

## Enhanced bone regeneration by *Aloe vera* gel conjugated barnacle cement protein composite hyaluronic acid hydrogel based hydroxyapatite derived from cuttlefish bone

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### Abstract

Injectable *Aloe vera* gel W (AGW®)-conjugated Barnacle Cement Protein (BCP) composite Hyaluronic Acid (HA) hydrogels provide local periodontal tissue for bone filling in periodontal surgery. We developed a novel type of injectable self-supported hydrogel (2 mg/ml of AGW®-BCP/HA) based cuttlefish bone derived hydroxyapatite (CBH) for dental graft, which could good handling property, biodegradation or biocompatibility with the hydrogel disassembly and provided efficient cell adhesion activity and no inflammatory responses. Herein, the aim of this work was to evaluate bone formation following implantation of CBH and collagen membrane in rabbit calvarias defects. Eight male New Zealand rabbits were used and four circular calvarias defects were created on each animal. Defects were filled with different graft materials: 1) collagen membrane, 2) collagen membrane with CBH, 3) collagen membrane with bovine bone hydroxyapatite (BBH), and 4) control. The animals were sacrificed after 2 and 8 weeks of healing periods for histologic analysis. Both sites receiving CBH and BBH showed statistically increased augmented volume and new bone formation ( $p < 0.05$ ). However, there was no statistical difference in new bone formation between the CBH, BBH and collagen membrane group at all healing periods. Within the limits of this study, collagen membrane with CBH was an effective material for bone formation and space maintaining in rabbit calvarias defects.

**Keywords:** Bone Regeneration, *Aloe vera* Gel, Barnacle Cement Protein, Hyaluronic Acid, Hydrogel, Cuttlefish Bone, Hydroxyapatite, Collagen Membrane, Calvarias

### Introduction

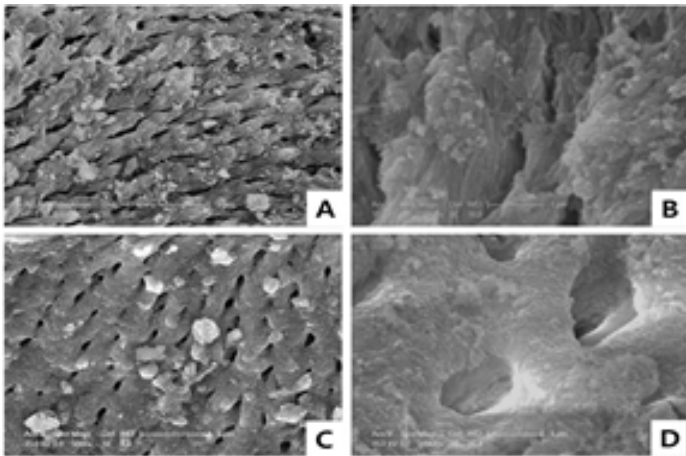
To replace bones with insufficient bone mass and osseous tissue, various graft materials and membranes such as autografts, allografts, xenografts, and synthetic bone grafts have been researched and successfully applied to bone regeneration and periodontal tissue repair [1-5].

Although autografts are acknowledged for being osteogenic while less immunogenic, they have limitations such as restricted donor site availability, difficulty of obtaining adequate amounts of bone, risk of infection, and a rapid resorption tendency [5-7]. To avoid or minimize the complications related to autografts, researchers have studied allografts such as Freeze-dried bone allografts (FDBA) and Demineralized freeze-dried bone allografts (DFDBA), xenografts such as bovine bone and porcine bone, and synthetic bone grafts such as calcium sulfate, hydroxyapatite (HA), and bioactive glasses. Contrary to autografts, allografts allow adequate amounts of bone to be easily obtained without a donor site. However, they carry risks of rejection by the immune system and increased morbidity from cross infection [8]. Synthetic bone grafts

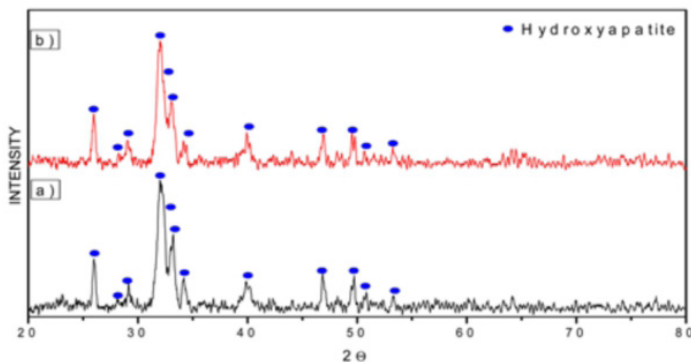
are less immunogenic, have no risks of transmissible diseases, and provide adequate amounts of bone at a low cost [9]. Despite many efforts to develop synthetic bone graft materials that are similar in structure to actual osseous tissue, current synthetic bone graft materials have low osteogenic abilities [9,10].

Xenografts are grafts obtained from animals and like allografts, allow adequate amounts to be easily obtained, but are limited as the risks of transmission of zoonotic diseases cannot be eliminated. The best known xenograft, demineralized bovine bone mineral (Bio-oss®, Geistlich-Pharma, Wolhusen, Switzerland) has a porous structure similar to human osseous tissue as well as a long resorption time, serving as an ideal scaffold for osteogenesis [11,12]. However, bovine bone carries the danger of transmitting bovine spongiform encephalopathy (BSE) which causes Creutzfeldt-Jakob Disease, and the transfer of the abnormal prion cannot be blocked from the graft material [13]. Cuttlefish bone is being considered as an alternative to bovine bone [14-18]. Like demineralized bovine bone, demineralized cuttlefish bone has a high porosity, with particles sized 0.25-1.0 mm in

average. X Ray Diffraction (XRD) results also show that the grafts share the same structure of hydroxyapatite (Figure 1 and 2) [15]. Demineralized cuttlefish bone has a long resorption tendency of over five months, and thus is capable of performing the functions of a scaffold for osteoconduction in the same manner as bovine bone [18]. Furthermore, cuttlefish bone is more similar to human osseous tissue than synthetic HA is in chemical and histological structure [14]. Thus it has a higher biocompatibility, better fusion on the transplant site, and a high bond strength, and is being used as an effective graft material for bone regeneration [16,19,20]. In clinical trials, promoting bone regeneration by covering the top of the graft materials with a membrane brings better results than using the graft materials alone. In addition to the advantages of biocompatible resorbable collagen membrane, the cross-linked resorbable collagen membrane has excellent abilities in space formation and maintenance, making it ideal for applications to defects [22,23].



**Figure 1:** SEM images of BBH (A,B) and CBH (C,D). (A,C, X 5000; B,D, X 20000).



**Figure 2:** XRD charts of CBH (a) and BBH (b).

Barnacle cement proteins have potential as environmentally friendly adhesives for use under aqueous conditions and may be of particular value in medical applications. During the last two decades, many efforts have been tried to develop adhesives from marine barnacles. However, practical applications of Dopa-containing barnacle cement proteins have been severely limited by uneconomical extraction and unsuccessful large-scale production

[31]. Availability of large quantities of recombinant barnacle cement proteins will enable to develop practical cement for diverse applications. Recent developed new hybrid types of barnacle inspired cement proteins might enable to realize this dream. Therefore, the researchers need to use several cement proteins simultaneously to develop cement materials with more practical and better properties, and based on these developed cements, they should find novel biological applications including gene and drug delivery, anti-biofouling coatings, medical device coatings, and surgical sealants [32-34].

Hyaluronic Acid (HA) is the most important hydrophilic carrier material used for oral controlled drug delivery systems and dental devices [37,38]. Handling properties, mechanical strength, hardening times, and swell ability were greatly improved by the addition of HA for them. Upon contact with water or biological fluid the latter diffuses into the device, resulting in polymer chain relaxation with volume expansion [39]. For some clinical indications, injectable biomaterials are preferable over macro porous blocks or particles of bone grafts [40,41]. Injectable bone grafts are convenient for filling complex shaped bone defects using a minimally invasive approach. To date, several injectable bone grafts have been developed.

We recently developed a biocompatible injectable bone binder which AGW®-conjugated barnacle cement protein (AGW®-BCP) composite HA hydrogel based cuttlefish bone hydroxyapatite provide local periodontal tissue for bone filling in periodontal surgery. And we developed a novel type of injectable self-supported hydrogel (2 mg/ml of AGW®-BCP/HA) based cuttlefish bone hydroxyapatite (CBH) for dental graft binder, which could good handling property, biodegradation or biocompatibility with the hydrogel disassembly and provided efficient cell adhesion activity and no inflammatory responses. From this results, in vitro cytotoxicity indicated no cell cytotoxicity was observed when the gel strength of CBH was up to 700 g bloom, and also no cytotoxic effects were observed [42-44].

The aim of this study was to evaluate bone regeneration following implantation of *Aloe vera* gel conjugated cuttlefish cancellous bone substitute and collagen membrane in rabbit calvarial defects. To achieve this purpose, cuttlefish bone hydroxyapatite (CBH) and bovine bone hydroxyapatite (BBH) covered by cross-linked resorbable collagen membrane were transplanted into calvarias defects in rabbits. Osteogenetic effects were comparatively analyzed histologically and histomorphometrically two weeks and eight weeks after transplantation.

## Materials and Methods

### Animals

Eight male New Zealand white rabbits (aged 9-20 months, weight 3.0-3.5 kg) were used in this study. The animal selection, management, and surgical protocol were approved by the Clinical Medicine Research Center at Jeju National University College of Medicine (Approval number: #2021-0046)

## Materials

The graft materials used in this experiment included 0.25-1.0 mm particles of AGW-BCP® hydrogel based cuttlefish bone hydroxyapatite (CBH, KJMBio, Co., Ltd, Seoul, Korea) with a total porosity of 72.4% and 0.25-1.0 mm particles of bovine bone hydroxyapatite (BBH, Bio-Oss®, Geistlich-Pharma, Wolhusen, Switzerland) with a total porosity of 63.5%. Cross-linked resorbable collagen membrane (EZ Cure®, Biomatlante, Co., Ltd, Vigneux de Bretagne, France) was used as the membrane.

## Experimental group set-up

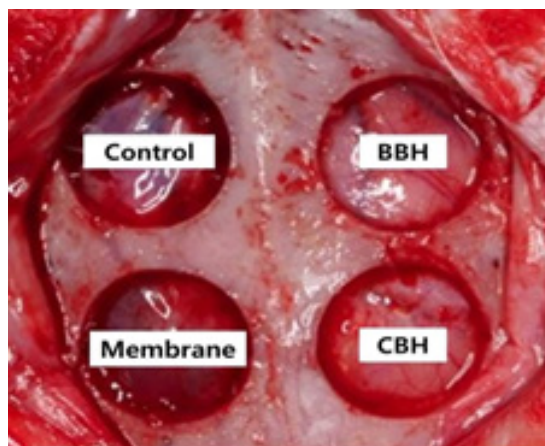
Four circular defects, each with a diameter of 8 mm, were prepared on the calvarias of each rabbit, and were categorized as shown below:

1. Control group: The bone defect was induced to be filled with blood clots.
2. Collagen membrane group: The bone defect was covered with a membrane to induce blood clots inside.
3. BBH transplant group, collagen membrane: BBH was transplanted in the bone defect and covered with a collagen membrane
4. CBH transplant group, collagen membrane: CBH was transplanted in the bone defect and covered with a collagen membrane.

## Surgical Procedure

All rabbits were put under general anesthesia with Ketamine hydrochloride (Ketalar®, Yuhan, Co., Seoul, Korea) and xylazine (Rumpun®, Bayer Korea Ltd., Seoul, Korea). The calvarias surgical site was depilated then disinfected with povidone iodine given infiltration anesthesia with 2% lidocaine (Lidocaine HCl, Huons Ltd, Seoul, Korea). The frontal bone was incised from the front portion to the back following the sagittal suture, and the calvarias was exposed by lifting the valves towards the periosteum. Using trephine bur with an external diameter of 8 mm, four circular defects, each with a diameter of 8 mm were made (Figure 3). The materials corresponding to the aforementioned experimental groups were inserted into the defects. The periosteum was sutured with 5-0 Vicryl® (Ethicon, Somerville, NJ, USA) and the scalp was sutured with 4-0 Monosyn® (B-Braun, Melsungen, Germany). During the week after the surgery, the antibiotic Gentamicin (5 mg/kg) was injected into the muscles, and the animals were released after one week. Two weeks and eight weeks after surgery, the

animals were sacrificed by injecting Phenobarbital (100 mg/kg) into their veins and their tissues were obtained.



**Figure 3:** Schematic diagram showing the histometric analysis.

## Evaluation Method

### 1. Clinical observations

The defect sites were checked for inflammation views, leakage of the graft material, significant changes, and complications two weeks and eight weeks after the surgery.

### 2. Histological observation

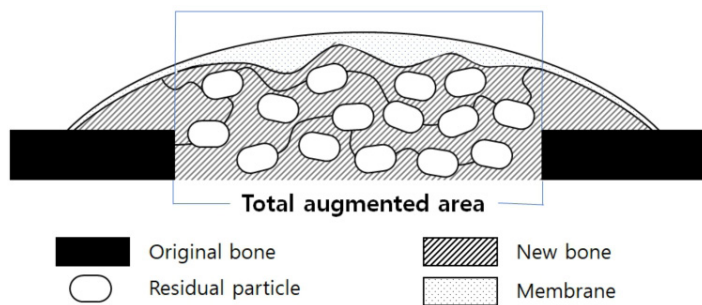
Samples of cranial tissue were fixed in 10% formalin for ten days, demineralized in 5% nitric acid for five days, and then embedded in paraffin. These were cut into four pieces, each with a width of 7  $\mu$ m, and dyed with hematoxylineosin (H&E) to be observed under an optical microscope at magnifications of X 40 and X 100.

### 3. Histomorphometric observations

The following were measured and calculated using an automatic image analysis software (Image-Pro Plus, Media cyber- netics, Silver Spring, Maryland, USA) (Figure 4).

1. Total augmented area ( $\text{mm}^2$ ): The total area covered by new bone, new connective tissue, residual graft material, adipose tissue, and blood vessels on the defect site,
2. New bone ( $\text{mm}^2$ ): The area of new bone on the defect site.
3. Residual particle ( $\text{mm}^2$ ): The area of the remaining graft material on the defect site.





**Figure 4:** Calabria defect formation and filled with bone materials.

### Statistical Analysis

The metrological value of each group was calculated using the statistical analysis software SPSS (SPSS 18.0; SPSS, Chicago, IL, USA). The non-parametric Kruskal-Wallis test ( $p < 0.05$ ) was used to analyze the significant differences between each group. The non-parametric Mann-Whitney test ( $p < 0.05$ ) was used to analyze the significant difference between the groups after two weeks and eight weeks.

### Results

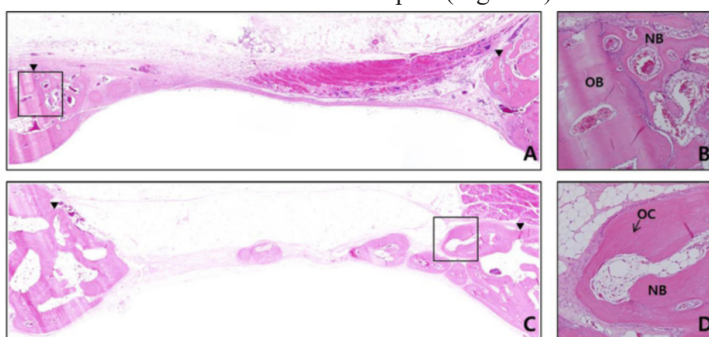
#### Clinical Observations

No significant infections, complications, or abnormal findings were observed in the animals during the healing period.

#### Histological Observations

##### 1. Control group

Two weeks after surgery, small amounts of immature new bone had formed on the defect margins. Coarse connective tissue filled most of the defect site. Chronic inflammatory cell infiltration and blood vessel proliferation were observed. At eight weeks, the defects had more new bone content and had a more mature bone structure in comparison to the bone defects at two weeks. Most of the new bone was adjacent to the defect margins. The connective tissue had further developed. Islet-like new bones were present in the center of the defect for some samples (Figure 5).

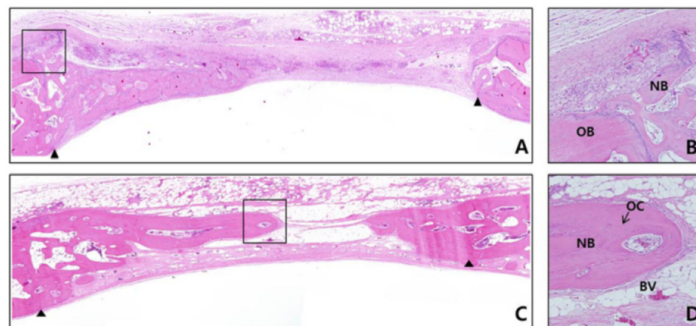


**Figure 5:** Transversal histologic presentation of control group at 2 weeks (A,B) and 8 weeks (C,D). Arrow head: defect margin, NB: new bone, OB: original bone, OC: osteocyte (H&E stain; original magnification: X 40 (A, C), X 100 (B,D)).

##### 2. Membrane group

Two weeks after surgery, the membrane was well maintained, and there was an increase in chronic inflammatory cell infiltration and blood vessel proliferation near the membrane. Immature new bone was present along the defect margins and the membrane and

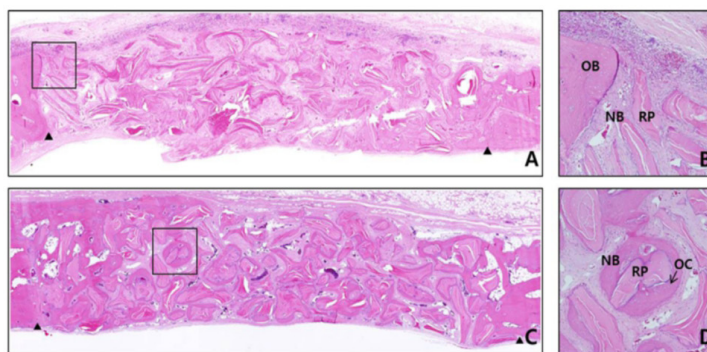
defect were mostly filled with coarse connective tissue. At eight weeks, the membrane in the margins was relatively uniform but more resorbed than it had been at two weeks. The concentration of inflammatory cell infiltration near the membrane had also decreased. Defects that were not supported by the graft material were pushed down by the force of the membrane and the soft tissues above, forming concave new bone along the defect margins and membrane (Figure 6).



**Figure 6:** Transversal histologic presentation of membrane group at 2 weeks (A,B) and 8 weeks (C,D). Arrow head: defect margin, NB: new bone, OB: original bone, OC: osteocyte, BV: blood vessel (H&E stain; original magnification: X 40 (A,C), X 100 (B,D)).

##### 3. BBH transplant group

Two weeks after surgery, chronic inflammatory cell infiltration and blood vessel proliferation were found near the membrane, which was well maintained. With the support of the bone graft material, the tissue grew larger than the membrane, and new bone was present around the defect margins and bone graft. At eight weeks, the membrane had gone through more resorption but almost none of the bone graft material had been resorbed. The bone graft supported the defect site, resulting in a relatively uniform tissue growth. Considerable amounts of mature bone could be observed not only in the defect margins, but also near the bone graft (Figure 7).

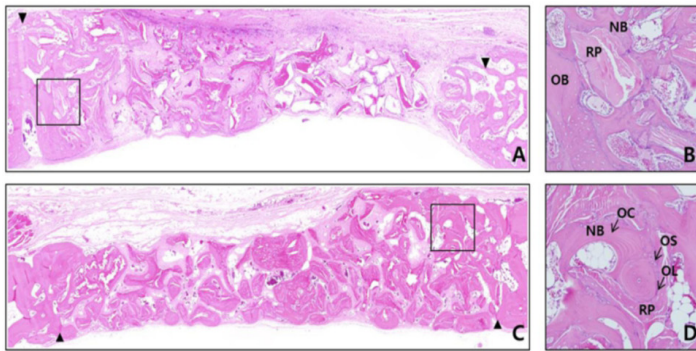


**Figure 7:** Transversal histologic presentation of BBH group at 2 weeks (A,B) and 8 weeks (C,D). Arrow head: defect margin, NB: new bone, OB: original bone, RP: residual particle, OC: osteocyte (H&E stain; original magnification: X 40 (A, C), X 100 (B,D)).

##### 4. CBH transplant group

Like the BBH transplant group, a well-maintained membrane and chronic inflammatory cell infiltration and blood vessel proliferation were observed two weeks after surgery. New bone

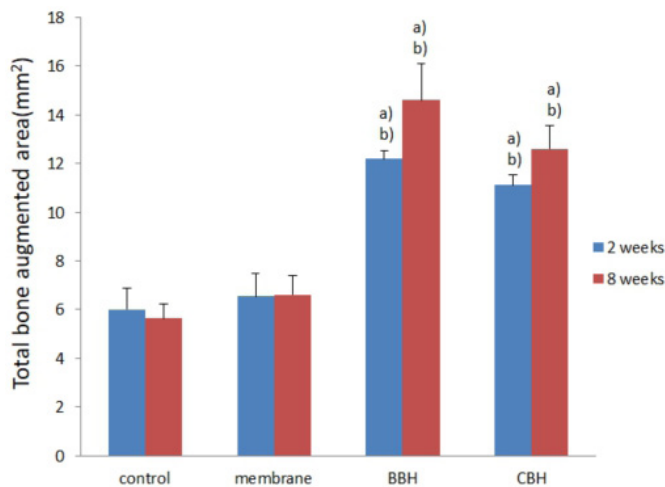
formation was found near the adjacent defect margins and bone graft. Most of the defect consisted of coarse connective tissue and bone graft material, and the observations of the tissue were similar to those of the BBH transplant group. At eight weeks, the amount of new bone around the bone graft and the number of osteoblasts increased. Although there was not as much membrane resorption as there had been at two weeks, there was no significant change in form or tissue growth in the defect, and mature bone cells had formed (Figure 8).



**Figure 8:** Transversal histologic presentation of CBH group at 2 weeks (A,B) and 8 weeks (C,D). Arrow head: defect margin, NB: new bone, OB: original bone, RP: residual particle, OC: osteocyte, OS: osteoblast, OL: Osteoblast lining (H&E stain; original magnification: X 40 (A, C), X 100 (B,D)).

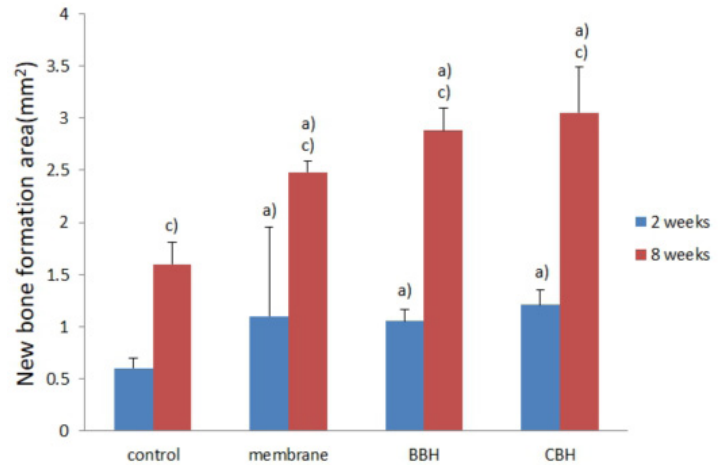
### 5. Histomorphometrical observations

After two weeks and eight weeks, the CBH and BBH transplant groups showed a more statistically significant increase in tissue area than the control group and membrane group ( $p < 0.05$ ). The order of tissue growth area in the defect from largest to smallest was the CBH group, BBH group, membrane group. Both the control and experimental groups had no statistical significance in tissue growth (Figure 9).



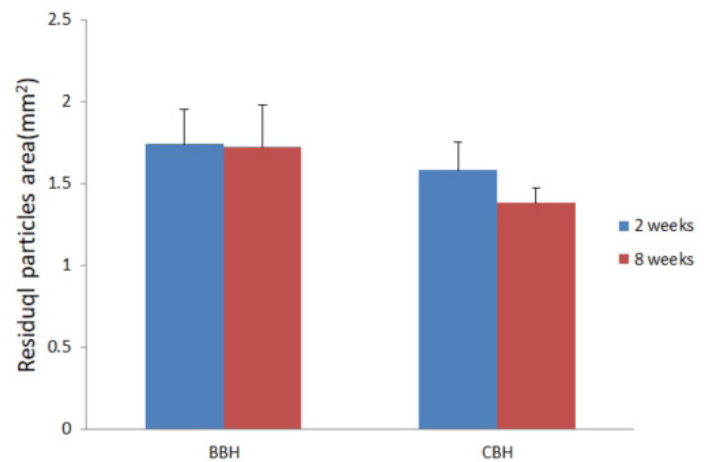
**Figure 9:** Total bone augmented area of histometric results at 2, 8 weeks. a) Significant statistical difference compared to control group at each week ( $p < 0.05$ ). b) Significant statistical difference compared to membrane group at each week ( $p < 0.05$ ).

The CBH, BBH, and membrane groups had a statistically significant new bone area increase from two weeks to eight weeks ( $p < 0.05$ ) but the control group did not. At eight weeks, the CBH transplant group formed the highest amount of bone, followed by the BBH transplant group and the membrane group, but there was no statistically significant difference between each group (Figure 10).



**Figure 10:** Total new bone formation of histometric results at 2, 8 weeks. a) Significant statistical difference compared to Control group at each week ( $p < 0.05$ ). c) Significant statistical difference compared to 2 weeks ( $p < 0.05$ ).

Compared to the defect after two weeks, the defect after eight weeks had less remaining bone graft material in the CBH and BBH groups but the decrease was statistically insignificant. (Figure 11 and Table 1).



**Figure 11:** Residual particles area of histometric results at 2, 8 weeks.

**Table 1:** Histometric results after 2 and 8 weeks of healing (mm<sup>2</sup>).

Parameters	Control	Membrane	BBH	CBH
2weeks (n=4)				
Augmented area	6.01 ± 1.75	6.56 ± 1.82	12.19 ± 0.66a)b)	11.12 ± 0.83a)b)
New bone area	0.60 ± 0.20	1.10 ± 0.16a)	1.05 ± 0.23a)	1.21 ± 0.28a)
Residual particles	-	-	1.74 ± 0.42	1.58 ± 0.34
8weeks (n=4)				
Augmented area	5.64 ± 1.17	6.61 ± 1.54	14.60 ± 2.97a)b)	12.61 ± 1.92a)b)
New bone area	1.60 ± 0.41c)	2.48 ± 0.22a)c)	2.88 ± 0.43a)c)	3.05 ± 0.89a)c)
Residual particles			1.72 ± 0.52	1.38 ± 0.19
Values are presented as mean ± SD				
a)Significant statistical difference compared to Control group at each week (p<0.05)				
b)Significant statistical difference compared to Membrane group at each week (p<0.05)				
c)Significant statistical difference compared to 2 weeks (p<0.05)				

## Discussion

In this study, the bone regeneration abilities of CBH and BBH were compared by making four 8 mm wide defects divided into the control group, membrane group, BBH transplant group and CBH transplant group, and evaluating their abilities in bone regeneration and osseous tissue growth after two weeks and eight weeks. Although a critical size of 10-15 mm is normally used to test bone regeneration in bone defects [22,24], 8 mm defects have also been shown to be useful in evaluating the bone regenerative abilities of bone graft materials [25]. The bone defects of rabbit calvarias form new bone three times as fast as human bone defects. Therefore, immature new bone forming in two weeks in rabbits corresponds to six weeks for humans, and mature bone forming in eight weeks corresponds to six months for humans [26]. Thus, this study analyzed the degree of osteogenesis during two weeks and eight weeks of bone healing, using bone defect models with a diameter of 8 mm each.

In the histological findings of this research, both the CHB and BBH groups were well maintained without any complications or abnormal findings after two and eight weeks. At two weeks, blood vessels, connective tissue, and immature bone cells were present in both groups. After eight weeks, many osteoblasts and new bone cells were found around the bone graft and membrane, and calcification was in progress. Compared to the control and membrane groups, the CHB and BBH groups promote osteogenesis with osteoconduction, acting as scaffolds that provide a suitable environment for osteoblasts to make bone instead of making bone themselves [18]. In the CBH group, the amount of remaining bone graft material after eight weeks was slightly less than the amount after two weeks, but there was no statistically significant difference. Both bovine bone and porcine bone both had slow resorption tendencies, corresponding with the results of the study by Barone et al. in 2005 [18].

Cuttlefish bone and bovine bone differ in micro morphological surface structure and chemical composition. At the micrometer

scale, cuttlefish bone has larger particles than bovine bone and has a calcium concentration of 19.9% whereas bovine bone has 18.4% [14]. In 2012, Park et al. transplanted cuttlefish bone and bovine bone into rabbit calvarias defects and recorded the formation of new bone after two and four weeks. There was no difference between the new bone formed by the two groups [15]. In 2010, when Yoo et al. transplanted cuttlefish bone and bovine bone to rat calvarias defects and evaluated them eight weeks after, cuttlefish bone had formed more uniform tissue, as well as a larger amount of bone. Although there was no statistically significant difference, the optical density of cuttlefish bone was also greater than that of bovine bone [27]. This supports the data from this study that the amount of new bone formed by cuttlefish bone is similar to that formed by bovine bone.

In all experimental groups including the control group, there was a statistically significant increase in new bone from two weeks to eight weeks. The formation of new blood vessels and new bone was present in all experimental groups. If the bone graft material had been used alone without the membrane, more fibrillary connective tissue would have formed than new bone, making the membrane limit interference from surrounding tissues and thus promoting vascularization and facilitating osteogenesis [2]. Although in 2003, Stravropoulos et al. found that using bovine bone with a membrane as opposed to the membrane alone hinders the resorption of the bone graft material, limiting and furthermore interfering with osteogenesis [28]. there was no statistically significant difference in bone formation between the membrane, CBH and BBH groups after two weeks and eight weeks in this study. The area of osseous tissue growth in the CBH and BBH groups increased significantly between two weeks and eight weeks compared to the membrane group, but the membrane group showed a slight but statistically insignificant increase in area of tissue growth, while the control group had a statistically insignificant decrease in area of tissue growth. This may be due to the resorption of the membrane and the absence of a tissue supporting the upper soft tissue of the defect [29]. From the samples in this study, it can be noted the



membrane group, which had the resorbable membrane alone, was not supported by the bone graft, resulting in soft tissue curving into the defect site, much like in the control group. Cross linked resorbable membranes has a slower resorbability than non-cross linked membranes, making them useful in bone regeneration [30]. However, using the membrane alone hinders space maintenance and osseous tissue growth. Since using a membrane in combination with bone graft material allows better space maintenance and tissue growth [31], using cross linked resorbable collagen membrane with bone graft material is recommended for bone regeneration and osseous tissue growth.

The results of this study suggest that cross linked resorbable collagen membrane and cuttlefish bone are useful materials for bone regeneration and osseous tissue growth. However, not many graft materials were used, and there have been no comparison studies of cross linked resorbable membranes and non-cross linked resorbable membranes. Further studies and comparison studies on membranes will be needed from many individuals.

### Conclusion

After histologically and histomorphometrically evaluating the bone regeneration and osseous tissue growth in rabbit calvarial defects using a cross-linked resorbable membrane exclusively, and using it in combination with bovine bone hydroxyapatite (BBH) or *Aloe vera* gel conjugated barnacle cement protein composite hyaluronic acid hydrogel based cuttlefish bone hydroxyapatite (CBH), the following conclusions were made:

1. CBH is an effective bone graft material with biocompatibility and abilities in osteogenesis and space maintenance.
2. The slow resorption of the cross-linked resorbable membrane facilitates osteogenesis but when used alone, space maintenance is poor. For effective bone regeneration and osseous tissue growth, a bone graft should be used with the membrane.

### Acknowledgement

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