A comparison of the osteoinductive potential of two calcium phosphate ceramics implanted intramuscularly in goats

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The osteoinductive potential, or bone induction potency, of two calcium phosphate ceramics was evaluated after intramuscular implantation in goats. The ceramics were comprised of hydroxyapatite (HA) and biphasic calcium phosphate (BCP), the later of which contained a 85/15 mixture of hydroxyapatite and tricalcium phosphate (TCP). Both ceramics had a similar macroporosity of around 55% and a pore distribution between 100 and 800 μm. Besides the difference in chemistry, BCP was also microporous and hence had a different surface microstructure. After implantation in the back muscles of four goats for 12 weeks, all 8 BCP samples ($7 \times 7 \times 7\,mm^3$) showed the presence of bone formation in the macropores ($1 \pm 1\%$), while no bone was found in any of the HA samples. The used BCP can therefore be characterized as an osteoinductive material. Having the ability to induce bone formation in soft tissues, the BCP presented herein may be a useful biomaterial for bone repair when combined with cultured osteogenic cells, growth factors or both.

1. Introduction

Potential synthetic bone grafts that may be used in bone repair can be evaluated based on several biological properties they elicit following implantation. Their potential to form a tight and intimate bond with bone tissue and ability to guide bone formation from the host bone bed along their surface into the material is seen as bioactivity or osteoconductivity and is desired to get rapid bone infiltration and implant stability. Another, less well understood and accepted property, is the ability of the implant material to induce bone formation when implanted in orthotopic, i.e. non-osseous, sites [1–3].

Bioactive or osteoconductive biomaterials all have in common that their surface is composed of calcium phosphate, the main inorganic component of bone, that is either present pre-operatively or that is formed post-implantation by mineralization processes. Such materials are relatively easily prepared by introducing calcium phosphate either into the biomaterials (e.g. calcium phosphate ceramics, calcium phosphate cements, glass ceramics containing calcium phosphates, composites with calcium phosphates and calcium phosphate coatings) [1, 3–4], or on their surface (plasma sprayed hydroxyapatite coatings, biomimetic coatings, etc.) [3]. At present, osteoinductive materials are generally prepared by introducing either growth factors, such as bone morphogenetic proteins (BMPs), osteogenic cells (bone tissue engineering) [3, 6], or both to the material. A current challenge is to synthesize osteoinductive biomaterials that can induce bone formation in soft tissues without the addition of any exogenous growth factors or osteogenic cells.

In general, biomaterials are believed not to be osteoinductive [1, 2, 4], or in other words, when biomaterials are implanted in soft tissue of animals, they usually do not induce bone formation. Winter and Simpson [7], however, were the first to report ectopic bone formation induced by a biomaterial, and over the past 10 years, an increasing number of reports have undeniably shown osteoinduction by a series of biomaterials in different animal models [3, 7–23]. Osteoinductive materials therefore do exist, although the reason for the elicited biological response after implantation is currently unknown, as is the question how to make such osteoinductive materials. In the present study, we produced two porous calcium phosphate ceramics and studied their osteoinductive potential after intramuscular implantation in goats.

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2. Materials and methods

2.1. Preparation of the ceramics

2.1.1. Hydroxyapatite

Hydroxyapatite (HA) ceramic was prepared from commercial HA powder (Merck, Amsterdam, The Netherlands). The processing route included the following steps: (1) preparation of an HA slurry (2/3 wt % calcined HA powder and 1/3 wt % water containing deflocculant (Dolapix CE 64, Germany) and binder (carboxyl–methyl cellulose, Pomosin BC, The Netherlands), (2) mixing two immiscible phases: water-based HA slurry and poly-methyl methacrylate (PMMA) resin with a volume ratio of HA/PMMA of 1 : 1. The PMMA resin was composed of PMMA powder, MMA monomer and an additional fugitive pore-maker (< 10 v/v%) such as naphthalene or wax particles. (3) After shaping in a mold and polymerization, the mixture was subjected to drying, pyrolyzing (to remove all organic phases) and final sintering in air at 1250 °C for 8 h.

2.1.2. Biphasic calcium phosphate

Biphasic calcium phosphate (BCP) ceramic was made from in-house made apatite powder as described briefly below. Under stirring, di-ammonium phosphate hydrate (Fluka Chemie, The Netherlands) solution (792 g di-ammonium phosphate hydrate in 2500 ml de-ionized water) and 3500 ml ammonia solution (Fluka Chemie, The Netherlands) were slowly added to a calcium nitrate tetrahydrate (Fluka Chemie, The Netherlands) solution (2360 g calcium nitrate tetrahydrate in 5000 ml de-ionized water) to form an apatite slurry. The slurry was then aged at respectively 80 °C for 96 h and at room temperature for another 30 days. Approximately 1 kg apatite powder was obtained by washing the slurry with boiling de-ionized water, drying at 80 °C, grinding and sifting through a 140-mesh sieve. Porous green bodies were prepared from the apatite powder with 2% H_2O_2 solution (at the ratio of 1.0 g powder/1.2 $\pm$ 0.05 ml solution) and naphthalene (Fluka Chemie, The Netherlands) particles (710–1400 μm, at the ratio of 100 g powder/30 g naphthalene particles) at 60 °C. The naphthalene was subsequently evaporated at 80 °C and the porous green bodies were dried. Finally, porous calcium phosphate ceramic was obtained by sintering the dried porous green bodies at 1200 °C for 8 h.

2.2. Analysis of the ceramics

The chemistry of the ceramics was analyzed by X-ray diffraction (XRD, Miniflex, Rigaku, Japan). The macro-porous structure was evaluated by stereo-microscopy and the microstructure was observed with a scanning electron microscope (XL30, ESEM-FEG, Philips).

2.3. Animal experiments

Ceramic cubes ($7 \times 7 \times 7\,mm^3$) were prepared from the HA and BCP ceramic blocks and washed with acetone, 70% ethanol and de-ionized water. After steam sterilization at 121 °C for 30 min, the ceramic cubes were implanted in the back muscle (M. Longissimus Lumborum) of adult Dutch milk goats under general anaesthesia and sterile conditions. A total of four goats were used that each received two HA samples and two BCP samples. After 12 weeks, the animals were sacrificed and the samples (eight implants per group) were harvested.

After retrieval, the samples were fixed, dehydrated and embedded in methyl methacrylate. Thin undecalcified sections were made on an innerlock diamond saw (Leica) and stained with methylene blue/basic fuchsin for histological observation. Histomorphometrical analysis was performed on the histological sections to obtain both the porosity (percentage of macropore area) and the percentage of newly formed bone in the implants. Back-scattered electron microscopical analysis (XL30, ESEM-FEG, Philips) was also performed on the polished block-faces of the embedded samples to evaluate newly formed bone.

3. Results

3.1. Calcium phosphate ceramics

X-ray diffraction (XRD) analysis of the produced materials showed that the HA ceramic was pure hydroxyapatite, while the BCP ceramic was bi-phasic in nature and contained 80–90% of HA and 20 $\pm$ 10% β-TCP (Fig. 1). Stereomicroscopy and (back-scattered) scanning electron microscopy, revealed that both HA and BCP were porous ceramics with more than approximately 80% of the macropores ranging from 100–800 μm (Fig. 2(a) and (b)). Histomorphometrical analysis of the histological sections showed a macropore area of 57 $\pm$ 2% for HA, and 53 $\pm$ 4% for BCP. Different microstructures were observed by SEM. The HA ceramic had a dense macropore surface without micropores on the macropore surface. The macroporous BCP ceramic

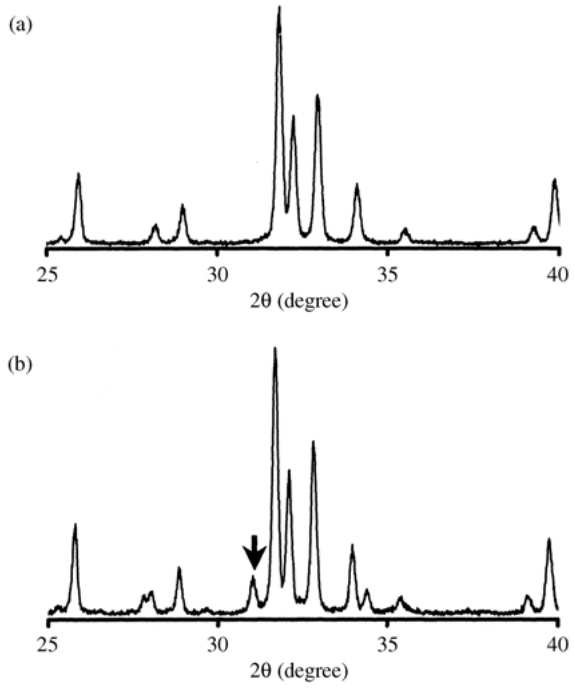

Figure 1 X-ray diffraction patterns of the HA ceramic (a) and the BCP ceramic (b). The arrowhead in B represents the main β-TCP peak of BCP.

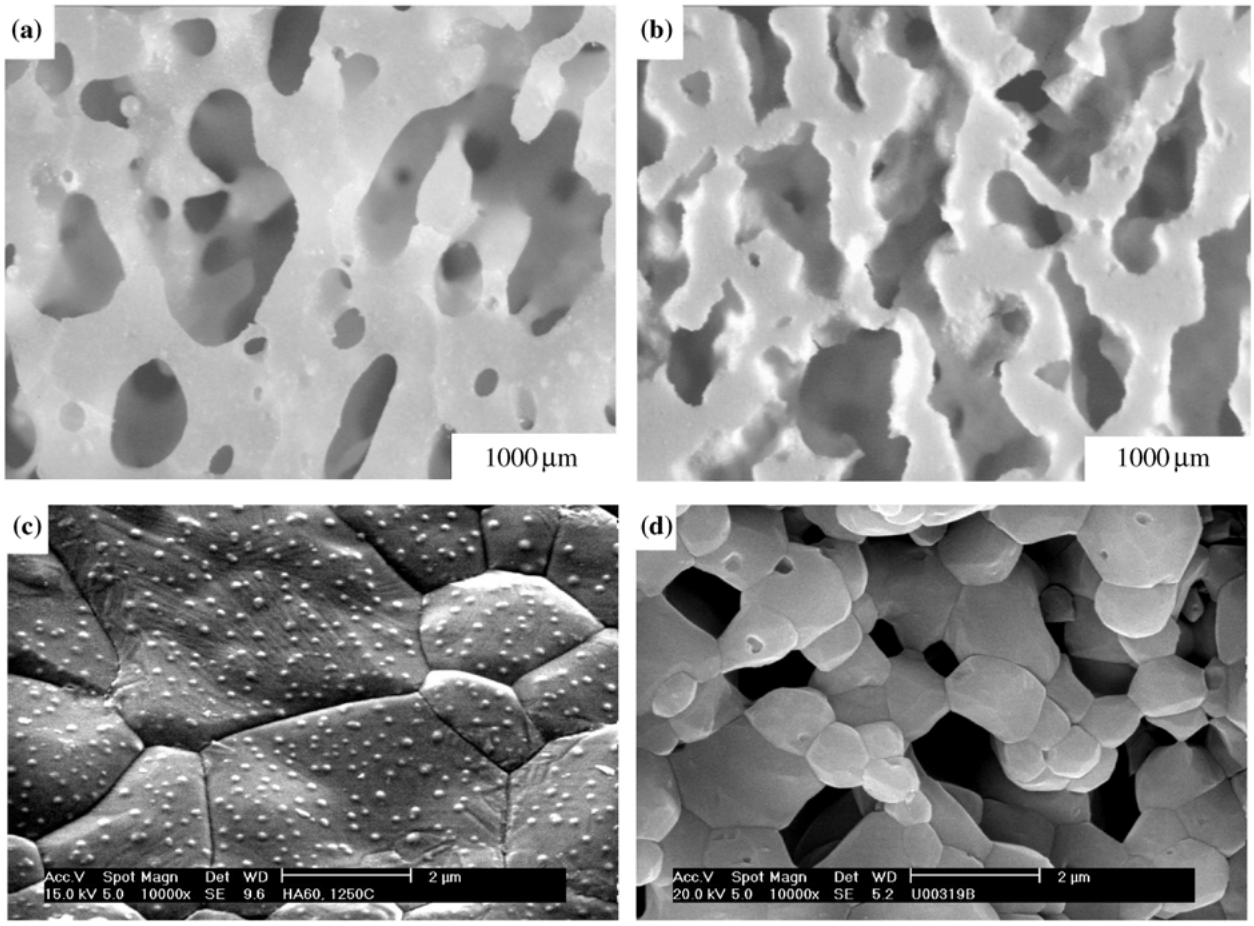

Figure 2 Stereo-micrographs (a, b) and scanning electron micrographs (c, d) of respectively the porous HA ceramic (a, c) and the BCP ceramic (b, d). Note the presence of micropores in BCP (d), while the surface of HA is dense (c).

on the other hand, was microporous throughout and contained abundant micropores (less than 2 µm) on the macropore surface (Fig. 2(c) and (d)).

3.2. Bone induction

After the 12-week intramuscular implantation period, bone formation was found in all BCP implants (8 out of 8), while no bone was found in any of the HA implants (0 out of 8). The macropores of the HA implants were filled with fibrous tissues (Fig. 3(a)), while in addition to fibrous tissue infiltration, bone tissue was sporadically found on the macropore surface of the BCP implants (Fig. 3(b)). Although the amount of bone was limited in quantity ($1 \pm 1\%$), it was only detected in the macropores inside the BCP implants and not on the outer surface of the implants or in the surrounding soft tissues. Cuboidal cells were frequently observed at the surface of the BCP that seem in close association with areas of early bone formation. In addition, examination of the sections under polarized light revealed that bundles of collagen-like tissue were often associated with the areas of bone condensation, and were mostly perpendicular aligned to the implant surface. The formed bone was normal in appearance with layers of secretory osteoblasts, osteoid, mineralized bone matrix and osteocytes (Fig. 3(c) and (d)). In addition, bone marrow formation was occasionally seen (Fig. 3(b) and (c)).

4. Discussion

The results of this study show that we have produced two calcium phosphate ceramics with different biological properties (HA and BCP). The BCP ceramic induced bone formation in the muscle of goats while the HA ceramic did not. These results not only suggest that bone induction by biomaterials is material-dependent but, more importantly, that osteoinductive biomaterials can be produced. The HA and BCP ceramics had not only a different chemistry, but were also different in microstructure or microporosity, and had slightly different macropore structures. Although the materials differed in more than one parameter, this is a good starting point for further studies to discern which factor is mainly responsible for the osteoinductive property of BCP.

With regard to bone ingrowth into biomaterials in orthotopic (bony implantation) sites, macroporous structures including macropore size and porosity are important material factors. Both the HA and BCP ceramics had macropores ranging from 100–800 µm, and the slight difference in porosity of $57 \pm 2\%$ for HA and $53 \pm 4\%$ for BCP is not expected to cause the difference in bone induction. As shown in SEM analysis, the HA used in this study had a dense wall without any micropores present, while the BCP had abundant micropores on its macropore surface. These different microstructures may be an important reason for the bone induction in BCP and the absence of bone induction in HA, which is further substantiated by our previous investigations [15–21]. We have previously shown that

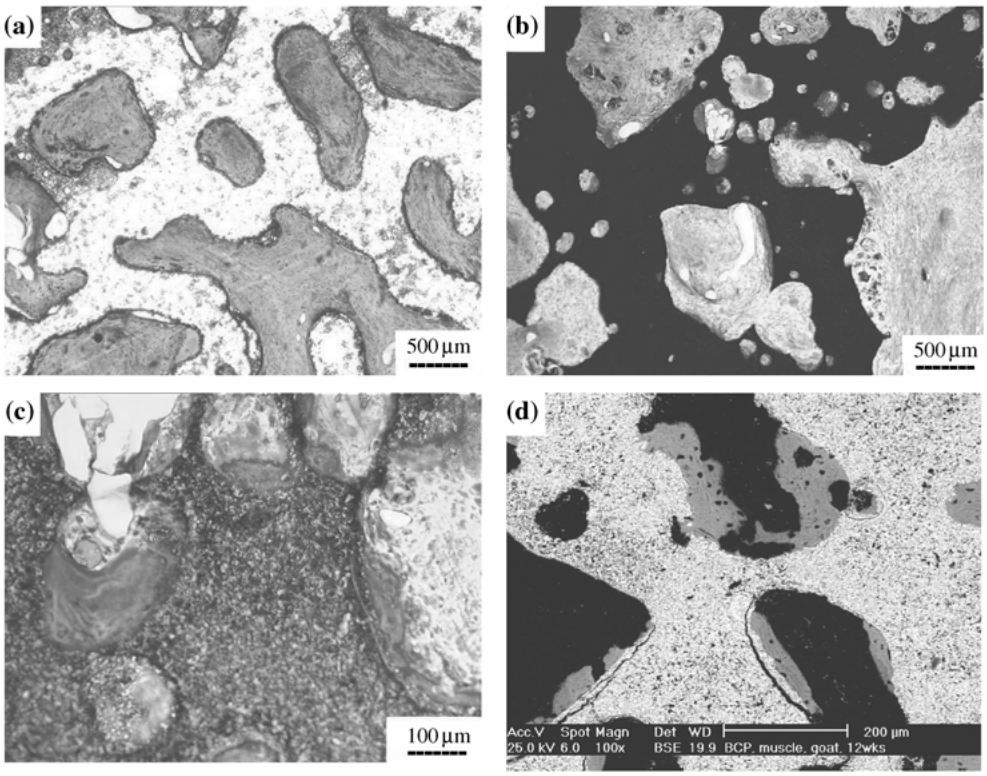

Figure 3 Undecalcified light micrographs (a, b, c) and back-scattered electron micrograph (d) showing the tissue response to HA ceramic (a) and BCP ceramic (b, c, d) after 12 weeks intramuscular implantation in goats. Fibrous tissue infiltration can be seen in all samples, while clear areas of bone formation can be seen with the BCP implants (b, c, d). The induced bone in the BCP samples has a normal morphology and is mineralized (d).

porous HA ceramic with micropores on the macropore surface can induce bone formation in soft tissues of dogs, while porous ceramic without micropores on macropore surface did not induce bone formation in this species [17]. We also reported that porous HA ceramic can induce bone formation to a limited extent in muscles of goats by increasing the surface microporosity through an acid-treatment [16].

Besides microstructure, the chemistry of calcium phosphate ceramics may be another important factor influencing their osteoinductive potential. Due to the mild dissolution of tricalcium phosphate (TCP) from BCP ceramic, this material has been suggested to be more active for bone formation than HA [15, 22]. Whether this is also the case for the osteoinductivity of this material, cannot be ruled out at present.

In the many reports over the past 10 years, the osteoinductive potential of biomaterials was shown to vary among biomaterials and species, while the highest osteoinductive potential was seen in large animals such as dogs, as compared to rabbits and mice [23]. We have now added the goat as another animal in which bone induction can occur. We have therefore indisputably shown that, contrary to what is generally thought, osteoinduction is a general phenomenon in mammals and related to the biomaterial used. Although the mechanism of bone induction by biomaterials is not clear as yet, much attention has been paid on BMPs [12]. It seems, however, that BMPs are not the only reason for bone induction by biomaterials, since BMP induced bone formation generally starts as cartilage (endochondral bone formation), while bone formation induced by biomaterials starts directly as bone [12, 13].

With the ability to form bone in soft tissues, an osteoinductive biomaterial may be useful for bone repair and speed up this process [23]. The limited amount of bone formation at relatively early implantation times may, however, be boosted by the addition of growth factors or osteogenic cells. As the results of the current study show that the osteoinductive material provides a good environment for bone formation and prepares the surface for pre-osteogenic cell attachment followed by osteogenic cell differentiation, this material may be excellently suited to function as a carrier of osteoinductive proteins [24] and/or (pre)osteogenic cells [25]. Initial studies in our laboratory have indeed shown a superior cell attachment to these osteoinductive materials as compared to non-osteoinductive materials. As a follow-up study, we are currently evaluating the effect of combining osteogenic cells to these HA and BCP implants on de novo bone formation after intramuscular implantation in goats.

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Received 24 May
and accepted 29 May 2002