行政院國家科學委員會專題研究計畫成果報告

計畫名稱：Application of artificial biomaterial in surgery of massive bone defect - II

（英、中文）人造替代骨於大量骨缺損病人手術之應用- II

計畫類別：☒ **個別型計畫** □ **整合型計畫**

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個別型計畫：計畫主持人：孫瑞昇

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執 行 單 位 ： 台 大 醫 學 院 骨 科

中 華 民 國 八 十 八 年 九 月 二十三 日

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英文：Application of artificial biomaterial in surgery of massive bone defect -I I

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中文摘要：

關鍵詞：生物適應性、磷酸鈣、顆粒、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。通常在手術後，骨骼細胞可緊密的長入殖入物間而沒有太多的纖維組織增生。可是位於殖入物旁之軟組織仍可能受到殖入物相關之因素所影響。過去幾年來，對於殖入物旁之骨組織影響已有多位學者研究；但對於殖入物旁之軟組織影響研究之學者並不多見。 宿主對這些殖入物之反應通常藉著在殖入部位附近型態及組織學上的變化來評估其生物適應性。通常於活體內評估某一特殊細胞對殖入物的反應，由於受到殖入物附近多種細胞及各種不同化學物質影響；造成其研究十分困難。為了解決此問題，利用細胞與生醫材料共同培養以探討其相互間之關係。本研究之主要目地即在澈定各種不同磷酸鈣對肌母細胞、骨母細胞之影響。

以四種不同磷酸鈣：包括β-**三磷酸鈣**(β-TCP),**氫氧基磷酸鹽**(HA)**，**β-**二磷酸鈣**(β-DCP)**，焦二磷酸鈣**(SDCP)**，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 **以促進肌母細胞之貼附。之後** 0.1%**四種不同磷酸鈣**(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。**

結果顯示控制組之細胞量及 TGF-β1 **濃度隨著時間呈現逐步增加之現象。在**β-TCP **及** HA **兩種粒子存在時，細胞量及** TGF-β1 **濃度在三天內均很低；爾後緩慢增加至七天時為止。在** DCP**、**SDCP **及** silica **三種粒子存在時，細胞量及** TGF-β1 **濃度之變化十分相近。在第一天內值很低；爾後緩慢增加至七天時已接近控制組。**

經由此實驗，各種不同磷酸鈣對骨母細胞之影響可以得到釐清。而可能的最有潛力之磷酸鈣也可以確定。我們認為β-TCP **及** HA**兩種粒子對細胞生長有抑制作用；此種作用可能影響到骨科手術後組織之癒合。**

英文摘要：

ABSTRACT

With advances in ceramics technology, calcium phosphate bioceramics have been applied as bone substitutes for several decades. The focus of this work is to elucidate the biocompatibility of the particulates of various calcium phosphate cytotoxicity. Four different kinds of calcium phosphate powders, including β-tricalcium phosphate (β-TCP), hydroxyapatite (HA), β-dicalcium pyrophosphate (β-DCP) and sintered β-dicalcium pyrophosphate (SDCP), were tested by osteoblasts cell culture. The results were analyzed by cell count, concentration of transforming growth factor-β1 (TGF-β1), alkaline phosphatase (ALP), and prostaglandin E_2 (PGE_2) in culture medium. The changes were most significant when osteoblasts cultured with β-TCP and HA bioceramics. The changes in cell population of the β-TCP and HA were quite low in the first 3 days, then increased gradually toward the 7th day. The changes of TGF-β1 concentration in culture medium inversely related to the changes of cell population. The ALP titer in culture medium of the β-TCP and HA were quite high in the first 3 days, then decreased rapidly during the 3rd and 7th day. The concentration of PGE_2 in culture medium tested were quite high in the first day, decreased rapidly to the 3rd day and then gradually till the 7th day. The changes in the β-DCP and SDCP were also quite similar to that of HA and β-TCP but much less significant. We conclude that HA and β-TCP is thought to have an inhibitory effect on the growth of osteoblasts. The inhibition effects of the HA and β-TCP powders on the osteoblast cell culture were possibly mediated by the increased synthesis of PGE_2.

Key words:

Biocompatibility, calcium phosphate, particle, osteoblast.

INTRODUCTION

With advances in ceramics technology, the application of calcium phosphate materials have been received considerable attention as bone substitutes for several decades. They are remarkable biocompatible, provoke little if any inflammatory response, and have a direct connection with bony structure.[1] Due to similar calcium/phosphate (Ca/P) ratios to that of nature bone, hydroxyapatite (HA) and tricalcium phosphate (TCP) are two calcium phosphates most commonly used in the clinical applications. HA are the well-known end products of the biological mineralization process. β-dicalcium phosphate (β-DCP) with formula of $Ca_2P_2O_7$ is one of the intermediate products in this process. [2] The biological response for new bone formation is quite similar to that of HA. [3,4] In our institute, sintered β-$Ca_2P_2O_7$ with 5% $Na_4P_2O_7 \bullet 10H_2O$ addition has been proved to have great potential as an in vivo biodegradable bone substitute. [5,6]

Continuous advances in the field of implantable calcium phosphate biomaterials have produced impressive progress concerning their biocompatibility and the ability to promote tissue formation. The nature and the degree of tissue response depend on the characteristics of the material such as its chemical composition,[7] surface texture,[8] porosity and density,[9] shape, and size.[10] However, it is difficult to compare the published results regarding the diversity of implantation sites,[11,12] implantation techniques, and animal species. Host tissue responses to these materials are generally assessed by morphological and histological examinations of the implant site in order to evaluate their biocompatibility. It is difficult to exam in-vivo reaction of a specific cell to the substrate because numerous cell populations and chemical factors are involved in implantation. In vitro methods are quite useful in examining cell interactions with biomaterials. [13-15] In order to determine the sequences of events and the parameters influencing the interactive process, the model of cell culture in the presence of biomaterials is of great interest. The aim of this study is to elucidate the interactions between 4 different calcium phosphate bioceramics and the osteoblasts.

MATERIALS AND METHODS

Material tested

Four different kinds of calcium phosphate powder, β-tricalcium phosphate (β-TCP: $Ca_3[P04]_2$, Merck, Germany), hydroxyapatite (HA: $Ca_5(P0_4)_6(OH)_6$, Merck, Germany), β-dicalcium pyrophosphate (β-DCP: $Ca_2P_20_7$, Sigma Chem. Co, USA), and sintered β-dicalcium pyrophosphate (SDCP: sintered β-$Ca_2P_20_7$ with $Na_4P_20_7{\cdot}10H_20$) were prepared as test materials. [16]

β-DCP powder with mean grain size about 0.1 μm was used in the experiment. The specific surface area determined by BET analysis is 51 ± 0.2 cm^2/g. Hydroxyapatite and β-tricalcium phosphate powders were spherical in shape and about 0.06 μm in grain size according to scanning electron microscopic observation, and their BET specific surface area were approximately 70 ± 0.2 cm^2/g and 59 ± 0.2 cm^2/g, respectively.

The powder of sintered β-dicalcium pyrophosphate (SDCP) with $Na_4P_2O_7{\cdot}10H_2O$ addition was prepared and briefly described as follows.[5] The β-DCP powder was mixed with 5 wt% $Na_4P_2O_7{\cdot}10H_2O$ 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 again into a mean grain size of about 1-5 μm. Hydroxyapatite, β-tricalcium phosphate, and dicalcium pyrophosphate particles were also prepared with the same procedures, but no $Na_4P_2O_710H_2O$ was added. The sintered temperature of the three ceramics were 1250°C, 1200°C, and 930°C, respectively, with the same heating rate of 3°C/min. After sintered, the particles were crashed into the same particle size of 1-5μm. All the materials were sterilized by autoclave in a temperature of 130°C and held for 40 minutes before experiment.

Osteoblast cell culture

Sequential digestion of newborn Wistar-rat calvaria was performed by using a modification of the methods described by Wong and Cohn.[17] The dissected calvaria was soaked in and washed with 4 mM Na_2-EDTA in a pre-warmed (37°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 then sequential digested with collagenase (180 U/ml, Sigma Co.) in solution A with EDTA. The sequential digestion consisted of four times' treatments each for 5 minutes and followed by two times' treatments each for 10 minutes. The cells released after each treatment were immediately harvested by centrifugation and resuspended in culture medium. Unambiguous identification of cell populations as osteoblasts is complex since none of the parameters used for defining osteoblasts-like cells are unique to this cell types. [18] The presence of alkaline phosphatase, an early marker of osteoblasts, [19] is used to assess the osteoblastic character of the isolated cells. [17,20-22]

Experimental procedures

In the experiment, confluent rat osteoblast cultures were passaged by trypsin-EDTA and then seeded into petri dishes (Corning-25810, NY, USA). The cell density was 1 x 10^5 cells / 940 mm^2 and 3ml medium was seeded for each petri dishes. The culture media used was Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Gibco, UK), penicillin G sodium 100 units/ml and streptomycin 100 mg/ml (Gibco, UK). The dishes were incubated at 37°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 to remove the possible interference of TGF-β1 existed in fetal calf serum. Insulin was added to supply the factor needed for the growth of osteoblasts. [23] Dulbecco modified Eagle 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 Corp.) were mixed with 0.1% of various tested materials (1 g/ l). One of the wells was added with the same concentration of silica (Silicone dioxide: SiO_2, Sigma Corp., size: 0.5 -10 μm). The day of plating was considered as the zero day of culture. After osteoblasts and bioceramics were incubated together for 3 hours, days 1, 3, 7, the test media were removed from wells, divided into section of 500 μl with eppendorf, and then deep frozen in -80°C for 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. Representative pictures were photographed 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). The mean cell population in four randomly selected high-power field (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). For the determination of cell population at zero time, ten separate wells were stained and measured after attachment of osteoblasts.

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

The transforming growth factor-β1 (TGF-β1) has been found in the highest concentration in mammalian bone and plays a critical role in the mediation of a number of biological response.[24] 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 incubated for 3 hours at room temperature on the benchtop. 200 μl of TGF-β1 (R & D System, Inc. USA) conjugate were added and then incubated 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 Corp, 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 Co. USA). An aliquot (20 μl) from the media was mixed with 1 ml alkaline phosphatase reagent, and 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. 50 μl of standard PGE_2 (Cayman Chemical Company, MI, USA) or sample were added per well. The tested samples were incubated for 18 hours at room temperature on the benchtop. 200 μl of Ellman Reagent were added to each well and then incubated for 1.5 hour at room temperature. The reaction was stopped and read by Microelisa reader (Emax Science Corp, 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 cell population at various tested periods.

Statistical analysis

The differences between various tested bioceramics were evaluated by an analysis of variances statistic method. The post hoc tests performed was Bonferroni's test. The level of statistical significance is defined as $P<0.05$.

RESULTS

Cell population

At time zero, the cell population was 157799.7 ± 5779.9 cells/well (mean ± standard error). The cell populations of control, silica, β-TCP, HA, β-DCP, SDCP at different times after incubation were summarized in Table 1. The changes of cell population of various preparations at 3 hr, 1 day, 3 days and 7 days were all statistically significant ($P < 0.0001$ by ANOVA test). Cells have been densely colonized in control, β-DCP, SDCP and silica well when cultured for 3 days.The cells were still sparsely colonized in the wells with TCP and HA even after 7 days' incubation.

The cell population of control persistently increased with the time passed. The cell population of silica had the same tendency with that of control; but at the initial 3 days of incubation, the cell population of silica were significantly lower than that of the control ($P<0.05$). The cell population of both the β-TCP and HA were quite low in the first 3 days, then slowly increased toward the 7th day. During the cultural period, the cell population of both the β-TCP and HA were persistently lower than that of control ($P < 0.0001$). The cell populations for β-DCP and SDCP in the first 3 days were significantly lower than that of control ($P<0.001$), thereafter, increased rapidly and even reached the cell count of the control at the 7th day of culture ($P > 0.05$).

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

The concentrations of TGF-β1 in culture medium of various preparations were shown in Table 2. The changes of TGF-β1 concentration in medium cultured for 3 hr was not statistically significant ($P=0.52$). The TGF-β1 concentration of medium on 1 day, 3 days and 7 days showed a statistically significant difference for each group ($P < 0.0001$ by ANOVA test).

The concentration of TGF-β1 in culture medium were quite similar between control and silica ($P > 0.05$) during the cultural period. The TGF-β1 titer in culture medium for β-TCP and HA were quite low at the first day after incubation, then increased rapidly during the 3rd and 7th day. After 3 days' culture, the TGF-β1 titer in culture medium of the β-TCP and HA took a turn up and both were significantly higher than that of control medium ($P < 0.0001$). The TGF-β1 concentration in medium for the β-DCP and SDCP were quite similar to that of control medium ($P >0.05$) but increased much higher than that of control after 3 days' culture ($P < 0.05$). The changes of TGF-β1 concentration in culture medium inversely related to the changes of cell population when osteoblasts cultured with the various calcium phosphate bioceramic particles.

Alkaline phosphatase (ALP) in culture medium

The titers of ALP in culture medium for each group were shown in Table 3. The changes of ALP titer in medium of various calcium phosphates during the tested periods were all statistically significant ($P<0.0001$). In the cultural period, the ALP concentration in cultural medium for silica, β-DCP, and SDCP ($P > 0.05$) had the same tendency in each other and near the baseline as shown in Fig 2B; which were quite close to that of control titer after cultured for 7th days. The ALP titer in culture medium of both the β-TCP and HA were always higher than that of the mentioned groups in the initial 3 days, but decreased rapidly afterward.

Prostaglandin E_2 (PGE_2) in culture medium

The concentrations of PGE_2 in culture medium for each group were shown in Table 4. The concentrations of PGE_2 in tested medium are always significantly higher than that of control medium ($P < 0.0001$ by ANOVA test). For all the groups tested, the PGE_2 concentration in the control medium increased at the 1st day, and then sharply decreased after 3 days. The changes of concentration of PGE_2 in culture medium for with silica and various calcium phosphates were quite the same. The concentrations of PGE_2 in the tested culture medium were increased with the incubation time at the initial 24 hours and then decreased rapidly. After cultured for 3 days, the PGE_2 concentrations were slowly decreased with the cultural time and retrogressed to the baseline as control group at the 7th day. The concentration of PGE_2 in culture medium of the β-TCP and HA were significantly higher than that of control medium ($P < 0.0001$). Similar results were observed in the culture medium of the silica, β-DCP and SDCP ($P < 0.05$), but always lower than that of the HA and β-TCP.

DISCUSSION

Biodegradable biomaterials have found a wide variety of applications in medicine. Certain biodegradable calcium phosphate has been used as bone substitutes to avoid the complications associated with bone grafts, such as shortage of supply, immunogenicity, and transfer of disease from the graft.[25-28] One of the main advantages of a biodegradable substances is that it obviates the need for the eventual surgical removal. Calcium phosphate ceramics are known as being particularly osteocompatible. However, the osteo-integration of these materials are not always very good. The failures of the osteo-integration of calcium phosphates had been reported.[29,30] It is very difficult to find a common cause for all these failures. The diversity of the specifications of the calcium phosphates make it difficult to link the biological result obtained with the characteristic of the material.[29]

Cell populations enzymatically isolated from the membranous bones of fetal or newborn rats are heterogenous but have served as a useful in vitro system for elucidating the responses of bone cells under variuos conditions. [17] By culturing osteoblast-like cells with calcium phosphates, Matsuda and Davies demonstrated the importance of the step-by-step interactions occurring at the cell-material interface. [31] There were many factors influence biodegradation. Material aspects include the size, the conditions under which bioceramic is synthesized, stability when subjected to body fluid, porosity of surface and condition of grain boundaries.[32,33] It is unclear whether phagocytosable particles originating from various biomaterials affect bone ingrowth. In this study, the main concern was to study the interactions of phagocytosable particles originating from various calcium phosphate bioceramics on the adjacent osteoblasts.

Several distinct regulatory mechanism can be identified by which cytokine networks regulate cellular events and interactions in bone tissue. The transforming growth factor-β1 (TGF-β1) has been found in the highest concentration in mammalian bone and has been shown to have stimulatory effects on osteoblasts.[34-37] The alkaline phosphotase is used to feature the osteoblastic characteristics. [17,20] A variety of bone cell products synthesis, such as prostaglandins and leukotrienes, were also mediated by cytokines. Cytokine-mediated effects on prostaglandin synthesis contribute to the local bone resorption. [38] In this study, TGF-β1, alkaline phosphotase, and prostaglandins were measured to monitore the effect of various calcium phosphate on the osteoblasts.

During manufacturing process, the physical and chemical characteristics of the material might be changed.[29] The results about the effect of various bioceramics' particulates on osteoblasts did not mean that the same result will be occurred in the block form. It does manifest the in vivo particulates behaviors of various calcium phosphate bioceramics on the adjacent ingrowing osteoblasts. As shown in this study, we may suggest the biocompatibility of the β-DCP and SDCP is superior to HA and β-TCP since the powders of β-DCP or SDCP were more tolerable by osteoblasts. In this study, the possible mechanism of particulates of various manufactured calcium phosphates upon the osteoblasts was further studied by measuring ALP and PGE_2 titer in the culture medium.

Alkaline phosphatase specific activities are associated with primary calcification in a number of tissue including endochondral bone, woven bone, and fracture callus.[39] Alkaline phosphatase production is regulated by factors affecting the cells both genomically and nongenomically.[40] The level of alkaline phosphatase activity in osteoblast lineage cells may reflect different degrees of differentiation. [41,42] In this study, the ALP concentration in control medium decreased gradually after 7 days' culture. The stationary results of ALP level suggested that there is no significant differentiation of the osteoblasts during this culture period. Although, collagenase-release methods generally give rise to enriched population of osteoblastic cells. [17] Fibroblastic overgrowth can become a serious problem with extended culture time. [43] This may also contribute to the gradual decrease in ALP titer of the control medium. The changes of ALP concentration were quite similar between that of silica, β-DCP, and SDCP ($P > 0.05$); which were quite close to that of control titer at the 7th days' culture (Table 3). The ALP titer in culture medium of the β-TCP and HA were quite high in the first 3 days after incubation and then rapidly decreased. The possible mechanism of this distinct changes in ALP titer is due to abrupt releases of intracellular enzymes of osteoblasts once β-TCP and HA particles being added. This result was supported by the obvious decrease in the osteoblasts cellularity in the first 3 days' culture.

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 cytokine is influenced by prostaglandins.[44] In this study, the changes of PGE_2 concentration in culture medium when osteoblasts cultured with various calcium phosphate bioceramics were also described. The concentrations of PGE_2 in culture medium for all the calcium phosphates were always significantly higher than that of control medium during the cultural period. The concentration of PGE_2 was up to the ceiling in the first day, and rapidly decreased. After cultured for 3 days, it turned into slowly decrease till the end of experiment. During the tested period, the PGE_2 concentration of the β-TCP and HA were persistently higher than that of silica, β-DCP and SDCP. The changes of PGE_2 concentration in culture medium reversely corresponded to the changes of

cell population when osteoblasts cultured with particles of various calcium phosphates. The higher PGE_2 concentration was associated with a decrease in cell population. On the contrary, the lower PGE_2 concentration was accompanied with the increase in cell population. This means that the low level of cellular PGE_2 synthesis did not affect the survival of osteoblasts, while increased cellular PGE_2 synthesis might affect the survival of osteoblasts. The inhibition effects of the HA and β-TCP particles on the osteoblast cell culture were possibly mediated by the increased synthesis of PGE_2.[44]

The inflammatory response of a host is mediated by endogenous compounds, including leukotrienes and prostaglandin. Prostaglandins are major contributors to the symptoms of inflammation and the prostaglandin E_2 and I_2 can promote leukocyte infiltration.[45] In this work, only some of the interactions between various calcium phosphates and osteoblasts were elucidated. Further studies on the relationships of various calcium phosphates to the leukocytes were now in progress.

Conclusion

Influence of calcium phosphate on osteoblast cells was surveyed by means of cell culture. Conclusions are as following:

(1) After 7 days culture in the presence of HA and β-TCP particulare, the osteoblasts population are lower than that of β-DCP and SDCP. Growth rate of osteoblasts in the presence of HA and β-TCP partculates was lower than that of β-DCP and SDCP.

(2) When osteoblasts cultured with β-TCP and HA, there was a significant increase in TGF-β1 and ALP concentration in culture medium. The increase in ALP level is due to abrupt release of intracellular enzymes from osteoblasts once β-TCP and HA particles being added.

(3) The concentration of PGE_2 in culture medium of the β-TCP and HA were persistently higher than that of silica, β-DCP and SDCP. The increase in PGE_2 synthesis was associated with the decrease in cell population.

(4) From these findings, HA and β-TCP is thought to have an inhibitory effect on the growth of osteoblasts. The inhibition effects of the HA and β-TCP particles on the osteoblast cell culture were possibly mediated by the increased synthesis of PGE_2.

REFERENCES

1. M. Jarcho, alcium phosphate ceramics as hard tissue prosthetics," Clin. Orthop., **157**, 259-278 (1981).

2. P. Ducheyne, ioceramics: material characteristics versus in vivo behavior," J. Biomed. Mater. Res.: Appl. Biomater., **21A**, 219-236 (1987).

3. T. Kitsugi, T. Yamamuro, T. Nakamura, S. Kotani, T. Kokubo, Four calcium phosphate ceramics as bone substitutes for non-weight-bearing," Biomaterials, **4**, 216-224 (1993).

4. F.H. Lin, and K.H. Hon, study on synthesized hydroxyapatite bioceramics," Ceram. Int., **5**, 530-536 (1989).

5. F.H. Lin, C.C. Lin, C.M. Lu, H.C. Liu, J.S. Sun, and C.Y. Wang, Mechanical properties and histological evaluation of sintered β-$Ca_2P_20_7$ with $Na_4P_20_7{\cdot}10H_20$ addition," Biomaterials, **16**, 793-802 (1995).

6. C.C. Lin, C.J. Liao, J.S. Sun, H.C. Liu, and F.H. Lin, Prevascularized bone graft cultured in sintered porous β-$Ca_2P_2O_7$ with 5 wt% $Na_4P_2O_7{\bullet}10H_2O$ addition ceramic chamber," Biomaterials, **17**, 1133-1140 (1996).

7. C.P.A.T. Klein, K. de Groot, A.A. Driessens, and H.B.M. van der Lubbe, A comparative study of different β-Whitlockite ceramics in rabbit cortical bone with regard to their biodegradation behaviour," Biomaterials, **7**, 144-146 (1986).

8. S.R. Taylor, and D.F. Gibbons, ffect of surface texture on the soft tissue response to polymer implants," J. Biomed. Mater. Res. **17**, 205-227 (1983).

9. J.C. Davila, E.V. Lautsch, and T.E. Palmer, Some physical factors affecting the acceptance of synthetic materials as tissue implants," Ann. N.Y. Acad. Sci., **146**, 138-147 (1968).

10. N.K. Wood, E.J. Kaminski, and R.J. Oglesby, The significance of implant shape in experimental testing of biological materials: Disc versus rod," J. Biomed. Mater. Res., **4**, 1-12 (1970).

11. D. Bakker, C.A. van Blitterswijk, S.C. Hesseling, I.J. Grote, and W.T. Daems, ffect of implantation site on phagocyte-polymer interaction and fibrous capsule formation," Biomaterials, **9**, 14-23 (1988).

12. E.J. Kaminski, R.J. Oglesby, N.K. Wood, and J. Sandrik, The behaviour of biological materials at different sites of implantation," J. Biomed. Mater. Res., **2**, 81-88 (1968).

13. J.E. Villanueva, and M.E. Nimni, Modulation of osteogenesis by isolated calvaria cells: a model for tissue interactions," Biomaterials, **11**, 13-15 (1990).

14. I.M. Brook, G.T. Craig, and D.J. Lamb, In vitro interaction between primary bone organ culture, glass-ionomer cements and hydroxyapatite/tricalcium phosphate ceramics, Biomaterials, **12**, 179-186 (1991).

15. H. Kodama, Y. Amagai, H. Sudo, S. Kasai, and S. Yamamoto, Establishment of a clonal osteogenic cell line from newborn mouse calvaria, Jpn. Oral Biol., **23**, 899-901 (1981).

16. J.S. Sun, Y.H. Tsuang, C.H. Yao, H.C. Liu, F.H. Lin, and Y.S. Hang, Effects of Calcium Phosphate Bioceramics on Skeletal Muscle Cells." J. Biomed. Mater. Res. (1996, in press).

17. G.L. Wong, and D.V. Cohn, Target cells in bone for parathormone and calcitonin are diferent: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces," Proc. Nat. Acad. Sci. USA, **72**,3167-3171, (1975).

18. C.G. Bellows, J.E. Aubin, J.N.M. Heersche, and M.E. Antosz, Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations," Calcif. Tissue Int., **38**, 143-154 (1986).

19. A.I. Caplan, and D.G. Pechak, The cellular and molecular embryology of bone formation," In: Peck C (ed.) Bone and mineral research, Vol. 5. Elsevier, Amsterdam, pp 117-183 (1987).

20. A.K. Hall, and I.R. Dickson, The effects of parathyroid hormone on osteoblast-like cell from embryonic chick calvaria," Acta Endocrinol., **108**, 217-223 (1985).

21. C.T. Begley, M.J. Doherty, D.P. Kankey, D.J. Wilson, The culture of human osteoblasts growth upon bone graft substitutes," Bone, **14**, 661-667 (1993).

22. M.J. Doherty, G. Schlag, N. Schwarz, R.A.B. Mollan, P.C. Nolan, D.J. Wilson, iocompatibility of xenogeneic bone, commercially available coral, a bioceramic and tissue sealant for human osteoblasts," Biomaterials, **15**, 601-608 (1994).

23. E. Canalis, M. Cenrtrella, W. Burch, and T.L. McCarthy, Insulin-like growth factor I mediates

selective anabolic effects of parathyroid hormone in bone cultures," J. Clin. Invest., **83**, 60-65 (1989).

24. M.B. Sporn, A.B. Roberts, L.M. Wakefield, B. de Crombrugghe, Some recent advances in the chemistry and biology of transforming growth factor-beta," J. Cell Biol., **105**,1039-1045 (1987).
25. G.D. Bos, V.M. Goldberg, J.M. Zika, K.G. Heiple, and A.E. Powell, Immune response of rats to frozen bone allografts," J. Bone Joint Surg., **65A**, 239-246 (1983).
26. J.O. Hollinger, and G.C. Battisone, iodegradable bone repair materials: Synthetic polymers and ceramics," Clin. Orthop., **207**, 290-305 (1986).
27. H.J. Mankin, M.C. Gebhardt, and W.W. Tomford, The use of frozen cadaveric allografts in the management of patients with bone tumors of the extremities," Orthop. Clin. N. Am., **18**, 275-289 (1987).
28. M.R. Urist, and E. Dawson, Intertransverse process fusion with aid of chemosterilized autolyzed antigen-extracted allogeneic (AAA) bone," Clin. Orthop., **154**, 97-113 (1981).
29. P. Frayssinet, J.L. Trouillet, N. Rouquet, E. Azimus, and A. Autefage, Osseointegration of macroporous calcium phosphate ceramic having a different chemical composition," Biomaterials, **14**, 423-429 (1993).
30. K.E. Salyer, and C.D. Hall, Porous hydroxyapatite as onlay bone-graft substitute for maxillofacial surgery," Plast. Reconstr. Surg., **84**, 236-244 (1989).
31. T. Matsuda, and J.E. Davies, he in vitro response of osteoblasts to bioactive glass," Biomaterials, **8**, 275-284 (1987).
32. M. Kohri, K. Miki, D.E. Waite, H. Nakajima, and T. Okabe, n vitro stability of biphasic calcium phosphate ceramic," Biomaterials, **14**, 299-394 (1993).
33. N.P. Ziats, K.M. Miller, and J.M. Anderson, In vitro and in vivo interactions of cells with biomaterials," Biomaterials, **9**, 5-13 (1988).
34. L.F. Bonewald, M.D. Murdy, " Role of transforming growth factor beta in bone remodeling." Clin. Orthop., **250**, 261-276 (1990).
35. M. Centrella, T.L. McCarthy, E. Canalis, " Transforming growth factor beta is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone." J. Biol. Chem., **262**, 2869-2874 (1987).
36. D.M. Rosen, S.A. Stempien, A.Y. Thompson, S.M. Seyedin, Transforming growth factor-beta modulates the expression of osteoblast and chondroblast phenotype in vitro. J. Cell Physiol., **134**, 337-346 (1988).
37. J.F. Wrana, M. Maeno, B. Hawrylyshyn, K.L. Yao, C. Domenicucci, J. Sodek, Differential effects ,of transforming growth factor β on the synthesis of extracellular matrix proteins by normal fetal rat calvarial bone cell populations. J. Cell Biol., **106**, 915-924 (1988).
38. S.R. Goldring, and M.B. Goldring, Cytokines and skeletal physiology," Clin. Orthop., **324**, 13-23 (1996).
39. A.L. Boskey, Current conceps of physiology and biochemistry of calcification," Clin. Orthop., **157**, 225-257 (1981).
40. Z. Schwartz, D. Schlader, L.D. Swain, B.D. Boyan, Direct effects of 1,25-dihydroxyvitamine D_3 and 24,25-dihydroxyvitamine D_3 on growth zone and resting zone chondrocyte membrane alkaline phosphatase and phospholipase A_2 specific activities," Endocrionlogy, **123**, 2878-2884 (1988).
41. K.H. Toshikatsu Matshyama, W. Lau, J.E. Wergedal, Monolayer cultures of normal human bone cells contain multiple subpopulations of alkaline phosphatase positive cells," Calcif. Tissue Int., **47**, 276-283 (1990).
42. K.H. Woldarski, Properties and origin of osteoblasts," Clin. Orthop. **252**, 276-293 (1990).
43. P. G. Robey, and J.D. Termine, Human bone cells in vitro," Calcif. Tissue Int., **37**, 453-460 (1985).
44. K.J. Ibbotson, D.R. Twardzic, S.M. D ouza, W.A. Hargreaves, G.K. Todaro, and G.R. Mundy, Stimulation of bone resorption in vitro by systemic transforming growth factor-alpha., Science, **228**, 1007 (1985).
45. Wingard L.B. Jr, Brody T.M., Larner J., Schwartz A.: Human Pharmacology: Molecular -to - Clinical. Wolfe publishing Ltd. London, 1991, p. 401.