Research Article

The Benefit Of Bone Marrow Concentrate In Addition To A Glass-Reinforced Hydroxyapatite For Bone Regeneration: An In Vivo Ovine Study†

Running Title:
The benefit in Bone Healing of Bone Marrow Concentrate and Hydroxyapatite in Sheep

Joao Torres[1];Manuel Gutierres[1];Luis Atayde[2];Paulo Cortez[2];M. Ascenção Lopes[3]; J. Domingos Santos[3]; A. T. Cabral[1]; Carola F. van Eck[4*]

1 - Faculty of Medicine, University of Porto, Alameda Hernani Monteiro, 4200-319 Porto, Portugal
2 –Departamento de Clinicas Veterinarias, Instituto de Ciencias Biomedicas de Abel Salazar (ICBAS), Universidade do Porto (UP), Rua de Jorge Viterbo Ferreira, n228, 4050-313 Porto, Portugal
3 - CEMUC, Faculdade de Engenharia, Universidade do Porto, Rua Doutor Roberto Frias, 4200-465 Porto, Portugal
4 - Department of Orthopaedic Surgery, University of Pittsburgh Medical Center, 3471 Fifth Avenue, Kaufman building suite 1011, Pittsburgh, PA 15213, USA

* Corresponding author

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ABSTRACT

Introduction - This study evaluates the ability of a Glass Reinforced Hydroxyapatite Composite (GRHC), in a new microporous pellet formulation with autologous bone marrow concentrate (BMC), to enhance bone regeneration and new bone formation.

Material and Methods - Ninety non-critical sized bone defects were created in the femurs of 9 *Merino breed* sheep and randomly left unfilled (group A), filled with GRHC pellets alone (group B) or filled with GRHC pellets combined with BMC (group C). The sheep were sacrificed at 3 weeks (3 sheep), 6 weeks (3 sheep) and 12 weeks (3 sheep) and histological analysis (Light Microscopy-LM), scanning electron microscopy (SEM) and histomorphometric analysis (HM) were performed.

Results -

At 3, 6 and 12 weeks, HM revealed an *average percentage of new bone* of 48, 72, 83%; 25, 73, 80% and 16, 38, 78% for Groups C, B and A respectively (significantly different only at 3 weeks - $p<0.05$). LM and SEM evaluation revealed earlier formation of well-organized mature lamellar bone in Group C.

Conclusion - This study demonstrates that the addition of a bone marrow concentrate to a glass reinforced hydroxyapatite composite in a pellet formulation promotes early bone healing.

Key words: bone healing, bone marrow, bone substitute

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INTRODUCTION

Historically, various materials have been used to assist in bone healing. Starting with a simple wooden splint and evolving to the complex and sophisticated implants used in modern day fracture care. Despite this significant evolution, implants alone have not been able to solve some of the issues related to fracture healing. Especially in circumstances such as delayed union, nonunion and large bony defects.[1] Autologous bone grafting is an option in those circumstances, but there is donor site morbidity associated with this.[2] In addition not all patients have adequate bone stock to provide autograft. Allograft bone is another option, but has the risk of disease transmission, and is not readily available in all countries.[1,3]

Tissue engineering has been focusing on alternatives to aid in bone healing.[3-6] Most of these developments have been guided by the *Diamond Concept*[7,8] which has four basic principles: *osteoconductivity* given by a scaffold; *osteogenicity* obtained from cell precursors; *osteoinductivity* dependent on growth factors; and *mechanical stimulus*.

Many types of tissue engineered bone substitutes have been described[9], both organic and inorganic, with different characteristics. Many of these are expensive and some rely on cell cultures, which are time- and labor- consuming, precluding their use in any urgent setting. Also, potential uncontrolled differentiation and possible malignancy after implantation of cultured cells can be an issue. In this study, the authors evaluate a Glass Reinforced Hydroxyapatite Composite (GRHC) in a new microporous pellet formulation[10,11] with autologous bone marrow concentrate (BMC).[31] This is a simple technique that can be performed in the operating room during a surgical procedure without the need for culture. In previous studies this specific bone substitute

has shown promising results.[31] It was therefore hypothesized that this GRHC/BMC construct would enhance bone regeneration and new bone formation in the *in vivo* setting .

MATERIAL AND METHODS

Prior to the start of the study, all experimental procedures were approved by the veterinary authorities and were found to be in accordance with the European Communities Council Directive 86/609/EEC. The study was approved by the Institutional Ethics Commission.

The used scaffold (GRHC) is a mixture of hydroxyapatite and bioglass. It has previously been studied in different *in vivo* and *in vitro* settings showing excellent osteoconductive properties.[14-20] In this study, a pellet formulation was used.[10-11] On scanning electron microscopy (SEM) observation this possesses a high surface roughness, thought to be essential for cell adhesion (Fig.1A and B) and high inter-pellet porosity, important for bone growth[12]

Nine *Merino breed* sheep were chosen as experimental models. This model has been shown to resemble the human bone healing cascade better than rabbits and other previously described animal models.[30] In addition, it allows for extrapolation of the results to the clinical setting. Adequate measures were taken to minimize pain and discomfort. Bone marrow was aspirated from the posterior iliac crest of the sheep (Fig.1C) and centrifuged for 15min at 3200 rpm (Fig.1D). It was then added to the GRHC pellets (Fig.1E).

Five bone defects were created with a burr in each femur (Fig.1F), accounting for a total of 90 bone defects. The bone defects were 5 mm in diameter and the depth was equal to the thickness of the cortex of the sheep. In each sheep, the defects were then randomly either left

unfilled (Group A: 3 defects/sheep x 3sheep = 9 defects)); filled with GRHC pellets alone (Group B: 3 defects/sheep x 3sheep = 9 defects) or filled with GRHC pellets combined with BMC (Group C: 4 defects/sheep x 3sheep = 12 defects; Fig.1G) (Table 1). After the procedure, the sheep were transferred to individual cages and allowed full weightbaring. At two weeks the sheep were transferred to grass fields and allowed activity as tolerated.

Antero-posterior lateral and radiographs of each femur were obtained at 0, 3, 6, and 12 weeks to evaluate bone healing and GRHC absorption (Fig.1H). The sheep were sacrificed at 3 weeks (3 sheep), 6 weeks (3 sheep) and 12 weeks (3 sheep).[30] At the time of sacrifice, macroscopic evaluation was performed. Soft tissues were stripped from the bones and the samples were fixed in a 10% neutral formaldehyde solution for 7 days, dehydrated in an alcohol solution and embedded in a methyl-methacrylate resin. They were then stored until analysis as completed.

Histological analysis

The methyl-methacrylate resin embedded specimens were sectioned with a diamond saw to a thickness of 40+/-10µm for histology slides. They were then stained with hematoxylin/eosin (HE) and Solochrome cyanine R, a proven method to evaluate newly formed bone[30]. A Nikon light microscope (Eclipse E600, Nikon, Tokyo, Japan) equipped with a calibrated digital camera (Nikon DS-5 M-L1 Digital Sight Camera System, Nikon, Tokyo, Japan) was used for evaluation of the slides.

Histomorphometric analysis

Osteointegration, bone apposition, degradation of GRHC granules and formation of new bone within the defects was evaluated using a method previously described by the authors[30]. Examination of unstained slices was performed using a scanning electron microscope (FEI

Quanta 400 FEG ESEM, Hillsboro, USA; EDAX Pegasus X4M, NJ, USA) operating in high vacuum mode at an accelerating voltage of 15 kV. The defect areas and adjacent cortical bone were photographed and the resulting digital images were combined to produce one high-resolution image of the whole defect (AutoStitch v2.2, British Columbia, Canada). These images were then imported into ImageJ software (v1.41o, National Institute of Health, USA) for histomorphometric analysis. The percentage of defect filling in relation to the surrounding non-operated cortical bone was determined[30]. A grid with a spacing of 200 x 200 μm was placed over each image. The tissue underlying each area of the grid was recorded as either: resorption cavity, new bone, or GRHC particles with the aid of 'point picker plug-in' for ImageJ. Because of the space occupied by the GRHC granules in the filled defects, the space available for new bone growth was smaller than in the empty samples. To compensate for this difference, empty space available for bone growth was calculated as the difference between the size of the defect and the space occupied by the granules (100% - granules area) and this number was used to further analysis.

Statistical Analyses

Statistical analysis was performed using the SPSS version 19.0 (SPSS, Chicago, IL, USA). Differences between time points and between groups were tested by repeated measures ANOVA, accounting for multiple comparisons per group. In case ANOVA revealed an overall significant main effect of experimental group, pair-wise comparisons between the groups was undertaken using the post hoc Tukey test. The P value for statistical significance was set at < 0.05.

RESULTS

Radiographic analysis

At time zero, radiographs of the filled defects showed radiopaque circles. At three weeks, the defects filled with GRHC or GRHC and BMC showed evidence of reduction of the radiopaque circle, which continued to show resolution up to 12 weeks. In the empty defects, an increase in radio-opacity was progressively observed at three weeks, which decreased slowly until 12 weeks (Fig.2).

Macroscopic Analysis

On evaluation at three weeks, the created femoral defects showed almost complete macroscopic bone healing in 10 out of the 12 defect filled with GRHC/BMC and in 4/9 defects filled with GRHC, while none of the unfilled defects had this appearance at 3 weeks. At 6 weeks, all Group B and C and 5/9 Group A defects showed complete macroscopic bone healing. At 12 weeks, all defects showed complete macroscopic bone healing (Fig.2).

Light Microscopy

Histologic analysis with Hematoxylin/Eosin staining revealed new bone formation at the walls of the defects in all groups (Fig.3). However, in the defects filled with GRHC and BMC (group C), new bone was formed both at the walls of the defects, as well as between the granules. This new bone showed embedded osteocytes adjacent to resorption lacunae, which surrounded the GRHC pellets. The presence of Haversian canals in close proximity to these lacunae indicates bone remodeling around the granules. There was a faster formation rate and earlier maturation of bone in the GRHC/BMC filled defects, with formation of normal periosteum and endosteum (Fig.4) when compared to GRHC filled defects. These differences

faded at 6 weeks, between Group B and C. Group A defects were not yet filled with new bone in this period. At 12 weeks, Group B and C were identical. Group A defects were completely filled. However, the bone was still histologically disorganized. Further examination of the samples with GRHC/BMC stained with Solochrome cyanine R confirmed a higher degree of bone maturity at 3 weeks compared to the other groups, with a well-organized mature lamellar pattern of bone throughout the defects. These differences faded with time between Group B and C at 6 weeks, and both groups were identical at 12 weeks. Despite fully filled with new bone tissue by 12 weeks, Group A samples still showed immature bone tissue at 12 weeks.

SEM

Scanning electron micrographs (Fig.5) at 3 weeks demonstrated more mature features of bone formation in the defects filled with GRHC/BMC. The pellets were surrounded by *de novo* mature bone without any intervening fibrous connective tissue. It was possible to observe bone tissue formed among the GRHC pellets with the presence of new osteons. Evaluation at 6 and 12 weeks between Groups A, B and C, showed similar results to the evaluation performed with Optic Microscopy. Histomorphometric analysis across the defects was performed in all groups, providing quantitative data. Results of the *average percentage of new bone* are shown in Table 2.

DISCUSSION

The most important finding of the present study is that there is an improved early biological behavior of the GRHC/BMC construct, when compared to the control groups with regards to bone healing. With this construct, bone tissue formed faster and resembled normal bone in all characteristics evaluated. This is most likely explained by the combination of the

osteoconductive properties of the bone substitute (given by its chemical composition, roughness and shape), with the *osteogenic* and *osteoinductive* properties of the bone marrow cells; along with early weight bearing (*mechanical stimulus*).

The principle of autologous bone marrow loaded implants in animals is not new, and has proven itself to be effective in previously published studies. [24,25,26,28,32] However, many of these studies used manipulated or cultured cells. These techniques require a two-stage procedure: one procedure for harvesting and a separate procedure for implantation after *in vitro* culture. In addition, the use of culture manipulated cells has significant concerns with regards to uncontrolled cell differentiation, contamination and associated ethical issues.[3-6]

Zhong et al.[35] concluded that the use of human bone marrow concentrate or platelet rich plasma (PRP) was superior to the use β-tricalcium phosphate alone in bone tissue engineering applications. However, they used the cranium of immunodeficient mice as a model, a flat bone without mechanical stimulus in a rejection free environment. This makes the results difficult to extrapolate to humans. Recently, Hakimi et al.[36] showed a benefit of adding a combination of bone marrow concentrate and PRP to calcium phosphate granules in critical-size bone defects in the proximal tibia of mini-pigs. However, calcium phosphate is a rapidly absorbed scaffold and the metaphysis of a long bone provides a very rich biological environment which can cause confounding of variables.

It is now widely accepted that the posterior iliac crest is a rich source of osteoprogenitor cells[33] and that concentration methods are effective in supplying enough cell quantity and quality for bone regeneration techniques.[27,31] Promising clinical results were recently presented by Le Nail et al. in forty three cases of late unions of open tibia fractures treated with percutaneous

grafting with bone marrow autologous concentrate.[37] However, in this study the authors did not use a scaffold and no quantitative evaluations or histological results were available. This illustrates it is challenging to evaluate the effect of bone regeneration techniques in the clinical setting, as human studies often lack histological and quantitative evaluations[31,34], which are easier to obtain using an animal model such as the one used in the present study.

There are several limitations to this study. The study is performed in an animal model, and so extrapolation of results to the *in vivo* human setting is necessary. However, the chosen large animal model using sheep, resembles the human bone healing cascade more than other previously used small animal models such as rabbits.[30] Secondly, non-critical size defects in cortical bone were used. Cortical bone is used less frequently as a model than cancellous bone. However, it accounts for 70–85% of long bone strength[30] and has the advantage of being less vascularized then cancellous bone. Therefore confounding biological variables, like osteogenic cells and growth factors from the metaphysis that may contribute to bone healing, are better controlled. Also, the lateral femoral diaphysis allows for more samples without compromising femur strength and an even distribution of weight bearing forces. This permits the number of sacrificed animals to be smaller. Lastly, the sample size of the present study is small. This is in part due by the strict Ethics Commission regulations at our institution in allowing for large animal studies. A post hoc power analysis was performed and showed a power of 0.55. This is not ideal, but still considered acceptable for large animal studies of this nature.

CONCLUSION

This study demonstrates that the addition of a bone marrow concentrate to a glass reinforced hydroxyapatite composite in a pellet formulation is effective in promoting early bone healing.

FIGURE LEGENDS

Fig1. A- SEM image of a GRHC pellet macrostructure; B- Surface roughness of a pellet; C- Harvesting of Bone Marrow Aspirate; D- Bone Marrow Concentrate; E- Colonization of GRHC pellets; F- Bone defects creation; G- Bone defects after filling with the pellets/cell concentrate construct; H- X-Ray image of a defect

Fig2. Radiograph in a lateral projection and respective femur preparation for different implantation times of 0, 3, 6 and 12 weeks. Arrow heads indicate the location of empty holes. White arrows indicate the presence of GRHC fiiled defects. Orange arrows indicate the presence of GRHC+BM Cells fiiled defects.

Fig3. Histology: Bone defect left unfilled at 3weeks (A) and 6weeks (D); Bone defect filled with GRHC pellets at 3weeks(B) and 6 weeks (E); Bone defect filled with GRHC pellets (*arrow*) and Bone marrow concentrate at 3weeks (C) and 6 weeks (F- periosteum (*arrow)* and endosseum formed – *arrow head*)

Fig4. Histology: bone defect filled with GRHC pellets and bone marrow concentrate at 3weeks (A) and 6 weeks (B); bone defect filled with GRHC pellets at 3weeks (C) and 6 weeks (D) (*arrow* - mature bone; *white arrow head* - pellet resorption; *red arrow head - ostocytes*).

Fig5. Histomorphometric analysis: SEM images of bone defects at 3,6 and 12 weeks. A – unfilled defects; B – GRHC pellets filled defects; C - GRHC pellets and bone marrow concentrate filled defects

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	GROUP A Unfilled N = 3 sheep	GROUP B GRHC N = 3 sheep	GROUP C GRHC+BMC N = 3 sheep
3 weeks	9 defects	9 defects	12 defects
6 weeks	9 defects	9 defects	12 defects
12 weeks	9 defects	9 defects	12 defects

Table 1. Experimental Groups and Time Points.

Unfilled – Empty defect; GRHC – Defect filled with Glass Reinforced Hydroxyapatite Composite; GRHC+BMC - Defect filled with Glass Reinforced Hydroxyapatite Composite and Bone Marrow Cells

Table 2. Histomorphometric analysis. Results of the *average percentage of new bone* for Group A,B and C at 3,6, and 12 weeks. AVG – average; SD – Standard Deviation; *Significantly different from A; #Significantly different from A; §Significantly different from B.

	3 weeks			6 weeks			12 weeks		
	A	B	C	A	B	C	A	B	C
AVG	15.76	25.15*	48.08#§	37.86	73.37*	71.73#	77.83	80.33	83.48
SD	2.29	3.29	6.39	2.46	6.88	6.77	5.15	3.89	3.39

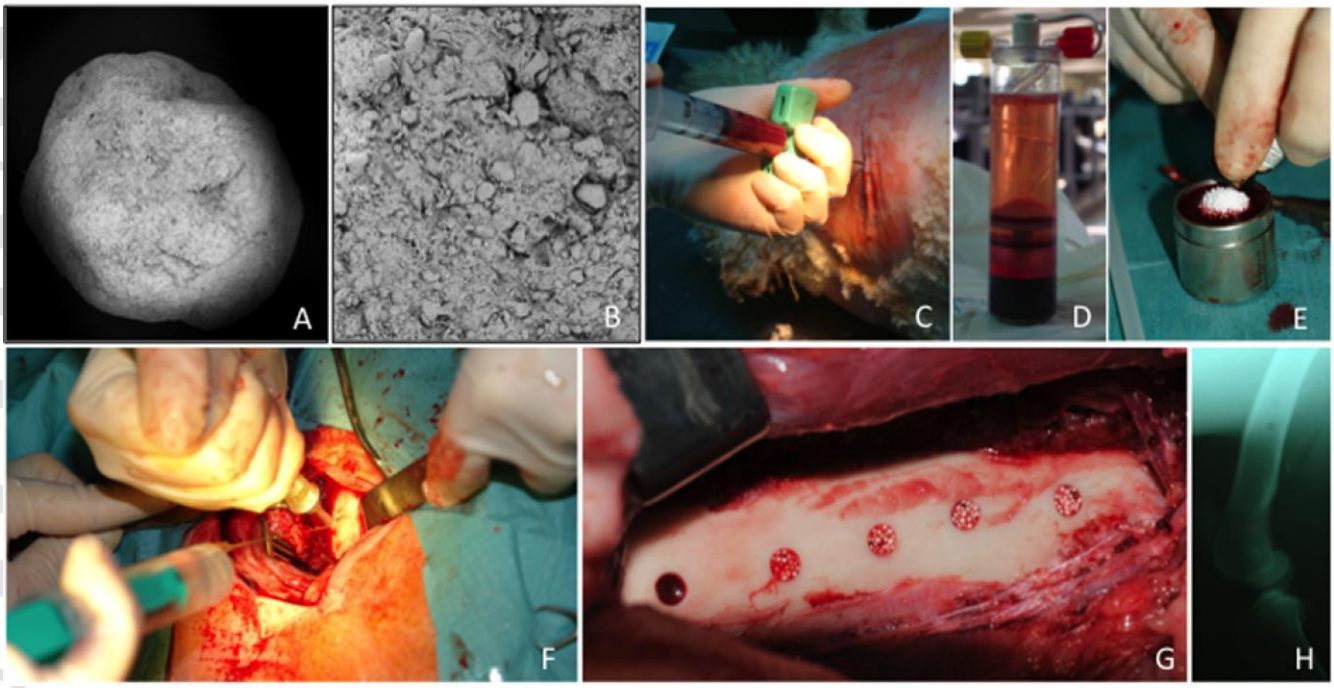

Figure 1

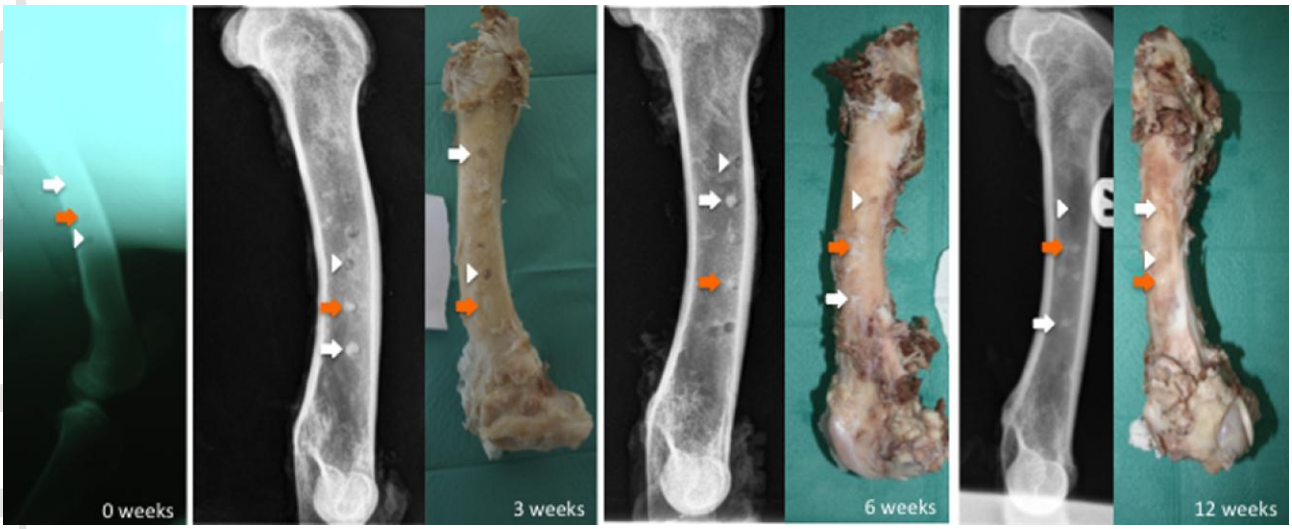

Figure 2

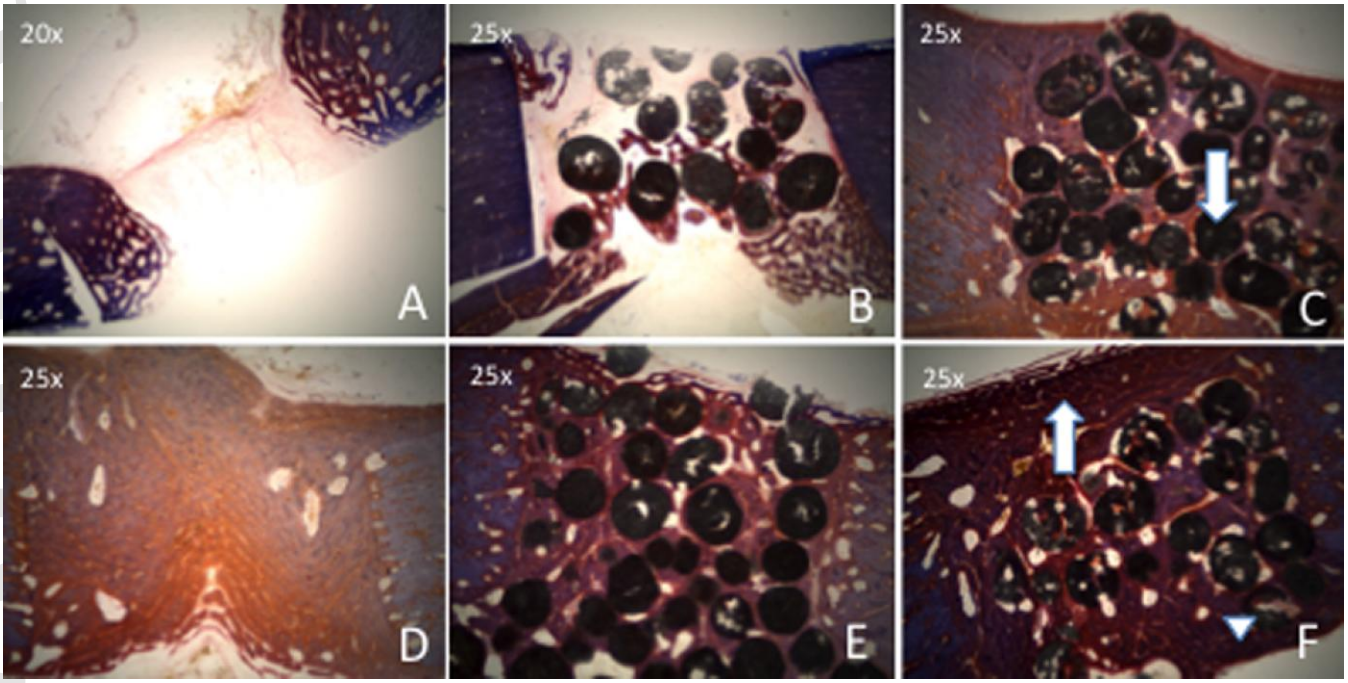

Figure 3

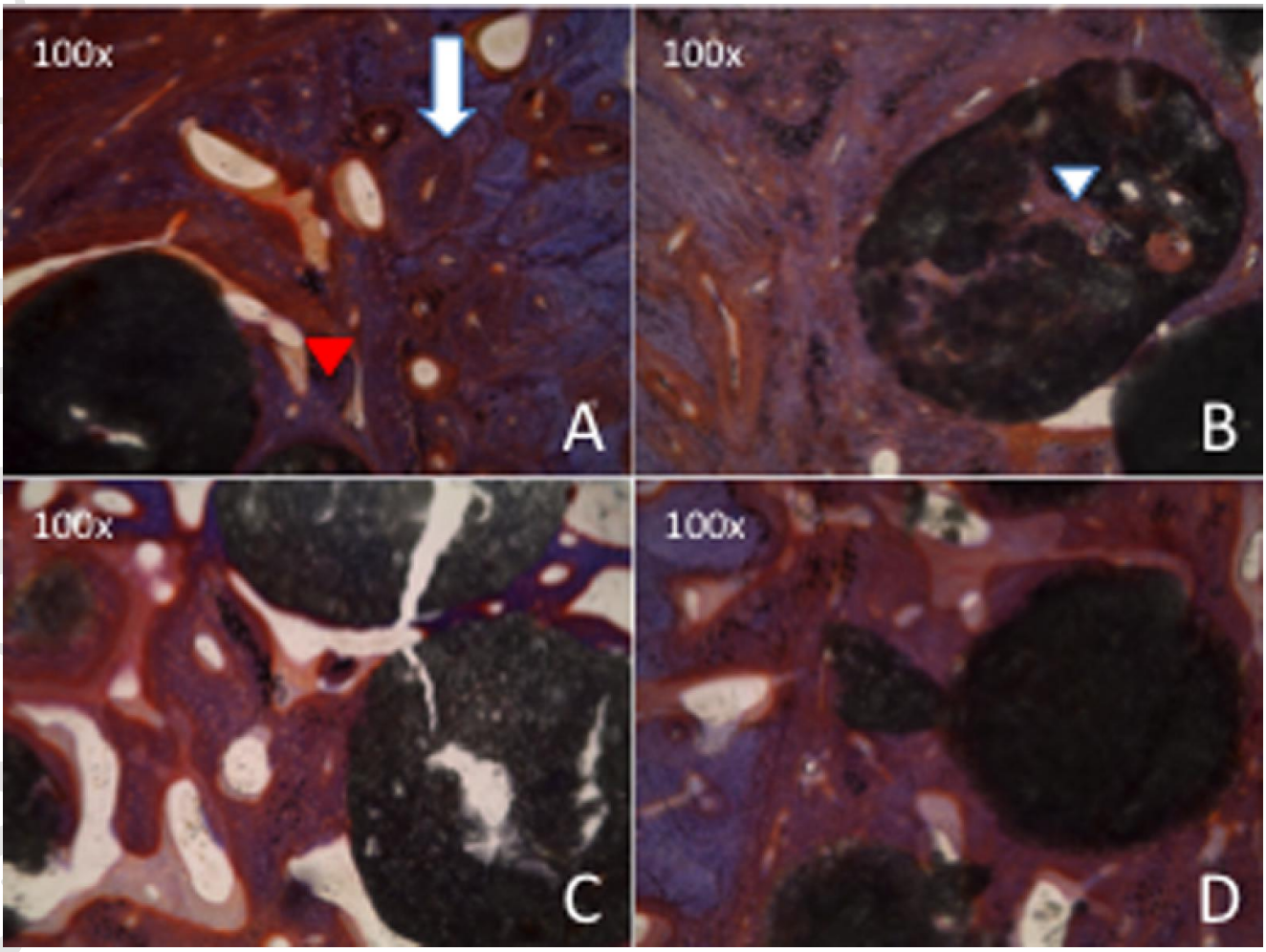

Figure 4

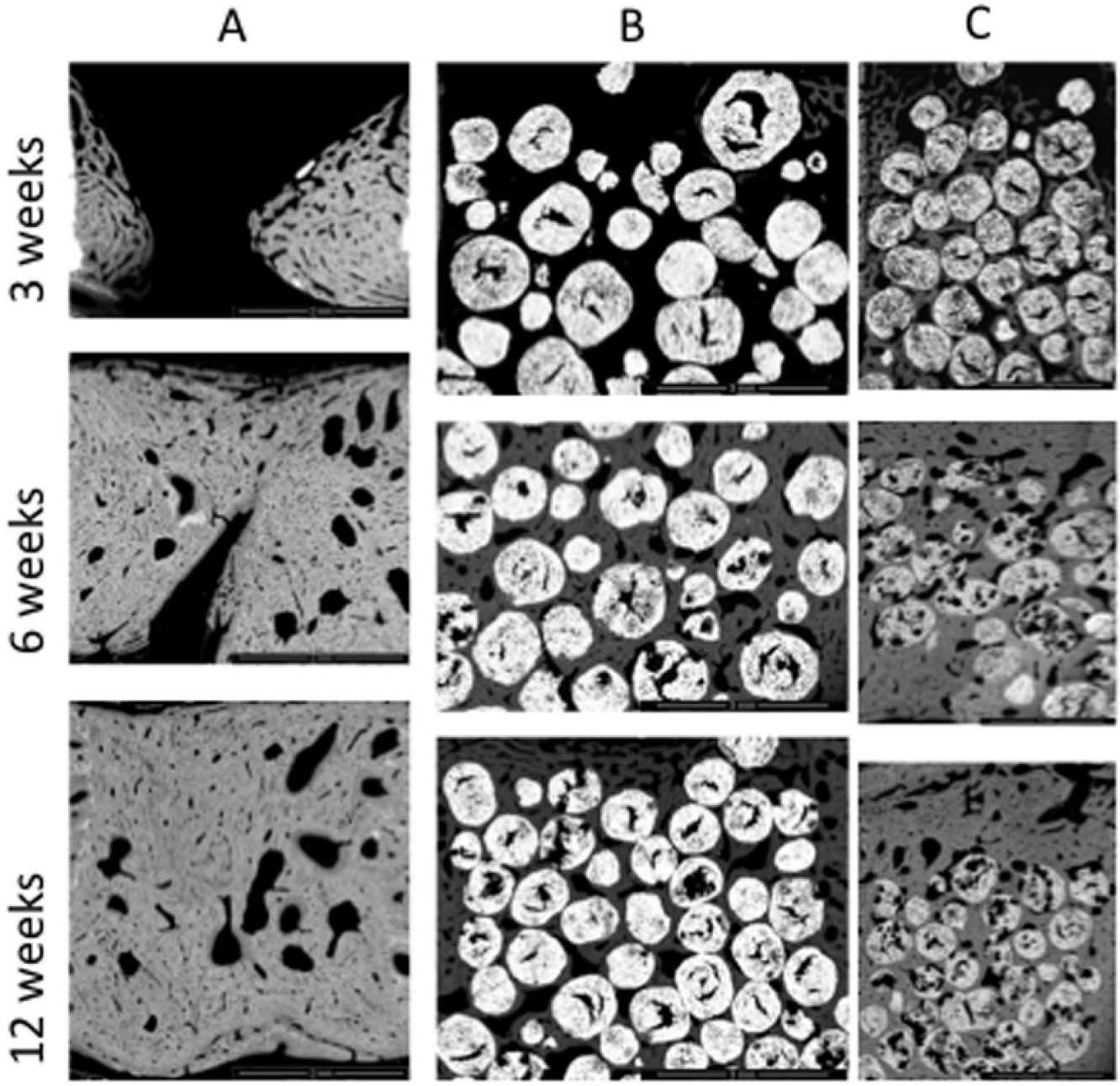

Figure 5