

Osteogenic Evaluation of Glutaraldehyde Crosslinked Gelatin Composite with Fetal Rat Calvarial Culture Model

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Abstract: The cytotoxicity of the synthetic bone substitute composed of tricalcium phosphate and glutaraldehyde crosslinked gelatin (GTG) were evaluated by osteoblast cell culture. In a previous study, the GTG composites were soaked in distilled water for 1, 2, 4, 7, 14, 28, and 42 days, and then the solutions (or extracts) were cocultured with osteoblasts to evaluate the cytotoxicity of GTG composites by alive cell counting. In this study, the extracts were cocultured with the osteoblasts; thereafter, the concentration of transforming growth factor- β (TGF- β_1) and prostaglandin E₂ (PGE₂) in the medium was analyzed to strictly reflect the biological effects of GTG composites on the growth of osteoblasts. In order to investigate the osteoconductive potential of the GTG composites on new bone formation in a relative short term, a model of neonatal rat calvarial organ culture was designed prior to animal experiments. Three experimental materials of 4, 8, and 12% GTG composites were evaluated by fetal rat calvarial organ culture for their ability for bone regeneration. Deproteinized bovine and porcine cancellous bone matrixes were used as the controlled materials. All the organ culture units were maintained in cultured medium for 5 weeks. Following the culture period, the morphology of tissue was observed under an optical microscope, and the quantitative evaluation of the new generation bone was determined by using a semiautomatic histomorphometric method. Except in the initial 4 days, the concentration of

TGF- β_1 of 4% and 8% GTG composites was higher than that of the blank group for all the other experimental time periods. The PGE₂ concentration for 4% and 8% GTG composites was lower than that of the blank group. It revealed that the 4% and 8% GTG composites would not lead to inflammation and would promote osteoblast growth. The morphology and activity of the osteoblasts were not transformed or changed by the 2 GTG composites. For the 12% GTG composite, the performance of the in vitro condition was inferior to the blank group and the other 2 GTG composites. Although the concentration of TGF- β_1 and PGE₂ was gradually back to normal after 14 days, the morphology of the osteoblasts was abnormal with features such as contracted cytoplasm structures. The osteoblast was damaged perhaps in the initial stage. We suggested that the 4% and 8% GTG composites should be soaked in distilled water at least for 4 days before medical applications. The 12% GTG composite and the composites with a concentration of glutaraldehyde solution higher than 12% were not recommended as a medical prostheses in any condition. The fetal rat calvaria culture also showed the same results with the analysis of TGF- β_1 and PGE₂. From the study, we could predict the results of animal experiments in the future. **Key Words:** Bioabsorbed bone graft—Glutaraldehyde—Tricalcium phosphate—Gelatin—Osteoblast—Rat calvarial tissue culture.

In recent years, a variety of materials has been researched and developed as hard tissue replacements, but there has been no adequate material for bone substitution in orthopedic surgery until now. In clinical orthopedic therapy, natural skeletal tissues

are widely used in surgery as transplants to serve many purposes. The grafts are usually derived from the tissues of patients as so-called autografts (1,2). An autograft is the best bone substitute with the properties of osteoconduction and osteoinduction. It contains bone morphogenic proteins to induce bone regeneration and has no risk of immunological response. However, some drawbacks limit fresh autogenous bone graft for clinical applications, such as uncontrolled resorption during healing, small

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amounts of supply, donor site morbidity, and so forth (3–5). Transplantation of allografts or xenografts represents only a minor part of reconstructive surgery because immunological reaction and risk of viral transmission happen frequently (6,7). The disadvantages of natural materials have stimulated the development of various artificial materials for bone substitutes, such as metals, polymers, and ceramics (8–11). However, we should emphasize that all these artificial materials have their limitations (12–15).

In a previous study, we described a biodegradable and biocompatible composite, glutaraldehyde crosslinked gelatin (GTG), which tricalcium phosphate (TCP) particulates dispersed in GTG matrix, as a bone substitute. In the cytotoxicity study, we found that the concentration of glutaraldehyde solution used as a crosslinking agent in the developed GTG composites should be in the range of 2–8%. It should be soaked in distilled water at least for 4 days before clinical applications. The gelatin molecular, calcium, and phosphorus ions would gradually be released from the composite, which could stimulate the proliferation and differentiation of osteoblasts.

Animal models have been used to provide the information for tissue response to implants, but their results were difficult to interpret at the cellular level because of the numerous and complex events that occurred upon insertion of a foreign material into a bleeding wound site. Cell and tissue culture models are, therefore, becoming prevalent in the investigation of tissue response to implants (17,18). Furthermore, the *in vivo* environment renders the task of deconvoluting the cascades of biological, material, and interfacial responses impossible without recourse to a more controlled experimental environment. *In vitro* approaches can provide ideal systems for studying tissue/implant interactions (19,20).

In this study, we designed a model of neonatal rat calvarial organ culture to assess the biological effects and osteoconductive potential of the GTG composites (21–24). We tried to understand whether GTG composites would promote new generation bone in the cultured calvaria. In this model, the tissue responses and feasibility of the GTG composites for clinical applications in a relatively short time were evaluated. If *in vitro* experiments can be used to imitate the perspective of the *in vivo* response to this composite, then it may be possible to use such a method to unravel the biological reactions that occur at the interface between bone tissue and the material. The GTG composites were evaluated by the growth of osteoblasts with alive cell counting in the previous study. It was supposedly too difficult to evaluate the cytotoxicity of the GTG composite. The

concentration of TGF- β_1 and PGE₂ in the medium was analyzed in the study to strictly estimate the biological effects of GTG composites on the osteoblasts.

MATERIALS AND METHODS

Composite preparation

The TCP [Ca₃(PO₄)₂] powder used in this study was supplied by Merck (Darmstadt, Germany). It was placed in a platinum crucible, sintered in a SiC element furnace at 1,100°C for 1 h, and then cooled down to room temperature. The sintered ceramic particles were crushed in an alumina grinding bowl and sieved in 30 to 40 mesh. The sieved TCP ceramic particles with 300 to 500 μm grain size were obtained for material preparation.

We prepared the matrix phase of the composite by adding 5 g of bovine gelatin (Sigma Chemical Co., St. Louis, MO, U.S.A.) to 15 ml of deionized distilled water. The mixture was stirred vigorously and kept at 65°C by water bath until a homogenous 16.7% gelatin solution was attained. Fifteen grams of TCP particles were then poured down into the gelatin solution. The mixture was stirred for 5 min to ensure a uniform consistency. Finally, 4%, 8%, and 12% glutaraldehyde solutions were added to the mixtures for crosslinking, respectively.

In order to obtain the homogeneous and higher crosslinking density of the composites, the temperature of the above mixture was cooled to 40°C before adding the glutaraldehyde solution for crosslinking reaction of the gelatin. After the mixture was crosslinked completely, the composite was molded uniformly into cylindrical plastic molds 6 mm in diameter. All composites used in this study were soaked in deionized distilled water for at least 4 days to ensure the unreactive glutaraldehyde in the composites was removed.

Osteoblasts cocultured with glutaraldehyde crosslinked gelatin composite extracts

Preparation of extracts from glutaraldehyde crosslinked gelatin composites

All the GTG composites, including GTG 4%, GTG 8%, and GTG 12%, were shaped into cylinder specimens 6 mm in diameter and 2 mm in length. Each sterilized GTG composite sample was placed in a capped plastic test tube with 20 ml deionized distilled water, and then kept in an incubator at a temperature of 37°C. After soaking for 1, 2, 4, 7, 14, 28, and 42 days, the GTG composite samples were removed from the test tubes, and the extracts were collected for use in cell culture examination. The composites soaked in distilled water for 4 days were

used in the later experiment of rat calvarial organ culture (16,25,26).

Cell culture methods and cytokine analysis

Neonatal Wistar rat calvaria were the source of the osteoblasts in the experiment. Under sterilized conditions, the parietal bone was removed from the dissected calvaria, stripped of soft tissue, and washed in phosphate buffered solution (PBS) 3 times. Then it was cut into fractions and digested in collagenase solution for 2 h. The cells from the digestion were pooled, washed, resuspended in tissue culture medium (Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 1% antibiotics), and then plated in plastic tissue culture dishes. The cells were cultured at 37°C in a humidified 5% CO₂ balance air incubator (27).

One milliliter of 1×10^4 cells/ml osteoblasts was seeded in individual wells of a 24 well tissue culture plate for evaluating the effects of extracts on the growth of osteoblasts. The complete medium was mixed with the extract at 1:1 volume ratio. In the blank group, the PBS was mixed with the complete medium in a volume ratio of 1:1 to have the same concentration as the experimental group (16,25,28, 29).

After 2 days of culture, the medium in each culture well was collected, and the osteoblasts attaching on the surface of the well were observed under optical microscopy to examine the morphology of osteoblasts after fixing by 2% glutaraldehyde solution. Then the concentrations of TGF- β_1 and PGE₂ in the medium collected from tissue culture dishes were analyzed by enzyme-linked immunosorbent assays (ELISAs). The ELISAs for each were identical using appropriate antibodies against the different cytokines and following the manufacture's protocol. Briefly, 100 μ l of osteoblasts culture medium or cytokine standards were added to wells of a microtiter plate coated with a murine monoclonal antibody against the appropriate cytokine. After a 2 h incubation at room temperature, wells were rinsed, and 200 μ l of anticytokine polyclonal antibody conjugate to horseradish peroxidase was added. Wells were incubated an additional 2 h at room temperature and rinsed. Next, 200 μ l of hydrogen peroxide and tetramethylbenzidine was added to each well as a substrate for 20 min. The reaction was stopped with 50 μ l of 2 N sulfuric acid. The optical density of each well was determined using an ELISA plate reader (ELX-800, Bio-Tek Instruments, Inc., Sunnyvale, CA, U.S.A.) with a 450 nm filter. Results were interpolated from the concentration versus absorption

curve generated with the cytokine standards, and the data presented as picograms per well (30,31).

In vitro neonatal calvarial organ culture

Control materials

In the model of neonatal calvarial organ culture experiments, deproteinized bovine and porcine cancellous bone matrices were used as the control materials. The bone matrices were derived from femoral condyle bovine and porcine cancellous bone, respectively. They were machined as spongy laminates with 6 mm \times 6 mm \times 0.5 mm volume. In order to get completely deproteinized and defatted bone matrices, the cancellous bone laminates were kept in boiling water for 12 h and then dehydrated with a series of alcohol solutions. The laminates were finally sintered at a temperature of 1,200°C for 1 h in a computer programmed SiC heating element furnace. The pore size and porosity of all the testing materials, GTG composites and controlled materials, were determined before being used in organ culture (32).

Organ culture method

Calvarias were harvested from neonatal Wistar rats about 4 days old. The rats were sacrificed with an overdose of Pentothal (0.5–1.0 ml), and then the calvaria was dissected from which the endocranial and extracranial periosteal were completely removed to minimize fibroblastic impurity. The calvaria was promptly put into the PBS. A hole 5 mm in diameter was then created in the central area of each parietal bone by a sterilized hollow steel tube. The testing material, such as GTG composites or controlled specimens, was placed on the hole area of these calvarias with the surface in contact as an organ culture unit. The organ culture unit was transferred to Fitton-Jackson's modification of Bigger's culture medium that was supplemented with 10% fetal calf serum, 20 μ l/ml 200 mM glutamine, 10 μ l/ml penicillin/streptomycin (500 U/ml), 25 μ l/ml HEPES 1 M solution, 10 μ l/ml β -glycerol-2-phosphate 1 M solution, and 50 μ g/ml ascorbic acid. The medium was replenished every 3 days, and the organ culture units were kept in such a condition for 5 weeks (21,22,33, 34). Each organ culture unit was observed under an optical microscope in every culture period of 1 week, 3 weeks, and 5 weeks to evaluate the area of new generation bone.

Quantitative evaluation

Quantitative evaluation of the new generation bone was performed by using a semiautomatic histomorphometric method. The system consisted of a microscope with crosspolarizing filters, digitizing

plate, digitizer, and microcomputer with a mini-floppy disk drive. The microscope was equipped with a photic drawing tube, through which the image of the digitizing plate was projected over the optical field. By moving a cursor on the digitizing plate, which was visible by its projection over the histological field, newly grown bone tissue was calculated and expressed as a percentage of the ingrowth bone tissue occupying the entire pore area in a calvarial bone cavity (35).

RESULTS

Osteoblast cell culture

In a previous study, we merely used the cell population to express the biological effects of the GTG composites. However, it was difficult to be sure whether the growth activity of osteoblasts was promoted by the GTG composite or not (16). Measurement of TGF- β_1 concentration in cell culture medium could more clearly illustrate the biological effect and cytotoxicity of GTG composites.

Figure 1 shows the relationship between the concentration of TGF- β_1 and soaking time after a series of GTG composite extracts cocultured with fetal rat osteoblasts for 2 days. The curves for all the GTG composites had a common tendency and could be divided into 3 stages. For the 4% and 8% GTG com-

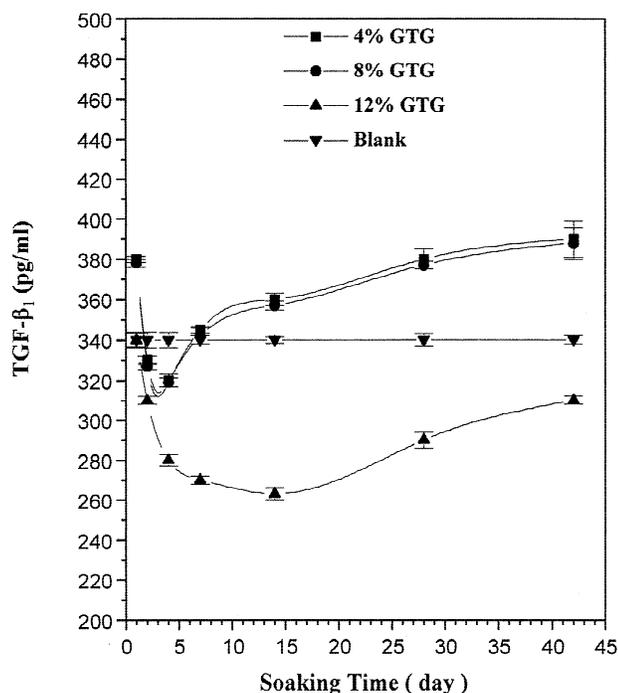


FIG. 1. The graph shows the relationship between the concentration of TGF- β_1 in the medium and soaking periods after a series of GTG composite extracts were cocultured with 1×10^4 cells/ml fetal rat osteoblasts for 2 days.

posites, the concentration of TGF- β_1 sharply decreased with the soaking time in the initial 4 days. But the minimum value of TGF- β_1 for the 12% composite was extended up to 14 days as shown in Fig. 1. As described in a previous study, we found that the concentration of residual glutaraldehyde in the extract of 4% GTG and 8% GTG composites was the highest and that the cell population showed a minimum value at the fourth day of the soaking period. The highest concentration of glutaraldehyde and the lowest cell population appeared at the 14th day of soaking time for the 12% GTG composite. TGF- β is a member of a family of growth and differentiation factors identified in a wide variety of organisms ranging from insects to humans, which was described as a factor to induce phenotypic transformation of some cell lines. It is now known to affect proliferation and differentiation in a wide variety of cell types and can act as a growth stimulator (36,37). TGF- β_1 in the cultured medium could be used to reflect the cell population. Glutaraldehyde was supposed to have a negative effect on the growth of osteoblasts, which would cause the cell population to decrease at a higher concentration.

In the second stage of the curves in Fig. 1, both concentrations of TGF- β_1 in medium for 4% and 8% GTG composites showed a great increase after soaking for 4 days. The 2 curves had a plateau area with a soaking time of up to 14 days and then maintained a constant value at the so-called third stage. The concentration of TGF- β_1 in medium for the 12% GTG composite also showed a turning point that appeared at the 14th day of the soaking period. The curve for the 12% GTG composite had an increasing tendency thereafter.

The concentration of TGF- β_1 in the blank group was 340 pg/ml after culture of 2 days. Compared with the blank group, the concentration of TGF- β_1 in the medium for the 4% and 8% GTG composite was over that of the blank group after 7 days of soaking. The concentration of 4% and 8% GTG composite was about 380 pg/ml at 28 days of soaking and even reached 400 pg/ml after soaking for 42 days. The concentration of TGF- β_1 in the medium for the 12% GTG composite was always lower than that of the blank group throughout the whole soaking period. The results of TGF- β_1 analysis in Fig. 1 were in agreement with the results of previous studies on cell population, chemical analysis, and cytotoxicity evaluation.

In this study, the determination of the PGE₂ concentration in cultural media was used as the estimation of cellular activity when the osteoblasts were cocultured with a series of GTG composites extracts

for 2 days. After osteoblasts cocultured with the extract of GTG composites for 2 days, the concentration of PGE₂ released from osteoblasts is shown in Fig. 2. In the blank group, the extract of GTG composites was replaced with PBS. The concentration of PGE₂ in cultural media of the blank group almost maintained at a constant value about 250 pg/ml after 1×10^4 cell/ml osteoblasts were cocultured with PBS for 2 days.

For the 4% and 8% GTG composites, the curves showed a rapid ascent where the concentration of PGE₂ increased with soaking time in the initial 4 days. The concentration for the 2 groups was in a range of 250–325 pg/ml in the initial 4 days. After 4 days, the curves for 4% and 8% GTG composites had a descending tendency. After 4% and 8% GTG composites were soaked in distilled water for 7 days, the concentration of PGE₂ for the 2 groups was even lower than that of the blank group.

The curve of the 12% GTG composite had the same tendency as the previous 2 curves, but a difference in PGE₂ concentration. The concentration of PGE₂ for the 12% GTG composite increased with the soaking time in the initial 14 days and then decreased thereafter. It showed an abnormally high concentration in a range of 350–475 pg/ml and was

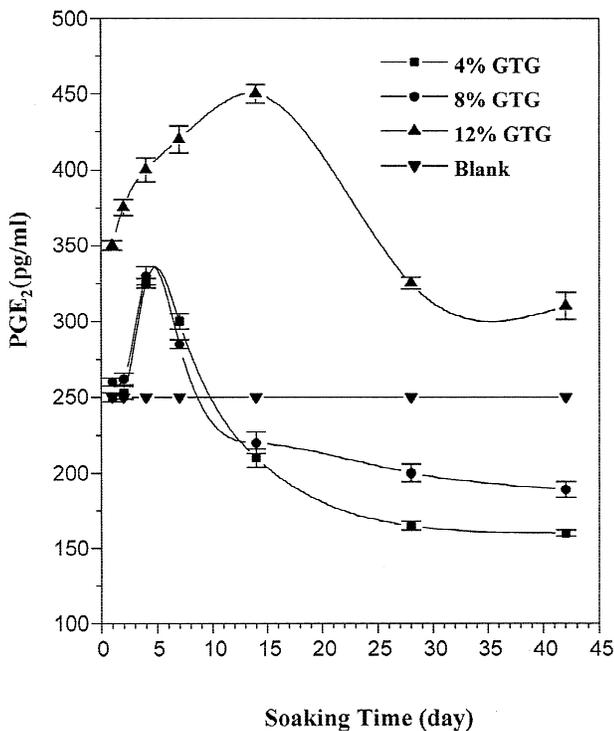


FIG. 2. The graph shows the relationship between the concentration of PGE₂ in the cultural medium and soaking periods after 1×10^4 cells/ml fetal rat osteoblasts were cocultured with a series of GTG composites extracts for 2 days.

higher than that of the blank group at each soaking period.

The actions of prostaglandins (PGs) on bone cells are complex and contradictory, depending on the class of PG and the species studied. The PGEs, especially PGE₂, reduced the activity of both osteoblasts and osteoclasts. Abundant PGE₂ would release from osteoblasts while the osteoblasts were damaged by injury (38,39). The results of the PGE₂ concentration in the cultural media implied that the residual glutaraldehyde existing in the extracts had a negative influence on the osteoblast but might do no significant harm to the extract with lower concentration of residual glutaraldehyde. The concentration of PGE₂ for the 3 experimental groups showed a sharp increase in the initial stage because the unreacted glutaraldehyde was fast released from the GTG composites. The PGE₂ concentration for all 3 experimental groups rapidly decreased after the concentration reached a highest value, which was due to the nutrient substances such as gelatin, phosphorous ion, and calcium ion releasing from the GTG composites. As described in the previous study, we found that at 4 days of soaking time for GTG composites treated with 4% and 8% glutaraldehyde, the concentration of residual glutaraldehyde in extracts reached the highest point, which led the concentration of PGE₂ to the maximum value at that time. It needed 14 days for the 12% GTG composite to remove the residual glutaraldehyde so that the concentration of PGE₂ reached the maximum value at the 14th day of the soaking time.

Compared with the curves of Figs. 1 and 2, there were obvious complementary distributions existing between TGF- β_1 and PGE₂ for the 3 experimental GTG composites. In the initial stage, the PGE₂ showed an upward curve while the curve for TGF- β_1 showed a downward tendency. Once it reached the lowest value, the curve of TGF- β_1 concentration turned into an ascent when the curve of PGE₂ went to an obvious descent after the highest value.

Several studies have evaluated the cytocompatibility of various biomaterials by observing the morphological changes of cells with an optical microscope, detecting different degrees of cell loss, patterns of injury, or nuclear and cytoplasm damage. The methods were described in our previous study to evaluate the effect of gelatin solution on cell proliferation with an optical microscope. All the 14 day soaking time extracts of the 3 experimental groups' material were tested in this study. After the 14 day extract of GTG composites was cocultured with osteoblasts for 2 days, the culture dishes were washed with 0.185 M sodium cacodylate buffer (pH 7.4) and fixed with 3%

formaldehyde solution for 30 min, and then they were stained with hematoxylin-eosin.

The morphologies of osteoblasts for the blank group are shown in Fig. 3A. Figures 3B to 3D show the morphologies of osteoblasts for 4%, 8%, and 12% GTG composites, respectively. The morphologies of nuclear and cytoplasm in osteoblasts were not significantly different between the blank group and the GTG composites with concentrations of 4% and 8%. Compared with the blank group, the 12% GTG composite showed a lower population of osteoblasts and had an abnormality of cellular morphologies such as contracted cytoplasm structure as observed in the dish. The osteoblasts in the 12% GTG composite just attached on the well ground, and there is no sign of a fusion process having occurred in the stage. It is possible that the high-concentration glutaraldehyde would have an adverse effect upon the growth of the osteoblasts.

Neonatal calvarial organ culture

In vitro assays will, of course, be even further removed from the reality of the ultimate implantation bed. Indeed, it is not possible to conduct in vitro experiments on the assumption that they can replace absolutely the complexity of an in vivo environment. However, if in vitro experiments can be used to mimic aspects of the known in vivo response to bio-

active materials, then it may be possible to use such methods to unravel the biological reactions that occur at material surfaces.

Figure 4 shows the average pore size for all the test materials. Deproteinized bovine and porcine bone were used as control groups in the experiment with an average pore size of about 850 and 500 μm in diameter, respectively. The pore size for the 3 experimental GTG composites was about 650 μm with no significant difference from each other. The porosity for the 5 tested materials is illustrated in Table 1. It shows that the porosity of the deproteinized bovine and porcine bone was about 46.67% and 36.66%, respectively. The porosity for the 3 GTG composites was about 20% with no distinct difference among them. This fact revealed that the pore size and porosity of the GTG composites had nothing to do with the crosslinking concentration of glutaraldehyde.

After culture for 1 week, the new generation bone was growing around the surface of the pores inside of the deproteinized bovine and porcine bone as shown in Figs. 5A and 6A, respectively. From the results of histomorphometric evaluation, the percentage of the new generation bone for the 2 control groups was not statistically different, about 1.74% for bovine and 4.49% for porcine (Table 2). For the 4%, 8%, and 12% GTG composite, the new generation bone was also growing around the surface of the pores and much more than that of the 2 control

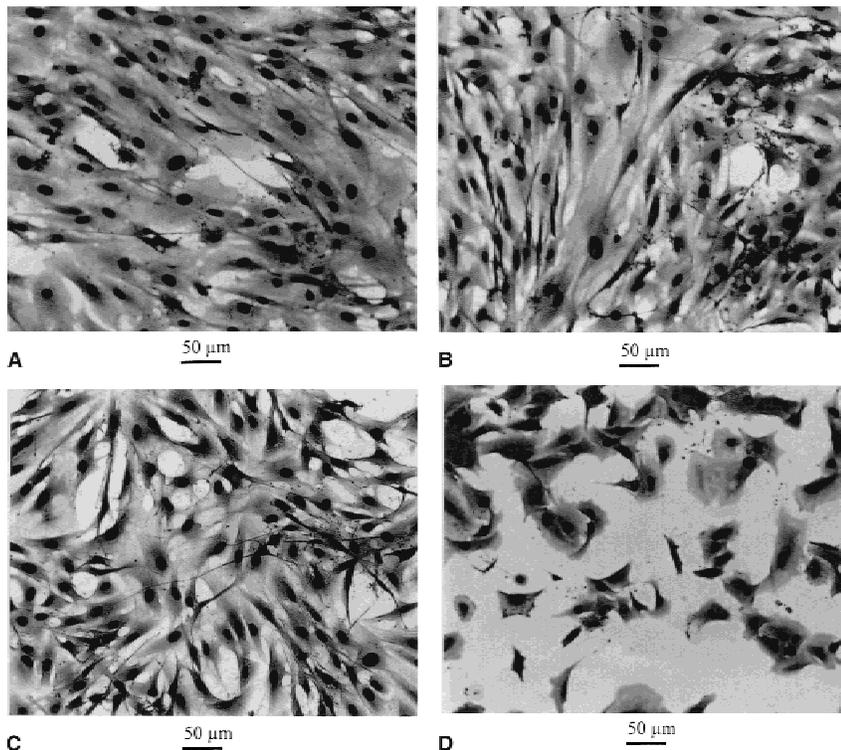


FIG. 3. The photographs show optical microscopic examination of the fetal rat osteoblasts cocultured for 2 days with 14-day soaking extracts of GTG composites: PBS (A), 4% GTG (B), 8% GTG (C), and 12% GTG (D); original magnification $\times 200$.

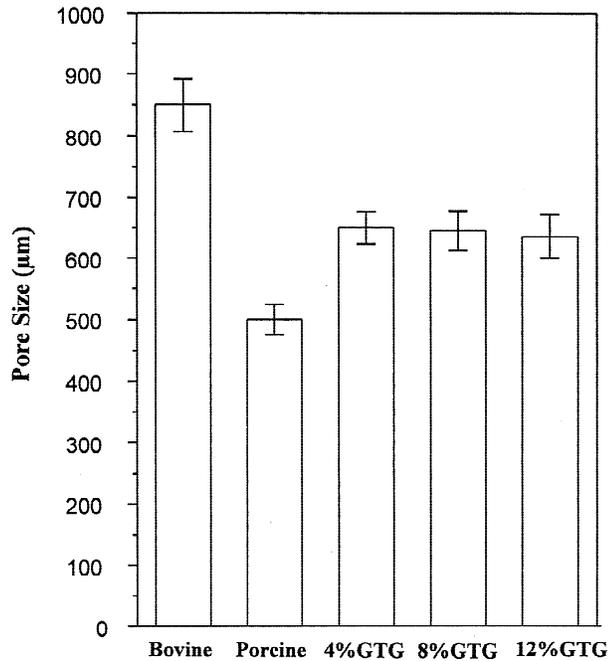


FIG. 4. The graph shows pore size in the 5 different tested materials. Values are mean \pm standard deviation ($n = 3$) (Bovine: deproteinized and defatted bovine cancellous bone matrix, Porcine: deproteinized and defatted porcine cancellous bone matrix, 4% GTG: GTG composite treated with 4% glutaraldehyde, 8% GTG: GTG composite treated with 8% glutaraldehyde, 12% GTG: GTG composite treated with 12% glutaraldehyde).

groups at the first week culture. The percentage of the new bone generation area for the 3 GTG composites was 53.46%, 33.36%, and 18.30% for 4%, 8%, and 12%, respectively. The appearance of the new bone growing into the pores of the 4% GTG composite is shown in Fig. 7. The new bone tissue was gradually growing around the peripheral area inside of the pore.

After culture for 3 weeks, the 2 control groups showed a slow growth on the new generation bone of about 23.30% and 36.05% for deproteinized bovine

TABLE 1. The porosity of 5 tested specimens ($n = 3$)

Material	% Mean (SD)	<i>t</i> -test ($p < 0.01$)
Bovine	46.67 (1.2)	<i>a</i>
Porcine	36.66 (2.1)	<i>b</i>
4% GTG	17.02 (1.5)	<i>c</i>
8% GTG	18.87 (2.5)	<i>c</i>
12% GTG	19.63 (2.0)	

a A significant difference existed in porosity between bovine and porcine.

b A significant difference existed in porosity between porcine and 4% GTG.

c No distinction in porosity appeared among 3 GTG composites.

GTG: glutaraldehyde crosslinked gelatin.

and porcine bone. At the same culture period, the percentage of new generation bone for the 3 experimental groups had a progressive increase of about 89.87%, 71.28%, and 37.84% for 4%, 8%, and 12% GTG composite, respectively (Table 2). For the 4% and 8% GTG composite, a significant amount of new bone formation could be observed from the optical photographs shown in Figs. 8 and 9.

At the fifth week, the 2 control groups showed a great amount of increase in the area of new generation bone of about 63.35% and 73.57% for deproteinized bovine and porcine bone. There was only a small amount of increase for the 4% and 8% GTG composite of about 95.58% and 94.48%, respectively, in area. For the 12% GTG composite, new generation bone showed a progressive growth at this period. The percentage of new bone area increased up to 90.10% for the 12% GTG composite. The dynamic observation for each culture period of all the test materials could be easily examined by the optical microscope as shown in Figs. 5 to 10. The statistical analysis of the new generation bone for all the test materials in each culture period is summarized in Table 2 in detail.

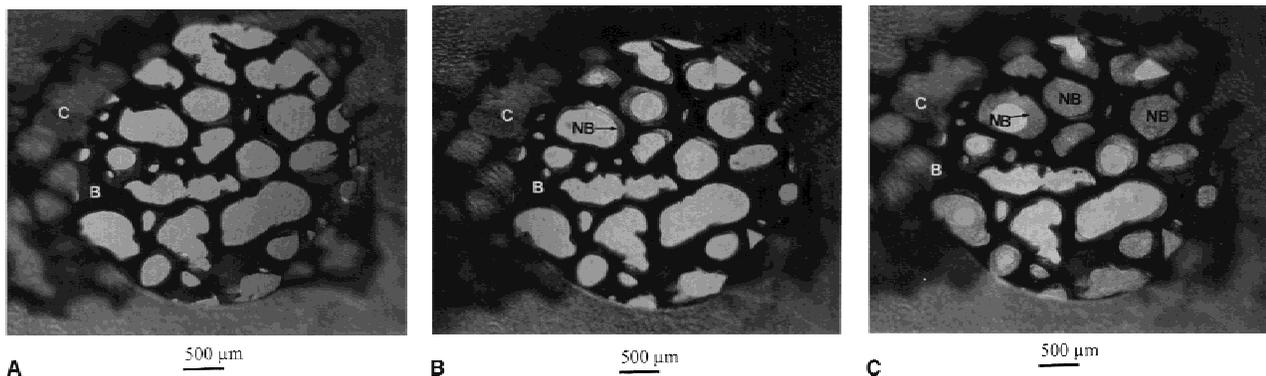


FIG. 5. Porosity in the different tested materials.

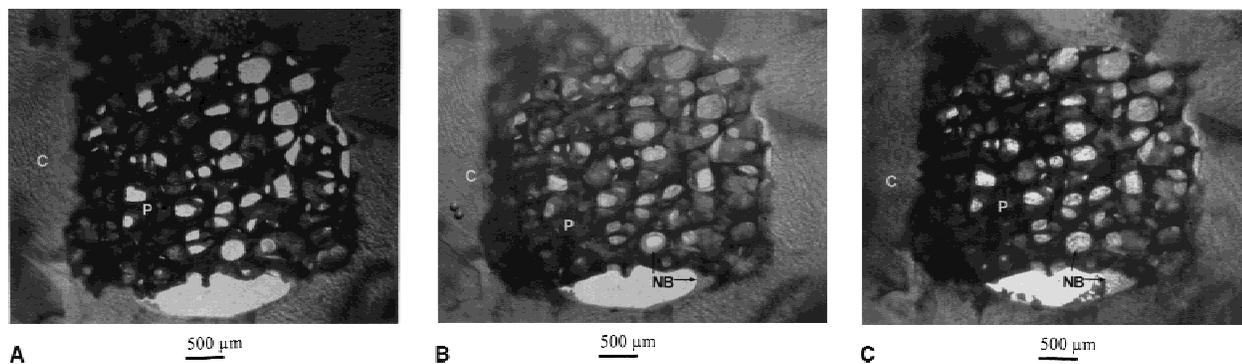


FIG. 6. Shown is percentage of new ingrowing bone tissue area in pore size.

DISCUSSION

There is no conclusive evidence that any of the porous calcium phosphate biomaterials are osteoinductive. Most are considered osteoconductive, that is, allowing for bone ingrowth from an osseous bed. No bone ingrowth will occur if the implant is inserted into muscle or subcutaneous tissue (39–41). Such potential testing procedures are of considerable importance in the biomaterials field since not only can they be adapted to provide a biological bath-testing assay for bioactive bone substitute materials, but they also provide a means of investigating the intimate step-by-step interactions occurring at the tissue-material interface using relatively simple techniques when compared with in vivo implantation. Indeed, while Greenlee et al. (42)

showed that in vivo systems could be used to study the effect on tissue behavior brought about by changing implant composition, they recommended that in vitro systems be devised for evaluating variables of materials, which would be less laborious than in vivo methods. In order to study the biocompatibility and osteoconductive potentiality of the GTG composites on the damaged bone tissue in vitro, we used a fetal rat calvarial organ culture model to study the effect of bony ingrowth on the material biological properties of the new bone substitute. Furthermore, the aim of this study was to observe the reaction of the fetal rat calvarial bone tissue to the GTG composites and attempt to reproduce in vitro some of the known in vivo characteristics of such materials.

The results of x-ray diffractometer analysis showed that both deproteinized bovine and porcine bone had the same crystallographic composition of about 93% hydroxyapatite and 7% TCP. However,

TABLE 2. The ratio of ingrowth to total pore area (n = 3)

Culture time	Material	% Mean (SD)	t-test (p < 0.01)	
			A ^a	B ^b
1 Week	Bovine	1.74 (0.54)	c	
	Porcine	4.49 (1.96)		
	4% GTG	53.46 (2.10)		
	8% GTG	33.36 (0.53)		
3 Weeks	12% GTG	4.31 (0.89)		d
	Bovine	23.30 (0.27)		
	Porcine	36.00 (2.22)		
	4% GTG	89.87 (0.05)		d
5 Weeks	8% GTG	71.28 (0.20)		d
	12% GTG	37.84 (0.48)		d
	Bovine	63.35 (0.02)		
	Porcine	73.57 (0.81)		
	4% GTG	95.58 (1.93)	c	d
	8% GTG	94.48 (0.14)		d
	12% GTG	90.10 (0.03)		d

^a t-test was done between 2 testing groups with random choice. Only no significant difference only is presented.

^b t-test was done between each experimental group and both of the control groups. Only the significant difference is presented.

^c Not significantly different.

^d Significantly different at p < 0.01.

GTG: glutaraldehyde crosslinked gelatin.

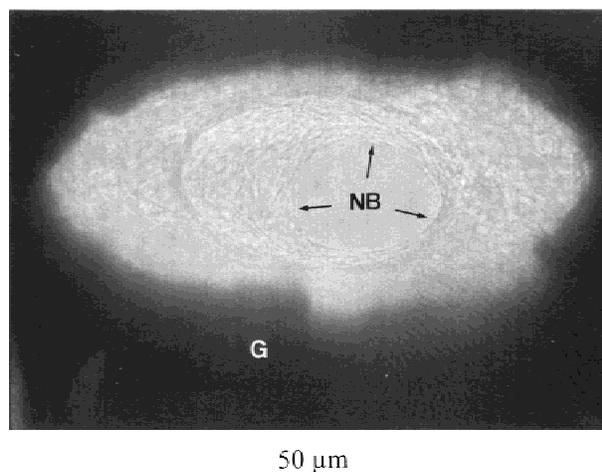


FIG. 7. Optical microscopic examination shows the appearance of new bone tissue growing increasingly along the edge of the pore (NB: new bone tissue, M: material; original magnification x40).

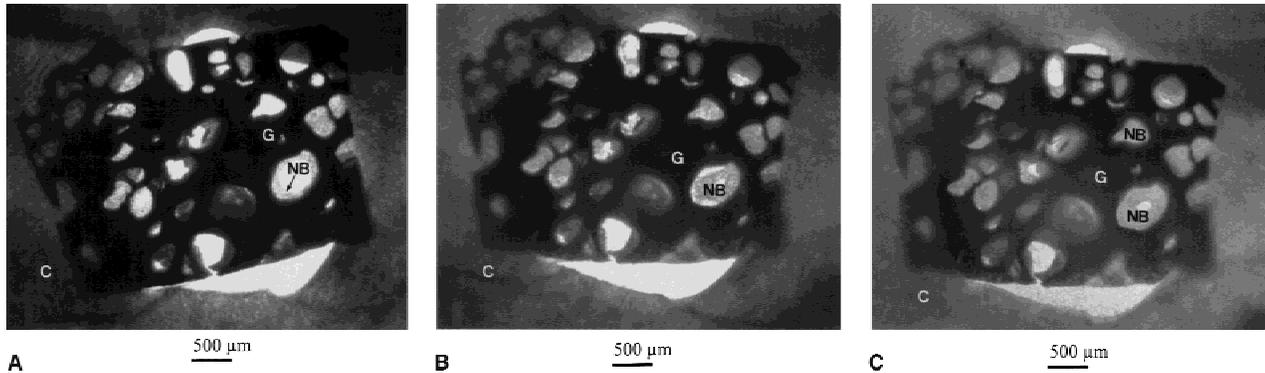


FIG. 8. Optical microscopic examination of 4% GTG composite cocultured with rat calvaria for (A) 1 week, (B) 3 weeks, and (C) 5 weeks (G: GTG composite, NB: new bone, C: calvaria; original magnification $\times 10$).

it revealed a difference in the amount of new bone tissue for the 2 control groups after culture periods up to 3 weeks and 5 weeks. The major difference between the 2 control groups was pore size. Several studies have been undertaken to evaluate the biological effects of various biomaterials with different pore size. However, that work cannot show the optimum pore size for the effective ingrowth of bone into porous ceramic structures. Hulbert et al. (43) reported that the optimum pore size required for the ingrowth of new bone was about 100 to 300 μm . K. de Groot (44) showed that the optimum pore size for bone ingrowth was about 200 to 500 μm . In that study, the pore size of deproteinized porcine bone was around 500 μm , which was the upper limit for the pore size of optimum bone in growth. The pore size of deproteinized bovine bone was about 800 μm , which was too large and difficult to colonize host bone tissue on the peripheral area inside of the pore. The difference of bone ingrowth between the 2 control groups was based on the difference of pore size.

GTG composite is a biodegradable biomaterial composed of TCP and GTG that was designed as a large bone substitute. At the first week of organ cul-

ture, the area of new generation bone for 4% and 8% GTG composite was much higher than that of the 2 control groups (Table 2). For the 12% GTG composite, it showed a lower percentage of new generation bone in the pores than the 2 control groups. As stated previously, the concentration of TGF- β_1 in the medium for the 4% and 8% GTG composite was higher than that of the blank group after 7 days of soaking. The phosphorus ions, calcium ions, and amino acids would gradually release from the composites that were thought to be the positive factors for bone regeneration. On the contrary, the concentration of TGF- β_1 for the 12% GTG composite was lower than that of the blank group. The unchained glutaraldehyde was released from the composite that would lead to lower colonized bone tissue from the fetal rat calvaria.

At the third week of organ culture, the new generation bone for all the test materials showed a progressive growth. The new bone areas for the 4% and 8% GTG composites were much higher than those of the other 3 groups. The result was in agreement with the results of TGF- β_1 and PGE₂ analysis. The concentration of TGF- β_1 for the 4% and 8% GTG

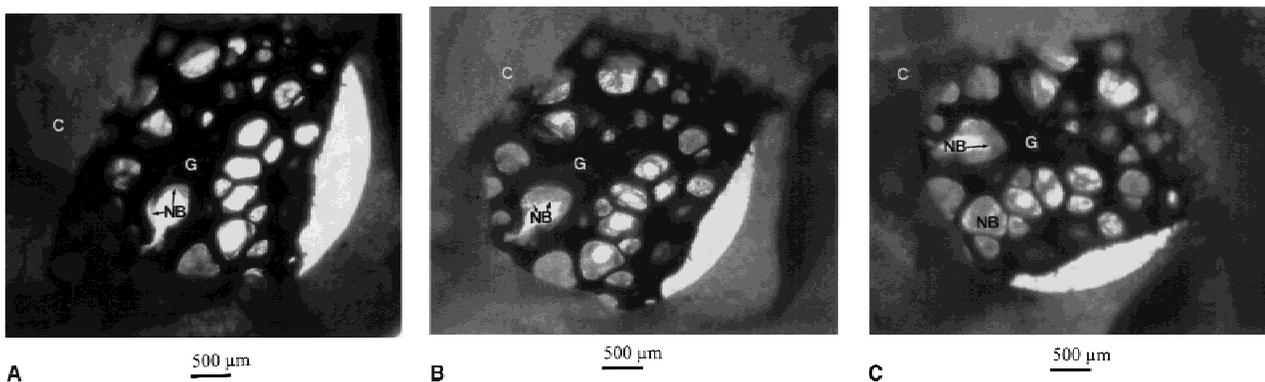


FIG. 9. Optical microscopic examination of 8% GTG composite cocultured with rat calvaria for (A) 1 week, (B) 3 weeks, and (C) 5 weeks (G: GTG composite, NB: new bone, C: calvaria; original magnification $\times 10$).

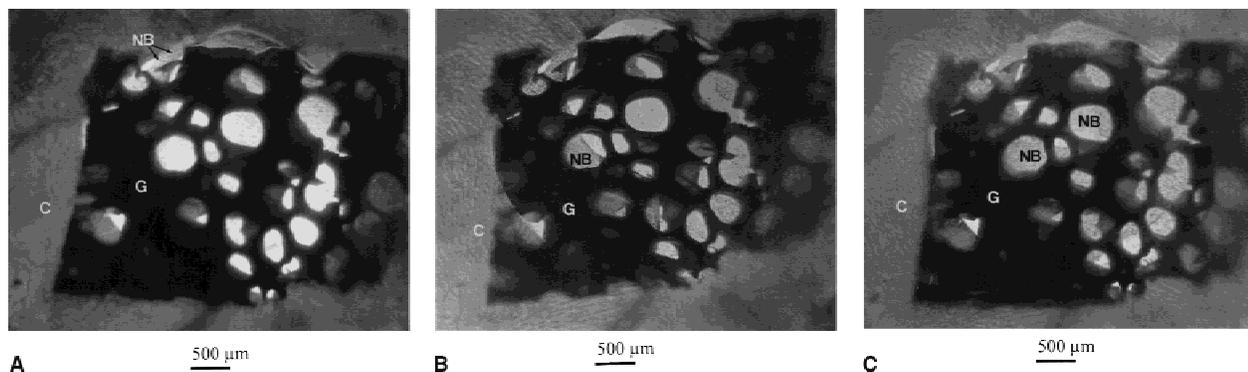


FIG. 10. Optical microscopic examination of 4% GTG composite cocultured with rat calvaria for (A) 1 week, (B) 3 weeks, and (C) 5 weeks (G; GTG composite, NB: new bone, C: calvaria; original magnification $\times 10$).

composite was higher than that of the blank group and the concentration PGE_2 was lower than that of the blank group. It reflected that the 4% and 8% GTG composite not only did no harm to the bone tissue but also promoted new bone formation.

After fetal rat calvaria was cultured for 5 weeks, the new generation bone almost covered the all the pores for the 4%, 8%, and 12% GTG composites. Although the new bone area increased with culture time for the 2 control groups, it was still far lower than that of the 3 GTG composites. In the initial 3 weeks, the new bone area for the 12% GTG composite kept the same pace as the 2 control groups. It was amazing that the new bone area for 12% GTG composite had a greater increase and was much higher than that of the 2 control groups at the fifth week of the organ culture. As described in the previous study, the unchained glutaraldehyde in the 12% GTG composite would be removed completely after being soaked in distilled water for 14 to 28 days. The nutrition elements such as gelatin and other amino acids would then flow out from the GTG composite because of the degradation in the medium. The residual glutaraldehyde was thought to be the main factor for the slow growth of new bone in 12% GTG composite at the initial 3 weeks. The nutrition elements released from the composite was the possible reason for the prosperous growth of new bone at the fifth week of calvaria culture.

CONCLUSIONS

Except in the initial 4 days, the analysis of $TGF-\beta_1$ for the 4% and 8% GTG composites was higher than that of the blank group for the other experimental time period. The PGE_2 analysis for the 4% and 8% GTG composites was lower than that of the blank group as it was for $TGF-\beta_1$. It revealed that the 4% and 8% GTG composites would not lead to inflam-

mation and would promote the osteoblast growth. The morphology and activity of the osteoblast were not transferred or changed by the 2 GTG composites. For the 12% GTG composite, the performance of the in vitro condition was far behind the blank group and the other 2 GTG composites. Although the concentration of $TGF-\beta_1$ and PGE_2 was gradually back to normal after 14 days, the morphology of the osteoblast showed an abnormality of cellular morphologies such as a contracted cytoplasm structure. The osteoblast was damaged perhaps somehow in the initial stage. We suggested that the 4% and 8% GTG composites should be soaked in distilled water at least for 4 days before medical applications. The 12% GTG composite and the composites with concentrations of glutaraldehyde solution higher than 12% were not recommended as the medical prostheses in any condition. The fetal rat calvaria culture also showed the same results as the analysis of $TGF-\beta_1$ and PGE_2 . From this study, we could expect to predict the results of animal experiments in the future.

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