Osteoinductive potential of a novel biphasic calcium phosphate bone graft in comparison with autographs, xenografts, and DFDBA

ARTICLE *in* CLINICAL ORAL IMPLANTS RESEARCH · JULY 2015

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Osteoinductive potential of a novel biphasic calcium phosphate bone graft in comparison with autographs, xenografts, and DFDBA

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Key words: bone grafts, natural bone mineral, osteoinduction, osteoinductive potential

Abstract

Objectives: Since the original description of osteoinduction in the early 20th century, the study and development of innovative biomaterials has emerged. Recently, novel synthetic bone grafts have been reported with potential to form ectopic bone *in vivo*. However, their full characterization in comparison with other leading bone grafts has not been investigated. The aim of this study was to determine the osteoinductive potential of bone grafts by comparing autogenous bone grafts, demineralized freeze-dried bone allografts (DFDBA), a commonly utilized natural bone mineral (NBM) from bovine origin (Bio-Oss), and a newly developed biphasic calcium phosphate (BCP).

Materials and methods: Grafts were compared *in vitro* for their ability to stimulate bone marrow stromal cell (BMSC) migration, proliferation, and differentiation as assessed by quantitative real-time PCR for genes coding for bone markers including Runx2, collagen I, and osteocalcin. Furthermore, bone grafts were implanted in the calf muscle of 12 beagle dogs to determine their potential to form ectopic bone *in vivo*.

Results: The *in vitro* results demonstrate that both autografts and DFDBA show potential for cell recruitment, whereas only autografts and BCP demonstrated the ability to differentiate BMSCs toward the osteoblast lineage. The *in vivo* ectopic bone model demonstrated that while NBM particles were not osteoinductive and autogenous bone grafts were resorbed quickly *in vivo*, ectopic bone formation was reported in DFDBA and in synthetic BCP grafts.

Conclusion: The modifications in nanotopography and chemical composition of the newly developed BCP bone grafts significantly promoted ectopic bone formation confirming their osteoinductive potential. In conclusion, the results from this study provide evidence that synthetic bone grafts not only serve as a three-dimensional scaffold but are also able to promote osteoinduction.

Bone grafting materials have extensively been used in the field of dentistry to fill bone defects caused by trauma, periodontal disease, infection, and a variety of biochemical and skeletal disorders. Over the years, the role of biomaterials has changed from a passive, structural supporting network to one that may provide and orchestrate the process of cell migration and/or differentiation down to a bone-forming lineage (Langer & Tirrell 2004; Miron & Zhang 2012). Typically, the regenerative potential of bone grafts is divided into three categories. The ideal grafting material should (1) provide an osteoconductive matrix, which allows vascular invasion and cellular infiltration, (2) demonstrate osteoinductive potential by recruiting and inducing mesenchymal cells to differentiate into mature bone-forming osteoblasts, and (3) contain osteogenic progenitor cells within the bone grafting scaffold capable of forming a new bone matrix.

Consequently, the gold standard of bone grafting materials is autogenous bone due to its excellent combination of the three important biological properties of osteoconduction, osteoinduction, and osteogenic cells (Miron et al. 2011). Despite the numerous benefits associated with the use of autogenous bone, limitations including extrasurgical time and clinician costs as well as additional donor site

Date:
Accepted 18 May 2015

To cite this article:
Miron RJ, Sculean A, Shuang Y, Bosshardt DD, Gruber R, Buser D, Chandad F, Zhang Y. Osteoinductive potential of a novel biphasic calcium phosphate bone graft in comparison with autographs, xenografts, and DFDBA.
Clin. Oral Impl. Res. **00**, 2015, 1–8
doi: 10.1111/clr.12647

morbidity have motivated the search for alternatives. These include demineralized freeze-dried bone allografts (DFDBA) from human donors, xenografts from animals, and an array of synthetic alloplasts including hydroxyapatite, β-tricalcium phosphates, biphasic calcium phosphates, polymers, and bioactive glass (Bender et al. 2005; Jensen et al. 2009; Emerton et al. 2011; Park et al. 2012; Buser et al. 2013). Although most of these materials are osteoconductive, only a limited number of osteoinductive materials are available with FDA approval and the great majority of them include recombinant growth factors with osteoinductive potential (Miron & Zhang 2012).

To optimize the osteoinductive potential of bone grafting materials, it was recently proposed that the osteoinduction phenomenon is divided into three principles (Miron & Zhang 2012). These include the ability for an osteoinductive material (1) to recruit mesenchymal osteoprogenitor cells, (2) to induce an undifferentiated mesenchymal cell into a mature bone-forming osteoblast, and (3) to induce ectopic bone formation when implanted in extraskeletal locations. The proposed combination of these three principles would ideally maximize the grafts osteoinductive potential and ability to contribute to new bone formation (Miron & Zhang 2012).

It was recently suggested that synthetically fabricated bone grafts sintered at low temperatures do possess osteoinductive potential (Fellah et al. 2008; Yuan et al. 2010). Despite novel technical approaches for the development of synthetic bone grafts, the ability of calcium phosphate ceramics to induce bone formation in comparison with other bone grafting materials has not yet been investigated. Therefore, the aim of this study was to compare the osteoinductive potential of four commonly used bone grafting materials including autogenous bone chips harvested with a trephine bur and processed by a bone mill, DFDBA, a commonly employed xenograft (a natural bone mineral, NBM) and a newly developed synthetic bone graft fabricated from biphasic calcium phosphate (BCP). The osteoinductive potential of each bone graft will be compared for its ability to induce *in vitro* cell recruitment, proliferation, and differentiation as well as ectopic bone formation in an animal model.

Material and Methods

Isolation of bone marrow stromal cells

Bone marrow stromal cells (BMSCs) aspirates were obtained from healthy beagle dogs (used in conjunction with the study animal model) approved by the ethics committee following the guidelines for the treatment of experimental animals at Wuhan University (China). The bone marrow contents were collected from the humorous using a 10 ml heparinized syringe and was first layered onto gradient density (Histopaque®-1077 density 1.077 g/ml) and then centrifuged at 400 × *g* for 30 min. The mononuclear cells at the interface between each of the bone marrow aspirate layers were used. Occasionally, remaining red blood cells were mixed and incubated with an equal volume of red blood cell lysis buffer for 5 min. The mixture was then centrifuged and resuspended with 1 ml of culture medium. Mesenchymal bone marrow cells were then plated in T-25 flasks containing DMEM medium supplemented with 15% fetal bovine serum (FBS) and antibiotics. Once confluent, BMSCs were subcultured using a trypsin solution [0.25% trypsin (Gibco, Zug, Switzerland), 0.1% glucose, citrate–saline buffer (pH 7.8)] until experimental seeding. All cells were used between passages 2 and 4.

Bone graft selection

Autogenous bone was harvested from beagle dogs using a trephine bur and bone mill as previously described (Miron et al. 2011).

Demineralized freeze-dried bone allograft (Straumann AG, Basel, Switzerland) was used as our laboratories previous handling (Miron et al. 2013a; Wei et al. 2015).

A natural bone mineral graft (NBM, Bio-Oss®, Geistlich AG, Wolhusen, Switzerland) was selected as a representative of xenograft largely used in the dental field.

Finally, a newly developed biphasic calcium phosphate (Straumann AG, Basel, Switzerland) was utilized as the alloplast material.

Scanning electron microscopy

Bone particles were fixed in 1% glutaraldehyde and 1% formaldehyde for 2 days for SEM. Following serial dehydration with ethanol, the samples were critical point dried (Type M.9202 Critical Point Dryer, Roth & Co. Hatfield, PA, USA) followed by overnight drying. The following day, the samples were sputter coated using a Balzers Union Sputtering Device (DCM-010, Balzers, Liechtenstein) with 10 nm of gold and analyzed microscopically using a Philips XL30 FEG scanning electron microscope (Philips, Amsterdam, the Netherlands) to determine surface variations between bone grafts.

Cell migration assay

The migration assay of BMSCs was performed with a Boyden chamber using a 24-well plate and polycarbonate filters (Transwell Costar, Corning, Acton, MA, USA) with a pore size of 8 μm. One hundred milligrams (100 mg) of each bone graft was transferred into the lower compartment of the wells. After prewetting with culture medium overnight, 10^4 BMSCs were suspended in 50 μl DMEM and seeded in the upper compartment of the transwell. The cells were allowed to migrate to the lower compartment over a period of 24 h at 37°C in a humidified 5% CO_2 atmosphere. After 24 h, nonmigrated cells in the upper compartment were removed and the remaining migrated cells in the lower side of the filter were counted using an MTS assay as previously described (Miron et al. 2013a). All analyses were performed in triplicate and within three independent experiments. For statistical testing, triplicates were averaged and compared for statistical variance using a one-way analysis of variance (ANOVA) with Bonferroni test ($P < 0.05$).

Cell proliferation assay

Bone marrow stromal cells were quantified using the CellTiter 96 One Solution Cell Assay (MTS) (Promega, Madison, WI, USA) as previously described (Miron et al. 2013b). Cells were seeded on 100 mg of bone graft per well in 24-well plates at a density of 10^4 cells per well. At 1, 3, and 5 days postseeding, cells were washed with PBS and incubated with 80 μl of CellTiter 96 aqueous solution dissolved in 400 μl of PBS. After 4 h of incubation, the cell number was determined by measuring the absorbance at 490 nm on a 96-well plate reader. Experiments were performed in triplicate with three independent experiments performed for each condition. For statistical testing, triplicates were averaged and compared for statistical variance using a one-way analysis of variance (ANOVA) with Bonferroni test ($P < 0.05$).

Quantitative Real-Time PCR (qRT-PCR) analysis

qRT-PCR was used to quantify the total mRNA of osteoblast differentiation markers. Total RNA was isolated using TRIzol reagent and RNAeasy Mini kit (QIAGEN, Basel, Switzerland) at 3 and 14 days postseeding to determine the osteoblast differentiation markers. Primers and probes for genes encoding Runx2, collagen1α1, osteocalcin, and GAPDH were purchased as predesigned gene expression assays (Taqman, Applied Biosystems, Rotkreuz, Switzerland). qRT-PCR was performed using 20 μl final reaction volume of One step Master Mix kit as previously described (Miron et al. 2010). RNA quantifi-

cation was performed using a Nanodrop 2000c and 100 ng of total RNA per sample well. All samples were assayed in triplicate, and three independent experiments were performed. The ΔΔCt method was used to calculate gene expression levels normalized to GAPDH values as internal control for gene expression. Data were log-transformed prior to analysis by one-way ANOVA with Bonferroni test using GraphPad Software v. 4 (La Jolla, CA, USA).

Alizarin red quantification

Alizarin red staining was performed to determine the presence of extracellular matrix mineralization after 14 days as previously described (Miron et al. 2011). Osteoblasts were seeded at a density of 10^4 cells per 24-well culture dish onto 100 mg of bone grafting material. After 14 days, cells were fixed in 96% ethanol for 15 min and stained with 0.2% alizarin red solution in water (pH 6.4) at room temperature for 1 h. Alizarin red was dissolved using a solution of 20% methanol and 10% acetic acid in water for 15 min. Liquid was then transferred to cuvettes and read on a spectrophotometer at a wavelength of 450 nm. The samples were normalized to DNA content as well as 100 mg of bone grafting material run in parallel plates without cells. For statistical testing, triplicates were averaged and compared for statistical variance using a one-way analysis of variance (ANOVA) with Bonferroni test ($P < 0.05$).

Animals and surgical procedures

Ectopic bone formation experiments were performed in accordance with the guidelines of the animal handling committee at the University of Wuhan (China) as well as the ARRIVE Guidelines. In total, 12 mature beagle dogs (male, 10–15 kg) were used for these experiments. A dog model was chosen due to previous experimental comparison of animal models demonstrating superior results in a dog model (Yuan et al. 2006a,b). After anaesthetizing the dogs by an intra-abdominal injection of pentobarbital sodium (30 mg/kg body weight; with subsequent injections given as required), the lateral aspect of the femur was shaved and the skin cleaned with iodine. Then, a longitudinal incision was made, and the femoral muscles were exposed by blunt separation. Longitudinal muscle incisions were subsequently made by scalpel, and two muscle pouches with the distance more than 2 cm were created by blunt separation as previously described (Yuan et al. 2006a,b). Autogenous bone grafts, DFDBA, NBM, and CaP bone grafts were then inserted into each animal in their respective pouches (2 pouches/bone grafts per muscle) and sealed with silk sutures. Following surgery, each dog received penicillin intramuscularly for three consecutive days to prevent infection. Six dogs were sacrificed with overdose pentobarbital sodium injection at each time period of 60 days and examined for ectopic bone formation following fixation in 4% buffered formaldehyde solution (pH 7.4).

All animals were subject to treatment with autogenous bone, DFDBA, NBM, and CaP ($n = 6$ replicates per time point (two time points) for a total of 12 samples per group). After 30 and 60 days postimplantation, the samples were removed and prepared for histological analysis.

Histological analysis

The samples were decalcified in 10% EDTA which was replaced twice weekly for 3 weeks at room temperature. Then, the samples were dehydrated in a series of graded concentrations of ethanol from 30% to 100% followed by embedding in paraffin as previously described (Zhang et al. 2012). Serial

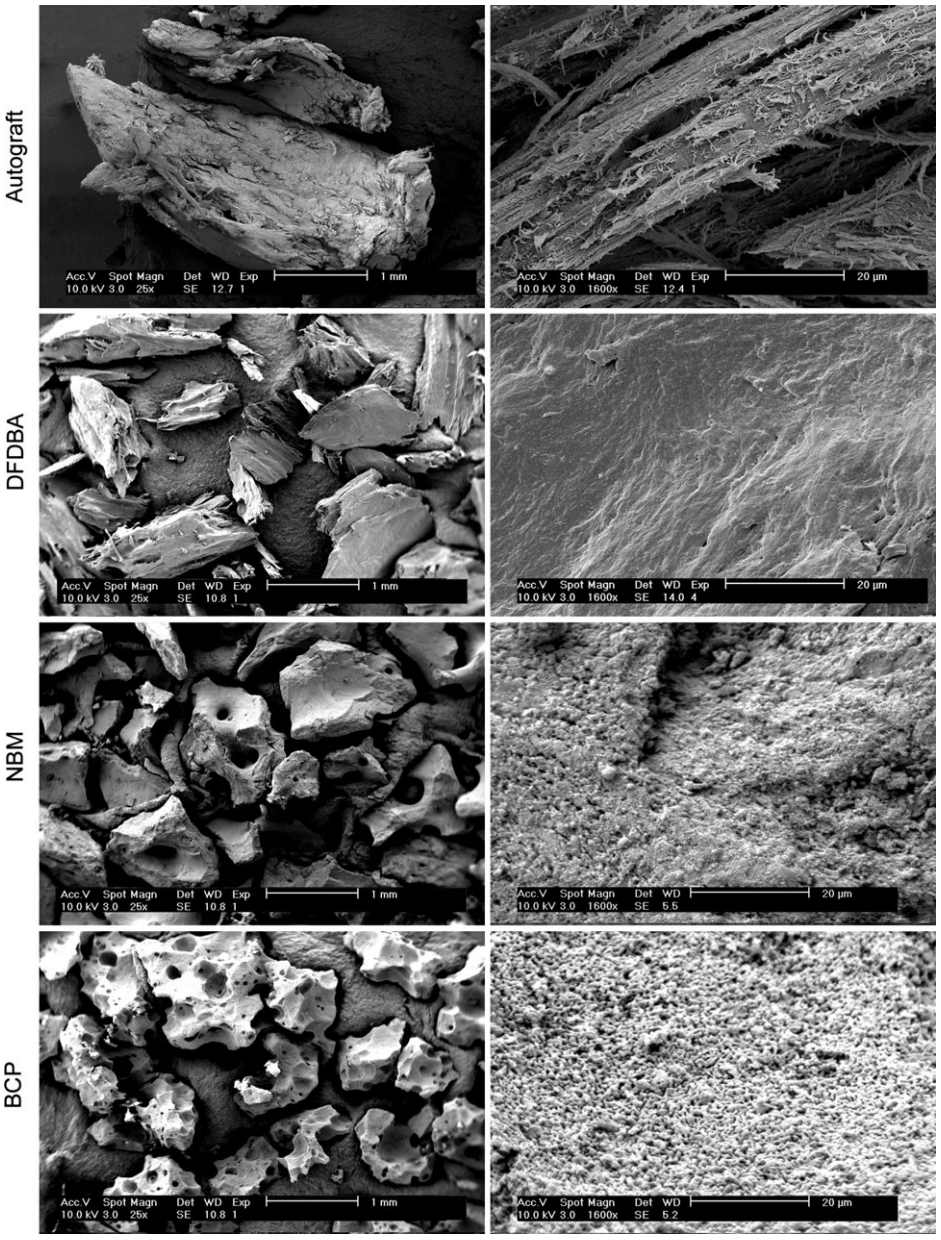

Fig. 1. Scanning electron microscopy of four commonly utilized bone grafting materials in dentistry including autogenous bone harvested with a bone mill, a demineralized freeze-dried bone allograft (DFDBA), a commonly employed xenograft of bovine origin (natural bone mineral, NBM), and a synthetically fabricated biphasic calcium phosphate.

sections of 5 μm were cut and mounted on polylysine-coated microscope slides and stained with H&E (Sigma #S2255; Sigma-Aldrich, St. Louis, MO, USA) in accordance with the manufacturer's protocol. All samples were observed for evidence of ectopic bone formation and remaining bone grafting particles. Furthermore, the ability of the samples to induce new ectopic bone formation was semi-qualitatively evaluated by two independent observers blinded to treatment and rated according to a previously published scheme (Boyan et al. 1999, 2006; Schwartz et al. 2000). As previously described, a score of 1 indicated the presence of particles without any bone; 2 indicated the production of new bone in one site within the section and covering less than 40% of the surface area examined; 3 indicated the production of new bone in more than one site, covering more than 40% but less than 70%, of the surface area examined; and 4 indicated the production of new bone in more than one site, covering more than 70% of the surface area examined. The overall grade for each implant was obtained by averaging the scores from all specimens in the group.

Results

Surface topography of various bone grafting particles

Scanning electron micrographs were utilized to visualize the surface variations of bone grafting particles at both 40 and 1600× magnifications (Fig. 1). Autogenous bone particles harvested with a bone mill demonstrated large particles with roughened surface containing visible protein adsorption and cells at the scaffold surface (Fig. 1a,b). In contrast, DFDBA particles demonstrate smooth non-mineralized tissues devoid of all cells and proteins (Fig. 1c,d). NBM bone grafting particles demonstrated a microrough surface with many macrorough and microrough topographies resembling native bone (Fig. 1e,f). The surface was completely devoid of proteins (Fig. 1f). Biphasic calcium phosphate grafting particles composed of hydroxyapatite and beta-tricalcium phosphate demonstrated both large-scaled, micro- and nanotopographies (Fig. 1g,h).

In vitro behavior of BMSCs seeded with bone grafting particles

First, each bone graft was tested for its ability to recruit BMSCs in Boyden chambers (Fig. 2a). The results demonstrated that both autogenous bone and DFDBA were able to significantly induce cell recruitment whereas both NBM and BCP demonstrated no potential of cell recruitment. Analysis of cell proliferation demonstrated the ability of BMSCs to proliferate on all bone grafts with a preference for cell proliferation on autogenous bone when compared to all other modalities at 3 and 5 days postseeding (Fig. 2b).

qRT-PCR was used to determine genes coding for bone differentiation markers including Runx2, collagen1, and osteocalcin at 3 and 14 days postseeding. Early changes in Runx2 were observed in BMSCs seeded on autogenous bone and BCP bone grafts when compared to DFDBA and NBM (Fig. 3a). Analysis of collagen 1 gene expression demonstrated over a 10 fold increase in BCP scaffolds when compared to the other modalities and significantly higher expression in DFDBA group (Fig. 3b). A 3-fold increase in osteocalcin gene expression, a late marker for osteoblast differentiation, was also observed in BCP scaffolds when compared to all other modalities (Fig. 3c). The results at 14 days postseeding further confirmed the ability for BCP scaffolds to induce genes coding for osteoblast cell differentiation markers by dem-

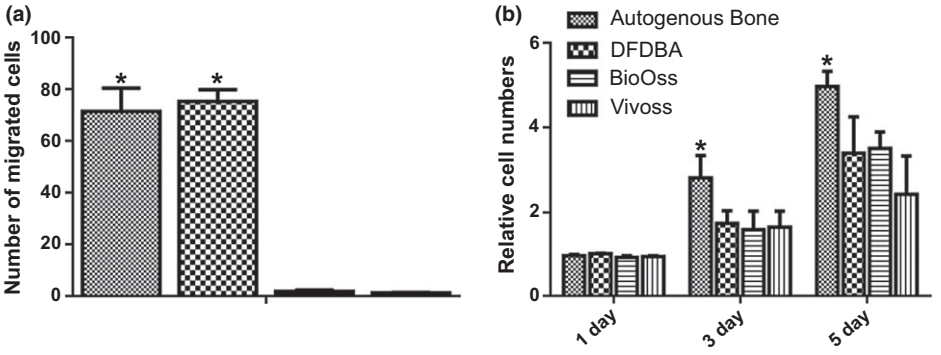

Fig. 2. (a) Migration assay using a Boyden chamber of bone marrow stromal cells (BMSCs) seeded in the presence of (1) autogenous bone harvested with a bone mill, (2) demineralized freeze-dried bone allografts (DFDBA), (3) natural bone mineral (NBM), and (4) Biphasic calcium phosphate (BCP). (b) Proliferation assay of BMSCs seeded on each bone grafting material and quantified for cell number following 1, 3, and 5 days postseeding. Data shown is the average value from three independent experiments (three replicates per experiment) ± SE. (*$P < 0.05$).

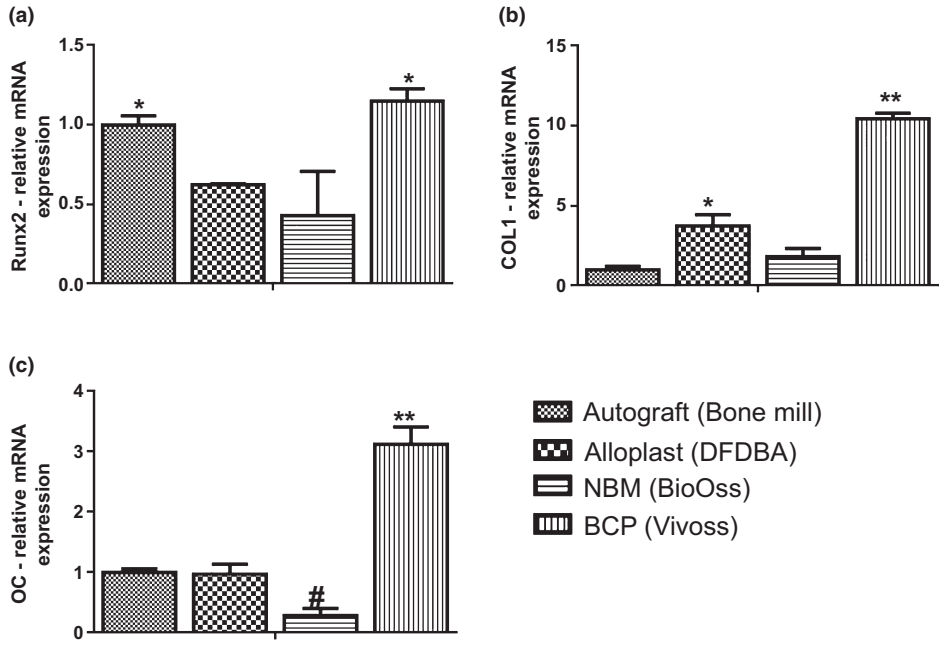

Fig. 3. Relative mRNA levels of Runx2, collagen1, alkaline phosphatase (ALP), and osteocalcin (OC) for bone marrow stromal cells (BMSCs) seeded on (1) autogenous bone harvested with a bone mill, (2) demineralized freeze-dried bone allografts (DFDBA), (3) natural bone mineral (NBM), and (4) Biphasic calcium phosphate (BCP) at 3 days postseeding. Data shown is the average value from three independent experiments (three replicates per experiment) ± SE. (*$P < 0.05$, **$P < 0.05$; group higher than all other groups, #$P < 0.05$; group lower than all other groups).

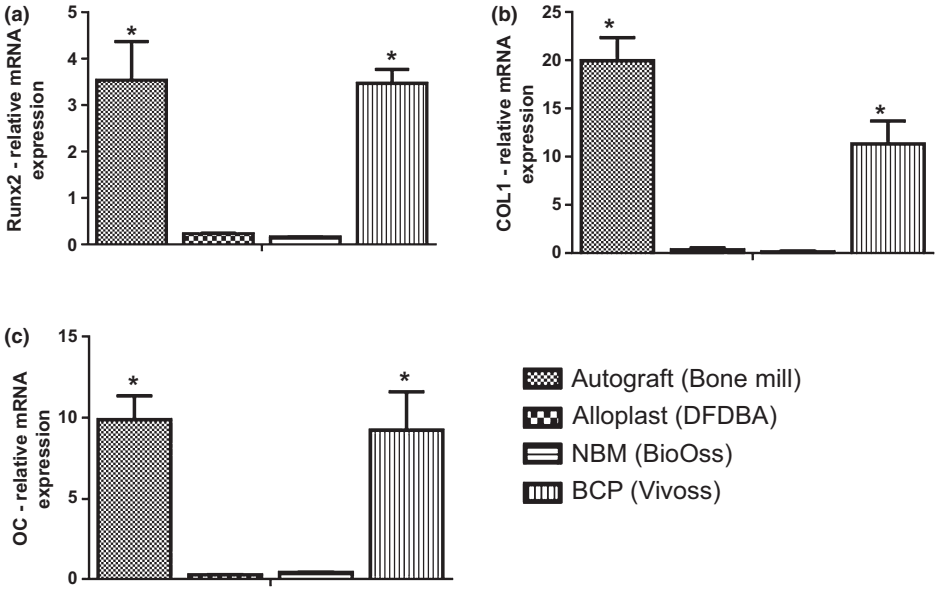

Fig. 4. Relative mRNA levels of Runx2, collagen1, alkaline phosphatase (ALP), and osteocalcin (OC) for bone marrow stromal cells (BMSCs) seeded on (1) autogenous bone harvested with a bone mill, (2) demineralized freeze-dried bone allografts (DFDBA), (3) natural bone mineral (NBM), and (4) biphasic calcium phosphate (BCP) at 14 days postseeding. Data shown is the average value from three independent experiments (three replicates per experiment) ± SE. (*$P < 0.05$).

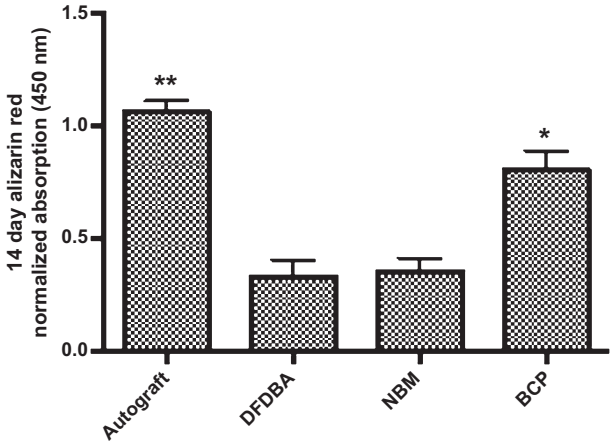

Fig. 5. Normalized alizarin red staining absorbance at 14 days postseeding. (three replicates per experiment) ± SE. (*$P < 0.05$, **$P < 0.05$; group higher than all other groups).

onstrating significantly higher expressions of genes coding for Runx2, collagen1, and OC when compared to DFDBA and NBM (Fig. 4). Furthermore, alizarin red staining was used to quantify the amount of newly mineralized tissue. It was found that both autogenous bone and BCP performed significantly better when compared to DFDBA and NBM (Fig. 5).

In vivo model for ectopic bone formation

Bone grafts were implanted into calf muscle of beagle dogs and examined for evidence of ectopic bone formation at 30 and 60 days (Fig. 6). All animals healed normally with no signs of infection. The implantation of autogenous bone led to rapid resorption of the graft material with no signs of either bone particles or ectopic bone formation found at either 30 or 60 days (data not shown). While autogenous bone resorbed quickly, DFDBA particles implanted in calf muscles demonstrated signs of new ectopic bone formation at 30 days near the particle surface in all samples (Fig. 6a). Following 60 days, most of the DFDBA particles had been fully resorbed although ectopic bone remained present within the defect sites (Fig. 6d). The implantation of a NBM from bovine origin demonstrated no signs of ectopic bone formation at either 30 or 60 days postimplantation (Figs 6b,e and 7). Little to no resorption of these scaffolds was visible within the defect sites (Fig. 6b,e). The implantation of BCP synthetic scaffolds demonstrated the ability for ectopic bone formation in all six samples (Figs 6f and 8). Interestingly, at 30 days, little bone formation was observed on the scaffold surface although a large number of infiltrating cells were found on the material surface (Fig. 6c). At 60 days, all samples demonstrated signs of ectopic bone formation adjacent to the material surface (Fig. 6f).

Discussion

In the present study, the osteoinductive potential of autografts, DFDBA, NBM, and BCP was compared both *in vitro* for their ability to induce cell migration, proliferation, and differentiation as well as *in vivo* for their ability to form ectopic bone. Not surprisingly, the ability of grafts to support cell migration was observed only in autografts and DFDBA. As both are known to contain growth factors within their matrix (Li et al. 2000; Miron et al. 2013b), the ability to recruit cells demonstrates that both derive some of their osteoinductive potential from the release of growth factors to their surrounding environment.

The results from the cell proliferation experiments demonstrate that the autogenous bone exhibits a faster rate of cell growth when compared to all other bone grafting materials used in the present study (Fig. 2b). Our laboratory has previously demonstrated that autografts are able to release a wide-array of growth factors from their matrix over time including bone morphogenetic proteins, transforming growth factors, insulin-like growth factors, and vascular endothelial growth factors (Miron et al. 2013b). Furthermore, it was recently demonstrated that the autografts are also able to release osteocyte-specific factors such as sclerostin and FGF-23 which may further contribute to cell growth (Brolese et al. 2014). However, the direct cause responsible for higher cell numbers on cells seeded on autografts, when compared to DFDBA, NBM, and BCP, remains to be investigated.

Analysis of cell differentiations revealed a significant advantage for BMSCs seeded on BCP scaffolds when compared to NBM and

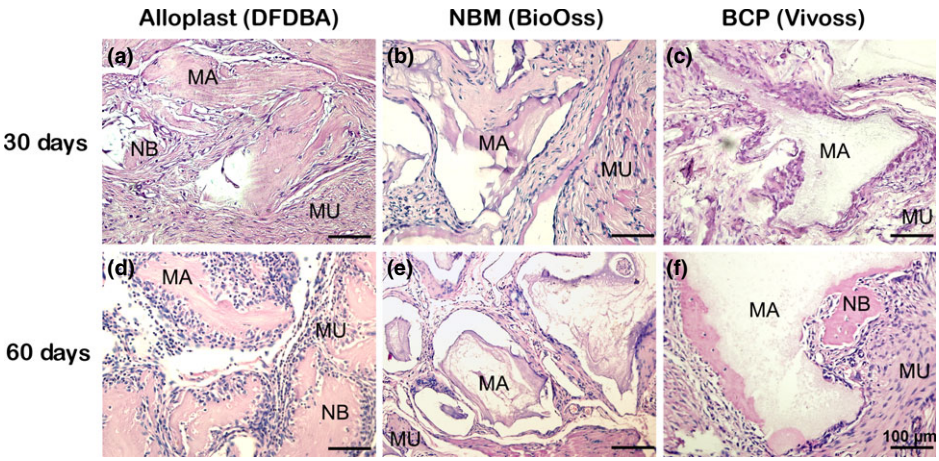

Fig. 6. H&E staining of representative figures of alloplasts (DFDBA), natural bone mineral (NBM), and a synthetical biphasic calcium phosphate (BCP) implanted into the calf muscles of beagle dogs at 30 and 60 days to analyze ectopic bone formation *in vivo*. MA = Material, MU = Muscle, NB = new bone, bar = 100 μm.

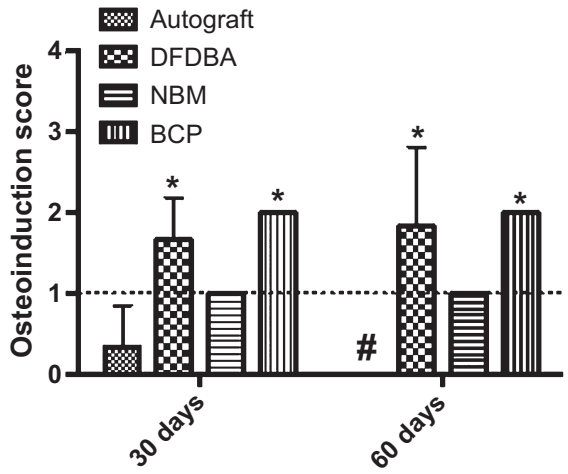

Fig. 7. The effect of different bone grafts on osteoinductivity using a qualitative scoring system: 0 = no evidence of any bone graft; 1 = only original bone graft present; 2 = one site with visible ectopic bone formation; 3 = two or more sites of ectopic bone formation; 4 = >70% of field at ×10 covered by new bone (*$P < 0.05$, #$P < 0.05$; group lower than all other groups).

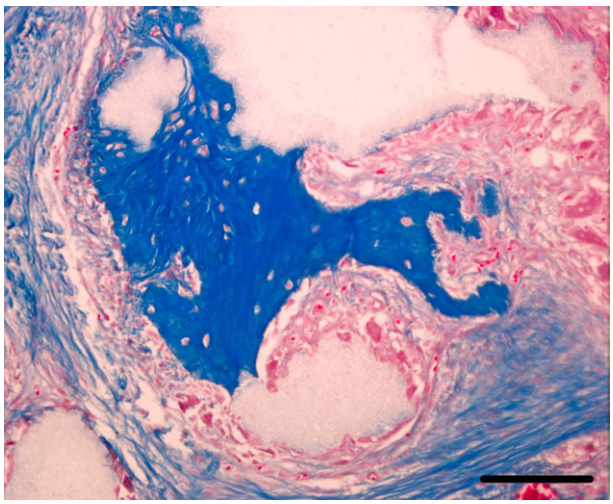

Fig. 8. Mason staining demonstrating ectopic bone formation for biphasic calcium phosphate scaffolds when implanted in the muscle of beagle dogs at 60 days (bar represents 100 μm).

DFDBA (Fig. 3). Furthermore, BMSCs seeded on BCP exhibited over a three fold significant increase in gene expression of osteocalcin, a late marker for osteoblast differentiation at 3 days postseeding (Fig. 3c). Thus, it is reasonable to assume that in the absence of osteoblast differentiation media, cells seeded on BCP scaffolds with specific and highly defined micro- and nanotopographies are able to stimulate the spontaneous differentiation from mesenchymal progenitor cell toward the osteoblast lineage. Much research has been performed on this topic within the past few years (Li et al. 2007; Barbieri et al. 2010; Yuan et al. 2010; Chan et al. 2012; Davison et al. 2014; Idowu et al. 2014; Luo et al. 2014). It has been proposed that ectopic bone formation of synthetic alloplasts might be a result of accumulation of growth factors such as BMPs to the surface of the bone grafts which are then able to induce mesenchymal differentiation to osteoblasts. In the present study, we demonstrate that the progenitor cells completely devoid of any osteoinductive growth factors are able to spontaneously increase the expression of bone markers including collagen 1 and osteocalcin suggesting that the auto-induction is likely governed by a process receiving much of its cues from surface topographical features and potential chemical signals from the dissolution of the scaffold material.

Bone grafts were then implanted into the muscle pouches of beagle dogs to examine their ability to form ectopic bone. While autografts were resorbed quickly *in vivo* and NBM demonstrated no potential to form ectopic bone formation, both DFDBA and BCP grafts were able to induce ectopic bone formation (Fig. 6). Interestingly, BCP grafts formed ectopic bone in all samples whereas DFDBA samples exhibited new bone formation in varying degrees, with some samples unable to induce osteoinduction. It has previously been reported that variability in osteoinductive potential does exist between samples, sterilization technique and donor age (Schwartz et al. 1998; Boyan et al. 2006; Yang et al. 2014; Wei et al. 2015). Contrarily to this, the BCP biomaterial is synthetically fabricated and a more homogenous scaffold topography is thus obtained likely contributing to reproducibility of the results between samples.

In conclusion, the results from the present study further illustrate the osteoinductive potential of various bone grafts. Although autografts did not form ectopic bone *in vivo* in this model, likely due to the presence of viable cells signaling their misplacement in

surrounding tissues, they demonstrate higher rates of cell migration and proliferation when compared to the bone grafting categories. Furthermore, their potential to differentiate BMSCs to osteoblasts is significantly upregulated when compared to DFDBA and NBM. Within the limits of the present study, we demonstrate that the novel synthetic BCP scaffolds were able to significantly and rapidly upregulate the expression of genes coding for bone-specific markers such as osteocalcin as early as 3 days postseeding, thus largely suggesting the ability for these scaffold topographies to dictate the differentiation down to the osteoblast lineage. Furthermore, their ability to form ectopic bone in all samples further confirms their osteoinductive potential and ability to act as a bone graft with osteoinductive potential with a large predictability as the materials are fabricated synthetically and do not necessitate human donors. Recent research further confirms the graft's ability to induce bone formation in bone defects created in animals (Dahlin et al. 2015; Yip et al. 2015). Future research comparing the potential of these new BCP scaffolds to other bone grafts for the healing of bone defects in clinical trials is now necessary to fully characterize their potential future use in regenerative dental procedures.

Acknowledgements: This study was funded by the International Team for Implantology (ITI) Foundation grant number (1000_2014). All authors declare no conflict of interests.

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