

**以頭顱骨缺陷模式評估生醫玻璃及戊二醛交聯複合材料作為
骨骼大缺陷之可行性分析**

**Osteogenic Evaluation of GTG 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 cross-linked gelatin (GTG) has been evaluated by osteoblasts cell culture. In the previous study, the GTG composites have been 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 the study, the extracts were cocultured with the osteoblasts; thereafter the concentration of TGF- β_1 and PGE $_2$ 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 experiment. 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 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 period. The PGE $_2$ 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 the osteoblast growth. The morphology and activity of the osteoblasts were not transformed or changed by the two GTG composites. For the 12% GTG composite, the performance of in vitro condition inferior to the blank group and the other two GTG composites. Though the concentration of TGF- β_1 and PGE $_2$ was gradually back to normal after 14 days, the morphology of the osteoblasts was abnormal with features such as contracted cytoplasm structure. The osteoblast perhaps be damaged 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 or the composites with concentration of glutaraldehyde solution higher than 12% was not recommended as the medical prostheses in any condition. The fetal rat calvaria culture also showed the same results with the analysis of TGF- β_1 and PGE $_2$. From the study, we could use to predict the results of animal experiment in the future.

Keywords: *bioabsorbed bone graft, glutaraldehyde, tricalcium phosphate, gelatin, osteoblast, rat calvarial tissue culture*

1 Introduction

In recent years, a variety of materials have been searched and developed as hard tissue replacement but there was no adequate material for bone substitute in orthopedic surgery till now. In clinical orthopaedic therapy, natural skeletal tissues are widely used in surgery as transplants to serve many purposes. The grafts are usually derived from the tissues of patient as so called autograft [1,2]. 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 amount of supply and donor site morbidity...etc [3-5]. Transplantation of allografts or xenografts represents only a minor part of reconstructive surgery because immunological reaction and risk of viral transmission are happened frequently [6,7]. These disadvantages of natural materials stimulate the development of various artificial materials for bone substitute, such as metals, polymers and ceramics [8-11]. However, we should emphasize that all these artificial materials have their limitations [12-15].

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

Animal models have been used to provide the information of 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 implant [17,18]. Furthermore, it is

very complicated in the in vivo environment which renders the task of deconvoluting the cascades of biological, material, and interfacial responses impossible without recourse to more controlled experimental environment. In vitro approaches can provide ideal system for studying tissue/implant interactions [19,20].

In the 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 try to understand whether GTG composites would promote new generation bone in the cultured calvaria? In the model, the tissue responses and feasibility of the GTG composites for clinical applications in a relative short time will be evaluated. If in vitro experiments can be used to imitate perspective of the in vivo response to this composite, then it may be possible to use such method to unravel the biological reactions which occur at interface between bone tissue and material. By the way, the GTG composites have been evaluated the cytotoxicity of GTG composites on the growth of osteoblast by alive cell counting in the previous study. It was supposed too rough 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.

2 Materials and Methods

2-1 Composite Preparation

The tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) powder used in this study was supplied by Merck, Germany. It was placed in a platinum crucible and sintered in a 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 with 300-500 μm in grain size was obtained for material preparation.

We prepared the matrix phase of the composite by adding 5 g of bovine gelatin (Sigma Chemical Co., USA) 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. 15 g tricalcium

phosphate particles were then poured down into the gelatin solution. The mixture was stirred for 5 minutes to ensure a uniform consistency. Finally, 4%, 8% and 12% glutaraldehyde solutions were added to the mixtures for cross-link, respectively.

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

2-2 Osteoblasts cocultured with GTG Composite extracts

2-2-1 Preparation of Extracts from GTG Composites

All the GTG composites, including GTG4%, GTG8%, and GTG12%, were shaped into a cylinder specimen with 6 mm in diameter and 2mm 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 soaked 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 uses in cell culture examination. The composites soaked in distilled water for 4 days were used in the latter experiment of rat calvarial organ culture [16,25,26].

2-2-2 Cell Culture Methods and Cytokine Analysis

Neonatal Wistar rats 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 buffer solution (PBS) for three times. Then it was cut into fractionlet and digested in collagenase solution for 2 hours. 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 humidified, 5% CO₂ balance air incubator[27].

1ml of 1×10^4 cells/ml osteoblasts were 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 extract of 1:1 in volume ratio. In blank group, the PBS was mixed with complete medium in a volume ratio of 1:1 to have the same concentration with experimental group [16,25,28,29].

After 2-day culture, the medium in each culture well was collected and the osteoblasts attaching on the surface of the well was observed under optical microscopy to examine the morphology of osteoblasts after fixed by 2% glutaraldehyde solution. Then the concentration of TGF- β_1 and PGE₂ in the medium collected from tissue culture dishes were analyzed by ELISA (enzyme-linked immunosorbent assays). 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 hours incubation at room temperature, wells were rinsed and 200 μ l of anticytokine polyclonal antibody conjugates to horseradish peroxidase was added. Wells were incubated an additional 2 hours at room temperature and rinsed. Next, 200 μ l of hydrogen peroxide and tetramethylbenzidine was added to each well as substrate for 20 min. The reaction was stopped with 50 μ l of 2N sulfuric acid. The optical density of each well was determined using an ELISA plate reader(ELX-800, Bio-Tek Instruments, INC.) 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].

2-3 In vitro Neonatal Calvarial Organ Culture

2-3-1 Control materials

In the model of neonatal calvarial organ culture experiments, deproteinized bovine and porcine cancellous bone matrixes were used as the control materials. The bone matrixes were derived from femoral condyle cancellous bone of bovine and porcine, respectively. They were machined as a spongy laminates with 6 mm x 6 mm x 0.5mm in volume. In order to get completely deproteinized and defatted bone matrix, the cancellous bone laminates was stayed in boiling water for 12 hours, then dehydrated with a series of alcohol solutions. The laminates were finally sintered at a temperature of 1200°C for 1 hour 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 used in organ culture [32].

2-3-2 Organ culture method

Calvarias were harvested from neonatal Wistar rats about 4-day old. The rat was killed with overdose 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. The central area of each parietal bone was then created a hole with 5mm in diameter by a sterilized hollow steel tube. The testing material, such as GTG composites or controlled specimens, was placed on the hole area of these calvarial with surface in contact as a 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, 20µl/ml 200mM glutamine, 10µl/ml penicillin/streptomycin (500 units/ml), 25µl/ml HEPES 1 M solution, 10µl/ml β-glycerol-2-phosphate 1M solution and 50µg/ml ascorbic acid. The medium was replenished every 3 days and the organ culture units were stayed in such a condition for 5 weeks [21,22,33,34]. Each organ culture unit was observed under optical microscope in every culture period of 1 week, 3 weeks, and 5 weeks to evaluate the area of new generation bone.

2-3-3 Quantitative Evaluation

Quantitative evaluation of the new generation bone was performed by using a semiautomatic histomorphometric method. The system consisted of a microscope with cross-polarizing 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 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 calvarial bone cavity [35].

3. Results

3-1. Osteoblast Cell Culture

In previous study, we merely used the cell population to express the biological effects of the GTG composites. However, it was difficult to ensure 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 outtell and more clearly illustrate the biological effect and cytotoxicity of GTG composites.

Figure 1 showed the relationship between the concentration of TGF- β_1 and soaking time after series of GTG composites extracts cocultured with fetal rat osteoblasts for 2 days. The curves for all the GTG composites had common tendency and could be divided into three stages. For the 4% and 8% GTG composites, the concentration of TGF- β_1 sharply decreased with the soaking time in initial 4 days. But the minimum value of TGF- β_1 for the 12% composite was extended up to 14 days as shown in Figure 1. As described in the previous study, we found that the concentration of residual glutaraldehyde in the extract of 4%GTG and 8%GTG composites was the highest and the cell population showed a minimum value at the 4th day of soaking period. The highest concentration of glutaraldehyde and the lowest cell population was 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 negative effect for the growth of osteoblast which would cause cell population decreased at a higher concentration. Since the concentration of TGF- β 1 is related with the cell population, it would be no doubt that the concentration of TGF- β 1 had the same tendency with the result of cell population and had in opposition with the result of the concentration of glutaraldehyde.

In the second stage of the curves in Figure 1, both concentration of TGF- β 1 in medium for 4% and 8% GTG composites showed a great increase after soaked for 4 days. The two curves had a plateau area when soaking time up to 14 days, and then maintained a constant value as so called the third stage. The concentration of TGF- β 1 in medium for the 12% GTG composite also showed a turning point but was appeared at the 14th day of soaking period. The curve for 12% GTG composite had a increasing tendency thereafter.

The concentration of TGF- β 1 in blank group was 340 pg/ml after cultured for 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 than that of the blank group after 7-day soaking. The concentration of 4% and 8% GTG composite was about 380 pg./ml at 28-day soaking, even reached to 400 pg/ml after soaked for 42 days. The concentration of TGF- β 1 in the medium for the 12% GTG composite was always lower than that of blank group throughout the whole soaking period. The results of TGF- β 1 analysis in Figure 1 was in agreement with the results of previous studies on cell population, chemical analysis and cytotoxic 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 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 was shown in Figure 2. In the blank group, 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 1x10⁴ cell/ml osteoblasts were cocultured with PBS for 2 days.

For the 4% and 8% GTG composites, the curves showed a rapid ascend where the concentration of PGE₂ increased with soaking time in the initial 4 days. The concentration for the two 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 an descendent tendency. After 4% and 8% GTG composite soaked in distilled water for 7 days, the concentration of PGE₂ for the two groups was even lower than that of blank group.

The curve of 12% GTG composite had the same tendency with the previous two curves but 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 abnormal high of the concentration in a range of 350~475 pg/ml and was higher than that of blank group at each soaking period.

The actions of PG (prostaglandins) on bone cells are complex and contradictory, depending on the class of PG and the species studied. The PGE, especially PGE₂, reduce the activity of both osteoblasts and osteoclasts. And abundant PGE₂ would release from osteoblasts, while the osteoblasts were damaged by injuring [38,39]. The results of the PGE₂ concentration in the cultural media implied that the residual glutaraldehyde existed in the extracts had a negative influence for the osteoblast but might be no significant harm for the extract with lower concentration of residual glutaraldehyde. The concentration of PGE₂ for the three 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 the three 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 lead the concentration of PGE₂ to the maximum value at that time. It needed 14 days for the 12% GTG composite to vanish 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 Figure 1 and Figure 2, there were obvious complementary distribution existed between TGF-β₁ and PGE₂ for the three experimental GTG composites. In the initial stage, the curve for the PGE₂ showed a upward while the curve for TGF-β₁ showed a downward tendency. Once reached a lowest value, the curve of TGF-β₁ concentration turned into a ascent when the curve of PGE₂ went to an obvious descent after highest value.

Several studies have been introduced to evaluate the cytocompatibility of various biomaterials by observing the morphological changes of cells with optical microscope, detecting different degrees of cell loss, patterns of injury, or nuclear and cytoplasm damage. The methods were described as the previous study to evaluate the effect of gelatin solution on cell proliferation with the optical microscope. All the 14-day soaking time extracts of the three experimental groups materials were tested in this study. After 14-day extract of GTG composites cocultured with osteoblast 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 minutes, and then they were stained with hematoxylin/eosin.

The morphologies of osteoblasts for the blank group was shown in Figure 3(a). Figure 3(b), Figure 3(c), and Figure 3(d) showed the morphology of osteoblasts for 4%, 8%, and 12% GTG composites, respectively. The morphology of nuclear and cytoplasm in osteoblasts were no significant difference between the blank group and the GTG composites of 4% and 8%. Compared with blank group, 12% GTG composite showed a lower population of osteoblast and had an abnormality of cellular morphologies such as contracted cytoplasm structure as observed in the dish. The osteoblasts in 12% GTG composite just attached on the well ground and there is no sign of 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.

3-2. 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 unable to conduct in vitro experiments on assumption that they can replace absolutely the complexity of in vivo environment. However, if in vitro experiments can be used to mimic aspects of the known in vivo response to bioactive materials, then it may be possible to use such methods to unravel the biological reactions which occur at material surfaces.

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

After cultured 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 Figure 5(a) and Figure 6(a), respectively. From the results of histomorphmetric evaluation, the percentage of the new generation bone for the two controlled groups was no statistical difference 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 two controlled groups at the first-week culture. The percentage of the new generation bone area for the three 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 4% GTG composite was shown in Figure 7. The new bone tissue was gradually growing around

the peripheral area inside of the pore.

After cultured for 3 weeks, the two controlled groups showed a slow growth on the new generation bone about 23.30% and 36.05% for deprotienized bovine and porcine bone. At the same cultured period, the percentage of new generation bone for the three experimental groups had a progressive increase 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 as shown in Figure 8 and Figure 9.

At the 5th weeks, the two controlled groups showed a great amount increase in the area of new generation bone about 63.35% and 73.57 for deprotienized bovine and porcine bone. There was only a small amount of increase for the 4% and 8% GTG composite 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 cultured period of all the test materials could easily examined by the optical microscope as shown in Figure 5~Figure 10. The statistical analysis of the new generation bone for all the test materials in each cultured period were summarized in Table 2 in detail.

4. 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 [41]. Such potential testing procedures are of considerable importance in the biomaterials field since, not only could they be adapted to provide a biological bath-testing assay for bioactive bone-substitute materials, but they also provide a means of investing 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 materials variables, 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 of deproteinized bovine and porcine bone had the same crystallographic composition about 93% of hydroxyapatite and 7% of tricalcium phosphate. However, it revealed a difference in the amount of new bone tissue for the two control groups after culture period up to 3 weeks and 5 weeks. The major difference between the two controlled groups was pore size. Several studies have been introduced to evaluate the biological effects of various biomaterials with different pore size. However, the works can not show the befitting pore size for the effective ingrowth of bone into porous ceramic structures. Klawitter and Hulbert[43] reported that the optimum pore size required for the ingrowth of new bone was about 100-300 μm . K. de Groot [44] showed that the optimum pore size for bone in growth was about 200-500 μm . In the 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 that was too large and difficult to colonize host bone tissue on the peripheral area inside of the pore. The author speculated that the difference of bone in growth between the two controlled groups was based on the difference of pore size.

GTG composites is a biodegradable biomaterial composed of tricalcium phosphate and glutaraldehyde cross-linked gelatin that was designed for large bone substitute. At the first week of organ culture, the area of new generation bone for 4% and 8% GTG composite was much higher

than that of the two controlled groups (Table 2). For the 12% GTG composite, it showed lower percentage of new generation bone in the pores as the two controlled groups. As stated in section 3-1, the concentration of TGF- β_1 in the medium for the 4% and 8% GTG composite was over than that of the blank group after 7-day soaking. The phosphorus ion, calcium ion 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 blank group. The unchained glutaraldehyde was releasing from the composite that would lead to lower colonized bone tissue from the fetal rat calvara.

At the 3rd weeks of organ culture, the new generation bone for all the test materials showed a progressive growth. The new bone area for the 4% and 8% GTG composites were much higher than that of the other three 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 composite was higher than that of the blank group and the concentration PGE2 was lower than that of the blank group. It reflected that the 4% and 8% GTG composite could not only be no harm for the bone tissue but also promote new bone formation.

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

for the new bone prosperous growth at the 5th week of calvaria culture.

5. Conclusion

Except in initial 4 days, the analysis of TGF- β_1 for the 4% and 8% GTG composites was higher than that of the blank group for the other experimental time period. The PGE2 analysis for the 4% and 8% GTG composites was lower than that of the blank group as did for TGF- β_1 . It revealed that the 4% and 8% GTG composites would not lead to inflammation and would promote the osteoblast growth. The morphology and activity of the osteoblast were not transferred or changed by the two GTG composites. For the 12% GTG composite, the performance of in vitro condition was far behind the blank group and the other two GTG composites. Though the concentration of TGF- β_1 and PGE2 was gradually back to normal after 14 days, the morphology of the osteoblast showed an abnormality of cellular morphologies such as contracted cytoplasm structure. The osteoblast perhaps be damaged 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 or the composites with concentration of glutaraldehyde solution higher than 12% was not recommended as the 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 expect to predict the results of animal experiment in the future.

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Captions

Figure 1

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

Figure 2

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

Figure 3

The optical microscopic examination of the fetal rat osteoblasts cocultured with the GTG composites 14-days soaking extracts for 2 days. (a) PBS, (b) 4% GTG, (c) 8% GTG, (d) 12% GTG. Original magnification x 200

Figure 4

Pore size in the five different tested materials. Values are mean $\pm$ standard deviation. (N=3). Pyrost = deproteinized and defatted bovine cancellous bone matrix, Porcine= deproteinized and defatted porcine cancellous bone matrix, GTG4%= GTG composite treated with 4% glutaraldehyde, GTG8%= GTG composite treated with 8% glutaraldehyde, GTG12%= GTG composite treated with 12% glutaraldehyde.

Figure 5

Porosity in the five different tested materials. Values are mean $\pm$ standard deviation. (N=3). Pyrost = deproteinized and defatted bovine cancellous bone matrix, Porcine= deproteinized and defatted porcine cancellous bone matrix.

Figure 6

Percentage of the new ingrowing bone tissue area in pore size. Following the concentration of glutaraldehyde solution used as cross-linking agent increases, the value gradually decreases.

Figure 7

Optical microscopic examination showing the appearance of new bone tissue increasingly ingrow along the edge of material pore.

NB: new bone tissue, M: material. Original magnification x40.

Figure 8

Optical microscopic view of calvaria after grafting Pyrost specimen. (a) culturing for one week, (b) culturing for three weeks, (c) culturing for five weeks.

C: calvaria, P: Pyrost, NB: new bone tissue. Original magnification x10

Figure 9

Optical microscopic view of calvaria after grafting Porcine specimen. (a) culturing for one week, (b) culturing for three weeks, (c) culturing for five weeks

C: calvaria, P: Porcine, NB: new bone tissue. Original magnification x10

Figure 10

Optical microscopic view of calvaria after grafting 4%GTG specimen. (a) culturing for one week, (b) culturing for three weeks, (c) culturing for five weeks

C: calvaria, G: 4%GTG, NB: new bone tissue. Original magnification x10

Figure 11

Optical microscopic view of calvaria after grafting 8%GTG specimen. (a) culturing for one week, (b) culturing for three weeks, (c) culturing for five weeks

C: calvaria, G: 8%GTG, NB: new bone tissue. Original magnification x10

Figure 12

Optical microscopic view of calvaria after grafting 12%GTG specimen. (a) culturing for one week, (b) culturing for three weeks, (c) culturing for five weeks

C: calvaria, G:12%GTG, NB: new bone tissue. Original magnification x10

Table 1:

The porosity of five tested specimens. Values are mean(standard deviation). (N=3).

Table 2:

Ratio of ingrowth to total pore area. Values are mean(standard deviation). (N=3).