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Biological effects and cytotoxicity of the composite composed by tricalcium phosphate and glutaraldehyde cross-linked gelatin

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Abstract

The purpose of this study was to prepare and evaluate the feasibility and cytocompatibility of a composite (GTG) as a large defect bone substitute. The composite is combined with tricalcium phosphate ceramic particles and glutaraldehyde cross-linked gelatin. Gelatin had been reported as an adhesive and biocompatible binder that could accelerate the recovery of damaged soft tissue, but the effects of gelatin when acting on the bone tissue is not clear. Thus, it is necessary to determine if the substances released from the GTG composite can facilitate the growth of bone cells. The substances released from the GTG composites after being soaked in deionized distilled water were analyzed by gas chromatography (GC), ultraviolet and visible absorption spectroscopy (UV-VIS), and inductive-coupled plasma-atomic emission spectrometry (ICP-AES). The cytotoxicity of the GTG composites was assessed by coculture of rat osteoblasts in vitro. Extracts were obtained by soaking the GTG composites in deionized distilled water for 1, 2, 4, 7, 14, 28 and 42 d. The extract mixed with complete medium in a ratio of 1:1 was added into the cell culture wells containing 1×10^4 cells ml^{-1} osteoblasts. After culturing for 2 days, the cells attached to the surface of wells were trypsinized and the number calculated by the Neubauer counting-chamber under the optical microscope. Finally, three samples in each GTG group were examined by scanning electron microscopy (SEM) to observe the morphology of the osteoblasts attached to the surfaces of GTG composites. The examinations of osteoblasts cocultured with the developed GTG composites were used to decide the ideal concentration of glutaraldehyde as a cross-linking agent. The results of extracts cocultured with osteoblasts showed that the extracts obtained from the 2, 4 and 8% glutaraldehyde cross-linked GTG composites would inhibit the growth of osteoblasts in the first 4 soaking days. During the 4–7 days soaking, the cell numbers quickly increased with the soaking time, thereafter, the cell numbers almost reached a constant value. In the analyses of substances released from the GTG composites, it was found that the gelatin and calcium were gradually released from the GTG composites, which were supposed to be nutritious for the growth of the osteoblast. The results of osteoblasts cocultured with the GTG composites showed that the concentration of glutaraldehyde used as a cross-linking agent should be lower than 8%. Compared to the GTF (composite combined with tricalcium phosphate ceramic particles and formaldehyde cross-linked gelatin), GTG composites were much suitable for a large defect bone substitute in the near future.

Keywords: Bioabsorbed bone graft; Glutaraldehyde; Cytotoxicity; Tricalcium phosphate; Gelatin; Osteoblast

1. Introduction

Various shapes and sizes of bone defects caused by trauma, tumors and infections must be filled with suitable substances to promote bone repair. The ideal function of the refilled biomaterial was to lead the migration of bone cells towards and then inside the biomaterial, to replace the implant by the production of induced cells, and to fill the cavity with natural bone [1–4].

A number of prosthetics for accelerating bone repair had been reported. The most common clinical bone substitutes are autografts, allografts, polymers such as polymethylmethacrylate (PMMA) and ceramics such as dense and porous hydroxyapatite (HA), tricalcium phosphate ceramics and bioglass ceramic in the system of Na_2O– CaO–SiO_2–P_2O_5 [5–7]. But none of the materials presently available is entirely suitable for orthopedic applications, each suffering specific disadvantages.

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Although the biocompatibility and osteoinductive potentiality of the autograft material is the preferred bone-substitute material, because its immune response is precluded and it contains bone morphogenic proteins, there are restrictions in its application [8–10]. These restrictions include limited donor bone supply and additional surgical trauma created by the donor site. Since eliciting an immunologic response due to genetic differences and the risk of viral transmission are frequent, transplantation of living tissue by means of allogenic or xenogenic grafts are also limited in their medical applications. Because of the disadvantages of natural grafts, the attention has been focused on the development of artificial materials in the last decades [11, 12].

Clinically, bioactive ceramics are most widely used and investigated. These bioactive ceramics scaffold for repairing large skeletal defects were usually used in dense and porous form [13–15]. The results of a large number of investigations confirm that bioactive ceramics have good biocompatibility and osteoconductive potentiality with the porous structure caused by resorption process or manual treatment. It has been reported that the use of block forms of porous hydroxyapatite (HA) to reconstruct atrophic residual mandibular ridges results in an unacceptably high failure rate in human clinical applications. The use of granular instead of block forms of HA has therefore been suggested. But whichever form is used, HA has problems due to lack of biodegradability. As a result, β-tricalcium phosphate (TCP) ceramics have been developed as a biodegradable bone replacement. However, it was difficult to be kept in the recipient site compared to the convenient fabrication and operation of block-type ceramics. Thus, it is necessary to mix the granular TCP with an adequate binder. Recently, the composites of ceramics powder with natural degradable polymers have attracted much interest as bone implants [16–22]. Fibrin, collagen and gelatin which have good biocompatibility, adhesiveness and plasticity properties are habitually considered as binders. Clinically, xenograft collagen or gelatin have been used as effective biomaterials [4, 22–24]. Indeed, in a recent study, Lin et al. [25, 26] showed implants of GTF (formaldehyde cross-linked gelatin and tricalcium phosphate) to be osteoconductive and bioabsorbable for filling small irregular defects. In the result of degradable rate determination of GTF, the complete loss of implant was found at the sixth week after operation in the rabbit condyle area. In order to repair large bone defects and to promote new bone formation, a new composite (GTG) combined with glutaraldehyde cross-linked gelatin and tricalcium phosphate (TCP) was developed in this study. The weight ratio of TCP and gelatin in the composite was 3:1 which was equivalent to that of inorganic/organic in natural bone [24, 25]. Gelatin cross-linked by glutaraldehyde has been used in clinical applications and has proved to be efficient for more than 20 years. For instance, valve bioprostheses made of bovine pericardium or porcine heart valve tissues were usually treated by glutaraldehyde [27, 30].

The clinical success of any implant is directly dependent upon the cellular behavior on the surface of the biomaterial or in the immediate vicinity of the interface between the host and implants [31–41]. Therefore, in order to study their biodegradability and to evaluate whether the composites can promote the growth of new bone, the composites degradation rate, the effects of gelatin on osteoblasts, and the attachment number as well as morphology of osteoblasts on the composite surfaces, were examined in the present study.

2. Materials and methods

2.1. Materials preparation

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 a SiC-element furnace at 1000°C for 1 h, and then cooled down to room temperature. The sintered ceramic powder was crushed in the alumina grinding bowl and sieved in the 40–60 mesh. The TCP ceramic particles of grain size 200–300 μm were obtained for material preparation. Fig. 1 shows the X-ray diffraction pattern of the ceramic which is pure and well-crystallized symbol β-TCP.

The gelatin powder, provided by Sigma Chemical Co., USA, was extracted and purified from the porcine skin with the average molecular weight of about 60 000–100 000 Da measured with SDS-PAGE. In the present study, the gelatin powder was weighted and dissolved in the deionized distilled water until a homogeneous 16.7% gelatin solution was attained. The dissolution process was kept at 65°C using a water bath. The gelatin solution must be stirred continuously, and then the sintered TCP ceramic particles were poured down and mixed with the gelatin solution at a constant temperature.

A series of glutaraldehyde solutions in different concentrations were added to the ceramic/gelatin mixture for gelatin matrix cross-linking. After the mixtures were cross-linked, the cylinder composite was molded and dried in an oven overnight for later experiments. 0.5 wt% KBr powder was added to both the GTG composite and the TCP powders and the mixture was then analyzed in the FTIR diffuse reflectance mode.

2.2. Preparation of extracts from GTG composites

The GTG composites were shaped into a cylinder specimen with 6 mm diameter and 2 mm height. The total surface of each cylinder specimen exposed to the soaking solution is about 4.71 $cm^2\,ml^{-1}$. Each sterilized

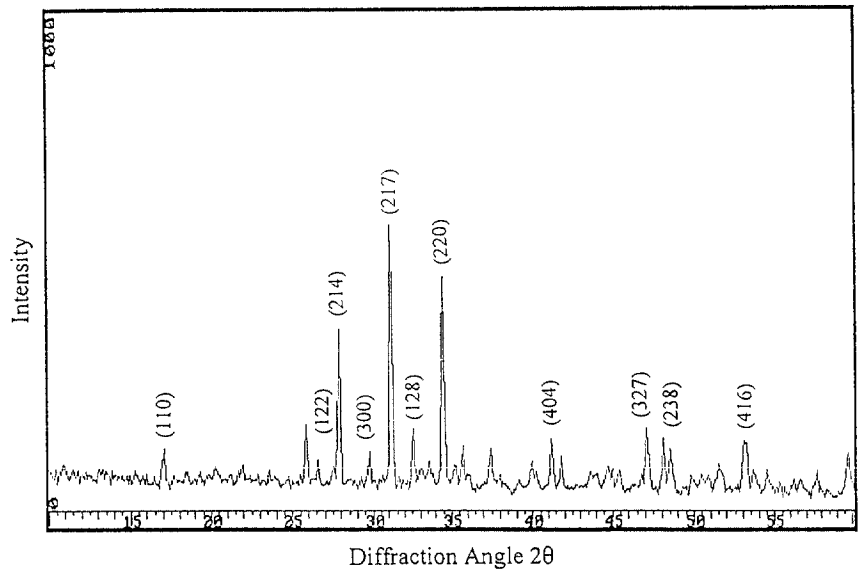

Fig. 1. X-ray diffraction pattern showed that the ceramic powder used in the study maintained its well-crystallized β-TCP after sintering in 1000°C for 1 h.

GTG composite sample was placed in a capped plastic test tube with 20 ml deionized distilled water, and all the test tubes were kept in an incubator at 37°C [33, 34]. After soaking for 1, 2, 4, 7, 14, 28 and 42 d, the extracts were collected for uses in cell culture examination, gas chromatography (GC), ultraviolet and visible absorption spectroscopy (UV/VIS), and inductive-coupled plasma-atomic emission spectrometry (ICP-AES).

2.3. Analysis of extracts from GTG composites

To determine the cytological effects of the extracts of GTG composite on osteoblast, the content of possible constituents in extracts was measured. The calcium concentration in individual extracts was determined by ICP-AES, and the gelatin content was measured by a UV-visible light spectrophotometer (Hitachi U-2000) at 194 nm [25, 26]. Gas chromatography (GC) was used to detect the amount of uncross-linked or unchained glutaraldehyde in the series of GTG composites.

2.4. Cell culture

Rat osteoblasts were enzymatically isolated from neonatal rat calvaria. The calvaria of Wistar rats (N.T.U., Taipei, ROC) was excised, stripped of soft tissue and washed three times in a phosphate buffer solution (PBS). Then it was digested in collagenase solution for 2 h. The cells from the digestion were pooled, washed, resuspended in a tissue culture medium (Eagle's minimum essential medium supplemented with 10% fetal calf serum and 1% antibiotics) and then plated in plastic culture dishes [40].

One milliliter of 1×10^4 cells ml^{-1} osteoblasts were seeded in individual wells of a 24-well tissue culture plate for testing the effects of gelatin and calcium on the growth of osteoblasts, and cytotoxicity of the extracts from GTG composites. In the present study, a series of gelatin and calcium solutions in different concentrations of 0.1–1 mg ml^{-1} with 0.1 mg ml^{-1} increments were prepared to test the effects of gelatin and calcium for osteoblasts.

To evaluate the effects of gelatin, calcium and extracts on the growth of osteoblasts, the complete medium was replaced with mixed solutions of the medium and evaluated solutions in a volume ratio of 1:1, thus giving the final evaluated solutions a concentration of 50% [33, 42]. In control group, the phosphate buffer solution (PBS) was mixed with the complete medium in a ratio of 1:1 for cell cultures. Cell cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. After culturing for 2 d, the cells attached to the surface of the wells were trypsinized and counted by the Neubauer counting-chamber under the optical microscope [43].

2.5. Osteoblasts cocultured with GTG composites

The effects of fetal rat osteoblasts cultured upon unsoaked and soaked GTG composites were used to evaluate directly the cytocompatibility of GTG, and to decide the necessary soaking time to competely remove the unreacted glutaraldehyde. The sterilized GTG samples were placed in 24-well culture dishes and then 3×10^6 osteoblasts seeded in each well with complete medium. After culturing for 6 h, the medium was

removed and the tested samples were transferred into another clean 24-well plates. The cells attached to the surface of GTG were washed three times with PBS and trypsinized, resuspended, and then 0.5 ml trypan blue was added to make sure that the cells were still alive and the cell number counted.

Osteoblasts attached to the surfaces of different GTG samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h. They were then dehydrated through an ascending alcohol series, commencing at a solution of 70% alcohol, then dried in a critical point drier and sputter-coated with gold. All the specimens were examined using a JOEL JXA-804A scanning electron microscope.

3. Results and discussions

3.1. Infrared analysis

The FTIR spectras of the TCP and GTG composite were shown in Fig. 2. The FTIR spectrum of Fig. 2a showed the characteristic features of TCP powder prior to the combination with cross-linked gelatin. The intensities of well-pronounced absorption bands are at 1040 and 3765 cm^{-1} (H–O–H). The observed 1040 cm^{-1} band was assigned to the vibration of PO_4^{3-}. It existed in the FTIR spectras of both, the TCP powder and the GTG composites.

The spectrum of Fig. 2b showed the characteristic features of the GTG composite. Two primary new additional absorption bands (1536–1562 and 1659 cm^{-1}) appeared in this FTIR spectrum of TCP powder mixed with cross-linked gelatin, thereby suggesting the formation of cross-linkages among gelatin matrix. The appearance of the 1532–1562 cm^{-1} absorption band was due to the N–H stretching of the –NH–R group, and the appearance of the 1659 cm^{-1} band was the stretching mode of C=N vibration. These two additional absorption bands in the IR spectra could be observed in the pattern of TCP combined with glutaraldehyde cross-linked gelatin. It was supposed that cross-linked reaction occurred between glutaraldehyde and gelatin molecules. The appearance of C=O band at 1462 cm^{-1} in the spectrum of GTG

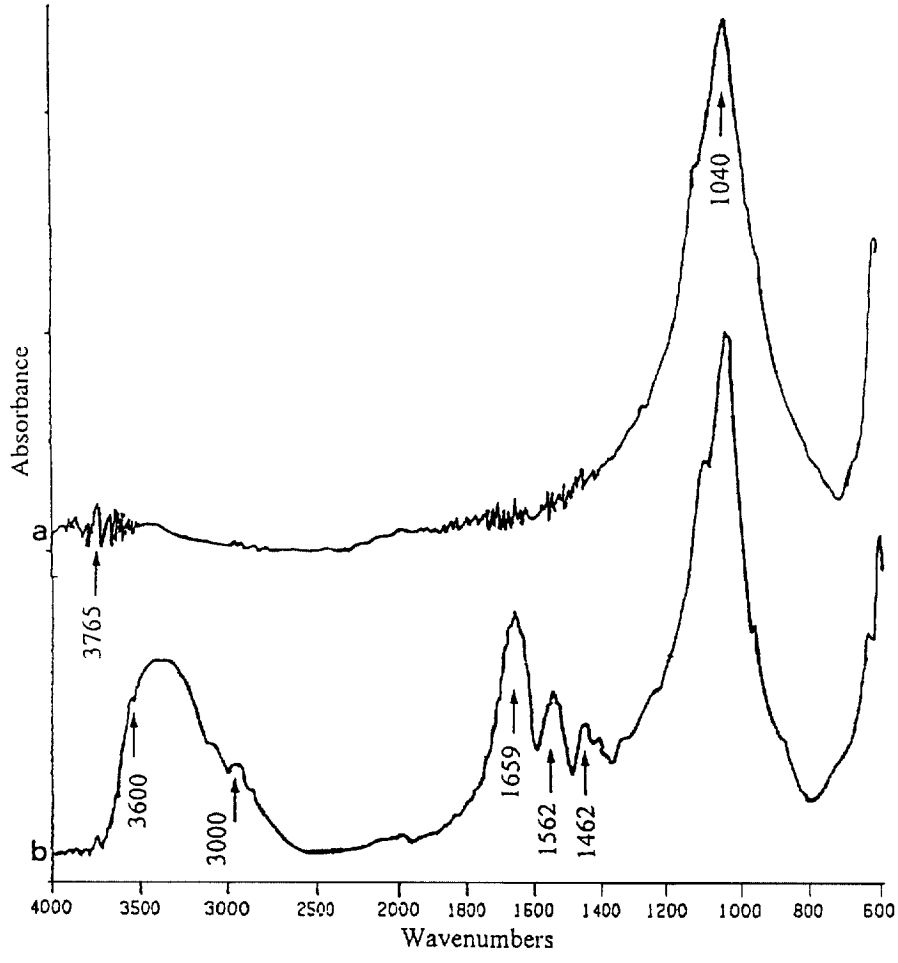

Fig. 2. The FTIR spectrum: (a) characteristic features of TCP powder prior to the combination with cross-linked gelatin, (b) characteristic features of GTG composite.

showed uncross-linked or unchained glutaraldehyde in the GTG composite. Another new broad band (3000–3600 cm^{-1}), not existing in the FTIR spectrum of TCP, corresponded to the stretching mode of the N–H vibration for aminoacids of gelatin. To sum up, cross-linkage would form after the gelatin combined with glutaraldehyde; however, some glutaraldehyde remained in the GTG composite.

3.2. *The residual glutaraldehyde in the GTG composites*

Fig. 3 showed the glutaraldehyde concentration in the extracts after a series at GTG composites were soaked in the deionized distilled water for 1, 2, 4, 7, 14, 28 and 42 d. The curves in Fig. 3 could be divided into three groups. In the first group, the glutaraldehyde concentrations in the extracts of GTG composite cross-linked by 1% glutaraldehyde were in a range of approx. 25–40 $\mu g\,ml^{-1}$. The second group was the extracts with a concentration of glutaraldehyde about 30 $\mu g\,ml^{-1}$ higher than that in the first group, which came from the GTG composites cross-linked with 2, 4 and 8% of glutaraldehyde. And the third group had the highest glutaraldehyde concentration in the range of approx. 65–90 $\mu g\,ml^{-1}$ in the extracts from the GTG composites cross-linked with 12% glutaraldehyde.

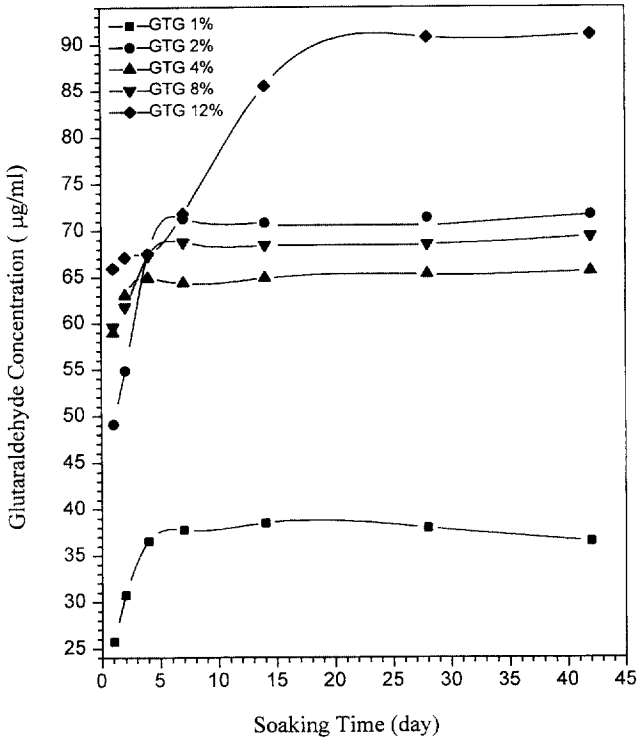

Fig. 3. The glutaraldehyde concentrations in the extracts after the series of GTG composites soaked in the deionized distilled water for 1, 2, 4, 7, 14, 28, and 42 d, respectively.

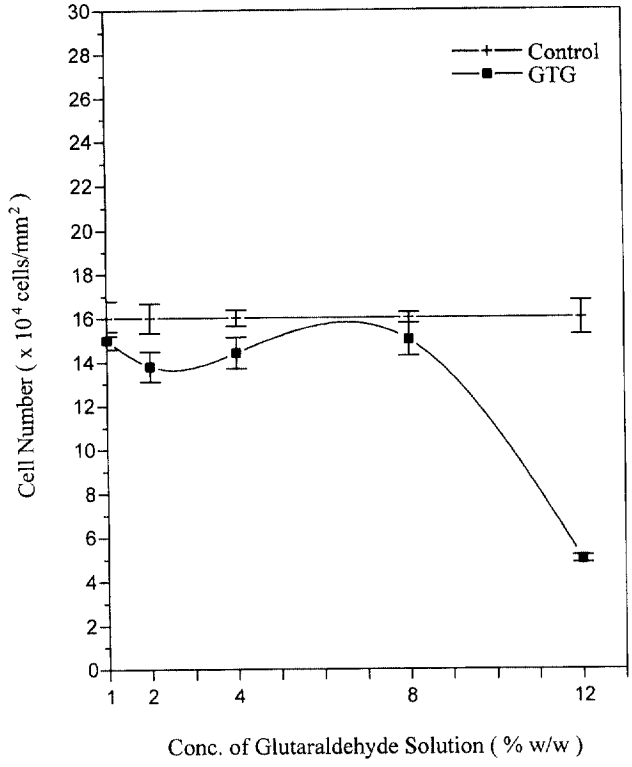

Fig. 4. The relationship between cell numbers being attached to the surface of GTG composites and glutaraldehyde concentrations used as a cross-linking agent for the preparation of GTG composites.

All the curves of the first and second groups in Fig. 3 demonstrated a common tendency, whereby the residual glutaraldehyde released from the various GTG composites reached a plateau after a soaking period of 4 d. The tendency reflected the fact that the residual or uncross-linked glutaraldehyde in the GTG composites would be completely released after the composites had been soaked in the deionized distilled water for 4 d. But the curve of the third group showed a significant difference in the soaking time period. It needed 14 days for the GTG composite cross-linked with 12% glutaraldehyde to completely release the residual glutaraldehyde. Moreover, the extracts of 12% glutaraldehyde cross-linked GTG composite always showed much higher glutaraldehyde concentration than that of the 1, 2, 4 and 8% glutaraldehyde cross-linked GTG composites in every soaking time period.

Fig. 4 showed the number of osteoblasts attached to the surface of various GTG composites without soaking treatment after culturing for 6 h. The cell numbers attached to the surfaces of 1, 2, 4 and 8% GTG composites and to the controlled group were almost with a constant with value of about 15×10^4 cells mm^{-2}, and the cell number for 12% GTG composites obviously decreased to 5×10^4 cells mm^{-2}. The higher the concentration of glutaraldehyde used as a cross-linking agent, lesser were

the number of osteoblasts that attached to the surface of the GTG composites. The result of a significant difference in cell attachment, between 12% GTG composite and 1, 2, 4 and 8% GTG composites indicated that the cytocompatibility was affected by the existence of these different concentrations of uncross-linked glutaraldehyde in the composites' surface. It is surprising that there were no significant differences in the number of osteoblasts present on the surfaces of 1, 2, 4 and 8% GTG composites. This finding could possibly suggest that the concentration of glutaraldehyde used as a cross-linked agent for GTG composites should be lower than 8%. Considering the cytocompatibility of these composites, the 1, 2, 4 and 8% glutaraldehyde solutions were considered to be harmless to osteoblasts and adequate to the cross-linked agent for GTG composites.

In order to determine the cytotoxicity of residual glutaraldehyde in the composites and how long it takes to completely remove the unchained glutaraldehyde during the soaking process, GTG composites were cocultured with osteoblasts for 6 h after the composites were soaked in deionized distilled water for a period of time. Cell attachment and the growth of osteoblasts on the surface of GTG composites were examined under an optical microscope. In Fig. 5 the curves showed the changes of cell number attached to the composite surfaces with different soaking days when treated with glutaraldehyde concentrations of 1, 2, 4, 8 and 12%, respectively. During the initial 4 d, the cell numbers for 1, 2, 4 and 8% GTG composites increased largely with soaking time. Also these four curves showed a common tendency that the cell number reached a plateau after a 4 d soaking period. The tendency reflected a fact that the residual glutaraldehyde in the GTG composites would be thoroughly released after the composites were soaked in distilled water for more than 4 d. The 12% GTG composite showed a lower cell number than that of the previous four groups, and the cell number reached a plateau after a 2-week soaking period. Since the cross-linking density was higher than that in the previous four groups, the authors supposed that a longer soaking time was needed to completely remove the residual glutaraldehyde from 12% GTG. The curves corresponding to GTG composites treated with a low concentration of glutaraldehyde showed no significant difference in cell numbers attached to the composite surfaces. However, the curve corresponding to the groups treated with a high concentration of high glutaraldehyde exhibited an apparent difference in cell attachment capacity when compared to groups treated with low concentrations of glutaraldehyde.

From the previous section, the authors could come to two important conclusions: (1) the glutaraldehyde cross-linking agent added into the GTG composite should be lower than 8% in order to abate the unreacted glutaraldehyde leaking out from the GTG composites; and (2) the glutaraldehyde cross-linked GTG composites should be soaked in distilled water for 4 d before preparing them for medical applications.

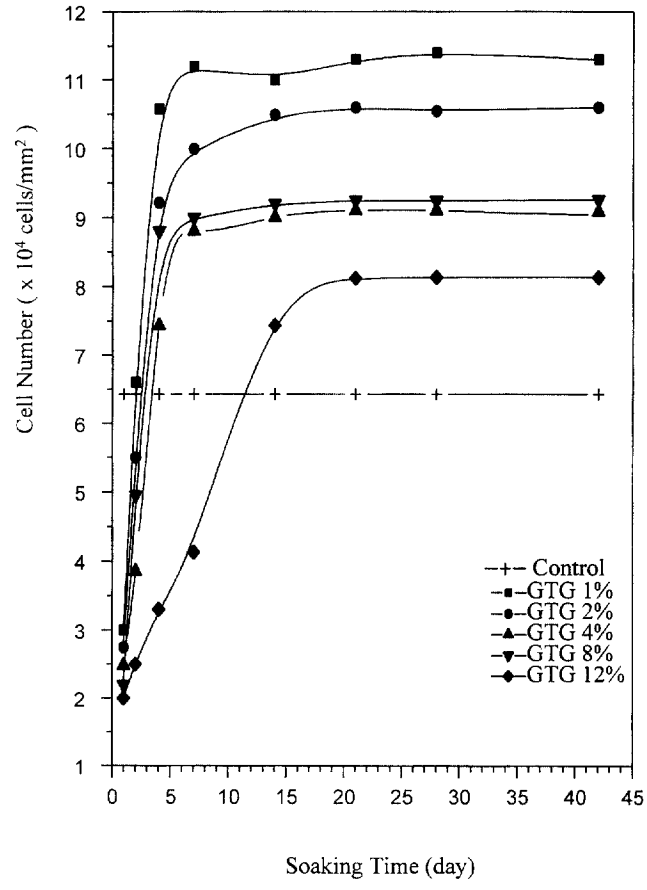

Fig. 5. The relationship between cell number being attached to the surface of the GTG composites and soaking time, after a series of GTG composites were soaked in deionized distilled water for a period of time and then cocultured with rat osteoblasts for 6 h.

3.3. *The effects of GTG extracts on osteoblast cell culture*

Fig. 6 showed the relationship between cell number and soaking periods after a series of GTG composites extracts were cocultured with 1×10^4 cells ml^{-1} fetal rat osteoblasts in each well of 24-well plates for 2 days. All the curves could be individually divided into three periods. In the first period, the cell numbers sharply decreased with the soaking time in the initial 4 d for 1, 2, 4, 8% groups and initial 14 d for the 12% group. At the end of the first period, all the cell numbers cocultured with the extracts reached a minimum value. As described in the previous section, we found that at 4 and 14 d of soaking time, the concentration of residual glutaraldehyde in extracts reached the highest point, which explained why the cell numbers reached the minimum value at that time. In addition, the results exhibited no significant difference in the cell numbers cocultured with the

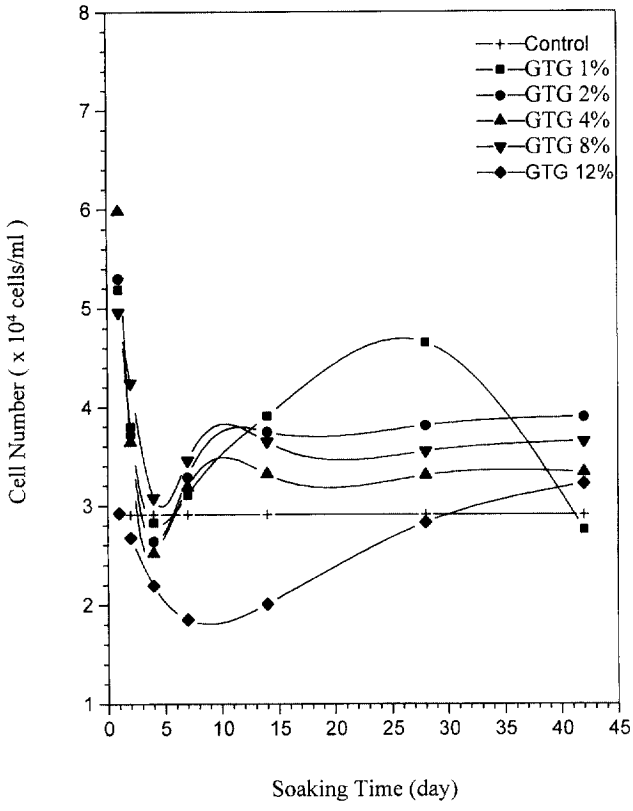

Fig. 6. The cell number in the extracts after a series of GTG composites extracts were cocultured with rat osteoblasts for 2 d.

4-d extracts of the GTG composites treated with 1, 2, 4 and 8% glutaraldehyde, but the 12% glutaraldehyde cross-linked GTG composite always showed less cell numbers than that of the previous four GTG composites.

In the second period of the 2, 4 and 8% glutaraldehyde cross-linked GTG composites, the curves exhibit an increase in the cell numbers and reach almost maximum values after the osteoblasts were cocultured with 7-day extracts for two days. The same tendency was observed in both curves of the 1 and 12% glutaraldehyde cross-linked GTG composites. However, the second period for the 1% glutaraldehyde cross-linked GTG composite was extended from the 4-day to 28-day, and for the 12% glutaraldehyde cross-linked GTG composite was from 14-day to 28-day much longer than that of the previous groups.

In the third period, the curves representing 2, 4, 8 and 12% glutaraldehyde cross-linked GTG composites, the cell numbers began to increase slowly after the soaking time over 7 or 28 days, and which were greater than that of the controlled group. The cell number of the controlled group was almost approx. 3×10^4, after being cultured for 2 d. An anomalous sharp decrease appeared in the curve of 1% glutaraldehyde cross-linked GTG composite after soaking for 28 days. The authors speculated that the growth of the osteoblasts was inhibited by the higher concentration of some elements released from the GTG composite.

As described in the previous section, the residual or unchained glutaraldehyde of the GTG composites were completely released after soaking in deionized distilled water for about 4 d for 1, 2, 4 and 8% groups and 14 d for the 12% group. The difference in growth of the osteoblasts reflected in Fig. 6 was in agreement with the previous observations as shown in Figs. 3 and 4. However, the osteoblasts show a progressive growth after coculturing with extracts soaked for over 4 or 14 d. It was speculated that some substances might be released from GTG composites during soaking in deionized distilled water for over 4 or 14 d and were possibly nutritious for the growth of osteoblasts.

3.4. The gelatin molecules released from the GTG composites and its effects on the growth of rat osteoblast

Fig. 7 shows the concentration of gelatin molecules in the extracts of a series of GTG composites soaked in deionized distilled water. All the curves in Fig. 7 demonstrated a rapid increment of the gelatin concentration in extracts, in the initial seven days. The gelatin concentration in the extracts was distributed between 0.1 and 0.4 mg ml^{-1}. All the series of GTG composites, except the

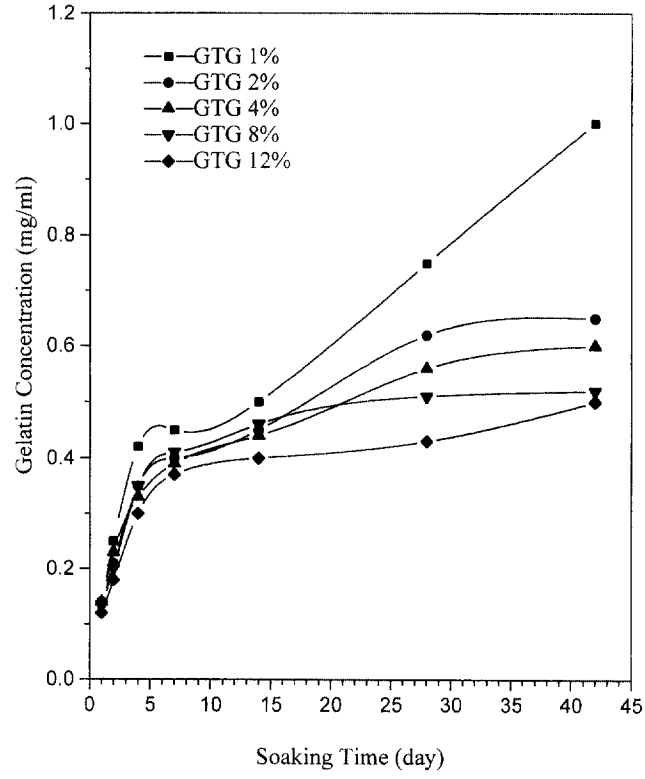

Fig. 7. The concentration of gelatin molecules in the extracts after a series of GTG composites were soaked in the deionized distilled water for a period of time.

1% glutaraldehyde cross-linked GTG composite, were soaked for over 7 d, and the gelatin concentration in the extracts increased to around 0.4–0.65 mg ml^{-1}. The gelatin concentration in the extracts of 1% glutaraldehyde cross-linked GTG composite soaked in deionized distilled water for 42 d reached approx. 1 mg ml^{-1}.

The results of the gelatin concentration in the extracts implied the relationship between the gelatin release rate and the soaking time of the GTG composites. The gelatin showed a regular release rate in the initial 7 d because the unchained gelatin was gradually released from the GTG composites during the period of soaking time. The gelatin concentration in the extracts, theoretically, should increase with the soaking time. The curves in Fig. 7, however, show a slower gelatin releasing rate. We thought that it might be related to the change in surface structure of the GTG composites. In the initial soaking stage, the gelatin covered on the GTG composites' surface gradually dissolved in the deionized distilled water and attributed to the gelatin concentration increase in the solution as shown in Fig. 7. The TCP ceramic particles appeared on the surface of the composites, thereafter. The surface of the TCP particles are covered with fine needle-like crystals when exposed to the deionized distilled water. It has been reported that the fine needle crystals precipitating on the surface of tricalcium phosphate were formed by the hydration of the calcium phosphate, and these crystals examined by XRD were identified as calcium-deficient HA [44]. HA crystal was supposed to be quite stable in distilled water and slowed the gelatin dissolution rate. We suggested that the degradable rate of the GTG composites might be attenuated by the effect of TCP gradually converting to HA in deionized distilled water.

The gelatin concentration in the extracts was in the range of 0.1–1.2 mg ml^{-1}. What would be the effect of gelatin concentration on the growth of rat osteoblasts? Fig. 8 showed the relationship between cell number and various concentrations of gelatin solutions. The curve could be divided into three different parts. In the first part, the concentrations of gelatin solution were in the range of 0.1–0.3 mg ml^{-1}. The cell numbers of the osteoblasts almost maintained a constant value of about 5.0×10^4 cells ml^{-1}, which was a little greater than that of the controlled groups (4.7×10^4 cells ml^{-1}). We found that the effect of these gelatin solutions within the range of 0.1–0.3 mg ml^{-1} on osteoblasts was not so distinct. The concentrations of these gelatin solutions were supposed to be too low to accelerate the growth of osteoblasts.

In the second part of Fig. 8, the cell numbers had a sharp positive tendency with the increase of gelatin concentration. The cell numbers in the gelatin solution with concentration from 0.4 to 0.9 mg ml^{-1} increased from 5.5×10^4 to 10×10^4 cells ml^{-1}, respectively. The results implies that the gelatin solution with concentration in the range of 0.4–0.9 mg ml^{-1} is a nutritious element for the osteoblast growth. When the GTG composites treated with 1, 2, 4 and 8% glutaraldehyde were soaked in the deionized distilled water for over 4 d, the gelatin concentration in the extracts are over 0.4 mg ml^{-1} as shown in Fig. 6. The results might give the answer why the cell number exhibited an increasing tendency with the soaking time when the soaking time was 4–7 d in the Fig. 6, and reached a maximum value afterwards.

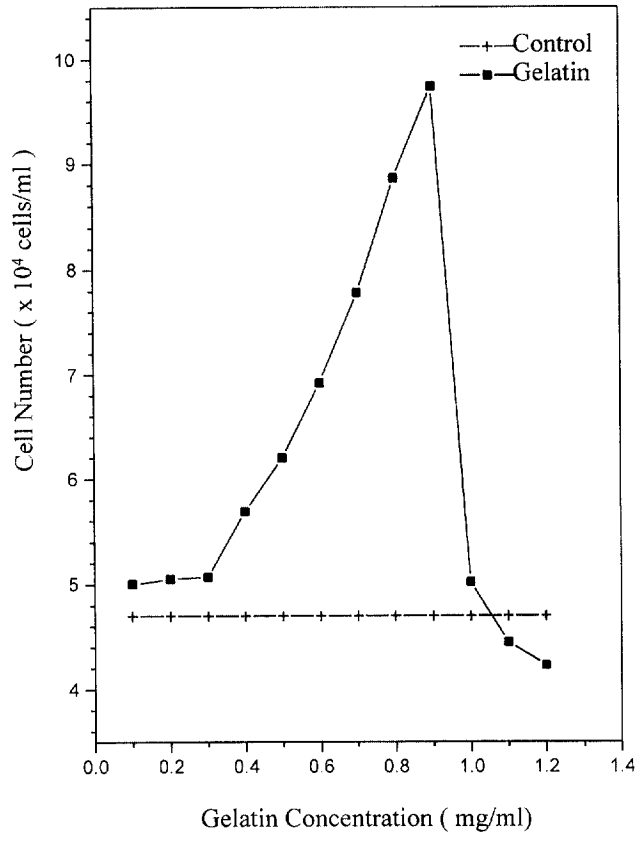

Fig. 8. The effect of gelatin concentration on the growth of fetal rat osteoblasts after culturing for 2 d.

If the gelatin concentration was over 0.9 mg ml^{-1}, what would be the effect of gelatin concentration on the growth of osteoblast? Is it promoted or inhibited? In the third part of Fig. 8, the curve showed that the cell number rapidly decreased when the gelatin concentration was above over 0.9 mg ml^{-1}, even lower than that of the controlled group for which the rapid decrease in the cell number occurs for a gelation concentration above 1.1 mg ml^{-1}. We supposed that it would turn into a negative factor for the osteoblast growth because osmotic pressure would exist between high concentration gelatin solution and osteoblasts, for gelatin concentrations over 0.9 mg ml^{-1}. The results showed that the concentration of gelatin less than 0.9 mg ml^{-1} would promote the growth of the osteoblasts and could be regarded as one of

the nutritious elements favorable to osteoblast growth. It was speculated that higher concentrations of gelatin, above 0.9 mg ml^{-1}, was the main contributing factor to inhibit the growth of osteoblasts cocultured with the extract of 1% glutaraldehyde cross-linked GTG composite soaked for 42 d in Fig. 6.

3.5. *The calcium ion released from the GTG composites and its effects on the growth of rat osteoblasts*

In the mechanism of bone-defect healing, calcium ion is a required element in the ensuing ossification process, because it is needed to reconstitute the mineral structure of renewal bone [2, 11].

The measurement of calcium content in the extracts was achieved by ICP-AES. Fig. 9 represents the calcium concentration in the extracts as a function of soaking time of GTG composites. We found that all the curves except the GTG composite cross-linked with 12% glutaraldehyde had the same tendency in calcium concentration at each soaking period. In the first 7 d, calcium ions are constantly released into the immersed solution for all the GTG composites. The calcium content in the extracts at this time period was about 20–40 μg ml^{-1}. Obviously, the presence of calcium ions in the extracts were due to the TCP ceramic particles dissolving in the deionized distilled water. Since gelatin decomposed and was released from the GTG composite as previously discussed, the TCP ceramic particles were then exposed to the solution. The unchained gelatin molecules were gradually released from the GTG composite, and then the exposed TCP particles kept dissolving in the solution. Thus would cause the calcium content to progressively increase in the extracts.

After 7 d, a lower calcium ion releasing rate was shown. The calcium ion concentration in the extracts even reached to a plateau by virtue of the HA crystals precipitating on the surface of tricalcium phosphate. We supposed that the TCP particles on the surface of the GTG composite would be converted into fine needle-like crystals which was thought to be stable in the deionized water. The phenomenon might be attributed to the slower calcium ion releasing from the GTG composites [44–46].

The same question should be raised: what would be the effects of calcium content in the extracts on the growth of the osteoblast? The authors also prepared a series of solutions differing in calcium content in the range of 10–60 μg ml^{-1}, with an increment of 10 μg ml^{-1}, and then cocultured them with the 1×10^4 cells ml^{-1} osteoblasts for 2 d. The result, as shown in Fig. 10, revealed that the cell numbers increased with the calcium content and the controlled group without calcium had a lowest value. It is possible that the calcium ion in the extracts would be beneficial to the growth of the osteoblasts.

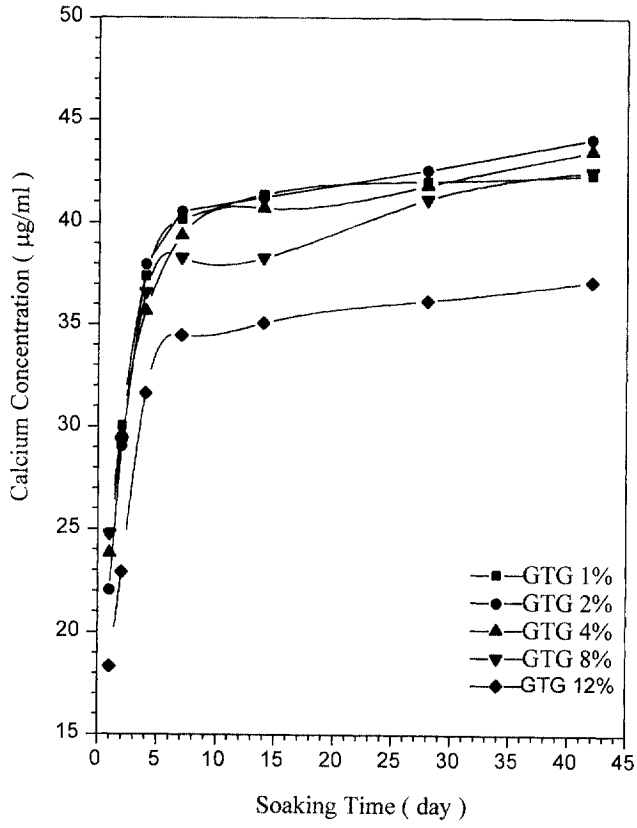

Fig. 9. The calcium concentration in the extracts after a series of GTG composites were soaked in deionized distilled water up to 45 d.

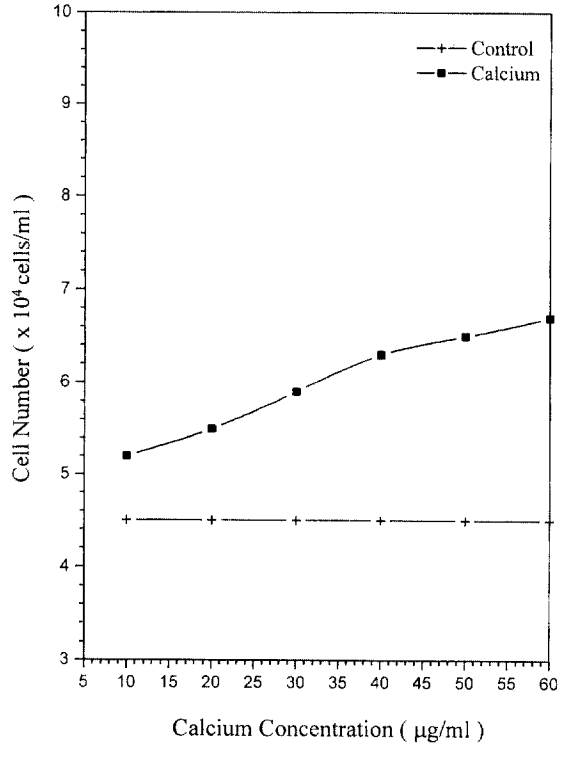

Fig. 10. The effect of calcium concentration on the growth of rat osteoblast after culturing for 2 d.

3.6. Morphology analysis

3.6.1. Morphological observation of effects of gelatin solution on osteoblasts

Several studies have been introduced to evaluate 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 [47–49]. After culturing for 2 d, 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 [42].

The effect of the gelatin solution on cell proliferation was observed under the optical microscope. Fig. 11a and b shows the morphology of the fetal rat osteoblast cocultured with PBS and a 0.5 mg ml^{-1} gelatin solution, espectively. The morphologies of nuclear and cytoplasm from osteoblasts did not show significant difference between the two tested groups. However, a higher population of osteoblasts were observed in the dishes with gelatin concentration of 0.5 mg ml^{-1} after 2 d of incubation. The cell number at 'time 0' is about 1×10^4 cells ml^{-1} in each well. After 2 d culture, the number for 0.5 mg ml^{-1} and the controlled group is about 5.6×10^4 and 4.7×10^4 cells ml^{-1}, respectively. The cells attached to the floor at the well were washed three times with PBS and trypsinized, resuspended, and then 0.5 ml trypan blue was added to make sure that the cells are still alive and the cell number was counted. The floor of the well was further examined under an optical

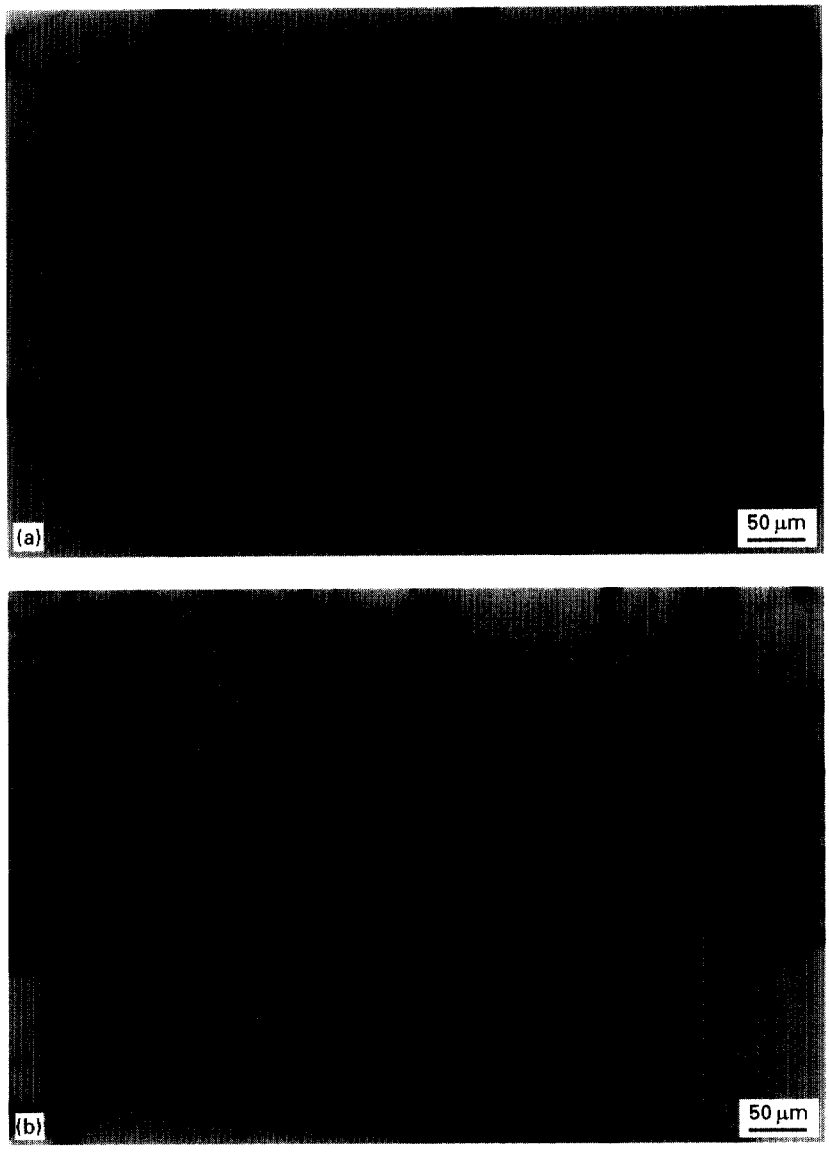

Fig. 11. The optical microscopic examination of the rat osteoblasts cocultured with gelatin concentration of (a) 0 mg ml^{-1} (PBS) and (b) 0.5 mg ml^{-1}.

microscope with a HE stain, to make sure no cells attached to it any more. Compared with control dishes, the dishes with 0.5 mg ml^{-1} gelatin concentration demonstrated a significant promotion of cell proliferation affected by gelatin.

3.6.2. Scanning electron microscopy

Cell morphology on the different types of material surfaces was usually used to determine the cytocompatibility of materials [50, 51]. SEM was used to investigate the phenomenological behavior of osteoblasts towards GTG composites in the present study. The whole process of adhesion and spreading consists of cell attachment, filopodial growth, cytoplasmic webbing, flattering of the cell mass and the ruffling of peripheral cytoplasm progression was in a sequential fashion [52]. Fig. 12a and b were the morphologies of the fetal rat osteoblasts attaching and adhering to the surfaces of unsoaked and 4 d soaked 4% glutaraldehyde cross-linked GTG composite, respectively. SEM examination revealed that the osteoblasts with flattened morphology were in the stage of ruffling of peripheral cytoplasm on the 4 d soaked 4% glutaraldehyde cross-linked GTG composite surface. However, the morphological type of osteoblasts on the surface of 4% glutaraldehyde cross-linked GTG composite without soaking treatment was just in the adhesion stage. The morphologies of osteoblasts attaching to the two tested composite surfaces in different adhesion and spreading stages were due to the residual glutaraldehyde existing in the GTG composites. The results showed that the residual glutaraldehyde in GTG composites would reduce the growth of the rat osteoblasts. We suggest that GTG composites need to be immersed in distilled water at least for 4 d to make sure all the residual glutaraldehyde is removed, before medical applications.

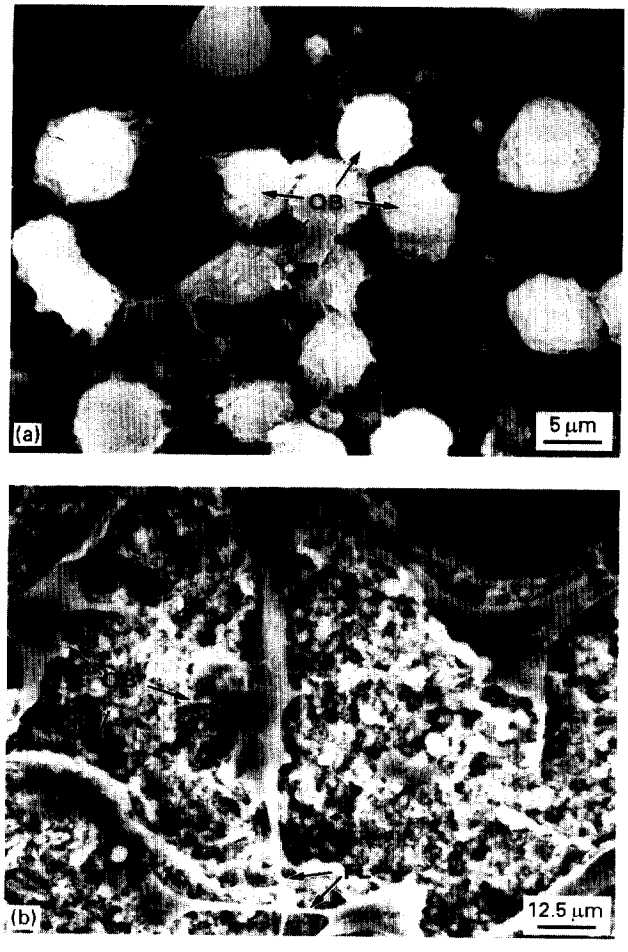

Fig. 12. SEM observation of osteoblasts attached and adhered to the surface of 4% GTG composites after culturing for 6 h. (a) 4% GTG without soaking treatment, (b) 4% GTG with 4 d soaking treatment. OB: osteoblast, GT: GTG composite, FL: filament.

4. Conclusion

We suggested that the glutaraldehyde solution used as a cross-linking agent and added to the developed GTG composites should be lower than 8% to decrease the toxicity to the growth of osteoblasts. The unchained or uncross-linked glutaraldehyde in the series of GTG composites, except 12% GTG composites, would be completely released by soaking in the deionized distilled water for 4 d. It was recommended to soak the developed GTG composites in the deionized distilled water at least for 4 d before clinical applications. After coculturing with 1–4 d extracts, the number of osteoblasts were lower than the control group. It was because residual glutaraldehyde was progressively released from the GTG composites and caused the concentration to increase. On the contrary, the cell numbers rapidly increased after the osteoblasts were cocultured with the extracts of GTG composites which were soaked for longer time. This was due to the release of calcium ions and gelatin from the GTG composites. Calcium ions and gelatin were released quickly from the GTG composites in the initial 7 d of their soaking in deionized distilled water, and the two concentrations reached a constant value thereafter. The gelatin concentration in the range 0.1–0.9 mg ml^{-1} showed a positive factor for the growth of rat osteoblasts. The calcium ion also played a positive role in the osteoblast growth, even at the concentration up to 60 μg ml^{-1}.

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