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Comparative study of biphasic calcium phosphate with beta-tricalcium phosphate in rat cranial defects—A molecular-biological and histological study

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SUMMARY

The aim of this study was to evaluate the *in vivo* biocompatibility of a biphasic calcium phosphate (BCP) bone graft substitute consisting of 60% hydroxyapatite and 40% β-tricalcium phosphate (β-TCP) in comparison to a pure β-TCP of identical shape and porosity.

The materials were evaluated using an established rat cranial defect model in 24 animals. One bone defect with a diameter of 5 mm was created per animal. The defects were filled with either BCP or β-TCP and left to heal for 4 weeks. Twelve samples (6 per material) were processed for histological evaluation and immunohistochemistry. The remaining 12 samples were processed for mRNA expression analysis.

No signs of inflammation or adverse material reactions were detected. New bone formation in the former defect site did not differ between the two groups (BCP: 49.2%; β-TCP: 52.4%). Osteoblast-like and TRAP-positive osteoclast-like cells were found at the surface of the bone graft substitute granules. The β-TCP group showed significantly higher mRNA levels for the bone resorption marker Acp5 and osteogenic differentiation marker Runx2. The expression of IGF1, IGF2, VEGF, Phex, Alpl, Col1, Col2, Bglap and MMP8 did not differ between the groups.

The *in vivo* biocompatibility of BCP is to a large part identical to those of TCP. Within the limitation of the animal model, the implantation study shows that BCP can be used as bone graft substitute, due to the fact that the material integrates into tissue, remains stable in the implantation bed and serves as an osteoconductive scaffold.

1. Introduction

Due to its osteoconductive and osteoinductive properties, autogenous bone still is the gold standard for the reconstruction of bone defects and augmentations. However, the amount of autogenous graft that can be harvested is limited. Furthermore, harvesting requires an additional surgical intervention with the inherent risks of pain and neural damage at the donor site (Cordaro et al., 2008). The use of synthetic bone substitutes is of increasing importance for reconstructive surgery, orthopaedics, dentistry, spinal arthrodesis, oral, and maxillofacial surgery as alternatives to autogenous bone grafts.

Alloplastic calcium phosphates, such as tricalcium phosphate (TCP) and hydroxyapatite (HA), have been widely applied as bone graft substitutes, implant coatings, bone cements, drug delivery systems and tissue engineering scaffolds (Lobo and Livingston, 2010). Due to their biocompatibility, biological safety, predictability, virtually unlimited availability, ease of sterilization and long shelf life, calcium phosphate ceramics are attractive as bone grafts substitutes (Schwartz et al., 1999; Le Nihouannen et al., 2007).

The *in vivo* resorption behaviour of bone graft substitutes is governed by their chemical composition, by their micro- and macrostructure and by host factors. Evidently, full resorption of the bone graft substitute during regeneration of the host bone is a desirable material property. The newly formed bone tissue may remodel, unhindered by integrated ceramic bone graft substitute remnants, to conform to biomechanical stimuli by the musculoskeletal system or dental and orthopaedic implants. However, resorption and new bone formation may not necessarily proceed in parallel, which may lead to incomplete bone regeneration in sites where bone regeneration does not keep pace with material resorption (Yuan et al., 2001; Kuemmerle et al., 2005; Habibovic et al., 2006). In clinical situations, bone regeneration depends on multiple, interdependent factors such as state of health, genetic background, medication, defect anatomy, size and location. All of these factors may as well influence the resorption rate of a bone graft substitute directly and indirectly, thus it is virtually impossible to choose bone graft substitutes with resorption behaviours

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precisely adapted to the individual patient's need. Furthermore, especially in dental applications, augmentations, i.e. creation of new bone beyond the skeletal anatomical envelope, is frequently attempted. These augmented sites are prone to atrophy if they lack mechanical stimulation by titanium fixtures or teeth (Schropp et al., 2003a,b; Caneva et al., 2011). In such situations, where the presence of an osteogenic scaffold is required during prolonged periods of healing and bony consolidation, partially or non-degradable bone graft substitutes are used. Such slow or non-resorbable materials may remain integrated in bone unchanged for decades (Mordenfeld et al., 2010).

Biphasic calcium phosphates (BCP's) are composed of two calcium phosphate phases at a defined ratio. Mostly, they are compounds made of HA and the β-TCP (Daculsi et al., 2003). Although HA and β-TCP have similar chemical compositions, they have different biological resorption capacities. HA is relatively less bioactive and virtually non-resorbable whereas β-TCP is a fully resorbed material (Daculsi et al., 1989). The radiographic remodelling and consolidation process of BCP was found to be different from that of β-TCP. β-TCP resorption with concomitant bone formation was reported to take place within 2 years (van Hemert et al., 2004; Gaasbeek et al., 2005). BCP materials, on the other hand were integrated in bone and lasted longer than 2 years (Xie et al., 2006; Ozalay et al., 2009).

It has been suggested that the resorb ability and bioactivity of BCP ceramics can be controlled by varying their β-TCP/HA ratios (Jensen et al., 2009). The combination of β-TCP with HA may lead to more complex biological and chemical events caused by both β-TCP and HA (Kamitakahara et al., 2008). The osteogenesis initiated by BCP was described as a sequence of the following reactions: dissolution, precipitation, ionic exchange, deposition of minerals, cell attachment, proliferation, differentiation, and extracellular matrix formation (Ducheyne and Qiu, 1999). A rapid bone in-growth in BCP particles has recently been shown in *in vivo* animal and clinical studies (Cong et al., 2000; Bourgeois et al., 2003; Manjubala et al., 2005).

The aim of the present study was to evaluate a new biphasic calcium phosphate bone graft substitute (calc-i-oss^{TM}CRYSTAL, Degradable Solutions AG, Schlieren, Switzerland) with an HA/TCP ratio of 60/40, a particle size of 450–1000 μm, and a total porosity of 70% in a well-documented animal model. The material was compared with a β-TCP bone graft substitute of the same particle size and very similar shape, micro- and macroporosity. The histological evaluation and gene expression analysis aimed to evaluate the *in vivo* biocompatibility, bone formation and graft degradation.

2. Materials and methods

2.1. Bone graft substitutes

The BCP test material (calc-i-oss^{TM}CRYSTAL 450–1000 μm, Degradable Solutions AG, Zurich, Switzerland) is a biphasic compound of 60% hydroxyapatite and 40% β-TCP, with each granule being composed of the two calcium phosphates. The round granules are microporous with a total porosity of about 70%. Sintering process and granule shape were developed for an optimal mechanic stability of the granules with minimal microparticle formation during transport and material application. The control material is composed of phase-pure β-TCP (calc-i-oss^{TM}CLASSIC 500–1000 μm, Degradable Solutions AG, Zurich, Switzerland). Both test and control materials are synthetic. Granule shape, size and porosity are virtually identical between the BCP and β-TCP granules.

2.2. Experimental design and surgical procedure

Twenty-four adult Lewis 1A rats (2-month old, body weight between 250 g and 350 g and of both sexes) were divided into two groups. All surgical and experimental procedures were approved by the Animal Welfare Committee on the State Government (LALLF M-V/TSD/7221.3-1.2-040/11). For surgery, each rat was anesthetized with intraperitoneal injection of Ketamine (10%; CEVA Tiergesundheit, Düsseldorf, Germany) and Rompun (2%; Bayer HealthCare, Leverkusen, Germany) at a ratio of 3:2 and an approximate dosage of 0.1 ml/100 g body weight. A midline skin incision was performed on the skull. Bone defects with a diameter of 5 mm size (using a pre-designed template) were created in each parietal region of the cranium with a trephine under constant irrigation. In the control group ($n = 12$) β-TCP was inserted into the defect. In the test group ($n = 12$), the bone defects were filled with the BCP material (Fig. 1). The animals were sacrificed at 4 weeks. The skulls were harvested and processed for gene expression analysis (6 per group) and histological examination (6 per group). For molecular-biological examination, dissected skulls were shock-frozen in liquid nitrogen. For histological examination, skulls were placed in 4% PBS-buffered formalin, dehydrated in a graded series of alcohol, and either embedded in methylmethacrylate (Technovit 9100 neu, Kulzer, Germany) as described previously (Allegrini et al., 2008; Gedrange et al., 2008; Kunert-Keil et al., 2009; Mai et al., 2009) or in paraffin after decalcification.

2.3. TaqMan RT-PCR

Homogenization of bone samples (in each case $n = 6$) was performed in QIAzol lysis reagent (RNeasy Lipid Tissue Mini Kit, Qiagen, Hilden, Germany) using a mortar and pestle. Total RNA was isolated using guanidinium-isothiocyanate (RNeasy Lipid Tissue Mini Kit, Qiagen, Hilden, Germany), RNA concentration was determined by UV absorbance measurements and an amount of 200 ng total RNA was reverse transcribed (TaqMan-reverse transcription kit, PE Applied Biosystems, Weiterstadt, Germany). To quantify the expression of different rat genes, gene-specific TaqMan PCR primers and probes were purchased from PE Applied Biosystems as described previously (Gredes et al., 2012a,b,c). The method was performed using a real-time PCR cycler (StepOne Plus, Applied Biosystems). The gene-specific transcript level was calculated using the standard curve method and is given in relation to those of the 18S cDNA. A "no-template control" with water was performed in parallel to all experiments. Each series of experiments was performed in duplicate.

2.4. Histology

For staining, the methylmethacrylate embedded bone specimens (in each case $n = 3$) were cut using a diamond saw and successively ground to a thickness of approximately 100 μm with a grinding system (Exakt Apparatebau, Norderstedt, Germany) as described by Donath and Breuner (1982). The specimens were then stained with Masson-Goldner trichrome for differentiation between collagen and bone tissue. The Masson-Goldner trichrome stained samples were used for histomorphometry. From paraffin-embedded bone specimens (in each case $n = 3$) serial longitudinal sections of about 5 μm were stained with haematoxylin/eosin (H.E.) and Masson Goldner trichrome for recognizing various tissue types. Additionally, in order to identify osteoclast-like cells, selected tissue sections were stained for tartrate-resistant acid phosphatase (TRAP) as described by Gerber et al. (2006) and Canullo et al. (2013).

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