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A New, Segmental, Critical-size Defect Model for Long Bones: A Sheep Study

V.Viateau**, G. Guillemin*, Y. Calando*,K. Oudina*, L. Sedel*, D. Hannouche, H. Petite*
Laboratoire de Recherches Orthopédiques, UMR-CNRS 7052, Paris, France.
**Ecole nationale Vétérinaire de Maison Alfort, France
veronique.viateau@wanadoo.fr , nguillem@ext.jussieu.fr,
calando@idf.ext.jussieu.fr valerie.bousson@lrb.ap-hop-paris.fr,
oudina@idf.ext.jussieu.fr, laurent.sedel@lrb.ap-hop-paris.fr
didier.hannouche@lrb.ap-hop-paris.fr, hpetite@infobiogen.fr

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Abstract
In the present study, we (i) established a large animal model in sheep of a two-step surgical procedure for successful reconstruction of large segmental bone defects, based on a concept that proved successful in humans and (ii) explored the possibility of using skeletal stem cells loaded onto a coral scaffolds to repair such defects.

Introduction

Reconstruction of large segmental bone defects can be achieved by a two-steps procedure using cancellous bone chips as described by Masquelet et al[1]. In a first surgical procedure a PMMA-cement spacer is inserted into the defect. The presence of this foreign body triggers the formation of a pseudo-synovial membrane. After 8 weeks, the cement spacer is removed leaving a cavity surrounded by a membrane. This cavity is then filled with autologous cancellous (ACG) graft. Successful repairs of bone losses up to 25 cm long have been reported in human with this technique[2]. It, however, requires large quantities of autograft and, sometimes successive surgeries need to be performed to achieve bone union. The discovery of skeletal stem cells have opened new avenues for designing highly efficient bone substitutes. In the present study, we set up an animal model that mimicked the surgical procedure described by Masquelet and explore the possibility of using skeletal stem cells loaded onto a coral scaffolds to repair such defects.

Materials and Methods

Coral Scaffolds: Three-dimensional scaffolds (3x3x3 mm3) of natural coral (Porites) were kindly donated by Biocoral, Inc. This material consisting mainly (99%) of calcium carbonate in the form of aragonite and 1% of organic material (specifically, amino acids) had been previously fully characterized and had open and communicating pores of average diameter 250 μm and a porosity volume of 49±2% [3,4]. Prior to cell seeding, these coral scaffolds were sterilized by autoclaving. 200 pieces of coral were used to fill each defect.

MSCs amplification: Bone marrow was aspirated from the iliac crest of ewes during the first surgery (as described in the surgical procedures section) according to established techniques[5]. Subsequently, the cells adhering on tissue culture plastic ware were cultured in α-Minimal Essential Medium (α-MEM) containing 10% fetal bovine serum (FBS) under standard cell culture conditions till they reached confluence. The cells were then passaged as, and when, needed using standard techniques.

Preparation of coral/MSCs: $5*10^6$ MSCs were seeded into 200 pieces of coral scaffolds under slight vacuum at room temperature in 10 ml. After 4 hours, they were transferred into a bioreactor (Wheaton Science; Magnaflex) containing 60 mL of α-MEM (with the same composition as the culture medium), and cultured in a humidified, 37°C, 5% CO2/ 95% air environment, under gentle stirring (20 rotations per minute) for 8 days prior to implantation in the donor animal (n=7).
Surgical procedure and experimental groups: The surgical procedure involved two interventions. In the first procedure, a 25-mm long midiaphyseal ostectomy was performed and was stabilized with a Dynamic Compression Plate. The resected bone (25-mm long and 13-mm wide) was replaced with a poly(methyl)methacrylate (PMMA) cylinder whose shape and dimensions matched the ones of the resected bone. In the second procedure, six weeks after the first one, the casts were removed the membrane surrounding the PMMA cylinder was incised lengthwise, sampled for histology and for immunohistochemistry and was elevated from the underlying cement with a periostal elevator. The PMMA cylinder was grabbed with bone-holding-forceps and removed as a single piece. The medullary canal was then drilled with a 3.5-mm diameter drill (Synthes) to re-establish its patency. and the defect was either left empty (Group 1; n = 4) or filled with coral scaffold (Group 2 n=5), coral/MSC construct (Group 3 n=7) or a morcellized, corticocancellous autograft sampled at the right side of the iliac crest (Group 4; n = 5). The incised edges of the membrane that surrounded the cavity were sutured over the defect with a simple continuous suture pattern using dec 3 polyglactin 910. Skin closure was accomplished according to routine procedures.
Follow-up: Standard craniocaudal and mediolateral oblique radiographs were taken when each surgery was completed. Animals in Groups 3 were x-rayed at monthly intervals. Radiograms of animals in Groups 1, 2 and 4, however, were taken only at the end of months 1, 2, 3 and 6.
Explant Specimen Analysis: Immediately upon animal sacrifice, the casts were removed and the metatarsal bone was excised, examined for evidence of implant loosening, X-rayed, fixed in 10% neutral buffered formalin for 2 weeks and embedded in poly methyl methacrylate resin. Explants were examined using tomoradiography, and longitudinal sections were cut, ground down to a thickness of 100 µm, polished, microradiographed, and stained with Stevenel Blue and van Gieson picro fushine. Quantitative information regarding the three-dimensional distribution of newly-formed bone volume was obtained using tomodensitometry.
The membranes encapsulating the temporary PMMA implants were analyzed using established histological and immunohistochemical techniques.

Results

Postoperative Follow-up and Necropsy Findings: The animals in the study recovered successfully form the surgical procedures, remained in good health and maintained stable implant fixations for the duration of the study with the following exceptions: one animal in Group 4, which died of pulmonary disease 10 days after the second surgery and one animal in Group 1, in which loosening of the fixation was detected two months after the second surgical procedure (but was due to a technical error during the first surgical procedure). These two animals were excluded from the study. In two animals of Group 2 the fixation plates broke sometime between the third and sixth months after the second surgery; these animals were also excluded from the study.

In vivo evaluation of the osteogenic potential
Group 1 : At the end of the study, bone deposition in the animals of Group 1 was scant and limited to the vicinity of the cut edges of the defects. No bony union was observed radiographically in any of the animals of this group (Figure 1, frame A and B). Histological analysis confirmed the radiography results regarding lack of bone and revealed the presence of fibrous tissue. The volume

of newly formed bone was low (161±214 mm3) and significantly inferior to the volume of newly formed bone of defects filled with coral ($p=0.018$), coral/MSC ($p<0.0001$) and autografts($p<0.0001$). (figure 3). Its apparent bone mineral density was significantly inferior to the apparent bone mineral density of the adjacent bone (635±88 versus 1392±42 mg HA/ml). Immunohistochemistry study of the membrane showed that it was a well vascularized type 1 collagenous membrane which contained cells positive for CBFA1and very few macrophages.

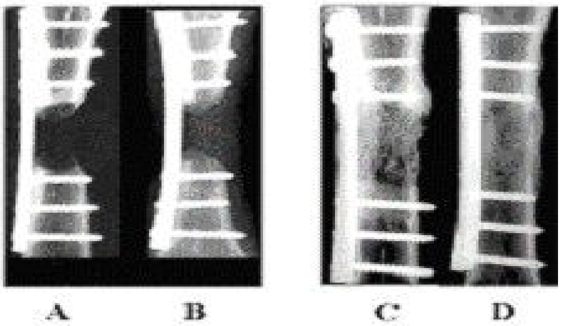

Figure 1

Group 2: By three months post-implantation, complete resorption of the plain, coral scaffolds had occurred but was not accompanied by new bone formation in any animal filled with coral scaffold alone. Despite some new bone formation, no union occurred in any animal of this group even at six months. As early as one month resorption of the coral scaffolds was evident in all animals filled with coral/MSC contructs (data not shown) and was complete by the end of the second month post implantation. The volume of defects filled with coral was statistically different from defects filled with coral/MSCs ($p=0.0239$) or autografts ($p=0.0015$). (figure 3).

Group 3: In contrast to lack of new bone in defects filled with coral scaffold alone (Figure 2), new bone was present in defects filled with coral/MSCs at three months. Sufficient bone was formed to bridge at least one cortice along the fixation in four (out of the seven) animals in this group. At 6 months, there was no differences between the volume of defects filled with coral/MSCs and autografts($p=0.0836$) (figure 3).

Group 4: The radio-opacity of bone autografts was initially weaker than the radio-opacity of the rest of the metatarsus but increased over the following months and almost matched that of the host bone at the end of the study, at which time bone union was evident in all defects filled with bone autografts. (Figure 3). No resorption of the autografts was observed in this group.

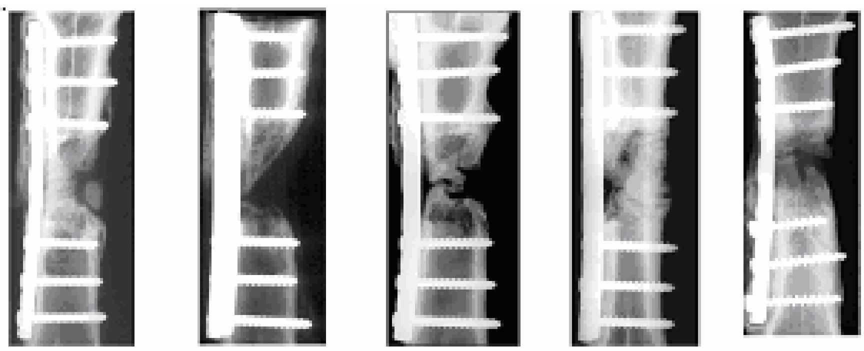
Figure 2

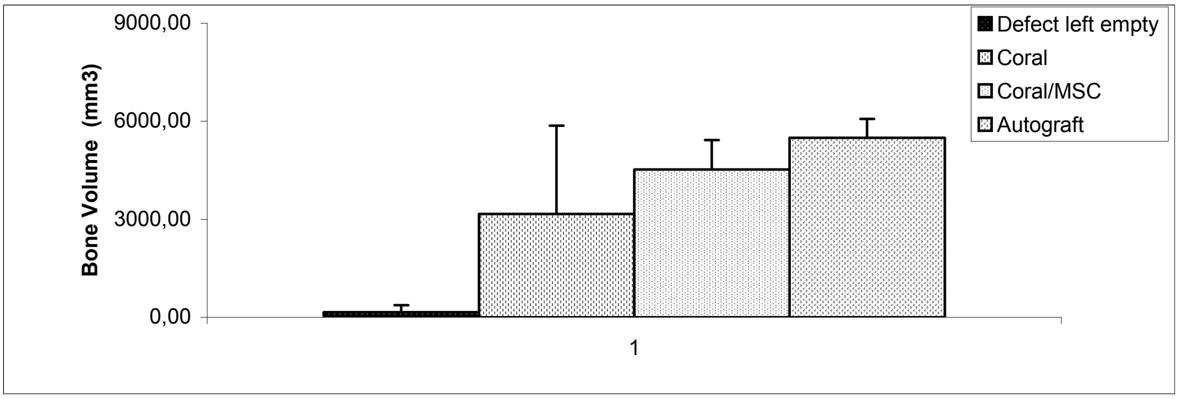

Figure 3

Conclusions

In this study, we developed an experimental defect model on weight bearing bone closing mimicking the clinical procedure. This model appears to be a critical size defect as defects left empty never healed. An inappropriate mechanical environment might led to delayed or non-union. This was not observed in this study when implanting autograft, the benchmark of bone reconstruction, which always healed. Interestingly, the immunohistochemical data suggest that the membrane was well vascularised and contained CBFA1 positive cells suggesting that the membrane could act as a local source of osteoprogenitor cells. Nevertheless, further investigations are needed to better understand the role of the membrane.

In this study, we showed that addition of MSCs to coral led to a significant increase in the volume of newly formed bone and at 6 months bone formation in defects filled with coral/MSCs was not different from defects filled with autografts. Other paremeters including organization of the newly formed bone and presence of a continuity of bone cortices have been affected by addition of MSCs to coral. Newly formed bone was found preferentially in the outer part of the defect when defects were filled with coral/MSC constructs. In addition, bone continuity was re-established in 4/7 animals treated with coral/MSC constructs whereas no bone continuity was observed in animals treated with coral alone. Although these results are very encouraging, cell therapy using adult stem cells is in its infancy and needs further improvements including improved scaffold coupling biomaterial resorption and bone formation before they could translate from the bench to the bedsite.

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