ORIGINAL ARTICLE

Mandibular Segmental Defect Regenerated With Macroporous Biphasic Calcium Phosphate, Collagen Membrane, and Bone Marrow Graft in Dogs

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Objective: To reconstruct segmental mandibulectomy using calcium phosphate ceramics and collagen membrane with a delayed bone marrow grafting in experimental animals.

Design: Defects of segmental mandibulectomy were filled with calcium phosphate granules and wrapped with a collagen membrane in 4 dogs and left empty as a control in 2 dogs. Two months later, a bone marrow graft was injected into the center of the implants. Animals were humanely killed after a 16-week delay.

Subjects: Six adult beagles were included in this study.

Intervention: Segmental mandibulectomy.

Main Outcome Measure: Bone ingrowth and material resorption in the reconstructed segment.

Results: Successful osseous colonization bridged the whole length of the defects. The good new bone formation at the center and the periosteum-like formation at the periphery suggest the osteoinductive role of the bone marrow graft and the healing scaffold role of the membrane.

Conclusions: This model succeeded in regenerating a large segmental defect in the mandible. An investigation with a postimplantation radiation delivery schedule is required with the use of this model, which should be considered as a preclinical study for a bone tissue engineering approach in patients with cancer-related bone defects.


TREATMENT OF ORAL CARCINOMAS usually requires surgical removal followed by postoperative radiotherapy.1 Because tumors are frequently bulky at the time of diagnosis, bone is often involved and surgical procedures frequently require creating a large bone defect that alters esthetic and functional outcomes. Free flaps still constitute the gold standard in mandibular reconstruction.2 However, donor site morbidity, prolonged general anesthesia, necessity to select patients with good general status and without vascular co-morbidity, selection of the surgical team, and poor results after radiotherapy have led to the need to investigate alternative therapies.3,4

Autologous bone grafting failed to provide enough harvesting material and has also been associated with donor site morbidity. A variety of osteoconductive biomaterials, such as ceramics and titanium, have been widely studied as replacements for autologous bone grafts and have been successful in repairing unloaded bone cavities and small bone defects. The most attractive feature of macroporous biphasic calcium phosphate (MBCP) ceramic is its ability to form direct bone bonding with the host bone, resulting in a strong interface.5-8 These ceramics are commercially available in blocks, particles, and customized designs. One of the main concerns is to ensure the stability of the calcium phosphate ceramic particles in the site before bone ingrowth has occurred. Cross-linked collagen membranes, acting as resorbable scaffolds, have been developed to solve this drawback and guide the healing process in the surrounding tissues. Furthermore, calcium phosphate biomaterials are osteoconductive but have little if any osteoinductive activity.9 They act as scaffold, and their microporous and macroporous structure supports the ingrowth of osteogenic cells from the host bone toward the center of the implant. Despite good osteoconductive properties, calcium phosphate biomaterials are unable to encourage the recruitment of mesenchymal-type cells from the surrounding tissue for differentiation into osteogenic precursor cells. This lack of osteoinductive properties explains their failure in the reconstruction of large bone defects. The following 3 methods have been proposed to add osteoinductive potential to biomaterials: addition...
of cytokines and growth factors (directly or by gene therapy), use of nanomaterials, and addition of osteogenic cells from bone marrow.10-12

Bone marrow is a source of osteoprogenitor cells that can be stimulated to proliferate under appropriate conditions to form bone.13 Most of the currently available models combine isolation from whole bone marrow aspirates, ex vivo expansion, and then an attachment of mesenchymal stem cells to the biomaterial. However, there are several critical questions regarding the quality of cell population isolation, the preservation of stem cell properties after expansion, and the reproducibility of the model for clinical and surgical applications owing to its complexity.14 Moreover, the severe adverse effects of radiation on the properties of bone marrow cells are well known, and the radiation delivery often required in the postoperative period for oncological defects would certainly impair the osteoinductive potential of such composites.15,16

The use of total bone marrow grafts as an osteoinductive material has been investigated in animals, as well as in clinical applications in human nonunions,17-19 along with various types of calcium phosphate ceramics in both small defects20 and large segmental defects.21 We have recently proposed the concept of an immediate postradiation bone marrow graft to add osteoinductive potential to small bony defects in dog and rat models.22,23 The delayed and percutaneous marrow graft prevents any detrimental effect of the radiation on the osteoinductive potential.

The aim of this study was to reconstruct larger and segmental critical size defects in canine mandibles to mimic human oncological situations. The procedure used 1-stage marrow injection was 2 months so that radiation delivery could take place within it in a further model.

COMPOSITE

The composite was made from MBCP+ (Biomatlante SAS, Vigneux de Bretagne, France) and a collagen membrane (EZ Cure; Biomatlante SAS). The MBCP ceramic was made from 5 mm3 of granules composed of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) in a 20:80 weight ratio, with macropore size ranging from 300 to 600 µm and global porosity of approximately 80% (macroporosity, 80%; microporosity, 20%). The cross-linked porcine collagen membrane was 30 × 40 mm in size.

ANIMALS AND SURGICAL PROCEDURE

Six mature female beagles were obtained from a certified breeding center (Domaine des Souches, Toucy, France). Animal care was provided at the National Veterinary School in Nantes, France, in accordance with European directive number 86/609/CEE regarding conducting animal experiments. The study complied with the rules of ethics of the Nantes Veterinary School. The composite was implanted in 4 animals, and 2 defects were left empty as a control.

Molars and premolars 406, 407, and 409 were removed from the right mandible of 6 adult female beagles (Figure 1). At the same time, bone marrow aspirate samples were obtained from the left proximal humerus epiphysis and immediately spread on to slides for a cytological myelographic analysis (Table). Bone defects were made while the 6 beagles were under general anesthesia. General anesthesia was induced by intravenous injection of 0.04 mg/kg of dog medetomidine (Domitor; Pfizer Santé Animale, Paris, France) and 8 mg/kg of ketamine (Imalgène1000; Merial SAS, Lyon, France) and preserved by means of an isoflurane inhalation after tracheal intubation. A perioperative injection of 0.4 mg/kg tolenuc acid (Tolidine 4%; Vétouquinol SA, Lure, France) was performed for analgesia. A blood injection of 1 g of cephalixin (Rilexine; Virbac, Carros, France) was performed as perioperative antibiotic prophylaxis. A skin incision was made 1 cm above the inferior edge of the horizontal branch and was raised at the internal face of the platysma, preserving the facial nerve. The external and internal sides of the mandible were exposed up to the upper edge, and the mucosa was incised at the attached gingival tissue surrounding molar 408 that was initially not extracted, serving as a location for the defect. A 15-mm-long segmental defect centered by the residual molar (408) was surgically removed in all animals. Osteosynthesis was performed using a 3.1-mm-thick steel plate (DCP 3D ref. 245.210; Synthes, Solothurn, Switzerland) for 2.7-mm-diameter cortical screws. The mucosal opening was closed using a separated inverted closure (Vicryl; Janssen, Issy-les-Moulineaux, France) (Figure 2).

Four dogs were implanted with the aforementioned composite. A 30 × 40-mm resorbable porcine collagen membrane (EZ) was placed around the defect and then completely filled with MBCP granules, which were disposed side by side along
the defect, before being totally rolled around itself and sutured with 3 separate resorbable sutures (Vicryl). Postoperative prophylactic antibiotic therapy was administered using cephalixin (Rilexine) (15 mg/kg twice a day for 5 days), intraoral spiramycin (75 000 IU/kg/d for 10 days), and metronidazole (Buccoval; Labo Sepval, Laval, France) (12.5 mg/kg/d, for 10 days). The operated areas and general conditions of the animals were checked daily according to standard veterinary postoperative care.

In 2 animals, defects were left empty as a control.

**BONE MARROW GRAFT**

Two months after implantation, an autologous bone marrow graft was injected into the implanted site while the dogs were under general anesthesia. This delay was chosen to allow a radiation delivery in a clinical situation. Using the same surgical procedure as described for bone marrow cytological analysis, we removed 1 mL of bone marrow aspirate from the right proximal humeral epiphysis with a previously heparinized 18-gauge needle (30 mg of heparin in 1000 mL of physiologic serum dilution) and then immediately injected it into the center of the implants by means of a transcutaneous puncture under radioscopic imaging. All animals (implanted and controls) received bone marrow grafts (Figure 1).

**EXPLANTATION**

Four months after the bone marrow injection, all the animals were humanely killed. Time after initial bone defect creation was 24 weeks. The implanted bone areas were dissected and removed and were then fixed in 4% paraformaldehyde phosphate-buffered saline (Seromed, Berlin, Germany).

**X-RAY MICROTOMOGRAPHY**

Images (2- and 3-dimensional) were obtained from an x-ray microtomograph (Micro CT SkyScan 1072; SkyScan, Kontich, Belgium) without any preparation. Establishing different grayscale level thresholds in the slides made it possible to differentiate between bone ingrowth and residual ceramics.

**HISTOLOGICAL EXAMINATION**

All samples were dehydrated with graded ethanol and acetone. Nondecalcified bone specimens were infiltrated and embedded in glycol-methacrylate (GMA) resin. For each sample, serial sections were cut perpendicular to the implant using a diamond saw (Reichert-Jung Supercut 2050; Cambridge Instruments GmbH, Vienna, Austria) for 7-µm sections and using a tungsten saw (Leitz, Wetzlar, Germany) for 100-µm sections. The 7-µm sections were stained with Movat pentachrome and then observed under a light microscope (Olympus BH2; Olympus, Tokyo, Japan). The 100-µm sections were observed under polarized light (Olympus BH2). Perpendicular sections were chosen to allow image analysis in different levels of the implant with comparisons between injection site of the bone marrow (center) and adjacent sites (cranial and caudal quarter), avoiding great axial section bias.

**SCANNING ELECTRONIC MICROSCOPY AND IMAGE ANALYSIS**

After histological sections, the GMA-embedded bone specimens were sanded on a DP 10 (Struers SAS, Champigny sur Marne, France) then carbon coated using a JVG N1 low-vacuum evaporator (Jeol, Tokyo, Japan). Scanning electronic microscopic studies were performed with backscattered electrons at 15 kV in conjunction with image analysis. Ceramic integration and connections between bone and implants were analyzed. The quantity of newly formed bone and ceramic degradation was determined as previously described in the different levels of the implant: center, anterior quarter, and posterior quarter.

**RESULTS**

**BONE MARROW MYELOGRAPHIC ANALYSIS**

A cytological myelographic analysis was performed to assess the quality of the bone marrow grafts obtained by humeral puncture. The different lineage physiological cells contained in bone marrow were found, that is, myeloblastic, myelocytic, proerythroblastic, erythroblastic, and megakaryocytic lineages, lymphocytes, plasmocytes, and monocytes. The results confirmed that the samples enclosed physiological bone marrow, without blood recovery (Table).

**POSTOPERATIVE CARE**

Spontaneous mucosal fistulas in regard to the bone defect occurred in all animals at the seventh postoperative day. All animals underwent a further surgical procedure, with 0.9% sodium chloride solution irrigation of the defect cavity. At this time, a thick, localized mucosal translational flap rising from the floor of the oral cavity adjacent to the defect was performed in all animals to cover the defect area by adding a new collagen membrane. The flap was sutured directly on to the mucosa at the periphery of the defect with special care to avoid any contact between the suture and the bone defect. After this new procedure, a good outcome was obtained, and all animals had adequate mucosal healing and normal oral feeding.

**X-RAY MICROCTOMOGRAPHIC ANALYSIS**

Three-dimensional imaging made possible global examination of the implants and showed a bony formation bridging the whole length of the defect in all the implanted animals (Figure 3). Two-dimensional imaging showed that ceramic granules had been colonized by calcified tis-
sue (Figure 4). The distribution of this newly formed bone was homogeneous at the different levels of the implants. In the control group, nonunions were observed and the defects had not healed.

HISTOLOGICAL AND SCANNING ELECTRONIC MICROSCOPIC ANALYSIS

The defects in the control group did not heal spontaneously, and no bone formation was observed in the center of the defect. A very small amount of calcified tissue could be observed at the expense of the defect (Figure 3). Scanning electron microscopic analysis from a section of the implant (3a, 4a, 5a, and 6a) revealed that new bone formation has surrounded the granules and colonized the macropores. In 3 samples (3b, 4b, and 6b), granules have been totally surrounded by newly formed bone, whereas a part of the remnant granules has not been colonized in the other (5b).

Figure 3. Three-dimensional reconstruction from x-ray microtomographic analysis with scanning electron microscopic section correspondence. Nonunion was observed in the control group (1 and 2) with a very small amount of calcified tissue arising from defect borders (scale bar = 15 mm). In the implanted group, complete bridging of the defect was observed with 3-dimensional reconstructions from x-ray microtomographs (3a, 4a, 5a, and 6a). Scanning electron microscopic analysis from a section of the implant (3b, 4b, 5b, and 6b) revealed that new bone formation has surrounded the granules and colonized the macropores. In 3 samples (3b, 4b, and 6b), granules have been totally surrounded by newly formed bone, whereas a part of the remnant granules has not been colonized in the other (5b).

Figure 4. Two-dimensional microtomographic analysis. New bone formation (gray) has colonized the defect surrounding the ceramics granules (white). Threshold sensibility of the microtomograph density does not allow the observation of new bone formation within the macropores (M), which is best observed with scanning electron microscopy.

microscopy and scanning electronic microscopy using backscattered electrons, the bone appeared well mineralized and organized. Lamellar bone was observed directly in contact with the MBCP granules and with no fibrous interposition. Neither acellular, avascular area nor fibrous encapsulation was observed in the implanted group. New bone formation had developed from each extremity of the host bone of the defect to its center, with osseous continuity. The defect was filled with woven bone composed of dense and lamellar structures, with few spaces for vessels and soft tissue. The soft tissue and mucosa at the periphery of the remnant ceramic, where the collagen membrane had initially been, showed no histological modifications (Figure 5). In the noncalcified spaces seen on the scanning electronic microscopy sections, marrow cells with little fibrosis were observed with Movat pentachrome staining.

COMMENT

The aim of this study was to produce a preclinical animal model for regeneration of a mandible segmental critical size defect that could be integrated into a postoperative radiation delivery schedule in oncological conditions. To our knowledge, this study is the first to evaluate a composite combining calcium phosphate ceramic granules with collagen membrane, followed by a delayed bone marrow graft in a segmental defect in dog mandibles.
Our experimental results indicate successful bone colonization with this model. In all the animals in the implanted group, the reconstruction of the defect allowed for normal oral feeding. Morbidity at the time of bone healing reconstruction was minimal, and there was only donor site morbidity from the bone marrow aspiration that was noted as an adverse effect of this experimental model. This model reproduces many of the surgical difficulties that are often encountered in human mandible reconstruction. The viability of a critical size segmental defect in dog mandibles is a challenge in itself; since osteosynthesis must support the biomechanical strength of physiological mastication, the defect will not heal spontaneously until the end of the implantation delay, and the proximity of the oral cavity increases the probability for infectious complications.

Osteosynthesis was chosen as a compromise, avoiding any intraosseous defect mobility and making possible biomechanical stimulation for oriented bone ingrowth. The absence of well-oriented bone organization with woven bone after such a long delay suggests that osteosynthesis may have limited the biomechanical stimulation in the defect and that less rigid osteosynthesis may have been chosen. The critical defect concept depends not only on healing delay, defect size, location and species, and periosteum preservation but also on the quality and stability of the osteosynthesis. Osteosynthesis that does not provide stability would lead to insufficient spontaneous bone healing and an inadequate critical size defect. A 15-mm-long defect has already been described as being a critical size defect at 24 weeks in dog mandibles. On the basis of this, the defect presented was considered to be critical, and for ethical consideration no control group without ceramics or bone marrow grafts was constituted for this study. The control group was injected percutaneously with bone marrow grafts into empty defects because the ceramic was considered to be the main parameter in bone regeneration. When used alone, ceramics have failed to reconstruct large segmental defects, whereas bone marrow grafts used alone have been considered to be an easy method for regeneration of nonunions in diaphyseal bone in both animals and humans.
mans. Bone and ceramic quantities were identical at the different levels of the implant, which is unusual in macroporous calcium phosphate bioeramics for which centripetal bone colonization is commonly observed even after 16 weeks. These observations suggest that bone marrow grafts at the center of the defect may have an osteoinductive effect.

The proximity of the oral cavity may result in serious sepsis, compromising the success of the bone reconstruction. Most experimental models used in mandible reconstruction studies do not usually include the mucosal aperture. Teeth are usually removed at a primary stage several weeks before the segmental bone defect is created via a cervical approach. During this delay, the mucosa was able to heal spontaneously, leading to a continuous layer over the defect that protected against salivary aggression. In our study, one of the molars was left in its socket as a permanent marker for the bone defect that was centered at this point. The final dental extraction was performed at the same time as the creation of the bone defect. It is likely that the mucosal aperture required for the extraction and the excessive tension from the filling volume on the sutured mucosa explain the early postoperative nonunion that occurred in all animals. This is a drawback that will need to be addressed in future experiments using this model. Another explanation would be that mucosal breakdown due to saliva aggravating the suture could have led to premature destruction of the collagen membrane and then exposition of the granule asperity to the mucosa. Local flaps completed with a new collagen membrane made it possible to restitute the composite coverage and its isolation from the oral cavity. Collagen membrane was used not only as a way of making the surgical management of the biomaterial easier but also as a guide for bone regeneration. It acts as a resorbable healing scaffold that can lead to a thick fibrous interposition between the mucosa and ceramic and also to periosteum-like tissue formation on the external bone surface. Many types of guided bone regeneration membrane have been studied in various mandible models that can lead to confusion. Collagen membrane has been used for guided bone regeneration with positive results in animal experiments as well as in controlled clinical studies in humans. Our experimental model thus reproduced the surgical and clinical difficulties frequently encountered in oral surgery in humans, and the results of this study demonstrated its feasibility.

In our study, the composite was made from MBCP granules composed of HA and ß-TCP in a 20:80 weight ratio combined with collagen membrane. To achieve a potentially clinically applicable procedure, a cross-linked porcine collagen membrane was used to make the surgical reconstruction process easier by maintaining granules in the defect. This biocompatible barrier acts as a resorbable healing scaffold and can lead to peristome-like tissue formation on the external bony surface when used in conjunction with calcium phosphate ceramics. It has also been used for guided bone regeneration with positive results in both animal experiments and human clinical studies. The aim of using a biphasic composition is to take advantage of the rapid resorption of the ß-TCP and the inert scaffold of dense hydroxyapatite. Livingston studied various HA to ß-TCP ratios from 100% HA to 100% ß-TCP in subcutaneous implantation in mouse backs. The highest amount of new bone formation was obtained with a 20:80 HA to ß-TCP ratio at 6 and 12 weeks, suggesting the potential of using faster resorbable ceramics in bone tissue engineering. The osteogenic potential of calcium phosphates is based on their osteoconductive properties. Such ceramics are used as a scaffold on which new bone leans to colonize the defect from the periphery to the center of the defect. In the case of large bone defects such as in our model, osteoconductive properties are unable to attain bone colonization, and peripheric osteogenic cells are not likely to be recruited in the center of such a large defect, leading to insufficient ceramic resorption and bone substitution. This may have been more critical in our model owing to the adverse effects of radiation. External radiotherapy after major bone removal and reconstruction is a common situation in oral oncology. The effects of radiation on normal bone are well known: bone marrow is deprived, and vascularity, bone ingrowth, and bone remodeling decrease.

For clinical oncological applications of this type, the biomaterials must be not only osteoconductive but also osteoinductive so as to provide osteogenic capacities. Bone marrow cells, such as the adjunction of mesenchymal stem cells, have been suggested as a means of adding osteoinductive properties. Mesenchymal stem cells are multipotent cells that can differentiate into various cells, such as the osteoblastic lineage. Their osteoinduction properties have been well demonstrated, and a wide range of experimental models have been used to successfully regenerate bone defects. Most of these models integrate isolation from whole bone marrow aspirates, ex vivo expansion, and attachment of the mesenchymal stem cell to the biomaterial. However, there are still several questions regarding the best donor site, the isolation procedure and expansion methods, and stem cell behavior after expansion and implantation; there may indeed be several factors that affect the success of bone marrow progenitor cell implantation. Moreover, such demanding protocols are less likely to be reproducible in bone defects of oncological origin owing to waiting times and high costs and because postimplantation irradiation, which may cause severe and irreversible bone marrow damage, may impair the osteoinductive properties of such composites.

The osteogenic effects of total bone marrow grafts have been known since 1868, and the percutaneous total bone marrow graft concept was used by Connolly and colleagues for healing nonunions in human tibias in 1986. The osteoinductive potential of total bone marrow grafts has been demonstrated, and composites combining autologous fresh marrow cells with calcium phosphate ceramics have provided good results in nonirradiated bone with various types of calcium phosphate ceramics in small defects as well as in large segmental defects. When used as an osteoinductive material, a number of investigators have reported good results with marrow as a source of substitute for bone-grafting material; and certain studies have shown that bone marrow is capable of promoting new bone formation, although the results of others are more controversial. The hypotheses...
advocated are that (1) bone marrow cells induce mesenchymal stem cells to migrate to the defect and differentiate into osteoblastic lineage and (2) undifferentiated cells from the bone marrow grafts themselves could be pushed toward osteoblastic lines by an adequate environment of calcium phosphate ceramic.

One limitation of this study is the lack of a control group in which the defect could have been filled with the ceramics without bone marrow injection. Cell quantity could have been insufficient to carry out bone regeneration function and justify the use of centrifuged bone marrow to enrich bone marrow stem cells. Recently, however, good results obtained with total bone marrow vs mesenchymal stem cells for regeneration in irradiated bone have raised the importance of parameters other than cell count. Biphasic calcium phosphate associated with total bone marrow appears to be the most efficient material for bone substitution in irradiated areas. Also, under irradiated conditions, total bone marrow grafts were found to enhance bone ingrowth when combined with MBCP granules in dogs or with injectable calcium phosphate in rats in small defects compared with ceramics alone.

The present study gives more information on the potential for total bone marrow and ceramic composite for larger reconstruction in low tropistic conditions.

In conclusion, this model was created to reproduce an oral oncological schedule that integrates surgical removal with first-stage reconstruction followed by 4 to 7 weeks of postoperative radiotherapy. To prevent the radiation causing irreversible damage to the osteoinductive potential of the composite, the previously reported older technique of bone marrow grafts was used and delayed in relation to implantation of the initial composite. This preliminary study was needed to differentiate the numerous factors that may influence bone regeneration. Since we obtained good bone reconstruction results with this composite in comparison with the control group, other experiments are needed to evaluate this engineering scheme with postreconstruction radiation delivery and by creating a soft tissue defect that closely resembles oncological conditions.

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