thillou, F., Brune, T., Berdal, A. & Wurtz, T. (2010) Tissue-engineered ligament: implant constructs for tooth replacement. *Journal of Clinical Periodontology* **7**, 750–758.

Gottlow, J., Nyman, S., Karring, T. & Lindhe, J. (1984) New attachment formation as the result of controlled tissue regeneration. *Journal of Clinical Periodontology* **11**, 494–503.

Grzesik, W. J. & Narayanan, A. S. (2002) Cementum and periodontal wound healing and regeneration. *Critical Reviews in Oral Biology and Medicine* **13**, 474–484.

Hiraga, T., Ninomiya, T., Hosoya, A., Takahashi, M. & Nakamura, H. (2009) Formation of bone-like mineralized matrix by periodontal ligament cells in vivo: a morphological study in rats. *Journal of Bone and Mineral Metabolism* **27**, 149–157.

Iwata, T., Yamato, M., Tsuchioka, H., Takagi, R., Mukobata, S., Washio, K., Okano, T. & Ishikawa, I. (2009) Periodontal regeneration with multi-layered periodontal ligament-derived cell sheets in a canine model. *Biomaterials* **30**, 2716–2723.

Iwata, T., Yamato, M., Zhang, Z., Mukobata, S., Washio, K., Ando, T., Feijen, J., Okano, T. & Ishikawa, I. (2010) Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. *Journal of Clinical Periodontology* **37**, 1088–1099.

Jepsen, S., Eberhard, J., Herrera, D. & Needleman, I. (2002) A systematic review of guided tissue regeneration for periodontal furcation defects. What is the effect of guided tissue regeneration compared with surgical debridement in the treatment of furcation defects? *Journal of Clinical Periodontology* (Suppl 3), 103–16.

Keles, G. C., Cetinkaya, B. O., Baris, S., Albayrak, D. & Simsek, S. B. (2009) Comparison of platelet pellet with or without guided tissue regeneration in the treatment of class II furcation defects in dogs. *Clinical Oral Investigation* **13**, 393–400.

Lang, H., Schuler, N. & Nolden, R. (1998) Attachment formation following replantation of cultured cells into periodontal defects–a study in minipigs. *Journal of Dental Research* **77**, 393–405.

Lian, J. B. & Stein, G. S. (1992) Concepts of osteoblast growth and differentiation: basis for modulation of bone cell development and tissue formation. *Critical Reviews in Oral Biology and Medicine* **3**, 269–305.

Murano, Y., Ota, M., Katayama, A., Sugito, H., Shibukawa, Y. & Yamada, S. (2006) Periodontal regeneration following transplantation of proliferating tissue derived from periodontal ligament into class III furcation defects in dogs. *Biomedical Research* **27**, 139–147.

Nakahara, T., Nakamura, T., Kobayashi, E., Kuremoto, K., Matsuno, T., Tabata, Y., Eto, K. & Shimizu, Y. (2004) In situ tissue engineering of periodontal tissues by seeding with periodontal ligament-derived cells. *Tissue Engineering* **10**, 537–544.

Niederman, R., Savitt, E. D., Heeley, J. D. & Duckworth, J. E. (1989) Regeneration of furca bone using Gore-Tex periodontal material. *International Journal of Periodontics Restorative Dentistry* **9**, 468–480.

Nohutcu, R. M., McCauley, L. K., Shigeyama, Y. & Somerman, M. J. (1996) Expression of mineral-associated proteins by periodontal ligament cells: in vitro vs. ex vivo. *Journal of Periodontal Research* **31**, 369–372.

O'Neal, R., Wang, H. L., MasNeil, R. L. & Somerman, M. J. (1994) Cells and materials involved in guided tissue regeneration. *Current Opinion Periodontology* 141–156.

Park, J. B., Matsuura, M., Han, K. Y., Norderyd, O., Lin, W. L., Genco, R. J. & Cho, M. I. (1995) Periodontal regeneration in class III furcation defects of beagle dogs using guided tissue regenerative therapy with platelet-derived growth factor. *Journal of Periodontology* **66**, 462–477.

Pepelassi, E. M., Bissada, N. F., Greenwell, H. & Farah, C. F. (1991) Doxycycline-tricalcium phosphate composite graft facilitates osseous healing in advanced periodontal furcation defects. *Journal of Periodontology* **62**, 106–115.

Pontoriero, R., Lindhe, J., Nyman, S., Karring, T., Rosemberg, E. & Sanavi, F. (1989) Guided tissue regeneration in the treatment of furcation defects in mandibular molars. A clinical study of degree III involvements. *Journal of Clinical Periodontology* **16**, 170–174.

Regazzini, P. F., Novaes, A. B. Jr., de Oliveira, P. T., Palioto, D. B., Taba, M. Jr., de Souza, S. L. & Grisi, M. (2004) Comparative study of enamel matrix derivative with or without GTR in the treatment of class II furcation lesions in dogs. *International Journal of Periodontics Restorative Dentistry* **24**, 476–487.

Ribeiro, F. V., Suaid, F. F., Ruiz, K. G., Rodrigues, T. L., Carvalho, M. D., Nociti, F. H., Sallum, E. A. & Casati, M. Z. (2010) Peri-implant reconstruction using autologous periosteum-derived cells and guided bone regeneration. *Journal of Clinical Periodontology* **37**, 1128–1136.

Ripamonti, U. & Reddi, A. H. (1997) Tissue engineering, morphogenesis, and regeneration of the periodontal tissues by bone morphogenetic proteins. *Critical Reviews in Oral Biology and Medicine* **8**, 154–163.

Sander, L. & Karring, T. (1995) Healing of periodontal lesions in monkeys following the guided tissue regeneration procedure. A histological study. *Journal of Clinical Periodontology* **22**, 332–337.

Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahim, J., Young, M., Robey, P. G., Wang, C. Y. & Shi, S. (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149–155.

Somerman, M. J., Young, M. F., Foster, R. A., Moehring, J. M., Imm, G. & Sauk, J. J. (1990) Characteristics of human periodontal ligament cells in vitro. *Archives of Oral Biology* **35**, 241–247.

Van Dijk, L. J., Schakenraad, J. M., van der Voort, H. M., Herkstroter, F. M. & Busscher, H. J. (1991) Cell-seeding of periodontal ligament fibroblasts. A novel technique to create new attachment. A pilot study. *Journal of Clinical Periodontology* **18**, 196–199.

Venezia, E., Goldstein, M., Boyan, B. D. & Schwartz, Z. (2004) The use of enamel matrix derivative in the treatment of periodontal defects: a literature review and meta-analysis. *Critical Reviews in Oral Biology and Medicine* **15**, 382–402.

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Clinical Relevance

Scientific rationale for the study: Some histological studies have demonstrated positive results after the use of autologous cells in tissue engineering. This study was designed to evaluate, histologically, the healing of surgically created class II furcation lesions in beagle dogs treated with autologous PDL cells associated with GTR.

Principal findings: Results showed that PDL cells were able to promote periodontal regeneration in class II furcation defects created in dogs.

Practical implications: PDL cells may improve periodontal regeneration; however, more studies are necessary to investigate the advantages and limitations of this therapy in the treatment of different types of periodontal defects.