바이오시스템공학 (*J. of Biosystems Eng.*)
Vol. 29, No. 4, pp. 347~356 (2004)

A Novel Bioresorbable Bone Cement Using Tooth Apatite, Chitosan, and Cyanoacrylate for Bone Tissue Engineering
- Handling & mechanical properties, cytotoxicity and biocompatibility -

J. H. Chung W. Lee P. H. Choung J. E. Davies

골 조직공학을 위한 치아인회석, 키토산, 시아노아크릴레이트를 이용한 새로운 생체흡수성 골시멘트

정종훈 이 원 정필훈 J. E. Davies

적 요

치아인회석, 키토산, 시아노아크릴레이트 등의 생분해성 생체재료를 이용해 새로운 생체흡수성 골시멘트를 개발하고자 하였다. 이들 골시멘트들에 대해서 중합온도, 응고시간 등의 조작특성과 압축강도, 전단강도 등의 물성을 분석하였다. 특히, 치아인회석과 키토산의 미립가루와 부틸 시아노아크릴레이트의 강력접착제를 이용한 시멘트(B)에 대해서는 직접 접촉방법과 XTT 방법을 통해 세포독성을 분석하였고, 또한 쥐를 이용한 동물실험에서 시멘트(B)의 처리그룹에 따라 생체적합성을 분석하였다.

시아노아크릴레이트를 이용한 골시멘트의 최대중합온도는 약 33℃, 조작(응고)시간은 3~6분, 압축강도는 약 15~25 MPa, 전단강도는 약 0.4~1.7 MPa를 나타내었다. 첨가제로 사용된 Lipiodol은 골시멘트의 주사성과 강력접착제의 초기 중합지연도를 높였고, 특히 송진가루는 시아노아크릴레이트의 초기중합을 지연시켰다. 시아노아크릴레이트를 이용한 시멘트(B)의 세포독성을 분석한 결과, 대분분의 처리 그룹에서 낮게 나타났고, 특히 키토산과 치아인회석을 사용한 경우 각각 세포 독성이 더 낮게 나타났다. 그리고 골시멘트(B)의 동물 생체적합성 실험의 방사선상 및 조직학적 분석에서도 뼈 형성 및 결합이 우수하게 나타났다.

Keywords : Bioresorbable bone cement, Bone tissue engineering, Tooth-apatite, Butyl-cyanoacrylate, Chitosan.

1. Introduction

A bioresorbable, biodegradable and injectable bone cement using biomaterials is needed for bone fracture, bone defects, bone loss due to trauma, and for bone scaffolds, bone screws and plates(Gresser et al., 1995). Also, the bone fixation using metal plates needs to be replaced by a bone cement with high strength. Bone cements consist of powders and liquid monomers. There are two typical bone cements; one is a bioresorbable bone cement, another is a nonbioresorbable bone cement. Polymethyl methacrylate(PMMA) cements and some ceramic bone cements with SiO_2 and Al_2O_3 are nonbioresorbable. Calcium phosphate cements(CPC) are bioresorbable(Takechi et al., 1998). Current bone cements have low mechanical properties, low biodegradation, low bio-compatibility, and low chemical binding to bone. Especially, a PMMA cement is currently used in orthopedic treatments, even though it has high polymerization heat of 70~80℃(maximum temp. 114.5℃), a carcinogenic monomer, no biodegradation and acidity of pH 4.7(Kuhn, 2000). Therefore, the PMMA cement needs to be replaced by a novel bioresorbable bone cement with low polymerization temperature. The calcium phosphate cements don't have strength enough for bone fracture and bone bonding, and hence much time in hardening.

The adhesives that would enable immediate and strong bone bonding have been the dream of many surgeons. Bone bonding agents could be useful as an adjunct to osteosynthesis by permitting to reduce the fracture in anatomic position even for the most comminuted fractures (Weber and Chapman, 1984). Butyl-2-cyanoacrylate(BCA) adhesive is easy to apply, bioresorbable, biocompatible, inexpensive, noninfective, as a possible alternative to

Corresponding Author: Jong Hoon Chung, Professor, Biosystems and Agricultural Engineering. Chonnam National University; W. Lee, Ph.D. MD. and P. H. Choung, Professor, College of Dentistry, Seoul National University; J. E. Davies, Professor, Institute for Biomaterials and Biomedical Engineering and Faculty of Dentistry, University of Toronto.

conventional rigid fixation techniques(Shermak et al., 1998). Amarante et al.(1995) recommended the use of cyanoacrylate as adhesive material of bone, except mandible and long bone, because the mandible was an area where structural and functional demands were very similar to that on long bones which were subjected to be greater than those in the craniofacial area. BCA adhesives are currently used as biodegradable glues for bonding skins. These cyanoacrylates harden so fast as soon as they contact with β-tricalcium phosphate(TCP) or tooth apatites, bones and body fluids without setting time. So, an inhibitor in the initial polymerization is necessarily required for the setting time of a bone cement. The butyl-cyanoacrylate is most bioresorbable among cyanoacrylates.

Calcium hydroxy apatite(HA) has now been clinically accepted in medicine and dentistry as one of the most important bone substitute. Alloplastic HA has taken from coral and bovine commonly but also from extracted human tooth source. Tooth is well-crystallized hard tissue containing larger mineral portion than bone. Tooth mineral is a structurally imperfect analogue of HA and the reactivity and biological behavior are different from the crystal of bone or synthetic HA. Some reports have introduced calcined teeth under certain temperature as an alternative material of the synthetic HA. Other researches stated it as 'tooth ash'(Hoh, 1984 and Bakos et al., 1999). The authors called it as 'tooth apatite(TA)' because it is almost composed of HA and whitelockite. Biocompatibility and osteoconductivity of TA have been approved *in vitro* and *in vivo* by some researchers. Choung and Kim(1997) indicated its disadvantages that it was powder form and unsuitable for handling and processed into different form as structural biomaterials.

Bone cements need biodegradable powders like water soluble chitosan for bone formation and revascularization. Chitosan is the product of the partial deacetylation of the naturally occurring polysaccharide chitin, which is found in the exoskeletons of insects and marine invertebrates. It is a linear copolymer of N-acetyl-D-glucosamine and glucosamine, with the latter constituting greater than 80% of the molecule. Chitosan has been suggested to possess biological and material properties suitable for clinical applications. Over the past 20 years, the biomedical applications of chitosan have been widely studied (Shepherd et al., 1977). The material has been evaluated as a wound-healing agent, bandage material, skin grafting template, cholesterol lowering agent, hemostatic agent, hemodialysis membrane, and drug-delivery vehicle(Hirano, 1996). Using chitosan plugs, Muzzarelli et al.(1994) succeeded in creating mineralized bone-like tissue in osseous defects in rats, sheep, and dogs. Chitosan is biologically renewable, biodegradable, biocompatible, non-antigenic, non-toxic, and biofunctional(Muzzarelli et al., 1989). It evokes a minimal foreign body reaction and fibrous encapsulation upon implantation(Sundararajan and Howard, 1999).

To prevent ingrowth of other cell types and rapidly regenerating tissue, the principle of guided tissue regeneration(GTR) using a membrane was introduced for periodontal application by Nyman et al.(1982). Dahlin et al.(1988) and Zellin et al.(1995) reported that the principle has also been used for bone defects. The clinical use of semipermeable membrane such as expanded polytetrafluoroethylene(e-PTFE) was established. Some investigators have attempted to avoid the necessity of second operation to remove the nonbiodegradable membrane. The biodegradable membrane systems are made from synthetic polymer such as lactid or glycolid polymers or from natural materials such as chitosan or collagen.

In addition, bone cements and bone substitutes should be injectable to bone defects and bone fracture with proper viscosity. As a liquid of bone cements, an seed (poppy or safflower) needed for the proper viscosity.

The purpose of this study was to develop a novel bioresorbable bone cement using biodegradable biomaterials such as β-tricalcium phosphate($Ca_3(PO_4)_6$, TCP), recycled tooth apatite(TA), chitosan and an adhesive of butyl-2-cyanoacrylate(BCA). The physical, handling and mechanical properties of bioresorbable bone cements were investigated, and cytotoxicity *in vitro* and animal tests *in vivo* on an acrylic bone cement were conducted.

2. Materials and Method

A. Materials

Bioresorbable and biodegradable biomaterials of β-tricalcium phosphate(TCP) and tooth apatite(TA) obtained

from extracted teeth were considered as a composite powder(TATCP) of a bone cement or a bone substitute. TCP was used with TA limited in amount as a main powder of a bone cement as TCP is more bioresorbable than TA ceramic. The main components of the TA obtained by sintering the extracted teeth were whitlockite. Zinc(ZnO) and a solution of di-sodium hydrogen orthophosphate(Na_2HPO_4, pH: 9.3) were used for the acceleration of a calcium phosphate bone cement. N-butyl cyanoacrylate(Histoacryl^{O} B|Braun, Tuttlingen, Germany) was used as a liquid monomer of a bone cement. Water soluble chitosan was used as an additive for biocompatibility and bone revascularization. An 40% iodized poppy seed oil, lipiodol, was used as an opacifier, an inhibitor of initial polymerization and an injectable. Edible pine resin powder was also considered as an additive of inhibitor in the initial polymerization of TCP and BCA. And as additives, slowly biodegradable poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid)(PHBV) and caprolactone with high plasticity were applied to increase the strength.

B. Method

1) Formulas for a bone cement using biodegradable biomaterials

Cements of seven types shown in Table 1 were tested to develop a bioresorbable and injectable bone cement. Physical and handling properties of the cements were analyzed in plasticity, setting and hardening times, etc. The Type A cement consisted of TCP, water soluble chitosan powder and ZnO without an adhesive of BCA. The Type B cement consisted of TA and BCA with an additive of chitosan. The Type C cement consisted of TCP, TA and BCA with additives of pine resin and lipiodol. The Type D cement consisted of TCP, TA, PHBV powders and BCA with additives of pine resin and lipiodol. Morphology of a bone cement was observed by a scanning electron microscope(SEM, Hitachi S-570, Tokyo, Japan) at 15 kV as a physical property. The specimen was coated by a SEM coating system(Polaron Co., USA) for the SEM test.

2) Handling properties of cements

The powder of each cement was mixed with a liquid monomer and additives. The handling properties of the mixed cement were investigated in views of setting temperature, setting time, hardening time, and injectability. The setting temperature and setting time of cements were measured according to ISO 5833(Kuhn, 2000).

3) Mechanical properties of cements

The mechanical properties of bone cements recommended in this study were investigated in a compressive strength and a 3-point bending strength. The compressive strength of specimen dried for 24 hours was measured by a Instron machine(model 8501, Instron Co., USA) with a cross-head speed of 20 mm/min. The cross-head of the plunger was a cylindrical type with a diameter of 5.6 mm. The diameter and height of a sample were 6 mm

Table 1 The formulars of bone cements tested in this study

Cement Type	Powder	Liquid Monomer	Additives	Remarks
A	TA	H_2O or Na_2HPO_4	Chitosan, ZnO	2M Na_2HPO_4 Solution
B	TA	BCA	Chitosan	
C	TA + TCP	BCA	Pine resin, Oil	Pine resin: powder Oil: Poppy seed oil
D	TA + TCP + PHBV	BCA	Pine resin, Oil	
E	TA + TCP	BCA	Pine resin, Oil, Caprolactone	Caprolactone:liquid
F	TA + TCP + PHBV	BCA	Pine resin, Oil Caprolactone	
G	TA + TCP + PHBV	BCA	Oil Caprolactone	No pine resin

Note : 1) TA : tooth apatite, 2) TCP : tri-calcium phosphate, 3) BCA : butyl-cyanoacrylate, 4) PHBV : poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid).

and 12 mm. The bending strength of a specimen was measured with a loadcell of 10 N and a cross-head speed of 0.2 mm/min.

4) Evaluation of the cytotoxicity

a. Direct contact cell culture evaluation

The cytotoxities of the Type B bone cement such as BCA, chitosan with BCA, tooth apatite with BCA, and membrane with BCA were investigated. L929 mouse fibroblasts(Korean Cell Line Bank, Seoul, Korea) were used. The suspensions of L929 fibroblasts(4.0×10^5 cells/mL in FBS-DMEM: fetal bovine serum-Dulbecco's modified Eagle Medium, GibcoBRL® Grand Island, USA) were evenly seeded into each Petri dish. There were 5 test specimens which were diameter of 5mm paper (Control), paper with BCA(Histoacryl, Group 1), membrane(Gore ResolutÔ, W.L. Gore & Associates, Flagstaff, USA) with BCA(Group 2), paper with chitosan (Jakwang, Ansung, Korea) and BCA(Group 3), paper with tooth apatite and BCA(Group 4). The test specimens were overlaid on the center of the plate where confluent monolayers of the fibroblasts were formed. After removal of the specimens from each plate, the plate was washed with phosphate-buffered saline(PBS) and stained with 0.2% crystal violet(CV)-ethanol solution for 20 minutes. The results were compared with the reference Controls(paper only) and interpreted by the value of zone index by American Society for Testing and Materials(ASTM, 2000) 813-83.

b. Preparation of extracts of test materials

Each 4 cm^2 blank filter paper(Control), paper with BCA(Group 1), membrane with BCA(Group 2), paper with chitosan and BCA(Group 3), paper with TA and BCA(Group 4) were steeped in extraction vial containing 4ml DMEM without phenol as extractant. The extraction vials were incubated at 37℃, and in an air atmosphere of 5% CO_2 for 72 hours.

c. XTT test

To make colorimetric assay based in XTT(sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid) for the quantification of cell proliferation, the cells were then seeded in 96-well cell culture clusters, at a density of 1.5×10^4 cells/well and incubated for 24 hours to enable attachment. After incubation, medium was aspirated from all wells and replaced with 100㎕ control media and samples(100%, 50%, 25%, 12.5%, 6.5% and 0% of the concentrations). The spectrophotometrical absorbances of the samples were measured by a microtiter plate(ELISA) reader(Bio-Rad Model 450 microplate reader, Bio-Rad Laboratories Inc., Hercules, USA). The wavelength to measure absorbance of the formazan product is 450nm according to the filters available for the ELISA reader. The reference wavelength is 655nm. The raw data was taken by Microplate Manager PC ver 4.0(Bio-Rad Laboratories Inc., Hercules, USA).

5) Animal Test for Biocompatibility

a. Animals and anesthesia

75 adults male Sprague-Dawley rats were used. Anesthesia was induced with an intramuscular injection. The animals were feeding soft diet soaked in water for 1 week after surgery.

b. Surgical procedure

The left mandibular angle was exposed by incisions on the left neck. The fracture of mandible was examined by separation gap of the fragments. The fixation was accomplished with no adhesives(Control), BCA only (Group 1), BCA with 3×6 mm membrane(Group 2), BCA with chitosan(Group 3), BCA with tooth apatite (Group 4). The adhesive was placed as minute droplet over the contact area of the fragments in such a way that it covered the fracture line and around bucca surface.

c. Tissue processing

(1) H/E staining procedures

After the healing periods of 2 weeks, 4 weeks and 6 weeks, all animals were sacrificed by an overdose of CO_2. Block biopsies, including the mandible and surrounding soft tissues, were taken from all operation sites carefully. The specimens were fixed by immersion in 10% buffered formalin, followed by decalcification in 5% nitric acid solution, and finally embedding in paraffin. Serial horizontal sections(4mm) were cut through the specimens, and they were stained with haematoxylin/ eosin.

(2) Immuno staining procedures

All immunostaining procedures were carried out at

room temperature. Sections were then incubated with primary antibodies for BMP-2(1 : 100, Santa Cruz Biotechnology Inc., Santa Cruz, USA), BMP-2/4(1:100, Santa Cruz Biotechnology Inc., Santa Cruz, USA), Osteocalcin(1:1000, Zymed laboratories Inc., South San Francisco, USA) for approximately 24 hours at 4℃. To allow comparisons between treatments, all sections were stained for each antibody at the same time.

d. Radiologic evaluation

To take the radiographic findings, the harvested blocks were placed on the radiographic film and exposured by X-ray(43 kVp, 3.2mA, 16ms, Philips, Best, The Netherlands). The experimental side was compared with the contralateral, unoperated side to take postoperative results. A grading system was developed on the basis of radiographic findings to compare results(Table 2).

Table 2 Grading system for radiologic evaluation

Grade	Radiographic findings
0	No gap at the fracture side
1	Radiolucent line at the cortical bone only
2	Radiolucent line at the cortical and marrow bone
3	Demonstrated radiolucent gap less than 1.0mm
4	Demonstrated radiolucent gap more than 1.0mm

e. Histologic evaluation

The three mid-section from all specimens were analyzed by light microscopy. In order to quantify the histological findings, such as blood clot organization, bone union and formation of compact bone, a grading(scoring) system, modified after Heiple et al. and Zellin et al. was used(Table 3).

Table 3. Numerical grades for histologic evaluation, modified after Heiple et al. and Zellin et al.

Blood clot	No organization of clot	0
	Organization well under way(vessels, fibers)	2
	Clot completely organized(coarser bundles)	4
Bone union	No sign of fibrous of other union	0
	Fibrous union	1
	Osteochondral union	2
	Bone union	3
Compact bone	None	0
	Beginning to appear	1
	Formation well under way	2
	Complete reorganiztion	4

3. Results and Discussion

A. Handling and mechanical properties of cements according to formulars

The physical, handling and mechanical properties of bone cements of Types A, B, C, D, E, F, and G were presented in Table 4. The cements of Types A and B are TA ceramic bone cements, and the cements of Types C, D, E, F and G are a kind of acrylic bone cements. The cement of Type A was made from tooth apatite and chitosan gel(chitosan + H_2O) with an additive of ZnO. The cement was a kind of ceramic bone cement, and the characteristics of TA was similar to those of TCP. Hardening of the cement was accelerated by ZnO. The hardening time of the specimen was about 20 min. The cement could be injectable by increasing the amount of water. The water soluble chitosan was used for biocompatibility and revascularization of the bone cement as a bone matrix needs pores with diameter of 300~500 μm for the penetration of cell growth. However, the chitosan decreased the strength a little. The cement didn't have enough strength due to no polymerization, compared with that of a bone cement with an adhesive of BCA. An adhesive was necessarily required for increasing the strength of a bone cement. An injectable ceramic bone cement was available by mixing TA and TCP with a solution of 0.2M Na_2HPO_4, which was hardened in about 20 min after mixing.

In the Type B cement, as soon as the powder of TA with little chitosan contacted with the BCA adhesive, the mixed powder was instantly hardened without a setting time. A injectable bone cement was not available. In this case an inhibitor of BCA was required for preventing the initial polymerization. This cement could be used for an instant bonding of bones.

bility of the Type C cements. The noninjectable C-1 cement had higher values in compressive and bending strengths, compared with those of the other cements in Type C. The setting time of C-1 cement was prolongated by the pine resin powder. The maximum setting temperature and the setting time of Type C-2 cements were 33 ℃ and 3 min as shown in Fig. 1. The

compressive strength(bioyield point) of the Type C cements ranged from 15 to 25 MPa(Fig 2). The 3-point bending strength of the Type C cements ranged from 0.4 to 1.7 MPa. The C-4 cement was considered as a good formula among Type C cements. In case of adding water soluble chitosan of 0.04 g to the C-3 cement, the cement had a setting time of about 4 min. and a hardening time of 7 min.

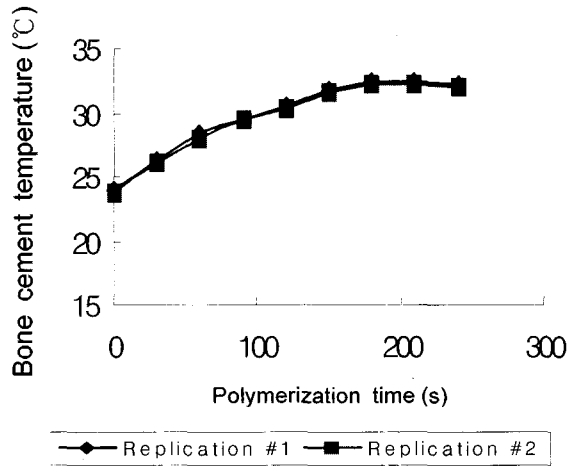

Fig. 1 The change of cement temperature according to polymerization time in case of C-2 cement.

The Type D cement with PHBV had higher compressive srength than the Type C cements, having same setting and hardening times like those of Type C cements.

The Type E cement with caprolactone showed compressive strength of 24 MPa with high plasticity(Fig. 3), having short setting and hardening times compared with those of Type C cements. In case that the amount of caprlactone was more than 20$\mu\ell$, the cement was not injectable due to instant polymerization and had very high plasticity. The PHBV increased a compressive strength in the cements. Caprolactone increased the plasticity and the strength, and reduced the polymerization time in the cements. However, the oil decreased the compressive strength of the cements.

The Type F cement with less caprolactone and more oil showed lower compressive and bending strengths compared with Type C and E cements. In case that the F cement without the oil polymerized instantly, which was not injectable.

The Type G cement without the pine resin powder was not polymerized due to the oil, which needed the pine resin powder as an additive necessarily.

Table 4 The handling and mechanical properties of bone cements

Cement Type	Components (powders, liquids, additives)	Max. Setting Temp.℃	Setting Time, min.	Hardening Time, min.	Compressive Strength,MPa (Bending S.)	Injectability or Remark
A-1	TA(0.4 g), Chitosan(0.04 g), H_2O(0.2 ml), ZnO(0.02 g)	Normal	10	20		Good
A-2	TATCP(0.8g), Na_2HPO_4(0.6 ml)	Normal	10	20		Very good
B	**BCA(0.2 ml), TA(0.2g), Chitosan**	**33**	**Instant**	**Instant**	**> 25**	**None**
C-1	BCA(0.2 ml), Pine resin powder(0.04g), TATCP(0.25g)	33	2	3	25 (1.7 MPa)	Not good
C-2	BCA(0.2 ml), Pine resin powder(0.06g), Oil(30 $\mu\ell$), TATCP(0.2 g)	33	3	5	17	Good
C-3	BCA(0.2 ml), Pine resin powder(0.06g), Oil(40 $\mu\ell$), TATCP(0.2 g)	33	6	10	15	Good
C-4	BCA(0.2 ml), Pine resin powder(0.04g), Oil(30 $\mu\ell$), TATCP(0.25 g)	33	5	7	20 (0.4 MPa)	Good
D	BCA(0.2 ml), Pine resin powder(0.04g), Oil(30 $\mu\ell$), TATCP(0.2g) + PHBV(0.05g)	33	5	7	25	Good
E	BCA(0.2 ml), Pine resin powder(0.04 g), Oil(30 $\mu\ell$), Caprolactone(10 $\mu\ell$), TATCP(0.2 g)	33	2	3	24	Good
F	BCA(0.2 ml), Pine resin powder(0.04 g), Oil(40 $\mu\ell$), Caprolactone(5 $\mu\ell$), TATCP(0.2g) + PHBV(0.05g)	33	5	7	15 (0.2 MPa)	Good
G	CA(0.2 ml), Oil(30 $\mu\ell$), Caprolactone(5 $\mu\ell$), TATCP(0.2g) + PHBV(0.05g)	33	No polymerization	-	-	

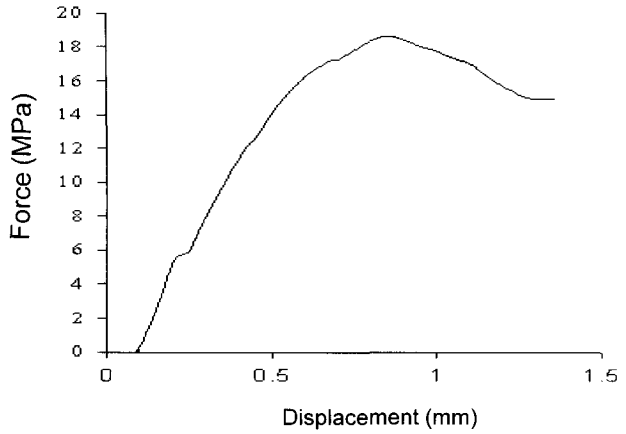

Fig. 2. Compressive strength of cement(Type C-3) with BCA, pine resin powder, oil and TATCP(tooth apatite + β-tricalcium phosphate).

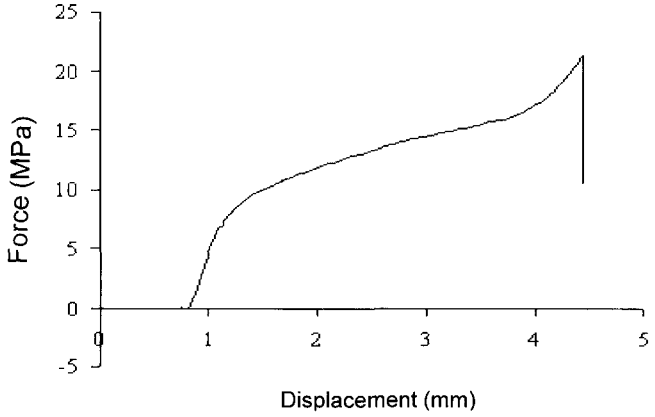

Fig. 3. Compressive strength of the cement(Type E) with BCA, pine resin powder, oil, caprolactone and TATCP(tooth apatite + β-tricalcium phosphate).

Through the above experiments, the maximum polymerization temperature of the BCA cements showed about 33℃ compared with 114.5℃ of PMMA cements, and the setting time could be prolongated to 3~6 min. The most BCA cements showed higher compressive strength than cancellous bones have a compressive strength of about 12 MPa.

B. Evaluation of the cytotoxicity

1) Direct contact cell culture evaluation

In vitro cytotoxicity tests by direct contact method were performed for 5 specimens. As a result of direct contact between L929 fibroblastic cells and the specimens, the extents of cell proliferation were shown in Fig. 4. The zone around the control was not detectable(grade 1, 5mm), but Group 1, Group 2, Group 3, and Group 4 produced zone around the specimens because of their toxicity that could promote neither cell proliferation nor cell adhesion. These results suggested that the BCA may be toxic to the cells and prevent the cell from growing in a single layer with favorable cellular attachment. The zone index of Group 1, Group 2, Group 3, and Group 4 are grade 2, which size was 6 mm(Fig. 4).

2) XTT test

The optical densities(ODs) of the specimens measured by a microtiter plate reader were calculated as an average of 4 replications and OD for 50% concentration inhibition (IC_{50}), which inhibited the cell growth 50%. The equation of the OD for IC_{50} was as follows;

$$\text{OD for } IC_{50} = (\text{OD of Control} - \text{OD of Blank})/2 \quad (1)$$

The ODs of L929 fibroblasts according to the dilution

Fig. 4 Direct contact cell culture after CV staining.

percentage of the specimen extracts were shown in Fig. 5. The values were obtained from regression equation to compare with the others. The values of IC_{50} of Control, Group 1, Group 2, Group 3, and Group 4 were calculated.

The value of IC_{50} of Group 1 was approximately 39.8% and that of Group 2 was about 70.8%. However, the values of IC_{50} of Control, Group 3 and Group 4 were not calculated. The Control and Group 3 and 4 had no calculated IC_{50} in experimental concentration. The Control and Group 3 and 4 were less cytotoxic than Group 1 and 2.

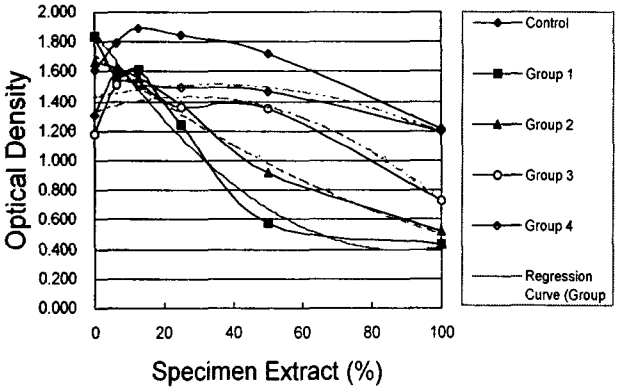

Fig. 5 The ODs of L929 fibroblasts according to the dilution percentage of the specimen extracts were shown. There were significant differences between the Control and the Group 1, Group 2, Group 3, and Group 4. (ODs: average values of 4 replications).

C. Animal Test

1) Radiologic evaluation

The gaps of fracture areas of the Groups 2, 3 and 4 were wider than those of Control and Group 1. Means of the grade at Week-2 were 2.0(SD : 0.71) in Control and Group 1, 2.6(SD : 1.14) in Group 2, 2.8(SD : 1.10) in Group 3 and 3.6(SD : 0.55) in Group 4. Their grades of gap decreased at Week-4 to 1.2(SD : 1.3) in Control, 1.6(SD : 1.52) in Group 1, 1.2(SD : 0.45) in Group 2, 1.4(SD : 1.14) in Group 3 and 2.8(SD : 1.10) in Group 4. At Week-6, all mandibles showed no gap and 56% of them showed radiolucent lines in cortical bone only(grade 1) or cortical bone and marrow bone(grade 2). The differences of the grade of the gap were significant between the gaps at Week-2 and Week-6($p < 0.01$). They are shown at Fig. 6.

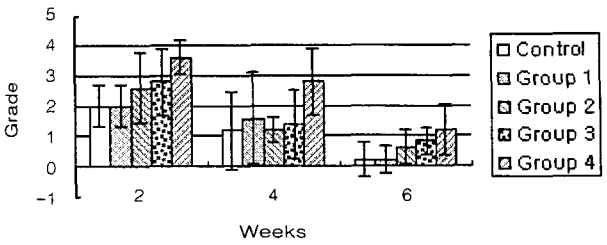

Fig. 6 Radiologic evaluation of the 5 groups after 2, 4, and 6 weeks. Graded by table 2. Control: No fixation, Group 1: n-butyl-2-cyanoacrylate (BCA) Only, Group 2: Membrane with BCA, Group 3: Chitosan with BCA, and Group 4: Tooth apatite with BCA.

2) Histologic evaluation

All animals recovered well after the surgical procedure and showed no macroscopic signs of infection. Blood clots were well organized in all at Week-2, Week-4, and Week-6. All their grades were-4. Bone unions at Week-6 were shown bony union(grade 3) in three Groups(Control, Group 1, and Group 2), some of the two Groups(Group 3 and Group 4) showed fibrous union. In case of the mandibles that were sacrificed at Week-2, Group 1 was higher grade than the other Groups including Control. They were summarized in Fig. 7.

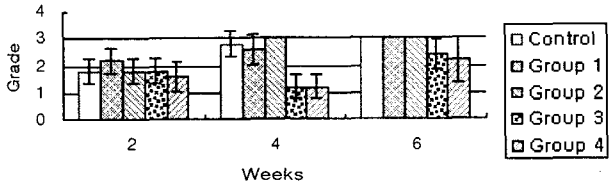

Fig. 7 Bone Union after 2, 4, and 6 weeks.

Reorganizations of compact bone were complete at Week-6 in two Groups(Group 1 and Group 2) except 1 of the Group 1, but Group 3 and Group 4 were poor. Half of the two Groups(Group 1 and Group 2) at Week-4 was complete reorganization of compact bone, but the compact bone began to appear in all of Group 4. 80% of the five Groups at Week-2, compact bone were formed well under way. Group 1, Group 2 and Group 3 were similar to control in reorganization of compact bone, but Group 4 was poor reorganization. The Group 1 was more reorganization of compact bone than Group 2, but there was no significance between two Groups. The Fig. 8 showed the grades of compact bone reorganization. Inflammatory responses were similar from Week-2 to

Week-6 in all Groups except Control. They showed small infiltration of inflammatory cell and chronic inflammation. Bone marrow cells in the fracture line increased as the healing periods increased.

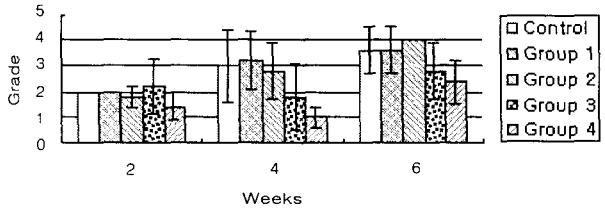

Fig. 8 Compact bone after 2, 4, and 6 weeks.

3) Immunohistochemistry

In immunohistochemistry at Week-2, BMP-2 was more reactive in Group 2 and Group 3 than in Control, Group 1 and Group 4. BMP-2/4 was more reactive in Control, Group 1 and Group 2 than in Group 3 and Group 4. Osteocalcin was more reactive in Control, Group 1, and Group 2. Control, Group 1, and Group 2 were similar in immunostaining of BMP-2, BMP-2/4, and osteocalcin, but they showed in local area and had no significant differences among them.

4. Conclusions

A study on the development of a novel bioresorbable and injectable bone cement using biodegradable biomaterials of chitosan, tooth apatite, and butyl-cyanoacrylate was carried out in views of handling and mechanical properties, cytotoxicity and animal tests. The developed BCA cements had lower polymerization heat, good bioresorbability, and high biocompatibility compared to the PMMA cements used currently.

(1) The cement of Type A was made from tooth apatite and chitosan gel(chitosan + H_2O) with an additive of ZnO. Hardening of the cement was accelerated by ZnO. The hardening time of the specimen was about 20 min. The cement could be injectable by increasing the amount of water. An injectable ceramic bone cement was also available by mixing TA and TCP with a solution of 0.2M Na_2HPO_4, which was hardened in about 20 min after mixing.

(2) In the Type B cement, as soon as the powders of TA with little chitosan contacted with the BCA adhesive, the mixed powder instantly hardened without a setting time. This cement was not injectable. In this case an inhibitor of BCA was required for preventing the initial polymerization. This cement could be used for an instant bonding of bones.

(3) In the Type C cement, pine resin known as an natural adhesive was used an inhibitor of BCA as its powder prolongated the initial polymerization of BCA in the powders of TA(50%) and TCP(50%)(TATCP). The lipiodol of poppy seed oil was used for the prolongation and injectability. The resin powder and the oil were very effective for prolongation and injectability of the Type C cements. The maximum setting temperature and the setting time of Type C cements were 33 ℃ and 3~6 min. The compressive strength(bioyield point) of the Type C cements ranged from 15 to 25 MPa. The 3-point bending strength of the Type C cements ranged from 0.4 to 1.7 MPa. The C-4 cement was considered as a good formula among Type C cements.

(4) The Type D cement with PHBV had higher compressive srength than the Type C cements, having same setting and hardening times like those of Type C cements.

(5) The Type E cement with caprolactone showed compressive strength of 24 MPa with high plasticity, having short setting and hardening times compared with those of Type C cements. The Type E cement with 20 $\mu\ell$ caprolactone showed a compressive strength of 34.7 MPa due to high plasticity. The PHBV increased a compressive strength in the cements. Caprolactone increased the plasticity and the strength, and reduced the polymerization time in the cements. However, the oil decreased the compressive strength of the cements.

(6) The max. polymerization temperature of the BCA cements showed about 33℃ compared with 114.5℃ of PMMA cements, and the setting time could be prolongated to 3~6 min. The most BCA cements showed higher compressive strength than the cancellous bone.

(7) The cytotoxicity tests in the Type B cement indicated that Group 1, 2, 3 and 4 were more toxic than Control. The Control showed grade 1 and all 4 Groups showed grade 2 in direct contact cell culture. The value

of IC_{50} of Group 1 by XTT assay was approximately 39.8%, that of Group 2 was about 70.8%, but the Control, Group 3, and Group 4 had no IC_{50} in experimental concentration.

(8) In radiologic evaluation, the gaps of fracture areas of Group 2, 3, and 4 were wider than those of Control and Group 1 at Week-2. But, all Groups had narrow gaps and radiolucent lines in compact bones.

(9) In histologic evaluation, blood clots were well organized in all Groups after 2, 4, 6 weeks. Bone union after 2 and 4 weeks were shown fibrous union in 80% of two Groups(Group 3, 4), but Control, Group 1, and Group 2 showed bony union after 6 weeks. Inflammatory responses were not severe in all periods of all Groups.

(10) In immunohistochemistry at Week-2, BMP-2, BMP-2/4 and osteocalcin responded in all Groups with little significance.

References

1. Amarante, M. T., M. A. Constantinescu, D. O'Connor, and M. J. Yaremchuk. 1993. Cyanoacrylate fixation of the craniofacial skeleton: an experimental study. Plast. Reconstr. Surg. Vol. 95:639-646.
2. ASTM F813-83. 2000. American Society for Testing and Materials. Standard practice for direct contact cell culture evaluation of materials for medical devices.
3. Bakos, D., M. Soldan and I. Hernandez-Fuentes. 1999. Hydroxyapatite-collagen-hyaluronic acid composite. Biomaterials. Vol. 20(2):191-195.
4. Choung, P. H. and E. S. Kim. 1997. Toothapatite as a bone substitute: An experimental study and clinical applications. J. Korean Dentist Ass. Vol. 35:180-188.
5. Dahlin, C., A. Linde, J. Gottlow and S. Nyman. 1988. Healing of bone defects by guided tissue regeneration. Plast. Reconstr. Surg. Vol. 81:672-676.
6. Gresser, J. D. et al. 1995. Analysis of a vinyl-pyrrolidone/poly(propylene fumurate) resorbable bone cement. J. of Biomedical Materials Research, Vol. 29, 1241-1247.
7. Hirano, S. 1996. Chitin biotechnology applications. Biotechnol Annu Rev. Vol. 2:237-58.
8. Hoh, K. Y. 1984. A study on the physical properties and cytotoxicity of tooth ash and dental porcelain. J. Kor. Acad. Prosth. Vol. 22(1):51-63.
9. Kuhn, K. D. 2000. Bone Cements. Published by Springer. New York. p. 30-45.
10. Muzzarelli, R. A., M. Mattioli-Belmonte, C. Tietz, R. Biagini, G.. Ferioli, M. A. Brunelli, M. Fini, R. Giardino, P. Ilari and G. Biagini. 1994. Stimulatory effect on bone formation exerted by a modified chitosan. Biomaterials. Vol. 15:1075-1081.
11. Muzzarelli R. A., G. Biagini, A. Pugnaloni, O. Filippini, V. Baldassarre, C. Castaldini and C. Rizzoli. 1989. Reconstruction of parodontal tissue with chitosan. Biomaterials. Vol. 10(9):598-603.
12. Nyman, S, J. Lindhe, T. Karring and H. Rylander. 1982. New attachment following surgical treatment of human periodontal disease. J. of Clin. Periodontol. Vol. 9:290-296.
13. Shepherd, R., S. Reader and A. Falshaw. 1977. Chitosan functional properties. Glycoconjugate J. Vol. 14:535-542.
14. Shermak, M. A., L. Wong, N. Inoue, E. Y. Chao and P. N. Manson. 1998. Butyl-2-cyanoacrylate fixation of mandibular osteotomies. Plast. Reconstr. Surg. Vol. 102:319-324.
15. Sundararajan, V. M. and W. T. M. Howard. 1999. Porous chitosan scaffolds for tissue engineering. Biomaterials. Vol. 20(12):1133-1142.
16. Takechi, M., Y. Miyamoto, K. Ishikawa, T. Toh, T. Yuasa, M. Nagayama and K. Suzuki. 1998. Initial histological evaluation of anti-washout type fast-setting calcium phosphate cement following subcutaneous implantation. Biomaterials Vol. 19(22):2057-2063.
17. Weber, S. C. and M. W. Chapman. 1984. Adhesives in orthopaedic surgery. A review of the literature and *in vitro* bonding strengths of bone bonding agents. Clin Orthop. Vol. 191:249-261.
18. Zellin, G., A. Gritli-Linde and A. Linde. 1995. Healing of mandibular defects with different biodegradable and nonbiodegradible membranes: an experimental study in rats. Biomaterials. Vol. 16(8):601-609.