Comparative evaluation of three calcium phosphate synthetic block bone graft materials for bone regeneration in rabbit calvaria

Jae-SubPark

Department of Dental Science

The Graduate School, Yonsei University

Comparative evaluation of three calcium phosphate synthetic block bone graft materials for bone regeneration in rabbit calvaria

Directed by Professor Seong-Ho Choi

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This certifies that the Master’s thesis of
Jae-Sub Park is approved.

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Thesis Supervisor：Seong-Ho Choi

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Chang-Sung Kim

_____

Ui-Won Jung

The Graduate School

Yonsei University

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감사의 글

이 논문이 완성되기까지 부족한 저에게 지도와 격려를 아끼지 않으신 최성호 교수님께 깊은 감사를 드립니다. 그리고 언제나 따뜻한 관심과 진심 어린 조언을 아끼지 않으셨던 채중규 교수님, 조규성 교수님, 김창성 교수님, 정의원 교수님, 이용근 교수님께도 깊은 감사를 드립니다.

연구 내내 많은 도움을 주신 이중석, 박정철 교수님, 그리고 황지완 선생과 조아란 연구원에게도 특별한 감사를 드립니다. 저를 항상 지켜봐 주셨던 치주과 선후배님들 에게도 고마운 마음을 전합니다.

논문 영작에 많은 도움을 준 동서에게도 진심으로 고맙다는 말을 하고 싶습니다.

마지막으로 항상 제게 힘이 되어 주고 따뜻한 격려를 해주신 양가 부모님께 깊은 사랑과 감사를 드리며, 저를 항상 이해해주고 사랑해 준 아내와 딸 시현에게 이 논문을 바칩니다.

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저자 씀

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ABSTRACT

Comparative evaluation of three calcium phosphate synthetic block bone graft materials for bone regeneration in rabbit calvaria

Jae-Sub Park, D.D.S.

Department of Dental Science
Graduate School, Yonsei University
(Directed by Professor Seong-Ho Choi, D.D.S., M.S.D., PhD.)

Various synthetic materials that maintain space and allow bone ingrowth have been developed for use in implant dentistry and periodontal treatments. Among various alloplastic bone substitutes, calcium phosphate ceramics have been investigated extensively because their mineral chemistry resembles that of human bone. In this work, we evaluated the bone formation of three calcium phosphate synthetic block-type bone grafts in rabbit calvarial defects. Four 8-mm-diameter defects were created in each of ten young adult New Zealand white rabbits. Each of three of the defects was randomly filled with one of three fabricated synthetic block-type bone graft materials: hydroxyapatite (HA), beta-tricalcium phosphate (β-TCP), and biphasic calcium phosphate (BCP). BCP is a mixture of HA and β-TCP at a ratio of 7:3. The fourth defect, a sham-surgery control, was filled with blood clots. The specimens

were harvested at 4 and 8 weeks postsurgery for histologic and histomorphometric evaluation.

Our results indicate that the amount of newly formed bone and bone density were increased more for the BCP block bone substitute than for the other two types. Furthermore, the histologic and histometric findings revealed that the space-maintaining ability was significantly better for all three calcium phosphate block bone graft materials than for the control group at both 4 and 8 weeks. However, measurement of the absorption of each particle revealed a significant decrease in the residual particles for β-TCP, while there were only small decreases for HA and BCP.

Key Words : rabbit calvarial defect, calcium phosphate, synthetic block bone

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I. Introduction

Clinicians often encounter an inadequate amount of bone during surgical procedures, which is due to several reasons, including injuries, periodontal diseases, and implant surgery. To overcome this difficulty, various bone substitutes have been used to reconstruct bony defects (Rojbani et al., 2011). Autogenous bone graft materials have been considered the gold standard in reconstructive surgery because of their osteogenic property (Bauer and Muschler, 2000). Despite this advantage, the autogenous bone graft has many disadvantages, including limited donor-site availability and associated morbidity. Conversely, allogenic bone graft and xenogenic bone graft materials can be obtained easily; however, they may provoke an immune response (Tudor et al., 2008). These limitations have led to extensive investigations

and the development of alloplastic materials. Alloplastic bone grafts are osteoconductive and do not induce immunogenicity (Daculsi et al., 2003).

Among various alloplastic bone substitutes, calcium phosphate ceramics have been investigated extensively because their mineral chemistry resembles that of human bone (Han et al., 1984; Trombelli et al., 2002). The first successful application of a calcium phosphate reagent [described as "triple calcium phosphate," or tricalcium phosphate (TCP)] in humans was reported in 1920 (Albee, 1920).The clinical use of a TCP preparation in surgically created periodontal defects in animals was first reported 50 years later (Nery et al., 1975). Furthermore, the application of dense hydroxyapatite (HA) as an immediate tooth root replacement has been reported (Denissen and de Groot, 1979); synthetic HA and beta-TCP (β-TCP) have been used as commercially available bone substitute materials for dental and medical fields largely through efforts made in the early 1980s (LeGeros, 1988; Metsger et al., 1982).

In general, HA has low osteoconductive activity but has a good space-maintaining capacity, whereas β-TCP is more bioresorbable and is rapidly replaced by new bone material. Biphasic calcium phosphate (BCP) ceramics, which comprise mixtures of HA and β-TCP at various ratios, allow the resorption rate to be controlled without distorting the osteoconductive property of the bone (Nery et al., 1992; Yamada et al., 1997b).

There are two types of calcium phosphate bone substitute: particulated and block. The better space-providing abilities of block-type bone substitutes result in them showing better stability of augmented areas during the healing period(Kim et al.,

2011). Another advantage of the block-type bone substitute is its higher efficiency, with new bone apposition occurring in association with progressive material degradation (Daculsi and Passuti, 1990; Merkx et al., 1999).

Based on results obtained in recent studies, it is proposed that HA blocks should be used as a prefabricated scaffold in cell transplantation for periodontal regeneration. The block-type bone grafts may be a better solution than particulated bone graft materials in cases of vertical augmentation (Chiapasco M et al., 1999; Cordaro L et al., 2002). However, few studies have assessed the performance of block-type bone grafts using different calcium phosphates. Hence, in the present study we evaluated the bone regeneration capabilities of three calcium phosphate synthetic block bone grafts in rabbit calvarial defects.

II. Materials and methods

1. Animals

Ten New Zealand white rabbits weighing 3.0–3.5 kg were used in this study. The animals were housed in separate cages under standard laboratory conditions and fed a standard diet. Animal selection, management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

2. Materials

Preparation of calcium phosphate glass

Calcium phosphate glass was prepared using the sol-gel process according to previous methods (Jang YJ et al,. 2011). In the present system CaO-CaF2-P2O5-MgO-ZnO was prepared using raw materials such as CaCO3, CaF2, H3PO4, MgO, and ZnO. The CaO: CaF2 molar ratio was fixed at 9. MgO and ZnO were both used at a concentration of 1%wt.

Mixed batches were dried for 12 h at 80ºC, calcined for 1 h at 450ºC, melted in a platinum crucible at 1250ºC in a Kanthal Super furnace, and then poured onto a

graphite plate at room temperature. As-quenched glass was crushed with an alumina pestle and attrition milled to an average size of less than 40 μm.

When preparing the glass the calcium: phosphorus ratio was 1.67 for HA and 1.5 for β-TCP. BCP was prepared with an HA: β-TCP glass ratio of 7:3.

Preparation of porous calcium phosphate blocks

The following procedure to prepare calcium phosphate blocks was applied to all HA, β-TCP, and BCP glasses. Porous calcium phosphate blocks were prepared using prefabricated calcium phosphate glass and a polymeric sponge according to previously described methods(Park YS et al., 2006). The reticulated polyurethane ester sponge used in this experiment (Regicell, Jehil Urethane Co., Korea), has 500 three-dimensionally interconnected open pores per linear millimeter. First, a calcium-phosphorus glass slurry was prepared by dispersing the prepared calcium phosphate glass powders into distilled water with organic additives such as binder, dispersant, and a drying chemical control additive (DCCA). The second procedure was infiltration. Prior to the infiltration process, the surface layer of the sponge was treated ultrasonically with a 2% solution of NaOH for 20 min to improve the surface layer's hydrophilicity. After cleaning and drying, the porous sponge was immersed into the glass slurry and taken back several times, and then rolled through twin Teflon rollers, the spacing between which was controlled to reduce the thickness of the sponge by 75% to as to remove the excess residual slurry from the sponge.

Compressed air was blown into the pores of the sponge to perforate any clogged pores. After infiltration, the sponge was dried at room temperature and then heat-treated in a Kanthalfurnace, wherein the condition of the heat treatment was based upon a thermal analysis. The temperature was increased to 600ºC at 1ºC/min in order to burn out the sponge entirely, and then held constant for 2 h to remove the volatile organic additives such as the binder, dispersant, and DCCA. The remaining calcium phosphate glass was then sintered for 2 h at various temperatures from 650ºC to 850ºC. The full procedure described above was repeated twice to thicken the framework of the porous block. The finished block was trimmed into a cylindrical shape (8 mm diameter and 3 mm thick).

3. Study design

Four circular defects with a diameter of 8 mm were created in the calvaria of ten young adult New Zealand white male rabbits, and then three were filled with a fabricated HA, β-TCP, or BCP synthetic block-type bone graft (8 mm in diameter and 3 mm thick). The remaining (control) defect was filled with a blood clot (Fig.1a,b)

4. Surgical protocol

The animals were anesthetized with an intramuscular injection of a mixture of ketamine hydrochloride (Ketalar, Yuhan, Seoul, Korea) and xylazine (Rompun, Bayer

Korea Co., Seoul, Korea). The surgical site was shaved and then wiped with alcohol and povidone iodine, followed by local anesthesia with 2% lidocaine (LidocaineHCl, Huons, Seoul, Korea). An incision was made along the sagittal midline from the frontal bone to the occipital bone. The four circular defects were then created in each animal using 8-mm trephines under cool saline irrigation. Each of three of the defects was filled with one of the three synthetic block-type grafting materials, while the fourth control defect was filled with blood clots (see Study Design). The soft tissue was repositioned and then sutured layer by layer with a resorbable suture material (Monosyn®). The rabbits were sacrificed at either 4 weeks (n=5) or 8 weeks (n=5) postsurgery.

5. Histologic processing

Block sections including the surgical sites were removed when the animals were killed. The sections were rinsed with sterile saline and fixed in 10% buffered formalin for 10 days. After being rinsed with water, the sections were decalcified in 5% formic acid for 14 days and then embedded in paraffin. Serial 5-μm-thick sections were cut through the center of the circular calvarial defects, as well as the subcutaneous sites. Two sections that contained the central portion were selected from each block, and stained with Goldner's Masson trichrome andhematoxylin and eosin.

6. Evaluating methods.

Clinical and Histologic analysis

The animals were observed carefully for allergic reactions, inflammation, and other complications around the surgical site throughout the 8-week healing period. The specimens were examined under a binocular microscope (DM LB, Leica Microsystems, Wetzlar, Germany) equipped with a camera (DC300F, Leica Microsystems, Heerburgg, Switzerland). Images of the slides were acquired and saved as digital files.

Histometric analysis

After conventional microscopic examination, computer-assisted histometric measurements of the newly formed bone in the calvarial defect model were performed using an automated image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). Four parameters were measured: the area of augmentation, the area of new bone, the new bone density, and the area of residual particles. Only the area with newly formed mineralized bone was measured as the new bone area (in mm2); marrow and fibrovascular tissues were excluded (Fig. 2).

7. Statistical analysis

The statistical analysis was performed using a commercially available software program (SPSS 15.0, SPSS, Chicago, IL, USA). Histomorphometric records from the calvarial defect samples were used to calculate the mean and standard deviation values of groups. The data were examined with the Kolmogorov-Smirnov test for conformance to a normal distribution. Analysis of variance was used to analyze the effects of time and experimental conditions. The Tukey test was used to analyze differences between the groups; these were considered significant when $p<0.05$. A two-sample t-test was carried out to analyze the differences in parameters between the 4- and 8-week groups. The level of statistical significance was set at $p<0.05$.

III. Results

1. Clinical findings

Healing during the postoperative period was uneventful for all animals. There were no complications (i.e., inflammatory reactions, exposure of graft materials, or allergic reactions).

2. Histologic findings

In the control group, we observed that the defect areas had become filled with loose fibrous tissue. A thin layer of new bone had formed in the 4-weeks group, while a thicker layer of new bone was evident in the 8-weeks group. Bony islands that appeared in a few specimens at the center of the defects contained moderate amounts of bone marrow in the 8-weeks group (Figs. 3a and 4a).

One of the synthetic block bone grafts, HA, had induced limited formation of new bone at the boundary of the defect area after 4 weeks of postoperative healing. After 8 weeks, significantly more bone had been formed, although it was not thicker than the original bone (Figs. 3c and 4c).

New bone formation was also observed in the β-TCP group, but a notable distinguishing feature of this group was the presence of chronic inflammatory cells

and fibrovascular tissue filling the majority of the defects. One interesting difference between the 4- and the 8-weeks groups for this synthetic block bone graft material was new bone forming a bony island in the upper areas of the defects only in the latter group.

Finally, impressive bone ingrowth into the BCP particle was observed in all BCP-filled defects in the BCP group, with no visible inflammatory reaction. Similar to other groups, there was more new bone formation in the 8-weeks group than in the 4-weeks group (Figs. 3g and 4g). However, one difference was that the new bone was thicker than the original bone in the BCP group, while this was not the case in the HA group.

3. Histometric analysis

The histometric measurements are summarized in Tables 1–4 . Throughout our observations, the augmented areas (in mm2)—including bone areas, residual materials, and soft tissue area—were significantly larger in the three experimental groups than in the control group (Table 1). The augmented areas did not differ significantly among the three experimental groups at either 4 or 8 weeks of healing.

We observed that the new bone area (in mm2) was greater at 8 weeks than at 4 weeks in all groups. The β-TCP group exhibited the smallest area of new bone at both 4 and 8 weeks. There was a significant difference between the HA group at 8 weeks and the β-TCP group at 4 weeks. Furthermore, there was a significant difference

between the BCP group at 8 weeks and the β-TCP group at 4 weeks. However, there were no statistically significant differences among the other experimental groups and healing periods.

The findings regarding bone density (Table 3) show that the bone density was significantly higher in the control group than in the other groups. This may be because the control group had very small areas of new bone formation and because the defects were not filled with synthetic block-type bone graft material. The bone density in the three synthetic block bone grafts was lowest in the β-TCP group and highest in the BCP group at 8 weeks.

Table 4 lists the amount of residual particles of the synthetic block bone graft materials, demonstrating that the findings at 4 and 8 weeks did not differ significantly among the three types of graft materials. The HA and BCP groups exhibited a small decrease in residual particles between 4 and 8 weeks. The β-TCP group had a relatively large decrease in residual particles.

IV. Discussion

In the field of bone regeneration, autogenous bone for augmentation of bone defects is the gold standard. However, autogenous bone is not always available in sufficient volumes, and hence various methods have been proposed as alternatives, such as graft materials.Calcium phosphate ceramics have long been investigated as biologically compatible materials for use in the treatment of periodontal osseous defects and implant surgery. Calcium phosphate biomaterials allow the attachment, migration, proliferation, and phenotypic expression of bone cells, leading to bone apposition in direct contact with the implanted biomaterials.

In the present study we assessed the effectiveness of three types of calcium phosphate synthetic block bone graft material in bone regeneration using defects in the rabbit calvarium. Rabbit cranial defect models have been used in numerous studies for evaluating newly developed biomaterials due to the adequate amount of bone marrow. An 8-mm defect is known to be smaller than the critical defect size in rabbits for evaluating reossification, but has been suggested as a useful defect model for determining the effects of osteoinduction. The use of four 8-mm defects allowed comparison of an early-phase healing response for several materials, while avoiding individual variations. In the present study, the late and intermediate healing responses were analyzed at 8 and 4 weeks postsurgery, respectively.

The histologic and histometric results obtained in this study show that the three calcium phosphate block bone graft materials maintained space more effectively than

the control condition, which is consistent with the results of previous studies. For example, it has been demonstrated that the better space-providing abilities of block-type bone substitutes allows more stable augmented areas than particulated-type bone substitutes.

Previous studies found that HA did not support new bone formation, while the opposite result was found in the present study. This discrepancy is probably due to the use of a block-type bone substitute, which not only helps in the formation of new bone but is also more efficient than the particulated type of calcium phosphate bone substitute. In addition, block-type bone graft materials allow new bone apposition to occur in parallel with progressive material degradation.The histometric results of our study show that HA particles are not easily absorbed, since the amount of residual HA particles shows only small decreases between 4 and 8 weeks postsurgery.

We found that the amount of new bone formation was lowest in the β-TCP experimental group. β-TCP is known to be more readily bioresorbed than HA, and indeed our findings revealed the drastic absorption of β-TCP particles at 8 weeks postsurgery.

The amount of new bone formation at 8 weeks postsurgery was largest in the BCP group, which is consistent with the findings of previous studies. Moreover, the histometric analysis conducted in this study demonstrates that the BCP particles were absorbed more slowly than the β-TCP particles. This may be because the BCP group has the advantages of both the HA and β-TCP groups.

We tested three different block bone graft materials (HA, β-TCP, and BCP) in this study, and found that HA and BCP allowed considerably more new bone formation than either the β-TCP or control conditions. Evaluations of the performance of BCP should take into account that its bioreactivity and degradability can be controlled by altering the HA:β-TCP ratio. In previous studies, the HA:β-TCP ratio affected the reactivity of the material, such that a lower ratio resulted in higher reactivity. However, the optimal HA:β-TCP ratio for osteoconductivity has yet to be determined. Hence, additional studies assessing the efficacies of different HA:β-TCP ratios for block-type graft materials are necessary.

V. CONCLUSION

All three calcium phosphate synthetic block bone graft materials provided more space than the control natural-bone-healing group. Among the three synthetic graft materials, the BCP block bone graft was found to provide the most effective new bone formation at the 4- and 8-week postoperative periods.

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Legends

Figure 1. Clinical photograph of the experiment.

(a) Four 8-mm-diameter defects were made in rabbit calvaria.

(b) HA, β-TCP, and BCP block bone graft materials in the defects.

Figure 2. Schematic drawing for the histometric analysis**.**

Figure 3. Representative photomicrographs obtained at 4 weeks postsurgery.

(a, b) Control group; (c, d) HA group; (e, f) β-TCP group; (g, f) BCP group.

Arrowheads = defect margin; NB = new bone; OB = original bone.

(a, c, e, g) Goldner's Masson trichrome stain, original magnification: ×40.

(b, d, f, h) Hematoxylin and eosin stain, original magnification: ×100.

Figure 4. Representative photomicrographs obtained at 8 weeks postsurgery.

(a, b) Control group; (c, d), HA group; (e, f) β-TCP group; (g, h) BCP group.

(a, c, e, g) Goldner's Masson trichrome stain, original magnification: ×40,

(b, d, f, h) Hematoxylin and eosin stain, original magnification: ×100.

Tables

Table 1. Area of augmented bone- mm^2

(group mean ±SD ; N=5)

Group	4weeks postsurgery	8weeks postsurgery
Control	10.78±2.54	10.51±4.11
Hydroxyapatite	39.26±12.16 *	46.55±7.59 *
β-Tricalciumphosphate	42.50±4.95 *	40.96±6.44 *
Biphasic calcium phosphate	48.51±6.04 *	46.20±4.98 *

* Significant different from sham control group (p<0.05)

Table 2.Area of new bone - mm^2

(group mean ±SD ; N=5)

Group	4weeks postsurgery	8weeks postsurgery
Control	3.99±1.54	4.55±2.50
Hydroxyapatite	4.97±1.91	6.95±3.51*
β-Tricalcium phosphate	1.56±1.32	4.04±1.39
Biphasic calcium phosphate	5.60±3.93	9.03±3.39*

* Significant different from 4 weeks β-Tricalcium phosphate group ($p< .05$)

Table 3.Bone density at each time interval - %

(group mean ±SD ; N=5)

Group	4weeks postsurgery	8weeks postsurgery
Control	36.27±7.56	42.04±14.51
Hydroxyapatite	12.36±2.03 *	14.54±5.92 *
β-Tricalcium phosphate	3.71±3.23 *	9.68±1.95 *
Biphasic calcium phosphate	11.33±7.82 *	19.40±6.19 *

* Significant different from sham control group ($p < .05$)

Table 4.Area of residual particle - mm^2

(group mean ±SD ; N=5)

Group	4weeks postsurgery	8weeks postsurgery
Control	N-A	N-A
Hydroxyapatite	7.39±2.52	6.84±1.14
β-Tricalcium phosphate	9.88±2.08	5.76±2.25
Biphasic calcium phosphate	7.03±2.19	6.09±1.10

Figures

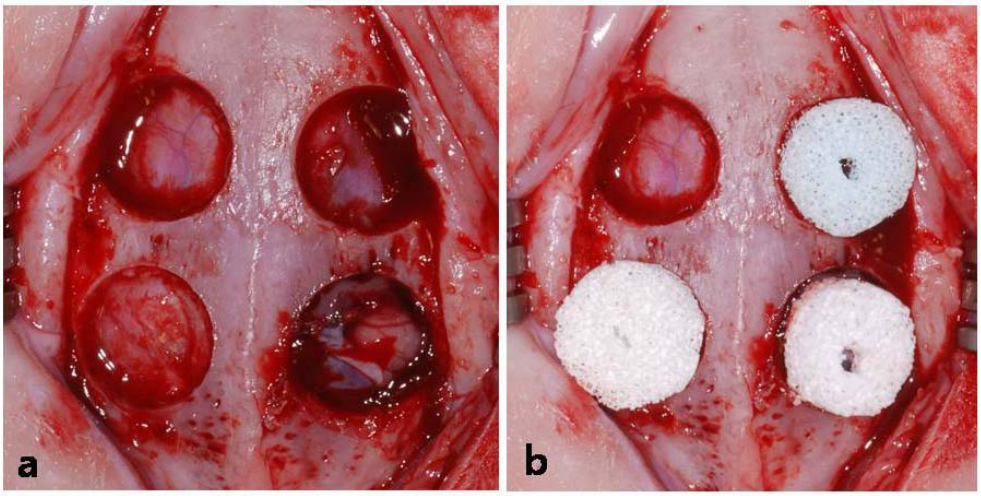

Figure 1

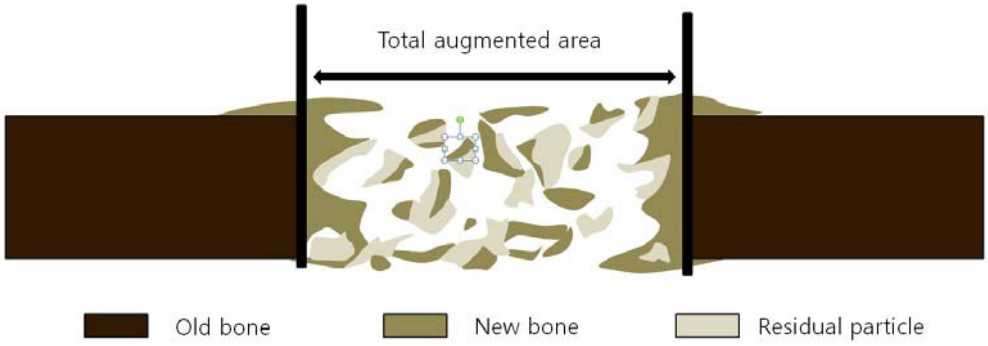

Figure 2

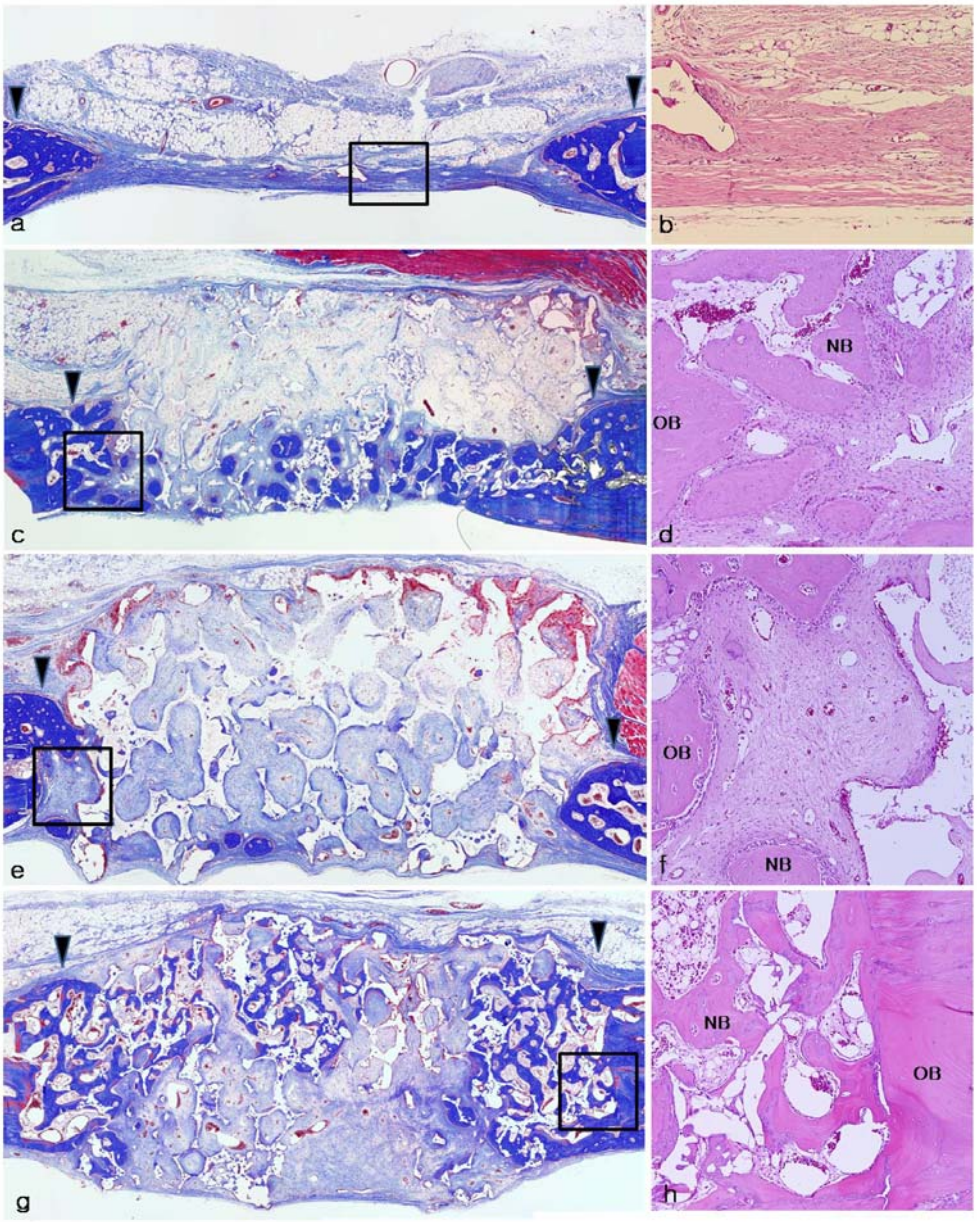

Figure 3

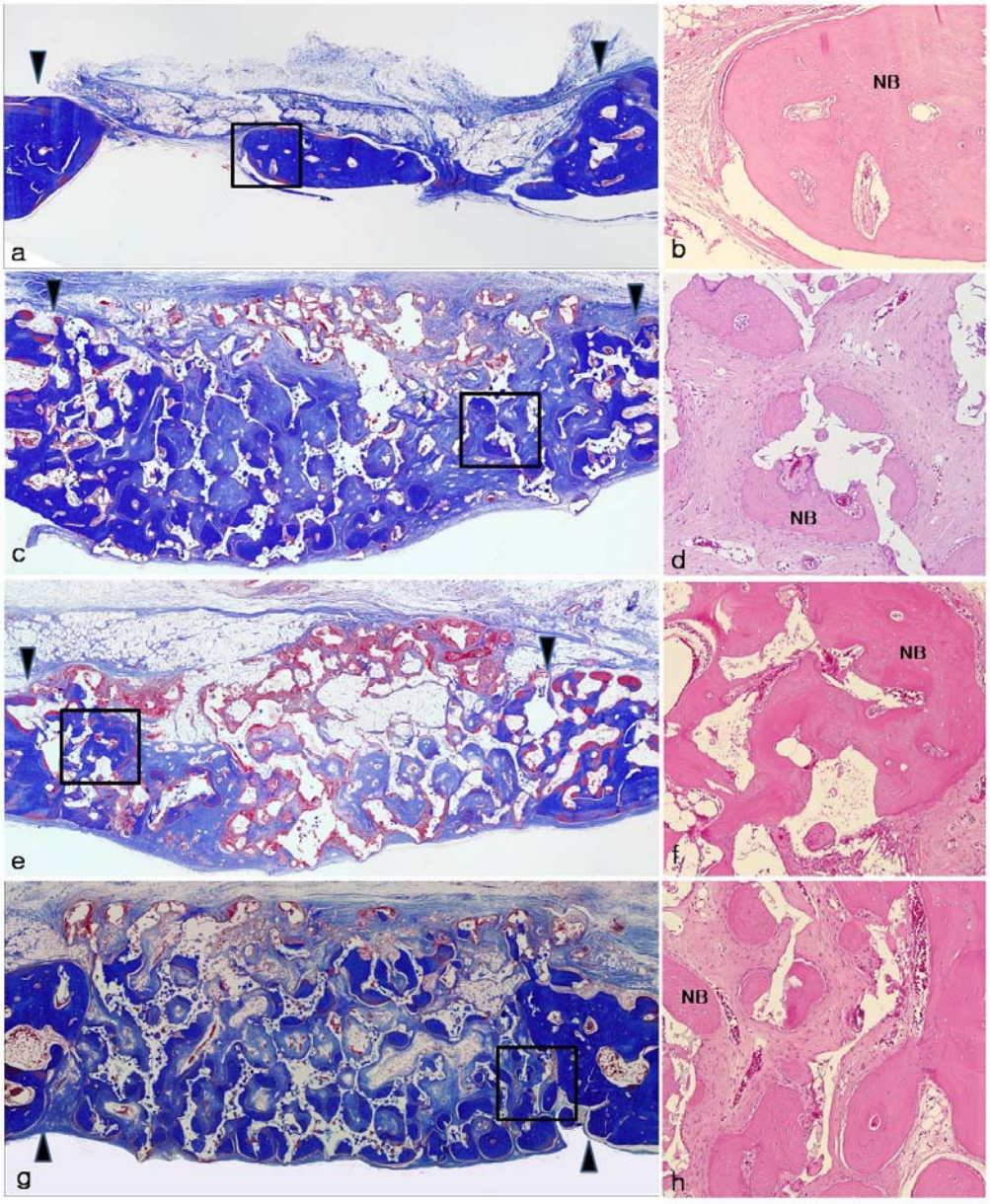

Figure 4.

국문요약

토끼 두개골 결손부에서세 가지 칼슘 포스패이트 합성 블록골이식재의 골형성 능력의 비교 연구

〈지도교수 최 성 호〉
연세대학교 대학원 치의학과
박 재 섭

하이드록시아파타이트 (HA)와 베타-트리칼슘포스페이트 (β-TCP)의 혼합물인 이상 (biphasic) 칼슘 포스페이트와 같은 합성골은 치주 치료와 임플란트시술시에사용되어진다. HA 는 이식 부위의 공간 유지 능력은 우수하나, 골형성 능력은 낮다. β-TCP 는 우수한 생체친화성을 가지고 골 유도 능력이 우수하나 생체에서 흡수가 잘 일어난다. 본 연구에서는 HA, β-TCP, BCP 세 가지의 블록본을 사용하여 각각의 골 형성 능력과 공간 유지 능력을 비교 평가하였다. 실험은 총 10 마리의 토끼의 두개골 각각에 네 개의 8mm 지름의 결손 형태를 만들어서 각각에 HA, β-TCP, BCP 블록본을 위치시켰으며, 나머지 한 부위는 대조군으로 남겨두었다. 수술 후 5 마리씩 분류하여 4 주와 8 주 후에 희생하였으며, 조직학적으로 신생골 형성 정도와 이식재의 공간 유지 능력에 대해 평가하였다. 신생골 형성과 골밀도 측정에서 세 가지 합성 블록골중에 BCP 군에서 가장 좋은 결과를 나타내었다. β-TCP 군에서는 골

형성 정도가 다른 두 가지 합성골보다 낮게 측정되었다. 이식 부위의 잔존 입자를 측정한 결과에서는 BCP 와 HA 군에서는 흡수가 조금 일어났지만, β-TCP 군에서는 4 주가 지나면서 급격한 흡수가 일어났다. 공간 유지 능력에 있어서는 세가지 실험군이대조군보다 높게 측정되었으며, 유의할만한 차이를 보였다.

핵심되는 말:토끼 두개골 결손부, 칼슘 포스페이트, 합성 블럭골