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Bonelike®/PLGA Hybrid Materials For Bone Regeneration: *In vivo* Evaluation

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Abstract. The biocompatibility and osteoconductive potential of Bonelike® and Bonelike®/PLGA hybrid materials were evaluated through subcutaneous and tibiae implantations for a 4 week period. Qualitative histology was performed to evaluate new bone formation and characterise bone-implant interface and results showed that Bonelike®/PLGA hybrid materials promote extensive bone growth. After the 4 week implantation period, Bonelike® resorption was recognized, which may be due to the presence of resorbable β and α-tricalcium phosphate phases.

Introduction

Reconstructive surgery has been a challenging clinical problem in terms of repairing large bone defects, such as in the cases of arthroplasty revision surgeries, facial reconstructions, healing of excised bone cysts and tumours [1]. There are a number of treatment strategies suitable for solving these clinical cases, which include utilization of autographs and allografts. However, there are limitations when using auto grafts due to restricted bone supply [2] and allografts due to the possibility of inducing transmissible diseases [3]. As an alternative, it has been proposed the use of alloplastic materials, which comprise the class of synthetic materials, such as hydroxyapatite (HA) and other calcium phosphate-based ceramics [4]. These biomaterials are similar to the inorganic phase of bone [5], are osteoconductive [6] and have been used clinically. A preventive treatment against post-operative infections is a common clinical procedure, which is based on systemic antibiotic therapy [7]. Nevertheless, this type of treatment has various systemic adverse effects, such as the development of bacterial resistance and the fact that only a small amount of the dose reaches the surgical site [7]. Therefore, much attention is being directed towards implantable calcium phosphates that locally deliver therapeutic molecules and therefore prevent bacterial infection [7] with simultaneous new bone formation. Bonelike® is a synthetic bone graft material that has been designed for clinical applications of bone regeneration. Its chemical composition mimics the mineral phase of bone tissues and also comprises some percentages of β-tricalcium phosphate (TCP), $Ca_3(PO_4)_2$, and α-TCP. These phases are known to degrade in physiological environment, however is difficult to chemically bind therapeutic molecules to the Bonelike® surface in a controlled manner. On the contrary, synthetic polymers, such as the poly (D,L-lactide-*co*-glycolide) (PLGA) are highly biodegradable and may be used in controlled drug delivery applications [8].

The purpose of this work was to assess the osteoconductive potential of novel Bonelike®/PLGA hybrid materials using an animal model. Qualitative histology was performed on undecalcified

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sections at the bone defect area to evaluate the extent of new bone formation. SEM analysis was additionally used to characterise the new bone-implant interface.

Materials and Methods

Implant materials. Bonelike® block samples were prepared with the size of 10 x 10 x 3mm^3 by mixing in methanol hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, supplied by Plasma Biotal (batch P201 R) with a 4wt% of P_2O_5-based glass as previously described by Santos *et al* [9]. The slip was dried, sieved to < 75μm and uniaxially pressed at 288MPa followed by sintering at 1300ºC with a ramp rate of 4ºC/min and 1h dwelling time. Bonelike® granules were also prepared for animal implantation with size ranging from 150 to 500μm using standard milling and sieving techniques. Bonelike® samples were degreased, washed with deionised water and coupled to poly(D,L-lactide-*co*-glycolide) 85:15 (PLGA) using a *post*-hybridisation method with solvent evaporation followed with heat treatment at 120ºC in vacuum for about 8hrs.

Implantation procedure. Prior to implantation Bonelike® samples were sterilized by soaking in several ethanol solutions (p.a.) and dried in a vacuum dessicator for 12hrs. *In vivo* studies were performed at the Japan SLC Inc. using a total of 10 White Japanese male rabbits weighting ~2kg at the time of implantation. A 50mg/ml pentobarbital sodium aqueous solution (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was used as the general anaesthetic and 1.0mL/kg was intravenous administrated in rabbit ear. The skin of the rabbit was shaved, cleaned and disinfected.
Two separate midline incisions, each with 20mm long, were made on the back of the animals using a standard procedure in aseptic conditions. Bonelike® samples were inserted through the incisions into the subcutaneous space without fixation and skin was closed with a Nesco suture (AZWELL Inc., Osaka, Japan).

For tibiae implantation Lidocaine supplied by Takeda Chemical Industries Ltd., Osaka, Japan was used as the local anaesthetic and it was administrated bellow the knee joint. After this procedure, longitudinal incisions with 20mm long were performed at the anterior medial rabbit tibiae. Holes of ϕ=2.12mm were drilled through cortex into bone medulla with simultaneous washing with physiological saline solution (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) to prevent tissue necrosis. After the haemorrhage has been controlled, holes were filled with Bonelike® granules and then periosteum and skin were closed in layers.
Animals were sacrificed with an overdose of general anaesthetic after the 4 week period. The rabbit skin and tibiae were removed from animal for histological examination.

Histological examination. The implanted area was sectioned, washed with physiological saline solution, fixed in 10% formalin/phosphate buffered solution, dehydrated with a graded series of alcohol solutions (70, 80, 90 and 100%) and embedded in methylmethacrylate resin. Thin sections with 150μm were cut perpendicularly to the tibiae axis with a diamond blade microtome (Struers Accutom) and hand-ground to approximately 70-80μm. Some slices were stained with Solo-Chrome R and haematoxylin-eosin for histological examination under light microscopy, while others were characterized by Scanning electron microscope (JEOL JSM-6301F) analysis. SEM specimens were coated with gold (Au) using a Fine Coater Ion Sputter (JEOL JFC-1100) before analysis. The elemental analysis was determined using an X-ray spectrometer (EDX) (NORAN-VOYAGER) attached to the JEOL 6301F microscope.

Results

After the 4 week period of subcutaneous implantation a fibrous capsule surrounding the implants was observed for both Bonelike® and Bonelike®/PLGA hybrid. No evidence of infection or inflammatory reaction was detected at the implantation sites.
Histological studies demonstrated that after 4 week implantation in rabbit tibiae, new bone was formed fully attached to Bonelike® and Bonelike®/PLGA hybrid granules (Fig. 1A-B), although

some gaps could be seen (Fig. 1A). The haematoxylin-eosin staining allowed observing new bone formation with various calcification densities in the surrounds of Bonelike® granules, i.e. non-mineralised or immature bone and mineralised bone as well as the vascularized *de novo* bone (Fig. 2A-B).

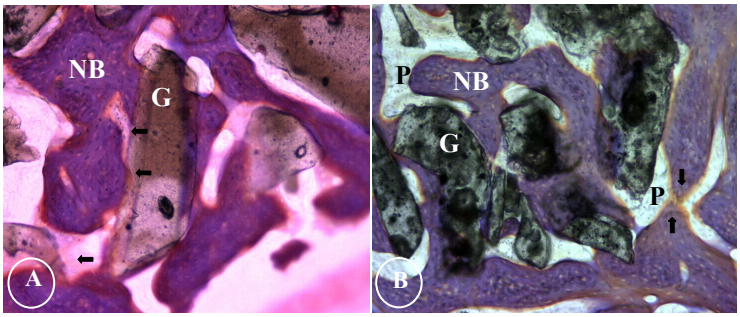

Fig. 1. Light microscopy photographs of Bonelike® granules (G) implanted in rabbit tibiae for 4 weeks at the bone defect area. Extensive new bone formation among granules may be observed for: Bonelike® (A) and Bonelike®/PLGA hybrid (B). New bone (NB), non-mineralised bone "*osteoid*" (arrows) and PLGA (P). (Undecalcified section, Solo-Chrome R stain, original magnification x100).

SEM studies showed new bone in perfect contact with Bonelike®/PLGA hybrid granules (Fig. 3A-B). Ingrowth of new bone was detected at resorption areas in Bonelike® granules (Fig. 3A). EDX analysis showed that new bone is slightly deficient in Ca and P elements compared to Bonelike® granules (Fig. 3B).

Discussion

Observation of the implant sections revealed extensive growth of newly formed bone tissue around Bonelike® granules confirming previous results, which showed that Bonelike® is highly osteoconductive biomaterial [11].

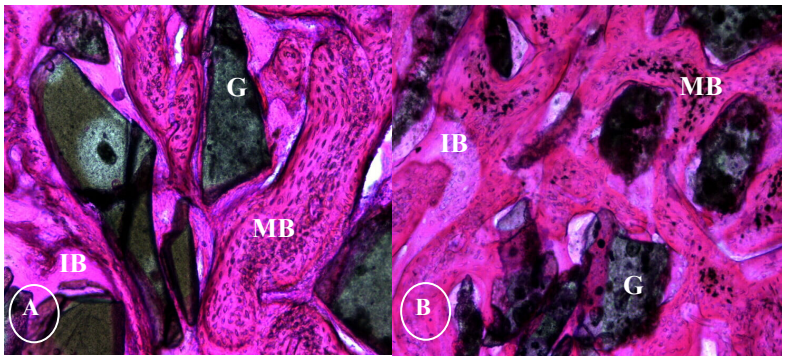

Fig. 2. Light microscopy photographs of Bonelike® granules (G) implanted in rabbit tibiae for 4-week at the bone defect area. Extensive new bone formation among granules may be seen for: Bonelike® (A) and Bonelike®/PLGA hybrid (B). Immature bone (IB) and mature bone (MB) recruits many multinuclear cells attached to the material (Undecalcified section, Haematoxylin-eosin stain, original magnification x100).

These results confirmed that the PLGA phase did not modified significantly the osteoconductive properties of Bonelike®. Bonelike® resorption was also observed with simultaneous new bone ingrowth, which may be due to the presence of degradable β-tricalcium phosphate (TCP), $Ca_3(PO_4)_2$, and α-TCP phases. This result is strongly encouraging, since the Bonelike®/PLGA hybrid materials have been designed for local drug delivery while supporting new bone formation. After the 4 week implantation period, Ca and P profile at implant-new bone interface showed that materials were perfectly osteointegrated.

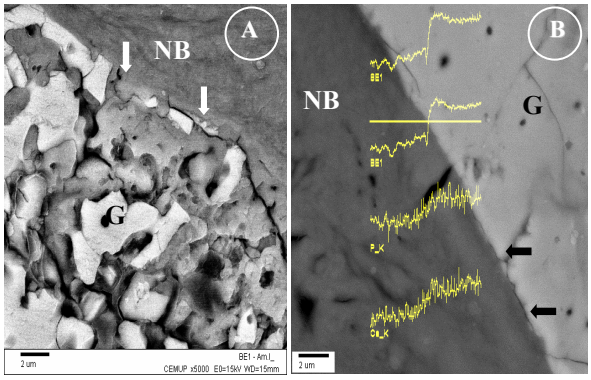

Fig. 3. (A) Backscattered scanning electron image of Bonelike®/PLGA hybrid granules, after a 4 week period, at implant (G)-new bone (NB) interface, showing areas of Bonelike® resorption with new bone ingrowth (white arrows) and (B) SEM/EDX elemental analysis of Ca and P distribution at new bone (NB)-implant (I) interface (black arrows) obtained at the line profile.

Conclusions

In vivo evaluation of Bonelike® and Bonelike®/PLGA hybrid for a 4-week period showed extensive formation of new bone and vascularization *de novo* bone. Bonelike® supports bone remodelling with simultaneous material degradation and new bone formation.

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