J Periodontal Implant Sci. 2022 Aug;52(4):312-324
https://doi.org/10.5051/jpis.2104700235
pISSN 2093-2278·eISSN 2093-2286

JPIS
Journal of Periodontal & Implant Science

Research Article
Periodontal Science

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# The effect of hard-type crosslinked hyaluronic acid with particulate bone substitute on bone regeneration: positive or negative?

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**Received:** Sep 10, 2021
**Revised:** Nov 19, 2021
**Accepted:** Jan 24, 2022
**Published online:** Mar 7, 2022

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## ABSTRACT

**Purpose:** The role of hard-type crosslinked hyaluronic acid (HA) with particulate bone substitutes in bone regeneration for combined inlay-onlay grafts has not been fully investigated. We aimed to evaluate the effect of hard-type crosslinked HA used with bone substitute in terms of new bone formation and space maintenance.
**Methods:** A 15-mm-diameter round defect was formed in the calvaria of 30 New Zealand White rabbits. All animals were randomly assigned to 1 of 3 groups: the control group (spontaneous healing without material, n=10), the biphasic calcium phosphate (BCP) graft group (BCP, n=10), and the BCP graft with HA group (BCP/HA, n=10). The animals were evaluated 4 and 12 weeks after surgery. Half of the animals from each group were sacrificed at 4 and 12 weeks after surgery. Samples were evaluated using micro-computed tomography, histology, and histomorphometry.
**Results:** The BCP group showed higher bone volume/tissue volume (BV/TV) values than the control and BCP/HA groups at both 4 and 12 weeks. The BCP and BCP/HA groups showed higher bone surface/tissue volume (BS/TV) values than the control group at both 4 and 12 weeks. The BCP group showed higher BS/TV values than the control and BCP/HA groups at both 4 and 12 weeks. No statistically significant difference in newly formed bone was found among the 3 groups at 4 weeks. The BCP group showed significantly higher new bone formation than the BCP/HA group at 12 weeks.
**Conclusions:** Hard-type crosslinked HA did not show a positive effect on new bone formation and space maintenance. The negative effect of hard-type crosslinked HA may be due to the physical properties of HA that impede osteogenic potential.

**Keywords:** Bone regeneration; Bone substitute; Hyaluronic acid; Osteogenesis

## INTRODUCTION

Hard tissue regeneration is significant in a variety of operative disciplines, including orthopedic, maxillofacial, and dental surgery. Scaffolds made of various materials, such as autogenous, allogenic, or alloplastic, have been suggested to initiate and promote bone

**Funding**

This study was supported by grant No. 08-2019-0002 from the Seoul National University Dental Hospital (SNUDH) Research Fund.

**Conflict of Interest**

No potential conflict of interest relevant to this article was reported.

**Author Contributions**

Conceptualization: Jungwon Lee, Yong-Moo Lee; Data curation: Jungwon Lee; Formal analysis: Junseob Yun, Sungtae Kim, Ki-Tae Koo, Yang-Jo Seol, Yong-Moo Lee; Investigation: Junseob Yun; Methodology: Jungwon Lee, Yong-Moo Lee; Project administration: Jungwon Lee, Yong-Moo Lee; Writing - original draft: Junseob Yun, Jungwon Lee; Writing - review & editing: Sungtae Kim, Ki-Tae Koo, Yang-Jo Seol, Yong-Moo Lee.

ingrowth by altering the mechanism of wound healing. An ideal bone graft material (GM) should be equipped with a scaffold and biomolecules that promote osteoinductive and angiogenic effects along with biological safety, low patient morbidity, and high volumetric stability [1-3]. Moreover, the grafted scaffold should incorporate into the recipient bone defect without complications.

Hyaluronic acid (HA) is a natural carbohydrate found commonly in the human body [4]. As a component of the extracellular matrix, it enhances wound healing and tissue regeneration [5]. A positive effect of HA on bone regeneration has been reported in the literature. A previous study reported osteogenic effects of HA, including cell proliferation and differentiation [6]. It was demonstrated that the high molecular weight of HA might facilitate bone formation with angiogenesis and mesenchymal cell differentiation at an early stage. Several recent preclinical animal studies demonstrated that the application of HA may stimulate osteoinduction and improve new bone formation [7-9]. Further, HA has been shown to exhibit anti-inflammatory, bacteriostatic, and anti-adhesive properties, which are important for wound healing [10-12].

In addition to healing potency, the mechanical stiffness of the grafted area can affect the results of guided bone regeneration. To enhance the mechanical properties, HA has been modified using cross-linkers such as 1,4-butanediol diglycidyl ether (BDDE), glutaraldehyde, and divinyl sulfone, forming covalent cross-links between HA molecules [13,14]. Cross-linked HA has been applied as a root coverage adjuvant, showing promotion of wound healing and regeneration in gingival recession defects [15]. However, it has not been fully investigated whether hard-type HA with improved mechanical properties can promote bone regeneration when used with particulate bone substitutes.

To investigate bone regeneration with biomaterials, such as bone GMs, membranes, or growth factors, the rabbit calvarium defect model has been utilized in various experiments [16-18]. Studies conducted in rabbit calvaria have discovered that bone defects larger than 15 mm could be deemed critical-size defects [19,20]. Bone regeneration analysis should be performed in both favorable and unfavorable environments, such as contained and non-contained defects, to distinguish the effect of a biomaterial. In a previous study, inlay grafts in contained defects demonstrated enhanced bone regeneration by promoting angiogenesis and osteogenic cell migration from the recipient bed, which is in direct contact with the defect; however, onlay grafts in non-contained defects showed decreased bone regeneration because of reduced levels of cytokines and/or biomolecules far from the area that is directly affected [21].

This study aimed to investigate the effects of hard-type cross-linked HA used with particulate bone substitute as a combined inlay-onlay graft in a rabbit calvarium model.

## MATERIALS AND METHODS

### Animals

The Seoul National University's Institutional Animal Care and Use Committee authorized the research protocols (SNU-210405-4). For this study, 30 New Zealand White rabbits (Duyeol Biotech, Seoul, Korea) aged 9 to 20 months and weighing over 3 kg were used. They were separated into 3 groups of 10. **Figure 1** depicts the experimental flow chart. The guidelines of the Institute of Laboratory Animal Resources, Seoul National University were followed in


https://doi.org/10.5051/jpis.2104700235

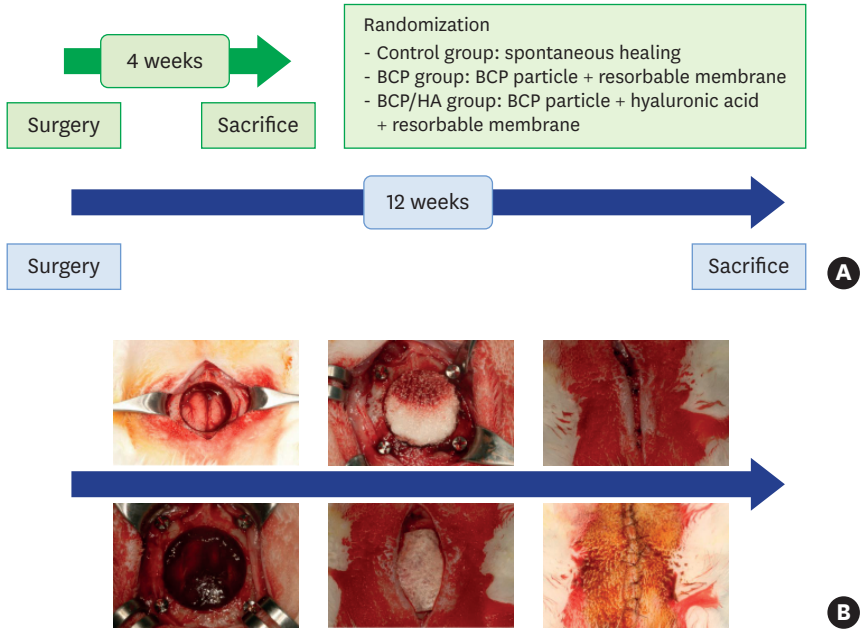


**Figure 1.** (A) Timeline of the study. (B) Surgical procedure.
BCP: biphasic calcium phosphate.

all experimental protocols. **Figure 1A** shows a flow chart of the experiment. The research was described according to the ARRIVE guidelines [22].

## Experimental design

The rabbits were randomly assigned to 1 of the 3 experimental groups:

(1) Control group: defect filled with blood clots without any materials and treatments (n=10).
(2) BCP group: biphasic calcium phosphate (BCP) (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genoss, Suwon, Korea) + absorbable membrane (Collagen Membrane-P, Genoss) (n=10).
(3) BCP/HA group: BCP (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genoss) + HA (Monalisa, hard type, approximately 600 μm granules, crosslinked with BDDE, Genoss) + absorbable membrane (Collagen Membrane-P, Genoss) (n=10).

## Surgical protocol

Intravenous anesthesia was used to anesthetize the New Zealand White rabbits. Zoletil (0.1 mg/kg, Virbac, Carros, France) and Rompun (2.3 mg/kg, Bater Korea, Ansan, Korea) were injected into a vein in the ear using a 24-gauge angiocatheter. The periosteum of the surgical site was subsequently injected with lidocaine (20 mg/kg, Huons, Sungnam, Korea).

Povidone-iodine was used to disinfect the operational site of the calvaria. An incision was made in the calvaria's midline, extending between the bi-pupillary line and the occipital process. A full-thickness flap was elevated with an elevator. A 15-mm diameter trephine bur with a 2-mm vertical stopper was used to create the calvarial defect, which included an interfrontal suture. Around the defect, 4 micro-fixing screws (4.0 mm in length, Dentium, Suwon, Korea) were inserted 2 mm higher than the surrounding calvaria.


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The experimental groups were randomly assigned after defect creation. Bone GMs were over-built by 2 mm above the superior border of the defect, up to the screw height. The GMs were covered with a cross-linked collagen membrane (Collagen Membrane-P, Genoss). The defect site was sutured using a 2-layered suture with 4/0 Vicryl and 5-0 Monosyn at the periosteum and skin, respectively.

Antibiotics (Metacam, Boehringer Ingelheim, Seoul, Korea) and analgesics (Cefazolin, Chong Kun Dang, Seoul, Korea) were injected intravenously after each group was disinfected with povidone-iodine.

Half of each group of rabbits was sacrificed 4 and 12 weeks following surgery, respectively, by intravenous potassium chloride injection (Jeil, Daegu, Korea).

## Micro-computed tomography analysis

The sample was analyzed by micro-computed tomography (SkyScan 1173, Bruker-CT) with a pixel size of 13.86 μm (130 kV, 60 μA). The volume of interest was the grafted area (**Figure 2A**). The horizontal margin was the rounded defect (diameter 15 mm) formed by the trephine bur. The vertical margin was 4 mm from the superior border of the dura mater to the superior margin of the GM. Bone tissue was applied as 52–250 grayscale values, and 8-bit grayscale values were used to analyze the defects.

In the micro-computed tomography analysis, software (CTAn, Bruker-CT) was used to analyze bone volume/tissue volume (BV/TV), bone surface/bone volume (BS/BV), and bone surface/tissue volume (BS/TV).

## Histological analysis

The histological preparation was performed with 10% buffered neutral formalin to fix the samples for 2 weeks. Subsequently, 5% formic acid was used for 10 days to demineralize the sample, and then all samples were embedded using paraffin. Tissue slides of 5-μm-thick

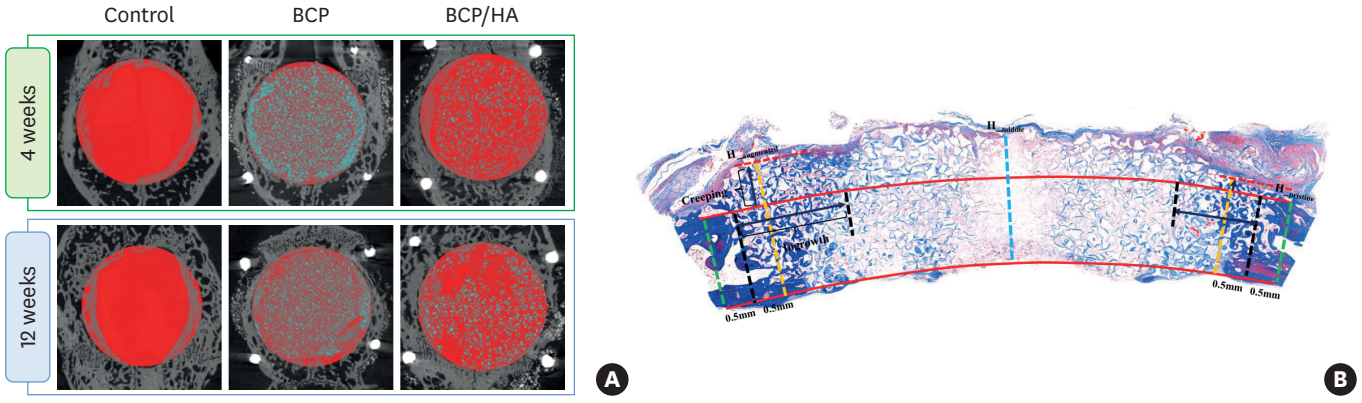


**Figure 2.** (A) Representative image of micro-computed tomography, including the control, BCP, and BCP/HA groups at 4 and 12 weeks after surgery. Red circles represent the cross section of the volume of interest. At 12 weeks, more new bone formation was observed than at 4 weeks. In the BCP/HA group, more space between particles is observed than in the other groups. This may have been caused by agglomerated undissolved hyaluronic acid. (B) Schematic diagram of the linear histomorphometric analysis. Green dotted line: $H_{\_pristine}$, yellow dotted line: $H_{\_augmented}$, blue dotted line: $H_{\_middle}$, vertical arrow: creeping distance, horizontal arrow: ingrowth distance.
BCP: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos, Suwon, Korea) + absorbable membrane (Collagen Membrane-P, Genoss), BCP/HA: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos) + hyaluronic acid (Monalisa, hard type, Genoss) + absorbable membrane (Collagen Membrane-P, Genoss), $H_{\_pristine}$: height of pristine bone, $H_{\_augmented}$: height of augmented bone, $H_{\_middle}$: height of bone in the midline.


https://doi.org/10.5051/jpis.2104700235

sections were stained with Masson trichrome. The sections were obtained from the center of the defect. Each slide was converted to a JPG file, and the region of interest (ROI) was applied using imaging software. **Figure 1B** shows the ROI and experimental parameters. Two imaginary horizontal lines were drawn on the upper and lower borders of the pristine cranial bone. Two imaginary vertical lines were drawn at the border of the defect. From these 4 imaginary lines, the following parameters were measured in the ROI:

- $H_{\_pristine}$: the vertical distance between the lower and upper pristine bone of the cranium.
- $H_{\_augmented}$: the vertical distance between the augmented border and the lower pristine bone line measured at the 0.5-mm medial site.
- $H_{\_middle}$: the vertical distance between the augmented border and the lower pristine bone line measured at the middle of the defect area.
- Creeping: the vertical distance between the maximum newly formed bone (NB) and upper pristine bone line measured at the 0.5-mm medial site.
- Ingrowth: the horizontal distance between the maximum NB and the vertical imaginary lines drawn at the border of the defect.

In addition, the area of NB, connective tissue (CT), and GM were analyzed using image measurement software (ImageJ Version 1.53a, National Institutes of Health, Bethesda, MD, USA). The schematic diagram of the linear histomorphometric analysis in the ROIs is shown in **Figure 2B**.

To investigate the space maintenance of augmented area at the defect margin and middle area of the defect, the $H_{\_augmented}/H_{\_pristine}$ and $H_{\_middle}/H_{\_pristine}$ ratios were measured. To investigate vertical bone formation capacity, the $Creeping/H_{\_pristine}$ ratio was measured.

### Statistical analysis

SPSS version 17 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The mean ± standard deviation was calculated for radiographic and histomorphometric measurements. The Mann-Whitney and Kruskal-Wallis tests were used for comparing 2 and 3 groups, respectively. Statistically significant differences were decided at a level of $P<0.05$. When the Kruskal-Wallis test showed statistically significant differences, the Mann-Whitney test (0.05/3=0.017) was used to perform Bonferroni multiple comparisons.

The sample size was calculated using G*Power software (version 3.1, Heinrich Heine University, Dusseldorf, Germany) based on a previous study [20]. The effect size was set at 1.25, and the type I and type II error probabilities were set at 0.05 and 0.20, respectively. Four samples were assigned per group at each healing period observation, and 10 animals were employed from each group for 4 and 12 weeks of healing period observations, applying a 25% rate of loss.

## RESULTS

### Clinical observation

Of a total of 30 animals, 1 in the control group scheduled for the 4-week observation period died before the scheduled euthanized date. Another animal in the control group scheduled for the 12-week observation period was sacrificed before the scheduled euthanized date because of blunt movements and poor feeding, which were presumed to be a complication from the operation. The remaining 28 animals showed no pathologic events, such as swelling, redness, or pus discharge. Finally, 4 animals in the control group, 5 in the BCP


https://doi.org/10.5051/jpis.2104700235

**Table 1.** Results of micro-computed tomography for the 3 groups

| Time | Group | n | BV/TV | BS/BV | BS/TV |
|---|---|---|---|---|---|
| 4 wk | Control | 4 | 7.37±1.95[a)] | 19.52±2.60[a)] | 1.43±0.34[a)] |
| | BCP | 5 | 17.30±1.71[b)] | 60.27±3.84[b)] | 10.41±0.98[b)] |
| | BCP/HA | 5 | 7.22±0.43[a)] | 72.56±14.59[b)] | 5.20±0.82[c)] |
| | *P* value | | 0.011 | 0.011 | 0.003 |
| 12 wk | Control | 4 | 9.45±0.59[a)] | 16.21±1.13[a)] | 1.53±0.08[a)] |
| | BCP | 5 | 15.48±3.13[b)] | 61.02±11.12[b)] | 9.18±0.95[b)] |
| | BCP/HA | 5 | 7.63±1.98[a)] | 68.39±15.20[b)] | 5.38±2.27[c)] |
| | *P* value | | 0.008 | 0.017 | 0.004 |

BCP: biphasic calcium phosphate (hydroxyapatite 60% + β- tricalcium phosphate 40%, Osteon III, Genos, Suwon, Korea) + absorbable membrane (Collagen Membrane-P, Genoss), BCP/HA: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos) + hyaluronic acid (Monalisa, hard type, Genoss) + absorbable membrane (Collagen Membrane-P, Genoss), BV/TV: bone volume/tissue volume, BS/BV: bone surface/bone volume, BS/TV: bone surface/tissue volume.
Different letters, a, b and c indicate statistical differences under the Bonferroni correction.

group, and 5 in the BCP/HA group were analyzed at 4 weeks, and 4 in the control group, 5 in the BCP group, and 5 in the BCP/HA group were analyzed at 12 weeks.

## Micro-computed tomography analysis

*At 4 weeks*

The BCP group showed higher BV/TV values than the control and BCP/HA groups. The BCP and BCP/HA groups showed higher BS/BV values than the control group. The BCP group showed higher BS/TV values than the control and BCP/HA groups (**Table 1**).

*At 12 weeks*

The BCP group showed higher BV/TV values than the control and BCP/HA groups. The BCP and BCP/HA groups showed significantly higher BS/BV values than the control group. The BCP group showed higher BS/TV values than the control and BCP/HA groups (**Table 1**).

## Histological analysis

Overall, NB was observed at the margin of the defect area. Clinical and histological inflammatory reactions were not observed. Undissolved HA was observed in the grafted area in the BCP/HA group (**Figure 3**), and the agglomerated form of undissolved HA was observed in some slides (**Appendix 1**).

## Histomorphometric analysis

*At 4 weeks*

The BCP group and BCP/HA group showed significantly higher $H_{\_augmented}$ and $H_{\_middle}$ values than the control group. Creeping was significantly higher in the BCP group than in the control group, but the BCP/HA group showed no statistically significant differences. The control and BCP groups showed significantly higher values of ingrowth than the BCP/HA group (**Table 2**).

The BCP and BCP/HA groups showed significantly higher $H_{\_augmented}/H_{\_pristine}$ and $H_{\_middle}/H_{\_pristine}$ values than the control group. Higher creeping/$H_{\_pristine}$ values were observed in the BCP group than in the control group; however, they were not significantly different from those in the BCP/HA group (**Table 2**).

There was no statistically significant difference in NB among the 3 groups. The control and BCP groups showed significantly higher values for CT than the BCP/HA group. The BCP


https://doi.org/10.5051/jpis.2104700235

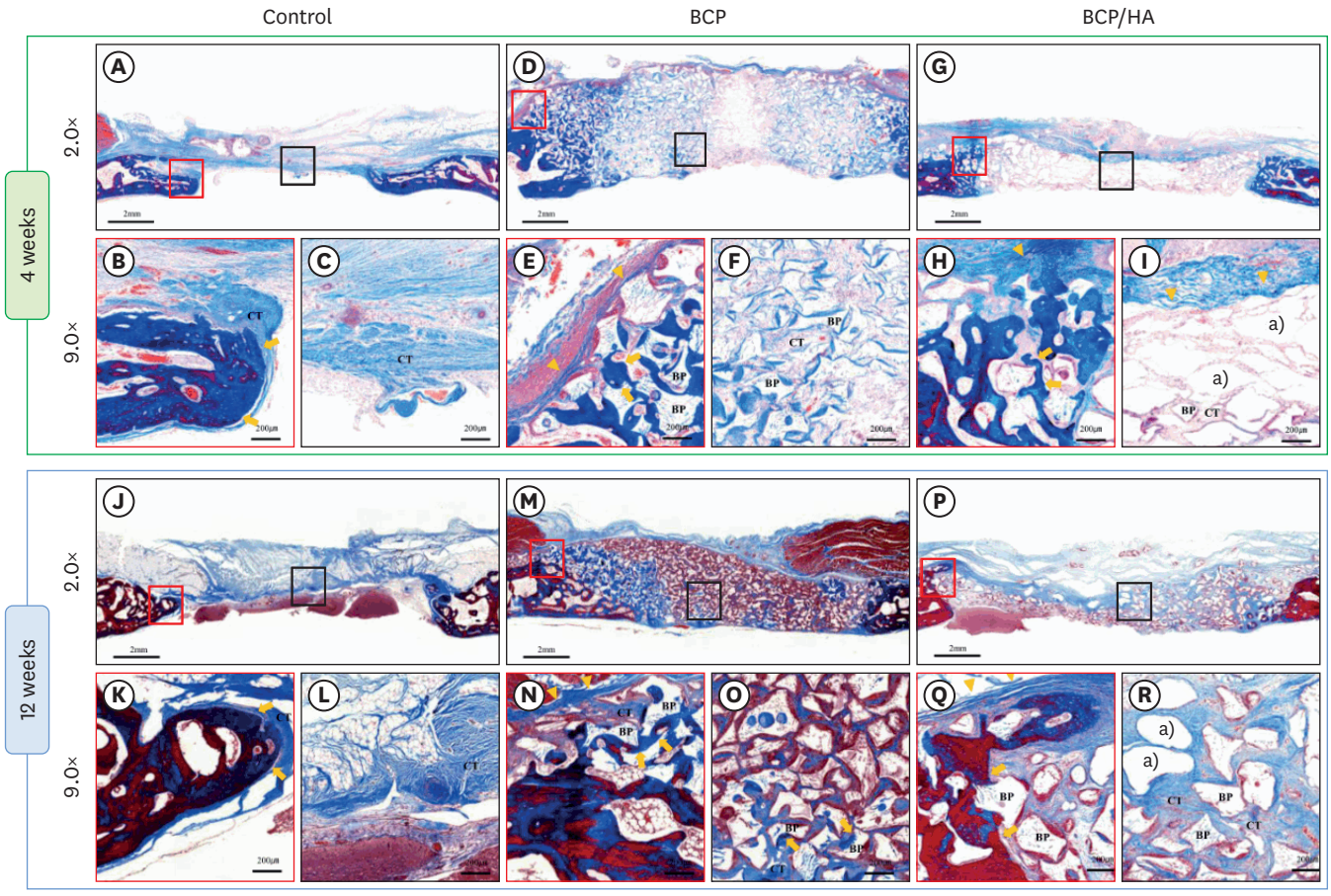


**Figure 3.** Representative microscopic views. Masson trichome staining was used 4 and 12 weeks after surgery. (A-C) The control group at 4 weeks with original magnification of 2.0×, 9.0×, and 9.0×. New bone formation (arrow) is observed in the defect margin. (D-F) The BCP group at 4 weeks with original magnifications of 2.0×, 9.0×, and 9.0×. New bone formation (arrow) is observed above the superior border of the defect, between the bone particles, and under the membrane (arrowhead). In the middle area, bone particles are surrounded by connective tissue. (G-H) The BCP/HA group at 4 weeks with original magnifications of 2.0×, 9.0×, and 9.0×. Undissolved HA agglomerate is observed in the middle area of the defect. (J-L) Control group at 12 weeks with original magnifications of 2.0×, 9.0×, and 9.0×. (M-O) The BCP group at 12 weeks with original magnifications of 2.0×, 9.0×, and 9.0×. New bone formation was observed in the middle of the defect, between the bone particles. (P-O) The BCP/HA group at 12 weeks with original magnifications of 2.0×, 9.0×, and 9.0×. Arrow: new bone; Arrowhead: membrane.
BP: bone particle, CT: connective tissue, BCP: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos, Suwon, Korea) + absorbable membrane (Collagen Membrane-P, Genoss), BCP/HA: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos) + hyaluronic acid (Monalisa, hard type, Genoss) + absorbable membrane (Collagen Membrane-P, Genoss).
[a]Undissolved HA.

group showed significantly higher GM than the BCP/HA group (**Table 3**). The percentage of undissolved HA in the BCP/HA group was 6.01%±3.96% (**Table 3**).

*At 12 weeks*

$H_{\_augmented}$ was significantly higher in the BCP and BCP/HA groups than in the control group. $H_{\_middle}$ was significantly higher in the BCP and BCP/HA groups than in the control group. Creeping was the highest in the BCP group, with statistically significant differences from the control and BCP/HA groups. The control and BCP groups showed significantly higher ingrowth than the BCP/HA group (**Table 2**).

The BCP group showed a significantly higher value of $H_{\_augmented}/H_{\_pristine}$ than the control and BCP/HA groups. The BCP group showed a significantly higher value of $H_{\_middle}/H_{\_pristine}$ than the control group; however, no significant difference was observed relative to the BCP/


https://doi.org/10.5051/jpis.2104700235

**Table 2.** Linear analysis for the 3 groups at 4 and 12 weeks

| Variable | Group | | | *P* value |
|---|---|---|---|---|
| | Control | BCP | BCP/HA | |
| 4 wk | | | | |
| Length (μm) | | | | |
| $H_{-pristine}$ | 1,571.21±345.65 | 1,742.63±186.42 | 1,686.55±234.26 | 0.553 |
| $H_{-augmented}$ | 993.99±257.03[a] | 2,867.33±421.42[b] | 2,385.71±213.81[b] | 0.008 |
| $H_{-middle}$ | 88.93±173.85[a] | 2,437.18±967.41[b] | 1,588.02±712.53[b] | 0.009 |
| Creeping | 0.00±0.00[a] | 520.42±111.53[b] | 233.93±222.20[a,b] | 0.009 |
| Ingrowth | 2,438.95±746.33[a] | 2,411.07±993.56[a] | 545.60±424.56[b] | 0.011 |
| Ratio | | | | |
| $H_{-augmented}/H_{-pristine}$ | 0.63±0.08[a] | 1.64±0.13[b] | 1.44±0.28[b] | 0.013 |
| $H_{-middle}/H_{-pristine}$ | 0.06±0.12[a] | 1.38±0.49[b] | 0.95±0.51[b] | 0.014 |
| Creeping/$H_{-pristine}$ | 0.00±0.00[a] | 0.30±0.08[b] | 0.14±0.14[a,b] | 0.012 |
| 12 wk | | | | |
| Length (μm) | | | | |
| $H_{-pristine}$ | 2,048.86±393.29 | 1,852.83±211.22 | 2,018.00±161.60 | 0.448 |
| $H_{-augmented}$ | 1,288.43±302.56[a] | 2,581.19±217.06[b] | 1,690.47±410.85[a] | 0.007 |
| $H_{-middle}$ | 401.95±290.51[a] | 2,391.14±669.56[b] | 1,640.98±484.67[b] | 0.010 |
| Creeping | 0.00±0.00[a] | 566.95±246.21[b] | 72.58±104.74[a] | 0.005 |
| Ingrowth | 3,026.41±644.26[a] | 4,101.20±993.65[a] | 1,170.74±518.33[b] | 0.006 |
| Ratio | | | | |
| $H_{-augmented}/H_{-pristine}$ | 0.63±0.06[a] | 1.41±0.22[b] | 0.84±0.20[a] | 0.006 |
| $H_{-middle}/H_{-pristine}$ | 0.22±0.19[a] | 1.31±0.41[b] | 0.81±0.22[a,b] | 0.011 |
| Creeping/$H_{-pristine}$ | 0.00±0.00[a] | 0.32±0.18[b] | 0.03±0.05[a] | 0.005 |

$H_{-pristine}$: height of pristine bone, $H_{-augmented}$: height of augmented bone, $H_{-middle}$: height of bone in the midline, BCP: biphasic calcium phosphate (hydroxyapatite 60% + β- tricalcium phosphate 40%, Osteon III, Genos, Suwon, Korea) + absorbable membrane (Collagen Membrane-P, Genoss), BCP/HA: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos) + hyaluronic acid (Monalisa, hard type, Genoss) + absorbable membrane (Collagen Membrane-P, Genoss).
Different letters, a and b indicate statistical differences under the Bonferroni correction.

**Table 3.** Histomorphometric results for the 3 groups at 4 and 12 weeks

| Variable | Group | | | *P* value |
|---|---|---|---|---|
| | Control | BCP | BCP/HA | |
| 4 wk | | | | |
| Area ($mm^2$) | | | | |
| NB | 4.35±1.27 | 5.55±2.10 | 4.45±1.25 | 0.566 |
| CT | 12.95±3.89[a] | 10.75±3.99[a] | 7.17±0.83[b] | 0.036 |
| GM | - | 22.35±4.62[a] | 14.22±6.90[b] | 0.005 |
| uHA | 0.00±0.00[a] | 0.00±0.00[a] | 6.01±3.96[b] | 0.002 |
| Total | 17.30±3.82[a] | 38.66±9.27[b] | 31.85±7.30[b] | 0.013 |
| 12 wk | | | | |
| Area ($mm^2$) | | | | |
| NB | 5.69±2.07[a,b] | 10.50±2.88[b] | 4.28±0.97[a] | 0.017 |
| CT | 14.10±1.39[a] | 11.65±1.08[b] | 9.29±2.89[b] | 0.015 |
| GM | - | 15.37±4.55 | 11.99±3.79 | 0.222 |
| uHA | 0.00±0.00[a] | 0.00±0.00[a] | 7.82±12.41[b] | 0.011 |
| Total | 26.53±2.41 | 37.52±5.60 | 32.77±13.62 | 0.087 |

BCP: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos, Suwon, Korea) + absorbable membrane (Collagen Membrane-P, Genoss), BCP/HA: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos) + hyaluronic acid (Monalisa, hard type, Genoss) + absorbable membrane (Collagen Membrane-P, Genoss), NB: newly formed bone, CT: connective tissue, GM: graft material, Total: total area of regenerated or augmented tissue in control and test groups, uHA: undissolved hyaluronic acid.

HA group. The creeping/$H_{-pristine}$ ratio was the highest in the BCP group, with statistically significant differences from the ratios in the other 2 groups (**Table 2**).

The BCP group showed significantly higher NB formation than the BCP/HA group; however, it did not show a significant difference from the control group in this regard. The control


https://doi.org/10.5051/jpis.2104700235

group showed a higher CT value than the BCP and BCP/HA groups. No significant difference was observed in GM between the BCP and the BCP/HA groups. The percentage of undissolved HA in the BCP/HA group was 7.82%±12.41% (**Table 3**).

## DISCUSSION

In this study, we investigated whether new bone formation and volume maintenance improved when cross-linked HA was applied for combined inlay-onlay bone grafts. To improve space maintenance in non-contained defects, cross-linked HA with high viscoelasticity was used, and the radiological and histological results of bone formation were observed. However, HA did not show a positive effect on new bone formation. The cross-linked HA with high viscoelasticity has 2 disadvantages in the guided bone regeneration procedure: 1) it is difficult to mix uniformly and remains in the agglomerated undissolved form, and 2) it is difficult to place bone particles as compactly as desired.

This study employed a hard-type HA with high viscoelasticity crosslinked with BDDE, which is a biocompatible crosslinking agent [23]. A recent study demonstrated that crosslinked HA with deproteinized bovine graft showed increased bone formation, which occurred more with high-viscosity HA than with low-viscosity HA [24]. In our study, a hard-type HA with high viscoelasticity was investigated, with the expectation that it would show enhanced space maintenance capacity. However, contrary to the expectations, the hard-type HA with extremely increased viscosity showed a negative effect on bone formation. An explanation for this result might be that the hard-type HA did not blend well with the bone GM. As histologic evidence, this study confirmed that the HA was agglomerated in a substantial proportion of the augmented area (**Appendix 1**). The ideal viscosity and elasticity of HA combined with the particle type of bone substitutes that would best promote new bone formation should be investigated in future studies.

The BCP/HA group showed significantly lower BV/TV values than were observed in the BCP group at 4 and 12 weeks (**Table 1**). Since the HA used in the BCP/HA group occupied a large amount of space, a relatively small portion of BV may have been located. Although a screw and membrane were used in this study, graft substitutes were observed outside the defect area on micro-computed tomography, demonstrating a limitation in guided bone regeneration using a particle-type bone substitute. A block-type bone substitute may be beneficial for vertical bone formation, which is advantageous for space maintenance in the process of new bone formation [25,26].

In order to investigate the osteogenic effect of HA, an unfavorable environment was created with an onlay graft model in this study. $H_{\_augmented}$ and $H_{\_augmented}/H_{\_pristine}$ showed similar values in the BCP and BCP/HA groups at 4 weeks, but significantly decreased in the BCP/HA group at 12 weeks (**Table 2**). $H_{\_middle}$ and $H_{\_middle}/H_{\_pristine}$ showed significantly lower values in the BCP/HA group than in the BCP group at 4 and 12 weeks of observation (**Table 2**). The BCP/HA group showed a disadvantage in terms of volume maintenance in an onlay graft. When performing guided bone regeneration, a compressive force can be applied to achieve a desirable bony contour. Furthermore, a previous study demonstrated that guided bone regeneration using a high compressive force can make a better bony contour and fewer voids in the augmented area [27]. The hard-type HA used in this study made it difficult to blend evenly with the bone substitute and fill in the defect due to the high viscoelasticity. As a result, insufficient bone substitute was present in the defect area and bone formation was reduced.


https://doi.org/10.5051/jpis.2104700235

This healing pattern was shown most conspicuously in the middle part of the defect due to the lack of volume maintenance using the bone substitute and far from the tenting screw placed near the defect margin. This can be confirmed through the fact that the BCP/HA group showed significantly lower values of $H_{\_middle}$ and $H_{\_middle}/H_{\_pristine}$ than the BCP group at both 4 and 12 weeks. The collapse of space maintenance may have diminished new bone formation. Creeping, the Creeping/$H_{\_pristine}$ ratio, and NB in the BCP group showed significantly higher values than those in the BCP/HA group at 12 weeks (**Tables 2** and **3**).

New bone formation tends to occur toward the center from the defect margin of the pristine bone. This ingrowth was observed in all groups; however, the BCP group showed significantly higher values than those of the other groups (**Table 2**). A previous study demonstrated that improved new bone formation was observed when deproteinized bovine bone mineral with 10% collagen soaked with HA was applied to compromised extraction sockets [28]. The results of the previous study are not consistent with those of this study, which showed reduced new bone formation in the BCP/HA group.

The possible reasons for low bone formation in BCP/HA are cytotoxicity of crosslinkers or the mechanical barrier effect of HA with extremely high viscoelasticity. Cytotoxicity of crosslinking agents used in the fabrication of crosslinked collagen membranes has been reported in the previous literature [29,30]. The biocompatibility and non-cytotoxicity of the crosslinker used in HA crosslinking should be ensured. A previous review demonstrated that BDDE, which was used as the HA crosslinker in this study, had no evidence of cytotoxicity, skin irritation, systemic toxicity, or genotoxicity [23]. Various data spanning more than several years support the favorable biologic safety profile of BDDE-crosslinked HA and its degradation product [23]. The evidence appears to be weak that the reduced bone formation in BCP/HA would be due to the cytotoxic effect of the crosslinker in HA. However, the extremely high viscoelasticity of HA, which did not mix with the bone substitutes, can act as a barrier to prevent the arrival of progenitor cells and cytokines for osteogenesis. The high ratio of undissolved HA and agglomerated HA in the BCP/HA group furnishes evidence of the mechanical barrier effect, resulting in a negative effect on new bone formation. Improved space maintenance using a 3D-printed scaffold might improve bone formation compared to the particle type of bone substitute. In addition, a less viscoelastic HA formulation might result in enhanced bone formation by mixing with bone substitute well. Further studies analyzing the effects of improvements in mechanical scaffolds or low-viscoelasticity HA on bone formation are needed to identify the reasons for slow bone formation in the BCP/HA group in this study.

This study has limitations related to the defect configuration and observation period. In this study, a circular contained defect was used. However, most bone defects in clinical situation have more complex configurations. Further research is needed on the effects of various defect configurations. In addition, the defect used in this study was an inlay-onlay combined defect. Because the potency for bone regeneration can be affected by the bone configuration, it will be necessary to examine the effect of HA in inlay or onlay defect models, respectively. The bone healing period was up to 3 months in this study, which is a somewhat short period to observe bone regeneration. Long term observation up to 6 months after the bone graft is needed in the future.

In conclusion, hard-type HA did not show a positive effect on new bone formation and space maintenance. The negative effects of hard-type HA may be due to the physical properties of HA that impede osteogenic potential.


https://doi.org/10.5051/jpis.2104700235

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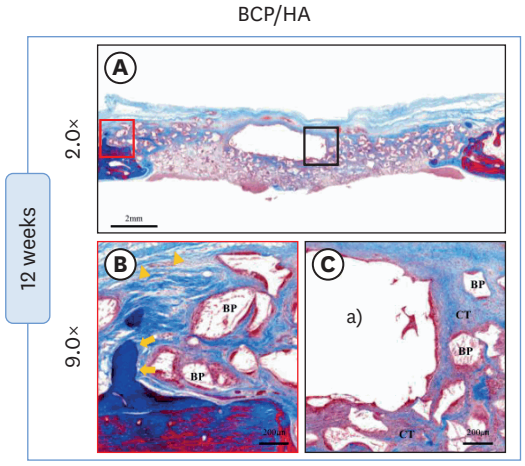


**Appendix 1.** Representative microscopic view with the agglomerated form of HA. Masson trichome staining was used 12 weeks after surgery. (A-C) The BCP/HA group at 12 weeks with original magnifications of 2.0×, 9.0×, and 9.0×. Undissolved HA agglomerated and functioned as a barrier obstructing new bone formation. Arrow: new bone; Arrowhead: membrane.
HA: hyaluronic acid, BP: bone particle, CT: connective tissue, BCP/HA: biphasic calcium phosphate (hydroxyapatite 60% + β-tricalcium phosphate 40%, Osteon III, Genos, Suwon, Korea) + hyaluronic acid (Monalisa, hard type, Genoss) + absorbable membrane (Collagen Membrane-P, Genoss).
[a]Undissolved HA.


https://doi.org/10.5051/jpis.2104700235