

Interaction between odontoblast and bio-calcium phosphate cement reinforced with chitosan

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Purpose: Calcium phosphate cement (CPC) is one of many useful materials for restoring tooth defects, periodontium and maxillofacial area. Chitosan is a biodegradable material that has been shown to promote the growth and differentiation of osteoblasts in culture. This study examined the interaction between odontoblasts and bio-calcium phosphate cement reinforced with chitosan.

Materials and Methods: 5×10^3 odontoblastic cells were seeded into each well. Various concentrations of bio-calcium phosphate cement reinforced with chitosan (10, 20, 50, 100, 200, 500 $\mu\text{g/ml}$, 1, 2, 4 mg/ml) were diluted and added to the wells. The well was incubated for 24 h, 48 h and 72 h. After incubation, the number of cells was assessed to determine the cell viability. A cytokinesis-block micronucleus assay and chromosomal aberration test were carried out to estimate the extent of chromosomal abnormalities. Microscopic photographs and RT-PCR were performed to examine the adhesion potential of bio-calcium phosphate cement reinforced with chitosan.

Results: Bio-CPC-reinforced chitosan did not show significant cytotoxicity. The number of damaged chromosomes in the cells treated with Bio-CPC-reinforced chitosan was similar to that in the control cells. There was no significant increase in the number of chromosomal aberrations in the Bio-CPC reinforced chitosan exposed cells. Microscopic photographs and RT-PCR confirmed the adhesive potential of bio-CPC reinforced chitosan to odontoblasts.

Conclusion: Bio-CPC-reinforced chitosan did not affect the odontoblastic cell viability, and had no significant cytotoxic effect. Bio-CPC-reinforced chitosan showed adhesive potential to odontoblasts. These results are expected form the basis of future studies on the effectiveness of dental restorative materials in Bio-CPC reinforced with chitosan.

Key words: Calcium phosphate cement, Chitosan, Odontoblast

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1. Introduction

Tooth is one of the most typical tissue that is not regenerated if its structure is destructed once in human's body. In addition, materials we used for damaged tooth are just replace the tooth structure, not related with substances consisting tooth. To regenerate tooth structure, materials should have ability to remineralize damaged portion. So many materials were investigated to restore destructed tooth structure.

One of useful materials to restore tooth defect is calcium phosphate cement (CPC). CPC is a well-known synthetic bone graft material. In dental area, CPC is used for pulp-capping material alternative to calcium hydroxide (CH). Traditionally, different formulations of calcium hydroxide (CH) have been

used for conservative pulp therapy¹. However, there are disadvantages associated with the use of CH, such as the presence of tunnels in the dentin bridge and the lack of adhesion and degradation after acid etching. CPC is superior to pure CH, which means that this material may have applications in pulp capping to induce reparative dentin formation or as a lining material². But, CPC is fabricated at 1500°C and pulverized. The frozen phase is not dissolved at 37°C. Thus, powder size is issue for cement reaction. Biomimetic calcium phosphate cement (Bio-CPC), in contrast, is made at 37°C, and it can be paste phase in 37°C. It is expected better properties than previous CPC.

Chitosan and chitosan derivatives appear to be good candidates for the elastomeric matrix. These natural biopolymers are biocompatible and biodegradable³, and osteoconductive⁴. They have been used in surgical interventions for the reduction of periodontal pockets⁵ in dental area. Chitosan has also been studied *in vitro* and *in vivo* with calcium phosphate compounds. Recently, chitosan/hydroxyapatite composites have been prepared with a homogeneous nanostructure using a coprecipitation method⁶.

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The aim of this study was to find basis on effectiveness of the Bio-CPC reinforced with chitosan. To investigate biological effects such as cell viability, cell proliferation, differentiation and adhesive potential of Bio-CPC reinforced with chitosan on odontoblast, interaction between Bio-CPC reinforced with chitosan and odontoblast.

II . Materials and Methods

Cell culture

We used the MDPC-23 mouse odontoblast-like cell. The cells were cultured in cell culture media (Dulbecco's modified Eagles medium, Gibco, NY, USA) supplemented with 10% heat-inactivated serum (fetal bovine serum (FBS), GIBCO) and 100 µg/ml penicillin/ streptomycin at 37°C in humidified incubator with 5% CO₂ atmosphere.

The effect of CPC-ch powders on MDPC-cell viability

5 × 10³ cells were seeded into each well of 96-well tissue culture plate and incubated for 24 h. The Powdered Bio-CPC reinforced chitosan was diluted in growth media. Each media was incubated for 24 h, 48 h and 72 h. After incubation, 10 µl of WST-1 solution was added to each well. Then incubating for 30 min, transfer 80 µl of solution to new plate and measure the O.D at 450 nm wavelength. For the control, 100 µl of Bio-CPC reinforced chitosan-diluted media was incubated for 24 h (without cells), and used for WST-1 assay.

Cytokinesis-block micronucleus assay

The MDPC 23 cells were seeded into a 100 mm dish at a density of 1 × 10⁶/well and incubated for 20 h. The cells were treated with Bio-CPC reinforced chitosan (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.

Chromosomal aberration test

The CHO-k1 cells were seeded into a 100 mm dish at a density of 1 × 10⁶/well and incubated for 24 h. The cells were treated with Bio-CPC reinforced chitosan (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 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.

Adhesion of Cells to CPC powders

3 × 10⁵ cells on each well were seeded into 6-well plates.(coated, or non-coated with adhesion molecules) At the same time, 1 mg/ml Bio-CPC reinforced chitosan powders was added to non-coated plate for experimental group. After 24 h 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 extraction, RT-PCR

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 Reverse transcription kit(Maxime RT PreMix kit, iNtRON, Daejon, 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.

Table 1. Reverse transcriptase-polymerase chain reaction (RT-PCR) primers sequence

Gene name	Sequence	Product size
Alkaline Phosphatase-1	TTTGGTGGATACACCCCC	176 bp
Integrin β1	GCCTGGTAGTTGTTGTGAGC TACACTGGCAGTGCATGT CTCTGCACTGAACACATTCT	199 bp
Integrin α1	GGAGCTGTGTACATTTATCA TGTAGCATTATGCATACTG	303 bp
Integrin α2	TTTGGTTCAGCAATTGCA TGGAATCCCCATTTAAATC	238 bp
CD 44	AGAAGGTGTGGGCAGAAG CCAATCTTCATGTCCACA	203 bp
β-actin	GACTACCTCATGAAGATC GATCCACATCTGCTGGAA	512 bp

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III. Results

Effects of cements on cell toxicity

Cell numbers assessed using Cell Proliferation Reagent WST-1, were represented in Figure 1. It was observed that all concentration of powdered Bio-CPC reinforced chitosan we studied in 24 h incubation has cytotoxicity against MDPC-23 cells. At 48 h after treatment, in contrast, Bio-CPC reinforced

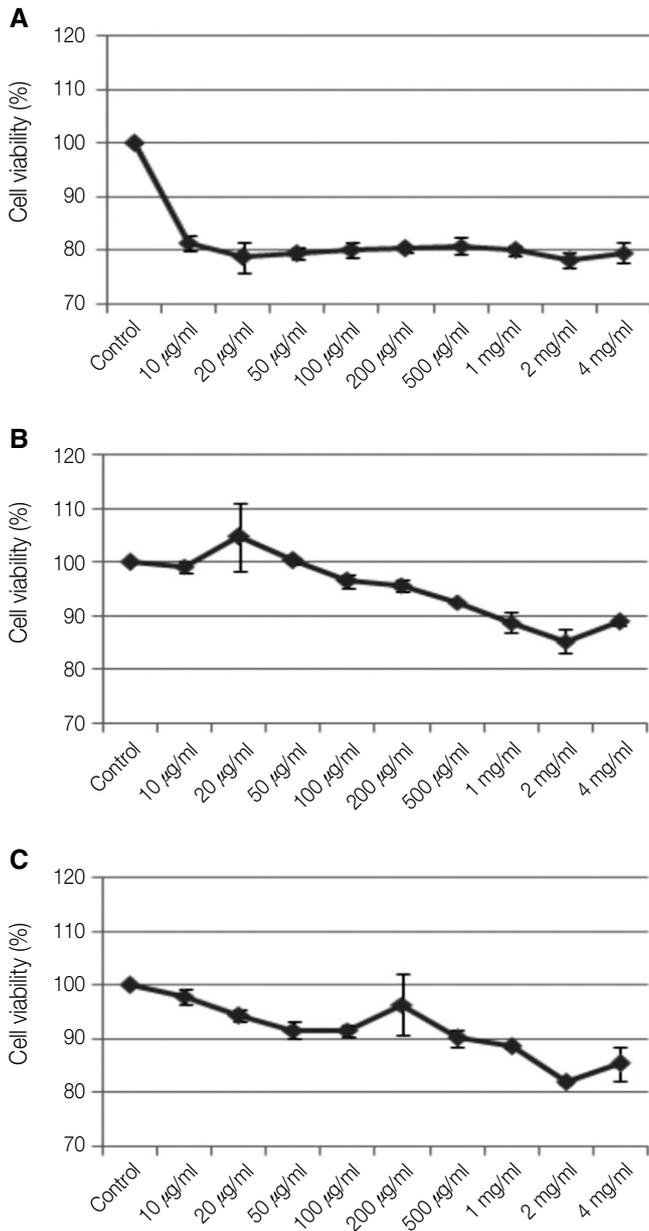


Fig. 1. The effects of cements on cell viability. *Byung-Do Chun et al: Interaction between odontoblast and bio-calcium phosphate cement reinforced with chitosan. J Korean Assoc Oral Maxillofac Surg 2011*

chitosan did not show significant cytotoxicity, especially it accelerated cell proliferation in 50 µg/ml. After 72 h, it didn't shows cytotoxicity significantly.

Cytokinesis-block micronucleus assay

The assay was performed in 100 µg/ml, because it was the concentration not disturbing for proliferation of MDPC23 cells. The numbers of damaged chromosomes in the cells treated with Bio-CPC reinforced chitosan were similar with in control cells.(Fig. 2) Total number of micronuclei (MN) in binucleated (BN) cells were similar with control group (5 in treated with CPC-ch, 3 in control group). But Other DNA damage events such as nucleoplasmic bridges (NPB) or nuclear buds (NBUD) were not shown in the present study. There was no BN cells with NPB in CPC-ch, 1 BN cell found in control group in contrast.

Chromosomal aberration test

There was no significant increase in chromosomal aberration in Bio-CPC reinforced chitosan treatment (100 µg/ml) compared with the control group.(Fig. 3) In chromatid level, 2 chromatid break (ctb) were found in Bio-CPC reinforced chitosan. Chromatid exchange (cte) was not seen. In chromosomal change, 3 chromosome break (csb) were shown in CPC-ch and no chromosome exchange (cse) was found. In control group, 4csb and 5cse were observed, but any other structural aberrations in chromatid level was not seen.

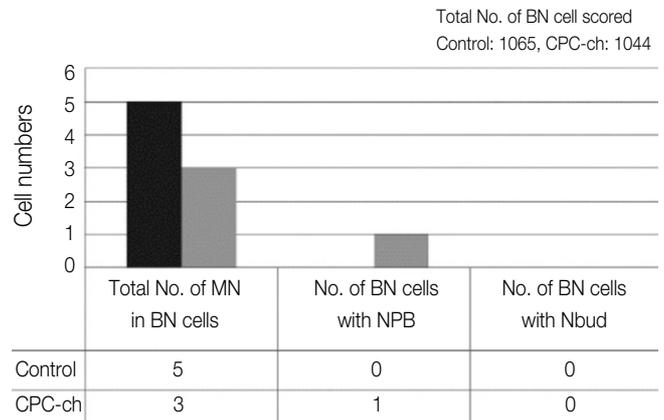


Fig. 2. Cytokinesis-block micronucleus assay. *Byung-Do Chun et al: Interaction between odontoblast and bio-calcium phosphate cement reinforced with chitosan. J Korean Assoc Oral Maxillofac Surg 2011*

Treatment	Time of treatment (h)	Concentration of treatment (µg/mL)	Chromosome NO.	NO. of chromosomal structure abnormality					
				Gap	Chromatid		Chromosome		Etc.
				g	ctb	cte	csb	cse	
Control	-	-	1072	0	0	0	4	5	0
CPC-ch	24	100	1152	0	2	0	3	0	0

Fig. 3. Chromosomal aberration test.

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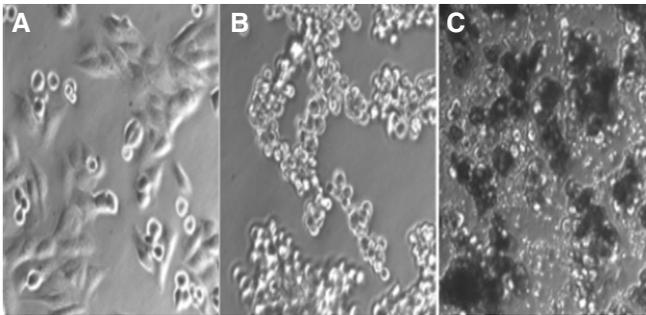


Fig. 4. Microscopic photography of the cell adhesion. A: coated plate, B: non-coated plate, C: non-coated plate with CPC-ch.
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Adhesion of cells to CPC powders & total RNA extraction, RT-PCR

The assay was also performed in 100 µg/ml concentration. Figure 4 shows the microscopic morphologies of cell adhesion on each well. In this study, cells in coated plate/non-coated plate were used as a positive/negative control. In coated plate, the cells stuck to adhesion molecules, whereas cells didn't in non-coated plate. In non-coated plate with Bio-CPC reinforced chitosan, cells were located around powder. Expression of Alkaline Phosphatase-1, Integrin β1, Integrin α1, Integrin α2, CD 44, and β-actin messenger RNA in cells on each sample was shown in Figure 5. In non-coated plate, cells expressed various adhesional molecules in order to adhere to something. But in non-coated plate with Bio-CPC reinforced chitosan, the expression of mRNAs was similar to that of cells in coated plate rather than non-coated plate's.

IV. Discussion

Calcium phosphate cement (CPC) is a synthetic bone graft material that was invented in 1986 by Chow and Brown, scientists at the American Dental Association. The cement is a white powder consisting of equimolar amounts of ground

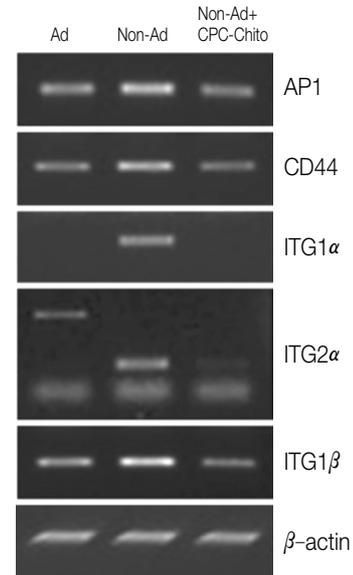


Fig. 5. The expression of mRNAs in MDPC-23 cells.

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Ca₄(PO₄)₂O (tetracalcium phosphate, TTCP) and CaHPO₄ (dicalcium phosphate anhydrous, DCPA). When mixed with water, the material forms a workable paste which can be shaped during surgery to fit the contours of bone defects. The cement hardens within 20 min allowing rapid closure of the wound. The hardening reaction, which forms nanocrystalline hydroxyapatite (HA) as the product, is isothermic and occurs at physiologic pH so tissue damage does not occur during the setting reaction.

Hydroxyapatite is an important biomaterial because of its similarity to the apatitic mineral in natural teeth and bones^{7,8}. CPC was developed with the advantage of being moldable and capable of in situ setting to form hydroxyapatite⁹. Several different cement compositions were developed. The CPC powder consisted of TTCP and DCPA, when mixed with water at a powder:liquid ratio of 4:1, the paste hardened in about 30 min and formed hydroxyapatite⁹⁻¹¹. CPC showed excellent biocompatibility and osteoconductivity, and was able to be resorbed and replaced by new bone^{10,11}. It was approved in 1996 by the Food and Drug Administration for repairing craniofacial defects in humans, thus

becoming the first CPC available for clinical use¹¹.

CPC showed promise for several dental applications, including root canal filler, sealer^{12,13} and base applications¹⁴. CPC-containing dental composites were also developed¹⁵. Unlike traditional composites with glassy fillers, the CPC powder as fillers in resins resulted in the release of Ca and PO₄ ions. These composites showed potential for pulp capping and cavity lining applications, and remineralized *in vitro* the demineralized dentin.

Recently, CPC has been also combined with a biopolymer chitosan to yield a strong and non-rigid hydroxyapatite composite envisioned for periodontal bone repair¹⁶⁻¹⁸. Chitosan is frequently proposed in the research field of tissue engineering¹⁹⁻²¹. This deacetylated derivative of chitin commonly found in shells of marine crustaceans and fungi is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine linked in a (1-4) manner. The glucosamine/N-acetyl glucosamine ratio is referred as the degree of de-acetylation, which can vary from 30% to 95%. In its crystalline form, chitosan is normally insoluble in aqueous solutions above pH 7; however in dilute acids (pH < 6.0), the protonated free amino groups on glucosamine facilitate solubility of the molecule. Chitosan is biodegradable, and has been shown to promote the growth and differentiation of osteoblasts in culture. It can be molded in porous structures and has been used in association with other biomaterials²² such as CPC. CPC set in situ to form hydroxyapatite are promising materials for a wide range of clinical applications especially in bone reconstruction due to their good osteoconductivity. Several groups have shown that chitosan can be used as adjuvant to CPC in order to enhance their mechanical properties without altering their chemical properties²³.

But previous CPC has some limitation. The tetracalcium phosphate powder was formed from a solid-state reaction between equimolar amounts of DCPA and CaCO₃, which were mixed and heated at 1500°C. The frozen phase of CPC is not well dissolved at 37°C. So powder size is an important issue for cement reaction and application of CPC needs water. On the other hand, Bio-CPC is produced at 37°C, 10.5 < pH < 12.0, it is confirmed through X-ray diffraction. It can be paste phase at 37°C. No water for dissolving CPC was needed, thus Bio-CPC may have resistance for resorption. Bio-CPC may be a polymeric molecular structure of TTCP, HA and Ca(OH)₂. Bio-CPC is mixed with a small amount of DCPA, HA formed. Bio-CPC 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. Bio-CPC may be used for bone cements because it makes HA like CPC. In this study, Bio-CPC is used because of this advantage. Addition of chitosan, enhanced the mechanical and biological properties of material.

Further studies are needed to investigate biocompatibility of biomimetic TTCP.

I tested the experimental Bio-CPC reinforced chitosan for their effects on MDPC-23 proliferation and odontogenesis. I found that Bio-CPC reinforced chitosan were cytotoxic for MDPC-23 at 24 h of cultivation. These results are supported by the data of Khashaba *et al.*²⁴ who reported that CPC decreased the proliferation of human gingival fibroblasts and a mouse fibroblast cell line. In the CPC setting, TTCP and DCPA dissolved in the liquid as Ca²⁺, PO₄³⁻, and OH-ions, which then reprecipitated to form hydroxyapatite²⁵. It seems likely that the ionic activities and pH changes that occurred during setting were responsible for the observed cytotoxicities²⁶. But relative cell viability showed over 80% at all concentrations in 24 h. Besides in 48 h and 72 h, it showed over 90% of control group and some showed over 100%. For chromosomal aberration, Bio-CPC reinforced chitosan also have good nature. In this study, only few cells were shown abnormalities in Bio-CPC reinforced chitosan group. Lee *et al.* (2010) studied toxicity of CPC to the human dental pulp cells.²⁷ 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. In this respect, Bio-CPC reinforced chitosan didn't seemed to have cytotoxicities.

Reverse transcription polymerase chain reaction (RT-PCR) is widely used in the diagnosis of genetic diseases and, semi-quantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression. In this study, RT-PCR was used to check expression of adhesion molecules. In non-coated plate with Bio-CPC reinforced chitosan powders (experimental group), several adhesion molecules were expressed like coated plate (positive control). Cell adhesion is important for bonding between dentin and Bio-CPC reinforced chitosan, the results shows Bio-CPC reinforced chitosan can be used for restorative material. In non-coated plate with Bio-CPC reinforced chitosan, the expression of mRNAs was similar to that of cells in coated plate rather than non-coated plate's. The expression of AP1, CD44, ITGβ1 and β-actin in experimental group was similar to positive control, besides ITGα2 expression was less than coated plate. ITG1α expression was found in negative control group, but experimental group and positive control group were not shown neither. It means Bio-CPC reinforced chitosan have good adhesive potential to odontoblast.

Thus, Bio-CPC reinforced chitosan is a good potential material to restore dental tissue. Further studies are needed to the effectiveness of dental restorative material in Bio-CPC reinforced chitosan.

V. Conclusion

Biomimetic calcium phosphate cement can be widely used to endodontic, periodontal and oral and maxillofacial area. Chitosan is useful for medical treatment by its biocompatibility and osteoconductibility. It was tested that interaction between Bio-CPC reinforced chitosan and odontoblast to investigate cell viability, adhesive potential and cytotoxicity of Bio-CPC.

In order to test the effect of Bio-CPC reinforced chitosan powders on MDPC-cell viability, cytokinesis-block micronucleus assay, chromosomal aberration test, adhesion of cells to powders and total RNA extraction, RT-PCR were performed. In cell viability test, mild decreased MDPC-23 cells were shown after 24 h incubation with Bio-CPC reinforced chitosan. But in 48 h and 72 h incubation, Bio-CPC reinforced chitosan didn't affect cell viability significantly. And chromosomal aberration was not occurred by Bio-CPC reinforced chitosan. In adhesive potential, it was shown that odontoblast attached around Bio-CPC reinforced chitosan. Also, the adhesive molecules were expressed similar to that of cells in coated plate in non-coated plate with Bio-CPC reinforced chitosan.

In conclusion,

1. Bio-CPC reinforced chitosan did not affect odontoblastic cell viability.
2. Bio-CPC reinforced chitosan did not show any remarkable cytotoxic effect .
3. Bio-CPC reinforced chitosan showed adhesive potential to odontoblast.

These results will form the basis of future studies on the effectiveness of dental restorative material in Bio-CPC reinforced with chitosan.

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