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Abstract

The healing mechanism of osteoconductive biphasic calcium phosphate granules was investigated by a histological assessment of early-stage bone deposition and remodeling. The deposition of *de novo* bone on the scaffold granules was observed to initiate at the defect periphery by week one and in the bulk of the defect incorporating the granules by week four. New bone tissue was deposited in the space provided by the macroporosity and was observed in direct apposition to the implanted material confirming the bioactivity of the biphasic calcium phosphate. The granules were removed through a cell-mediated resorption process that was observed to begin as early as week two following surgery. Mature lamellar bone, fatty bone marrow, and vascularization was observed throughout the bulk of the defect with the cortical shell healed by week twelve. This healing mechanism was found to balance bone formation and implant resorption resulting in complete healing of the corticocancellous defect in the rabbit femoral condyle.

Introduction

Biphasic calcium phosphate scaffolds comprised of hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) have been reported to be bioactive materials [1] that promote healing following an osteoconductive mechanism that balances new bone formation with device resorption [2]. However, the early-stage healing sequence is not well described for these ceramics particularly formulations with low HA content. HA has been extensively used alone in skeletal reconstruction over the past three decades as a bone graft substitute due to its chemical similarity to the mineral phase of natural bone tissue. This long history of clinical use has established HA as a highly biocompatible, bioactive [1, 3], and osteoconductive [1] material that when prepared as a porous scaffold may be used as a delivery vehicle for osteogenic cells and osteoinductive growth factors [4]. However, HA is remarkably stable under physiological conductions, and can persist in the body for many years after implantation. In contrast to the stability of HA, β -TCP, another widely used synthetic bone grafting material, is removed more quickly through both dissolution and osteoclastic resorption processes [4].

An optimal bone graft substitute design should provide both bioactive and resorptive properties such that the material supports new bone formation and then is gradually removed as healing progresses. This balance can be achieved by combining HA and β -TCP ceramic phases into a biphasic formulation [2, 5, 6]. As these two calcium phosphates contribute different functionality, the ideal ratio in which the biphasic chemistry is prepared is critical to how the bone graft substitute performs, and various formulations have been studied over the past two decades.

Biphasic calcium phosphates with a HA/ β -TCP ratio of 25/75wt% were found to maximize osteoclastic activity and resorb more extensively in comparison to compositions with high HA contents, and also exhibited resorption lacunae resembling those found on natural mineralized tissue [5]. Additionally, this study reported that phase pure β -TCP, although more soluble than the biphasic formulations investigated, did not demonstrate significant osteoclastic resorption, suggesting that there may be an inherit benefit in the use of a biphasic composition with regard to eliciting osteoclastic activity.

In another study investigating the differences between biphasic and phase pure calcium phosphate chemistries, bone formation was observed to be maximized by a formulation with a 20/80wt% HA/ β-TCP phase ratio and found that phase pure HA as well as pure β -TCP consistently produced the least amount of new bone [2, 6]. In yet another investigation, significant bone deposition from human Mesenchymal Stem Cells (hMSCs) was observed only on scaffolds with a minimum of 15wt% HA [7]. Interestingly, this study found that while phase pure β -TCP scaffolds displayed capacity to form bone with ovine MSCs, only scaffolds with an HA component formed significant bone with human cells.

These independent studies demonstrate the optimal balance between new bone formation and resorption was achieved by biphasic formulations with a high β -TCP content such as that present in the MASTERGRAFT™ family of products. The MASTERGRAFT resorbable ceramic is a synthetic, biphasic calcium phosphate technology comprised of a mixture of 15wt% hydroxyapatite and 85wt% β -tricalcium phosphate. This ceramic chemistry is formed into highly porous granules (80%±10%) that offer both micro and macropore size features. Magnification to examine the surface morphology reveals a microporous surface structure (pore size < 10µm, Figure 1a) for attachment, retention, and proliferation of bone cells. The interconnected macroporosity (avg. pore size: 500µm ± 100; interconnecting pore size: 125µm, Figure 1b) creates space for bone deposition and provides a pathway for cell and vascular infiltration throughout the bulk of the construct. This high concentration of large interconnected pores is critical for both bone ingrowth and granule resorption as highlighted by a recent study [8] examining human biopsies on implants with comparably low porosity (35% ±5%; avg. pore size: 100µm). The authors of this study found that even though these implants were prepared with the resorbable phase pure β -TCP chemistry, ongoing remodeling was still observed five years post-implantation in large defects [8].

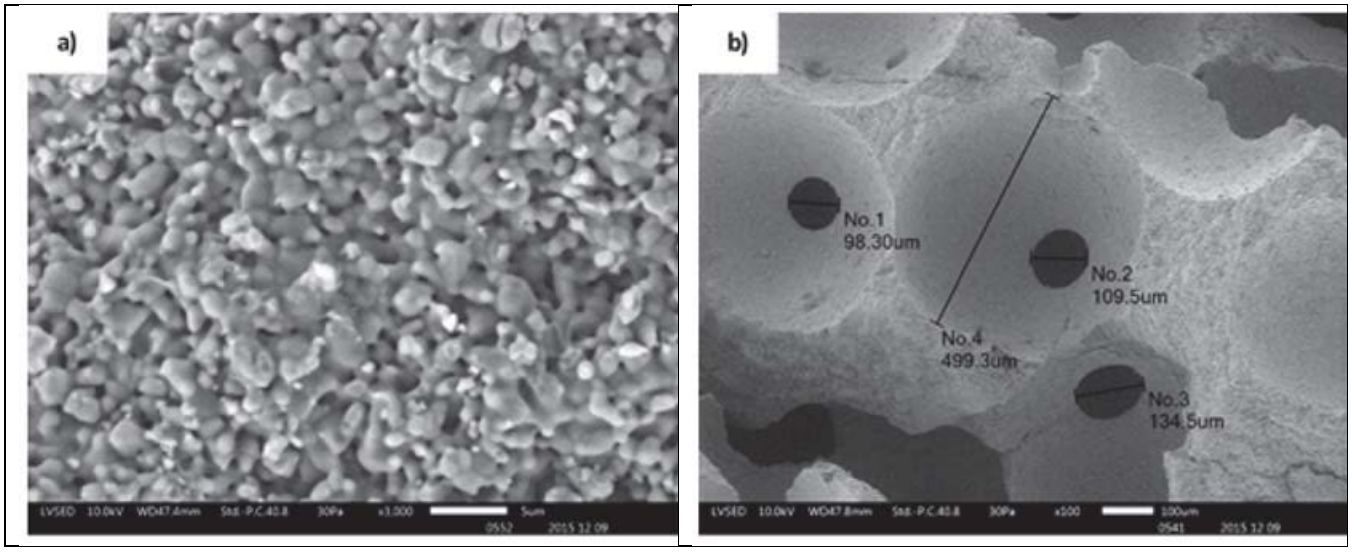


Figure 1: SEM images of MASTERGRAFT a) microporosity, scale bar = 5µm; b) macroporosity, scale bar = 100µm

The design characteristics of the MASTERGRAFT technology incorporate concepts accumulated from decades of scientific research to deliver an optimized synthetic scaffold for bone grafting. In order to demonstrate that the technology delivers on this functionality, an *in vivo* study was performed

employing the rabbit femoral condyle model to determine the mechanism of action of the early-stage bone healing following implantation of MASTERGRAFT granules.

Materials and Methods

Under an IACUC approved study protocol, MASTERGRAFT Mini Granules were implanted into critically-sized corticocancellous defects (n=4, drill hole, 6mm diameter and 8-10mm depth) in the lateral femoral condyle of New Zealand White rabbits for 1, 2, 4, and 12 weeks. NZ White rabbits are frequently used for studies investigating healing with synthetic bone void fillers, and the 6mm diameter and 10mm deep drill defect used in this study has been demonstrated to constitute a critically-sized defect in this animal model [9-14]. Therefore, no unfilled defect control group was included in the study design. Test articles were implanted as received without combination with autograft or autologous fluids (e.g. BMA, PRP, blood) through an open barrel 1cc syringe. Radiographs to confirm implant placement were taken immediately after surgery and again at necropsy. Explants were fixed in 10% formalin and then grossed for histological assessment examining new bone formation, bone remodeling, implant resorption, and cellular response. During embedding, the defect was positioned in paraffin such that the defect was sectioned in the coronal plane to produce a rectangular cross-section of the defect. This sectioning orientation was specifically chosen to show both the cortical and cancellous aspects of the defect allowing for healing in these different regions to be assessed. Two tissue blocks were prepared from each defect. The tissue samples were rinsed in water after sufficient time had passed to effect fixation, decalcified in formic acid (Immunocal, American MasterTech, Lodi, CA) and held in 70% ethyl alcohol. The tissue samples were then processed using a paraffin infiltrator (Leica ASP300 S Fully Enclosed Tissue Processor, Leica Incorporated, Buffalo Grove, IL) to dehydrate the specimens in graded alcohols, clear the specimens with xylene, and infiltrate the tissues with paraffin. The blocks were then cut on a rotary microtome producing thin sections of 6-8mm in thickness. These sections were then stained with Hematoxylin & Eosin (H&E) or Mallory Aniline Blue connective tissue stain (MH). Histological assessments were conducted using Image Pro Plus software (v 5.1, Media Cybernetics, Silver Spring, MD). A video camera (Model DFC 295, Leica Microsystems, Cambridge, UK) coupled to a zoom camera lens (Vivitar 100 mm, F3.5 zoom lens) through a c- mount adapter was used to acquire digital images of the slides. The corticocancellous defects were assessed for the presence and extent of incorporation of residual ceramic granules, the cellular activity adjacent to the ceramic, and types of cells (if present). Quantitative histomorphometric analysis was performed to measure the percentage area of *de novo* bone (not including viable marrow) at each study period.

Results

New bone formation in direct apposition with the biphasic ceramic granules was observed at the periphery of implants harvested at the one-week time point. This new bone integrated seamlessly with the peri-implant native bone creating a bony bond to the surrounding tissue. As shown by Figure 2, *de novo* bone deposition was observed in all samples at week one and progressed to a maximum of approximately 35% of the defect area by week 4.

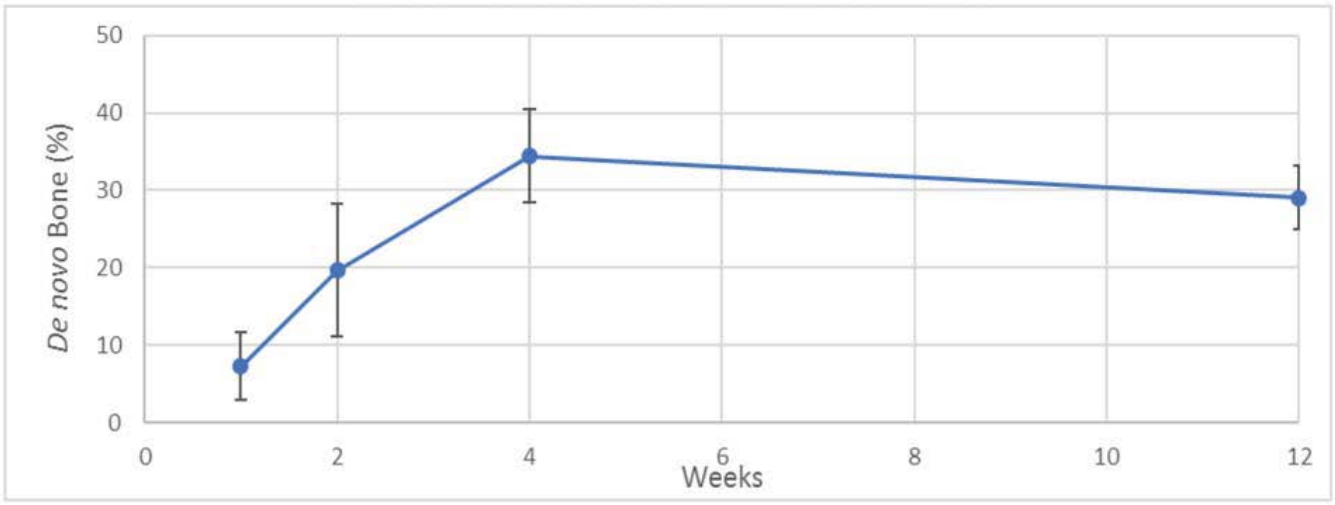
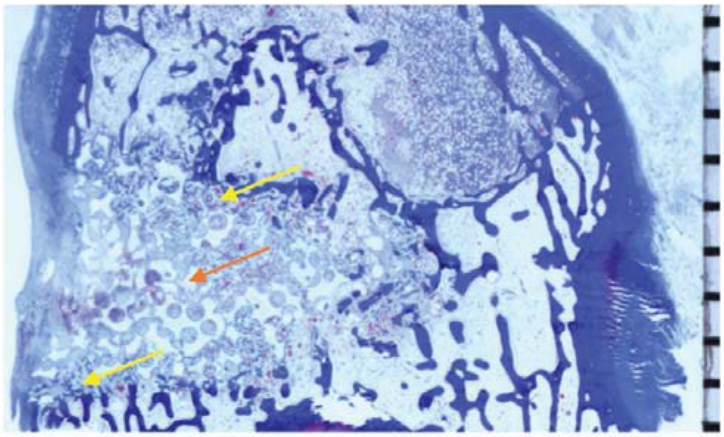


Figure 2: The progression of *de novo* bone deposition on MASTERGRAFT Granules. New bone is observed at the periphery of implants at week 1 and advances throughout the bulk of the implant reaching a maximum by week 4. Importantly, *de novo* bone in this figure indicates percent of the def formed trabeculae and does not account for marrow space associated with this *de novo* bone

New bone formed at week one was mostly limited to the periphery of the implant, as shown by the macroscopic histology imagine in Figure 3. This indicated that cells and growth factors provided by bleeding bone at the surrounding tissue are required to initiate the healing cascade. Also, at this time, the original defect was easily identifiable, and the cortical surface remained unhealed. As shown in Figure 4, new bone deposition advanced along the granule surface further fillig the defect volume by week two and the first evidence of cortical bone healing was observed.



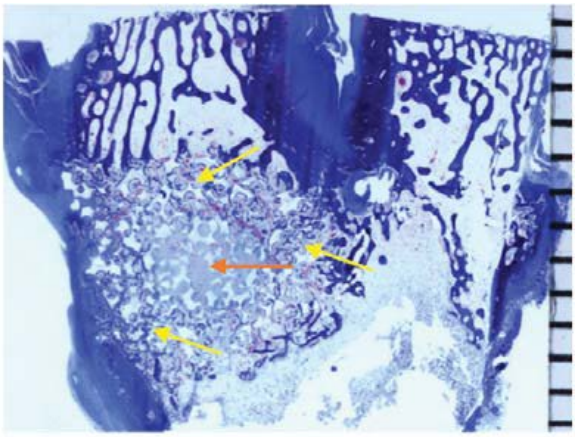


Figure 3: WEEK 1: Macro histological image of implant site (MH Stain). As shown by the yellow arrows, d*e novo* bone is restricted to the implant periphery showing the center of the defect free of new bone at this time, orange arrow (bars = 1mm increments)

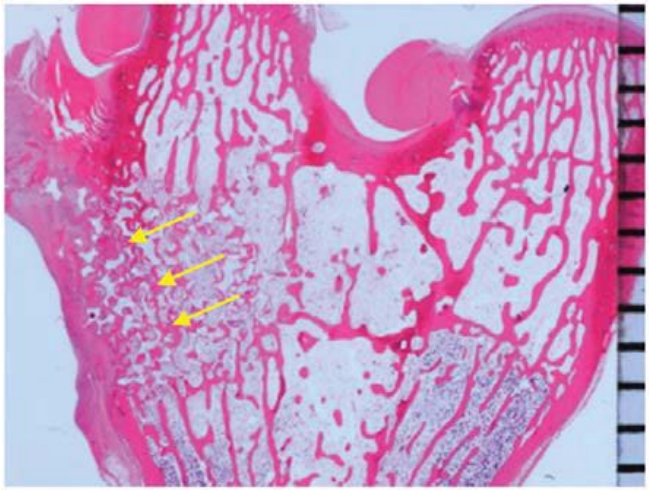
Figure 4: WEEK 2: Macro histological image of implant site (MH Stain). Yellow arrows show d*e novo* bone is advancing into the defect from the implant periphery. The center of the defect remains free of new bone at this time, orange arrow (bars = 1mm increments)

The absence of discreet bone foci in the interior of the implant at weeks one and two confirms the bone formation is not induced by the scaffold, but that new bone formation is developed following

an osteoconductive mechanism. Histological evidence of complete *de novo* bone deposition throughout the bulk of the implant by week 4 is provided in Figure 5. At this time, new bone had filled the defect and a cortical shell is forming at the defect opening.

At week 12, mature lamellar bone was observed throughout the interior of the implant and the cortical shell now spanned the defect (Figure 6). Additionally, the granules persisted throughout the defect ensuring the scaffold was present until the defect had healed.

The histological assessment of the implanted MASTERGRAFT granules confirmed that the biphasic calcium phosphate material is osteoconductive. *De novo* bone was observed as early as the first week after implantation and was found seamlessly integrated with the surrounding native mineralized tissue.



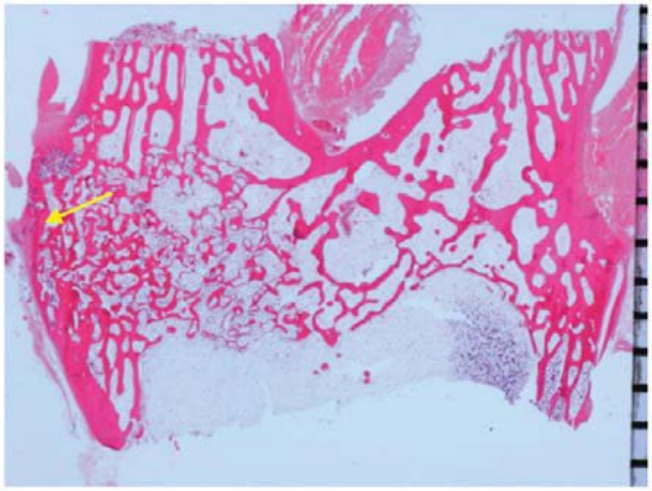


Figure 5: WEEK 4: Macro histological image of implant site (H&E stain). New bone has advanced throughout the bulk of the defect, yellow arrows (bars = 1mm increments)
Figure 6: WEEK 12: Macro histological image of implant site (H&E stain). The yellow arrow shows a cortical shell bridgin at week 12 (bars = 1mm increments)

In terms of bone grafting, the concept of bioactivity was originally defined by Hench as a material:

> *"…that elicits a specific biological response at the interface of the material which results in the formation of a bond between the tissues and the material." [15]*

This bonding characteristic was observed between newly formed bone deposited in direct apposition to the implant ceramic and native bone and confirms the inherent bioactivity of the HA phase was maintained even though it was present at a low concentration in the biphasic MASTERGRAFT formulation.

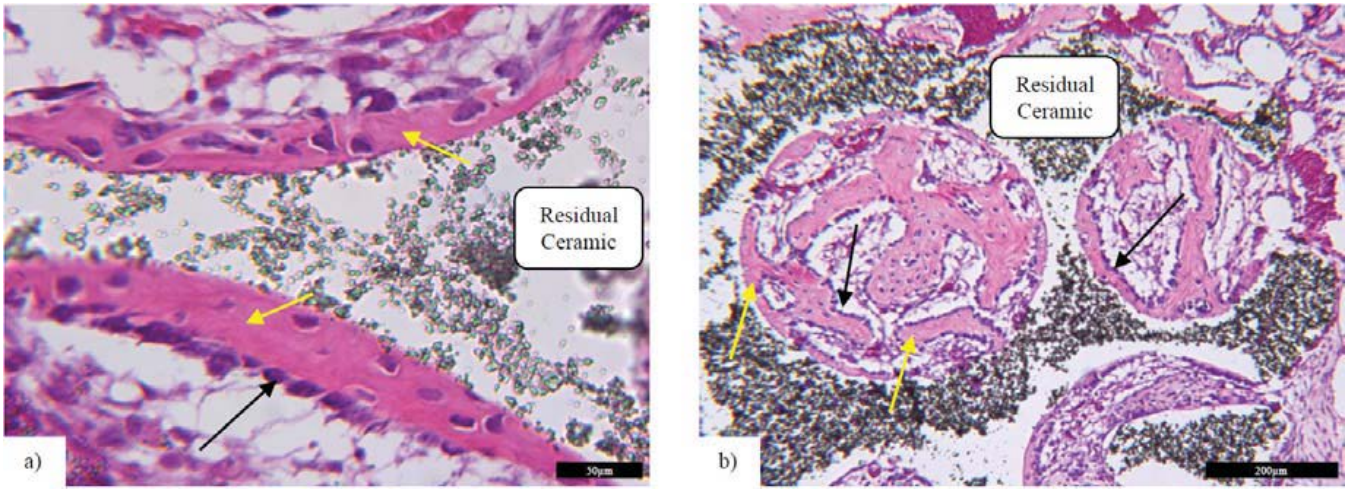


Figure 7: WEEK 1: Magnified histological images of the periphery of the implant site showing *de novo* bone deposition (yellow arrows) and osteoblasts (black arrows) lining new bone tissue directing growth away from the ceramic surface into the macropore space (H&E stain). a) 200x; b) 79x

Histological analysis performed on magnified sections from the periphery of the implant at week one found osteoblast cells lining the newly deposited tissue directing the growth outward away from the granular surface (Figure 7) into the available space provided by the macropores. The ceramic surface remained comparatively smooth at the one-week time point indicating that negligible resorption had occurred by this time.

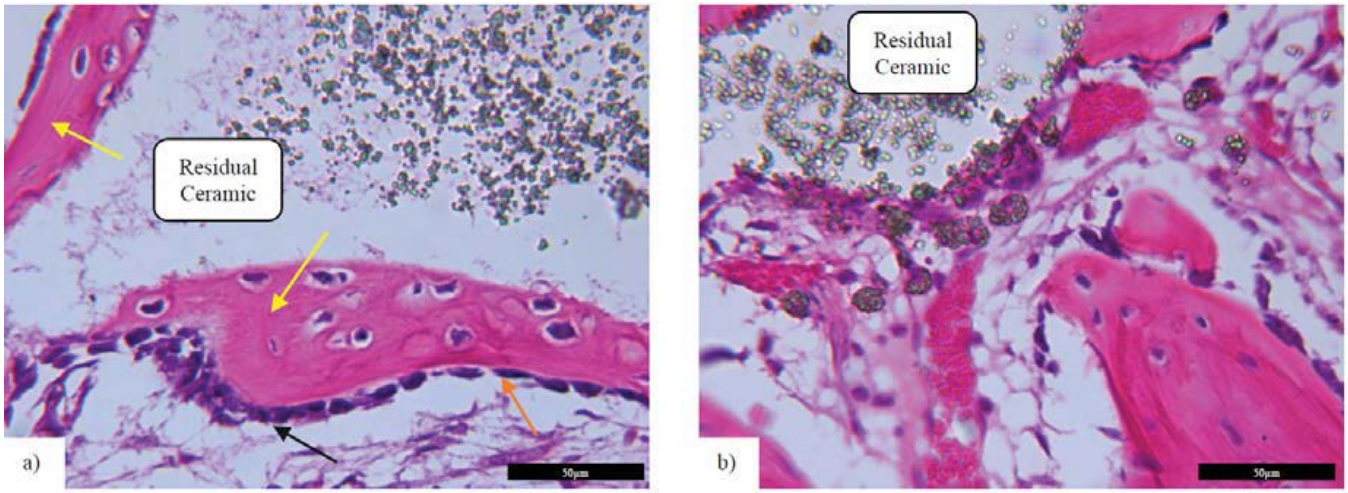


Figure 8: WEEK 2: a) Magnified histological image of implant site showing new bone deposition (yellow arrows) and osteoblasts (black arrows) as well as the initial formation of bone lining cells (orange arrows); b) Macrophages with intracellular ceramic particulate is observed confirming the cell-mediated resorption process (H&E Stain) a) and b) 313x

By week 2, bone formation had progressed further into the interior of the defect. The initial appearance of bone lining cells and mature lamellar bone was observed at peripheral sites indicating that remodeling had begun, as shown in Figure 8a. Additionally, the granular surface began to roughen, and ceramic particulate was detected inside macrophages adjacent to the ceramic surface (Figure 8b). This confirmed that the cell-mediated resorption process begins at the edge of the implant within two

weeks after implantation. At week 4, the granules were completely incorporated into the matrix of new bone throughout the bulk of the implant, and crevices in the ceramic scaffold were found to be infiltrated with *de novo* bone deposits, as shown by the yellow arrows in Figure 9. Osteocytes were also observed within the *de novo* tissue, and red blood cells were found indicating a neo-vascularization of the defect site.

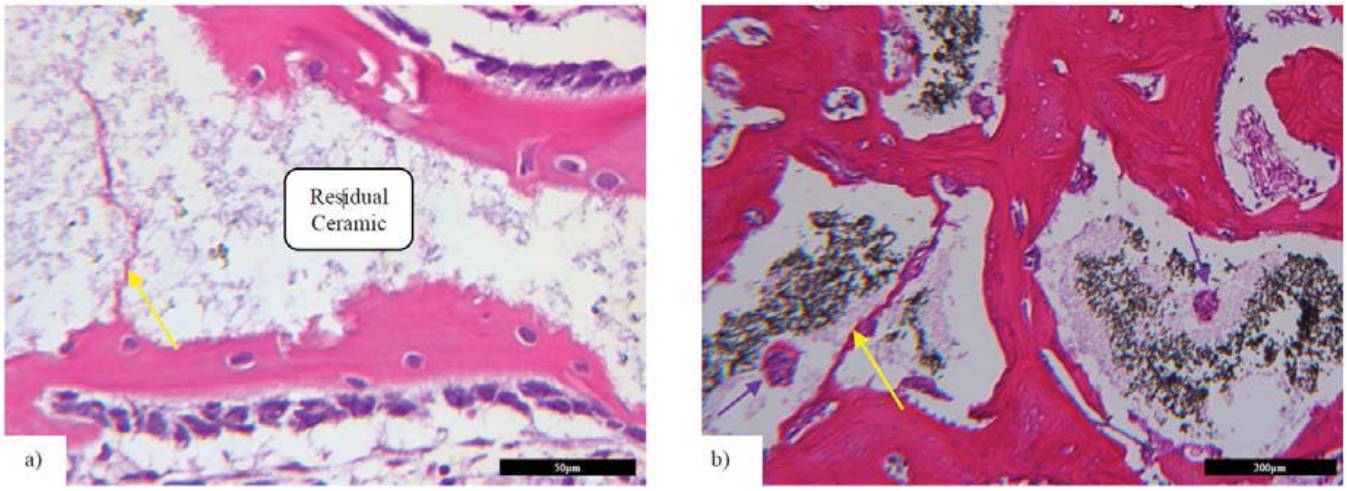


Figure 9: WEEK 4: Magnified histological images of implant site showing new bone penetrating into crevices (yellow arrows) in the granules and evidence of neo-vascularization (purple arrows) (H&E Stain) a) 313x; b) 79x

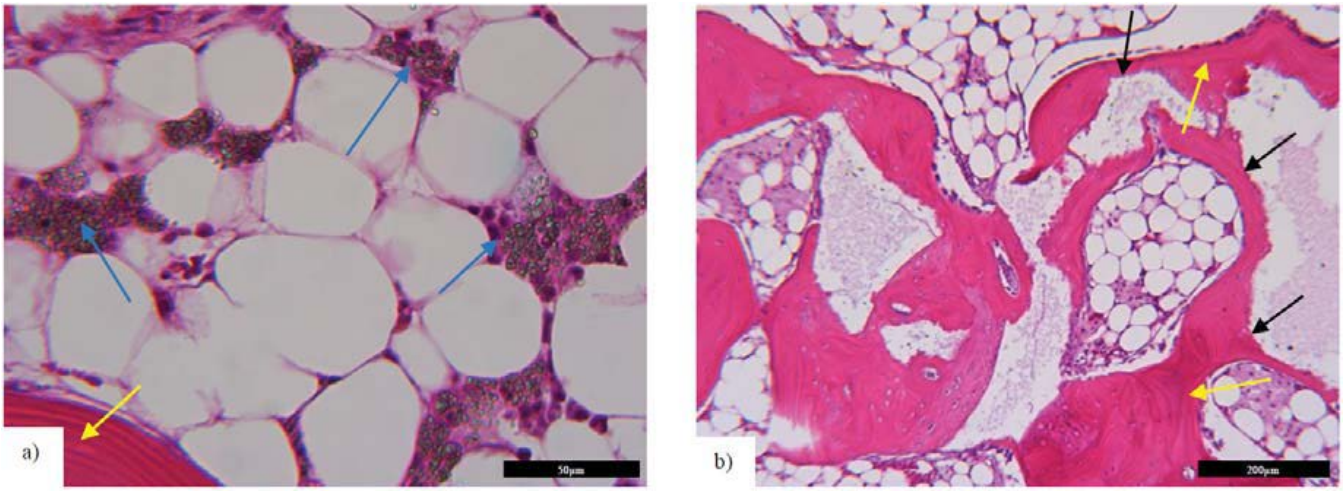


Figure 10: WEEK 12: Magnified histological image of implant site showing mature lamellar bone (yellow arrows) in the bulk of the defect and cell-mediated ceramic resorption (blue arrows). Roughened ceramic surfaces are also observed (black arrows) (H&E stain) a) 313x; b) 79x

By week 12, fatty bone marrow deposits filled much of the macroporosity not already occupied by bone, as shown in Figure 10. The ceramic particulate inside the macrophages had migrated within the fatty marrow space from the now visibly rough granule surfaces.

Discussion

This *in vivo* rabbit study demonstrated that the deposition of new bone on MASTERGRAFT granules was initiated by week one and peaked at 35% *de novo* bone present in the defect space by week four. The healing mechanism then transitioned into a phase dominated by remodeling in which

the biphasic ceramic granules were removed through cellular activity. This osteoconductive mechanism of action requires the scaffold to possess a surface that is conducive to cell attachment and proliferation such that new bone tissue can penetrate the interior of the defect. As the implanted test articles were not pre-loaded with any cells (e.g. MSCs, blood, bone marrow aspirate), the histological assessment revealed that the biphasic formulation in the MASTERGRAFT granules was conducive to the deposition of new bone by facilitating cellular infiltration and neo- vascularization/angiogenesis. The role of macroporosity is also highlighted in the histological assessment as these large pores provide the space for new bone formation through creeping deposition. The controlled and interconnected pore design provided by MASTERGRAFT scaffold was shown to facilitate this infiltration such that the defect was bridged by new bone. The characteristic of the MASTERGRAFT technology to promote bone formation throughout the defect prior to scaffold removal prevents the treated bone void from reforming through premature resorption, which represents a potential complication with faster resorbing materials such as calcium sulfate, calcium carbonate, Bioglass®, and synthetic polymer formulations. This is especially pertinent for patients with comprised healing or who require a bone graft to fill large defects. In contrast, the balanced remodeling process engineered into the MASTERGRAFT technology ensures the scaffold supports both new bone formation and implant resorption.

Conclusion

Histological assessment in an *in vivo* rabbit model illustrated the mechanism of action of bone formation on biphasic calcium phosphate granules. Histology demonstrated that bony healing was initiated at the defect periphery within the first week. At subsequent time points, new bone tissue was then observed to progress along the implant surface filling the space made available by the interconnected porosity and spanning the defect. By week 12, a cortical shell bridging the defect opening formed, and bone, fatty marrow deposits, and vascularization were observed within the healing defect site. This sequence produced mature lamellar bone throughout the femoral corticocancellous defect ensuring the healing was complete prior to substantial removal of the granules. Additionally, as the initial deposits of *de novo* bone tissue was observed primarily within the macropore space, the importance of designing granules with open and interconnected porosity was determined to be critical to the early-stages of bone healing. These results demonstrated that the biphasic calcium phosphate ceramic in MASTERGRAFT is osteoconductive, bioactive, and is easily remodeled as part of the natural healing process.

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