

# Tissue Response of Calcium Polyphosphate in Beagle Dog

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## I. Introduction

Most bone grafts performed today utilize autograft, allograft, and alloplast with moderate clinical success<sup>1-3</sup>). Autografts have the advantage of optimal biologic incorporation, histocompatibility and little chance of disease transmission. But it has lack of availability in clinical use, and the harvesting of graft tissue may cause patient morbidity. In contrast, allografts get much wider availability and little patient morbidity associated with graft procurement. However, problems with unreliable graft incorporation, immune response, and possible disease transmission represent clear drawbacks to their use. While several synthetic graft materials have also been introduced, most alloplastic materials function primarily as a biocompatible defect filler<sup>4</sup>). Several attempts have been made to resolve these problem use of biodegradable polymers and combination of ceramics with bioactive polymers such as collagen and polylactides<sup>5-7</sup>). Chitosan is a biodegradable

cationic polysaccharide composed of N-acetylglucosamine residues which is known to accelerate wound healing and bone formation<sup>8</sup>). Many previous reports corroborated enhanced wound healing<sup>9-12</sup>) and hemostatic effect<sup>13-17</sup>) of chitosan. It is hypothesized that the major path for chitosan breakdown in vivo is through lysozyme, which acts slowly to depolymerize the polysaccharide<sup>18</sup>). The biodegradation rate of the polymer is determined by the amount of residual acetyl content, a parameter that can easily be varied<sup>8</sup>).

One possible approach toward addressing the respective problems inherent in autograft and allograft material is employing tissue engineering which is actively researched today. Tissue engineering is a science in which material properties of synthetic compounds are manipulated to enable delivery of an aggregate of dissociated cells into host in a manner that result in the formation of new tissue. Thus the major goal of tissue engineering is in vitro construction of transplantable vital tissue<sup>19</sup>).

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The engineering of bone tissue requires appropriate carriers that allow a 3-dimensional distribution of cells. Ishaug et al. suggested several prerequisites for a scaffold material for bone formation<sup>20)</sup>. First, the scaffolding material for bone formation must allow for the attachment of osteoblasts because they are anchorage dependent cells that require a supportive matrix in order to survive. Second the scaffold must provide an appropriate environment for proliferation and function of osteoblasts. Third the scaffold should allow for ingrowth of vascular tissue to ensure the survival of transplanted cells. Fourth, the materials should be biodegradable and its degraded molecule should be easily metabolized and excreted. Finally it should be processable into irregular 3-dimensional shapes.

Porous alloplastic implants have been studied extensively for their use in oral and maxillofacial applications<sup>21)</sup>. The use of these materials allows for recovery of the cosmetics and continuity of the surrounding bony structures without the concerns associated with the use of autogenic implants. These include but are not limited to increased potential for graft resorption donor site morbidity, and immunogenic reaction to bank bone. Other advantages of porous alloplastic material in periodontal and craniofacial applications include an increase in resistance to separation<sup>22)</sup>. Ceramics, porous block hydroxyapatite(HA), which is one such alloplastic material, has been shown to be an effective implant material in short- and long-term application<sup>23)</sup>. With advances in ceramics technology, the application of calcium phosphate materials have received considerable attention as bone substitutes for several decades. Calcium phosphate as bone substitutes are believed to be biocompatible and osteoconductive when implanted in bone defects<sup>24-27)</sup>. Numerous animal studies provide histologic evidence of the long-term biocompatibility of porous HA and of its favorable

interaction with soft tissue and bone<sup>28,29)</sup>. In addition, these studies indicate the lack of an inflammatory response to HA implants<sup>30,31)</sup>.

A substrate such as hydroxyapatite that provides a three-dimensional guideline for bone shape facilitates bony ingrowth and subsequent positional stability as discussed in Wolford et al<sup>32,33)</sup>. The porous structure of HA provides a template for fibrovascular ingrowth which is followed by osteoblast differentiation that results in the deposition of new lamellar bone. Porous material are highly favorable over nonporous materials owing to the accessibility of the interior of the material to tissue ingrowth. If the pores appropriately sized, they can provide a framework for bone growth into the matrix of the material. Thus, matrices with a normal pore size of  $200\mu\text{m}$ <sup>34)</sup>, resulted in occlusion of pores by migrating cells, while similar scaffolds of  $500\mu\text{m}$  nominal pore size permitted three -dimensional tissue growth in vitro which has led to the convincing demonstrating of new bone growth on the pore walls in vivo by Yoshikawa et al.

Calcium phosphates are generally considered materials of choice as bone substitutes. While calcium phosphate ceramics meet some of the needs for bone replacement they are limited by their inherent stiffness, brittleness and low fatigue properties relative to bone<sup>35)</sup> and are generally not resorbed during bone remodeling<sup>36)</sup>. The condensed phosphates are very numerous and exist both as crystalline salts and as amorphous glasses. According to the ratio of Ca and P, condensed phosphates can be divided into three groups: linear polyphosphates, metaphosphates, and ultraphosphates. All varieties are formed by repeated condensation of tetrahedral  $\text{PO}_4^{3-}$  groups. Among the many candidates for bioabsorbable or transient implants, the use of bioabsorbable Calcium Polyphosphate(CPP) has twofold advantages over that of the other

bioabsorbable polymeric materials, that is, CPP is chemistry similar to natural bone and has higher stiffness. The CPP are controlled the rates of biodegradability by method of preparation and type of CPP<sup>37</sup>.

The purpose of this study was to investigate the tissue response and osteo-conduction capability of Calcium Polyphosphate(CPP) as bone graft material and to compare with the semi-automatic histomorphoric new bone formation between CPP and demineralized freeze-dried bone .

## II. Materials and Methods

### 1. Manufacturing Calcium Polyphosphate

Interconnected porous calcium polyphosphate (CPP) blocks were prepared by condensation of anhydrous  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ (Duksan Chemical Co., Inc.) to form non-crystalline  $\text{Ca}(\text{PO}_3)_2$ . From the latter, an homogenous melt was created by thermal treatment, quenched in distilled water, and the block was then milled to produce CPP powder<sup>38</sup>. Which was added with  $\text{Na}_2\text{O}$  addition of  $\text{Na}_2\text{O}$ .

CPP granules were prepared according to the pore size 45ppi. Pore size of CPP(45ppi) is approximately 450-550  $\mu\text{m}$ .

The CPP granules with chitosan was prepared by use of CPP powder. The each powder of CPP,  $\text{CaSO}_4$ , and chitosan were mixed into weight ratio 5:1:1 in 5% chitosan solution as binder. The mixed compound was penetrated into mesh which pore size is 800  $\mu\text{m}$  and then dried in the fan oven. The size of CPP granule with chitosan was 300-500  $\mu\text{m}$ .

### 2. Animal experiments

The 3 year-old female adult beagle dog used were bred exclusively for biomedical studies and bucco-

gingival health was checked before experimentation, and all teeth of the dog were preliminarily scaled and polished under general anesthesia. Antibiotic treatment with spiramycin and metronidazole was given for 5days.

**surgical procedures<sup>39</sup>.** All surgical procedures were performed under general anesthesia with intramuscular rompun<sup>®</sup>(1.5mL/kg) followed by anesthesia with ketar<sup>®</sup> (5mg/kg). During surgery, the animals received lactated Ringer's solution and 1g of cephalosporin antibiotic perfused intravenously. The extractions were performed on right and left second, third and fourth mandibular premolars and on right and left second and third maxillary premolars.

Thus, 10 teeth were extracted per animal, as premolar teeth are biradicular, 20 alveolar extraction sites were available for bone filling. After sulcular incisions, full- thickness buccal and lingual mucoperiosteal flaps were raised. Gingival incisions were extended mesially from the canine and distally to the molar teeth. A vertical interradicular section was performed with a dental bur under sterile irrigation to separate all teeth into 2 pieces and avoid root and alveolar cortical bone fracture during extraction. Each root was carefully mobilized with a dental elevator and then gently extracted with a dental forceps.

All alveolar sites were checked after extraction and thoroughly debrided with a dental curet to remove the periodontal ligament. Extraction sites were grafted with biomaterial or left unfilled; i.e, the mesial socket of a tooth was left unfilled and the distal socket filled with the composite biomaterial. The CPP granules with chitosan and CPP granules with  $\text{Na}_2\text{O}$  were injected into the extraction socket in a retrograde manner from the bottom of the socket to the top of the alveolar crest. Material was gently packet into place (no overpacking), and excess

material was removed from unfilled sites. The connective tissue surfaces of the buccal and lingual flap margins were carefully joined together, and non-resorbable hermetic sutures were performed.

Antibiotic treatment by intramuscular injection of cephalosporin( 15mg/kg, b.i.d )was continued for 48hours after surgery. The animals were checked daily and fed with a soft consistency diet. Sutures were removed under short general anesthesia 2 weeks after implantation ( day 14) and a normal diet was then given.

The animals were sacrificed 3months after implantation (day 90) by intravenous injection of over-dosed sodium pentobarbital.

**Sample preparation.** Mandibular and maxillary osseous segments were immediately dissected from the animals and fixed in paraformaldehyde solution. They were fixed with thin metal pins before radiographs were performed to localize each socket. The sockets were individually separated by cutting into the intra and interdental septa with a diamond saw, according to the pins' position. Each socket was dehydrated in graded ethanol and embedded in

a glycolmethylmethacrylate resin.

### 3. Histological evaluation and Bone ingrowth measurements

Both treated and control mandibular and maxillary sites were histologically evaluated with light microscopy. For each socket, 30  $\mu$ m thick sections were cut with a hard tissue microtome along the long axis of the root implantaton site and then multiple-stained for light microscopy observations.

The surface of the resin block obtained from the centarl area of the socket was prepared for LM observations Bone ingrowth was investigated and compared in filled and control mandibular and maxillary sites and quantitatively evaluated using a Image Access (Bildanalysis System, Sweden) . Results are given as the percentages of newly formed bone in mandibular and maxillary extraction sites.

### 4. Statistical analysis

Differences in new bone formation between CPP

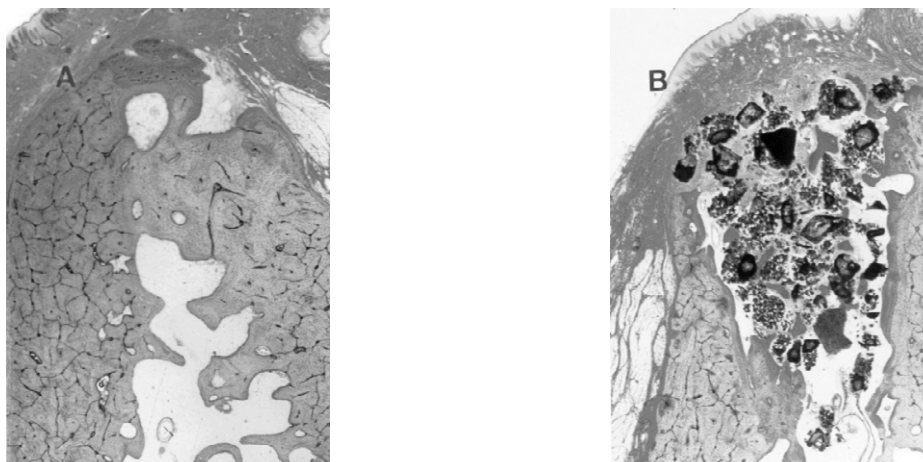


Figure1. CPP granules with Na<sub>2</sub>O and control after 3months of healing in extraction site. Figure illustrates the whole defect. (A) control site (multiple stain, original magnification x20) (B) CPP granules with Na<sub>2</sub>O

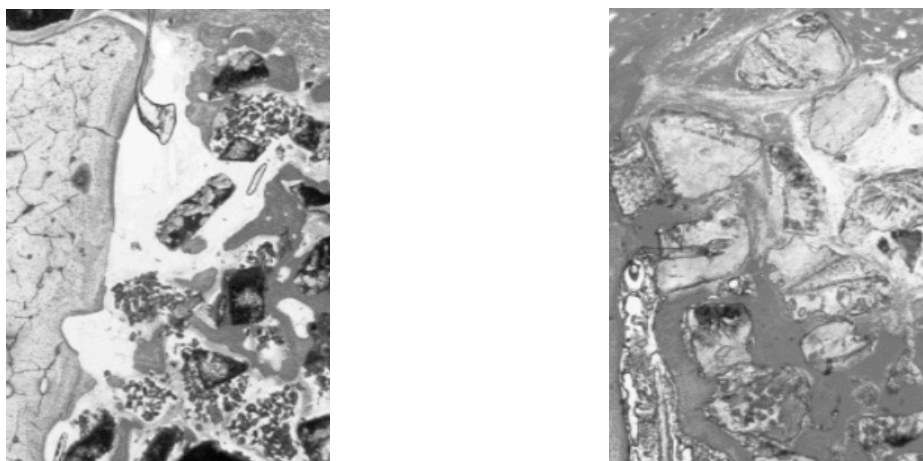


Figure2. bone formation around CPP granules (A) CPP granules with chitosan and(B) CPP granules with  $\text{Na}_2\text{O}$ . (multiple stain, original magnification x20)

granules with chitosan and CPP granules with  $\text{Na}_2\text{O}$  and demineralized freeze-dried bone sites were studied for statistical purposes with one-way ANOVA test, P values  $<0.05$  were considered statistically significant.

### III. Results

#### 1. Histologic findings

All control and experimental sites healed uneventfully with no clinical evidence of inflammatory response to the CPP implants and DFDB. Histologically, although no quantitative measurements were made, the amount of newly formed bone appear to be greater in specimens with CPP granules with  $\text{Na}_2\text{O}$  as compared to the control sites. This is shown in figure 1 which depicts accelerated new bone formation around the CPP granules and no fibrous encapsulation (fig2).

However, the tissue appears to demonstrate a decrease in osteoblastic activity and some of the CPP granules were simply surrounded with fibrous

connective tissue.

The unresorbed CPP granules with  $\text{Na}_2\text{O}$  and CPP granules with chitosan showed an irregular surface indicating that resorption or dissolution process of the CPP granules took place prior to laying down of mineralized bone matrix. This is in contrast with the relatively smooth appearing surface which contacted directly newly formed bone around CPP granules (fig3). The bone showed large narrow spaces with no evidence of active bone formation. As the proportion of  $\text{Na}_2\text{O}$  increases, osteoclastic activity also

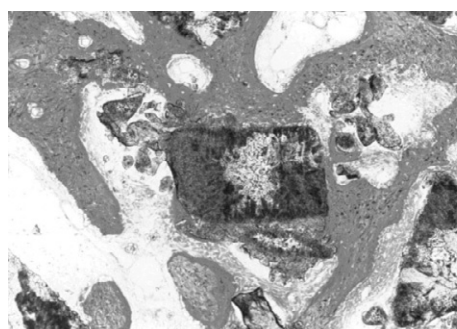


Figure3. CPP granules have smooth and irregular surface (multiple stain, original magnification x 100)

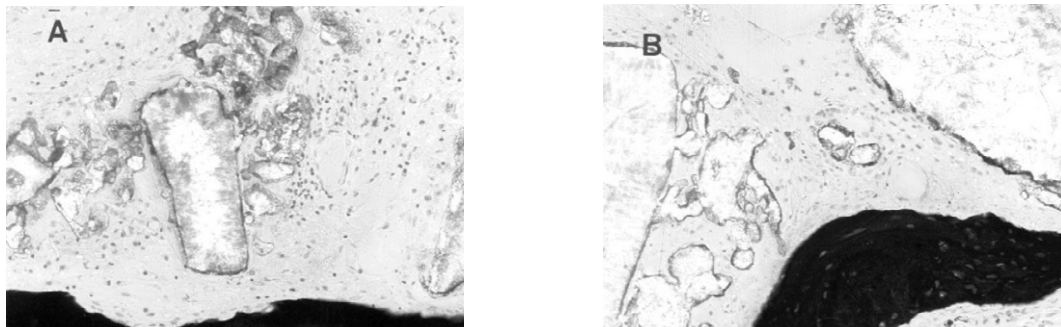


Figure 4. Tissue response of CPP in extraction site. A few inflammatory cells infiltrate but no significant adverse reaction is seen. Multinucleated giant cells were shown. (A) CPP granules with chitosan (B) CPP granules with Na<sub>2</sub>O. (H&E stain, original magnification x200)

increases with concomitant CPP granules resorption as evidenced by the presence of numerous multinucleated giant cells (fig4) and CPP granules which have rough surface present in the implant site.

## 2. Measurement of newly formed bone

The one-way ANOVA showed that all the treatments produced statistically significant higher gain in new bone formation than did the control groups ( $p < 0.05$ ) (table 1). But the analysis showed that, for implanted sites comparisons, there is no significant difference between experimental sites with implant

material (fig. 5). Between the CPP granules with chitosan and CPP granules with Na<sub>2</sub>O, there is also no significant difference. In experimental sites, DFDB, CPP granules with chitosan and CPP granules with Na<sub>2</sub>O are also showed new bone formation but, there is no significant difference between groups.

## IV. Discussion

Periodontal regeneration therapy with bone-substituting materials has gained favorable clinical efficacy by enhancing osseous regeneration in peri-

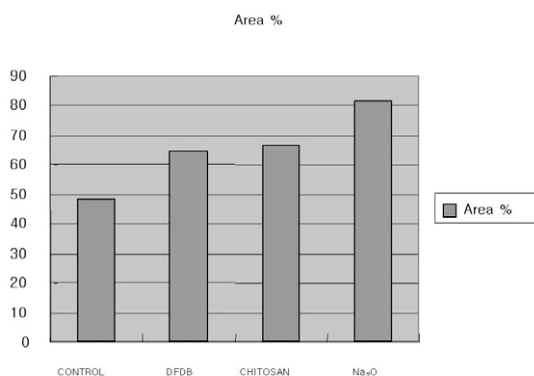


Figure 5. Area % of new bone formation in groups.

Table 1.

Group	% of Regenerated Area
1	44.04 ± 4.7*
2	72.64 ± 6.1#
3	66.48 ± 1.0#
4	80.46 ± 5.7#

\*: Mean ± S.D.

# : There were significant differences % area of new bone formation between group 1 and the other groups ( $p < 0.05$ ).

Group 1 : Control

Group 2 : Demineralized freeze dried bone (DFDB)

Group 3 : CPP granule with chitosan

Group 4 : CPP granule with Na<sub>2</sub>O

odontal bony defect<sup>(40-48)</sup>. As bone substituting materials, bone powder<sup>(40,41)</sup>, calcium phosphate ceramic<sup>(42,43)</sup>, modified forms of hydroxyapatite<sup>(44,45)</sup>, and hard tissue replacement polymer<sup>(46-48)</sup> have demonstrated their periodontal bony regenerative potency. Bone-substituting materials should fulfill several requirements such as biocompatibility, osteogenicity, malleability, biodegradability<sup>(40-42,46)</sup>. However, these materials revealed some drawbacks including bone resorption, immune response, disease transmission, low biodegradability, poor adaptation.

Many investigators reported encouraging clinical results of several synthetic grafts, but histologically the grafts tend to be encapsulated by connective tissue with little bone formation. This is likely to be true with not only non-absorbable materials but also absorbable materials. At present, therefore, most of synthetic graft materials were considered primarily as biocompatible defect filler<sup>(4)</sup>. In this point, regardless of additional component, CPP granules might exhibit biocompatible, highly osteoconductive characteristics. They might be also considered promising candidates as a scaffold for tissue engineered bone formation. But they exhibited very slow degradation rate. That may pose a problem for the replacement of these devices with new bone and may alter the mechanical properties of the newly formed bone. In this study, there is no result about bioabsorbable properties of CPP granules, so further investigations are to fulfilled.

Since chitosan can be well adapted to inorganic materials such as calcium phosphate, tricalcium phosphate and hydroxyapatite containing chitosan paste has been introduced as a self-setting material for bony lesion<sup>(50-55)</sup>. Also in present study CPP granules were employed for enhancement of material properties and osteoconductivity of chitosan matrix. In histologic finding and histomorphometric finding,

no significant differences between CPP granules with Na<sub>2</sub>O and CPP granules with chitosan were found in matrix degradation and the amount of induced new bone by the two. These findings indicate that the addition of chitosan to CPP granules probably does not evoke any adverse effects to degradation of the matrix or healing of defect, and also does not serve any additional osteoconductive effect.

The susceptibility of chitosan to depolymerization under the catalytic action exerted by lysozyme is dependent on the molecular size of the polymer or degree of deacetylation. Thus, biodegradation rate of the chitosan matrix could be varied by controlling the amount of residual acetyl content. In present histologic examination of extraction sites healing, newly formed osseous tissues infiltrated into and were mingled with the matrices being degraded simultaneously. In this points, these matrices might show acceptable degradation rate as bone replacement graft materials, compared to CPP granules itself.

The knowledge of the degradation mechanism is far from complete, but it is generally known that the hydrolytic degradation of CPP is due to the disruption of hydrolyzable -P-O-P bonds in CPP by water molecules<sup>(56)</sup>. Bunker reported on the mechanism and kinetics of dissolution for polyphosphate ceramics and that the dissolution of polyphosphate ceramics is congruent independent of ceramic composition, the pH, or the sample surface/solution volume ratio(S/V). It occurs by the hydration of the glass surface and release of polyphosphate chains into the solution. It is known that protons enhance the dissolution rates of polyphosphate ceramics, but the hydrated polyphosphate released in solution limits the dissolution<sup>(57)</sup>. Owing to the progressive increase in ionic strength, an increase in electrostatic interactions in the hydrated layer occurs, that results in a

decrease of the dissolution rate. In this study, addition of Na<sub>2</sub>O may increase the biodegradation rate initially, but during the degradation, that retards the biodegradation. So optimal percentage of Na<sub>2</sub>O will be investigate in future study.

In this study, regardless of its additive components, CPP granules showed osteoconductivity and biocompatibility, but they exhibited very slow degradation rate. CPP is available in various forms and its degradation rate is controllable, so in future study, we investigate the optimal forms of CPP and additive components to increase the degradation rate.

## V. Conclusion

1. The CPP granules contact to new bone directly.
2. The new bone formation was found in CPP granules with chitosan , CPP granules with Na<sub>2</sub>O and DFDB group
3. There is significant difference between CPP granules with chitosan, CPP granules with Na<sub>2</sub>O and control group ( $p < 0.05$ ).
4. There is no significant difference between CPP granules with chitosan and CPP granules with Na<sub>2</sub>O ( $p > 0.05$ ).

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## 비글견에서의 Calcium Polyphosphate의 조직반응에 관한 연구

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치주조직 재생을 도모하기 위한 전통적인 시술방법으로 여러가지 골이식재를 이용한 골이식술이 이용되고 있다. 이번 실험의 목적은 골형성을 위한 재료로 Calcium Polyphosphate(CPP)를 사용하여 비글견에서의 조직 반응과 골유도성을 보는 것이고 또한 다른 이식재들간에 신생골의 형성에 어느정도 영향을 주는 가를 알아보는 것이다. 이번 실험에 사용된 CPP는 무수  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ 를 condensation하여 무결정의  $\text{Ca}(\text{PO}_3)_2$ 를 얻고 이를 용출하고 냉각시킨 후 분쇄하여 얻은 것으로 3세된 비글견에 이식하여 관찰하였다. 조직학적으로 CPP granule의 경우는 키토산이나  $\text{Na}_2\text{O}$ 를 넣은 경우 모두 bone 의 ingrowth가 관찰되었고 다른 섬유조직의 개재를 볼수 없었다. 동결탈회건조골을 넣은 경우에는 주위로 골형성 보이지 않았고 단지 섬유성 조직이 관찰 되었다. 아무것도 넣지 않은 경우에 비해서 동결탈회건조골이나 키토산,  $\text{Na}_2\text{O}$ 를 넣은 CPP granules 경우에 더 많은 비율로 신생골의 양이 나타나는 것을 볼수 있었다. 아무것도 넣지 않은 대조군과 이식재를 넣은 군간에는 유의성이 있는 것으로 나타났고( $p < 0.05$ ). 또한 CPP granules with chitosan과 CPP granules with  $\text{Na}_2\text{O}$  사이에는 신생골의 형성에 유의성이 없었다. 이것으로 보아 CPP granules with chitosan과 CPP granules with  $\text{Na}_2\text{O}$ 는 모두 골유도성이 있고 신생골의 형성을 촉진하므로 치주질환으로 인한 골결손 부위에 사용할 수 있는 재료로 우수한 특성을 지닌다고 사료된다.

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주요어 : Calcium Polyphosphate, 골 유도성, 키토산,  $\text{Na}_2\text{O}$ , 신생골 형성, 조직공학