Cell therapy with allogenic canine periodontal ligament-derived cells in periodontal regeneration of critical size defects.

Núñez, J¹, Sanchez N¹, Vignoletti F¹, Sanz-Martin I¹, Caffesse R¹, Santamaria S², Garcia-Sanz JA² & Sanz, M¹.

¹ Section of Periodontology, Faculty of Odontology, University Complutense of Madrid, Madrid, Spain.

² Department of Cellular and Molecular Medicine, Centro de Investigaciones Biológicas (CIB-CSIC), Madrid, Spain

Corresponding Author:

Prof. Mariano Sanz

Facultad de Odontología - Universidad Complutense de Madrid

Plaza Ramón y Cajal ; 28040 Madrid

e mail: marianosanz@odon.ucm.es

Key words: Bone remodeling/regeneration, periodontal ligament, cell biology, cementum, regenerative medicine .

CONFLICT OF INTEREST AND SOURCE OF FOUNGING STAMENT

The authors declare that the research is original and it has not been published elsewhere.

The authors have stated explicitly that there are no conflicts of interest in connection with this article.”

This study was partially supported through an Osteology Foundation Advanced Resesarch Grant (10-012)

Total word count: 3227

Number of figures and tables: 2 figures, 3 tables

Abstract

Aim: The objective of this in-vivo experimental study to evaluate the regenerative potential of a cell therapy combining allogenic periodontal ligament derived cells with in a xenogeneic bone substitute in a similar experimental model.

Methods: In 9 beagle dogs, critical-size 6 mm supra-alveolar periodontal defects were created around the PIII and PIV. The resulting supra-alveolar defects were randomly treated with either 1.4×10^6 allogenic canine periodontal ligament-derived cells seeded on de-proteinized bovine bone mineral with 10% collagen (DBBM-C) (test group), or DBBM-C without cells (control group). Specimens were obtained at 3 months and histological outcomes were studied.

Results: The histological analysis showed that total furcation closure occurred very seldom in both groups, being the extent of periodontal regeneration located in the apical third of the defect. The calculated amount of periodontal regeneration at the furcation area were comparable in both the test and control groups (1.93 ± 1.14 mm (17%) vs. 2.35 ± 1.74 mm (22%), respectively ($p=0.37$). Similarly, there were no significant differences in the amount of new cementum formation 4.49 ± 1.56 mm (41%) versus 4.97 ± 1.05 mm (47%), respectively ($p=0.45$).

Conclusions: This experimental study was unable to demonstrate the added value of allogenic cell therapy in supra-crestal periodontal regeneration.

CLINICAL RELEVANCE

Scientific rationale for the study: the search for new periodontal regenerative t to reconstruct the supra-alveolar periodontal lesion has focused on cell therapies using mesenchymal stem cells. The rational of this experimental investigation was to evaluate the regenerative potential of allogenic mesenchymal cells seeded in a bone replacement graft.

Principal findings: Cell therapy using allogenic canine periodontal ligament derived cells promoted partial regeneration of supra-alveolar periodontal defects in dogs.

Practical implications: This study suggests that the combination of cells with appropriate scaffolds may contribute to develop novel therapies for the treatment of supra-alveolar periodontal defects.

Introduction

The periodontal ligament (PDL) contains mesenchymal stem cells (MSCs) responsible for the maintenance and regeneration of periodontal tissues. These cells, termed PDLSCs, were first isolated by Seo et al. (2004) and their use as cell therapy has been evaluated in experimental studies (Núñez et al. 2010) demonstrating regenerative potential when seeded in a collagen sponge and implanted in a three-wall self-contained intrabony periodontal defect model in beagle dogs (Núñez et al. 2012). Other attempts to promote periodontal regeneration by bio-engineered approaches have used collagen sponges mixed with recombinant human bone morphogenetic protein-2 in three-wall intrabony defects (Choi et al. 2002) and growth/differentiation factor-5 in one-wall intrabony periodontal defects (Kim et al. 2009b). Collagen sponges as scaffold biomaterial, however, were unable to provide space maintenance in non-contained periodontal lesions. Recent investigations have used the furcation experimental model as a challenging model to evaluate the potential of cell therapies for periodontal regeneration, either using autologous periodontal ligament cells (Suaïd et al. 2012) or embryonic stem cells (Yang et al. 2013). These studies demonstrated the capability of this cell therapy in regenerating the furcation lesion, although without achieving a true supra-crestal periodontal regeneration. To demonstrate this goal, a critical size supra-alveolar defect models was developed (Wikesjö et al. 1994) and showed its ability to assess the biological potential of new regenerative therapies, as well as the space maintenance ability of the scaffold used (Pellegrini et al. 2009, Seol et al. 2010).

Harvesting autologous PDLSCs requires a prior tooth extraction, what is an added limiting factor of using this source of cells for periodontal regenerative cell therapies,

and hence, there is an aspiration to find effective allogeneic cell-based regenerative approaches, mainly for the treatment of those lesions, such as the supracrestal periodontal defects where traditional periodontal regenerative approaches are not predictable (Ding et al. 2010). It was, therefore, the purpose of this pre-clinical experimental in vivo study to assess the regenerative potential of an allogenic cell therapy, using canine periodontal ligament-derived cells seeded in a xenogeneic porous bone replacement graft implanted in critical size supra-alveolar furcation defects in dogs.

Material and Methods

Cell Culture

Allogenic canine periodontal ligament-derived cells (canine PDLDCs) were harvested from extracted premolars in dogs using previously reported protocol (Núñez et al. 2012) and characterization by immunocytochemistry (with antibodies to STRO-1, Osteocalcin, CEMP-1 and CD44); flow cytometry to CD73, CD90, CD44, CD105, CD24, CD45, CD11b, CD80, CD19; and mineralization assays (Núñez et al. 2012).

De-proteinized bovine bone mineral with 10% collagen (DBBM-C; Bio-Oss® Collagen, Geistlich Pharma AG, Wolhusen, Switzerland) was tested as scaffold and carrier of the allogeneic cells. In 100 mg of scaffold 2×10^6 cells in 200 μ l of culture medium were seeded and incubated for 45 min at 37° C in a 5% CO₂ / 95% air incubator, to allow adhesion of the cells to the matrix. The same scaffold, seeded with 200 μ l PBS without cells, was used as control. The loading cell capacity of the scaffold was determined with a MTT assay that measures the amount of living cells in/out of the scaffold, when compared against a standard curve containing a known number of cells. This MTT assay uses on a non-radioactive cell proliferation test, which measures the conversion of

a tetrazolium salt into a formazan product. Only viable cells were able to catalyze this reaction detected colorimetrically at an absorbance of 490nm. Results from these assays showed that after 45 min of incubating the cells in DBBM-C more than 80% remained alive for at least 2 hours. After seeding the allogeneic cells on DBMM, as described earlier, the scaffold was transferred to a fresh plate, covered with media to avoid drying and was implanted in the animal experimental defects within 2 hours.

Animals

This pre-clinical in vivo investigation was designed following to the modified ARRIVE guidelines for pre-clinical research (Vignoletti & Abrahamsson 2012). Nine male beagle dogs, weighting approximately 14 kg (12-18 kg), 12-14 months old, were selected and subjected to the prescribed quarantine period. All surgical procedures were performed in the Animal Experimentation Service Facility at the Veterinary Teaching Hospital Rof Codina of Lugo (Spain) once the Institutional Animal Ethical Committee had approved the protocol. This experimental study was conducted according to Spanish and European Union regulations and European Community Council Directive 86/609/EEC.

Surgery

All surgical procedures were performed under general anesthesia and sterile conditions in an operating room using a mixture of isoflurane at a concentration of 2,5-4% (Isoba-Vet, Schering-Plough, Madrid, Spain) and oxygen, with a mechanical respirator throughout the surgical procedure. Antibiotic prophylaxis was administrated using cefovecin sodium (8 mg/kg/s.i.d./s.c., Convenia, Zoetis, Spain). Dogs had free access to drinking water and were fed with a soft diet for reducing the potential mechanical trauma. Plaque control consisted in tooth cleaning three times a week with gauzes embedded in chlorhexidine solution (0.12%) during the first two weeks, and then a brush and chlorhexidine gluconate gel until euthanasia.

Surgery 1 (creation of the supra-alveolar defect)

A full mouth prophylaxis using ultrasonic scalers and manual curettes was carried out before the surgical intervention. Mucoperiosteal flaps were raised in both posterior lower jaws and the teeth PI, PII, the mesial root of MI were extracted. Critical-size 6 mm supra-alveolar periodontal defects around were created around teeth PIII and PIV (Wikesjö et al. 1994, Kwon et al. 2010). Using chisels and a hand-piece with fissure burs under copious water irrigation; alveolar bone was removed around the circumference of PIII and PIV. Then the roots of were planed with curettes and water-cooled rotating diamonds to remove the cementum. Orthodontic wire ligatures were then placed and secured around the affected teeth and inserted into the furcation defects before the flaps were repositioned and sutured with absorbable interrupted sutures (Vicryl" 4.0, Johnson & Johnson, St-Stevens- Woluwe, Belgium) (Fig. 1.1). The animals were then fed with a soft diet that allowed plaque accumulation and ensured the chronification of the created defects.

Surgery 2 (supra-alveolar regeneration)

After seven weeks of plaque accumulation, animals were sedated with 25 µg/kg of Medetomidine and reverted with 75 µg/kg of Atipamezol (Antisedan, Esteve, Barcelona, Spain). Ligatures were removed together with the accumulated plaque and calculus using ultrasonic scalers (Fig. 1.1). Seven days later (eight weeks after the creation of the supra-alveolar defects) the regenerative surgeries were carried out using the same anesthetic protocol described for the first surgical intervention. After raising buccal and lingual mucoperiosteal flaps the supra-alveolar periodontal defects were carefully debrided and the roots were planed with curettes (Figure 1.2). Reference notches were placed at the level of the bone crest at the buccal and inter-radicular surfaces of the two roots to serve as histological landmarks. Each quadrant was

randomized with either test or control treatments using a computer generated list (n=9) (Appendix Table 1). Supra-alveolar defects around PIII and PIV were treated with either 200 mg. DBBM-C (Bioss-Collagen®) seeded with 1.4×10^6 canine PDLDCs cells in 400 µl (Test) or 200 mg. DBBM-C (Bioss-Collagen®) seeded with the same volume of phosphate buffer saline (control) (Fig. 1.2).

Following the protocol described by Lindhe et al. (1995) the crowns of the treated teeth were reduced to approximately 2 mm coronal to the CEJ in order to avoid flap margin recession during the first weeks of healing. Furthermore, the coronal position of the flaps was assured by a coronal suture locked in a bucco-lingual groove created in the coronal portion of the tooth. The periosteum was released at the base of the mucogingival flaps to allow a tension-free closure of the flaps when sutured with Cytoplast™ PCS061 18PREM PTFE monofilament sutures (Osteogenic Biomedical, Inc. Lubbock, TX, USA) (Fig. 1.2).

Histological Processing

Three-month post-surgery, the animals were sedated and euthanized using an overdose of sodium pentobarbital (40-60 mg/kg/i.v., Dolethal, Vetoquinol, France). Four specimens of 36 (2 control and two test) were immersed in a commercial solution containing EDTA di-sodium salt (volume ratio specimen/decalcifier 1:100, Osteodec, Bio-Optica, Milano, Italy) until complete decalcification. Mesio-distal sections of 5 µm in thickness were obtained, stained with haematoxylin and eosin (HE) and Mallory's trichrome and evaluated by light microscopy. The other 32 specimens were processed for ground sectioning according to the method described by Donath & Breuner (1982). Mesio-distal sections of the most central mesial-distal aspect of the supra-alveolar defect were obtained and reduced to a thickness of approximately 40 µm using a grinding machine (Exakt® Apparatebau, Norderstedt, Germany). The slides were

finally stained using the Levai Laczkó technique (Laczkó & Levai 1975).

Histological Evaluation

All histometric measurements were performed blindly by a single calibrated examiner (IS) (in pre-study calibration exercises the inter-examiner agreement was > 96%), using a Nikon Eclipse Ti microscope (Nikon, Heidelberg Germany) equipped with image analysis software (Q-500 MC; Nikon, Heidelberg, Germany) and a computerized image analysis system (NIS Elements BR, Nikon DS-Ri1; Amstelveen, The Netherlands).

Linear Measurements

The following linear measurements (mm) were obtained on the mesial and distal roots of the furcation side (Appendix Fig.1) (Caffesse et al. 1990):

1- Total defect length (TDL): total length of the root surface between notches on the mesial and distal roots; 2- Tissue-free defect length (TFL): portion of the TDL with the absence of any new tissue formation; 3- New cementum (NC): linear extension of the root surface covered by new cementum; 4- Periodontal regeneration (R): linear extension of the root surface covered by NC adjacent to newly formed bone, with functionally-oriented collagen fibers; 5- Epithelium/connective tissue extension (ECT): 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 measurements (mm²) were collected (Caffesse et al. 1990) using the point counting technique:

1-Total defect area (TDA): area limited by the apical line and the root surface in the furcation region; 2- Non-filled area (NFA): portion of the TDA not filled with any tissue; 3- Total epithelial fill (TEF): portion of the TDA filled with epithelium 4- Total

connective tissue fill (TCTF): portion of the TDA filled with connective tissue;
5-Residual graft particles (RGP): portion of the TDA filled with DBBM-C (Bio-Oss®);
6- New bone area (NBA): portion of the TDA filled with new bone.

Statistical analysis

The means and standard deviations for each histological parameter were calculated for both experimental and control groups, being the animal the experimental unit for all the comparisons (n=9). Differences between groups were analyzed with the Mann–Whitney test for both the histological and clinical outcome variables.

Results

Clinical observations

During the postoperative period after the regenerative surgery, healing occurred uneventfully, without the occurrence of relevant adverse effects, such as allergies or other immunologic reactions; formation of abscesses or rejection of the grafting material. In the majority of the cases, however, there was soft tissue recession in the furcation area with early exposure of the furcation fornix, although soft tissues presented healthy clinical conditions without signs of overt inflammation.

Histological observations

No significant differences in any of the histological parameters were observed when treatment groups were compared. All defects exhibited new bone formation extending at varying distances from the basal bone, which was easily identified due to its lower calcification. Presence of biomaterial particles were evident in different quantities depending on the defect. In most cases the particles in the most coronal area were surrounded by dense connective tissue, but deeper the granules were covered and incorporated in the newly formed bone (Fig. 2.1 and 2.2). A PDL space between the

tooth and the newly formed bone was clearly observed, but occasionally small areas of ankylosis were observed in both treatment groups. Abundant collagen fibers between the newly created acellular cementum and bone were observed. These new collagen fibers were similar to the adjacent periodontal ligament containing numerous fibroblasts in a matrix composed of collagen fibers and blood vessels. New cellular cementum was clearly identified coronally to the notch, with similar characteristics and dimensions to the primary cementum. Crestal bone remodeling was also identified. A well-differentiated multilayer gingival epithelium was present in all samples without ulcers or lesions. The underlying connective tissue was well vascularized and free of inflammation (Appendix Fig. 2).

Histometric analysis

The histometric results are depicted in tables 1-3. There were no statistically significant differences between the treatment groups for any of the outcome variables measured. The analysis of the furcation areas showed in the treatment group a mean length of new cementum of 4.49 ± 1.56 mm (41% of total defect) versus 4.97 ± 1.05 mm (47% of total defect) in the control group ($p=0.45$). The calculated amount of periodontal regeneration was 1.93 ± 1.14 mm (22%) in the test group compared to 2.35 ± 1.74 mm (17%) in the control group, ($p=0.37$). The root surface covered by epithelium and connective tissue was similar in the test group when compared to the control group (3.08 ± 1.07 mm (28%) vs. 3.75 ± 1.35 mm (35%) ($p=0.31$)) (Table 1). Additionally, the analysis of the interproximal areas showed that the test group presented a mean length of new cementum of 0.7 ± 0.52 mm (6%) versus 1.17 ± 0.49 mm (11%) in the control group ($p=0.06$). The root surface covered by epithelium and connective tissue was also similar in the test and control groups (2.45 ± 1.05 mm (22%) vs. 2.75 ± 0.83 mm (26%) ($p=0.5$)). The calculated amount of periodontal regeneration was 0.27 ± 0.34

mm (2%) in the test group compared to 0.42 ± 0.45 mm in the control group (4%), ($p=0.46$) (Table 2).

In the control group, the furcation defects presented a mean fill of 21% and 2.25 mm^2 of connective tissue and gingival epithelium, while the corresponding values in the test group were 25%, and 2.78 mm^2 (Table 3). The mean hard tissue values were 43% (4.47 mm^2) for the control and 30% (3.27 mm^2) for the test group. The demineralized bovine bone mineral with 10% collagen (DBBM-C; Bio-Oss® Collagen) underwent a considerable degree of reduction, only 7% (0.77 mm^2) of residual DBBM-C was observed in the control furcations and 5% (0.57 mm^2) in the test group. These residual graft particles were mostly located in the proximity of the residual alveolar bone and were surrounded by new bone formation. Root resorption was not found, but minute areas of ankylosis were observed in both groups, representing the 3% (0.35 mm) of the total defect length. Two control furcations (12.5%) were filled completely and two others with more than 50% filling (12.5%), In the test group only 2 specimens (12.5%) presented partial filling of the furcation area.

Discussion

The aim of this study was to evaluate whether a cell therapy based on the implantation of allogenic canine periodontal ligament-derived cells seeded in a deproteinized bovine bone mineral with 10% collagen scaffold would result in periodontal regeneration in supra-alveolar chronic periodontal defects in beagle dogs.

The healing after the implantation of this bio-engineered intervention occurred uneventfully, without any adverse effects or rejection of the grafted materials, although the added value of the allogeneic cells was not demonstrated since there were no significant differences between the tested groups for any of the outcome measurements.

In spite of the allogenic source of the PDLDCs there was no evident inflammatory reaction, probably due to the demonstrated immunosuppressive properties of mesenchymal stem cells (Wada et al. 2009). The histological findings demonstrated that both tested regenerative therapies were able to promote similar partial periodontal regeneration with formation of new cementum, new bone and connective tissue attachment, although the extent of this supra-crestal regeneration was limited in these periodontal defects affecting the furcation. Similar outcomes have been reported in experimental studies using autologous PDL-MSC together with barrier membranes in the treatment of supra-alveolar acute periodontal defects, without significant differences between the cell therapy and control groups for new bone, cementum and PDL regeneration (Tsumanuma et al. 2016). One possible explanation for the lack of added value could be the experimental model used, since the use of large size degree III furcations (opening $> 4\text{-}5\text{ mm}^2$) combined with supra-alveolar periodontal defects may not provide the clot stabilization and wound stability needed for allowing the biological activity of the tested cell therapies. The absence of appropriate wound stability may have contributed to the early exfoliation of the biomaterial and treatment failure in both groups regarding furcation closure. This hypothesis is supported by the fact that after three months of healing the mean residual DBBM-C was only 0.57 mm^2 representing 5% of the defect area.

Using a similar experimental model with class III furcation defects resulted in similar results attaining only partial periodontal regeneration (Susin et al. 2015, Wikesjö & Nilvéus 1990). When assessing the extent of periodontal regeneration at the interproximal areas, the results were even more limited in both tested groups, what emphasizes the difficulty to stabilize the blood clot supra-crestally in this location during healing.

Supra-crestal periodontal regeneration not only needs wound stability but also space provision protected from the contaminated oral environment which allows the blood clot maturation and the differentiation to mature periodontal tissues during early healing. In this experimental study, we have used a block of de-proteinized bovine bone mineral combined with 10% collagen as scaffold biomaterial that has shown space maintenance capability and high osteoconductivity in experimental models aimed for bone regeneration (Araújo & Lindhe 2009). The results obtained in this investigation were similar to those reported in a randomized clinical trial for the treatment of periodontal 2-3 walled infrabony defects using autologous PDLSCs seeded in the same biomaterial (DBBM) and compared with the use of the biomaterial alone. No statistically significant differences were reported between test and control groups in terms of clinical attachment level gains and vertical bone fill (Chen et al. 2016). Other similar experimental studies have used as scaffolds for delivering stem cells synthetic ceramic biomaterials combining hydroxyapatite and β -tricalcium phosphate, also reporting similar outcomes (Iwata et al. 2009, Kim et al. 2009a).

The amount of periodontal regeneration achieved in the present investigation was significant with new cementum at the furcation area amounting 4.97 mm. (47% of total defect) and 4.49 mm. (41% of total defect) in control and test groups, respectively. These results were comparable with another experimental study where periodontal ligament cells were placed in acute furcation defects, reporting a mean 58% regeneration of the defect (Suaid et al. 2012). The referred experiment used acute periodontal defects, which have a significantly greater capacity to self-regenerate as compared to chronic supracrestal defects utilized in this study. In another experimental study using bone marrow mesenchymal stem cells (BMMSCs) seeded on

chitosan/anorganic bovine bone for treating acute surgically created one-wall critical size infrabony periodontal reported a regeneration of 38% of total defect (Zang et al. 2016). The amount of periodontal regeneration achieved within the furcation (2.35 mm and 1.93 mm in control and test groups, respectively) can also be comparable with other experimental studies evaluating guided tissue regeneration with barrier membranes of expanded polytetrafluoroethylene (e-PTFE) (Lindhe et al. 1995, Polimeri et al. 2004) or with a combination of enamel matrix proteins and a bioabsorbable membrane (Donos et al. 2003).

Extensive periodontal regeneration has also been reported in an experimental study using BMMSCs seeded in a β -tricalcium phosphate scaffold in class III furcation defects, with 89.2 % of new cementum (Nagahara et al. 2015). In this model, however, the adjacent teeth were not extracted and therefore, the defects were only limited to the furcation area, resulting in a defect anatomy more prone to space maintenance (keyhole defects). Similarly, experimental studies using similar cell therapies in well-contained defects have demonstrated extensive periodontal regeneration when treating intrabony defects (Núñez et al. 2012) as well as in furcation defects (Suaid et al. 2012). These differences in defect anatomy and morphology, as well as the acuteness of the model may well explain the observed differences in the regenerative outcomes achieved at the different experimental studies.

The supra-crestal furcation model used in this investigation required the coronally advancement of the flaps to attempt for the full coverage of the bio-engineered therapy during healing. The fact that only in two cases in the control group the full closure of the furcation was attained, underscores the difficulty of maintaining regenerated

supracrestal defects covered with soft tissues and well protected from the oral contaminated environment. It is well established that bacterial contamination will shift from a regenerative to an inflammatory phenotype, hence limiting the amount of tissue regeneration (Larsson et al. 2016).

Within the limits of this experimental investigation, the results indicate that the use of DBBM-C with or without allogenic PDLDCs cells promoted periodontal regeneration in experimental critical-size supra-alveolar chronic periodontal defects. Further research is needed to explore the use of cell therapies with efficient methods to avoid soft tissue recession and early exposure of the furcation (Chiu et al. 2013). Furthermore, tested cellular therapies might be enhanced by the addition of osteoinductive reagents or differentiating factors (Susin et al. 2015).

Acknowledgments

The authors gratefully acknowledge the help of Dr Fernando Muñoz (School of Veterinary, University of Santiago de Compostela, Santiago de Compostela, Spain) for the veterinary services and histological processing. We highly appreciate the collaboration of Dr. Nicola Discepoli (from School of Dentistry, Complutense University of Madrid, Madrid, Spain) as participating surgeon in the experimental surgeries. The authors declare have not any conflict of interest associated with this investigation and was partially financed through an Osteology Foundation Advanced Researcher Grant (grant # 10-012).

References

- Araújo, M.G. & Lindhe, J. (2009) Ridge preservation with the use of Bio-Oss collagen: a 6-month study in the dog. *Clinical Oral Implant Research* **20**: 433–440.
- Caffesse, R.G., Dominguez, L.E., Nasjleti, C.E., Castelli, W.A., Morrison, E.C. & Smith, B.A. (1990) Furcation Defects in Dogs Treated by Guided Tissue Regeneration (GTR). *Journal of Periodontol* **61**: 45–50.
- Chen, F.M., Gao, L.N., Tian, B.M., Zhang, X.Y., Zhang, Y.J., Dong, G.Y., Lu, H., Chu, Q., Xu, J., Yu, Y., Wu, R.X., Yin, Y., Shi, S. & Jin, Y. (2016) Treatment of periodontal intrabony defects using autologous periodontal ligament stem cells: a randomized clinical trial. *Stem Cell Research Therapy* **19**: 7:33.
- Chiu, H.C., Shen, E.C., Lin, S.J., Susin, C., Wikesjö, U.M.E. & Fu, E. (2013) Periodontal repair in dogs: space supports alveolar bone and cementum formation. *Journal of Clinical Periodontology* **40**: 358–363.
- Choi, S. H., Kim, C. K., Cho, K. S., Huh, J. S., Sorensen, R. G., Wozney, J. M. & Wikesjö, U. M. (2002) Effect of recombinant human bone morphogenetic protein-2/absorbable collagen sponge (rhBMP-2/ACS) on healing in 3-wall intrabony defects in dogs. *Journal of Periodontology* **73**, 63–72.
- Ding, G., Liu, Y., Wang, W., Wei, F., Liu, D., Fan, X., An, Y., Zhang, C. & Wang, S. (2010). Allogeneic Periodontal Ligament Stem Cell Therapy for Periodontitis in Swine. *Stem Cells*. **28**: 1829–1838.
- Donath, K. & Breuner, G.A. (1982) Method for the study of undecalcified bones and teeth with attached soft tissue. The Sage-Schliff (sawing and grinding) techniques. *Journal of Oral Pathology* **11**: 318–326.

- Donos, N., Sculean, A., Glavind, L., Reich, E. & Karring, T. (2003) Wound healing of degree III furcation involvements following guided tissue regeneration and/or Emdogain® A histologic study. *Journal of Clinical Periodontology* **30**: 1061–1068.
- Iwata., T., Yamato, M., Tsuchioka, H., Takagi, H., Mukobata, S., Washio, K., Okano, T. & Ishikawa, I. (2009) Periodontal regeneration with multi-layered periodontal ligament-derived cells sheets in a canine model. *Biomaterials* **30**: 2716–2723.
- Kim, S. H., Kim, K. H., Seo, B. M., Koo, K. T., Kim, T. I., Seol, Y. J., Ku, Y., Rhyu, Y. C., Chung, C. P. & Lee, Y. M. (2009a) Alveolar bone regeneration by transplantation of periodontal ligament stem cells and bone marrow stem cells in a canine peri-implant defect model: A pilot study. *Journal of Periodontology* **80**, 1815–1823.
- Kim, T. G., Wikesjö, U. M., Cho, K. S., Chai, J. K., Pippig, S. D., Sietler, M. & Kim, C. K. (2009b) Periodontal wound healing/regeneration following implantation of recombinant human growth/differentiation factor-5 (rhGDF-5) in an absorbable collagen sponge carrier into one-wall intrabony defects in dogs: a dose-range study. *Journal of Clinical Periodontology* **36**, 589–597.
- Kwon, D.H., Bisch, F.C., Herold, R.W., Pompe, C., Bastone, P., Rodriguez, N.A., Susin, C. & Wikesjö, U.M. (2010) Periodontal wound healing/regeneration following application of rhGDF-5 in a b-TCP/PLGA carrier in critical-size supra-alveolar periodontal defects in dogs. *Journal of Clinical Periodontology* **37**: 667–674.
- Laczkó, J. & Levai, G. (1975) A simple differential staining method for semi-thin sections of ossifying cartilage and bone tissues embedded in epoxy resin. *Mikroskopie* **31**: 1–4.
- Larsson, L., Decker, A.M., Nibali, L., Pilipchuck, S.P., Berglundh, T. & Giannobile, W.V. (2016) Regenerative Medicine for Periodontal and Peri-implant Diseases. *Journal of Dental Research* **95**: 255–266.

- Lindhe, J., Pontoriero, R., Berglundh, T. & Araújo, M. (1995) The effect of flap management and bioresorbable occlusive devices in GTR treatment of degree III furcation defects: An experimental study in dogs. *Journal of Clinical Periodontology* **22**: 276–283.
- Nagahara, T., Yoshimatsu, S., Shiba, H., Kawaguchi, H., Takeda, K., Iwata, T., Mizuno, N., Fujita, T. & Kurihara, H. (2015) Introduction of a mixture of β -tricalcium phosphate into a complex of bone marrow mesenchymal stem cells and type I collagen can augment the volume of alveolar bone without impairing cementum regeneration. *Journal of Periodontology* **86**: 456–464.
- Núñez, J., Sanz-Blasco, S., Vignoletti, F., Muñoz, F., Arzate, H., Villalobos, C., Núñez, L., Caffesse, R. & Sanz, M. (2012) Periodontal Regeneration Following Implantation of Cementum-Derived and Periodontal Ligament-Derived Cells. *Journal of Periodontal Research* **47**: 33–44.
- Núñez, J., Sanz-Blasco, S., Vignoletti, F., Muñoz, F., Caffesse, R., Sanz, M., Villalobos, C. & Núñez, L. (2010) 17beta-estradiol promotes cementoblast proliferation and cementum formation in experimental periodontitis. *Journal of Periodontology* **81**: 1064–1074 .
- Pellegrini, G., Seol, Y.J., Gruber, R. & Giannobile, W.V. (2009) Pre-clinical models for oral and Periodontal reconstructive therapies. *Journal of Dental Research* **88**: 1065–1076.
- Polimeri, G., Koo, K.T., Qahash, M., Xiropaidis, A.V., Albandar, J.M. & Wikesjö, U.M.E. (2004) Prognostic factors for alveolar regeneration: bone formation at teeth and titanium implants. *Journal of Clinical Periodontology* **31**: 927–932.

- Seo, B.M., Miura, M., Gronthos S., Bartold, P.M., Batouli, S., Brahimi, 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.
- Seol, Y.J., Pellegrini, G., Franco, L.M., Chang, P.C., Park, C.H. & Giannobile WV. (2010) Preclinical methods for the evaluation of periodontal regeneration in vivo. *Methods in molecular biology* **666**: 285–307.
- Suaib, F.F., Ribeiro, F.V., Gomes, T.R., Silvério, K.G., Carvalho, M.D., Nociti, F.H. Jr, Casati, M.Z. & Sallum, E.A. (2012) Autologous periodontal ligament cells in the treatment of Class III furcation defects: a study in dogs. *Journal of Clinical Periodontology* **39**: 377–384.
- Susin, C., Fiorini, T., Lee, J., De Stefano, J.A., Dickinson, D.P. & Wikesjö, U.M. (2015). Wound healing following surgical and regenerative periodontal therapy. *Periodontol 2000*. **68**: 83–98.
- Tsumanuma, Y., Iwata, T., Kinoshita, A., Washio, K., Yoshida, T., Yamada, A., Takagi, R., Yamato, M., Okano, T. & Izumi, Y. (2016) Allogeneic Transplantation of Periodontal Ligament-Derived Multipotent Mesenchymal Stromal Cell Sheets in Canine Critical-Size Supra-Alveolar Periodontal Defect Model. *BioResearch Open Access* **5**: 22–36.
- Vignoletti, F. & Abrahamsson, I. (2012) Quality of reporting of experimental research in implant dentistry. Critical aspects in design, outcome assessment and model validation. *Journal of Clinical Periodontology* **39** Suppl 12: 6–27.
- Wada, N., Menicanin, D., Shi, S., Bartold, P.M. & Gronthos, S. (2009) Immunomodulatory properties of human periodontal ligament stem cells. *Journal of Cellular Physiology* **219**: 667–676.

- Wikesjö, U. & Nilvéus, R. (1990). Periodontal repair in dogs: effect of wound stabilization on healing. *Journal of Periodontology* **61**: 719–724.
- Wikesjö, U.M., Kean, C.J. & Zimmerman, G.J. (1994) Periodontal repair in dogs: supraalveolar defect models for evaluation of safety and efficacy of periodontal reconstructive therapy. *Journal of Periodontology* **65**: 1151–1157.
- Yang, J.R., Hsu, C.W., Liao, S.C., Lin, Y.T., Chen, L.R. & Yuan K. (2013) Transplantation of embryonic stem cells improves the regeneration of periodontal furcation defects in a porcine model. *Journal of Clinical Periodontology* **40**: 364–371.
- Zang, S., Jin, L., Kang, S., Hu, X., Wang, M., Wang, J., Chen, B., Peng, B. & Wang, Q. (2016) Periodontal Wound Healing by Transplantation of Jaw Bone Marrow-Derived Mesenchymal Stem Cells in Chitosan/Anorganic Bovine Bone Carrier Into One-Wall Infrabony Defects in Beagles. *Journal of Periodontology* **87**: 971–981.

Table 1. Histometric results at 3 months post-surgery of linear parameters at furcation area of the supra-alveolar periodontal defects.

Group	TDL	TFDL	NC	PR	E/CTE
Control	10.49 (1.38)	4.23 (1.97)	4.97 (1.05)	2.35 (1.74)	3.75 (1.35)
Test	10.91 (1.27)	5.83 (1.77)	4.49 (1.56)	1.93 (1.14)	3.08 (1.07)
P VALUE	P=0.50	P=0.06	P=0.45	P=0.37	P=0.31

Mean ($\pm$ SD), in mm of the linear histometric measurements of the total Defect Length (TDL); Tissue Free Defect Length (TFDL); New Cementum (NC); Periodontal Regeneration (PR) and Epithelium/Connective Tissue Extension (E/CTE).

Table 2. Histometric results at 3 months post-surgery of linear parameters at interproximal area of the supra-alveolar periodontal defects.

Group	TDL	NC	PR	E/CTE
Control	10.49 (1.38)	1.17 (0.49)	0.42 (0.45)	2.75 (0.83)
Test	10.91 (1.27)	0.7 (0.52)	0.27 (0.34)	2.45 (1.05)
P VALUE	P=0.50	P=0.06	P=0.46	P=0.5

Mean ($\pm$ SD), in mm of the linear histometric measurements of the total Defect Length (TDL); New Cementum (NC); Periodontal Regeneration (PR) and Epithelium/Connective Tissue Extension (E/CTE).

Table 3. Histometric results at 3 months post-surgery of Area parameters at furcation area of the supra-alveolar periodontal defects.

Group	TDA	NFA	TEF	TCTF	THTF	RGP
Control	10.32 (2.76)	2.46 (1.88)	1.13 (0.43)	2.25 (1.49)	4.47 (2.42)	0.77 (0.59)
Test	10.85 (2.25)	3.44 (1.69)	1.34 (0.31)	2.78 (1.02)	3.27 (2.30)	0.57 (0.17)
P VALUE	P=0.79	P=0.16	P=0.2	P=0.45	P=0.35	P=0.75

Mean ($\pm$ SD), in mm² of the histometric measurements of the total defect area (TDA): area limited by the apical line and the root surface in the furcation region; Non-filled area (NFA): portion of the TDA not filled with any tissue; Total epithelial fill (TEF): portion of the TDA filled with epithelium; Total connective tissue fill (TCTF): portion of the TDA filled with connective tissue; Total hard tissue fill (THTF): portion of the TDA filled with hard tissue. Residual graft particules (RGP): portion of the TDA filled with DBBM-C (Bioss-Collagen®).

LEGENDS

Figure 1.1: (a). Generation of critical-size: supra-alveolar periodontal defects of 5-6 mm in third and fourth premolars were surgically created. These defects were made chronic by placing orthodontic ligatures around these teeth and allowing dental plaque accumulation. (b-c) Clinical and radiographic situation after two months showing that periodontal defects remained with evident loss of attachment and furcation exposure. **Figure 1.2:** (a) Clinical situation after Mucoperiosteal flaps were raised, the roots of PIII and PIV planed with curettes and water-cooled rotating diamonds to remove the cementum and the crowns of these teeth reduced to approximately 2 mm. A notch was performed as histological landmark, at the base of the defect with a round bur. (b) In the test group the supra-alveolar defects were treated with Bioss-Collagen® seeded each one with 1.4×10^6 allogenic canine periodontal ligament-derived cells (canine PDLDCs). (c) The periostium was fenestrated at the base of the mucogingival flaps to allow a tension-free closure when sutured with Cytoplast™ PCS061 18PREM PTFE monofilament sutures (Osteogenic Biomedical, Inc. Lubbock, TX, USA).

Figure 2.1: (a) Cross section of the furcation representing the control group with absence of total furcation closure. Periodontal regeneration was mainly located at the most apical areas. The xenograft material underwent a considerable degree of reduction. Toluidine Blue (TB) stain. Original magnification x 20. (b) Left detail of (a). The connective tissue fibers, coronal to the residual osseous crest, are inserted between new alveolar bone and the newly formed cementum (Original magnification x100; TB). (c) Central detail of (a). Residual graft particles were located mostly in the proximity of the basal bone and with new bone around them (Original magnification x100; TB). **Figure 2.2:** (a) Furcation representing the test group after 3 months of healing, showing

absence of total furcation closure. Periodontal regeneration was mainly located at the most apical areas. The xenograft material is significantly reduced. Toluidine Blue (TB) stain. Original magnification x 20. (b) Left detail of (a). The connective tissue fibers, coronal to the residual osseous crest, are inserted between new alveolar crest and the newly formed cementum (Original magnification x100; TB). (c) Central detail of (a). The bone was not as calcified as the resident alveolar bone. (Original magnification x 100; TB).

Appendix

Table 1. Clinical measurements at furcation area of the supra-alveolar periodontal defects.

Parameter	Surgery 1	Surgery 2
CEJ-AB	5.18 (0.37)	3.98 (0.67)
Fornix-AB	4.63 (0.47)	3.64 (0.47)

Surgery 1 representing at time of defect creation. Surgery 2 measurements were taken two months after chronification, prior to performing to regenerative surgery. CEJ-AB: Distance between cemento-enamel junction and alveolar bone. Fornix-AB: Distance between furcation fornix and alveolar bone. Mean (SD) mm.

Figure 1: The histo-morphometric analysis assessed the total length of the root surface; the amount of new cementum formation (NC); the linear extension of the root surface covered by NC adjacent to newly formed bone, with functionally-oriented collagen fibers (PR) and the extension of the root surface covered by epithelium and connective tissue.

Figure 2 of Histological observations: (a) *Fig. 6.* (a) Notch area of a test specimen, depicting a line of newly created cellular cementum with similar characteristics and dimensions as the rest of the cementum line. Haematoxylin and eosin (HE) and Mallory's trichrome stain. Original magnification x 40. (b) Right detail of (a). A PDL space between tooth and alveolar bone without signs of ankylosis, with collagen fibers between the cellular cementum and the newly formed bone, similar to the adjacent periodontal ligament including numerous fibroblasts in a matrix composed by collagen fibers and blood vessels. Haematoxylin and eosin (HE) and Mallory's trichrome stain. Original magnification x 200.