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Development and evaluation of tetrapod-shaped granular artificial bones

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ABSTRACT

We have developed a novel form of granular artificial bone "Tetrabones" with a homogeneous tetrapod shape and uniform size. Tetrabones are four armed structures that accumulate to form the intergranular pores that allow invasion of cells and blood vessels. In this study we evaluated the physicochemical characteristics of Tetrabones in vitro, and compared their biological and biomechanical properties in vivo to those of conventional β-tricalcium phosphate (β-TCP) granule artificial bone. Both the rupture strength and elastic modulus of Tetrabone particles were higher than those of β-TCP granules in vitro. The connectivity of intergranular pores 100, 300, and 400 μm in size were higher in Tetrabones than in the β-TCP granules. Tetrabones showed similar osteoconductivity and biomechanical stiffness to β-TCP at 2 months after implantation in an in vivo study of canine bone defects. These results suggest that Tetrabones may be a good bone graft material in bone reconstruction.

1. Introduction

Trauma, disease, and developmental abnormalities resulting in skeletal defects often incur considerable morbidity. Although the use of autogenous bone, as blocks or in granular form, has long been considered the gold standard in terms of grafting material, this approach has several disadvantages, including donor site morbidity and the restricted quantity and shape of the tissue [1,2]. For these reasons calcium phosphate-based artificial bone materials such as hydroxyapatite and tricalcium phosphate (TCP) have been widely used in clinical practice [3–6]. These materials are used in various forms, including blocks, pastes, and granules, depending on the indication and type of bone defect.

The ideal granular artificial bone should be biocompatible and biodegradable, and exhibit controlled porosity, good pore interconnectivity, and biomechanical strength [7]. However, it has not yet been established which type of granular calcium phosphate-based artificial bone materials possess the best osteoconductive potential and biomechanical properties [8]. One problem with conventional granular calcium phosphate-based artificial bones is that they have irregular shapes and sizes, which may compromise their performance. To circumvent this problem we have designed and fabricated a novel granular artificial bone taking advantage of its tetrapod shape.

In the field of civil engineering tetrapods are used to protect harbors against the force of the ocean and the consequent erosion, capitalizing on their high mechanical strength, low center of gravity, and stability to external forces [9]. These advantages led us to hypothesize that tetrapods could be scaled down for application as artificial bone. We expected that their structural characteristics would provide better mechanical stability and control over intergranular pores.

In this study we fabricated novel tetrapod shaped granular artificial bone (hereafter referred to as "Tetrabones") by injection molding using microparticles of α-tricalcium phosphate (α-TCP). We first studied the physicochemical characteristics of Tetrabones in vitro, and then evaluated its biological and biomechanical properties in a canine model *in vivo* in comparison with β-TCP granules, which are widely used in clinical practice.

2. Materials and methods

2.1. Fabrication of Tetrabones

2.1.1. Materials

A mix of 60/40 wt.% α-TCP powder (Taihei Chemical Industrial Co., Tokyo, Japan) and binder (composed of 55% olefin resin, 30%

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wax, and 15% plasticizing materials) were compounded in a tumbler mixer (Neo TecsK03P), and then mixed at room temperature in a rotating drum tumbler mixer for 5 h.

2.1.2. Fabrication of Tetrabones

Injection molds were prepared to fabricate 1 mm sized tetrapods, and the α-TCP powders were molded using an injection molding machine (J34AD, Japan Steel Works, Tokyo, Japan), Molded products were then degreased and calcined. The detailed parameters for the injection molding, degreasing, and calcination processes are given in Table 1.

The degreased and calcined products were soaked in 0.2 M succinic acid for 24 h to form octacalcium phosphate (OCP), rinsed twice with distilled water, dried under reduced pressure, and sterilized by electron beam irradiation at 25 kGy to give the final Tetrabone product.

β-TCP granules (Osferion®, size range 0.5–1.5 mm, porosity 75%; Olympus Biomaterial Corp., Tokyo, Japan) were used as the control material.

2.2. Material properties of Tetrabones

2.2.1. X-ray diffraction analysis

X-ray diffraction analysis (XRD) was performed using an X-ray diffractometer (Mini Flex 2, Rigaku, Japan) equipped with a CuK_α radiation source at 20 mA, scanning from $2\theta = 4$ to 60 °C. All samples were crushed before analysis. The results were compared with the International Center for Diffraction Data (ICDD) database.

2.2.2. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on pure α-TCP powders and the surface of injectionmolded products before and after succinic acid treatment, using a JCM-5700 scanning electron microscope (JEOL, Tokyo, Japan). Images were obtained at 1.2 keV accelerating voltage and 20 mA current.

2.2.3. Mechanical testing

The rupture strength of single Tetrabones and single β-TCP granules were measured with a rheometer (CR-500DX, Sun scientific Co., Japan). A single particle of each artificial bone was placed on the slab of the rheometer. The rod was loaded at 3 mm min^{-1} until the particle ruptured, and the rupture strength when each specimen broke was measured ($n = 4$).

For elastic modulus evaluation Tetrabones and β-TCP granules were embedded in cylindrical molds 5 mm in diameter and 10 mm long, and each mold was placed on the slab of an Instron universal testing machine (Instron-3365, Instron Corp., Norwood, MA). Arod 5 mm in diameter was loaded into the mold at 0.5 mm min^{-1}, and the elastic modulus measured ($n = 4$).

2.2.4. Analysis of size and connectivity of intergranular pores

Polymer beads used to simulate cells and blood vessels were provided by the Sekisui Plastics Corporation (Osaka, Japan). The beads were composed of cross-linked polymethyl methacrylate and had diameters of 100, 300, 400, and 600 μm.

The end of a 2.5 ml syringe barrel was cut to create a cylindrical tube and sealed with mesh. The plunger was pulled back and the rubber cap removed. The barrel was filled with 0.5 ml of Tetrabones or β-TCP granules, overlaid with 1.5 ml of the beads, and the plunger pushed back into the barrel. The end of the plunger was loaded with a 500 g weight and the syringe was vibrated using a vibrator for 2 min. The beads were collected as they exited the syringe and their weight measured ($n = 3$, Fig. 1). Additionally, mercury porosimetry was performed using a Micromeritics Autopore III 9510 mercury porosimeter (Micromeritics Instrument Corp., Norcross, GA) to compare the values of these methods.

Table 1
Detailed parameters of the Tetrabone fabrication process.

Injection molding	Degreasing	Calcination
Cylinder temperature 170–190 °C	Maximum temperature 500 °C	Maximum temperature 700 °C
Mold temperature 25–40 °C	Rate of temperature rise 87°Ch^{-1}	Rate of temperature rise 87°Ch^{-1}
Injection pressure 30–50 MPa	Holding time 1 h	Holding time 1 h
Injection velocity 0.3–0.5 s		
Screw revolution speed 1000 r.p.m.		

2.2.5. Cell viability

MC3T3-E1 cells were cultured on Tetrabones or β-TCP granules in standard medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 vol.% fetal bovine serum (FBS), 50 U ml^{-1} penicillin, and 50 mg ml^{-1} streptomycin) at 37 °C in a 5% CO_2 atmosphere. When the cells reached confluence the medium was changed to osteogenic medium (standard medium supplemented with 0.1 μM dexamethasone, 50 mM β-glycerophosphate and 50 μg ml^{-1} ascorbic acid). 7 days later the Tetrabones and β-TCP granules were stained with an alkaline phosphatasestaining kit (Takara Bio, Tokyo, Japan). The numbers of cells growing on the surface of the Tetrabones and β-TCP granules werecounted using a stereoscopic microscope.

2.3. In vivo experiments

2.3.1. Canine bone defect model

Seven healthy beagle dogs (10–12 kg body weight, 1–2 years of age) were purchased from Nosan Corporation (Kanagawa, Japan). General anesthesia was maintained with isoflurane, and fentanyl hydrate was continuously administered during and after surgery. The femoral medial condyle was exposed, and a tunnel defect 10 mm in diameter extending to the lateral cortex was created in the bilateral femur using a power surgery drill (IMEX™ Veterinary Inc., Longview, TX). After irrigation of the defect with sterile saline, Tetrabonesor β-TCP granules were implanted into the defect ($n = 5$ each), or, in the control group, nothing was implanted ($n = 4$). After implantation the joint capsule, fascia lata, subcutaneous tissue, and skin were sutured. An antibiotic (cefazolin, 20 mg kg^{-1} subcutaneously twice daily) and an analgesic (buprenorpine, 15 μg kg^{-1} intramuscularly twice daily) were administered for 3 days after implantation. This study was conducted under the Guidelines of the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo.

2.3.2. Biomechanical analysis

8 weeks after implantation the distal part of the femur was excised, and the surrounding tissue removed. The normal femur was used as the positive control ($n = 5$). The specimes was fixed with bone cement with the longitudinal axis of the bone defect vertical to the slab of a rheometer (CR-500DX, Sun Scientific Co., Japan), and a rod 5 mm in diameter was preloaded on the surface of the defect site at 1 N force. The specimen was loaded at 3 mm min^{-1}, and stopped when displacement reached a depth of 0.25 mm to avoid destruction of the specimen. Force–displacement changes in the bone defect were observed and the stiffness calculated from the slope of the linear region of the resulting force–displacement curve.

2.3.3. Histological analysis

After the biomechanical analysis the bone around the implant sites was trimmed and fixed with 10% neutralized formaldehyde