Journal of Hard Tissue Biology 21[1] (2012) pp - 8
© 2012 The Hard Tissue Biology Network Association
Printed in Japan, All rights reserved.
CODEN-JHTBFF, ISSN 1341-7649

Original

Induction of Insulin-Like Growth Factors Expression in Dog Mandibles by β-TCP

Kung-Liang Chao[1], Ujjal K. Bhawal[1], Takao Watanabe[2], Tsuneo Takahashi[2], Wei-Jen Chang[3], Sheng-Yang Lee[3] and Yoshimitsu Abiko[1]

1) Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo, Chiba , Japan
2) Department of Oral anatomy, Kanagawa Dental College, Kanagawa, Japan
3) Graduate Institute of Oral Rehabilitation Sciences and School of Dentistry, Taipei Medical University, Taipei, Taiwan
(Accepted for publication, September 21, 2011)

Abstract: To understand the usefulness of β-TCP for development of biomaterials implants, β-TCP was implanted into bone defects of dog mandibles, and gene expression profiles were examined using DNA microarray. An implant drill was used to make bone defects in Beagle dog mandibles, and then β-TCP was filled into bone defects. Total RNA was isolated from all specimens, and mRNA levels were analyzed using Affymetrix GeneChip. Higher mRNA levels of insulin-like growth factors, IGF1 and IGF2, were observed in β-TCP-implanted samples compared with controls. The enhancement of IGF1 and IGF2 mRNA levels by β-TCP was confirmed by RT-PCR and real-time RT-PCR. Immunohistochemical staining revealed increased IGF1 and IGF2 protein expression in β-TCP-implanted bone. Taken together, the stimulation of IGF1 and IGF2 expression by β-TCP might be a mechanism of accelerating bone formation.

Key words : Dog mandible, β-TCP, Insulin-like growth factor (IGF), Gene expression

Introduction

Repair of bone fractures or defects is achieved by local new bone formation. However, the regenerative repair of bone is often impaired when the damage is severe as seen in comminuted open fractures or large bone defects associated with bone tumor resection. The osteogenic potential of autogenous bone graft is due to the retention of osteogenic precursor cells with the ability to proliferate and differentiate to osteoblasts. Additionally, the grafted bone is resorbed and replaced by newly formed bone, thereby reestablishing a level of structural integrity at the grafted site[1].

The number of clinicians performing endosseous dental implants whether immediately following tooth extraction or after a period of time is rapidly increasing. However, on many occasions, clinicians encounter the lack of adequate amount of bone due to a number of reasons including injury, eradicated tumor masses, or progressive periodontal diseases. To overcome this difficulty, many methods have been developed and introduced using a variety of grafts such as autologous bone grafts, allografts, alloplasts, and xenografts[2]. Autologous bone is regarded as one of the gold standard of graft materials[3-5]. However, there are several problems in autologous bone grafts including morbidity of the donor site and limitation of amount of bone that can be harvested[6,7]. It is becoming a demand to identify different types of bone substitutes. Calcium phosphates, such as tricalcium phosphate (TCP), and hydroxyapatite (HA) are widely used as substitutes for autologous bone[8]. These materials show high biocompatibility and osteoconductivity[9]. HA is a biomaterial with a lower degradation rate than new bone formation *in vivo*[10,11]. On the other hand, the degradation rate of TCP is higher than HA *in vivo*. It is important for a bone substitute material to have the character of degradation rate equal to the process of bone formation.

The β -TCP scaffold contains interconnected pores which facilitate the infiltration of osteogenic cells, and this material is strong enough to maintain the implant's shape during bone formation[12]. Moreover, β -TCP is resorbed and replaced by host bone within 24 weeks with no apparent adverse effects[13]. Horch et al. described the favourable solubility and biocompatibility of β -TCP, as evidenced by the almost complete bony regeneration after 12 months without foreign body reactions. They also reported that filling defects with β -TCP stabilized the blood clot within

Correspondence to: Yoshimitsu Abiko, DDS, PhD, Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo 2-870-1, Sakaecho-nishi, Matsudo, Chiba, Japan
Tel: 81-473-360-9322 , Fax: 81-473-360-9329, E-mail: abiko.yoshimitsu@nihon-u.ac.jp

the defect, thereby facilitating bone regeneration[14]. In addition, histological and histomorphometric comparison in the same patients revealed that there was no significant difference between β-TCP and autogenous bone grafts in terms of the quantity and rate of ossification[14-16].

The process of osteogenesis can be stimulated by applying bone formation stimulating molecules[17,18].

We previously reported that β-TCP can stimulate bone formation at an early stage after implantation[19]. Using microarray and Ingenuity Pathway Analysis (IPA), we identified the potential involvement of large numbers of genes, particularly IGF signaling, perhaps identifying candidate genes for implant research. IGF1 and IGF2 are also the most abundant growth factors produced by osteoblasts, and exert important effects on the proliferation, differentiation, and apoptosis of osteoblasts[20]. IGF1 and IGF2 increase bone collagen synthesis and decrease collagen degradation in cultures of intact calvariae, the effect on collagen synthesis correlates with an increase in transcript levels in osteoblast cells[21].

However, very little is known about the IGF signaling enhancing the bone formation by β-TCP. To understand the usefulness of β-TCP for development of biomaterials implants, β-TCP were implanted into bone defects after extracting teeth in beagle dogs, and IGF gene expression profiles were examined using Affymetrix GeneChip system. Altered mRNA levels in GeneChip analysis were confirmed by reverse transcription polymerase chain reaction (RT-PCR), real-time RT-PCR and immunofluorescence analysis.

Materials and methods

Implantation of β-TCP

Beagle dogs (body weight 13±2 kg; 10 dogs) were purchased from Japan SLC (Shizuoka, Japan). The dogs were allowed access to food and water *ad libitum* at all times and were maintained on a 12 h light/dark cycle (lights on 8:00 to 20:00) at 23±1°C. All beagle dogs were maintained and used in accordance with the guidelines of the care and use of laboratory animals of Kanagawa Dental College (Approval #: 08.12.TTH). There were two experimental groups: the β-TCP scaffold implant group and the no implant control. The left and right mandibles of beagle dogs were divided randomly into three time groups according to the expected time when they would be sacrificed.

All beagle dogs were injected with sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 35 mg/kg. The dogs then had their premolar teeth extracted. After three months' healing, the sockets of mandible defects (4.5 mm diameter, 8 mm length) were made in the mandible bone using an implant drill with physiologic saline cooling under anaesthesia. The β-TCP was purchased (β-TCP-100, >99% pure, Taihei Chemicals Limited, Japan) and crushed to 500-800 μm diameter particles, and was filled into left or right mandible bone defects (200 μg per one defect). After surgery, each beagle dog received an intramuscular injection of sodium ampicillin (Viccillin®, Meiji, Tokyo, Japan) at a dose of 100 mg per kg body weight. All specimens (4.5 mm diameter, 8 mm length) were taken out in a cylinder at day 4 and day 7 after surgery, and immersed into RNA stabilization solution (RNA later®, Applied Biosystems, Ambion, USA).

RNA preparation

Total RNA was extracted from each bone biopsy using an optimised RNA extraction protocol based on the RNeasy® Fibrous Tissue Midi Kit Isolation System (Qiagen, CA, USA) according to the manufacturer's protocol. One piece of a biopsy (a maximum of a 3 mm cube=25-35 mg of tissue) was placed into 350 mL of lysis buffer (Buffer RLT, from the isolation kit) in a Lysing Matrix A tube (FastPrep® System, MP Biomedicals, Tokyo, Japan) and homogenised six times at 6.0 m/s for 45 s each. Samples were processed using the RNeasy Isolation System following the NanoDrop manufacturer's instructions. RNA quality was confirmed by calculating the $OD_{260/280}$ ratio via a spectrophotometer, and its integrity was verified using Agilent RNA 6000 nano kits (Agilent, Santa Clara, CA, USA).

DNA microarray analysis

The protocol for DNA microarray processing was carried out according to the GeneChip® 3' IVT express kit user manual (Affymetrix, Santa Clara, CA, USA). Total RNA samples (100 ng) were subjected to two-cycle target labelling according to the Affymetrix instructions. Antisense complimentary RNA (cRNA) derived from double-strand complimentary DNA (cDNA) was labelled in the presence of biotinylated deoxyribonucleotide triphosphate (dNTP) derivatives to produce cRNA probes. The probes were then fragmented and hybridized on the GeneChip® Canine Genome 2.0 Array (Affymetrix; 38,000 genes). After hybridization, the arrays were scanned using the GeneChip® Scanner 3000 and the scanned images were analyzed using GeneChip® operating software (Affymetrix). Flag analysis, to demonstrate the presence (P) or absence (A) of signals, was evaluated and intensity normalization was performed. Data analysis was then performed using the GeneSpring (Silicone Genetics, Redwood, CA, USA) software package.

RT-PCR and real-time RT-PCR analysis

RT-PCR and real-time RT-PCR reactions were carried out using a DNA thermal analyser (RCX43-Gene™ 6000; Corbett Life Science, Sydney, Australia). Amplification by PCR was started with an initial incubation at 95°C for 15 sec to activate the Taq DNA polymerase, and then performed at 95°C for 5 s and 56°C for 15 s for the appropriate number of cycles. RT-PCR

products were electrophorezed on a 1.5% agarose gel, followed by staining with ethidium bromide to examine the intensity and size of PCR products. Each assay was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels.

Real-time PCR was carried out with SYBR Premix Ex Taq™ (Perfect Real-Time PCR, Takara, Japan) and a green PCR kit (Qiagen). To calculate gene expression fold changes, the initial template concentration was derived from the cycle number at which the fluorescent signal crossed the threshold in the exponential phase of the real-time PCR reaction. The mRNA copy unit was given by the cycle threshold value from the fluorescent signal of all the samples, including the standard curve and target genes, following the method provided by Corbett Life Science using the RCX43-Gene™ 6000 software. Details were described in an operation manual (version 1.7.40, 2006).

The DNA primer sequences were: for *IGF1*, 5'-cctcgcacctcttctacctg-3' (forward); 5'-gcctcctcagatcacagctc-3' (reverse), predicted size=228 bp; for *IGF2*, 5'-accctccagtttgtctgtgg-3' (forward); 5'-ggggtatctggggaagttgt-3' (reverse), predicted size=213 bp; for *GAPDH* 5'-atctccatcttccaggag-3' (forward); and 5'-atggacggtggtcatgag-3' (reverse), predicted size=318 bp. Values were calculated as means±standard deviation (SD), (n=3). Comparisons were made between the two groups using a Student's t test.

Immunofluorescence staining

Formalin-fixed, paraffin-embedded specimens were subjected to antigen retrieval and endogeneous peroxidase blocking (30 min), and rinsed with phosphate-buffered saline (PBS). Rabbit polyclonal primary antibodies (IGF-I, Millipore, Temecula, CA; IGF-II, abcam, Cambridge, MA) were used. After overnight incubation at 4C, the specimens were rinsed with PBS and incubated at room temperature for 1 h with secondary antibody conjugated to Alexa Flour 488 donkey anti-rabbit IgG (H+L) (Molecular Probes, Inc., OR, USA). After rinsing with PBS, all specimens were color-developed with ProLong Gold Antifade Reagent with DAPI (Molecular Probes, Inc., OR, USA). The immunostaining of all specimens was performed simultaneously to ensure the same antibody reaction and DAPI exposure conditions.

Statistical analyses

Significant differences were analyzed by Fisher's exact test. P<0.05 was considered to be statistically significant and P<0.01 highly significant.

Results

DNA microarray analysis

The scatter plot of gene expression between control and ß–TCP implanted beagle dog mandibles on 4 and 7 days after ß-TCP implantation are shown in Fig.1. Beta-TCP altered many genes expression. Among those genes, the elevated mRNA level of IGF1 and IGF2 were found.

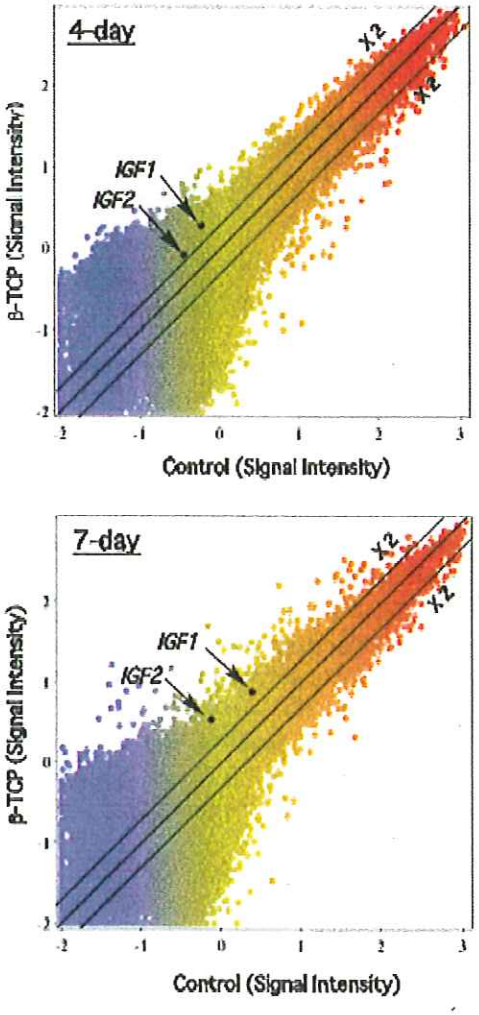

Figure1. Scatter plot of gene expression profiles between control and ß–TCP implanted beagle dog mandibles

Validation of microarray data by RT-PCR

Since the expression of IGF1 and IGF2 was up-regulated in β-TCP group by GeneChip analysis, we examined the expression of these genes by RT-PCR and Real-time RT-PCR. The same RNA samples used for GeneChip were examined. We have confirmed increased mRNA levels of IGF1 and IGF2 in β-TCP samples (Fig. 2).

Real-time RT-PCR analysis of IGF1 and IGF2 gene expression

To determine the quantity of mRNA levels, real-time RT-PCR was performed. The results were converted to relative mRNA copy units, and the significant increase in IGF1 and IGF2 gene expression was shown in β-TCP implanted samples (Fig. 3).

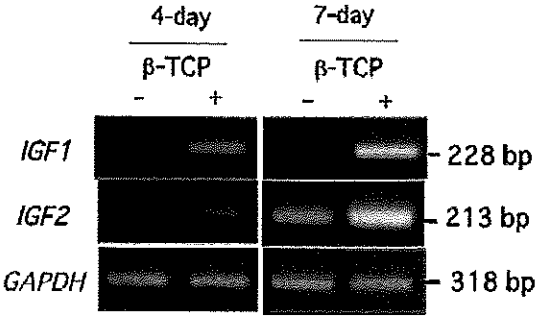

Figure 2. RT-PCR analysis of *IGF1* and *IGF2* mRNA levels. The amplification of each DNA band intensity of *IGF1* and *IGF2* were greater in ß-TCT implanted dog mandibles than in the control. Each GAPDH was used as an internal control.

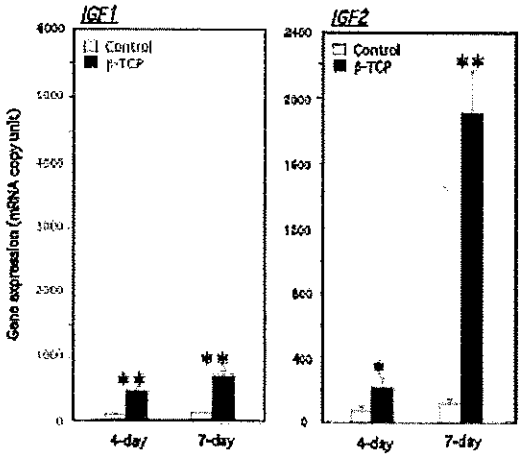

Figure 3 The quantified levels of gene expression relative to the level of GAPDH mRNA expression were performed. The data was converted to mRNA copy unit. Differences between control and β -TCP group were determined using Student's t-test. *, $p<0.05$; **, $p<0.01$, n=3.

Expression of IGF1 and IGF2 in β-TCP implanted tissues

To examine the phenotypic expression of IGF1 and IGF2, we performed immunofluorescene staining in dog mandible tissues. The experimental group with β-TCP implants showed more IGF1 and IGF2-positive cells than the control group. Staining for IGF1 and IGF2 was found in granulation tissue-forming processes in the mandibles, which showed markedly positive immunostaining in the β-TCP implanted samples, whereas weak staining was observed in the control group (Fig. 4).

Discussion

The rate of bone formation versus particle degradation is an important factor. Therefore, the ideal bone substitute material can be defined as the one that degrades in synchronous manner with the bone formation. It has been reported that the ideal graft substitute should reabsorb with time to allow and encourage new bone formation whilst maintaining its properties as an osteoconductive scaffold until it is no longer required[22].

Although autologous graft is the golden standard for the graft materials[23,24], it is not always possible to harvest sufficient amount of bone due to factors such as the limitation of donor sites[25-27]. Therefore, many researchers have investigated calcium phosphate as bone graft material because of its good biocompatibility[28].

β -TCP is widely used in clinical orthopedic surgery due to its high biodegradability, osteoconductivity, easy manipulation and lack of histotoxicity. It has also been used for bone regeneration, including dental implant therapy. When β -TCP is mixed with the blood clot and surrounded by the bony walls of the alveolar socket, osteogenic cells, including undifferentiated mesenchymal cells, start migrating from the bone surface between and over the surface of β -TCP. The TCP particles attracted osteoprogenitor cells that migrated into the interconnecting micropores of the bone substitute material by 6 months[29]. β -TCP particles in the extraction sockets are clinically osteoconductive. In our previous study, the β -TCP group showed a higher bone formation rate at 4 and 7 days, although this difference between the β -TCP and control groups seemed to disappear by day 14[19]. These findings suggest that tissue healing and bone formation occurred at a relatively early stage. The gene expression of COL1A1, ALP, OPN and TGF- β_2 was increased and RANKL and IFN expression decreased in β -TCP-implanted group[19].

In this study, the expression level of IGF-1 and IGF2 was increased in β -TCP-implanted group compared to the control, as determined by GeneChip analysis. IGF1 and IGF2 are important regulators of bone formation and promotes both osteoblast precursor proliferation and differentiation. Body weight was decreased by 67%, and 40%, respectively, in mice deficient in IGF1 and IGF2 . Peak bone mineral density (BMD) was decreased by 56%, and 18%, respectively, in mice deficient in IGF1 and IGF2 [30]. The patients with disruption of IGF-I exhibit the peak BMD of 4.78 SD below the mean value for age-matched normal subjects[31]. Mice lacking the type I IGF receptor exhibited delayed appearance of their ossification centers and delay in epiphyseal maturation, and they usually died at birth due to generalized hypoplasia of all muscles, including those responsible for respiration[32]. In vivo IGF-II increased the bone mineral density in rats[33]. Gene knockout studies revealed that a primary function of the IGF-II receptor is to regulate IGF-II levels, which can be lethal if too high[34]. These findings suggest that IGFs play important roles in bone formation and remodeling. The complex of IGF-I and its specific binding protein, IGFBP-3, may stimulate the osteoblastic activities via prolonged serum half life and increased cellular association of IGF-I[35]. The use of IGF-I/IGFBP-

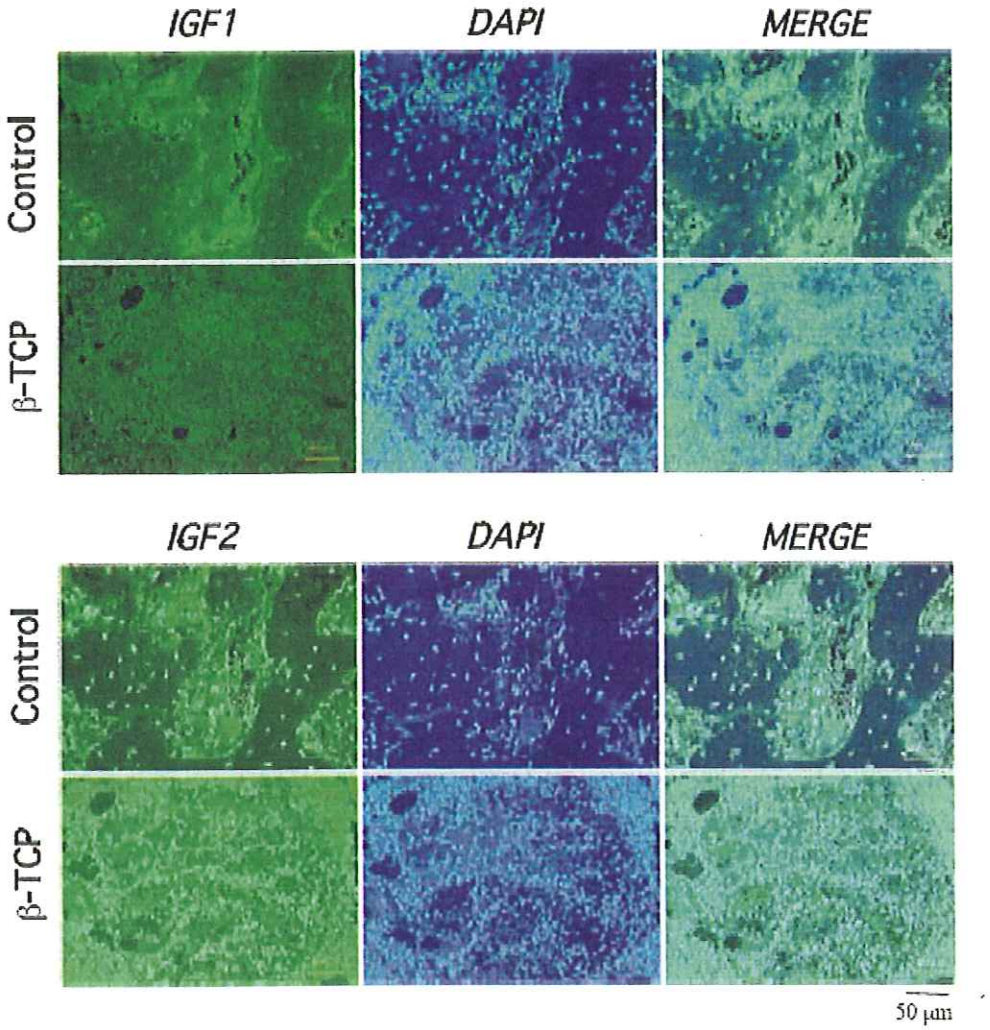

Figure 4. The immunostaining results for IGF1 and IGF2 at day 4 and day 7 after surgery. The nuclei were counter-stained with ProLong® Gold antifade reagent with DAPI in accordance with the manufacturer's instructions. The cells were visualized using confocal laser scanning microscopy (BZ-8000, Keyence Corporation, Osaka, Japan). The merge image represents the localization of IGF1 and IGF2 in cells. Original magnification = X20; Bar = 50 μm.

3 complex seems to be very useful and safe in older women with recent hip fracture[36]. Taking these reports together with our findings, we propose that β-TCP may be involved in bone remodeling and that the biodegradability of β-TCP is mediated through IGF expression.

Conclusion

Gene therapy and tissue engineering both offer the potential for treating clinical conditions that are now considered extremely difficult to manage by conventional therapeutic measures and increase our focus on the β-TCP coated with growth factors. This work contributes to a better basic understanding of the IGF signaling after implantation of β-TCP at an early stage, and the characteristics of β-TCP in bone tissue.

Acknowledgements

This study was supported in part by the "Academic Frontier" Project for Private Universities: a matching fund subsidy from Ministry of Education, Culture, Sports, Science and Technology, 2007-2011 and by Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (B21390497). We thank Dr. Jian Zhao for her excellent technical assistance.

References

1. Matsushita N, Terai H, Okada T, Nozaki K, Inoue H, Miyamoto S and Takaoka K. A new bone-inducing biodegradable porous beta-tricalcium phosphate. J Biomed Mater Res A 70:450-458, 2004
2. John HD and WENZ B. Histomorphometric analysis of

natural bone mineral for maxillary sinus augmentation. *Int J Oral Maxillofac Implants* 19:199-207, 2004

3. Boyne PJ and James RA. Grafting of the maxillary sinus floor with autogenous marrow and bone. J Oral Surg 38:613–616, 1980
4. Brugnami F, Caiazzo A and Leone C. Local intraoral autologous bone harvesting for dental implant treatment: Alternative sources and criteria of choice. Keio J Med 58:24-28, 2009
5. Moy PK, Lundgren S and Holmes RE. Maxillary sinus augmentation: Histomorphometric analysis of graft materials for maxillary sinus floor augmentation. J Oral Maxillofac Surg 51:857-862, 1993
6. Younger EM and Chapman MW. Morbidity at bone graft donor sites. J Orthop Trauma 3:192-195, 1989
7. Yuan H, Yang Z, De Bruij JD, De Groot K and Zhang X. Material-dependent bone induction by calcium phosphate ceramics: A 2.5-year study in dog. Biomaterials 22:2617-2623, 2001
8. Santos FA, Pochapski MT, Martins MC, Zenobio EG, Spolidoro LC and Marcantonio E. Comparison of biomaterial implants in the dental socket: Histological analysis in dogs. Clin Implant Dent Relat Res 12:18-25, 2010
9. Alcaide M, Serrano MC, Pagani R, Sanchez-Salcedo S, Vallet-Rehi M and Portoles MT. Biocompatibility markers for the study of interactions between osteoblasts and composite biomaterials. *Biomaterials* 30:45-51, 2009
10. Kihara H, Shiota M, Yamashita Y and Kasugai S. Biodegradation process of alpha-TCP particles and new bone formation in a rabbit cranial defect model. J Biomed Mater Res B Appl Biomater 79:284-291, 2006
11. Yamaguchi K, Hirano T, Yoshida G and Iwasaki K. Degradation-resistant character of synthetic hydroxyapatite blocks filled in bone defects. Biomaterials 16:983-985, 1995
12. Ozawa M. Experimental study on bone conductivity and absorbability of the pure β-TCP. J Jpn Soc Biomater 13:167-175, 1995
13. Yoneda M, Terai H, Imai Y, Okada T, Nozaki K, Inoue H, Miyamoto S and Takaoka K. Repair of an intercalated long bone defect with a synthetic biodegradable bone-inducing implant. Biomaterials 26:5145-5152, 2005
14. Horch HH, Sader R, Pautke C, Neff A, Deppe H and Kolk A. Synthetic, pure-phase beta-tricalcium phosphate ceramic granules (Cerasorb) for bone regeneration in the reconstructive surgery of the jaws. Int J Oral Maxillofac Surg 35:708-713, 2006
15. Brandoff JF, Silber JS and Vaccaro AR. Contemporary alternatives to synthetic bone grafts for spine surgery. Am J Orthop 37:410–414, 2008
16. Szabó G, Huys L, Coulthard P, Maiorana C, Garagiola U, Barabás J, Németh Z, Hrabák K and Suba Z. A prospective multicenter randomized clinical trial of autogenous bone versus beta-tricalcium phosphate graft alone for bilateral sinus elevation: Histologic and histomorphometric evaluation. Int J Oral Maxillofac Implants 20:371-381, 2005
17. Kasugai S. Local application of statin for bone augmentation. Clin Calcium 15:67-72, 2005
18. Kasugai S, Fujisawa R, Waki Y, Miyamoto K and Ohya K. Selective drug delivery system to bone: Small peptide (Asp)6 conjugation. J Bone Miner Res 15:936-943, 2000
19. Zhao J, Watanabe T, Bhawal UK, Kubota E and Abiko Y. Transcriptome analysis of β-TCP implanted in dog mandible. Bone 48:864-877, 2011
20. Govoni KE, Baylink DJ and Mohan S. The multi-functional role of insulin-like growth factor binding proteins in bone. Pediatr Nephrol 20:261-268, 2005
21. McCarthy TL, Centrella M and Canalis E. Regulatory effects of insulin-like growth factors I and II on bone collagen synthesis in rat calvarial cultures. Endocrinology 124: 301-309, 1989
22. Walsh WR, Vizesi F, Michael D, Auld J, Langdown A, Oliver R, Yu Y, Irie H and Bruce W. Beta-TCP bone graft substitutes in a bilateral rabbit tibial defect model. Biomaterials 29:266-271, 2008
23. Giannoudis PV, Dinopoulos H and Tsiridis E. Bone substitutes. An update, Injury 36:S20-S27, 2005
24. Rueger JM. Bone substitution materials. Current status and prospects. Orthopade 27:72-79, 1998
25. Baqain ZH, Anabtawi M, Karaky AA and Malkawi Z. Morbidity from anterior iliac crest bone harvesting for secondary alveolar bone grafting: An outcome assessment study. J Oral Maxillofac Surg 67:570-575, 2009
26. Beirne JC, Barry HJ, Brady FA and Morris VB. Donor site morbidity of the anterior iliac crest following cancellous bone harvest. Int J Oral Maxillofac Surg 25:268-271, 1996
27. Swan MC and Goodacre TEE. Morbidity at the iliac crest donor site following bone grafting of the cleft alveolus. Br J Oral Maxillofac Surg. 44:129-133, 2006
28. Yoshimine Y, Akamine A, Mukai M, Maeda K, Matsukura M, Kimura Y and Makishima T. Biocompatibility of tetracalcium phosphate cement when used as a bone substitute. Biomaterials 14:403-406, 1993
29. Zerbo IR, Bronckers AL, De Lange G and Burger EH. Localisation of osteogenic and osteoclastic cells in porous beta-tricalcium phosphate particles used for human maxillary sinus floor elevation. Biomaterials 26:1445-1451, 2005.
30. Mohan S, Richman C, Guo R, Amaar Y, Donahue LR, Wergedal J and Baylink DJ. Insulin-like growth factor regulates peak bone mineral density in mice by both growth hormone-dependent and -independent mechanisms.

Endocrinology 144:929-936, 2003

31. Woods KA, Camacho-Hübner C, Savage MO and Clark AJ. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N Engl J Med 335:1363-1367,1996
32. Van Wyk JJ and Smith EP. Insulin-like growth factors and skeletal growth: Possibilities for therapeutic interventions. J Clin Endocrinol Metab 84:4349-4354, 1999
33. Ishibe M, Ishibashi T, Kaneda K, Koda T, Rosier RN and Puzas JE. Stimulation of bone formation in vivo by insulin-like growth factor-II in rats. Calcif Tissue Int 63:36-38, 1998
34. Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P and Stewart CL. Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. Genes Dev 8:2953-2963, 1994
35. Tanaka H, Moriwake T, Matsuoka Y, Nakamura T and Seino Y. Potential role of rhIGF-I/IGFBP-3 in maintaining skeletal mass in space. Bone 22:145S-147S, 1998
36. Agnusdei D and Gentilella R. GH and IGF-I as therapeutic agents for osteoporosis. J Endocrinol Invest 28:32-36, 2005