J Korean Assoc Maxillofac Plast Reconstr Surg 2011;33(4):301-307

Original Article

Response of Odontoblast to the Bio-Calcium Phosphate Cement

Jin-Woo Kim[1], Sung-Won Kim[2], Gyoo-Cheon Kim[3],
Yong-Deok Kim[2], Cheol-Hun Kim[1], Bok-Joo Kim[1], Uk-Kyu Kim[2]

[1]Department of Dentistry, College of Medicine, Dong-A University, Departments of [2]Oral and Maxillofacial Surgery, [3]Oral Anatomy, School of Dentistry, Pusan National University

Abstract

Purpose: If the tooth structure is damaged, then it is impossible to regenerate the tooth. The materials used to restore the tooth structure are not related to the composition of the tooth. The materials used to restore the structure can't replace the natural tooth because they just fill the defective structure. Calcium phosphate cement remineralizes the dentin and almost replaces the natural tooth, but there are some disadvantages. We conducted basic tests with Biomimetic CPC (Bio-CPC) to make sure of the possibility of the biomaterial to remineralize the defective tooth structure.

Methods: In this study, the bioactivity and biocompatibility of Bio-CPC were evaluated for its potential value as the bio-material for regeneration of damaged tooth structure by conducting a cell toxicity assay (WST-1 assay), a cytokinesis-block micronucleus assay, a chromosomal aberration test, total RNA extraction and RT-PCR on MDPC-23 mouse odontoblast-like cells.

Results: The in vitro cytotoxicity test showed that the Bio-CPC was fairly cytocompatible for the MDPC-23 mouse odontoblast-like cells.

Conclusion: Bio-CPC has a possibility to be a new biomaterial and further study of Bio-CPC is needed.

Key words: Calcium phosphate cement (CPC), Odontoblast

Introduction

To recover damaged human body and function, lots of studies are in progress over the various fields. In dental clinics, many materials have been used for damaged tooth. Once tooth structure is damaged, it is impossible to regenerate original structure. In addition, materials to restore are unrelated with substances consisting tooth. As the restoration is just replaced in defected structure, it is impossible to work equally like natural teeth.

Calcium phosphate, one of composition in human body, have been researched in bone generation[1] and used to reconstruct bone defect in trauma or lesion and to strengthen porous bone[2]. Calcium Phosphate Cement (CPC) is also useful for broken tooth structure. CPC pastes have been used to remineralize defected tooth structure[3].

CPC contained an equimolar mixture of finely ground tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA) or dicalcium phosphate dihydrate (DCPD) as the solid phase[4]. When mixed with water,

RECEIVED March 29, 2011, ACCEPTED May 12, 2011

Correspondence to Uk-Kyu Kim
Department of Oral and Maxillofacial Surgery, School of Dentistry, Pusan National University
Beomeo-ri, Mulgeum-eup, Yangsan 626-770, Korea
Tel: 82-55-360-5112, Fax: 82-55-360-5104, E-mail: kuksjs@pusan.ac.kr

the paste was formed and it can reconstruct bone defects. After hardening reaction, the cement forms nanocrystalline hydroxyapatite (HA) as the only end-product.

Normal HA is very small rod-like crystals, and it has similar size with the HA crystalline in human tooth enamel. HA, CPC is nearly insoluble in water, but it is readily soluble under strong acidic. Since CPC has a neutral pH and contains only calcium phosphates, it was found to be highly biocompatible and osteoconductive. Due to its good biocompatibility and its similarity to the mineral phase of natural bone tissue, many studies have reported usefulness as bone cements[5,6]. Today, a number of bone cements from CPC and some restorative material for endodontic treatment also are currently available[7-16].

Because CPC with traditional TTCP has some disadvantages, it need to be improved. Biomimetic CPC (Bio-CPC) is the CPC using Biomimetic TTCP (Biomimetic tetracalcium phosphate, $Ca_4(PO_4)_2O$, developed by coworker, Prof. Chang and DeLong[17]), resolving the disadvantage of CPC. In this study, we had basic test with Bio-CPC to make sure the possibility of the biomaterial for remineralizing defected tooth structure.

Materials and Methods

1. Cell culture - MDPC-23 mouse odontoblast-like cell

The cells were cultured in Dulbecco's modified Eagles medium (Gibco, New York, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, New York, NY, USA) and 100 μg/ml penicillin/streptomycin at 37°C in humidified incubator with 5% CO_2 atmosphere.

2. Cell toxicity assay

5×10^3 cells were seeded into each well of 96-well tissue culture plate. After 24 h of incubation, the cells were treated with various concentrations of powdered CPC containing media and incubated for 24 h, 48 h, 72 h respectively. At the end of incubation, cell numbers were assessed using Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals, Rotkreuz, Zug, Switzerland). Add 10 μl of WST-1 solution to each well and incubate for 30 minutes. 80 μl of solution were transfer to new plate. The O.D were measured at 450 nm wavelength. For the control, 100 μl of CPC-diluted media was incubated for 24 (without cells), and used for WST-1 assay.

3. Cytokinesis-block micronucleus assay

The MDPC 23 cells were seeded into a 100 mm dish at a density of 1×10^6/well and incubated for 20 h. The cells were treated with Bio - CPC (100 μg/ml) for 24 h. Cytochalasin B (3 μg/ml) was added 44 h after the start of the culture, and incubation was continued for an additional 28 h. After culturing for 72 h, the cells were harvested and incubated in PBS for 5 min. After fixing carnoy solution (a mixture of methanol and acetic acid; 3 : 1) for 20 min at 4°C. The cell solution was dropped onto cold glass slides. Air-dried cell preparations were stained with 8% Giemsa solution for 15 min.

4. Chromosomal aberration test

The CHO-k1 cells were seeded into a 100 mm dish at a density of 1×10^6/well and incubated for 24 h. The cells were treated with Bio - CPC (100 μg/ml) for 24 h. Added Colchicine was a final concentration of 4 μg/ml and incubated for 2 h. After the cells were harvested and incubated in 0.075 M KCL for 20 min at 37°C. After fixing carnoy solution (a mixture of methanol and acetic acid; 3 : 1) for 20 min for 4°C. The cell solution was dropped onto cold glass slides. Air-dried cell preparations were stained with 8% Giemsa solution for 15 min.

5. Adhesion of Cells to CPC powders & total RNA extraction, RT-PCR

3×10^5 cells/well were seeded into 6-well plates (coated, or non-coated with adhesion molecules). At the same time, 1 mg/ml CPC powders was added to non-coated plate for experimental group. After 24 hours of incubation, microscopic photographs were taken and then cells were gathered, and subjected to RNA extraction procedure using Trizol reagent. 1 μg of extracted total RNA was used for cDNA synthesis. 3 μl of each cDNA was used for PCR against adhesion proteins and functional markers.

Total RNA was extracted from each sample using the Trizol reagents (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Promega, Madison, WI, USA). First strand complementary DNA

(cDNA) was reverse transcribed using the Maxime RT PreMix kit (iNtRON, Seongnam, Korea). The PCR products were resolved by electrophoresis on 1.5% ethidium bromide stained agarose gel. Detailed information of primers used in this article was subscribed on Table 1.

Results

1. Cell toxicity assay

After treatment, Cell Proliferation Reagnet WST-1 binds strongly to odontoblast. Cell viability evaluated by WST-1 assay or by direct counting after enzymatic dissociation (Fig. 1~3). After 24 h treatment, cell viability was decreased. After 48 h treatment, viability was increased. But at 72 h after treatment, viability was decreased again.

In 10 μg/ml of concentration, cell viability is recovered after 48 h treatment and maintained. In higher concentration, cell viability is not recovered after 72 h.

Table 1. Primer sequences of ALP, CD44, Integrin α1, α2, β1

Gene name	Sequence	Product size
Alkaline phosphatase-1	TTTGGTGGATACACCCCC GCCTGGTAGTTGTTGTGAGC	176 bp
CD 44	AGAAGGTGTGGGCAGAAG CCAATCTTCATGTCCACA	203 bp
Integrin α1 (ITG 1α)	GGAGCTGTGTACATTTATCA TGTAGCATTTATGCATACTG	303 bp
Integrin α2 (ITG 2α)	TTTGGTTCAGCAATTGCA TGGAATCCCCATTTAAATC	238 bp
Integrin β1 (ITG 1β)	TACACTGGCAGTGCATGT CTCTGCACTGAACACATTCT	199 bp
β-actin	GACTACCTCATGAAGATC GATCCACATCTGCTGGAA	512 bp

Cell viability is over than 80% at 24 h, 48 h, 72 h after treatment in 10 μg/ml, therefore 10 μg/ml is the best condition for MDPC-23 mouse odontoblast-like cell in cell toxicity assay.

2. Cytokinesis-block micronucleus assay

The numbers of damaged chromosomes in the cells treated with Bio-CPC were similar with in control cells (Fig. 4). Total number of MN in BN cells no different from control group (4 in treated with Bio-CPC, 3 in control group), but there was no BN cells with NPB (1 in control group).

3. Chromosomal aberration test

The number of chromosomal structure abnormality is quite small and shows no gap between both groups (Fig.

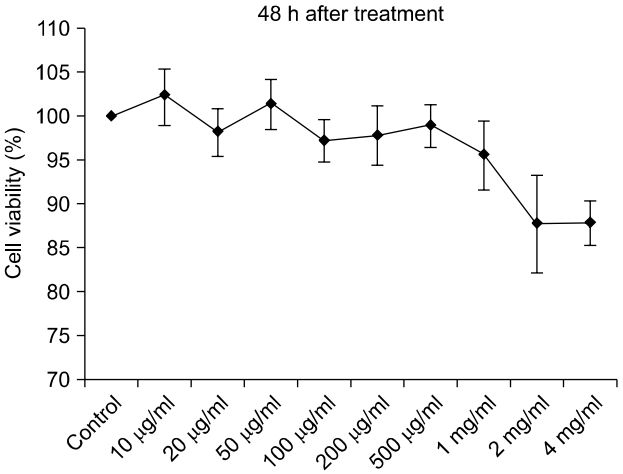

Fig. 2. Cell viability test by WST-1 assay after 48 h treatment.

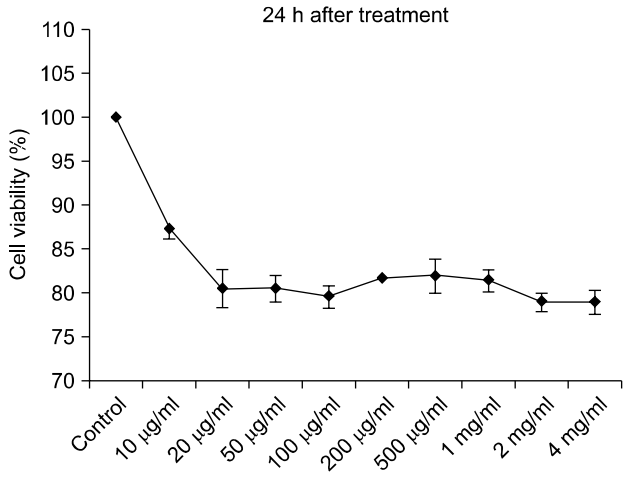

Fig. 1. Cell viability test by WST-1 assay after 24 h treatment.

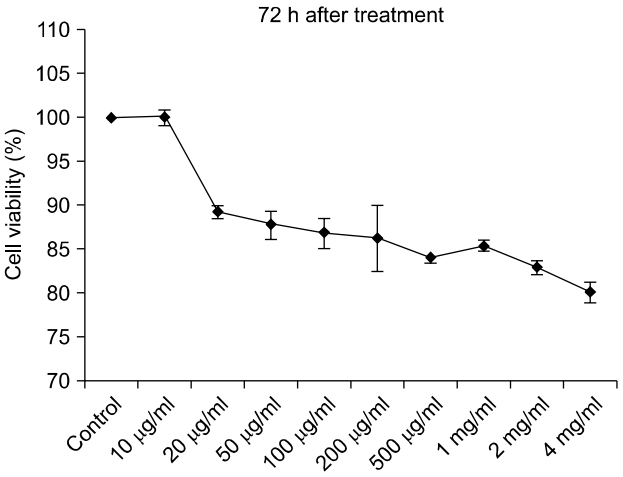

Fig. 3. Cell viability test by WST-1 assay after 72 h treatment.

	Total no. of BN cells scored	Total no. of MN in BN cells	No. of BN cells with NPB	No. of BN cells with Nbud
Bio-CPC	1015	4	0	0
Control	1044	3	1	0

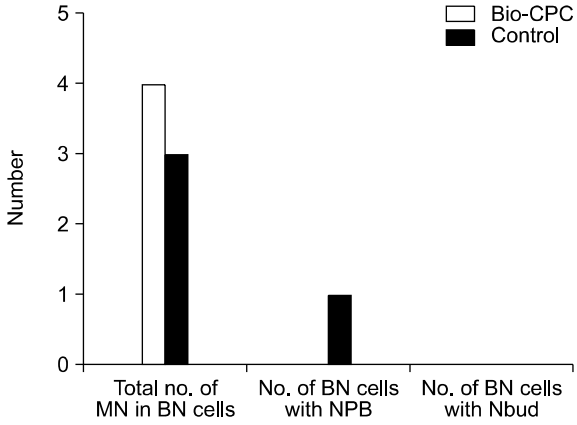

Fig. 4. Cytokinesis-block micronucleus assay in 100 μg/ml. MN, micronucleus; BN, binucleated; NPB, nucleoplasmic bridge; Nbud, nuclear bud.

5). In chromatid level, it showed no abnormality. In chromosome level, only small gap were exist in csb, moreover, the number of chromosomal structure abnormality is small than control group in cse.

4. Adhesion of Cells to CPC powders & total RNA extraction, RT-PCR

On coated plate, cells adhered on the plate. On non-coated plate, cells couldn't adhered on the plate, adhered adjacent cells (Fig. 6).

The results of RT-PCR showed mRNAs for adhesion molecules formed on cells in each plates (Fig. 7). Alkaline phosphatase 1 was the bone marker and CD44 is antibody. They were used for checking genetic disorder in MDPC-23. β-actin was used as a loading control for among groups and it expressed similar level. On coated plate, adhesive glycoprotein molecule was weak because adhesion molecule was not necessary. On non-coated plate without Bio-CPC, band of adhesion molecule was definite than

Treatment	Time of treatment (h)	Concentration of treatment (μg/ml)	Chromosome no.	No. of chromosmal structure abnormality					
				Gap	Chromatid		Chromosome		Etc.
				g	ctb	cte	csb	cse	
Control	-	-	1072	0	0	0	4	5	0
Bio-CPC	24	100	1307	0	0	0	6	1	0

Fig. 5. Chromosomal aberration test in 100 μg/ml. ctb, chromatid breakage; cte, chromatid exchange; csb, chromosome breakage; cse, chromosome exchange.

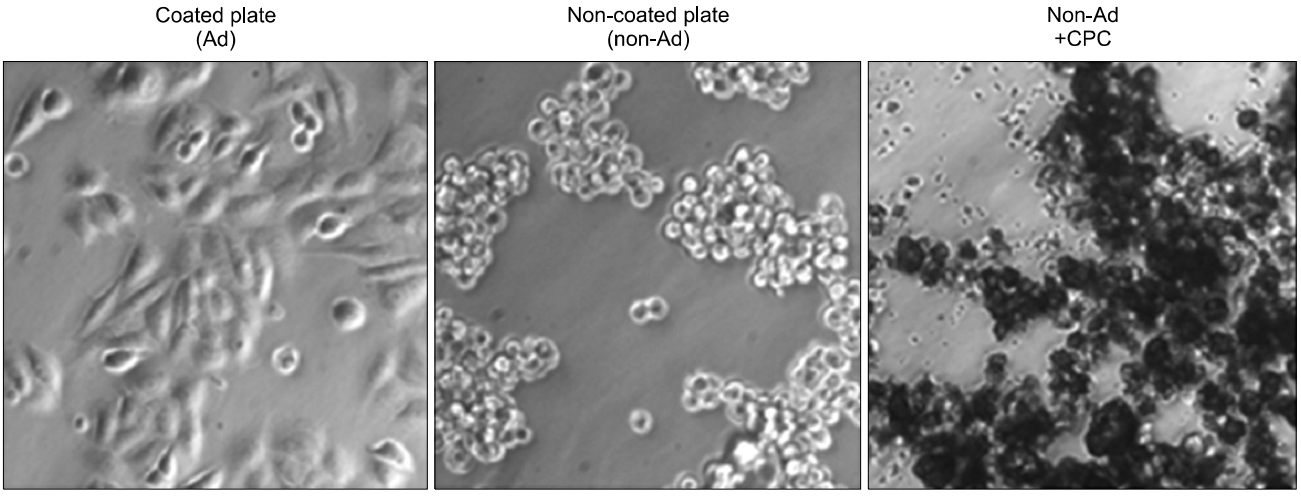

Fig. 6. Microscopic photographs of cell adhesion in various conditions.

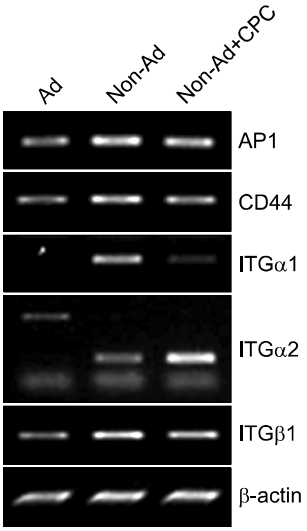

Fig. 7. mRNAs for adhesion molecules were seen in RT-PCR. It shows molecules formed on cells in each plates. Ad, Adhesion culture media; Non-Ad, non-adhesion culture media.

coated plate. On non-coated plate with Bio-CPC, CD44, ITG 1α, and ITG 1β were weaker than without Bio-CPC. But ITG 2β was more definite expressed.

Discussion

The tetracalcium phosphate powder was synthesized from a solid-state reaction between equimolar amounts of dicalcium phosphate anhydrous (DCPA) and $CaCO_3$, which were mixed and heated at 1,500°C for 6 hours in a furnace (Model 51333, Lindberg, Watertown, WI, USA)[18]. CPC is synthesized with the TTCP. As previously indicated, the synthesis needs water. HA is formed in an aqueous environment and has a relatively low crystallinity, similar to biological apatite, and it seems to be responsible for CPC's in vivo resorption characteristics.

Biomimetic TTCP (Bio-TTCP) is developed to improve this problem. Bio-TTCP is the TTCP made by new method. Bio-TTCP is produced at 37°C, 10.5<pH<12.0. Bio-CPC used Bio-TTCP is similar with CPC but no water needed for dissolving. The amount of DAPA for the reaction depends on the Bio-CPC paste at from 11.6 to 11.7 in pH for suspension and emulsion, respectively. Because phase of Bio-CPC is paste phase at 37°C, 10.5<pH<12.0, and it is confirmed through X-ray diffraction, Bio-CPC may have resistance for resorption. Bio-CPC may be a polymeric molecular structure of TTCP, HA and $Ca(OH)_2$. Bio-CPC may be used for bone cements because it makes HA like CPC.

Mechanical property of Bio-CPC is important, because bone and teeth receive strong power. Compressive strength (CS) of trabecular bone is 10 MPa[19]. CS of Bio-CPC is 70 MPa and diametral tensile strength (DTS) of Bio-CPC is 9 MPa.

The role of CPC is "bone repairing." High biocompatibility and self hardening is unique properties of CPC in vivo. Those properties makes gradual resorption and replacement by new bone formation with no loss in volume. To make sure these issues, further biomechanical tests for Bio-CPC are undergoing by Prof. Chang and DeLong[17].

There are many studies to make sure that CPC is not toxic in human body. Lee et al.[20] studied toxicity of CPC to the human dental pulp cells. The results showed CPC decreased viability of cells but it's not critical. They also showed CPC containing chitosan was less toxic to the cells. The biocompatibiliity of CPC was systematically investigated by Liu et al.[21]. The text included systemic injection acute toxicity assay, cell culture cytotoxicity assay, gene mutation assay (Ames test), chromosome aberration assay (micronucleus test), DNA damage assay (unscheduled DNA synthesis test) and implant histological evaluation. The investigation on the inherited toxicology of Magnesium phosphate cement including gene mutation assay (Ames test), chromosome aberration assay (micronucleus test), and DNA damage assay (unscheduled DNA synthesis test) were carried out by Yu et al.[22]. So the study for toxicity of Bio-CPC with chitosan in progress. In this study, results of cell toxicity assay shows cell viability is over than 75% at 24 h, 48 h, 72 h on all concentration after treatment and it means Bio-CPC has low toxicity for odontoblast.

Cytokinesis-block micronucleus assay is a sensitive and simple indicator of chromosome damage, both chromosome loss and chromosome breakage, which also provides information on cell cycle progression and cytotoxicity. Chromosomes of odontoblast were not damaged by Bio-CPC. Therefore, though we didn't observe Bio-CPC enhanced cell proliferation and differentiation, at least Bio-CPC was not harm to cell proliferation.

Structural chromosome aberrations may be induced via DNA breaks by various types of mutagens. Such DNA breaks may either rejoin such that the chromosome is re-

stored to its original state, rejoin incorrectly or not rejoin at all. These last two cases may be observable on microscopic preparations of metaphase cells. However, many of these gross changes probably will not allow cell survival after division, but they serve as indicators for the induction of smaller, not readily observable changes, which do allow cell survival but may have deleterious consequences for the organism. The result of chromosomal-aberration test in this study showed low possibility for Bio-CPC damaging odontoblasts.

Zhang et al.[23] evaluated the effect of a calcium phosphate material equipped with poly (lactic-co-glycolic acid) microspheres for pulp capping, and to measure the dentin bridge formation. Boland et al.[24] checked the cytotoxic effect to odontoblast and pulp cells by resin-based calcium phosphate cement. Many studies showed CPC has the potential for remineralizing defected tooth and has not severe toxicity to odontoblast.

Tang et al.[25] found out the structure and components of CPC are similar to those of the normal human bone, and bone marrow stem cells grow well on the surface of it, so it is a suitable scaffold for tissue engineering artificial rib. However, the cell adhesion ability is to be further improved. In this study, odontoblast with Bio-CPC formed adhesion molecules less than negative control group, more than positive control group. This showed Bio-CPC has an adhesive ability for the cells.

Reverse transcription polymerase chain reaction (RT-PCR) is widely used in the diagnosis of genetic diseases and, semiquantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression. Lee et al.[20] studied whether CPC and CPC-chitosan promoted odontoblastic differentiation of HDPCs, as evidenced by the formation of mineralized nodules. They found out chitosan did not enhance adhesion, growth and differentiation by RT-PCR. In this study, RT-PCR was also used to check expression of adhesion molecules like integrin on odontoblast.

Integrin (ITG) is obligate heterodimers containing two distinct chains, called the alpha (α) and beta (β) subunits. In mammals, eighteen α and eight β subunits have been characterized. In addition, variants of some of the subunits are formed by differential splicing; for example four variants of the $\beta 1$ subunit exist. Through different combinations of these α and β subunits, some 24 unique integrins are generated, although the number varies according to different studies[26].

In coated plate with CPC powders ITG $\alpha 1$, ITG $\beta 1$ were expressed weaker than non-coated plate without Bio-CPC (control group). But ITG $\alpha 2$ was expressed stronger in plate with Bio-CPC. ITG $\alpha 1$ and $\alpha 2$ are combined with ITG $\beta 1$ (integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$) in vertebrates[27], it means that integrin $\alpha 1\beta 1$ was rarely expressed, and intergrin $\alpha 2\beta 1$ was expressed much. Two integrins have ligands for collagens and laminins. Therefore Bio-CPC has adhesional potential with odontoblast.

This study demonstrates Bio-CPC has a possibility to be regenerative material for defected teeth as a biocompatible material.

Conclusions

Calcium phosphate cement (CPC) remineralize defected tooth structure in endodontic treatment. It is useful but has some disadvantages. In this study, we performed cell toxicity assay, cytokinesis-block micronucleus assay, chromosomal aberration test, total RNA extraction, RT-PCR with Bio-CPC in odontoblast to find out clinical value of Bio-CPC. Bio-CPC was not severe harmful effect compared with CPC. Bio-CPC showed adhesion with odontoblast. Therefore Bio-CPC was considered as the potential material for regenerating tooth structure. Bio-CPC has possibility to be new biomaterial and continuous study is needed.

References

1. Xu HH, Weir MD, Simon CG. Injectable and strong nano-apatite scaffolds for cell/growth factor delivery and bone regeneration. Dent Mater 2008;24:1212-22.
2. Ryu KS, Shim JH, Heo HY, Park CK. Therapeutic efficacy of injectable calcium phosphate cement in osteoporotic vertebral compression fractures: prospective nonrandomized controlled study at 6-month follow-up. World Neurosurg 2010; 73:408-11.
3. Chau JY, Hutter JW, Mork TO, Nicoll BK. An in vitro study of furcation perforation repair using calcium phosphate cement. J Endod 1997;23:588-92.
4. Brown WE, Chow LC. A new calcium phosphate water-setting cement. In: Brown PW, editor. Cements research progress 1986. Westerville: American Ceramic Society, OH; 1987. p.352-79.

5. Xu HH, Carey LE, Simon CG Jr, Takagi S, Chow LC. Premixed calcium phosphate cements: synthesis, physical properties, and cell cytotoxicity. Dent Mater 2007;23:433-41.
6. Carey LE, Xu HH, Simon CG Jr, Takagi S, Chow LC. Premixed rapid-setting calcium phosphate composites for bone repair. Biomaterials 2005;26:5002-14.
7. Chohayeb AA, Chow LC, Tsaknis PJ. Evaluation of calcium phosphate as a root canal sealer-filler material. J Endod 1987;13:384-7.
8. Hong YC, Wang JT, Hong CY, Brown WE, Chow LC. The periapical tissue reactions to a calcium phosphate cement in the teeth of monkeys. J Biomed Mater Res 1991;25:485-98.
9. Friedman CD, Costantino PD, Jones K, Chow LC, Pelzer HJ, Sisson GA Sr. Hydroxyapatite cement. II. Obliteration and reconstruction of the cat frontal sinus. Arch Otolaryngol Head Neck Surg 1991;117:385-9.
10. Sugawara A, Nishiyama M, Kusama K, et al. Histopathological reactions of calcium phosphate cement. Dent Mater J 1992;11:11-6.
11. Costantino PD, Friedman CD, Jones K, Chow LC, Sisson GA. Experimental hydroxyapatite cement cranioplasty. Plast Reconstr Surg 1992;90:174-85; discussion 186-91.
12. Sugawara A, Kusama K, Nishimura S, et al. Histolopathologycal reactions to calcium phosphate cement for bone filling. Dent Mater J 1993;12:691-8.
13. Costantino PD, Friedman CD. Synthetic bone graft substitutes. Otolaryngol Clin North Am 1994;27:1037-74.
14. Bohner M. Calcium orthophosphates in medicine: from ceramics to calcium phosphate cements. Injury 2000;31(Suppl 4):37-47.
15. Friedman CD, Costantino PD, Takagi S, Chow LC. BoneSource hydroxyapatite cement: a novel biomaterial for craniofacial skeletal tissue engineering and reconstruction. J Biomed Mater Res 1998;43:428-32.
16. Schmitz JP, Hollinger JO, Milam SB. Reconstruction of bone using calcium phosphate bone cements: a critical review. J Oral Maxillofac Surg 1999;57:1122-6.
17. Chang MC, DeLong R. Calcium phosphate formation in gelatin matrix using free ion precursors of Ca2+ and phosphate ions. Dent Mater 2009;25:261-8.
18. Xu HH, Quinn JB, Takagi S, Chow LC. Processing and properties of strong and non-rigid calcium phosphate cement. J Dent Res 2002;81:219-24.
19. Carter DR, Hayes WC. Bone compressive strength: the influence of density and strain rate. Science 1976;194:1174-6.
20. Lee SK, Lee SK, Lee SI, et al. Effect of calcium phosphate cements on growth and odontoblastic differentiation in human dental pulp cells. J Endod 2010;36:1537-42.
21. Liu C, Wang W, Shen W, Chen T, Hu L, Chen Z. Evaluation of the biocompatibility of a nonceramic hydroxyapatite. J Endod 1997;23:490-3.
22. Yu Y, Wang J, Liu C, et al. Evaluation of inherent toxicology and biocompatibility of magnesium phosphate bone cement. Colloids Surf B Biointerfaces 2010;76:496-504.
23. Zhang W, Walboomers XF, Jansen JA. The formation of tertiary dentin after pulp capping with a calcium phosphate cement, loaded with PLGA microparticles containing TGF-beta1. J Biomed Mater Res A 2008;85:439-44.
24. Boland EJ, MacDougall M, Carnes DL, Dickens SH. In vitro cytotoxicity of a remineralizing resin-based calcium phosphate cement. Dent Mater 2006;22:338-45.
25. Tang H, Xu Z, Liu C, Qin X, Wu B, Wu L. Preparation of CPC for tissue engineering artificial rib and a study on proliferation and adhesion of BMSCs on CPC. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2008;22:1238-41.
26. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673-87.
27. Harvey Lodish, editor. Molecular cell biology. 5th ed. New York: WH Freeman WH and Co.; 2003. p.973.