QUT Digital Repository:
http://eprints.qut.edu.au/34479

Jiang, Jun and Wu, Xiaohong and Lin, Minkui and Doan, Nghiem and Xiao, Yin and Yan, Fuhua (2010) *Application of autologous periosteal cells for the regeneration of class III furcation defects in Beagle dogs*. Cytotechnology, 62(3). pp. 235-243.

© Copyright 2010 Springer.

Application of Autologous Periosteal Cells for the Regeneration of Class III furcation defects in Beagle Dogs

Jun Jiang[1], Xiaohong Wu[1], Minkui Lin[1], Nghiem Doan[2], Yin Xiao[2], Fuhua Yan[1,3]

[1]School and Hospital of Stomatology, Fujian Medical University, China

[2]Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Qld. Australia

[3]To whom correspondence should be addressed at School and Hospital of Stomatology, Fujian Medical University, 246 Yangqiao Zhong Road, Fuzhou, Fujian 350002, People's Republic of China. E-mail: fhyan2005@126.com

Abbreviations

MSC: Mesenchymal stem cells

DMEM: Dubbleco's-modified Eagle's memoleculem

SEM: Scanning electron microscope

β-TCP: β-tricalcium phosphate

e-PTFE: e-polytetrafluoroethylene

Abstract

Objective*: ** The aim of this study was to evaluate the healing of class III furcation defects following transplantation of autogenous periosteal cells combined with β-tricalcium phosphate (β-TCP). ***Methods: Periosteal cells obtained from Beagle dogs' periosteum explant cultures, were inoculated onto the surface of β-TCP. Class III furcation defects were created in the mandibular premolars. Three experimental groups were used to test the defects' healing: Group A, β-TCP seeded with periosteal cells were transplanted into the defects; Group B, β-TCP alone was used for defect filling; and Group C, the defect was without filling materials. Twelve weeks post surgery, the tissue samples were collected for histology,, immunohistology and X-ray examination. ***Result:*** It was found that both the length of newly formed periodontal ligament and the area of newly formed alveolar bone in Group A, were significantly increased compared with both Group B and C. Furthermore, the proportion of newly formed periodontal ligament and newly formed alveolar bone in the defects, were much higher in both controls than tested. The quantity of cementum and its percentage in the defects (group A) were also significantly higher than those of group C. ***Conclusion:*** These results indicate that autogenous periosteal cells combined with β-TCP application can improve periodontal tissue regeneration in class III furcation defects.

Key Words

Autologous periosteal cells; Alkaline phosphatase; Class III furcation defects; Periodontal regeneration; Tissue engineering; *β*-tricalcium phosphate.

Introduction

Periodontitis is considered as subgingival inflammation caused by bacterial infection Shimizu et al. (2009). It affects the periodontal supporting tissues including periodontal ligament, cementum, and alveolar bone. Periodontitis affects the junction of multi-rooted tooth, initially with tissue destructions then gradually with further bone loss and eventually furcation involvement will be occurred. Aukhil (1991) pointed out that periodontal regeneration aims at restitution of supporting periodontal tissues loss due to periodontal diseases . However, furcation involvement is difficult to treat properly by conventional periodontal therapy or surgery. Jepsen et al. (2002) and Needleman et al. (2002) found that guided tissue regeneration (GTR) could promote periodontal regeneration and new attachment formation, especially in class II furcation defects and bone base pockets . But its therapeutic effect on class III furcation defects was limited. Pontoriero et al. (1992) reported that GTR was capable of closing class III furcation defects successfully in a dog model. However, the regeneration of class III furcation defects was always incomplete. It is suggested that this treatment is not effective for large class III furcation defects. De Bari C and his partners (2006) found that periosteal cells were clonogenic, displayed long telomeres and expressed markers of MSCs, regardless of donor age. Under specific conditions, both parental and single-cell-derived clonal cell populations could be differentiated to chondrocyte, osteoblast, adipocyte, and skeletal myocyte lineages *in vitro* and *in vivo*. Groeneveld et al. (1994) have also approved that periosteum cells could form

cartilage or bone *in vivo*. When periosteal was cultured in direct contact with bovine dentin slices in the presence of 10 mmol l^{-1} β-glycerophosphate, a fibrillar acellular cementum could be formed in these slices after two weeks. Steiner et al. (2007) indicated that periosteum was able to form alveolar bone, cementum and periodontal ligament when it was transplanted into periodontal defects. Mizuno et al. (2006) repaired Class III furcation defects in Beagle dogs by grafting autologous periosteal cells which was cultured from membrane derived from periosteum. They found that these membranoid promoted regeneration of periodontal tissue, and was able to form bone and worked as a barrier membrane in the regeneration of periodontal tissue. In the study conducted by Matsumoto et al. (2008), where β-TCP was used as scaffold material, they have proven that these membranoid are biodegradable and osteoconductive. To investigate the potential application of periosteal cells in periodontal regeneration, Beagle dogs were served as models for class III furcation defects in this study. Autogenous periosteal cells were harvested from the same dog, and then combined with β-TCP for the transplantation into class III furcation defects of the same Beagle dog. After 12 weeks, periodontal tissue regeneration was assessed by histology and histomorphometry.

MATERIALS AND METHODS

Preparation of periosteum cells and β-TCP composite

Four healthy Beagle dogs (Sichuan Academy of Medical Sciences Sichuan People's

Hospital Experimental Animal Research Institute) weighted between 9.5 and 10.5 kg were used for this study. Small pieces of periosteum around 5 mm × 4 mm in size, were harvested from the mandible of each dog during the time of establishing the furcation defects (detailed in the surgical procedure). These periosteal explants were then cut into smaller pieces of about 1 mm^2, and placed into the bottom of 6-well plates which were pre-wetted with Dulbecco′modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), and penicillin-streptomycin (100 IU ml^{-1}). The samples were then covered with 25 mm × 20 mm cover glass. The periosteal was cultured in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every 3 days until the outgrown cells were subconfluent, after that non-adherent cells were washed off with PBS and the adherent cells detached with trypsin-EDTA (Gibco, USA) and sub-cultured in a 10-cm dish.

β-tricalcium phosphate (*β*-TCP) (Shanghai Bio-lu Biomaterials Company Limited, China) was used as scaffold material in this study. *β*-TCP is composed of CaO and P_2O_5 at the ratio of 1.5 for Ca/P, similar to the inorganic composition of bone. The *β*-TCP used in this study was granular porous ceramic and their diameters were less than 1mm. Prior to *in vivo* transplantation, the *β*-TCP was sterilized with ethylene oxide gas, soaked in DMEM in 6-well plates (0.6g per well, overspread in the well with 1mm in thickness) for 24 hours to facilitate cell-scaffold incubation. The third passaged periosteal cells were incubated with *β*-TCP at 5×10^6 cells ml^{-1} in 6-well plates in 1ml culture media per well. The cell and *β*-TCP complex was cultured in

DMEM, supplemented with 10% FBS, and cultured at 37 °C with 5% CO_2 for 48 hours.

Surgical procedure

Six weeks prior to the cell transplantation, class III furcation defects were created surgically at the second, third and fourth mandibular premolars of Beagle dogs by using slow-rotation diamond burs. The alveolar bone from each premolar ($_2P_2$, $_3P_3$, $_4P_4$) was removed, creating a "horizontal" pattern of bone loss. According to the protocols by Yan et al. (2003) and Fernandes et al. (2005), the furcation defects were approximately 3 mm wide and 4 mm high and were filled with gutta-percha to promote inflammative reaction. Granulation tissue was removed and the root surface was thoroughly debrided by curets. Notches were placed on either side of the root at the top of the bone defects. Defects were then covered with e-polytetrafluoroethylene (e-PTFE) membrane completely. The e-PTFE membranes were fixed with surgical gel (Suncon, China). Mucoperiosteal flaps were subsequently repositioned and sutured. All dogs were fed on soft food to prevent traumas of mastication to the surgical areas. Six weeks later, the animal model of class III furcation defects were confirmed (Fig.1).

All teeth were randomly divided into three groups. Each furcally involved premolar was then assigned to one of three treatment groups: Group A (e-PTFE plus β-TCP and periosteal cells), Group B (e-PTFE plus β-TCP), Group C (e-PTFE). Mucoperiosteal flaps were raised and sutured with coronally reposition flap. No oral hygiene was instituted during the treatment. Six weeks later, the e-PTFE membranes were removed

and assessed by scanning electron microscope with JEOL JSM-6330F field emission (Philips XL30, NLD).

Histological processing

The animals were euthanized twelve weeks after surgery. The jaw of each animal was removed and specimens containing the experimental areas were harvested and placed in 20% buffered formalin. The radiolucency and density of the experimental defects in alveolar bone were assessed by X-rays. Specimens were then decalcified with 10% ethylenediaminetetra acetic acid (EDTA Gibco USA) for 2 months and then dehydrated in ethanol, embedded in paraffin, and serially sectioned (to 4 μm thickness) in the mesio-distal direction. Five sections collected from the central area of each furcation were selected from for further analyses. All sections were stained with hematoxylin-eosin and Mallory trichrome dye. The histological specimens were analyzed by a light microscope fitted with a digital camera. The linear distance and area, were measured by an analytic software (Olysia Bioreport3.2; Olympus, Japan). The measurements include: (1) TDL (total defect length): the distance between the notches on the mesial and distal roots. (2) NC (new cementum formation): the distance of root surface which was covered by newly formed cementum on the mesial and distal roots. (3) NP (new periodontal formation): the distance of root surface that periodontal ligament fibers had penetrated in between the notches between the mesial and distal roots. (4) TDA (total defect area): total defect area between the notches on the mesial and distal roots. (5) NBA (new bone area): the area filled with mineralized tissue. (6) STA (soft tissue area): the area filled with connective tissue and/or

epithelial tissue. (7) NFA (non-filled area): the area filled with nothing or occupied by dental plaque in the furcation defects. All distances were measured in millimeters (mm), and areas were measured in square millimeter (mm^2). (Fig. 2).

Statistical Analysis

Data obtained from linear measurements, area measurements and the percentage of regenerated tissue in the original defects, were expressed as means and standard deviation. One-way analysis of variance (ANOVA) was used for statistical evaluation. Student-Newman-Keuls (SNK) test was used to compare differences between groups. P-values less than 0.05 were considered statistically significant. These analyses were performed using Statistical Package for Social Science 10.5 for windows (SPSS 10.5, USA).

RESULTS

1. Clinical observations

Visual inspection: Upon visual inspection, parts of the barrier membranes appeared to separate from tooth cervix in all experimental animals, the coronal areas were not covered completely with gingiva while the unexposed membranes adhered tightly to tooth cervix. Closer observation of the gingiva adjacent to the membrane revealed that the gingiva attached and grew well into the central portion of the membranes. Once the e-PTFE membranes were removed, it became apparent that newly formed granulation tissues was detected in the defects. ***SEM inspection:*** At the coronal

portion of the tested site, evidence of orderly arranged collagen bundles, and bacterial colonies were found on the membranes (Fig. 3A). In the middle portion of the defects, it was obvious that a large quantity of fibre bundles adhered on the surface of the membranes in conjunction with well distributed and scattering erythrocytes were observed (Fig. 3B). In the lowest portion of the defect, the microfibrils on the membrane appeared to join to each other and there were clear evidence of porous structures between the microfibrils, as well as a few inflammatory cells.(Fig. 3C).

2. Efficacy of treatment

All animals survived from surgery without any adverse events. However, furcal perforation happened to a few teeth during preparation of defect model as well as during treatment (one in Group A and one in Group C). Furcation over preparation occurred in a number of teeth (one in Group A, two in Group B, and one in Group C). The furcation defects, which were over prepared and fail to meet the inclusion criteria in this study, were excluded. For all teeth included in this study, their furcation defect appeared to heal reasonably well with healthy gingival attachment. However, in a number of teeth, a small amount of calculus and plaque were detected in conjunction with minimal gingival recession (< 2mm).

3. Radiographical analyses

Dental radiographs were employed to assess mandibular bone defects 12 weeks after implantation. These radiographs showed evidences of bone regeneration as well as resorption. Areas of radiolucencies were clearly visible at bone-defect sites in Group B and C. In group A, the largest amount of bone regeneration was detected by x-rays

at the bone-defect sites (Fig. 4).

4. Histological analyses

Histological observation of the defect sites showed that the epithelium was found to surround all regenerating tissue, and the newly formed connective tissue was detected in the root furcation. Greater amount of newly-formed alveolar bone were observed in Group A than those in both Group B and C. Group C exhibited moderate or severe soft tissue inflammation. The newly formed cementum containing cementoblasts, distributed non-uniformly in all groups. These, in turn, helped uneven and collagen fibers insert into the newly formed cementum. In addition, immature newly formed bone including osteoblasts was observed in all three groups. The newly formed reverse lines showed that there were obvious boundaries between the newly formed bone and the existing bone (Fig. 5).

5. Results of linear measurements

The one-way analysis of variance and multiple comparison test indicated that the TDL among these three groups had no significant difference ($P>0.05$). The length of NC and NP had no significant difference between Group A and B, neither between Group B and C ($P>0.05$). But the length of NC and NP in Group A were significantly higher than Group C ($P<0.05$) (Table 1).

6. Results of area measurements

The analysis of variance and multiple comparison test showed that there was no significant difference for the TDA among these groups ($P>0.05$). The NBA in Group A was significantly higher than that of Group B and C ($P<0.05$), the NFA in Group B

was significantly lower than that of Group C (*P*<0.05) (Table 2).

7. The percentage of regenerated tissue in the original defect

The percentages of the length or area of all newly formed tissues in the original defect were calculated. The analysis of variance and multiple comparison test showed that NP% and NBA% in Group A were significantly higher than that in Group B and C, respectively (P <0.05); NC% in Group A was significantly higher than that of Group C; STA% in Group A was lower than that of Group B and C (*P* <0.05); NFA% in Group A was significantly lower than that of Group C (*P*<0.05). NBA% in Group B was significantly higher than that of Group C (*P* <0.05), but STA% and NFA% in Group B were significantly lower than that of Group C, respectively (*P* <0.05) (Table 3).

DISCUSSION

Hutmacher and Sittinger (2003) pointed out that periosteal cells could differentiate to bone when implanted *in vivo*, and had the potential for tissue engineering in bone reconstruction. Furthermore, De Bari C et al. (2006) demonstrated that, periosteal cells could also differentiate into chondrocyte, adipocyte、skeletal muscle cells and seem to be a favorable kind of cells for periodontal tissue engineering. In this study, the periosteal cell-induced periodontal regeneration were evaluated for the feasibility of autologous periosteal cells transplantation, in repairing class III furcation defects.

In all six experimental teeth of each treatment, it was noted that no significant

difference was found in average defect length and area of furcation defects in each group (*P*>0.05). Lindhe et al. (1995) demonstrated that the periodontal defects were difficult to repair completely if they were larger than 4 mm. In this study, the heights of defects were more than 4mm, and the average area in the three groups was 10.62 mm^2. Pontoriero et al. (1992) pointed out that, in the "horizontal" defects only the bone wall in the bottom supported cells to induce periodontal regeneration. Hovey et al. (2006) demonstrated that plaque was detrimental to traditional surgery and periodontal regeneration. In this study certain amount of plaque accumulation in the surgical sites was found due to the difficulties to keep oral hygiene for the animals, which may be responsible for the interruption of periodontal tissue regeneration and induce incomplete periodontal defect healing.

Regazzini et al. (2004) pointed out that, in GTR, the barrier membrane preserves a space for coronal migration of periodontal ligament cells and endosteal cells from the defect base. Tamai et al. (2007) reported that the β-TCP was widely used as a filling material in bone and cartilage repair. In this study, β-TCP with certain plasticity could prevent subsidence of e-PTFE membrane when it was combined with blood and culture medium, which preserves a space for proliferation of cells. However, β-TCP was displaced in some teeth in this study and the sealability of e-PTFE membrane was compromised, which allowed epithelial cells and connective tissue to grow into the defects. In each group it was found that epithelial cells and connective tissue were detectable in all the defects.

In the newly regenerated periodontal tissues, there were evidences of newly formed

alveolar bones, cementum and periodontal membrane. This indicated that the most likely pathway for periodontal recovering was to regenerate the functioning periodontium. Our results showed that the regeneration effect of periosteal cells in combination with β-TCP was better than the application of β-TCP alone. Compared with the defect without β-TCP filling, periodontal regeneration was detectable in β-TCP treatment group. This implies the ability to repair class III furcation defects by simple e-PTFE membrane is limited. The implantation of β-TCP served as space maker for the proliferation and differentiation of precursor cells. Furthermore, the osteoconductive nature of β-TCP facilitates bony tissue and blood vessels to grow into the implanted material efficiently. This subsequently promoted bone regeneration as well as restored functions of the defected teeth. Declercq et al. (2005) have shown that periosteum cells could differentiate into bone and play an important role in bone regeneration and the healing of bony defect or fracture. The same authors also indicated that periosteal cells express markers of mesenchymal stem cells and under specific conditions, they can differentiate to the chondrocyte, osteoblast, adipocyte, and skeletal myocyte lineages *in vitro* and *in vivo.* De Bari C et al. (2006) and Steiner et al. (2007) have demonstrated that the periosteum cells can take part in the regeneration of cementum, periodontal ligament and alveolar bone. Mizuno et al. (2006) indicated that when the membranoid substance combined periostium block and periosteal cells, then transplanted into class III furcation defects, could accelerate the regeneration of periodontal tissue. When combined with GTR membrane, Steiner et al. (2007) found that periosteum transplanted to furcation defects could help the

regeneration of alveolar bone, cementum and periodontal ligament. Similar results were reported in the application of the periodontal ligament fibroblasts and BMSCs by Yan et al. (2005); Hovey et al. (2006); Gay et al. (2007) and Li et al. (2008). Although the multiple differentiation potential of periosteum cells has been shown, no study has confirmed that periosteum cells can differentiate to cementoblasts. The newly formed periodontal ligament and cementum, might be differentiated from transplanted periosteal cells or derived from intrinsic periodontal ligament fibroblasts in the defects. Clearly, this study has demonstrated that the implantation of periosteum cells into the defects can significantly increase the quantity and biological activity of the tissue healing process in the type III furcation model.

CONCLUSION

Periosteal cells transplanted into the class III furcation defects in dog model showed an accelerated regeneration process of periodontal tissues. However, due to the challenge of cell delivery in the contaminated oral environment, ideal regeneration techniques need to be further developed and transplanted cell differentiation in periodontal defects also requires further investigation..

Acknowledgements

This study was supported by National Natural Science Foundation of China (30471892) and Key Item of Science and Technology Bureau of Fujian Province (2001Z021). We are grateful to Drs. Kai Luo, Xuan Zhan, and Xin Zhao for their

assistance in animal work and laboratory experiments.

References

Aukhil I (1991) Biology of tooth-cell adhesion. Dent Clin North Am 35:459-467.

De Bari C, Dell'Accio F, Vanlauwe J, Eyckmans J, Khan IM, Archer CW, Jones EA, McGonagle D, Mitsiadis TA, Pitzalis C & Luyten FP (2006) Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. Arthritis Rheum 54:1209-1221.

Declercq HA, Verbeeck RM, De Ridder LI, Schacht EH & Cornelissen MJ (2005) Calcification as an indicator of osteoinductive capacity of biomaterials in osteoblastic cell cultures. Biomaterials 26:4964-4974.

Fernandes JM, Rego RO, Spolidorio LC, Marcantonio RA, Marcantonio Junior E & Cirelli JA (2005) Enamel matrix proteins associated with GTR and bioactive glass in the treatment of class III furcation in dogs. Braz Oral Res 19:169-175.

Gay IC, Chen S & MacDougall M (2007) Isolation and characterization of multipotent human periodontal ligament stem cells. Orthod Craniofac Res 10:149-160.

Groeneveld MC, Everts V & Beertsen W (1994) Formation of afibrillar acellular cementum-like layers induced by alkaline phosphatase activity from periodontal ligament explants maintained *in vitro*. J Dent Res 73:1588-1592.

Hovey LR, Jones AA, McGuire M, Mellonig JT, Schoolfield J & Cochran DL (2006) Application of periodontal tissue engineering using enamel matrix derivative and a human fibroblast-derived dermal substitute to stimulate periodontal wound healing in Class III furcation defects. J

Periodontol 77:790-799.

Hutmacher DW & Sittinger M (2003) Periosteal cells in bone tissue engineering. Tissue Eng 9 Suppl 1:S45-64.

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? J Clin Periodontol 29 Suppl 3:103-116; discussion 160-102.

Li H, Yan F, Lei L, Li Y & Xiao Y (2008) Application of Autologous Cryopreserved Bone Marrow Mesenchymal Stem Cells for Periodontal Regeneration in Dogs. Cells Tissues Organs

Lindhe J, Pontoriero R, Berglundh T & Araujo M (1995) The effect of flap management and bioresorbable occlusive devices in GTR treatment of degree III furcation defects. An experimental study in dogs. J Clin Periodontol 22:276-283.

Matsumoto G, Omi Y, Kubota E, Ozono S, Tsuzuki H, Kinoshita Y, Yamamoto M & Tabata Y (2008) Enhanced Regeneration of Critical Bone Defects Using a Biodegradable Gelatin Sponge and {beta}-Tricalcium Phosphate with Bone Morphogenetic Protein-2. J Biomater Appl.

Mizuno H, Hata K, Kojima K, Bonassar LJ, Vacanti CA & Ueda M (2006) A novel approach to regenerating periodontal tissue by grafting autologous cultured periosteum. Tissue Eng 12:1227-1335.

Needleman I, Tucker R, Giedrys-Leeper E & Worthington H (2002) A systematic review of guided tissue regeneration for periodontal infrabony defects. J Periodontal Res 37:380-388.

Pontoriero R, Nyman S, Ericsson I & Lindhe J (1992) Guided tissue regeneration in surgically-produced furcation defects. An experimental study in the beagle dog. J Clin

Periodontol 19:159-163.

Regazzini PF, Novaes AB Jr, de Oliveira PT, Palioto DB, Taba M Jr, de Souza SL & Grisi MF (2004) Comparative study of enamel matrix derivative with or without GTR in the treatment of class II furcation lesions in dogs. Int J Periodontics Restorative Dent 24:476-487.

Shimizu H, Nakagami H, Morita S, Tsukamoto I, Osako MK, Nakagami F, Shimosato T, Minobe N & Morishita R (2009) New Treatment of Periodontal Diseases Using NFkB Decoy Oligodeoxunuclotides via Prevention of Bone Resorption and Promotion of Wound Healing. Antioxid Redox Signal.

Steiner GG, Kallet MP, Steiner DM & Roulet DN (2007) The inverted periosteal graft. Compend Contin Educ Dent 28:154-161.

Tamai M, Isama K, Nakaoka R & Tsuchiya T (2007) Synthesis of a novel beta-tricalcium phosphate/hydroxyapatite biphasic calcium phosphate containing niobium ions and evaluation of its osteogenic properties. J Artif Organs 10:22-28.

Yan F, Zheng B, Lin M & Xiao Y (2005) Tissue Regeneration of Chronic Periodontal Defects Using Tissue Engineering Approaches in a Dog Model. [J]. Journal of Oral Science Research 21:593-597.（in Chinese）

Yan F, Zheng Y, Fu S, Yao L, Chen H & Li L (2003) Experimental class II furcation defects preparation in mongrel dogs. J Clin Stomatol 19:456-458.（in Chinese）