Assessment of Bone Regeneration Using Adipose-Derived Stem Cells in Critical Size Alveolar Ridge Defects: An Experimental Study in a Dog Model

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Purpose: To assess bone regeneration potential of a fibronectin- and adipose-derived stem cell–covered ceramic biomaterial in three-wall critical size alveolar ridge defects. Materials and methods: In 18 dogs, four dehiscence-type and critical size defects were created surgically in the edentulous alveolar ridge. Defects were randomly regenerated using biomaterials coated with particulate ß-tricalcium phosphate (ß-TCP), ß-TCP with fibronectin (Fn) (ß-TCP-Fn), and ß-TCP with a combination of Fn and autologous adipose-derived stem cells (ADSCs) (ß-TCP-Fn-ADSCs), leaving one defect as control. The animals were divided into three groups according to the time of euthanasia (1, 2, or 3 months of healing). Results: At the time of sacrifice, statistically significant differences between the four types of defects in the total area of bone regeneration, percentage of neoformed bone matrix, medullary space, or contact between particulate biomaterial and neoformed bone matrix were not found. All defects showed a significant increase in neoformed bone matrix as sacrifice was delayed, but a uniform pattern was not followed. Only defects treated with ß-TCP-Fn-ADSCs showed a significant increase in the bone regeneration area when animals sacrificed at 3 months were compared to those sacrificed at 1 month (P = .006). Conclusion: The use of ADSCs in bone regeneration processes of critical size defects of the alveolar ridge did not entail an advantage regarding greater bone regeneration as compared with other biomaterials. However, the use of ß-TCP coated with a combination of Fn and ADSCs appeared to favor stabilization of the regenerated area, allowing a more efficient maintenance of the space at 3 months of healing. Int J Oral Maxillofac Implants 2016;31:xxx–xxx. doi: 10.11607/4190

Key words: adipose-derived stem cells, dehiscence-type defect, fibronectin, guided bone regeneration, stem cells

Implant placement is sometimes limited by critical defects in the edentulous alveolar ridge that occur following traumatic tooth extraction, tooth infections, or postextraction alveolar bone resorption. Large buccal cortical bone defects compromise both placement of implants and esthetic rehabilitation.1 Various techniques have been used for restoration of bone defects, including autografts, xenografts, allografts, and alloplasts with and without barrier membranes. Although autogenous bone grafting is accepted as
the gold standard of care,\textsuperscript{2–7} this method is associated with substantial morbidity and is limited in supply. Tissue engineering is a clinical alternative to autogenous bone grafts, and involves the morphogenesis of new tissue using constructs formed from isolated cells with biocompatible scaffolds and growth factors.\textsuperscript{2,6} Bone marrow–derived mesenchymal stem cells (MSCs) have been the main source for bioengineering. However, the clinical use of MSCs has presented problems, including morbidity and low cell number upon harvest. This has led many researchers to investigate alternate sources for MSCs with similar potential to differentiate into lineages of mesenchymal tissue.\textsuperscript{5,8–13}

Adipose-derived stem cells may represent a viable alternative option to bone marrow–derived MSCs since both are multipotent (capacity to give rise to a variety of other differentiated cell types, including osteocytes, chondrocytes, adipocytes, and myoblasts) and share common specific cell protein expressions and biomarkers.\textsuperscript{8,14–19} Also, bone regeneration may be enhanced by other factors, such as the use of fibronectin. Fibronectin is a glycoprotein of extracellular matrix that favors cell adherence, differentiation, and expansion. The use of biomaterials or titanium surfaces coated with fibronectin has shown satisfactory results in bone regeneration processes and osseointegration of dental implants.\textsuperscript{20–22}

The objective of the present experimental study was to assess bone regeneration potential of a ceramic biomaterial (ß-tricalcium phosphate, ß-TCP) alone or coated with fibronectin or the combination of fibronectin and adipose-derived stem cells (ADSCs) in three-wall critical size alveolar ridge defects covered with collagen, as compared with a control defect (without biomaterial filling).

**MATERIALS AND METHODS**

**Material**

The study was approved by the Ethics Committee on Animal Research (CEEA 227-109) of the University of Barcelona. A total of 18 somatically homogeneous female Beagle dogs in which a quarantine period was previously completed were included in the study. Animals were divided into three study groups and sacrificed at 1 (T1), 2 (T2), and 3 (T3) months postoperatively. Each animal was identified with an ear tattoo and microchip implant.

**Surgical Protocol**

The surgical protocol was designed into two phases in which animals were submitted to the same anesthetic technique, postoperative analgesia and antibiotic regimens, and aseptic conditions.

**Phase 1.** The first, second, and third premolars and the first molar of both mandibular hemiarches were extracted under general anesthesia. Dogs were premedicated with acepromazine, 2.5 mg/10 kg subcutaneously (s.c.) (Pharmavet) and atropine sulphate, 0.05 mg/kg s.c. (John Martin). Anesthesia was induced with sodium thiopental, 10 mg/kg intravenously (i.v.) (Pentovet, Richmond Vet Pharma) and inhaled 1.5% to 2% isoflurane (Soﬂoran, Pisa Agropecuaria) with subsequent endotracheal intubation. Local anesthetic infiltration, 1.8 mL per arch (articaine hydrochloride 4% and epinephrine 1:100,000, Ultracain, Normon) was also administered. Dental extractions were performed by odontosections to ensure preservation of the outer cortical bone, using round burs No. 6 tungsten carbide mounted handpieces and under constant irrigation with sterile saline. Alveolar mucosa was sutured with 4-0 silk sutures on a curved needle (Aragó). Sutures were removed 10 days later.

**Phase 2.** After a healing period of 3 months, four cylindrical bone defects (7 × 7 × 7 mm) were prepared after elevation of a mucoperiosteal flap, using a trephine bur (7 mm outer diameter), causing complete destruction of the buccal cortical plate of the alveolar ridge. Surgically created critical bone defects were similar to those reported by other authors.\textsuperscript{23–26} The position of the first premolar was assigned to the control group. The three remaining defects were filled at random with (1) 0.25 to 1 mm of particulate ß-TCP (KeraOs, Keramat), (2) particulate ß-TCP coated with fibronectin (ß-TCP-Fn), and (3) particulate ß-TCP coated with a combination of fibronectin and ADSCs (ß-TCP-Fn-ADSCs) (Fig 1). Defects were then covered with 30 × 40-mm bovine collagen membrane (Bio-Gide, Laboratorios INIBSA), and the surgical field was closed by primary intent with 4-0 silk sutures (Aragó).

**Postoperative Care.** Animals were kept on a soft diet and treated with amoxicillin trihydrate (Clamoxy, Pfizer), 15 mg/kg intramuscularly (i.m.) every 48 hours, starting 24 hours before surgery (five doses, total 10 days) and 0.2 mg/kg/day of meloxicam (Meloxicam Syntex, Syntex S.A.). Oral hygiene included daily brushing and irrigation with 0.2% aqueous solution of chlorhexidine.

Animals were sacrificed by a lethal dose of anesthetics at T1, T2, and T3. Both hemi-mandibles were dissected and immersed in 40% formaldehyde solution in codified containers for histomorphometric analysis.

**Bone Histomorphometry**

The samples were processed for study using the technique of embedding in methacrylate described by Donath and Breuner.\textsuperscript{27} Hemi-mandibles were divided into blocks, taking into account the study areas using the Exakt precision cutting and grinding system (Exakt 400...
Fig 1  (a) Preoperative view of the three study defects filled at random with bone regeneration biomaterial (β-tricalcium phosphate: β-TCP; β-TCP with fibronectin [Fn]: β-TCP-Fn; and β-TCP with a combination of Fn and adipose-derived stem cells [ADSCs]: β-TCP-Fn-ADSCs) and unfilled control defect. (b) Periapical radiographic image of the area after 3 months of healing. [Au: Please provide higher resolution images for figure 1.]

System, Apparatebau), dehydrated in graded alcohol, and embedded in glycol methacrylate (Technovit 7200 VLC, Heraeus Kulzer). Blocks were then divided along the long axis, and thickness was reduced until approximately 50-µm study samples were obtained.

Samples were stained following the method of Laczkó and Leval [Au: Reference 28 is Jenő and Géza. Should this be changed?] and were examined under a light microscope (BX51, Olympus) connected to a camera (DP71, Olympus). Twenty-five images at ×40 (×10 ocular, ×4 objective) were obtained and assembled using the Olympus Cell®D digital image system (Olympus). Reconstructed images were treated with Adobe Photoshop C S3 (Adobe Systems), and the areas of biomaterial and bone neoformation were colored (Fig 2). The following study variables were analyzed using the Microlmage 4.0 software (Olympus): surface area of bone regeneration (mm²), percentage of collapsed surface, percentage of neoformed bone matrix, percentage of medullary space, percentage of particulate biomaterial in relation to the original alveolar ridge area (the mean area of the original alveolar ridge was calculated according to the dimensions of the surgically created defect [Fig 3]), total perimeter of the biomaterial, and percentage of the perimeter of biomaterial in contact with the neoformed bone matrix.

Canine Adipose-Derived Stem Cells

Canine adipose-derived stem cells (cADSCs) were obtained from abdominal subcutaneous adipose tissue following a modified method described by Zuk et al. Twenty-four hours before the surgery, 500 μL of fibronectin solution (10 μg/mL in DMEM 1 g/L) was added per gram of bone graft and incubated at 37ºC for 24 hours. Finally, the coating solution was eliminated, and the grafts were washed with DPBS. One week before the surgery, the cells were thawed and plated at 1 × 10⁵ cells/cm² in a T75 flask in PM or osteogenic medium (OM) consisting of PM supplemented with 50 μg/mL ascorbic acid (Sigma), 1 μmol/L dexamethasone (Sigma) and 10 mmol/L glycerol 2P (Sigma). The osteogenesis was demonstrated by accumulation of mineralized calcium phosphate assessed by alizarin red staining.

Coating of β-TCP with Fibronectin (Fn) and ADSCs

Twenty-four hours before the surgery, 500 μL of fibronectin solution (10 μg/mL in DMEM 1 g/L) was added per gram of bone graft and incubated at 37ºC for 24 hours. Finally, the coating solution was eliminated, and the grafts were washed with DPBS. One week before the surgery, the cells were thawed and plated at 1 × 10⁵ cells/cm² in a T75 flask in PM medium and incubated at 37ºC in 5% CO₂. After 24 hours, the samples
were washed with DPBS to eliminate nonadhesive cells and kept in a PM. The cells were maintained with PM medium, which was changed three times per week. On the day of surgery, cells were harvested with trypsin-EDTA (Sigma) and seeded on β-TCP bone graft with or without fibronectin coating (5 × 10^5 cells/1 g β-TCP bone graft). To promote the adhesion, the cells seeded on the bone graft were maintained at 37 °C and 5% CO₂ for 2 hours.

**Statistical Analysis**
The Statistical Package for the Social Sciences (SPSS, SPSS Inc) version 15.0 for Windows was used for the analysis of data. Bone histomorphometric variables were expressed as mean and standard deviation (SD). Differences of histomorphometric variables between the control group and the three study groups (β-TCP, β-TCP-Fn, and β-TCP-Fn-ADSCs) at T1, T2, and T3 were assessed with the analysis of variance (ANOVA), and differences between data at T1, T2, and T3 with the t test for paired samples. Statistical significance was set at \( P < .05 \).

**RESULTS**
Of the 18 animals included in the study, two were excluded because of dehiscence of the operated area with important loss of regeneration material. Small wound dehiscence occurred in another two animals, which were sutured immediately, minimizing the loss of biomaterial. Therefore, data from 16 animals were analyzed with four histologic samples for each dog (control, β-TCP, β-TCP-Fn, and β-TCP-Fn-ADSCs), a total of 64 samples, which were grouped according to the euthanasia time (1 month, T1; 2 months, T2; 3 months, T3).

**Bone Formation and Degree of Collapse**
As shown in Table 1, there were no statistically significant differences in the mean area of bone regeneration (and, therefore, the percentage of collapsed surface) at T1, T2, and T3 time points in all study groups. However, as sacrifice of the animals was delayed, a progressive increase in the bone regeneration area was observed in all groups. Differences were only statistically significant for the group of animals treated with β-TCP-Fn-ADSCs when the bone regeneration area at T3 was compared to T1 (mean [SD] 26.21 [6.22] vs 14.24 [4.51] mm²) (\( P = .006 \)).

**Neoformed Bone Matrix and Medullary Space**
The percentages of neoformed bone matrix and medullary space in controls and the three study groups at different time points are shown in Table 1. When data
from controls and the three biomaterials (β-TCP, β-TCP-Fn, and β-TCP-Fn-ADSCs) at T1, T2, and T3 euthanasia times were compared, statistically significant differences were not found. However, the percentage of neoformed bone matrix increased significantly in all study groups as sacrifice of the animals was delayed (Table 1).

### DISCUSSION

The use of mesenchymal stem cells for regenerative purposes has been shown to be a predictable and promising technique, with reconstruction of critical defects of the arches as the main objective in the field of oral surgery and implantology. Differentiation of mesenchymal stem cells into osteoblasts takes advantage of osteoinduction and osteoconduction properties of autologous bone grafting, avoiding the need to use a donor area and harvesting autogenous bone grafts. More recently, much interest has developed in the use of ADSC, with similar differentiation capabilities to bone marrow cells and the benefits that can be easily harvested and cultured. Tissue engineering technology in combination with marrow-derived mesenchymal stem cells have been successfully used for alveolar bone regeneration.

### Table 1  Surface Area of Bone Regeneration in the Study Groups According to the Time of Euthanasia

| Data                              | Study groups |  |  |  |
|-----------------------------------|--------------|---|---|---|
| Bone regeneration area, mm², mean (SD) | Control | β-TCP | β-TCP-Fn | β-TCP-Fn-ADSCs |
| T1                                | 15.62 (6.73) | 16.77 (6.11) | 16.32 (1.85) | 14.24 (4.51) | .875 |
| T2                                | 18.54 (3.78) | 19.03 (6.28) | 21.71 (4.44) | 19.28 (3.47) | .708 |
| T3                                | 24.23 (13.14) | 24.55 (7.01) | 23.78 (9.64) | 26.21 (6.22) | .972 |
| Collapsed surface, %, mean (SD)   | T1          | 54.41 (17.5) | 56.41 (15.86) | 57.58 (4.82) | 62.98 (11.74) |
| T2                                | 51.81 (9.83) | 50.53 (16.31) | 43.56 (11.53) | 49.89 (9.02) |
| T3                                | 37.02 (24.15) | 36.20 (18.21) | 38.19 (25.05) | 31.87 (16.18) |
| Neoformed bone matrix, %, mean (SD)| T1          | 11.29† (3.42) | 14.33‡ (5.30) | 13.03 (3.39) | 10.08 (6.24) | .524 |
| T2                                | 20.61 (6.02) | 19.97 (7.86) | 19.51§ (5.36) | 12.33¶ (1.82) | .115 |
| T3                                | 32.12 (15.25) | 30.1 (4.08) | 29.6 (11.53) | 28.20 (6.59) | .929 |
| Medullary space, %, mean (SD)     | T1          | 22.52 (8.75) | 20.31 (8.01) | 21.23 (4.92) | 18.92 (7.69) | .891 |
| T2                                | 21.63 (5.88) | 15.94 (6.32) | 21.17 (5.91) | 21.87 (4.79) | .341 |
| T3                                | 22.32 (18.53) | 20.48 (7.37) | 20.52 (11.18) | 25.37 (9.92) | .893 |
| Biomaterial in contact with neoformed bone matrix, %, mean (SD) | T1          | NA | 31.43 (16.10) | 22.78 (9.69) | 16.50 (14.48) | .263 |
| T2                                | NA | 27.95 (3.49) | 28.71 (17.42) | 20.03 (11.02) | .475 |
| T3                                | NA | 41.48 (13.63) | 43.95 (21.26) | 27.33 (19.73) | .275 |

NA = not applicable.

*P = .006; †P < .05 for the comparison of T1 vs T2 and T1 vs T3; ‡P < .01 for the comparison of T1 vs T3; §P < .05 for the comparison of T2 vs T3; ¶P < .01 for the comparison of T2 v. T3.
favorable results of bone marrow stem cell-scaffold constructs to regenerate bone in significant osseous defects as compared with scaffolds without stem cells, with bone formation indexes similar to those obtained with autografts.

However, much controversy exists as to how stem cells efficiently differentiate and regenerate as well as how stem cell origin affects optimal differentiation and regeneration. Although ADSCs differentiate directly into osteoblasts less often than do bone marrow stem cells, the total amount of regenerated bone is almost the same. Moreover, experimental studies support the efficiency of mesenchymal stem cells derived from adipose tissue, periosteum, or umbilical cord blood for regeneration of bone defects.

Tissue-engineered bone regeneration using ADSCs provides an acceptable alternative to autologous bone graft but the regenerative capacity of ADSCs does not seem to be superior to autologous bone or other bone substitutes in the same conditions, as the authors found in the present study. A combination of β-TCP-Fn-ADSCs was used as a positive control instead of β-TCP-ADSCs because of the limited benefits seen in the results previously obtained by other authors when comparing adipose stem cells alone (or with a scaffold) over other bone substitutes or bone marrow stem cells. In addition, the size of the defects and the space available in the hemi-mandible limited the number of study groups, considering β-TCP-Fn-ADSCs as a more valuable group. The other hemi-mandible was used for another study, whose results will be reported shortly. In a canine maxillary alveolar cleft model, Pourebrahim et al showed less bone formation of ADSCs seeded onto hydroxyapatite/β-tricalcium phosphate-coated scaffolds than autografts. In the study of reconstruction of rat calvaria defects, Bohnenblust et al showed that the presence of osteogenic differentiated adipose stromal cells did not increase overall bone density compared with bone graft only. Han et al showed in cranial defects in rabbits, that the use of β-TCP-Fn-ADSCs as compared with other particulate biomaterials (β-TCP or β-TCP-Fn) or control defects in each of the T1, T2, and T3 study times. However, although there was a significant increase in bone formation as the healing was more advanced, this process was not homogeneous in all groups, with significant increases at 2 months after surgery in controls and at 3 months in β-TCP and β-TCP-Fn-ADSCs. The β-TCP-Fn group showed a progressive increase in neoformed bone matrix, with significant differences between animals sacrificed at 1 and 3 months of healing.

The use of agents as transfer vehicles of stem cells may have a positive effect in the process of bone regeneration of critical defects as well as acting as an osteoconductor element. Promising experiences have been reported with the combination of stem cells with platelet-rich plasma (PRP), fibrin glue, poly-lactic acid (PLA) polymers, synthetic extracellular matrices, or the use of allogeneic or synthetic bone substitutes. Tricalcium phosphate appears to be a biomaterial of choice for tissue-engineered bone regeneration because of its properties of biocompatibility, high conductivity, and lack of immunogenicity. Although there is insufficient evidence of the ideal biocompatible scaffold, frequently related to the defect size, tricalcium phosphate combined with stem cells has shown satisfactory results in the treatment of bone defects.

Contamination of control defects with regenerative material from adjacent bone defects, probably due to the use of particulate biomaterial, is a limitation of the study. Khoshzaban et al suggested that the material of the experimental defect got transferred to its adjacent empty defect from circulation and animal movement, especially at the operation area, after periosteal approximation. Takahashi et al concluded that the use of β-TCP and a collagen sponge could provide better intraoral manipulation capability than TCP granules alone, being particularly indicated in alveolar preservation procedures in bone defects with buccal dehiscence.

Other authors, such as Mankani et al, have shown that the size of the particles within bone marrow stromal cells appears to determine the extent of bone formation, with particles of 0.1 to 0.25 mm size demonstrating the greatest bone formation. In the present study, the use of biomaterial of higher particle size (between 0.25 and 1 mm) together with a high rate of contamination (80%) of control defects may be confounding factors for the lack of statistically significant differences in neoformed bone matrix between particulate biomaterial-treated defects (β-TCP, β-TCP-Fn, and β-TCP-Fn-ADSCs) and controls.

Fibronectin is an extracellular matrix glycoprotein that promotes cell adhesion, differentiation, and expansion. Jo et al showed that fibronectin in combination with a xenograft or a ceramic biomaterial (calcium phosphate) have a favorable effect on cell adhesion, especially within the first hours after culture. The adhesion-promoting property of fibronectin is particularly relevant in regeneration procedures of bone defects favoring adhesion of stem cells or osteoblasts when combined with some biomaterials.

In the present study, the use of a particulate β-TCP...
biomaterial coated with fibronectin or a combination of fibronectin and ADSCs was not associated with a better outcome in terms of bone formation or total area of bone regeneration as compared with the other study groups (β-TCP alone and controls). However, it should be noted that only bone defects treated with β-TCP-Fn-ADSCs showed a significant increase in bone regeneration as part of the period of the healing process increased. This enhancing effect of fibronectin on bone regeneration processes together with inherent properties of adipose stem cells may contribute to stabilize the regenerated tissue more rapidly, counteracting compression forces of surrounding soft tissue, with a space-maintaining effect that prevents collapse of critical defects. However, given that studies using a similar model of ADSCs have not been previously reported, comparative data are lacking.

CONCLUSIONS

The use of ADSCs in bone regeneration processes of critical size defects of the alveolar ridge did not entail an advantage regarding greater bone regeneration as compared with other biomaterials. However, the use of β-TCP coated with a combination of fibronectin and ADSCs appeared to favor stabilization of the regenerated area, allowing a more efficient maintenance of the space at 3 months of healing. The heterogeneity of experimental models in bone regeneration procedures using stem cells of different origins is an important drawback in the assessment of the advantages of the different scaffolds as well as determining the most adequate construct for each type of defect. Further studies are needed to determine the osteogenic ability of ADSCs in the reconstruction of bony defects.

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