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Fabrication and Evaluation of a New Composite Composed of Tricalcium Phosphate, Gelatin and Chi-Li-Saan as a Bone Substitute

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Abstract: The purpose of this study was to prepare and evaluate the feasibility and biocompatibility of a new composite as a bone substitute. The new composite (GTGC) was mainly composed of tricalcium phosphate ceramics and gelatin to which Chi-Li-Saan, a Chinese medicinal remedy was added. The GTGC composite was manually packed into cylindrical Teflon molds, dried overnight in an oven and sterilized by γ-ray prior to use. Mature New Zealand rabbits, weighting 3–3.5 kg, underwent full-thickness excision of the parietal bone. In the experimental group, bone defects of 12 animals were filled with the GTGC composites and another 12 unreconstructed rabbits were considered as controls. Three rabbits were examined for each group in every time period at 2, 4, 8 and 12 weeks after operation. There was no evidence of adverse tissue reaction to the GTGC composite. In addition, examination with light and fluorescent microscopy revealed a significantly greater amount of new bone ingrowth in the GTGC group at the same implantion time as compared with the controls. Therefore, the GTGC composite could serve as a useful substitute when repairing bone defects.

Keywords: Biomedical Composite; Chi-Li-Saan; Gelatin; Tricalcium Phosphate.

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Introduction

Bone defects resulting from trauma, tumor and infection have been conventionally repaired using autologous bone grafts (Burwell, 1994). However, under progressive, uncontrolled resorption during healing, limited donor bone supply and donor site morbidity are all the irrefragable drawbacks in the clinical use of fresh autogenous bone (Heppenstall, 1980; Damien and Parson, 1991; Kocialkowaki *et al*., 1994). Similarly, since rejection phenomena and risk of viral transmission are frequent, transplantation of allografts or xenografts represents only a minor part of reconstructive surgery (Buck and Malinin, 1989; Tomford and Mankin, 1994). To overcome these problems, a variety of artificial materials have been researched and developed as hard tissue substitutes in recent years. But none of the prosthetics presently available are entirely suitable for orthopedic applications till now.

Clinically, bioactive ceramics scaffold for repairing large skeletal defects are usually used in dense and porous form. Several investigators have conformed that bioactive ceramics with porous structure caused by resorption process or manual treatment have good biocompatibility and osteoconductive potentiality (Lin *et al*., 1991a; Holmes *et al*., 1986; Lin *et al*., 1991b; Lin *et al*., 1996). However, these ceramics, which are unable to be molded to fill irregular bone cavity have failed to achieve wide clinical use. Therefore, particulate tricalcium phosphate and hydroxyapatite have been studied as alternatives to bone grafts in the filling of irregular-shaped skeletal defects (Ricci *et al*., 1992). Unfortunately, the usefulness of loose particulate tricalcium phosphate or hydroxyapatite in orthopedic applications is limited by a tendency for particle migration away from the implant site. Attempts have been made to overcome this problem by binding the particulate together using matrices consisting of such materials as fiber, polylactic acid and collagen (TenHuisen *et al*., 1995; Ono *et al*., 1988; Verheyen *et al*., 1993).

For a large bone substitute, an ideal composite should meet the following criteria. First, the composite must be biodegradable, so that it does not act as a barrier to bone remodeling, but rather is replaced by host bone over time. Second, this composite must be highly biocompatible; preferably osteoconduction can be an inherent property of this bone substitute. We have previously described a biodegradable and biocompatible particulate composite bone substitute named the GTG (Lin *et al.*, 1998). It consisted of a tricalcium phosphate particulate phase bound together by a gelatin base matrix phase set by glutaraldehyde mediated cross-linking. In the results of previous biocompatibility and cytotoxicity studies, we found that the concentration of glutaraldehyde solution used as cross-linking agent in the developed composites should be in the range of 2–8%. And it was suggested to be soaked in the distilled water for at least 4 days before clinical applications. The concentration of calcium ion gradually increased the gelatin matrix degraded *in vitro*. The growth of osteoblasts was obviously enhanced after they were co-cultured with the extracts of the composites and soaked for over the required period of time.

Chi-Li-Saan has been reported as an effective Chinese medicine for rapid recovery of damaged bone tissue. A mixture of GTG and Chi-Li-Saan was then used as bone defect filler in this study. However, it is not clear about the pathological effects of the Chi-Li-Saan as it acts directly on the bone cell or tissue. Therefore, the present study was designed to

evaluate the effect of the mixture of Chi-Li-Saan and GTG composite on the repair of calvarial defects in rabbits.

Materials and Methods

Preparation of GTG and GTGC Composites

The tricalcium phosphate ($Ca_3(PO_4)_2$) powder used in this study was supplied by Merck, Germany. It was placed in a platinum crucible and sintered in an SiC-element furnace at 1100°C for 1 hour, then cooled down to the room temperature. The sintered ceramic particles were crushed in the alumina-grinding bowl and sieved in the 30−40 mesh. The sieved TCP ceramic particle of grain size 300−500 μm was obtained for material preparation.

We prepared the matrix phase of the GTGC composite by adding 5 g of bovine gelatin (Sigma Chemical Co., USA) to 50 ml of 0.2 wt% Chi-Li-Saan solution (Table 1). The mixture of this composite was stirred vigorously and kept at 65°C by water bath until a homogenous solution was attained. Then, 15 g of tricalcium phosphate particles was added into the mixture solution. The mixture was stirred for 5 minutes to ensure a uniform consistency. Finally, 4% glutaraldehyde solution was added to the mixture for cross-linking. In order to obtain a more homogeneous and higher cross-linking density of composite, the temperature of the mixture was cooled down to 40°C prior to adding glutaraldehyde solution for the cross-linking reaction of gelatin. After the mixture was completely cross-linked, the composite was molded uniformly using cylindrical plastics molds of 15 mm in diameter. All composites were then soaked in deionized distilled water for at least 4 days to ensure the complete removal of glutaraldehyde remnants.

Table 1. Composition of Chi-Li-Saan

Ingredients	Weight (g)
Resina Draconis	30.0
Moschus	0.4
Borneolum Syntheticum	0.4
Olibanum	5.0
Myrrhae	5.0
Flos Carthami	5.0
Cinnabaris	4.0
Cathechu	7.5

Chi-Li-Saan was provided by Sheng Chun Tang Pharmaceutical Industrial Co., Ltd. (Tainan, Taiwan). Moschus was synthesized (molecular formula $C_{16}H_{30}O$) by CPL Aromas (Far East) Ltd.

Experimental Procedure

GTGC composites were implanted in the defects of calvarial bones in mature New Zealand rabbits to test their osteogenerative properties. All animals were anesthetized with intramuscular injection of the combination of Ketamine hydrochloride and Combelen

(Bayer). The head of each rabbit was shaved and prepared for surgery in an aseptic animal operation room. The cranial surface was exposed by a midline incision and the overlying parietal periosteum was excised. Circular (15×15 mm), full-thickness defect of the parietal bone was created using a drilling burr in a slow-speed dental handpiece supplemented with 0.9% physiological saline without violating the dural and superior sagittal sinus (Fig. 1). GTGC composites were then filled into the defects of 12 rabbits (Fig. 2). Another 12 rabbits were ungrafted to monitor the spontaneous regeneration potential of calvarial bone defects. After operation, the periosteum was closed with 5-0 vicryl and the skin was closed with 4-0 nylon.

Post-operative analgesics and antibiotics were not required. To examine the new bone formation at multiple time periods, each animal was fluorescently labeled with oxytetracycline (30 mg/kg) and calcien green (15 mg/kg).

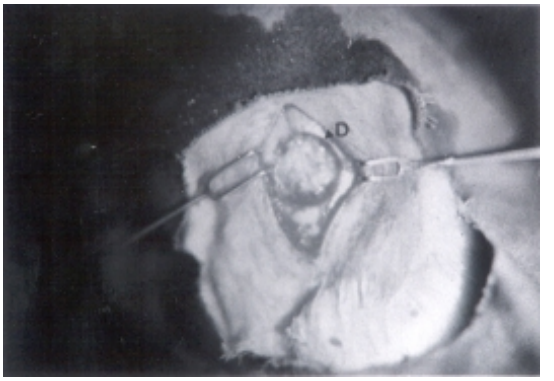

Figure 1. A 15×15 mm, full-thickness, parietal calvarial bone defect. The dural and superior sagittal sinus were not violated. D: Bone defect.

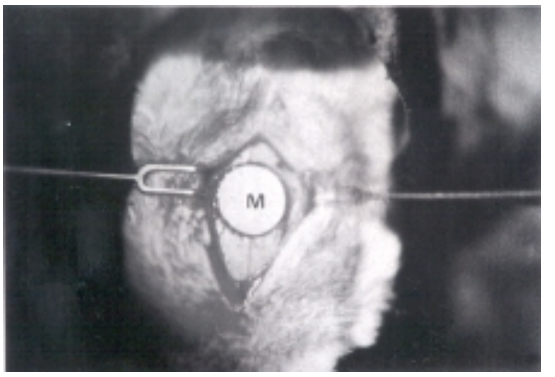

Figure 2. The GTGC composite was easily filled into the defect. No fixation treatment was necessary. M: GTGC.

Tissue Harvest, Radiomorphometry and Histomorphometry

Anesthetized animals were killed by an overdose of sodium pentobarbital at 2 weeks, 1 month, 2 months, and 3 months after operation. Craniectomy sites with 2–3 mm contiguous bone were removed from each skull. Specimens were immediately placed into vials with 70% ethanol and prepared for analysis. After 24 hours of immersion in 70% ethanol, specimens were radiographed in a cabinet X-ray machine (Ohmic OM-603, Tokyo) using the high contrast X-ray film at 28 KVp, 3 mA for 40 seconds. The specimens were then decalcified, embedded in paraffin, cut into 5 μm coronal sections, and stained with hematoxylin and eosin (H&E) for transmitted light microscopy observation.

In the undecalcified treatment, the specimen blocks dehydrated in ascending grades of ethanol, and embedded in polymethylmethacrylate. Once polymerized, sections were cut using a low-speed diamond saw for fluorescent microscopy examination.

Results

Gross Examination

There was no evidence of adverse tissue reaction to the GTGC. On the gross observation of the whole calvarium after 1-month operation, the GTGC composites were intimately incorporated with the surrounding host bone, and there was no wound infection, scalp effusion or hematoma in the implanted site (Fig. 3a). In the same implanted period, sites of the control animals did not reveal any new bone regeneration and the untreated defects were only filled with the fibrous connective tissues (Fig. 3b).

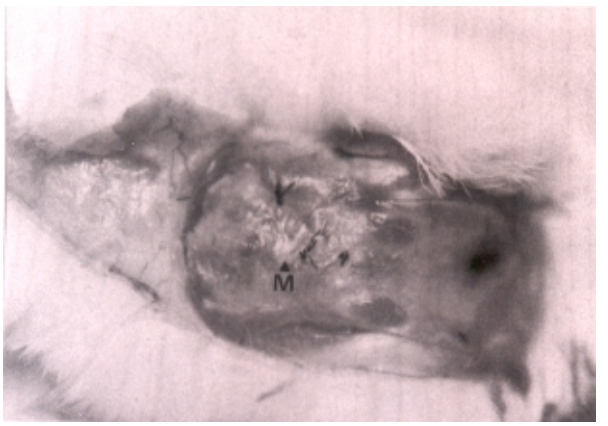

Figure 3a. One month after implantation of composite. GTGC composite was intimately incorporated with the surrounding host bone, and there was no wound infection, scalp effusion or hematoma in the implanted site. M: GTGC.

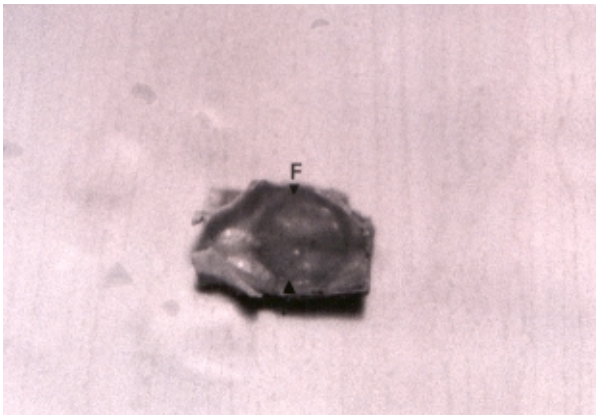

Figure 3b. One month after implantation of composite. Site of the control animal did not reveal any new bone regeneration and the untreated defect was only filled with the fibrous connective tissues. F: Fibrous connective tissue.

Histological Examination

At 1 month, the histological observation of unfilled defects did not significantly demonstrate new bone formation. Only a bridge of fibrous connective tissue was seen across the bone defect with a minimal amount of new bone formation around the edge of the defect (Fig. 4).

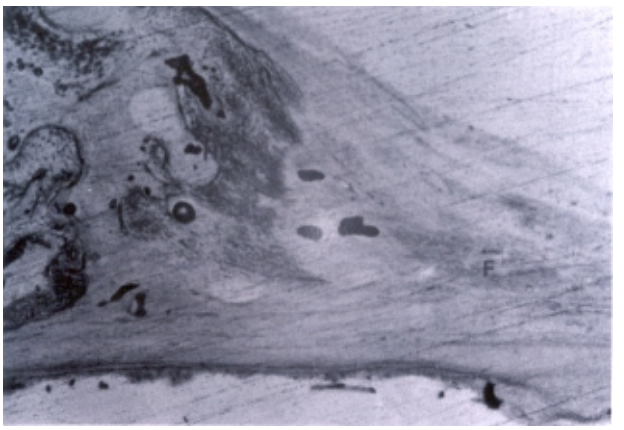

Figure 4. Histological section of unreconstructed defect, 1 month after operation. Only a bridge of fibrous connective tissue was seen across the bone defect (X40). HB: Host bone and F: Fibrous connective tissue.

As compared with the control, examination of the light and fluorescent microscopy revealed a significantly greater amount of new bone in-growth in the GTGC group at the same

implanted time (Fig. 5a). At the same view of histological section, clear fluorescent lines were observed under fluorescent microscopy. These results demonstrated a progressive growth of new bone into the bone defect from the margin toward the center of the bone defect (Fig. 5b).

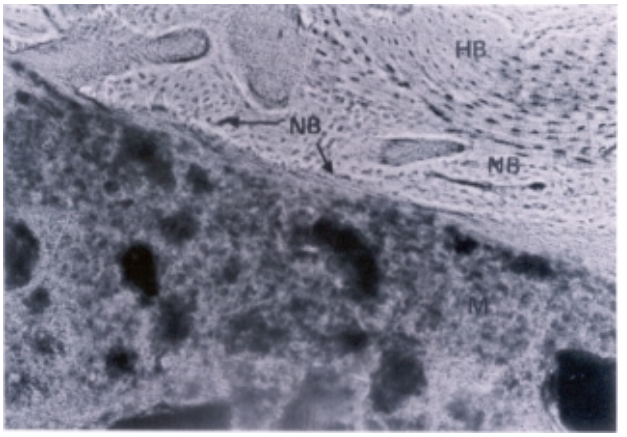

Figure 5a. Histological section of reconstructed defect, 1 month after implantation. Microscopy revealed a significantly greater amount of new bone ingrowth (X100). HB: Host bone, NB: new bone, and M: GTGC.

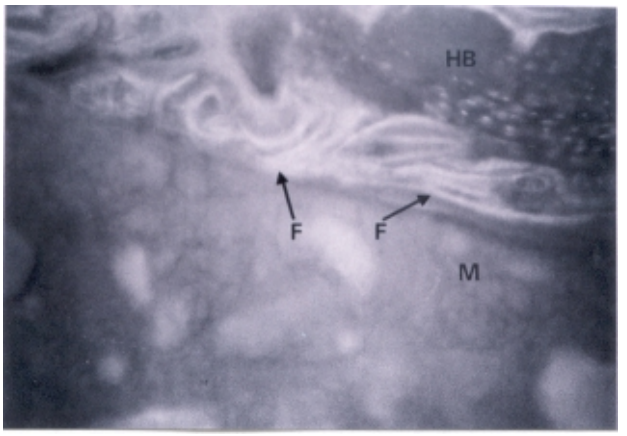

Figure 5b. At the same view of histological section of Fig. 5a, clear fluorescent lines were observed under fluorescent microscopy (X100). These results demonstrated a progressive growth of new bone into the bone defect from the margin toward the center of the bone defect. HB: Host bone and F: Fluorescent lines. M: GTGC.

The examination of the H&E stained sections of the craniectomy sites also revealed that the process of new bone replacement of the GTGC composite began with new bone appearing

near the bone-composite interface (Fig. 6a). The bone regeneration process was more advanced with time, and at three months after implantation, a significant amount of GTGC composite had been replaced by the new bone (Fig. 6b).

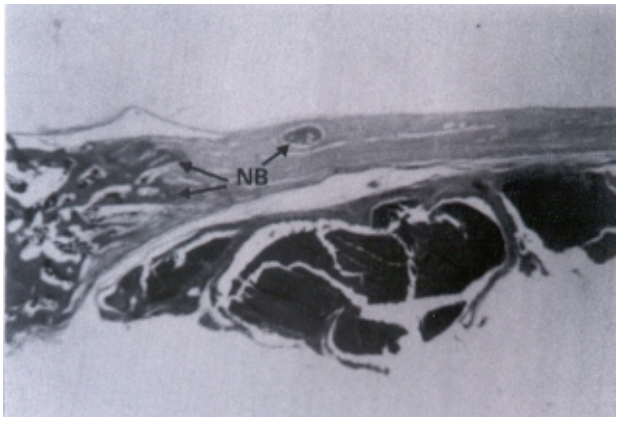

Figure 6a. H&E stained (X10) section of reconstructed defect, 1 month after implantation. New bone replacement of the GTGC composite began with new bone appearing near the bone-composite interface. HB: Host bone, NB: New bone, and M: GTGC.

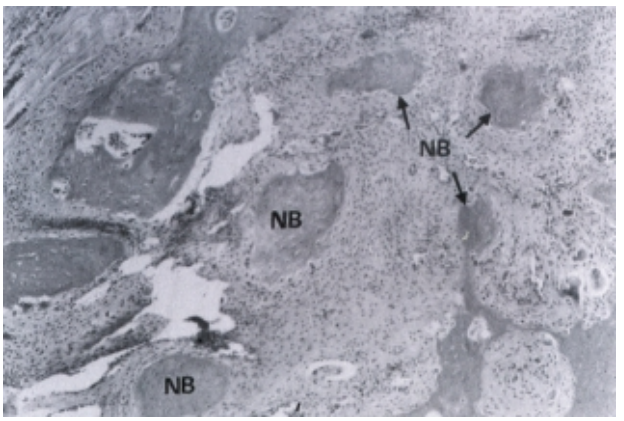

Figure 6b. H&E stained (X40) section of reconstructed defect, 3 months after implantation. Significant amount of GTGC composite was replaced by the new bone. NB: New bone.

Radiographic Analysis

At 3 months postoperatively, radiographs only revealed sparse radiopaque areas within the control implant site (Fig. 7). However, sites treated with the GTGC disks at 1 month displayed a dense pattern of radiopacity, and regenerated bone tissues with irregular edges had

incorporated with the GTGC composites. The GTGC composites were biodegraded with time and the rate of degradation was coupled with new bone generation. Therefore, defects treated with the GTGC showed almost complete radiopacity across the wounds at 1 month post-operative (Fig. 8).

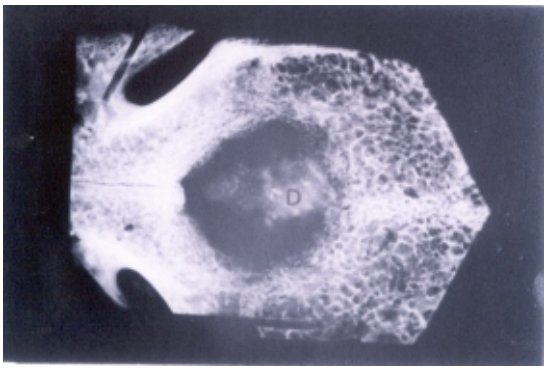

Figure 7. At 3 months postoperatively, radiographs only revealed sparse radiopaque areas within the control implant site. D: Calvarial defect.

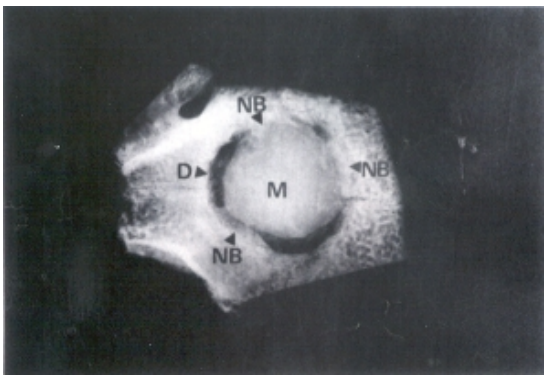

Figure 8. Radiograph of calvarial defect 1 month after implantation of composite. D: Calvarial defect, NB: New bone, and M: GTGC.

At 3 months post-operative, essentially the same pattern of radiographs was found. Defects treated with the GTGC displayed a diffuse pattern. The regenerated bone tissue was laid down from the edge of defect in the centripetal direction, obscuring the original interface of host bone and material (Fig. 9).

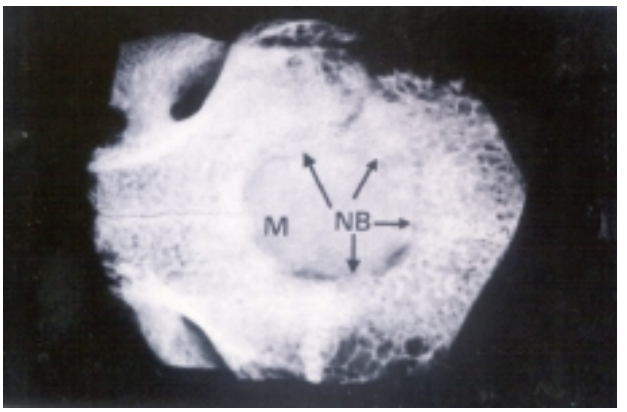

Figure 9. Radiograph of calvarial defect 3 months after implantation of composite. NB: New bone and M: GTGC.

Discussion

The calvarial model described in this study represents an important tool to evaluate the mixture containing the GTG and the Chi-Li-Saan on its bone growth-promoting capability. The cranial site is of interest, because many bone graft substitute materials have been used clinically in craniomaxillofacial applications (Manson *et al*., 1986; Holmes and Hagler, 1988; Harvey *et al*., 1993; Rawling *et al*., 1988). Another important reason of the calvarial model is to prepare a non-union large bone defect. Several researches have showed that the trephine defects larger than 8 mm in the skull can heal spontaneously only by soft tissue invasion and not by bony bridging in the time periods chosen for this study, and thereby act as good delayed-healing models (Frame, 1980; Schmitz and Hollinger, 1975). In order to be suitable for a large bone substitute, GTG which is composed mainly of calcium and phosphate ions, has been used in our previous study (Lin *et al*., 1998). In this previous study, we also found that bone formation around or in the pores of GTG could be resulted from its osteoconductive activity.

To further promote the bone regeneration, Chi-Li-Saan which has long been used in China to treat bone injury, was mixed with the GTG in the present study to fill a large defect in the calvarial bone. The results of this study revealed that the new composite demonstrates good osteoconductive activity. Radiographic and histological evaluations confirm progressive growth of new bone into the calvarium defect in the composite-reconstructed group. In addition, the composite is biodegradable as well as biocompatible since the calcium, phosphate, gelatin and Chi-Li-Saan released from the composite can supply nutrients for the regenerating new bone. Therefore, we believe this composite is a potential material, which could be used to repair large bone defect in clinics.

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