Effect of Mesenchymal Stem Cells and Platelet-Rich Plasma on the Healing of Standardized Bone Defects in the Alveolar Ridge: A Comparative Histomorphometric Study in Minipigs

Francesco Pieri, DDS, PhD,* Enrico Lucarelli, BS,† Giuseppe Corinaldesi, MD, DDS,‡ Milena Fini, MD,§ Nicolò Nicoli Aldini, MD,‖ Roberto Giardino, MD,¶ Davide Donati, MD,# and Claudio Marchetti, MD, DDS**

Purpose: The purpose of this study was to test the effect of the combination of mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP) incorporated into a fluorohydroxyapatite (FHA) scaffold on bone regeneration in cylindrical defects in the edentulous mandibular ridge of minipigs.

Materials and Methods: Two mandibular premolar teeth were extracted bilaterally in 8 adult minipigs. After 2 months, 4 standardized defects of 3.5 mm diameter and 8 mm depth were created in each root site. The defects were randomly grafted with autogenous mandibular bone, FHA alone, PRP-FHA, or MSCs-PRP-FHA. A resorbable collagen membrane was placed over the defect area and the flaps were sutured. The animals were sacrificed 3 months later and biopsy samples were taken from the defect sites for histologic and histomorphometric assessment.

Results: There was no evidence of inflammation or adverse tissue reaction with either treatment. MSCs-PRP-FHA-treated sites showed new vital bone between residual grafting particles. PRP-FHA- and FHA-treated sites showed residual particles in a background of marrow soft tissue with a moderate quantity of newly formed bone. Autogenous bone (46.97%) and MSCs-PRP-FHA (45.28%) produced a significantly higher amount of vital bone than PRP-FHA (37.95%), or FHA alone (36.03%). Further, the MSCs-PRP-FHA-treated defects showed a significantly higher percentage of contact between graft particles and newly formed bone compared with PRP-FHA and FHA group (59.23% vs 48.37% and 46.43%, respectively).

Conclusions: Our results suggest that, in this animal model, the addition of MSCs to PRP-FHA enhances bone formation after 3 months.

A satisfactory functional and esthetic outcome of an implant-supported restoration depends on implant placement in a favorable prosthetic position. From a surgical standpoint, the height, buccal-lingual position, and dimensions of the alveolar ridge influence appropriate implant placement. Because tooth extraction is usually followed by partial bone resorption, it is important to preserve the ideal horizontal and vertical dimensions of the alveolar ridge using bone grafting techniques. Augmentation of alveolar defects using...
promote osteogenesis in vivo. Several studies have shown that MSCs can effectively differentiate into cells with an osteogenic phenotype. Accordingly, MSCs have multi-lineage differentiation potential and can differentiate into cells with an osteogenic phenotype. Mesenchymal stem cells (MSCs) have multiple osteoinductive growth factors, and biocompatible scaffolds. Mesenchymal stem cells (MSCs) have multi-lineage differentiation potential and can differentiate into cells with an osteogenic phenotype. Accordingly, several studies have shown that MSCs can effectively promote osteogenesis in vivo.

A viable alternative to these procedures could be a new discipline that combines cell therapy and tissue engineering to develop a more effective and safer therapeutic system for bone regeneration. This new therapeutic technology induces bone regeneration by using a combination of osteogenic cells, various osteoinductive growth factors, and biocompatible scaffolds. Mesenchymal stem cells (MSCs) have multi-lineage differentiation potential and can differentiate into cells with an osteogenic phenotype. Accordingly, several studies have shown that MSCs can effectively promote osteogenesis in vivo.

Platelet-rich plasma (PRP) has been reported to accelerate bone formation and contains a large number of platelets that release significant quantities of growth factors known to promote bone regeneration. The factors released by the platelets are strong angiogenic inducers and are known to be mitogenic for MSCs in vitro. However, their capacity to improve the osteogenicity of MSCs in vivo has not been well characterized. Recent studies have suggested positive, synergistic effects of MSCs and PRP mixtures in enhancing bone formation in the oral and maxillofacial regions.

Bone regeneration with the use of MSCs is influenced by the fact that during their implantation, appropriate scaffold material is added, which is necessary for delivering cells and growth factors and maintaining temporary mechanical function. Recently, results of several experimental studies indicate that a natural macroporous hydroxyapatite (Algipore, Friadent GmbH, Mannheim, Germany) (FHA), calcified from red algae (Corallina officinalis), may provide a matrix that supports the proliferation and differentiation of human osteoblast-like cells on its surface and may be suitable for use as a scaffold material in tissue engineering strategies in vivo.

To date, co-transplantation of MSCs and PRP in combination with a FHA scaffold to repair alveolar bone defects has not been reported. Thus, the aim of the present study was to evaluate the synergic effect of MSCs and PRP incorporated into a FHA scaffold on bone formation in surgically created bone defects in the edentulous mandible of minipigs.

### Materials and Methods

#### MINIPIG ANIMAL MODEL

The study was carried out according to the provisions of European and Italian laws on animal experimentation and the Animal Welfare Assurance No. A5424-01 of the National Institutes of Health (NIH, Bethesda, MD). The animal research protocol was approved by the appropriate public authorities, as required by Italian law, according to the European Community rules (Legislative Decree, 27 January 1992, no. 116).

Eight adult minipigs (18 months old) weighing 45 ± 4.5 kg were used. The minipigs were housed separately in metal cages throughout the study at a temperature of 24 ± 2°C and a relative humidity of 55%. They were provided semiliquid food (Mucedola, Settimo Milanese, Milan, Italy) and tap water ad lib.

#### PREPARATION OF PRP

The PRP used in the present study was prepared according to a previous protocol, adjusted for the use of minipig blood. Platelets were obtained from the venous blood of the minipigs. Blood was drawn from the jugular vein into a bag containing CPD [100 mL of CPD contains citric acid monohydrate (327 mg), sodium citrate dehydrate (2.63 g), glucose monohydrate (2.55 g), and sodium dihydrogen phosphate dehydrate (251 mg)] as an anticoagulant (1 mL CPD/7 mL blood). Blood was centrifuged twice, first at 1,000 g for 15 min at 20°C to remove red blood cells, and then at 3,000 g for 10 min at 20°C to obtain PRP, resulting in a platelet number of 1 × 10^6/mL as reported by Weibrich et al. On addition of 330 μL of calcium gluconate (100 mg/mL) to 10 mL of plasma, autologous thrombin was released. By mixing 4 mL thrombin and 16 mL PRP, platelets released their granular content and platelet gel was obtained. Thrombin was added to PRP immediately before use.

#### ISOLATION AND EXPANSION OF MSC

From each minipig, a 10-mL sample of bone marrow was aspirated from the posterior iliac crest. Mononucleated cells were isolated by a density gradient and resuspended in modified essential media (α-MEM; Sigma Chemical Co, St Louis, MO) containing 20% fetal calf serum (FCS; Euroclone, Wetherby, UK), penicillin (10 U/mL), streptomycin (100 mg/mL), and glutamine (2 mM) (Euroclone). Non-adherent cells were discarded 1 week later and adherent cells were cultured for further expansion. The day before the surgical procedure, 4 × 10^7 cells were harvested and mixed in the scaffold, composed of 1:1:1 platelet gel, FHA, and sterile, rat tail-derived collagen (Roche GmbH, Mannheim, Germany), so that the final volume was 5 mL.
ANIMAL MODEL AND SURGICAL PROCEDURE

All surgical procedures were carried out under aseptic conditions and general anesthesia. The animals were premedicated with ketamine and xylazine and anesthetized with halothane. The perioral hair was cut and both the perioral tissues and gingiva were disinfected with povidone iodine (Betadine, Purdue Pharma, Norwalk, CT). Surgery was conducted with due attention to aseptic technique and under continuous ECG monitoring.

At the beginning of the experiment, the second and fourth premolar teeth on both sides of the mandible were hemisected using a multiblade bur and they were carefully extracted. The mucoperiosteal flaps were closed with interrupted sutures, and the extraction sockets were left to assess spontaneous healing. After 2 months, the extraction sites were exposed using a mucoperiosteal flap, and 2 standardized cylindrical bone defects were made in each root site bilaterally using a trephine bur (Cizeta Surgical, Bologna, Italy) under copious saline irrigation. The defects measured 3.5 mm in diameter and 8 mm in depth. The bone defects were then randomly filled with: 1) FHA granules alone (negative control); 2) autogenous mandibular bone (positive control); 3) FHA granules mixed with PRP; and 4) FHA granules mixed with MSCs and PRP. The autogenous bone specimens were harvested while creating the defects and particulated granules alone (negative control);

HISTOLOGY AND HISTOMORPHOMETRY

The animals were pharmacologically euthanized after 3 months with intravenous injection of Tanax (Hoechst AG, Frankfurt am Main, Germany) under general anesthesia. A bone core was harvested from each graft site using a 3-mm trephine drill (Cizeta Surgical, Bologna, Italy), after scraping away the overlying soft tissues. The bone specimens were fixed in 4% para-formaldehyde for 7 days for undecalcified bone processing; the samples were then dehydrated in graded series of alcohols until the absolute was reached. After a 24-hour infiltration period in methylmethacrylate, they were finally embedded in poly-methylmethacrylate (Merck, Schuchardt, Hohenbrunn, Germany). Blocks were sectioned using Leica 1600 diamond saw microtome (Leica SpA, Milan, Italy). A series of sections of 150 ± 10 μm in thickness were obtained and then thinned and polished (Struers Dap-7, Struers Tech, Denmark) to a thickness of 50 ± 10 μm. A total of 3 consecutive sections for each biopsy was obtained, stained with toluidine blue, acid fucsin and fast green, and analyzed. Histologic and histomorphometric analyses were carried out by means of a light optic Olympus BX41 microscope (Olympus, Melville, NY) and the QWIN image analysis software (Leica Imaging Systems Ltd, Cambridge, UK). Bone histomorphometry linear measurements were taken by an experienced, blinded investigator at ×100 magnification according to criteria described previously. In each section, linear measurements were carried out to determine the percentage of the circumference of the FHA particles that was in direct contact with newly formed bone. Furthermore, the area fractions (%) within the bone specimens occupied by newly formed bone, soft tissue, and residual grafting particles were determined. The mean values of the 3 measurements were used for statistical analysis.

STATISTICAL ANALYSIS

Statistical evaluation of data was carried out using the SPSS/PC+ Statistics software package (version 10.1; SPSS Inc, Chicago, IL). Group means ± standard deviations (SD) were calculated for each measured parameter. The significance of the differences between the experimental groups was determined using a Student’s t test for paired samples. Differences were deemed statistically significant at P less than .05.

Results

The healing process was uneventful in all the animals over the 3-month implantation period. No infections occurred at the surgical sites.

HISTOLOGIC EVALUATION

Histologic examination of the PRP-FHA (Fig 1) and FHA (Fig 2) samples showed moderate new bone formation and separated particles of residual graft material in both groups. Only around some particles was it possible to observe the presence of newly formed bone without an intervening marrow soft tissue layer. In all specimens, the newly formed bone consisted mainly of woven bone with some more mature lamellar bone apparent. No inflammatory cell infiltrate was present. MSCs + PRP + FHA samples showed a more pronounced new bone formation with only few marrow spaces (Fig 3). Most of the FHA particles were surrounded by newly formed bone with wide osteocytic lacunae, whereas a very limited number of particles were in contact with biologic fluids and marrow spaces. Generally, the grafted particles were more integrated into the newly formed
bone, which was observed bridging the particles in a trabecular form. Bone morphology was more mature and well-organized presenting a lamellar pattern compared with PRP-FHA and FHA specimens. Higher magnification of the bone tissue around the grafted particles showed that no gaps were present at the bone-particle interface, and the bone seemed to always be in close contact with the particles. In some fields, osteoblasts were observed in the process of apposing bone directly on the particle surface. Although FHA particles were well-identified, in a few areas at the junction between the biomaterial and the newly formed bone, it was possible to observe the presence of resorbed portions of the FHA particles substituted by new bone. No acute inflammatory cell infiltrate or foreign body reactions were present around the particles or at the interface with the bone.

In specimens taken from the autogenous bone group, significant bone formation can be observed after 3 months. All defects were filled with newly formed dense bone and bone marrow. The newly formed bone tissue had parallel-fibered and lamellar structures. No remaining bone grafted particles were evident in any of the specimens (Fig 4).

**HISTOMORPHOMETRIC EVALUATION**

The first histomorphometric analysis evaluated the mean area percent (±SD) of new bone tissue, marrow space, and remaining FHA particles (Table 1). Among the evaluated treatment options, autogenous bone (46.97% ± 2.57%) and MSCs-PRP-FHA (45.28% ± 2.01%) produced a statistically significant ($P < .05$)

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**FIGURE 1.** A, Histologic section from a PRP-FHA-treated site showing 28.4% new bone, 33.9% FHA particles, and 37.7% marrow soft tissue. Foci of vital bone formation are observed between the FHA particles (H) dispersed among large areas of marrow soft tissue (Toluidine Blue/Fast Green; scale bar = 500 μm). B, High-power image of a single FHA particle (H) with new bone formation (*) on its surface (Toluidine Blue/Fast Green; scale bar = 100 μm).

**FIGURE 2.** A, Histologic section from a FHA-treated site showing 23.9% new bone, 38.5% FHA particles, and 37.6% marrow soft tissue. FHA particles (H) were surrounded by marrow soft tissue and little bone formation (Toluidine Blue/Fast Green; scale bar = 500 μm). B, High-power image of residual FHA particles (arrows) in the process of being substituted by newly formed bone (Toluidine Blue/Fast Green; scale bar = 100 μm).

higher amount of vital bone than PRP-FHA (37.95% ± 2.23%) or FHA (36.03% ± 1.86%) (Fig 5). Furthermore, there were no significant differences in the amount of residual graft particles between MSCs-PRP-FHA (30.73% ± 1.61%), PRP-FHA (32.83% ± 1.39%), and FHA alone (33.80% ± 1.40%).

The second histomorphometric analysis evaluated the mean percentage (±SD) of newly formed bone in direct contact with the FHA particle surface. MSCs-PRP-FHA showed a significantly higher result ($P < .05$), with a mean contact of 59.23% ± 5.53%, compare to PRP-FHA (48.37% ± 4.11%) and FHA (46.43% ± 5.20%) (Fig 6).

**Discussion**

The present study showed that MSCs-PRP-FHA group resulted in a significantly greater bone formation in alveolar bone defects in the minipig mandible than PRP-FHA and FHA groups, and implicated that MSCs can produce synergic effect with PRP-FHA. Our findings confirm previous reports in which the use of a cell transplantation approach combining different types of scaffolds with osteogenic cells is comparable to autogenous bone graft for the repair of surgically created defects.27,28 The minipig was chosen as an animal model because of its similarities to humans in terms of platelet count, clotting parameters, metabolic rate, bone structure, and characteristics of their MSCs.29-31 An
alveolar defect model offers several advantages for the histologic evaluation of bone tissue-engineered constructs. The surgical procedure is simple, with limited risk of infection, and a similar intervention by grafting is advocated clinically. The main disadvantage of this model is spontaneous bone regeneration, as the alveolar socket is not a critical sized defect. A true critical-sized mandibular defect in the minipig model is more than 5 cm³. Therefore, 4 critical-sized defects cannot be prepared in the minipig mandible due to the small size of the mandible. In this study, we chose an alveolar defect model because we sought to determine the potential ability of MSCs to enhance bone formation when it was added to PRP and a FHA scaffold. Each minipig served as its own control.

In our study, FHA-alone was used as scaffold material for testing MSCs-PRP bone induction. The reason for selecting this particular biomaterial was that FHA has proven to be a suitable carrier for osteoblast-like cells, bone morphogenic proteins, and growth factors. The observation made in this study that the FHA particles placed in the surgically created defects became properly integrated in the newly formed network of lamellar bone during healing indicated that the material was osteoconductive and acted as a natural scaffold for new bone formation. Furthermore, the complete closure of all defects filled with FHA-alone indicated that porous FHA particles did not hamper the physiologic bone healing response. Bio-compatibility of porous FHA was confirmed by histological analysis of the sections, and the formation of fibrous capsule around the particles did not occur in any section. In this respect, the current findings are consistent with observations made in human histologic studies, which showed that an intimate contact is always established between FHA particles and newly formed mineralized bone. The morphometric findings of the present study indicated that FHA particles were present in similar amounts in the MSCs-PRP-FHA, PRP-FHA, and FHA-filled sites (30.73%, 32.83%, and 33.8%, respectively). Thus, it is suggested that in the MSCs-PRP-FHA sites, the greater amount of new bone occupied space at the expense of bone marrow because we observed signs of resorption of the FHA particles in only a few areas. This is consistent with previous reports that FHA particles are slowly resorbed during bone remodeling by multinucleated giant cells. Our histologic findings suggest that complete resorption of the FHA particles can be expected over longer observation periods, but long-term studies are needed for confirmation.

The histomorphometric results showed that 36.03% ± 1.86% of new bone area was achieved in FHA-filled control defects, 37.95% ± 2.23% in the PRP-FHA-filled defects, and 45.28% ± 2.01% in the MSCs-PRP-FHA-filled defects. The tissue-engineered bone showed a significant improvement in bone regeneration compared with the other groups assessed and is similar to autogenous bone. Further, MSCs-PRP-FHA had the highest mean percentage of newly formed bone covering the surface of FHA particles. Contradictory results have been published regarding the effectiveness of the additional transplantation of MSCs for improving osteogenesis in an osteoconductive scaffold. The elevated percentage of new bone obtained in the MSCs + PRP + FHA-treated defects is consistent with previous studies in various animal models. In all studies, the osteoconductive carriers alone resulted in a limited degree of bone formation in segmental mandibular defects, whereas additional transplantation of cultured MSCs significantly increased the amount of newly formed bone inside the porous carriers. Mankani et al. reported enhanced bone formation in

Table 1. MEAN AREA FRACTIONS (±SD) OF NEWLY FORMED BONE, SOFT TISSUE, AND REMAINING GRAFTING MATERIAL AFTER 12-WEEK HEALING PERIOD

<table>
<thead>
<tr>
<th>Group (n = 8 for each)</th>
<th>Bone (%)</th>
<th>Soft Tissue (%)</th>
<th>Residual Grafting Particles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSCs + PRP + FHA</td>
<td>45.28 ± 2.01</td>
<td>23.99 ± 1.71</td>
<td>30.75 ± 1.61</td>
</tr>
<tr>
<td>PRP + FHA</td>
<td>37.95 ± 2.23</td>
<td>29.22 ± 1.85</td>
<td>32.83 ± 1.39</td>
</tr>
<tr>
<td>FHA</td>
<td>36.05 ± 1.86</td>
<td>30.17 ± 1.98</td>
<td>33.80 ± 1.40</td>
</tr>
<tr>
<td>Autogenous bone</td>
<td>46.97 ± 2.57</td>
<td>53.03 ± 2.38</td>
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MSCs-containing transplants as early as 6 weeks after implantation in a mouse mandible compared with MSCs-free transplants. In contrast, Henkel et al\textsuperscript{12} grafted minipig mandibular defects with a bioactive matrix (60% hydroxyapatite and 40% b-tricalcium phosphate) alone or mixed with MSCs and found that the addition of MSCs did not enhance new bone formation after an implantation period of 5 weeks. The authors observed that the nutrition of the cultured osteoblasts seeded in the carrier material was insufficient in their experiment and thus the stimulation of ossification was small. Recently, to provide effective enhancement of proliferation and stabilization of MSCs differentiation, PRP, as a source of autologous growth factors, has increasingly been used in combination with transplanted cells at the time of seeding, both in animals and humans\textsuperscript{10,22,39}. The rationale behind the combination of PRP to MSCs is that a high concentration of platelets in a bone defect will increase the local concentration of secreted growth factors and subsequently enhance initial bone regeneration.\textsuperscript{12} Some in vivo studies have assessed the efficacy of the combination of MSCs-PRP in the healing of bone defects\textsuperscript{16,17}, suggesting that MSCs exhibit a positive effect when combined with PRP. Moreover, Ito et al\textsuperscript{40} observed that after 2, 4, 8, and 12 weeks of healing, MSCs combined with PRP, compared with the PRP alone, increased new bone formation and biomechanical stability of mechanically produced defects in the mandibular ridge of dogs. The histomorphometric data of the present study indicated a positive influence of MSCs and PRP on bone formation. A possible explanation for the increase in newly formed bone is that the MSCs have the potential to differentiate from immature cells to osteoblast-like cells in the presence of the several growth factors (PDGF, TGF, IGF, VEGF) introduced through the contemporary application of PRP.\textsuperscript{39} Interestingly, enhanced bone formation stimulated by a combination of MSCs and PRP observed in this study has been described in several studies in humans and animals in which PRP was added both to autologous bone grafts\textsuperscript{40,41} and human MSCs.\textsuperscript{42} In contrast, PRP alone, with or without bone substitute materials, showed little positive effect on bone regeneration.\textsuperscript{43,44} However, the mechanism used by MSCs contained in PRP-FHA constructs to enhance bone repair still need to be clarified. Moreover, our study design did not allow for evaluation of the bone forming capacity of MSCs alone, as it did not include an MSCs-FHA group. Therefore, the improved results observed with the MSC-PRP-FHA group may in fact be solely due to the bone forming capacity of MSCs, without any synergistic effect from PRP or FHA.

Within the limit of this study, it can be concluded that after a 3-month healing period the addition of MSCs in combination with PRP-FHA enhanced the amount of newly formed bone in the minipig mandible compared with PRP-FHA and FHA alone, with a similar effect to autogenous bone graft. Our data show the healing pattern in an animal model, but further research is needed for human applications.

Acknowledgment

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References