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Structure, properties and animal study of a calcium phosphate/calcium sulfate composite cement

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ABSTRACT

In-vitro and in-vivo studies have been conducted on an in-house-developed tetracalcium phosphate (TTCP)/dicalcium phosphate anhydrous (DCPA)/calcium sulfate hemihydrate (CSH)-derived composite cement. Unlike most commercial calcium-based cement pastes, the investigated cement paste can be directly injected into water and harden without dispersion. The viability value of cells incubated with a conditioned medium of cement extraction is >90% that of Al_2O_3 control and >80% that of blank medium. Histological examination reveals excellent bonding between host bone and cement without interposition of fibrous tissues. At 12 weeks-post implantation, significant remodeling activities are found and a new bone network is developed within the femoral defect. The 26-week samples show that the newly formed bone becomes more mature, while the interface between residual cement and the new bone appears less identifiable. Image analysis indicates that the resorption rate of the present cement is much higher than that of TTCP or TTCP/DCPA-derived cement under similar implantation conditions.

1. Introduction

Bioresorbable bioceramic has become one of the most promising bone substitute materials today. Calcium phosphate and calcium sulfate are two typical bioresorbable materials of such category [1–6]. It is known that when the resorption rate of a bioresorbable implant material is adjusted to be similar to the growth rate of natural bone, the implanted material can be gradually replaced by a new bone [7]. Furthermore, when appropriate kinds and amounts of calcium-based powder and setting solution are mixed, an injectable calcium-based cement paste may be formed which can be used in bonding, filling and repairing damaged natural bone for orthopedic, dental, maxillofacial and other applications via minimally invasive procedures [8–10].

Despite their many advantages, such as being highly biocompatible, osteoconductive, non-exothermic and X-ray detectable, most currently-used calcium phosphate and calcium sulfate bone substitute materials have their respective disadvantages. For example, calcium phosphates demonstrate bioresorption rates which are often clinically too low [11,12], leading to a long time (even longer than a year [13,14]) for the resorption process to be completed. On the other hand, calcium sulfates often show dissolution rates which are too high to allow new bone to grow into bone cavities in the most effective way ("one-to-one" resorption) [6,7]. It seems logical to combine calcium phosphate and calcium sulfate into a composite formula and, if appropriately processed, expect to see the inherent benefits from each component of the composite. One major advantage for making such a composite cement implant may be its easy adjustment in resorption rate by adjusting the weight or volume ratio of the two components. An adjustable resorption rate is desirable considering the fact that the resorption rate of a calcium-based implant is usually related to many material factors, such as chemical composition, phase content, degree of crystallization, and porosity, and that different resorption rates are often recommended for different applications/implantation sites [15–17].

Following this philosophy, a series of tetracalcium phosphate (TTCP)/dicalcium phosphate anhydrous (DCPA)/calcium sulfate hemihydrate (CSH)-derived composite cements have recently been developed in the present authors' laboratory [18]. Preliminary tests indicated that one of the most promising candidates of this series of calcium-based composite cements is comprised of a TTCP/DCPA/CSH mixed powder with a weight ratio of 2.69:1:4.51. Reported in the present study are experimental results of this particular calcium-based cement, including crystal structure/phases, morphology, some

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physical and mechanical properties, along with cytotoxicity and rabbit implantation study.

2. Materials and methods

2.1. Cement preparation and working time/setting time measurement

The TTCP powder used for the study was fabricated in-house using a method suggested by Brown and Epstein [19]. To prepare the composite cement for the study, this TTCP powder, a commercial DCPA powder and a commercial CSH powder were uniformly mixed with a weight ratio of 2.69:1:4.51. The composite powder was then mixed uniformly with a 0.6 M $(NH_4)_2HPO_4$ setting solution at a liquid/powder (L/P) ratio of 0.38 ml/g to form a cement paste.

Working time of the cement paste was determined by the time after that the cement paste was no longer workable, while the setting time was measured according to the standard method set forth in ISO 1566 for dental zinc phosphate cements. The cement was considered set when a 400 g weight loaded onto a Vicat needle with a 1 mm dia. tip failed to make a perceptible circular indentation on the surface of the cement. In the course of the test, the cement was kept in a 50–70% relative humidity environment at 24 °C. Each average working time or setting time value was obtained from 4 measurements taken under the same testing conditions.

To evaluate the cement dispersion behavior in water, the cement paste after being mixed for 1 min was quickly loaded into a 5 ml syringe with needle removed and directly injected into 37 °C deionized water. In so doing, whether or when the cement was dispersed in water could be easily determined by the naked eye.

2.2. pH measurement

The pH values of the Hanks' solution [20], a widely used SBF [35,36], was used wherein hardened composite cement samples were immersed for 1, 3, 7, 14, 28 and 42 days, were measured using a pH meter (Suntex Instruments SP-2300, Taipei, Taiwan). After the powder and setting solution were mixed for 5 min, 2 g of cement was taken and immersed in 20 ml of daily-refreshed Hanks' solution with a pH value of 7.4. Each average pH value was obtained from 4 measurements taken under the same testing conditions.

2.3. X-ray diffraction and microstructural examination

To study crystal structure/phases of the hardened cement immersed in Hanks' solution for the designated series of time, X-ray diffraction (XRD) was conducted using a Rigaku D-MAX B X-ray diffractometer (Tokyo, Japan) with Ni-filtered CuKα radiation operated at 30 kV and 20 mA at a scanning speed of 1°/min. The various phases of the samples being analyzed were identified by matching their characteristic peaks with data compiled in the JCPDS files. The compression-fractured surface morphology of the hardened cement was examined using a FEI Quanta 400 F environmental scanning electron microscope (ESEM) operated at 5 kV in secondary electron mode. Energy dispersive spectroscopy (EDS) point analysis was conducted on selected samples using an EDS system (INCA x-act, Oxford instrument, UK) operated at 10 kV with a spot size of 1 μm. To eliminate the charging effect, the surface for examination was coated with a thin layer of gold using an ion sputtering system (JFC-1100, JEOL, Japan) to facilitate electric conduction of the sample.

2.4. Porosity, weight loss and compressive strength measurement

Porosity values of the hardened cement immersed in Hanks' solution for different periods of time were determined according to the ASTM C830-00 (2006) method. The weight loss values were measured using an electronic balance with an accuracy of 0.001 g. Samples for the weight loss measurement were dried in anhydrous ethanol at 50 °C for 1 day after being removed from the Hanks' solution. The weight loss ratios were obtained from the equation,

$$\text{Weight loss ratio}(\%) = (W_0 - W_t)/W_0 \times 100,$$

where W_0 is the weight of the cement before immersion and W_t is the cement weight after immersion. Each average porosity or weight loss value was obtained from 6 measurements taken under the same testing conditions.

The compressive strength (CS) of the hardened cement was determined according to the ASTM 451-99a method using a desk-top mechanical tester (Shimadzu AG-10KNX, Tokyo, Japan) at a crosshead speed of 1.0 mm/min. To conduct the test, the cement paste after being mixed for 1 min was packed into a 6 mm diameter, 12 mm deep (ASTM 451-99a) cylindrical stainless steel mold under a pressure of 1.4 MPa for 30 min. After being removed from the mold, the hardened cement was immersed in Hanks' solution at 37 °C for various periods of time. After immersion, the samples were removed from the solution for CS measurement while they were still wet. Each average CS value was obtained from 6 measurements taken under the same testing conditions.

2.5. Cytotoxicity test

The cytotoxicity test was performed according to ISO 10993-5 methods, wherein an extraction method was used for this study. NIH/3 T3 fibroblasts, which are frequently used for the cytotoxicity test of orthopedic implant materials [39,40] with a seeding density of 5000 per well were pre-cultured for 24 h in Dulbecco's modified essential medium (DMEM) supplemented with bovine serum (10%) and PSF (1%). The extract was prepared by immersing hardened cement in the culture medium at a ratio of 0.2 g/ml at 37 °C for 24 h, followed by the collection of the liquid by centrifugation. Preparation of the extracts of negative control (Al_2O_3 powder) and positive control (0.3% phenol solution) followed the same extraction procedure. The extracts were added into a 96 well microplate (100 μl per well) incubated in a 5% CO_2 humidified atmosphere at 37 °C. After 24 h, the extract was removed from the microplate and a mixture of the culture medium (100 μl) and WST-1 (10 μl) was added to the wells and incubated for 1 h at 37 °C. Cell viability was measured using the WST-1 assay, which is a colorimetric assay of mitochondrial dehydrogenase activity where the absorbance at 450 nm is proportional to the amount of dehydrogenase activity in the cell. After 1 h incubation, the mixture of medium and WST-1 was transferred to a 96 well microplate and the absorbance at 450 nm was measured using an ELISA reader. Four bars were tested for each sample ($n = 4$).

2.6. Animal implantation and histological examination

Animal study was performed at National Cheng-Kung University Medical College Animal Center, Tainan, Taiwan. Adult (weighing 2.8–3.5 kg), healthy, male New Zealand white rabbits were used as the experimental animals. The rabbits were housed individually in stainless steel cages with free access to food and water. An acclimation period of a minimum of 7 days was allowed between receipt of the animals and the start of the study. Injection sites were shaved and cleansed with 70% ethanol and Betadine™ (povidone iodine 10%). All animals were operated under general anesthesia. Zoletil 50 (0.05 ml/100 g, Virbac, France) was used as general anesthesia, while xylocaine (AstraZeneca, England) was used as local anesthesia. To implant cement paste in the medial condyle of femur, a longitudinal incision was made on the anterior surface of the femur. The inner side of the knee joint was cut to expose the femur. After exposure of the femur, the periosteum was reflected and a 2 mm pilot hole was drilled. The hole was sequentially widened with drills of increasing size until a final diameter of 5 mm was reached.