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Reconstruction of calvarial defect using a tricalcium phosphate-oligomeric proanthocyanidins cross-linked gelatin composite

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ABSTRACT

A biodegradable GTP composite which was composed of oligomeric proanthocyanidins (OPCs) cross-linked gelatin mixed with tricalcium phosphate was developed as a bone substitute. The subcutaneous implantation in rats was examined to determine the *in vivo* degradation and biocompatibility of the GTP composites with various cross-linking densities. Experimental results indicated that the rate of *in vivo* degradation was markedly attenuated as the concentration of OPCs increased above 5.0 wt%. Furthermore, this study examined the biological response of rabbit calvarial bone to GTP composite to evaluate its potential for use as an osteoconductive bone substitute. Bone defects (10 mm in diameter) in New Zealand white rabbits were filled with the GTP composite. The de-protenized bovine cancellous bone matrix was employed as the control material. The results of radiographic analyses demonstrated obviously greater new bone ingrowth in the GTP composite than in the de-proteinized bovine bone at the same implantation time. Progressive replacement of the GTP composite by new bone proceeded by a combination of osteoconduction and biodegradation. The biodegradable GTP composite thus has great potential for improving bone repair.

1. Introduction

An ideal substitute material to be used in bone defects must be able to be reabsorbed or dissolved fairly naturally as bone growth proceeds, yielding a newly remodeled bone. Granular tricalcium phosphate ($Ca_3(PO_4)_2$), a synthetic bone-promoting biomaterial, has been extensively applied and investigated as a biodegradable bone replacement to repair bone defects with various shapes and sizes, which are caused by trauma, tumor resection or skeletal abnormalities [1]. However, tricalcium phosphate is difficult to maintain in the defect site and lacks the structural stability. To solve these problems, the tricalcium phosphate must be mixed with an adequate binder. Recently, a wide range of degradable polymers has been developed for use as binders, including natural polymers, gelatin and collagen, or synthetic polymers, poly(lactic) acid and poly(lactide-*co*-glycolide), to prevent migration of granules from the reconstructed site [2,3]. Gelatin is partially hydrolyzed collagen and has numerous characteristics that make it appealing for a wide variety of biomedical applications, such as wound dressing, bioadhesive, sealant for vascular prostheses and in drug delivery [4,5].

Since gelatin is easily resorbable *in vivo*, synthetic cross-linking agents are added to prolong the absorption of the gelatin in the living tissue and improve the mechanical properties of the composites [4]. However, most of synthetic cross-linking reagents, such as formaldehyde, glutaraldehyde, polyepoxy compounds, tannic acid, dimethylsuberimidate, carbodiimides and acyl azide, are highly cytotoxic [6].

Our previous study developed a novel biodegradable composite that comprised genipin, a natural cross-linking reagent extracted from the fruits of *Gardenia jasminoides Ellis*, gelatin and tricalcium phosphate for filling bone defects. The cross-linking reaction in the composite was complete when 0.5 wt% genipin had been added [7,8]. However, the concentration of genipin should be <0.5 wt% to

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prevent cytotoxicity [9]. Low-toxicity cross-linking agents are thus required to enable implants to form stable and biocompatible cross-linked products without cytotoxicity. Recently, oligomeric proanthocyanidins (OPCs), naturally occurring plant metabolites, have been used to fix biologic tissues and biomaterials [10–13]. The results demonstrated that OPCs can stabilize biomaterials effectively without cytotoxicity.

The authors' recent study therefore developed a novel biodegradable composite that comprised OPCs cross-linked gelatin mixed with tricalcium phosphate (GTP) for use as a bone substitute [14]. The cross-linking reaction between gelatin and OPCs was more complete when the concentration of OPCs was 5.0 wt%. Cytotoxicity evaluation indicated that OPCs, gelatin and calcium ion were gradually released from the GTP composite promoted the proliferation of MG-63 cells. Additionally, a preliminary *in vitro* MG-63 cells co-culture experiment revealed good adhesion and spreading of MG-63 cells on the surface of the GTP composite. In this study, the degradation and biocompatibility of the GTP composites with various concentrations of OPCs were further investigated *in vivo* by subcutaneous implantation in rats. Moreover, the biological responses of rabbit calvarial bone after various implanted intervals were observed to evaluate the tissue compatibility, biodegradation and the osteoconductive potential of the GTP composite as a bone defect filler. The evaluation involves serial post-operative gross examinations and radiographic analysis.

2. Materials and methods

2.1. Preparation of implant materials

The GTP composite was prepared as described elsewhere [14]. Briefly, a homogenous 18% gelatin solution was prepared by dissolving gelatin powder (Bloom number 300, Sigma) in distilled water in a water bath at 70 °C. β-Tricalcium phosphate ceramic particles (Merck) with grain sizes of 200–300 μm were mixed with the gelatin solution at 70 °C. The ratio of the weight of tricalcium phosphate to that of gelatin was 1:1. While the gelatin/tricalcium phosphate mixture cooled to 45 °C, OPCs solutions (Compson Trading Co., Taichung, Taiwan) with various concentrations (1.0, 2.5, 5.0, 7.5 and 10.0 wt%) were added to induce a cross-linking reaction. After stirring the solution for 20 min, the gelatin/tricalcium phosphate/OPCs mixtures were transferred to plastic dishes to solidify in a refrigerator at 4 °C for 24 h. The solidified mixtures were then frozen at −80 °C for 24 h and freeze-dried for a further 24 h to yield GTP composites. Before they were used in experiments, all samples were sterilized using a 10 kGy dose of ^{60}Co gamma ray irradiation and then soaked in physiological saline at room temperature.

2.2. In vivo evaluation of the subcutaneous implantation in rats

2.2.1. Implantation procedure

The *in vivo* degradability and biocompatibility of different GTP composites as subcutaneous implants in rat were examined. Sixteen adult male Sprague-Dawley rats, purchased from the National Laboratory Animal Center, Taiwan, weighing 300–350 g were used as experimental animals. All animals were housed in a manner consistent with national animal care guidelines. Before the beginning of the study, the ethical committee for animal experiments at the Central Taiwan University of Science and Technology, Taichung, Taiwan, approved the protocols. Rats were anaesthetized intramuscularly with Zoletil 50 (Virbac, France) and 2% Rompun solution (Bayer, Germany) (1:2 ratio, 1 ml/kg). The dorsal surface of each animal was shaved, sterilized with 10% povidone-iodine solution (Chou Jen Pharmaceutical Co., Nantou, Taiwan), and prepared for surgery, which was performed in an aseptic animal operation room. To insert of the implants, incisions (10 mm in length) were made and the GTP composites were implanted subcutaneously on both sides of the rats. Each side consisted of random 1.0, 2.5, 5.0, 7.5 and 10.0 wt% GTP composites. Each rat received ten subcutaneous implants, which were removed upon sacrifice at various time points: 1, 2, 4 and 8 weeks. At each implantation time, four rats were operated on. The implants were removed for *in vivo* degradation studies from one side and the tissue-covered implants were prepared for histological evaluation from the other side.

2.2.2. In vivo degradation studies

For *in vivo* degradation studies, the final dry weights of the GTP composites were compared with original sample weights and the percentage original weight remaining was calculated. To prevent any inaccuracies in the final weight of *in vivo* implant, adhered tissue was removed by scraping. All the samples were then frozen, dried (using the same aforementioned procedures) and weighed. The degradation rate for each sample was determined using the relationship between its weight loss percentage and the implantation time.

2.2.3. Histological observations

Following retrieval, the implants with surrounding tissue were immediately immersion-fixed in phosphate-buffered 10% formalin (Merck, Whitehouse Station, NJ). They were then washed with phosphate buffer solution and dehydrated using a graded series of ethanol solutions. The dehydrated samples were embedded in paraffin wax (Merck, Whitehouse Station, NJ), thin-sectioned (12 μm) and stained with hematoxylin-eosin (H&E) reagent (Sigma, St. Louis, MO) for histological observations. The reactions of the tissue to the test samples were evaluated on the basis of the uniformity and thickness of the foreign body capsule under an optical microscope (Olympus IX70, Japan).

2.3. Biological response of rabbit calvarial bone

2.3.1. Control material

The control material was de-protenized bovine cancellous bone matrix [15]. The bone matrix was derived from femoral condyle bovine cancellous bone. To obtain a completely de-proteinized and defatted bone matrix, the cancellous bone laminates were maintained in boiling water for 12 h and then dehydrated in a series of ascending alcohol. The laminates were finally sintered at 1200 °C for 1 h in a computer-programmed SiC heating element furnace. All the test samples were manually cut and shaped to a diameter of 10 mm.

2.3.2. Surgical procedures

Twelve mature New Zealand white rabbits, purchased from the National Laboratory Animal Center, Taiwan, weighing 3.0–3.5 kg were used in experimental cranial implantation. All animals were anaesthetized using intramuscular injections of a combination of Zoletil 50 and 2% Rompun solution (1:2 ratio, 1 ml/kg). The head of each rabbit was shaved, disinfected with 10% povidone-iodine solution and prepared for surgery that was conducted in an aseptic animal operation room. The cranial surface was exposed by making a midline incision, and the overlying parietal periosteum was then excised. A full-thickness circular defect of the parietal bone with a diameter of 10 mm was created using a drilling burr on a slow-speed dental handpiece that had been supplemented with 0.9% physiological saline without violating the dural and superior sagittal sinus. Two calvarial bone defects were produced in each rabbit. One defect was filled with the sterile de-proteinized bovine bone (control group) and the other was filled with the sterile GTP composite (experimental group) to evaluate their tissue compatibility and osteogenerative characteristics. The testing material was easily molded to the calvarial bone defect and did not require any fixation.

2.3.3. Harvesting and radiomorphometry of tissue

Bone defect repair was radiographically evaluated. Anesthetized animals were sacrificed by administering an overdose of sodium pentobarbital at 4 and 8 weeks post-operatively, respectively. Craniectomy sites with 2–3 mm of contiguous bone were removed from each skull. Specimens were fixed in phosphate-buffered 10% formalin for 24 h. They were then radiographed using an X-ray apparatus (MGU 100A, TOSHIBA Co.) with a high contrast X-ray film at 22 keV, 10 mA for 40 s. New bone was revealed by the radiographic appearance of a calcified mass.

2.4. Statistical analysis

Numerical data were presented as mean ± standard deviation. Statistical differences among samples were evaluated by one-way analysis of variance (ANOVA) followed by *post hoc* Fisher's LSD multiple comparison test. Statistical significance was considered at a probability $P < 0.05$.

3. Results

3.1. Subcutaneous implantation in rats

3.1.1. Determination of biodegradation rate

The rat subcutaneous implantation model was investigated to determine the biodegradation rate of the GTP composites *in vivo*. Fig. 1 presents weight losses following various periods of implantation of GTP composites with various concentrations of OPCs. The degradation rates of the GTP composites could be divided into two groups. When the gelatin was cross-linked with 1.0 and 2.5 wt% of OPCs, materials were completely degraded after 7 days of implantation. The degradation was significantly reduced when the concentration of OPCs exceeded 5.0 wt%.