The Influences of Different Ratios of Biphasic Calcium Phosphate and Collagen Augmentation on Posterior Lumbar Spinal Fusion
; A Comparative Study Using Rats` Posterior Lumbar Fusion Model

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Directed by Professor : Sung-Uk Kuh

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It was a great accomplishment of mine that I broaden my academic knowledge and upgrading my experimental skill while completing the thesis. Based on it, I hope it will contribute to the advance of the fusion based spinal surgery.

Kyung Hyun Kim

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ABSTRACT

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The purpose of this study was to determine the influence of the different ratio of Hydroxyapatite (HA) / beta tricalcium phosphate (β-TCP) and collagen augmentation for posterior lumbar fusion using rat model and to find ideal composition of two materials for various fusion situation. We generated a posterior fusion model of lumbar vertebrae in 50 rats and divided it into five groups with equal number by implanting the following materials between transverse processes of vertebrae, (1) Autologous bone graft as group A, (2) 70% HA + 30% β-TCP as group B, (3) 70% HA + 30% β-TCP + Collagen as group C, (4) 30% HA + 70% β-TCP as group D, and (5) 30% HA + 70% β-TCP + Collagen as group E. Rats were euthanized at 12 weeks after surgery and fusion was assessed by manual palpation, quantitative analysis using Micro CT and histology. The score of manual palpation was significantly higher in group C than group E (3.1±1.1 vs. 1.8±0.8, respectively, p=0.033) although no significant difference was noted between group B and group D (3.2±1.3 vs. 2±1.9, respectively, p=0.164). However, in terms of Micro CT analysis, Group D showed significant higher score than group B (5.5±0.8 vs. 3.1±1.1, respectively, $p=0.021$) although there was no difference between group C and group E (4.8±1.1 vs. 5.4±0.8, respectively, $p=0.073$). According to quantitative volumetric analysis, 30% HA + 70% β-TCP groups (group D and E) showed significantly reduced fusion mass at 12 weeks after surgery (123 ± 14.2, 117 ± 46.3 vs. 151 ± 27.3, $p=0.008$, $p=0.003$, respectively). Collagen augmentation groups revealed superior results in terms of both Micro CT score and histologic grade. Given our results, 70% HA + 30% β-TCP rather than 30% HA + 70% β-TCP could be more favorable graft substitute for lumbar spinal fusion. Because appropriate HA proportion should be needed for initial stabilizing the fusion mass. There was some positive role of collagen as adjunct for spinal bone fusion process in our study. Future study should be needed to determine the exact effect of collagen on spinal bone fusion.

Key words: biphasic calcium phosphate, collagen, spinal fusion, rat, β-TCP, hydroxyapatite

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I. INTRODUCTION

Spinal bone fusion based surgery is a fundamental treatment for degenerative or deformed spinal disorder.[1-4] For this purpose, autogenous bone grafting is considered golden standard as these show osteoinductive, osteoconductive, and osteogenic properties.[5] However recently because of various complication after autograft such as donor site pain, infection, and hematoma.[6-10] And also there is several reason that we could not use autograft due to poor bone quality of elderly patients, osteoporosis, malignancy, and previous extracted donor site for primary fusion based operation.[11,12] Thus, for several decades we explore materials to substitute autograft such as growth factors, allograft, demineralized bone matrix (DBM), bone morphogenic proteins (BMPs), and stem cell transplantation.[13-17]

Biphasic calcium phosphates (BCPs) were introduced in the late 1980s[18]. They are composed of Hydroxyapatite (HA), the most stable calcium orthophosphate and β -Tricalcium phosphate (β -TCP), a more soluble compound, with varying HA/ β -TCP ratios.[19-21] Bioactivity and resorption of BCPs can be different according to the HA/ β -TCP ratio and the crystallinity of the ceramic.[22-24] Researches with regard to different effect on bone fusion according to various HA/ β -TCP ratios were reported especially in calvarial bone defect, maxillary bony sinus.[22-24] Lim HC et al.[25] reported that the volume of new bone formation was not different between 7:3 HA/ β -TCP group and 3:7 HA/ β -TCP group and thus they concluded that 3:7 HA/ β -TCP ratio BCPs can be successfully used for sinus augmentation.[25] Mehdi Ebrahimi et al.[26] also concluded that higher β -TCP ratios had beneficial effects during the early phase of cell proliferation and differentiation, whereas high HA ratio performed better in the later stage.[26]

However, there were few researches about the effect on spinal fusion of different ratio of BCPs with Micro CT and histologic analysis although numerous articles about the efficacy for filling bony defect of calvarial, long bone, and maxillary sinus.[1-4,26-33] Spinal fusion is not same as calvarial or maxillary sinus bone fusion due to its segmental mobility during the fusion process and demands enough strength to support body weight. Although recent trends tend to make lower HA proportion and higher β -TCP proportion for more new bone formation in case of filling the bone defect, early excessive absorption of β -TCP could be the worse effect on bone fusion especially between the mobile separate segments like consecutive vertebrae.

This animal study using rat fusion model was designed to investigate the effect of two different HA/ β-TCP ratios (7:3 vs. 3:7) and the influence of collagen augmentation on posterior spinal fusion.

II. MATERIALS AND METHODS

1. Grouping and materials

All procedures were conducted in accordance with the National Institute of Health’s Guide for the Care and Use of Animals and were approved by the local ethical committee. All fifty male Sprague Dawley rats were used for a posterior spinal fusion model of lumbar vertebrae and divided it into five groups by implanting the following materials between transverse processes of vertebrae, (1) Autologous bone extracted rat tail bone as group A, (2) 70% Hydroxyapatite + 30% beta tricalcium phosphate (β-TCP) as group B, (3) 70% Hydroxyapatite + 30% beta tricalcium phosphate (β-TCP) + Collagen as group C, (4) 30% Hydroxyapatite + 70% beta tricalcium phosphate (β-TCP) as group D, and (5) 30% Hydroxyapatite + 70% beta tricalcium phosphate (β-TCP) + Collagen as group E. Detailed data of each group were descripted in Table 1.

Table 1. Detailed descriptions of group

Group	Graft materials	Number of rats
Group A	Autologous bone graft from tail bone	10
Group B	7:3 HA: β -TCP	10
Group C	7:3 HA: β -TCP + Collagen	10
Group D	3:7 HA: β -TCP	10
Group E	3:7 HA: β -TCP + Collagen	10

2. Characteristics of biphasic calcium phosphate

All 7:3 HA: β –TCP materials used in group B and 3:7 HA: β –TCP materials used in group D were HA coated with β –TCP with 0.3-0.5 mm sized particles and 300-500 μm porosity. 7:3 HA: β -TCP used in group B was granule type with 0.5 cc (approx. 500 mm^3), and 7:3 HA: β -TCP + Collagen, 3:7 HA: β –TCP, and 3:7 HA: β -TCP + Collagen used in group C, D, and E were manufactured by the company which has same sized cylindrical shape as 6 mm diameter with 10 mm height (Ø 6 x 10 mm^3 x 2 pieces = 565 mm^3), 0.3-0.5 mm sized particles, and 250 μm sized porosity. Augmented collagen was composed of type I collagen originated from bovine tendon.

3. Surgical techniques

A. Preparation of Tail bone for autologous bone graft

Animals were anesthetized with intraperitoneal ketamine injection (80-100mg/kg). A single dose of antibiotic (Gentamicin at the dose of 0.05ml/kg) was injected immediately after surgery prophylactically. Then, the animals were placed prone to a small operating table with warm heated and shaved and the operation side was sterilized with a 10% povidone iodine solution. To harvest a bone graft from the tail, the tail was amputated and all soft tissue was removed from the bone using forceps, rongeurs and scalpels (Figure 1A). Four vertebrae were separated from the intervertebral discs and all periosteum was removed (Figure 1B). The bone was morselized with rongeurs and weighed to create a homogenous distribution of the grafts between the groups (Figure 1C). Approximate volume of autologous tail bone used in group A was 474.9 mm^3 (Ø mean 5.5 mm x 5 mm height x 4 pieces).

Figure 1A. The tail was amputated for autobone graft.

Figure 1B. Four vertebrae were separated from the intervertebral discs and all periosteum was removed.

Figure 1C. The bone was morselized with rongeurs and weighed to create a homogenous distribution of the grafts between the groups.

B. Surgical procedure

After harvesting the tail bone graft, shaving, and disinfecting, a dorsal midline skin incision and a median fascial incision was made by scalpel and the paraspinal muscles were retracted to expose spinous process, lamina, and facet joint. We did not use electric drill and use scalpel for decortication. The implant was cylindrical shape manufactured by company thus all the volume of the implant were exactly same. For group A, autogenously morselized bone harvested from the tail described above was placed on the posterior decorticated lamina surface at both sides (Figure 2). For BCP groups (group B, C, D, and E), two cylindrical materials were implanted on the posterior decorticated lamina surface at both side (Figure 3). The wound were closed with 3-0 nylon sutures. The rats recovered from anesthesia in the warm basket for 15 minutes and were returned to home cages. For postoperative pain, lidocaine 0.1 mg/kg was given every 6 to 12 hours for the first 2 postoperative days. Then, animals were observed twice daily throughout the post-surgical study period for general attitude, appetite, appearance of the surgical site, neurological signs and respiratory stress. Twelve weeks after surgery, the rats were euthanized by overdose of ketamine. After euthanasia, the lumbar spines were explanted and the soft tissues removed. Fusion was assessed by manual palpation, Micro CT evaluation (SkyScan1173, Kartuizersweg 3B 2550 Kontich, Belgium) and histology.

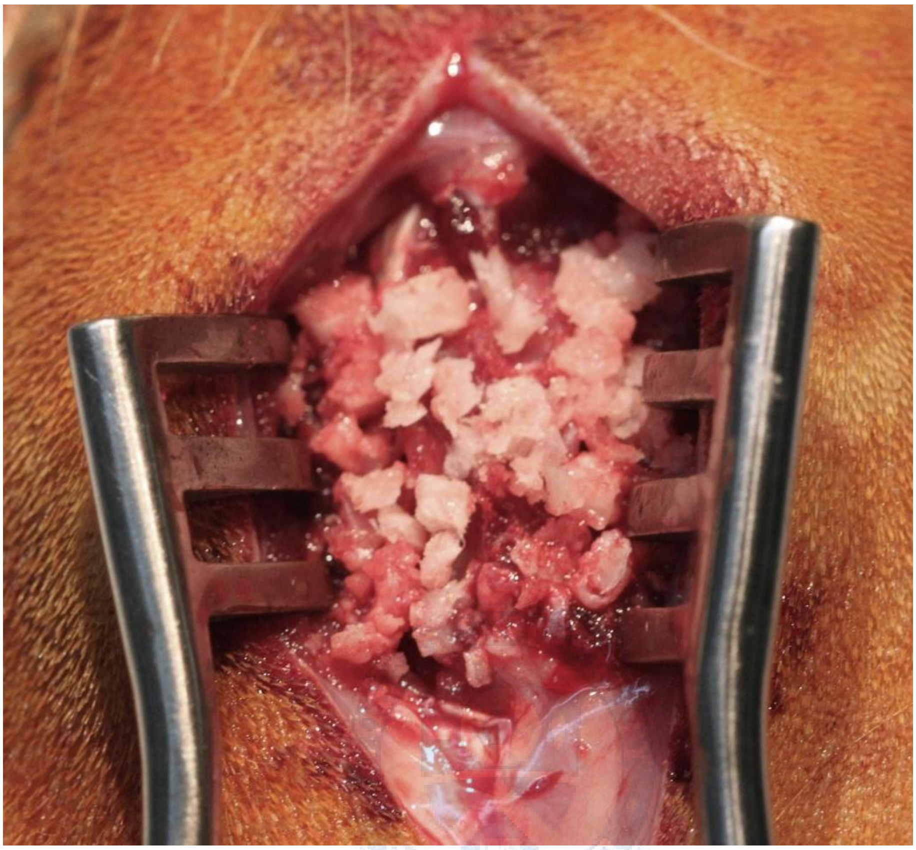


Figure 2. For group A, autogenously morselized bone harvested from the tail described above was placed on the posterior decorticated lamina surface at both sides.

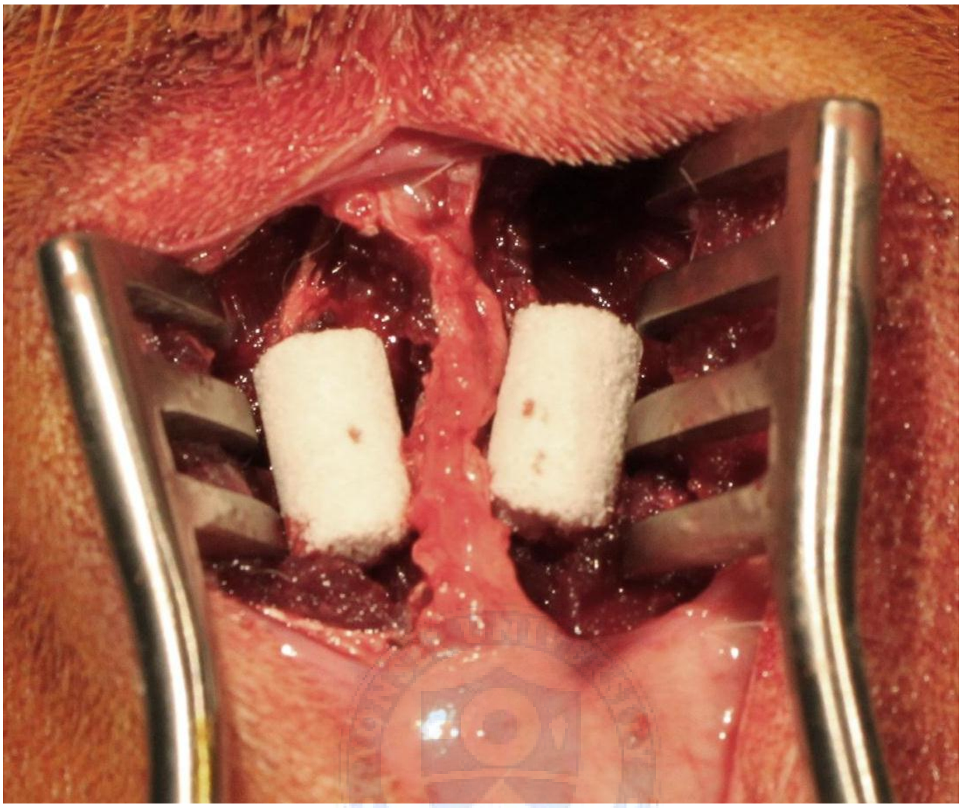


Figure 3. Two cylindrical materials were implanted on the posterior decorticated lamina surface at both side for BCP groups (group B, C, D, and E).

4. Manual Palpation

Harvested lumbar spines were manually palpated by flexion and extension at the fusion level and solidity was compared with the adjacent level by four different observers (Figure 4). Any motion detected on either side between the segments was considered nonunion and it`s score was 0. The absence of motion in every direction was determined as union and its score was 1. Four observers judged the scores according to the above rule. The four reviewers' scores were summed (maximum score, 4) and use it variable for statistical analysis. Detailed description of grading rules demonstrated in table 2.

Table 2. Description of Manual Palpation Grading

Grade	Score	Definition
Union	1	The absence of motion in every direction
Nonunion	0	Any motion detected on either side between the segments

Figure 4. Extracted lumbar spine at 12 weeks after surgery for fusion assessment.

5. Micro CT analysis

Micro CT scanning and analyses were performed on SkyScan1173 using the manufacturer's analysis software. Serial axial Micro CT images from each specimen were graded, in a masked fashion, by three independent scorers according to the following scoring criteria (0; minimal or no evidence of bone formation was observed, 1; immature bone formation with questionable fusion was observed and 2; solid appearing bone with fusion likely).[34] The 3 reviewers' scores were summed (maximum score, 6). A score of 5 or 6 was considered as indicating fusion.[34]

6. Volumetric analysis of fusion mass using Micro CT images

The scan was performed in the long axis of the spine with energy of 130 kV and a current of 30 μA, and a 250-ms exposure time producing 1200 axial images with 27.70 μm pixel sizes. To measure the total bone (new bone and residual graft material) and new bone volumes at the region of posterior spinal fusion, the axial image was converted to 3D by Digital Imaging and Communications in Medicine software (Lucion; Infinite, Seoul, Korea). We created three-dimensional image reconstructions to measure the total volume of fusion mass in both sides for each specimen.

7. Histological analysis

After Micro CT analysis and manual palpation test, explanted spine were decalcified by 10% decalcifying solution HCL (Cal- Ex) (Fischer Scientific, Fairlawn, NJ) and were fixed in 75% ethanol solution. Three blinded independent observers graded goldner`s trichrome stained sections on a scale from 1 to 10 based on the histologic ratio of fibrous tissue, cartilage, and mature bone visualized on a low-power field followed by the previous established method (Table 3).[35]

Table 3. Histologic grade

Grade	Histological findings
1	Fibrous tissue
2	Predominantly fibrous tissue with some cartilage
3	Equal amount of fibrous tissue with some cartilage
4	All cartilage
5	Predominantly cartilage with some woven bone
6	Equal amount of cartilage and woven bone
7	Predominantly woven bone with some cartilage
8	Entirely woven bone
9	Woven bone with some mature bone
10	Lamellar (mature) bone

8. Statistical analysis

Statistical analysis was performed using SPSS v.18.0.0 software (SPSS, Inc., Chicago, Illinois). Values were recorded as mean±standard deviation. Mann-Whitney U test was used for comparing values between groups. $P < 0.05$ was considered statistically significant.

III. RESULTS

1. Comparison BCP groups to autologous bone graft

Data are provided in Table 4. With regard to manual palpation, Group E showed significantly lower score (1.8±0.8) compared to Group A. There were no significant differences in manual palpation score between autologous bone group (Group A) and Other groups (Group B, C, and D). According to the results of Micro CT scan, the score of group B and C were significantly lower than group A and there were no significant differences between other groups (D and E) and Group A. As a score of 5 or 6 was considered as indicating fusion in this study, mean value over 5 points can be seen only in Group D and E which β –TCP proportion was 70% with or without collagen. Mean histologic grade of group B, C, D, and E were significantly lower than group A.

Table 4. Results of Manual palpation, Micro CT, and Histologic grade which compare all (group B, C, D, and E) to autobone graft (group A).

	Group A	Group B	Group C	Group D	Group E
Manual Palpation (Mean±SD)	3.6±0.5	3.2±1.3	3.1±1.1	2±1.9	1.8±0.8*
Micro CT (Mean±SD)	5.7 ±2.1	3.1±1.1*	4.8±1.1*	5.1±0.9	5.4±0.8
Histologic Grade (Mean±SD)	8.3±1.0	4.3±1.3*	5.5±1.4*	5.5.±0.8*	6.3±0.5*

* p<0.05, mann-whitney U test , all variables of groups compared to Group A

2. Influence of different ratios of HA/ β -TCP ratio on fusion

Data are provided in Table 3. To evaluate the effect of HA/ β –TCP ratio, we perform intergroup comparison between Group B and D, and C and E. The score of manual palpation was significantly higher in group C than group E although no significant difference was noted between group B and group D. However, in terms of Micro CT analysis, Group D showed significant higher score than group B although there was no difference between group C and group E. There were no significant differences between intergroup comparisons in histologic grade.

Table 5. Intergroup comparison for the influence of combination ratio of HA: β-TCP on Fusion

	Group B	Group D	p-value	Group C	Group E	p-value
Manual Palpation (Mean±SD)	3.2±1.3	2±1.9	0.164	3.1±1.1	1.8±0.8	0.033
Micro CT (Mean±SD)	3.1±1.1	5.1±0.9	0.021	4.8±1.1	5.4±0.8	0.073
Histologic Grade (Mean±SD)	4.3±1.3	5.5.±0.8	0.683	5.5±1.4	6.3±0.5	0.785

3. Influence of collagen on fusion

Data are provided in Table 4. To evaluate the effect of collagen on fusion, we did intergroup comparison between Group B and C, and D and E. There were no significant differences between intergroup comparisons in manual palpation scores. Regarding Micro CT analysis, the score was significantly higher in group C than group B although no significant difference was found between group D and group E. In terms of histologic grade, Group C and E showed significant higher score than group B and D.

Table 6. Intergroup comparison for the influence of collagen on fusion

	Group B	Group C	p-value	Group D	Group E	p-value
Manual Palpation (Mean±SD)	3.2±1.3	3.1±1.1	0.666	2±1.9	1.8±0.8	0.948
Micro CT (Mean±SD)	3.1±1.1	4.8±1.1	0.011	5.1±0.9	5.4±0.8	0.044
Histologic Grade (Mean±SD)	4.3±1.3	5.5±1.4	0.026	5.5.±0.8	6.3±0.5	0.038

4. Quantitative Analysis of Fusion Masses

At 12 weeks, bone volume on the fusion bed of group B and C showed no significant differences compared with group A (152 ± 16.6 vs. 158 ± 28.3 and 151 ± 27.3 vs. 158 ± 28.3, 0.458 and 0.365, respectively). However group D and E had significantly lower fusion mass volume than group A (123 ± 14.2 vs. 158 ± 28.3 and 117 ± 46.3 vs. 158 ± 28.3, P=0.008, and 0.003 respectively) (Table 7). Therefore, according to 3D reconstructive volumetric analysis, 7:3 HA: β –TCP graft group (group 2 and 3) had significantly higher fusion mass volume than autologous bone graft group and 3:7 HA: β –TCP graft group had significantly lower fusion mass volume than autologous bone graft group (Figure 5A, B, C, D, and E).

Table 7. Total volume of fusion mass of each group at 12 weeks after graft implantation.

Parameters	Postoperative time	Group A	Group B	Group C	Group D	Group E
Total Volume of Fusion mass (mm3, Mean±SD)	12 weeks	158 ± 28.3	152 ± 16.6	151 ± 27.3	123 ± 14.2	117 ± 46.3
P-value (Versus group A)			0.458	0.365	0.008	0.003

Figure 5. Representative Micro CT axial and sagittal view sections of fusion masses from each group.

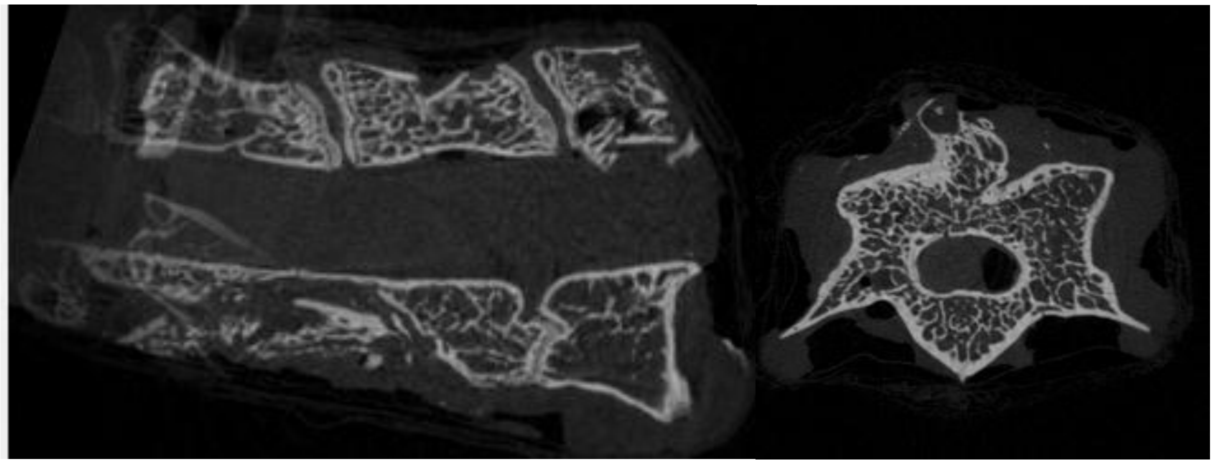


Figure 5A. Autobone (group A) showed complete union without graft-bone interface.

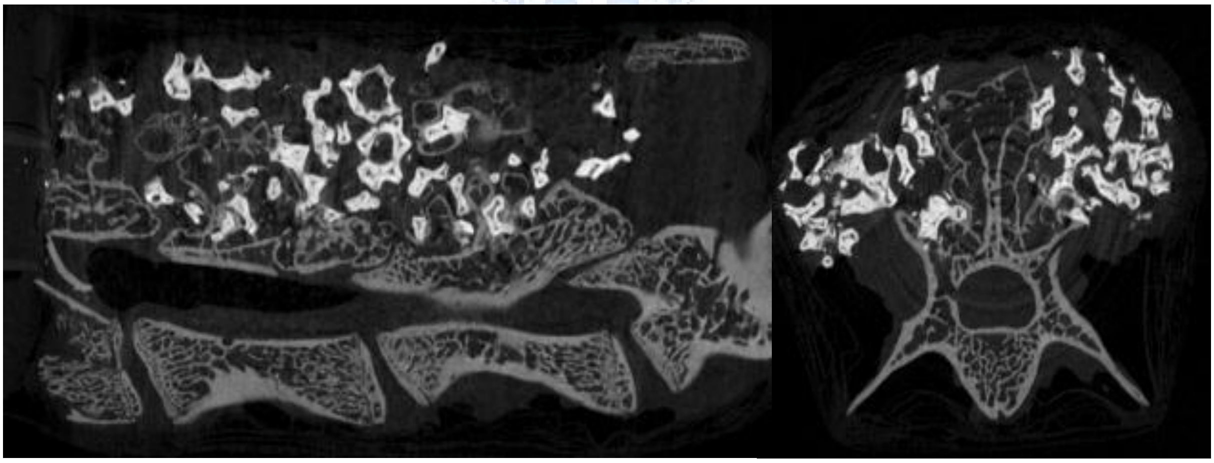


Figure 5B. 7:3 HA: β –TCP (group B) revealed relatively larger fusion mass but less incorporated into recipient lamina.

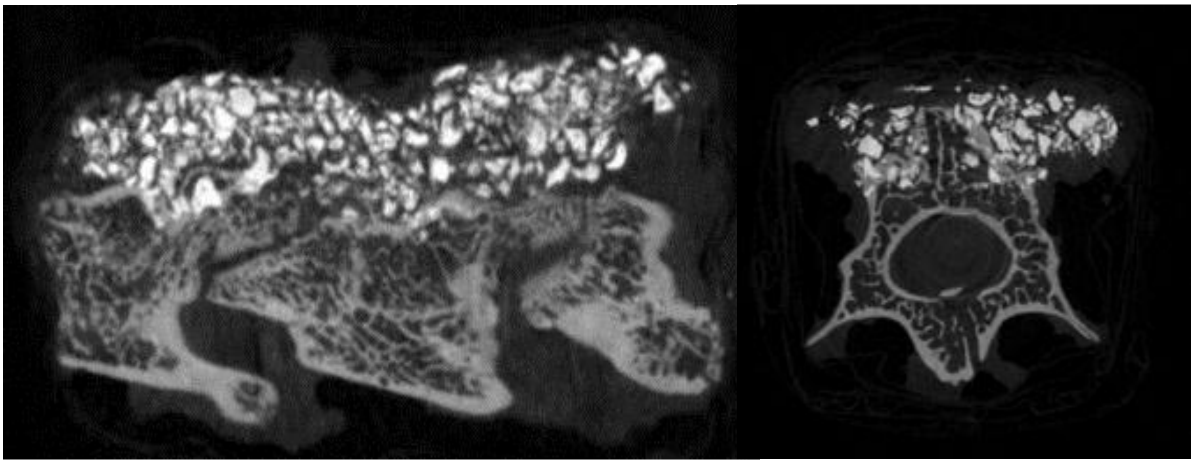


Figure 5C. 7:3 HA: β –TCP + Collagen (group C) showed little incorporation into lamina but more remained fusion mass.

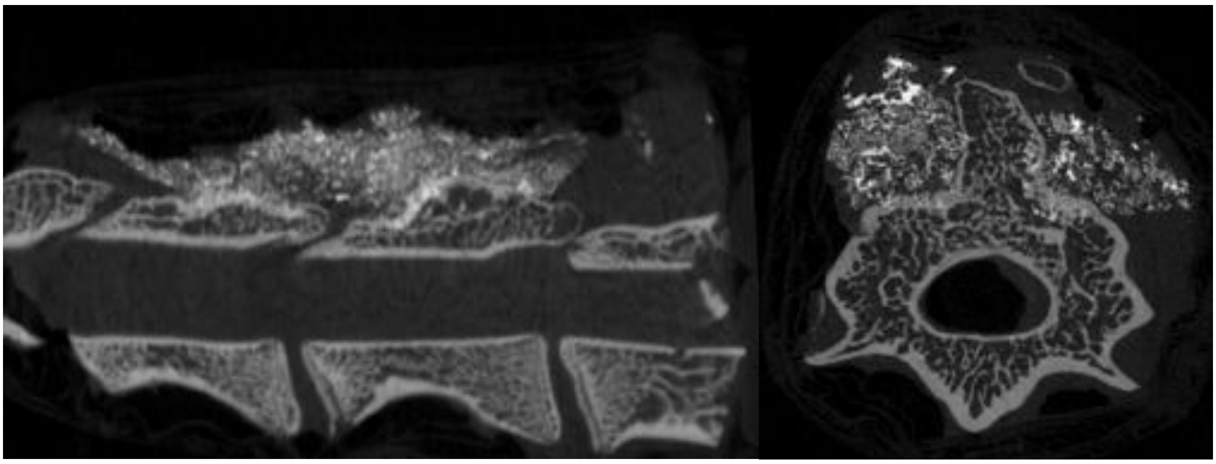


Figure 5D. 3:7 HA: β –TCP (group D) showed reduced fusion mass but revealed good graft incorporation into recipient lamina.

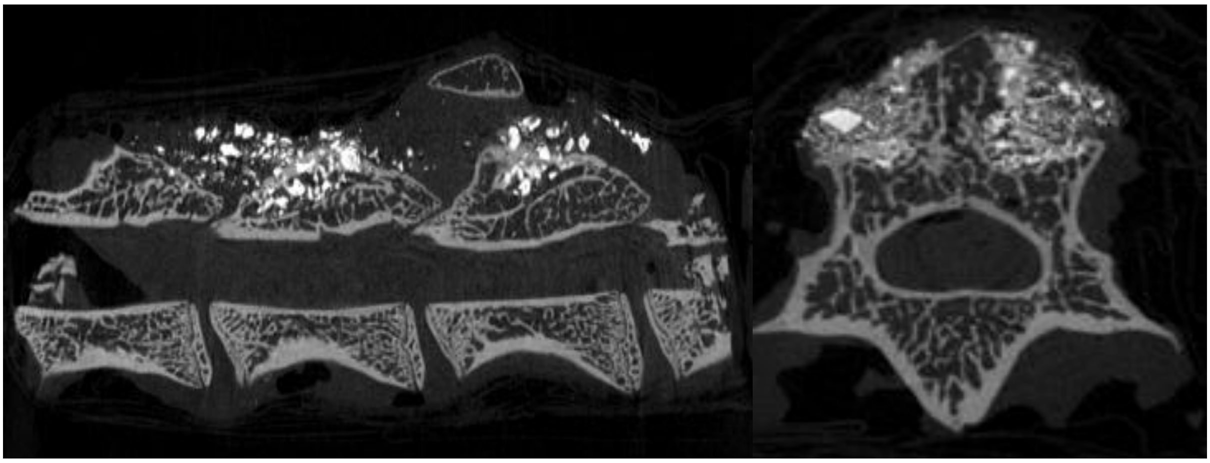


Figure 5E. 3:7 HA: β –TCP + Collagen (group E) demonstrated excellent graft incorporation into recipient lamina.

5. Histologic finding

Representative photos of each group were illustrated in Figure 6. Analysis of the histological sections of each material was performed at 12 weeks after surgical procedure. None of the grafted materials revealed a significant inflammatory reaction. Woven bone was identified around and close to the material in rat posterior laminar decorticated bone in all section of fusion mass. The volume of the autologous tail bone in group A decreased progressively as bone formation increased at the periphery and within the block, leading to its virtual disappearance and almost complete closure of the cortex at 12 weeks. It is also proved in Micro CT scan of this fusion mass which showed no distinction between graft and laminar bone. (Figure 6A) In group B with HA 70%, graft mass showed slower resorption than the other groups thus the volume of fusion mass was larger than others as well. Histological results showed that after implantation, changes to residual block content, peripheral bone resorption, new bone formation, and closure of the cortex were minimal in comparison with the other graft compositions (Figure 6B). In 7:3 HA/β -TCP implanted groups (B and C), there was intermediate structural stability of fusion mass, much less resorption than in 3:7 HA/β -TCP implanted groups (4 and 5). However 3:7 HA/β -TCP implanted groups (D and E) revealed less distinctive features between graft and laminar bone and superior integration into recipient laminar bone although much more resorbed fusion mass and naturally reduced the material volume were noted. According to histological comparison between groups with collagen and groups without collagen (B versus C, D versus E), collagenous membrane which might be expected can be found between materials and host bone. However definite histological findings supportive of enhanced contact or incorporation could not be detected in this research.

Figure 6 Representative histologic features of fusion mass on goldner`s trichrome staining.

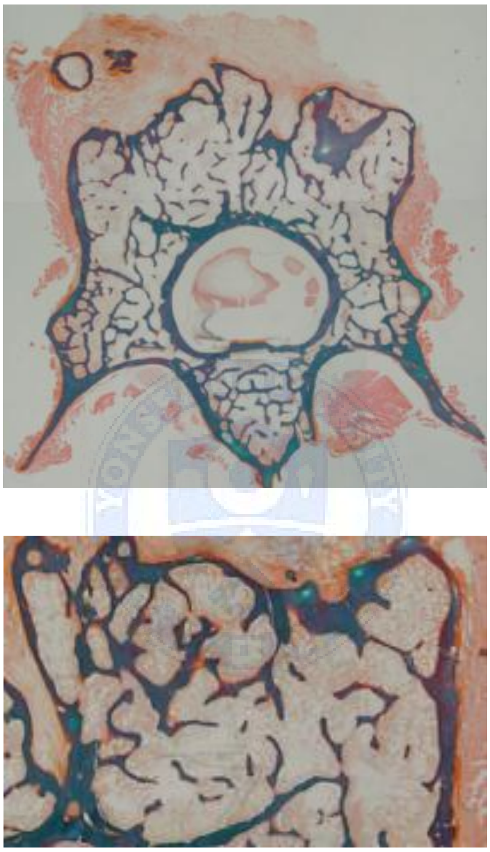
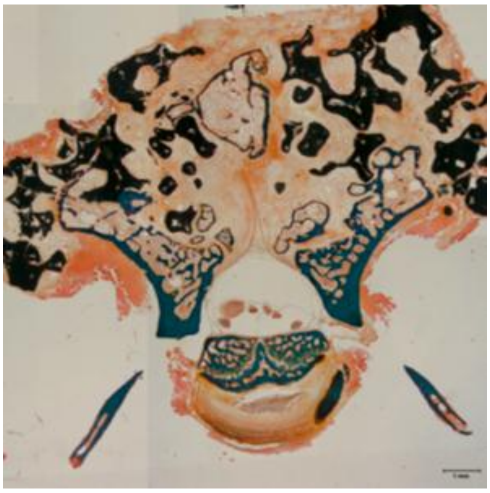


Figure 6A. Immature bone with narrow trabecular was formed along the cutting surface of bone defect (Group A).



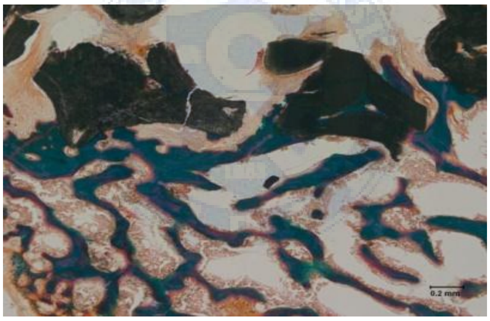


Figure 6B. Peripheral bone resorption, new bone formation, and closure of cortex were minimal in comparison with other group (Group B)

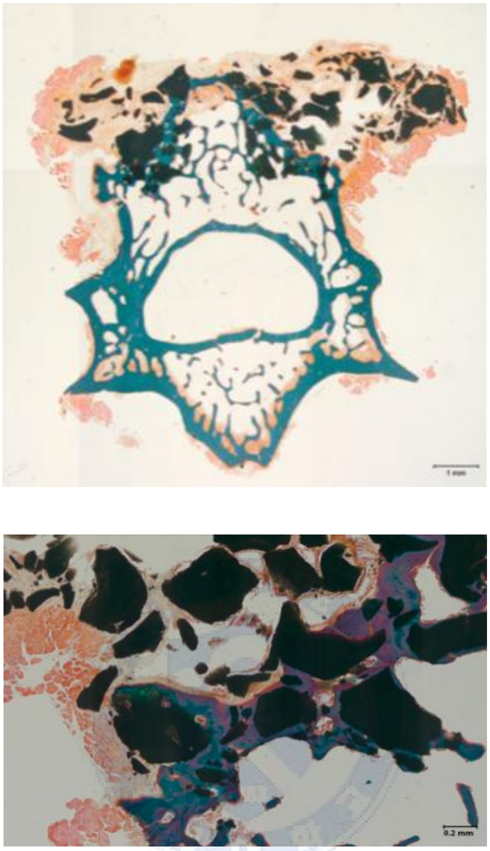


Figure 6C. Intermediate structural stability of fusion mass was noted but revealed less resorption than group D and E (Group C).

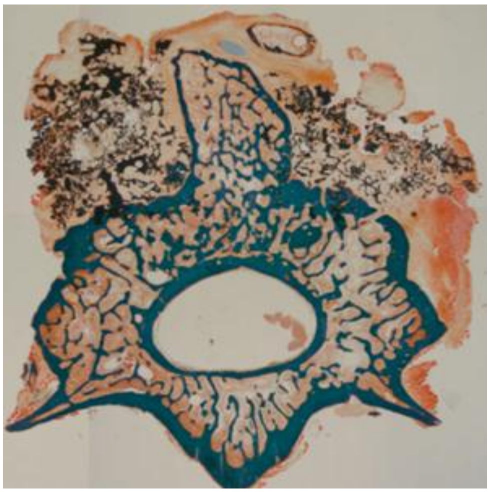


Figure 6D. Less distinctive features between graft and laminar bone and superior integration into recipient bone was shown and reduced volume of fusion mass was also noted (Group D)

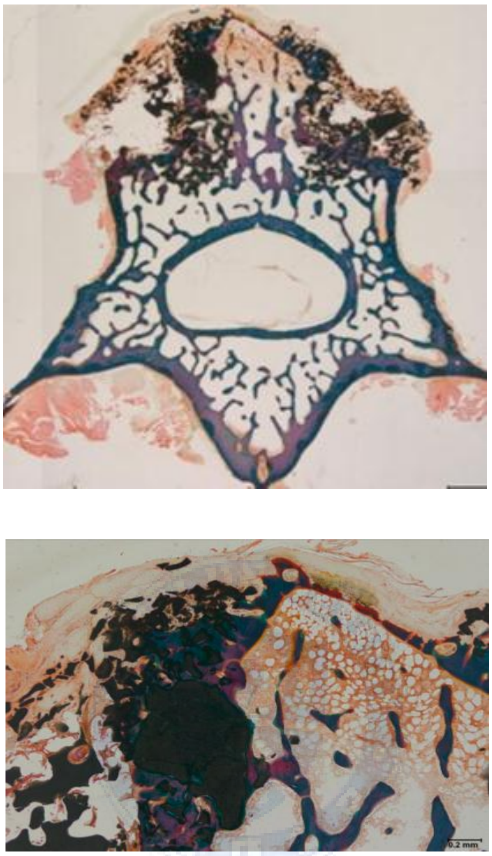


Figure 6E. There was excellent incorporation of graft into laminar bone but much less volume of fusion mass due to excessive resorption (Group E)

IV. DISCUSSION

Solid fusion is the most important prognostic factor for clinical success in fusion-based spinal surgery. Especially, geriatric population have more limitation for solid fusion even though autograft were used because they have more frequently diabetes, malnutrition and poor bone quality due to osteoporosis. Therefore, synthetic graft substitutes using various human bone morphogenic protein-2, β-TCP, hydroxyapatite, and collagen as an adjunct or a delivery system has been studied and customized as an alternative treatment for conventional autogenous bone grafting.[36,37]

This study was carried out to investigate the effect of different ratio of BCPs and collagen on posterior spinal fusion such as new bone formation, osteoinductivity, and biologic response between bone and material. Different ratio of HA/ β -TCP with or without collagen could produce different biologic responses, thus ideal composition of two materials for various fusion situation should be determined for various situations.[38] There was a difference of HA to β-TCP ratios between Groups in this study; HA portion was 70% or 30%. Although β-TCP degraded rapidly after the graft, HA remained for a long time and most of them was encapsulated by fibrous tissue in histologic findings.[38,39] As previously reported, β-TCP is biodegradable and more easily resorbed than HA.[40] In an ideal situation, it is better that a biodegradable bone substitute is slowly resorbed and replaced by natural bone.[40] In cases that percentage of HA particle was excessive, new bone formation within graft materials might be partly inhibited.[38,39] Therefore, appropriate BCP ratio was important but yet determined and unsettled.[39] In previous studies, 20/80 was the optimal ratio of HA to β -TCP in the graft material for biodegradation and sufficient bone formation.[39] On the contrary, Nery EB et al.[33] reported in their results about periodontal osseous defects that 85:15 HA/ β -TCP was the ideal ratio for new periodontal tissue attachment and bone regeneration.[33] However, unlike calvarial bone defect or craniomaxillofacial bony area, spine segment which has to be fused is mobile during the fusion process. In that situation, thus, immediate posterior screw fixation which makes it stable is the important for fusion. Our rat fusion model includes posterior spinal fusion without instrumentation which means still mobile after implantation of bone. Thus, Stabilized unreduced fusion mass by less resorbed HA might be needed in case of fusion between the separate vertebrae. We thought this is the different point of this study from other previous researches and the results about bone filling effect.

According to the our results (Table 2), Manual palpation score showed significantly lower in group E and the result of group D is also similar to group E although it is not proved statistically. And Group B and C showed similar strength of manual palpation compared to autologous bone graft (group A) BCP with 70% HA proportion produce more structural stable fusion mass than 30% HA proportion. Early biodegradable and resorption of β -TCP could reduce the volume of graft material during the early phase of fusion process and might be the one of cause. However, Micro CT score and histologic grade of BCP with 3:7 HA/ β -TCP ratio (Group D and E) was higher than BCP with 7:3 HA/ β -TCP ratio groups (group B and C). Especially group D was significantly superior results than group B in Micro CT analysis (5.5±0.8, 3.1±1.1 respectively, P=0.001). It means that bone-graft adherence and incorporation could be more superior in 3:7 HA/ β -TCP ratio than 7:3 HA/ β -TCP ratio.

In terms of the influence of collagen, it was already reported that collagen and biphasic calcium phosphate ceramic bone graft is as effective as autogenous graft in long bone fractures and traumatic osseous defects less than 30 mL in size and even in over 30 ml defect after tumor excision.[41] Multicenter clinical trials also have shown collagen and biphasic calcium phosphate synthetic bone graft to be as effective as autogenous graft in long bone fractures[41] However, Muschler et al reported in 1996 that composites of purified bovine Type I fibrillar skin collagen gel and granules of a biphasic calcium phosphate ceramic are ineffective as graft materials in this model when combined with autogenous cancellous bone or autogenous bone marrow.[42] However, In this study, although there were no significant differences between group B and C or group D and E in manual palpation score. In 7:3 HA/ β -TCP ratio intergroup comparison for collagen influence, the score of Micro CT of group C (collagen added) was higher significantly than group B (without collagen). And also significant differences was found between group D and E in 3:7 HA/ β -TCP ratio intergroup comparison. The histologic grade of groups with collagen (group C and E) were higher than the score of groups without collagen (group B and D). Our research showed that there were positive role of collagen for posterior lumbar spinal fusion and these results were expected based on previous results. Because majority of previous reports were investigated influence of collagen membrane wrapping on bone fusion as both bone formation enhancement by allowing osteoblasts to enter and the barrier for fibrous tissue growing into bony defect.[24,43] We can speculate that collagen added BCP has relatively smoother texture to adhere the mobile segment and act as a barrier for inhibiting fibroblast migration into the BCP while allowing osteoblasts.

Our research has several limitations. First, comparison by manual palpation score with unblinded fashion is obviously inferior to biomechanical bending test by machine. Thus we tried to exclude examiners` subject feeling by summation of multiple scores and binary simplification of score system. Second, inconsistent surgical method could be one of bias for fusion outcome. We focused on uneven size of transverse process among rats and decided to use posterior laminar surface for graft bed.

V. CONCLUSION

Given our results, 7:3 HA/ β -TCP ratio with collagen augmentation rather than 3:7 HA/ β -TCP ratio could be more favorable graft substitute for lumbar spinal fusion. because appropriate HA proportion should be needed for initial stabilizing the fusion mass, and there was some positive role of collagen as adjunct for spinal bone fusion process in our study. Future study should be needed to determine the exact effect of collagen on spinal bone fusion.

VI. REFERENCES

1. Zurbriggen C, Markwalder TM, Wyss S. Long-term results in patients treated with posterior instrumentation and fusion for degenerative scoliosis of the lumbar spine. *Acta Neurochir (Wien)* 1999; **141**(1): 21-6.
2. Gertzbein SD, Hollopeter M, Hall SD. Analysis of circumferential lumbar fusion outcome in the treatment of degenerative disc disease of the lumbar spine. *J Spinal Disord* 1998; **11**(6): 472-8.
3. Rompe JD, Eysel P, Hopf C. Clinical efficacy of pedicle instrumentation and posterolateral fusion in the symptomatic degenerative lumbar spine. *Eur Spine J* 1995; **4**(4): 231-7.
4. Herkowitz HN, Sidhu KS. Lumbar Spine Fusion in the Treatment of Degenerative Conditions: Current Indications and Recommendations. *J Am Acad Orthop Surg* 1995; **3**(3): 123-35.
5. Flouzat-Lachaniette CH, Ghazanfari A, Bouthors C, Poignard A, Hernigou P, Allain J. Bone union rate with recombinant human bone morphogenic protein-2 versus autologous iliac bone in PEEK cages for anterior lumbar interbody fusion. *Int Orthop* 2014; **38**(9): 2001-7.
6. Kim DH, Rhim R, Li L, et al. Prospective study of iliac crest bone graft harvest site pain and morbidity. *Spine J* 2009; **9**(11): 886-92.
7. Al-Sayyad MJ, Abdulmajeed TM. Fracture of the anterior iliac crest following autogenous bone grafting. *Saudi Med J* 2006; **27**(2): 254-8.
8. Pitzen T, Kranzlein K, Steudel WI, Strowitzki M. [Complaints and findings at the iliac crest donor site following anterior cervical fusion]. *Zentralbl Neurochir* 2004; **65**(1): 7-12.
9. Delawi D, Dhert WJ, Castelein RM, Verbout AJ, Oner FC. The incidence of donor site pain after bone graft harvesting from the posterior iliac crest may be overestimated: a study on spine fracture patients. *Spine (Phila Pa 1976)* 2007; **32**(17): 1865-8.
10. Ito Z, Imagama S, Kanemura T, et al. Bone union rate with autologous iliac bone versus local bone graft in posterior lumbar interbody fusion (PLIF): a multicenter study. *Eur Spine J* 2013; **22**(5): 1158-63.
11. Lippman CR, Salehi SA, Liu JC, Ondra SL. Salvage technique of posterior iliac bolt placement in

long-segment spinal constructs with a previous posterior iliac crest harvest: technical note. *Neurosurgery* 2006; **58**(1 Suppl): ONS-E178; discussion ONS-E.

12. Emery SE, Hughes SS, Junglas WA, Herrington SJ, Pathria MN. The fate of anterior vertebral bone grafts in patients irradiated for neoplasm. *Clin Orthop Relat Res* 1994; (300): 207-12.

13. Taghavi CE, Lee KB, Keorochana G, Tzeng ST, Yoo JH, Wang JC. Bone morphogenetic protein-2 and bone marrow aspirate with allograft as alternatives to autograft in instrumented revision posterolateral lumbar spinal fusion: a minimum two-year follow-up study. *Spine (Phila Pa 1976)* 2010; **35**(11): 1144-50.

14. Ajiboye RM, Hamamoto JT, Eckardt MA, Wang JC. Clinical and radiographic outcomes of concentrated bone marrow aspirate with allograft and demineralized bone matrix for posterolateral and interbody lumbar fusion in elderly patients. *Eur Spine J* 2015.

15. Virk S, Sandhu HS, Khan SN. Cost effectiveness analysis of graft options in spinal fusion surgery using a Markov model. *J Spinal Disord Tech* 2012; **25**(7): E204-10.

16. Hoffmann MF, Jones CB, Sietsema DL. Adjuncts in posterior lumbar spine fusion: comparison of complications and efficacy. *Arch Orthop Trauma Surg* 2012; **132**(8): 1105-10.

17. Thalgott JS, Giuffre JM, Fritts K, Timlin M, Klezl Z. Instrumented posterolateral lumbar fusion using coralline hydroxyapatite with or without demineralized bone matrix, as an adjunct to autologous bone. *Spine J* 2001; **1**(2): 131-7.

18. Martin S, Daculsi G, Passuti N, Le Nihouannen JC, Lajat Y, Resche F. [Bone integration within calcium phosphate ceramic by creation of posterior inter-articular lumbar spine fusions in dogs]. *Neurochirurgie* 1989; **35**(4): 209-15.

19. Yi J, Lee GW, Nam WD, et al. A Prospective Randomized Clinical Trial Comparing Bone Union Rate Following Anterior Cervical Discectomy and Fusion Using a Polyetheretherketone Cage: Hydroxyapatite/B-Tricalcium Phosphate Mixture versus Hydroxyapatite/Demineralized Bone Matrix Mixture. *Asian Spine J* 2015; **9**(1): 30-8.

20. Sugawara T, Itoh Y, Hirano Y, Higashiyama N, Mizoi K. beta-Tricalcium phosphate promotes bony fusion after anterior cervical discectomy and fusion using titanium cages. *Spine (Phila Pa*

1976) 2011; **36**(23): E1509-14.

21. Cosar M, Ozer AF, Iplikcioglu AC, et al. The results of beta-tricalcium phosphate coated hydroxyapatite (beta-TCP/HA) grafts for interbody fusion after anterior cervical discectomy. *J Spinal Disord Tech* 2008; **21**(6): 436-41.

22. Miron RJ, Sculean A, Shuang Y, et al. Osteoinductive potential of a novel biphasic calcium phosphate bone graft in comparison with autographs, xenografts, and DFDBA. *Clin Oral Implants Res* 2015.

23. Lobo SE, Glickman R, da Silva WN, Arinzeh TL, Kerkis I. Response of stem cells from different origins to biphasic calcium phosphate bioceramics. *Cell Tissue Res* 2015; **361**(2): 477-95.

24. Calvo-Guirado JL, Ramirez-Fernandez MP, Delgado-Ruiz RA, Mate-Sanchez JE, Velasquez P, de Aza PN. Influence of biphasic beta-TCP with and without the use of collagen membranes on bone healing of surgically critical size defects. A radiological, histological, and histomorphometric study. *Clin Oral Implants Res* 2014; **25**(11): 1228-38.

25. Lim HC, Zhang ML, Lee JS, Jung UW, Choi SH. Effect of different hydroxyapatite:beta-tricalcium phosphate ratios on the osteoconductivity of biphasic calcium phosphate in the rabbit sinus model. *Int J Oral Maxillofac Implants* 2015; **30**(1): 65-72.

26. Ebrahimi M, Pripatnanont P, Suttapreyasri S, Monmaturapoj N. In vitro biocompatibility analysis of novel nano-biphasic calcium phosphate scaffolds in different composition ratios. *J Biomed Mater Res B Appl Biomater* 2014; **102**(1): 52-61.

27. Nevins M, Nevins ML, Schupbach P, Kim SW, Lin Z, Kim DM. A prospective, randomized controlled preclinical trial to evaluate different formulations of biphasic calcium phosphate in combination with a hydroxyapatite collagen membrane to reconstruct deficient alveolar ridges. *J Oral Implantol* 2013; **39**(2): 133-9.

28. Ebrahimi M, Pripatnanont P, Monmaturapoj N, Suttapreyasri S. Fabrication and characterization of novel nano hydroxyapatite/beta-tricalcium phosphate scaffolds in three different composition ratios. *J Biomed Mater Res A* 2012; **100**(9): 2260-8.

29. Jensen SS, Bornstein MM, Dard M, Bosshardt DD, Buser D. Comparative study of biphasic

calcium phosphates with different HA/TCP ratios in mandibular bone defects. A long-term histomorphometric study in minipigs. *J Biomed Mater Res B Appl Biomater* 2009; **90**(1): 171-81.

30. Friedmann A, Dard M, Kleber BM, Bernimoulin JP, Bosshardt DD. Ridge augmentation and maxillary sinus grafting with a biphasic calcium phosphate: histologic and histomorphometric observations. *Clin Oral Implants Res* 2009; **20**(7): 708-14.

31. LeGeros RZ, Lin S, Rohanizadeh R, Mijares D, LeGeros JP. Biphasic calcium phosphate bioceramics: preparation, properties and applications. *J Mater Sci Mater Med* 2003; **14**(3): 201-9.

32. Bouler JM, LeGeros RZ, Daculsi G. Biphasic calcium phosphates: influence of three synthesis parameters on the HA/beta-TCP ratio. *J Biomed Mater Res* 2000; **51**(4): 680-4.

33. Nery EB, LeGeros RZ, Lynch KL, Lee K. Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/beta TCP in periodontal osseous defects. *J Periodontol* 1992; **63**(9): 729-35.

34. Peterson B, Whang PG, Iglesias R, Wang JC, Lieberman JR. Osteoinductivity of commercially available demineralized bone matrix. Preparations in a spine fusion model. *J Bone Joint Surg Am* 2004; **86-A**(10): 2243-50.

35. Biswas D, Bible JE, Whang PH, et al. Augmented demineralized bone matrix: a potential alternative for posterolateral lumbar spinal fusion. *Am J Orthop (Belle Mead NJ)* 2010; **39**(11): 531-8.

36. Drespe IH, Polzhofer GK, Turner AS, Grauer JN. Animal models for spinal fusion. *Spine J* 2005; **5**(6 Suppl): 209S-16S.

37. Bostan B, Gunes T, Asci M, et al. Simvastatin improves spinal fusion in rats. *Acta Orthop Traumatol Turc* 2011; **45**(4): 270-5.

38. Mate-Sanchez de Val JE, Mazon P, Guirado JL, et al. Comparison of three hydroxyapatite/beta-tricalcium phosphate/collagen ceramic scaffolds: an in vivo study. *J Biomed Mater Res A* 2014; **102**(4): 1037-46.

39. Yun PY, Kim YK, Jeong KI, Park JC, Choi YJ. Influence of bone morphogenetic protein and proportion of hydroxyapatite on new bone formation in biphasic calcium phosphate graft: two pilot

studies in animal bony defect model. *J Craniomaxillofac Surg* 2014; **42**(8): 1909-17.

40. Eftekhari H, Farahpour MR, Rabiee SM. Histopathological evaluation of potential impact of β-tricalcium phosphate (HA+ β-TCP) granules on healing of segmental femur bone defect. *Bratislava Medical Journal* 2015; **116**(01): 30-4.

41. Grimes JS, Bocklage TJ, Pitcher JD. Collagen and biphasic calcium phosphate bone graft in large osseous defects. *Orthopedics* 2006; **29**(2): 145-8.

42. Muschler GF, Negami S, Hyodo A, Gaisser D, Easley K, Kambic H. Evaluation of collagen ceramic composite graft materials in a spinal fusion model. *Clin Orthop Relat Res* 1996; (328): 250-60.

43. Jegoux F, Goyenvalle E, Cognet R, et al. Mandibular segmental defect regenerated with macroporous biphasic calcium phosphate, collagen membrane, and bone marrow graft in dogs. *Arch Otolaryngol Head Neck Surg* 2010; **136**(10): 971-8.

ABSTRACT(in Korean)

인산화 칼슘과 콜라겐의 서로 다른 비율이 후방 요추 체간 유합술에 미치는 영향 ; 백서 후방 요추 유합 모델을 이용한 비교 연구

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김 경 현

서로 다른 비율의 HA β-TCP 와 Collagen으로 만들어진 세라믹은 생물학적으로 다른 영향을 미칠 것이며, 이를 비교 분석하여 가장 척추 유합에 적합한 비율의 물질을 찾는 것이 필요하다. 따라서 본 연구는 백서 후방 요추 체간 유합 모델을 이용하여 HA/β-TCP 의 서로 다른 비율과 Collagen이 척추 유합에 미치는 영향을 알아보고자 하였다. 위 실험을 위하여 총 50마리의 백서에서 요추 후방 유합술 모델을 만들어 이를 5 그룹으로 나누었다. 그룹 1은 자가골이식 이며, 그룹 2는 7:3 Ha/β-TCP ratio를 가진 그룹이며 그룹 3은 그룹 2에 Collagen만 첨가되었으며, 그룹 4는 3:7 HA/β-TCP Ratio를 가진 그룹이며 이에 collagen을 추가하여 그룹 5를 생성하였다. 이들을 수술 후 12주에 희생하여 도수검사(등급 0-4), 마이크로 컴퓨터단층촬영 (스코어 0-6점), 유합조직의 부피 그리고 병리학적인 분석을 진행하였다. 도수 검사 결과 그룹 C의 스코어가 그룹 E보다 통계적으로 의미 있게 높았으나 (3.1±1.1 vs. 1.8±0.8, p=0.033), 그룹 B와 D의 비교에서는 두 그룹간의 차이가 없었다 (3.2±1.3 vs. 2±1.9, p=0.164). 하지만 마이크로 컴퓨터단층촬영 분석 결과상 그룹 D가 B보다 통계적으로 의미 있는 우수하였으나

(5.5±0.8 vs. 3.1±1.1, p=0.021) 그룹 C과 E사이에서는 의미 있는 차이는 없었다 (4.8±1.1 vs. 5.4±0.8, p=0.073). 유합 조직의 양적인 분석에서 그룹 D와 E에서 그룹 A와 비교하여 통계적으로 유의하게 유합조직의 부피가 감소함을 알 수 있었다 (123 ± 14.2, 117 ± 46.3 vs. 151 ± 27.3, p=0.008, p=0.003). 콜라겐을 첨가한 그룹에서 마이크로 컴퓨터 단층촬영과 조직검사상에서 콜라겐을 첨가하지 않은 그룹에 비하여 우수한 결과를 보였다. 7:3 비율의 HA/β-TCP에 콜라겐을 첨가한 그룹이 3:7 비율의 HA/β-TCP 그룹에 비하여 후방 요추 유합술에서 더 적합한 이식재임을 알 수 있었다. 적절한 HA 비율이 안정적인 유합 구조물을 유지하는데 중요한 역할을 하며, 본 연구 결과상 콜라겐의 첨가는 후방 요추 유합술에서 일부 긍정적인 결과를 보였다. 콜라겐이 요추 유합의 긍정적인 영향을 미치는 기전을 분석하는 연구가 필요할 것으로 생각된다.

핵심 되는 말: 이상성 인산화 칼슘, 콜라겐, 백서, 요추 유합술, 수산화 인회석, 베타-인삼산 석회