行政院國家科學委員會專題研究計畫成果報告

計畫名稱：The Effect of Sintered β-Dicalcium Pyrophosphate Particle Size on Newborn Wistar Rats Osteoblasts

（英、中文）不同大小焦二磷酸鈣顆粒對骨母細胞之影響

計畫類別：☒ **個別型計畫** □ **整合型計畫**

計畫編號：ＮＳＣ89－2320－B002－099

執行期間：88 **年** 08 **月** 01 **日至** 89 **年** 07 **月** 31 **日**

個別型計畫：計畫主持人：孫瑞昇

共同主持人：韓毅雄

整合型計畫：總計畫主持人：

子計畫主持人：

註： 整合型計畫總報告與子計畫成果報告請分開編印各成一冊，彙整一起繳送過國科會。

處理方式： ☒ **可立即對外提供參考**

□ **一年後可對外提供參考**

□ **兩年後可對外提供參考**

(必要時，本會得展延發表時限)

執 行 單 位 ： 台 大 醫 學 院 骨 科

中 華 民 國 八 十 九 年 十 月 十 三 日

計劃名稱：不同大小焦二磷酸鈣顆粒對骨母細胞之影響

英文：The Effect of Sintered β-Dicalcium Pyrophosphate Particle Size on Newborn Wistar Rats Osteoblasts

計劃編號：88－2320－B002－099

執行期限：88 **年** 8 **月** 1 **日至** 89 **年** 7 **月** 31 **日**

主持人：孫瑞昇

中文摘要：

關鍵詞：焦二磷酸鈣、顆粒大小、前列腺素、生長因子、骨母細胞。

由於近年來在生醫材料上的進步使的骨科手術亦有長足的進展。在本實驗室先期動物研究中：通常在手術後，骨骼細胞可緊密的長入焦二磷酸鈣殖入物間而沒有太多的纖維組織增生。本研究之主要目地即在澈定各種不同顆粒大小焦二磷酸鈣對骨母細胞之影響。

以不同顆粒大小焦二磷酸鈣(0.5 - 3.0 μm, 37 - 63 μm, 177 - 250 μm, 420 - 841 μm)**與大白鼠骨母細胞以** EDTA-Trypsin **打下後以** 1 x 10^5 cells/well **濃度置於** 3.0 x 3.0 cm **培養盤中。以** DMED + 10% FBS + penicilline & streptomycin **於** 37^oC **以** 5% CO_2 **培養** 24h **以促進骨母細胞之貼附。之後** 0.1%**四種不同顆粒大小焦二磷酸鈣**(1gm/ml)**與** DMEM + ITS **之混合培養液一同培養。於** 3h, 1, 3, 7 days **分別取出培養液，以** 500μl **分裝入** eppendorff **中，冰凍於零下** 80^oC**。並對細胞數、**TGF-β1**、**PGE_2**、等加以分析。**

結果顯示小顆粒焦二磷酸鈣(0.5 - 3.0 μm and 37 - 63 μm)**可明顯影響骨母細胞之細胞量而** TGF-β1, ALP **及** PGE2 **濃度呈現增加之現象。而此濃度呈現增加之現象在較小顆粒之焦二磷酸鈣更加明顯。**

經由此實驗，各種不同顆粒大小焦二磷酸鈣對骨母細胞之影響可以得到釐清。我們認為小顆粒焦二磷酸鈣粒子對骨母細胞細胞生長有抑制作用；此種作用可能經由抑制影骨母細胞之分化及PGE2**之合成而達成。**

英文摘要：

ABSTRACT

During the recent years, sintered dicalcium phosphate (SDCP) has been shown as an effective artificial bone fillers for repairing bone defects. The aim of this study is to elucidate the effect of SDCP particles size on the osteoblasts.

The osteoblasts were mixed and cultured with various sized SDCP particles (0.5 - 3.0 μm, 37 - 63 μm, 177 - 250 μm, 420 - 841 μm) for 1 hour, 3 hours, 1 day, 3 days, and 7 days, and then analyzed.

The results show that the adding of smaller sized SDCP particles (0.5 - 3.0 μm and 37 - 63 μm) into osteoblasts culture can significantly affect the cell counts of osteoblasts. The secretion of transforming growth factor-β1, alkaline phosphatase, and prostaglandin E_2 in culture medium increased significantly. The changes were most significant and persisted longer in smaller particle groups.

The small sintered dicalcium phosphate particles can inhibit the proliferation of the osteoblasts. The inhibitory effects of the smaller sized SDCP particles on the osteoblasts were mediated by the promotion of osteoblasts differentiation and the increased synthesis of prostaglandin E_2.

Keywords: sintered dicalcium phosphate, particle size, prostaglandin E_2, transforming growth factor, osteoblasts.

INTRODUCTION

Bone tissue damage, caused by trauma, tumor or infection, that creates bone defects must be filled with suitable substance to accelerate healing or enable them to heal. Autografts are preferably used because of their superior efficacy and capability of avoiding transmission of infection. However, it does have drawbacks including longer operation and anesthesia, higher blood loss, donor site morbidity and limited availability, particularly in children (1). The effectiveness of allogenous bone graft is limited by the problems of high cost of bone banking, potential of graft-related disease transfer, the high rates of nonunion and infection, and allograft fractures (1). To overcome these problems of natural grafts, research on bone-substitute materials has been developed. However, the biocompatibility and mechanical strength of synthetic materials have limited the success of these artificial biomaterials (2).

With advances in ceramics technology, the application of calcium phosphate materials as bone substitute has recently received considerable attention. They are remarkable biocompatible, provoke little, if any, inflammatory response, and have a bioactive property (3). During the last decade, a large number of biomaterials have been proposed as artificial bone fillers for repairing bone defects. Because similar calcium/phosphate (Ca/P) ratios to that of nature bone, hydroxyapatite (HA) and tricalcium phosphate (TCP) are two calcium phosphate most commonly used in the clinical medicine. HA and other complex calcium phosphate salts are the end products of the biological mineralization process. β-dicalcium phosphate (β-DCP), formula $Ca_2P_2O_7$, is one of the intermediate products in this process (4). The biological response for new bone formation is quite similar between β-DCP and HA (5). Sintered β-$Ca_2P_2O_7$ with $Na_4P_2O_7 \bullet 10H_2O$ addition (SDCP) was proved to has great potential as an in vivo biodegradable bone substitute (6,7).

The inertness of biomaterials is relative: even materials considered inert in bulk form are capable of eliciting inflammatory reactions in particulate form. It has been recognized that particulate debris around an orthopedic implant has an adverse effect on the surrounding tissues. It has been suggested that the adverse effects depend more on the particulate nature of the material than its chemical biocompatibility (8). In our previous study it has been demonstrated that the adding of HA particles into osteoblasts culture can significantly affect the behavior of the osteoblasts. The inhibitory effects on the osteoblasts were more significant and persisted longer in smaller particle groups (9). However, there is not yet general understanding whether SDCP particles might exert a similar harmful effect that is greater than that of their parent material. The aim of our investigation is to study the possible mechanism of various sized SDCP particles on the osteoblasts.

MATERIALS AND METHODS

Material tested

The powder of sintered β-dicalcium pyrophosphate (SDCP) with $Na_4P_2O_7 \bullet 10H_2O$ addition was prepared and briefly described as follows (6). The β-DCP powder (β-DCP: $Ca_2P_20_7$, Sigma, St. Louis, MO, USA) was mixed with 5 wt% $Na_4P_2O_7 \bullet 10H_2O$ in water and dried at 70°C for 3 days. The well mixed and dried cake was ground and sieved into 40-60 mesh particles. The sieved particles were placed in a platinum crucible and heated upto 930°C at a heating rate of 3°C/min in a conventional Ni-Cr coiled furnace and then maintained in air for 1 hour after the sintering temperature of 930°C was reached. The sintered particles were ground and separated with mesh into various grain size of about 0.5 - 3.0 μm, 37 - 63 μm, 177 - 250 μm, and 420 - 841 μm. SDCP particles were spherical in shape at various grain sizes according to scanning electron microscopic observation. Trace elements that might be connected with biocompatibility were detected by atomic absorption analysis. The concentrations of the trace elements were much lower than the maximum tolerable level. Prior to use, they were processed by dry heat sterilization (130^{o}C, 40 min) as previously reported (9).

Osteoblasts-like cell culture

Sequential digestion of newborn Wistar-rat calvaria was performed by using a modification of the methods previously described (9). Briefly, after pretreatment of the dissected calvaria with 4 mM Na_2-EDTA in a pre-warmed (37^{o}C) solution containing 137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4, pH 7.2 (solution A), for 10 minutes three times, the fragments were sequential digested with collagenase (180 U/ml, Sigma, St. Louis, MO, USA) in solution A with EDTA. The sequential digestion consisted of four times' treatments for 5 minutes followed by two times' treatments for 10 minutes. The cells released after each treatment were immediately harvested by centrifugation and re-suspended in culture medium. The osteoblastic phenotype of the cells was assessed by the tests of alkaline phosphatase assay.

Experimental procedures

In the experiment, confluent rat osteoblasts cultures were passage by trypsin-EDTA and then seeded into six 3.0 x 3.0 mm tissue culture wells (seeding density of 1 x 10^5 cells / well). The culture media used was Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY, USA), penicillin G sodium 100 units/ml and streptomycin 100 mg/ml (Gibco BRL, Grand Island, NY, USA). The dishes were incubated at 37^{o}C in an atmosphere supplemented with 5% CO_2 for 24 hours to facilitate the attachment of osteoblasts. The medium was then removed and washed twice with PBS solution. Dulbecco's modified Eagle's medium supplemented with ITS (Insulin, 10 μg/ml medium; Transferrin, 5 μg/ml medium and sodium selenite 5 x 10^{-3} μg/ml medium; Sigma, St. Louis, MO, USA) were mixed with 0.1% (1 mg/ ml) of various sized SDCP particles. The day of plating was considered as the zero day of culture. The test media were removed from wells at 1 hour, 3 hours, days 1, 3, 7. Divided into section of 500 μl with eppendorf, deep frozen in -80^{o}C till further analysis.

Cell count

After removal of the medium, the wells were fixed with 3% formaldehyde in 0.1 M PBS buffer (pH 7.4). The samples were stained with Hematoxyline-Eosin and then observed by inverted microscopy (Olympus, IMT-II, Japan). The cellularity of control and experimental wells were determined by MICD image analyzing system (MICD Software Series, Image Research Inc. Ontario, Canada). Briefly, the mean cell population in four randomly selected high-power fields (0.06 mm^2) were measured by counting the number of nuclei caught by CCD-72 camera (Dage-MII Inc., Michigan,

USA) through the microscope. Then the cell count per well was calculated by multiplying the area of each well (940 mm^2/well).

Analysis of transforming growth factor- 1 (TGF- 1) in culture medium

The production of TGF-β1 in culture medium was analyzed by ELISA methods. Briefly, 200 μl of standard or sample were added per well. The tested samples were incubate for 3 hours at room temperature on the benchtop. Then 200 μl of TGF-β1 (R & D System, Inc., MN, USA) conjugate was added. Incubate for 1.5 hour at room temperature. Two hundred μl of substrate solution were added to each well. The reaction was stopped and read by Microelisa reader (Emax Science, Sunnyvale, California, USA) at 450 nm after 20 minutes' Incubation.

Analysis of alkaline phosphatase (ALP) in culture medium

Alkaline phosphatase (ALP) activity released from the cells into the medium was measured with a commercially available assay kit (Procedure no. ALP-10, Sigma, St. Louis, MO, USA). Briefly, an aliquot (20 μl) from the media was mixed with 1 ml alkaline phosphatase reagent. The absorbance at 405 nm caused by p-nitrophenol production was followed for 5 minutes at 30°C. The change in rate of absorbance is directly proportional to alkaline phosphatase activity.

Analysis of prostaglandin E_2 (PGE_2) in culture medium

The production of prostaglandin E_2 (PGE_2) in culture medium was also analyzed by ELISA methods. Briefly, 50 μl of standard PGE_2 (Cayman Chemical Company, MI, USA) or sample was added per well. The tested samples were incubate for 18 hours at room temperature on the benchtop. Then 200 μl of Ellman's Reagent were added to each well. Incubate for 1.5 hour at room temperature. The reaction was stopped and read by Microelisa reader (Emax Science, Sunnyvale, California, USA) at 405 nm. The synthesis of PGE_2 by osteoblasts was measured by multiplying the concentration of PGE_2 with the volume of the medium then divided by the calculated cell population at various tested periods.

Statistical analysis

The differences between various sized SDCP particles were evaluated by an analysis of variances statistic method. The post hoc tests performed was Bonferroni's t test. The level of statistical significance is defined as $P<0.05$.

RESULTS

Cell count

The adding of SDCP particles into osteoblasts culture can significantly affect the cell counts of osteoblasts. The cell populations of control and that cultured with various sized SDCP particles were summarized in Table 1. The changes of cell population of various preparations at 1 hour, 3 hours, 1 day, 3 days and 7 days were all statistically significant ($P < 0.0001$ by ANOVA test) (Table 1).

The changes in cell population were quite similar between control and the groups with larger particle sized SDCP (i.e.: 177-250 μm and 420 - 841 μm), they increased in the first 3 days' culture and then reached a plateau at the 7 days' culture. When cultured with SDCP with smaller particle size (i.e. 0.5 - 3.0 μm and 37 - 83 μm), the osteoblasts populations were significantly lower than that of the control ($P<0.05$) (Table 1). The cell population of the smaller sized SDCP has less cell populations.

Transforming growth factor- 1 (TGF- 1) in culture medium

The transforming growth factor-β1 (TGF-β1) concentrations in the culture medium were not significantly affected, while the changes in the secretion of transforming growth factor-β1 (TGF-β1) by osteoblast-like cells were significantly affected by the addition of SDCP particles, especially when the particles are smaller in size. (Table 2).

The secretion of TGF-β1 by osteoblast-like cells in medium at 1 hour was low and not statistically significant ($P=0.4298$). The changes on 3 hours, 1 day, 3 days and 7 days were statistically significant ($P < 0.05$ by ANOVA test) (Table 2).

The secretion of TGF-β1 by osteoblast-like cells in control medium tested were quite similar between that of experimental medium with SDCP in 177 - 250 μm and 420 - 841 μm ($P > 0.05$). The TGF-β1 secretion by osteoblast-like cells in culture medium of the smaller sized SDCP (0.5 - 3.0 μm and 37 - 63 μm) were always significantly higher than that of the control medium after the 3rd hour of implantation and persisted till the 7th days' culture (Table 2). The increases of TGF-β1 secretion in culture medium was most significant in the groups with the smallest particle (i.e. 0.5 - 3.0 μm SDCP).

Alkaline phosphatase (ALP) in culture medium

The concentrations of alkaline phosphatase (ALP) in culture medium and secretion of ALP by osteoblast-like cells of various preparations were shown in Table 3. There was no statistically significant changes in ALP concentration of various sized SDCP particles while the changes of ALP secretion of various sized SDCP particles were all statistically significant except the 1st hour's culture ($P=0.08578$) (Table 3).

The secretion of ALP by osteoblast-like cells in control medium tested were quite similar between that with SDCP in 177 - 250 μm and 420 - 841 μm ($P > 0.05$), it decreased gradually till the end of 7th day's culture. The ALP secretion by osteoblast-loke cells in culture medium of the smaller sized SDCP (0.5 - 3.0 μm and 37 - 63 μm) were always significantly higher than that of the control after the 3rd hour of implantation and persisted till the 7th days' culture (Table 3). Similar to the changes of TGF-β1 secretion, increases of ALP secretion in culture medium was most significant in the groups with the smallest particle (i.e. 0.5 - 3.0 μm SDCP).

Prostaglandin E_2 (PGE_2) in culture medium

The concentrations of prostaglandin E_2 (PGE_2) in culture medium and secretion PGE_2 of by osteoblast-like cells of various preparations were shown in Table 4. The concentration of PGE_2 in the culture medium increased significantly immediately after adding of the SDCP particles and the changes were most significant in the smallest SDCP particles (Table 4).

The changes of PGE_2 secretion by osteoblast-like cells were also statistically significant ($P < 0.005$ by ANOVA test). The PGE_2 secretion by osteoblast-like cells

increased in the initial 3 hours, then rapidly decreased after 1 days' culture (Table 4). The changes of PGE_2 secretion by osteoblast-like cells tested with particle size 177 - 250 μm and 420 - 841 μm were quite similar to that of the control medium but at a little higher level (Table 4). The changes of PGE_2 secretion by osteoblast-like cells tested with smaller particle size (37 - 63 μm and 0.5 - 3.0 μm) was quite obvious and always higher than that other three groups (Table 4). The changes of PGE_2 secretion by osteoblast-like cells corresponded to the changes of cell population, TGF-β1 and ALP secretion when osteoblasts cultured with various sized SDCP particles.

DISCUSSION

During the last decade, a large number of biomaterials have been proposed as artificial bone fillers for repairing bone defects. The materials most widely used in clinical medicine have been hydroxyapatite (HA) ceramics (3). However, the use of block forms of porous HA results in an unacceptably high failure rate in clinical applications (10). We have investigated size effect of HA particles on the osteoblasts. We found that the adding of HA particles into osteoblasts culture can significantly affect the cellular behavior of osteoblasts and this effect was most significant and persisted longer in smaller particle groups (9).

The β-dicalcium phosphate is one of intermediate products in biological mineralization process of bone formation (4). Sintered β-$Ca_2P_2O_7$ with $Na_4P_2O_7 \bullet 10H_2O$ addition (SDCP) was proved to has great potential as an in vivo biodegradable bone substitute (6,7). Histological examinations after implantation to the rabbit's femur condyle showed direct deposition of new bone on the surface of the material and the implant decreased in volume gradually (6,7). We also investigated the biocompatibility of various calcium phosphate and found that HA has an inhibitory effect on the growth of osteoblasts while SDCP is more biocompatible to the osteoblasts (11). However, the effect of SDCP particles on the osteoblasts is not yet well elucidated. For the quantification of the effects of specific particles on cell metabolism, osteoblasts cell culture model in the presence of SDCP particles is performed.

The decrease in cell population after exposure can be used as marker of cytotoxicity of the tested material (12). It has been reported that that toxicity due to direct contact of particles with cells occurs only when the particles is smaller than 5 μm in diameter (13). In our previous study, the adding of HA particles into osteoblasts culture can significantly affect the cell counts and the decrease in cell populations occurs in all experimental groups of various sized HA particles; although the cell population of the smallest sized HA has the fewest cell populations (9). In this study, the adding of larger sized SDCP particles (i.e.: 177-250 μm and 420 - 841 μm) into osteoblasts culture did not affect the cell population; while when osteoblasts cultured with SDCP of smaller particle size (i.e. 0.5 - 3.0 μm and 37 - 83 μm), the osteoblasts populations were significantly lower than that of the control ($P<0.05$) (Table 1). The osteoblasts population were only affected by the smaller sized SDCP particles. The SDCP particles are more bio-inert than that of the hydroxyapatite. The mechanism of the cell damage in vitro can be depends on either a direct interaction between cells and particles (8) or leaching of toxic ions to inhibit the normal cell function (12). The effects of SDCP on the synthesis and secretion of specific protein were also evaluated in this study.

In this study, although the transforming growth factor-β1 (TGF-β1) concentrations in the culture medium were quite similar between all experiments, the secretion of transforming growth factor-β1 (TGF-β1) by osteoblast-like cells were significantly affected by the addition of SDCP particles (Table 2). In this study, the secretion of transforming growth factor-β1 (TGF-β1) by osteoblast-like cells were quite similar between that of experimental medium with SDCP in 177 - 250 μm and 420 - 841 μm (Table 2). In the 7 days culture of osteoblasts, we also did not observe any increase on secretion of alkaline phosphatase in these groups (Table 3). This fact reflected that the larger sized SDCP particles are not only bio-inert to the osteoblasts but also that our culturing condition was not optimal for a mineralization process. Similar to the changes of TGF-β1 secretion, increases of ALP secretion by osteoblast-like cells was most significant in the groups with the smallest particle (i.e. 0.5 - 3.0 μm SDCP). The TGF-β1 secretion of osteoblasts of the smaller sized SDCP (0.5 - 3.0 μm and 37 - 63 μm)

were always significantly higher than that of the control medium after the 3rd hour of implantation and persisted till the 7th days' culture (Table 2). The secretion of alkaline phosphatase (ALP) into culture medium of various preparations were quite similar to that of TGF-β1, it was always significantly higher than that of the control in culture medium of the smaller sized SDCP (i.e.: 0.5 - 3.0 μm and 37 - 63 μm) (Table 3).

The transforming growth factor-β1 has been shown to have stimulatory effects on osteoblasts (14), and the autoinductive processes can amplify and extend the activity of TGF-β1 (15). In the present study, the increases in the osteoblasts secretion of TGF-β1 by SDCP particles may reflect the fact that SDCP is not only bio-inert but also biocompatible to the osteoblasts. The osteoblasts have traditionally been considered to be responsible for bone formation; however, the origin of osteoblasts from pluripotential stem cells present in the bone marrow stroma exhibits various stages of differentiation features (16), and the ALP is expressed in the intermediate process of the osteoblasts differentiation (13, 17). As noted in the present study, the secretion of alkaline phosphatase (ALP) into culture medium of various preparations were quite similar to that of TGF-β1, it was always significantly higher than that of the control in culture medium of the smaller sized SDCP (i.e.: 0.5 - 3.0 μm and 37 - 63 μm) (Table 3). This means that the adding of the smaller sized SDCP particles not only stimulate but also promote differentiation of the osteoblasts. The growth rate of osteoblasts were determined by the death rate and their mitotic rate (16). The promotion of the osteoblasts differentiation by the biocompatible SDCP particles can partly explained the reason for the stationary changes in osteoblasts population after the adding of smaller sized SDCP particles (Table 1).

It is reported that smaller bone cement particles (less than twenty micrometers) resulted in more inflammation than large particles (fifty to 350 micrometers). Large particles induced a more intense rise in the white blood-cell count and in the production of prostaglandin E_2 (18). In the present study, the changes of PGE_2 concentration in culture medium corresponded to the changes of cell population, TGF-β1 and ALP secretion when osteoblasts cultured with various sized SDCP particles. The secretion of PGE_2 to culture medium tested with smaller particle size (37 - 63 μm and 0.5 - 3.0 μm) was always higher than that of the control and larger SDCP particles (Table 4). The increased synthesis and release of prostaglandin E_2 may act as an autocrine or paracrine factor. Also, the administration of prostaglandin E_2 in various in vitro and in vivo models has led to increased bone-remodeling of turnover (19), attributable to increased bone resorption (20, 21) or bone formation (22,23), or both. In this study, the changes of PGE_2 concentration in culture medium and secretion by osteoblast-like cells closely related to the changes of cell population when osteoblasts cultured with the various sized SDCP particles. It is reported that the ability of various cytokines (including interleukine-I, tumor necrotic factor and transforming growth factor-α) to stimulate bone resorption is mediated by increased prostaglandin E_2 synthesis and production of these cytokines is influenced by prostaglandins (24). The inhibition effects of the smaller sized SDCP particles on the osteoblasts cell culture were possibly mediated by the increases in the synthesis and secretion of prostaglandin E_2 by osteoblasts.

Sintered β-dicalcium phosphate is one of the bioceramics which has favorable biological response and great potential as an in vivo biodegradable bone substitute. In this study, the results about the effect of various sized SDCP particles on osteoblast-like cells manifest the possible in-vivo effect of degradation product of SDCP on the adjacent ingrowing osteoblasts. They suggest that after implantation of SDCP products which later break down into a fine powder fom will possibly promote osteoblast-like cells differentiation and alkaline phosphatase secretion. In this work, only the

biocompatibility of various sized SDCP particles to the osteoblasts were elucidated. Further studies on the the relationships of various sized SDCP particles to the osteoclasts were now in progress.

REFERENCES

1. Stupp SI, Hanson JA, Eurell JA, Ciegler GW, Johnson A. Organoapatites: materials for artificial bone. III. Biological testing. J Biomed Mater Res 1993; **27**: 301-311.
2. Yao CH, Sun JS, Lin FH, Liao CJ, Huang CW. Biological effects and cytotoxicity of tricalcium phosphate and formaldehyde cross-linked gelatin composite. Mater Chem Phys 1996; **45**: 6-14.
3. Jarcho M. Calcium phosphates as ceramics as hard tissue prosthetics. Clin Orthop 1981; **157**: 259-278.
4. Ducheyne P. Bioceramics: material characteristics versus in vivo behavior. J Biomedl Mater Res 1987; **21**: S219-S236.
5. Kitsugi T, Yamamuro T, Nakamura T, Kotani S, Kokubo T, Takeuchi H. Four calcium phosphate ceramics as bone substitutes for non-weight-bearing. Biomaterials 1993; **14**: 216-224.
6. Lin FH, Lin CC, Lu CM, Liu HC, Sun JS, Wang CY. Mechanical properties and histological evaluation of sintered beta-$Ca_2P_2O_7$ with $Na_4P_2O_7 \bullet 10H_2O$ addition. Biomaterials 1995; **16**: 793-802.
7. Lin CC, Liao CJ, Sun JS, Liu HC, Lin FH. Prevascularized bone graft cultured in sintered porous beta-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7 \bullet 10H_2O$ addition ceramic chamber. Biomaterials 1996; **17**: 1133-1140.
8. Evans EJ. Cell damage in vitro following direct contact with fine particles of titanium, titanium alloy and cobalt-chrome-molybdenum alloy. Biomaterials 1994; **15**: 713-717.
9. Sun JS, Liu HC, Chang WHS, Li J, Lin FH, Tai HC: The influence of Hydroxyapatite Particle Size on Bone cell activities: An In Vitro Study. J Biomed Mater Res 1998, 39: 390-397.
10. Hupp JR, McKenna SJ. Use of porous hydroxyapatite blocks for augmentation of atrophic mandibles. J Oral Maxillofac Surg 1988; **46**: 533-545.
11. Sun JS, Tsuang TH, Liao CJ, Liu HC, Hang YS, Lin FH. The Effects of Calcium Phosphates Particles on the Growth of Osteoblast. J Biomed Mater Res 1997; **37**:324-334.
12. Cheung HA, Haak MH. Growth of osteoblasts on porous calcium phosphate ceramic: an in vitro model for biocompatibility study. Biomaterials 1989; **10**: 63- 67.
13. Evans EJ, Clarke-Smith EMH. Studies on the mechanism of cell damage by finely ground hydroxyapatite particles in vitro. Clin Materials 1991; **7**: 241-245.
14. Bonewald LF, Murdy MD. Role of transforming growth factor beta in bone remodeling. Clin Orthop 1990; **250**: 261-276.
15. Sporn MB, Robert AB. Transforming growth factor-beta. Multiple actions and potential clinical applications. J A M A 1989; **262**: 938-941.
16. Martin TJ, Ng KW. Mechanism by which cells of the osteobast lineage control osteoblast formation and activity. J Cell Biochem 1994; **56**: 357 -366.
17. Zhou H, Choong P, McCarthy R, Chou ST, Martin TJ, Ng KW. In situ hybridization to show sequential expression of osteoblast gene markers during bone formation in vivo. J Bone Miner Res 1994; **9**: 1489 - 1499.
18. Gelb H, Schumacher HR, Cuckler J, Baker DG. In vivo inflammatory response to polymethylmethacrylate particulate debris: effect of size, morphology, and surface area. J Orthop Res 1994; **12**: 83-92.
19. Jee WS, Mori S, Li XJ, Chan S. Prostaglandin E sub 2 enhances cortical bone mass and activates intracortical bone remodeling in intact and ovariectomized female rats. Bone 1990; **11**: 253-266.
20. Saffar JL, Leroux P. Role of prostaglandins in bone resorption in a synchronized remodeling sequence in the rat. Bone 1988; **9**: 141-145.
21. Collins DA, Chambers TJ. Prostaglandin E sub 2 promotes osteoclast formation in

murine hematopoietic cultures through an action on hematopoietic cells. J Bone Min Res 1992; **7**: 555-561.
22. Jee WS, Veno K, Deng YP, Woodbury DM. The effects of prostaglandin E sub 2 in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. Calcif Tissue Internat 1985; **37**: 148-157.
23. Rasmussen H. The calcium messenger system (part 2). New England J Med 1986; **314**: 1164-1170.
24. Ibbotson KJ, Twardzic DR, D'Souza SM, Hargreaves WA, Todaro GK, Mundy GR. Stimulation of bone resorption in vitro by systemic transforming growth factor-alpha. Science 1985; **228**: 1007.

Table 1. The changes of cell population when osteoblasts cultured with various-sized synthetic Sintered β-Dicalcium Pyrophosphate (SDCP) bioceramics at the concentration of 1 mg/ml (N=10).

Cell count (x 10^4 / well)	I hour [a]	3 hour [b]	1 day [c]	3 day [d]	7 day [e]
A. (0.5 - 3.0 μm)	25.39 * (S.D. 8.06)	34.82 * (S.D. 12.35)	16.90 *** (S.D. 4.23)	15.37 *** (S.D. 8.76)	20.81 *** (S.D. 10.72)
B. (37 - 63 μm)	26.53 * (S.D. 8.93)	31.14 * (S.D. 14.43)	16.39 *** (S.D. 4.26)	33.30 *** (S.D. 14.55)	28.87 *** (S.D. 15.26)
C. (177 - 250 μm)	35.84 (S.D. 14.90)	44.56 (S.D. 13.93)	55.32 (S.D. 16.27)	137.92 (S.D. 19.71)	130.21 (S.D. 19.22)
D. (420 - 841 μm)	39.48 (S.D. 15.54)	49.81 (S.D. 17.95)	57.59 (S.D. 20.71)	146.68 (S.D. 27.57)	142.77 (S.D. 46.33)
E. Control	44.05 (S.D. 19.84)	47.46 (S.D. 13.31)	47.26 (S.D. 16.60)	147.03 (S.D. 24.88)	140.62 (S.D. 49.06)
P Value	0.0188	0.0229	7.86×10^{-10}	3.01×10^{-22}	8.19×10^{-12}

Note:
All data were analyzed by ANOVA test. S.D.: standard deviation.
[a] : $P < 0.05$ existed between Groups A, B and Groups C, D, E.
[b] : $P < 0.05$ existed between Groups A, B and Groups C, D, E.
[c] : $P < 0.0005$ existed between Groups A, B and Groups C, D, E.
[d] : $P < 0.0005$ existed between Groups A, B and Groups C, D, E; $0.005 < P < 0.05$ were existed between Group A and B.
[e] : $P < 0.0005$ existed between Groups A, B and Groups C, D, E.
*: $P< 0.05$; **: $P<0.005$; ***: $P<0.0005$ when compared with control group.

Table 2. The changes of TGF-β1 concentration in culture medium and TGF-β1 secretion when osteoblasts cultured with various-sized synthetic Sintered β-Dicalcium Pyrophosphate (SDCP) bioceramics at the concentration of 1 mg/ml (N=10).

TGF-β1 concentration (pg. /ml)	I hour	3 hour	1 day	3 day	7 day
A. (0.5 - 3.0 μm)	12.65 (S.D. 11.67)	11.69 * (S.D. 0.93)	69.84 * (S.D. 25.62)	216.36 (S.D. 19.51)	193.96 (S.D. 45.90)
B. (37 - 63 μm)	9.63 (S.D. 9.32)	14.04 ** (S.D. 6.22)	69.67 * (S.D. 18.04)	209.28 (S.D. 18.57)	184.89 (S.D. 48.88)
C. (177 - 250 μm)	11.56 (S.D. 5.51)	8.60 (S.D. 7.17)	72.32 * (S.D. 13.24)	227.60 (S.D. 31.39)	214.56 (S.D. 40.45)
D. (420 - 841 μm)	12.57 (S.D. 7.11)	14.96 ** (S.D. 4.72)	69.85 * (S.D. 14.47)	222.45 (S.D. 35.70)	230.90 (S.D. 41.19)
E. Control	8.60 (S.D. 7.00)	8.48 (S.D. 5.13)	90.25 (S.D. 13.15)	231.74 (S.D. 28.68)	233.91 (S.D. 50.64)
P Value	0.6926	0.0127	0.0344	0.3056	0.0764

TGF-β1 secretion (pg. / x 10^4 cells)	I hour	3 hour [a]	1 day [b]	3 day [c]	7 day [d]
A. (0.5 - 3.0 μm)	1.19 (S.D. 1.09)	1.12 ** (S.D. 0.40)	13.43 ** (S.D. 6.43)	49.92 *** (S.D. 20.08)	88.18 ** (S.D. 44.20)
B. (37 - 63 μm)	1.34 (S.D. 1.05)	1.59 ** (S.D. 1.16)	13.62 ** (S.D. 3.86)	25.09 ** (S.D. 18.50)	60.26 ** (S.D. 28.23)
C. (177 - 250 μm)	1.26 (S.D. 0.99)	0.64 (S.D. 0.42)	4.52 (S.D. 2.72)	5.02 * (S.D. 1.02)	5.16 (S.D. 1.07)
D. (420 - 841 μm)	1.03 (S.D. 0.74)	0.67 (S.D. 0.31)	4.11 (S.D. 1.61)	4.66 * (S.D. 0.94)	5.37 (S.D. 2.42)
E. Control	0.74 (S.D. 0.49)	0.54 (S.D. 0.31)	9.13 (S.D. 6.04)	4.02 (S.D. 0.70)	5.56 (S.D. 2.13)
P Value	0.4298	0.0033593	0.002014	1.95×10^{-11}	0.000178

Note:
All data were analyzed by ANOVA test. S.D.: standard deviation.
[a] : No significant difference existed between all measurements.
[b] : $P < 0.005$ existed between Groups A, B and Groups C, D, E.
[c] : $P < 0.005$ existed between Groups A, B and Groups C, D, E.
[d] : $P < 0.0005$ existed between Group A and Group B; $P < 0.005$ existed between Groups A, B and Groups C, D, E; $P < 0.05$ existed between Groups C, D and Group E; $P > 0.05$ between Group C and Group D.
[e] : $P < 0.005$ existed between Groups A,B and Groups C, D, E.
*: $P < 0.05$; **: $P < 0.005$; ***: $P < 0.0005$ when compared with control group.

Table 3. The changes of alkaline phosphatase (ALP) concentration in culture medium and secretion of ALP when osteoblasts cultured with various-sized Sintered β-Dicalcium Pyrophosphate (SDCP) bioceramics at the concentration of 1 mg/ml (N=10).

ALP concentration (Unit / ml)	I hour	3 hour	1 day	3 day	7 day
A. (0.5 - 3.0 μm)	42.01 (S.D. 4.28)	46.16 (S.D. 6.12)	46.16 (S.D. 7.14)	45.88 (S.D. 4.55)	39.53 (S.D. 5.98)
B. (37 - 63 μm)	38.14 (S.D. 6.99)	40.63 (S.D. 5.98)	40.91 (S.D. 7.80)	38.42 (S.D. 8.59)	36.49 (S.D. 8.82)
C. (177 - 250 μm)	37.04 (S.D. 7.51)	39.25 (S.D. 8.01)	41.18 (S.D. 4.21)	36.21 (S.D. 6.17)	35.66 (S.D. 6.17)
D. (420 - 841 μm)	37.04 (S.D. 6.92)	40.63 (S.D. 7.71)	38.42 (S.D. 8.08)	38.42 (S.D. 11.24)	37.31 (S.D. 7.95)
E. Control	40.35 (S.D. 6.80)	36.49 (S.D. 8.22)	40.35 (S.D. 7.84)	37.59 (S.D. 4.91)	38.14 (S.D. 6.87)
P Value	0.364	0.0687	0.1894	0.0533	0.7883

ALP secretion (Unit / 10^4 cells)	I hour [a]	3 hour [b]	1 day [c]	3 day [d]	7 day [e]
A. (0.5 - 3.0 μm)	4.09 (S.D. 2.37)	3.68 ** (S.D. 1.37)	7.50 ** (S.D. 3.15)	8.71 *** (S.D. 3.30)	16.67 ** (S.D. 8.14)
B. (37 - 63 μm)	4.03 (S.D. 1.54)	3.63 ** (S.D. 1.09)	6.74 ** (S.D. 2.46)	3.91 ** (S.D. 1.49)	10.97 ** (S.D. 5.08)
C. (177 - 250 μm)	3.24 (S.D. 1.99)	2.41 (S.D. 0.90)	2.12 (S.D. 1.13)	0.66 (S.D. 0.10)	0.73 (S.D. 0.23)
D. (420 - 841 μm)	2.67 (S.D. 1.07)	2.21 (S.D. 0.70)	1.85 (S.D. 0.63)	0.66 (S.D. 0.16)	0.71 (S.D. 0.28)
E. Control	2.82 (S.D. 1.57)	2.05 (S.D. 0.70)	3.08 (S.D. 1.62)	0.65 (S.D. 0.10)	0.75 (S.D. 0.27)
P Value	0.08578	0.0002948	7.17×10^{-7}	1.73×10^{-12}	9.77×10^{-5}

Note:
All data were analyzed by ANOVA test. S.D.: standard deviation.
[a] :P > 0.05 existed between all measurements.
[b] :P < 0.005 existed between Groups A,B and Groups C, D, E.
[c] :P < 0.005 existed between Groups A,B and Groups C, D, E.
[d] :P < 0.005 existed between Groups A,B and Groups C, D, E.; P < 0.005 existed between Group A and Group B.
[e] :P < 0.005 existed between Groups A,B and Groups C, D, E.
*: P< 0.05; **: P<0.005; ***: P<0.0005 when compared with control group.

Table 4. The changes of prostaglandin-E_2 (PGE_2) concentration in culture medium and secretion of PGE_2 when osteoblasts cultured with various-sized Sintered β-Dicalcium Pyrophosphate (SDCP) bioceramics at the concentration of 1 mg/ml (N=10).

PGE_2 concentration (pg / ml)	I hour	3 hour	1 day	3 day	7 day
A. (0.5 - 3.0 μm)	282.83 ** (S.D. 111.72)	278.52 * (S.D. 93.92)	202.07 (S.D. 105.14)	240.19 * (S.D. 127.73)	242.6 ** (S.D. 80.44)
B. (37 - 63 μm)	242.15 * (S.D. 88.20)	204.12 (S.D. 72.95)	213.16 (S.D. 135.93)	171.36 (S.D. 79.92)	260.81 *** (S.D. 85.90)
C. (177 - 250 μm)	145.31 (S.D. 81.03)	163.70 (S.D. 64.26)	171.82 (S.D. 139.16)	117.20 (S.D. 82.36)	124.66 (S.D. 39.60)
D. (420 - 841 μm)	138.43 (S.D. 40.25)	175.75 (S.D. 40.46)	133.47 (S.D. 80.98)	146.87 (S.D. 39.11)	121.24 (S.D. 44.71)
E. Control	163.95 (S.D. 49.32)	211.90 (S.D. 90.52)	194.27 (S.D. 66.06)	110.33 (S.D. 53.69)	109.21 (S.D. 31.45)
P Value	0.0003	0.0183	0.5316	0.0341	9.42×10^{-8}

PGE_2 secretion (pg / 10^4 cells)	I hour [a]	3 hour [b]	1 day [c]	3 day [d]	7 day [e]
A. (0.5 - 3.0 μm)	33.93 ** (S.D. 8.22)	26.19 * (S.D. 10.93)	32.92 ** (S.D. 19.56)	41.43 ** (S.D. 20.83)	112.91 *** (S.D. 55.76)
B. (37 - 63 μm)	28.78 ** (S.D. 10.81)	22.71 * (S.D. 9.22)	38.96 ** (S.D. 21.37)	16.45 ** (S.D. 7.38)	104.04 *** (S.D. 52.36)
C. (177 - 250 μm)	13.10 (S.D. 8.27)	13.83 (S.D. 8.26)	9.36 (S.D. 7.02)	2.02 (S.D. 1.88)	3.05 (S.D. 1.30)
D. (420 - 841 μm)	11.30 (S.D. 6.29)	13.38 (S.D. 6.47)	7.74 (S.D. 5.33)	2.61 (S.D. 1.33)	2.94 (S.D. 1.76)
E. Control	12.59 (S.D. 7.23)	13.61 (S.D. 4.52)	9.36 (S.D. 4.85)	1.16 (S.D. 0.98)	2.52 (S.D. 0.94)
P Value	4.96×10^{-8}	0.001097	0.002054	0.000335	0.000146

Note:
All data were analyzed by ANOVA test. S.D.: standard deviation.
[a] : $P < 0.005$ existed between Groups A, B and Groups C, D, E.
[b] : $P < 0.05$ existed between Groups A, B and Groups C, D, E.
[c] : $P < 0.005$ existed between Groups A, B and Groups C, D, E.
[d] : $P < 0.005$ existed between Groups A, B and Groups C, D, E.
[e] : $P < 0.005$ existed between Groups A, B and Groups C, D, E.
*: $P< 0.05$; **: $P<0.005$; ***: $P<0.0005$ when compared with control group.