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Combined effects of a chemically cross-linked porcine collagen membrane and highly soluble biphasic calcium phosphate on localized bone regeneration

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ABSTRACT

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Objectives: Aim of this study was to evaluate bone regenerative efficacy of a chemically cross-linked porcine collagen membrane (CM) when used in combination with highly soluble biphasic calcium phosphate (BCP).

Materials and methods: Physiochemical properties of the experimental collagen membrane were analyzed. Four circumferential defects with diameter of 8 mm were created in each calvarium of New Zealand white rabbits (n = 10). Defects were randomly allocated to one of following 4 groups: 1) BCP-CM (BCP (20% hydroxyapatite/80% β-tricalcium phosphate) covered with the prepared collagen membrane), 2) BCP (only BCP used), 3) CM (only the prepared collagen membrane used), and 4) C (control; only blood clot). After 2 weeks (n = 5) and 8 weeks (n = 5), histologic and histomorphometric analyses were performed.

Results: The experimental collagen membrane exhibited dense and compact structure, relatively high tensile strength and lower degradability. Histologic analyses revealed that new bone increased rapidly at 2 weeks, while defect was preserved at 8 weeks. Histomorphometric analyses revealed that the new bone areas increased in the BCP-grafted groups over 8 weeks, with BCP-CM exhibiting greater total augmented area than that of BCP group both at 2 weeks (27.12 ± 3.99 versus 21.97 ± 2.27 ㎟) and 8 weeks (25.75 ± 1.82 versus 22.48 ± 1.10 ㎟) ($P < 0.05$).

Conclusions: The experimental collagen membrane successfully preserved localized defect for 8 weeks despite early rapid resorption of BCP. Within the study limitations, combined use of the chemically cross-linked porcine collagen membrane and highly soluble BCP aided localized bone regeneration.

Key words : Biodegradation, Biphasic calcium phosphate, Bone regeneration, Bone substitute, Collagen membrane

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Ⅰ. Introduction

Guided bone regeneration (GBR) is a well-documented and routinely used technique for maintaining space for osseous proliferation in periodontal and peri-implant defects[1]. In the GBR technique, a barrier membrane is placed over the bone-substitute material for the following two reasons: 1) to prevent the down-growth of epithelial and connective tissue into the defect and 2) to maintain a space where osteogenic cells from the host bone can proliferate and subsequently promote bone regeneration[2]. For this purpose, various non-resorbable and resorbable membranes have been developed and tested in numerous preclinical and clinical studies. However, non-resorbable membranes such as those of expanded polytetrafluorethylene have several drawbacks. For instance, they result in early exposure to the oral environment and require a second surgical intervention for retrieval[3, 4]. In order to overcome these drawbacks, resorbable membranes have been suggested as alternatives.

Among the different resorbable membranes being explored, those of collagen are considered to be the best suited because of the biodegradability, biocompatibility, and favorable wound healing rate of collagen and given that these membranes do not require surgical removal[5, 6]. Recently, membranes derived from type I and III porcine or bovine collagen have been investigated for enhancing bone and periodontal regeneration in various animal and human studies[7, 8]. Porcine collagen is more similar to human collagen so allergies are less common[9]. However, the rapid degradation of native collagen by the enzymatic activity of macrophages and its lack of mechanical stiffness led to the early collapse of the barrier as well as insufficient space maintenance for tissue regeneration[10]. Therefore, various cross-linking techniques for delaying the degradation of collagen membranes and subsequently prolonging the regenerative period of defects have been investigated[11, 12]. Rothamel et al.[13] reported that chemically cross-linked porcine type I and III collagen membranes show moderately prolonged degradation periods (4-24 weeks) compared to a non-cross-linked collagen membrane (2-4 weeks). In addition, the former also show higher biocompatibility than collagen membranes that have been fabricated by other methods such as glutaraldehyde or enzymatic cross-linking. Based on these findings, it can be assumed that chemically cross-linked porcine type I collagen membranes would be suitable for use in bone regeneration for preventing the early collapse of the barrier and allowing enough time for osseous proliferation with biocompatibility.

A bone substitute material is another constituent used in GBR. Autogenous bone has been the gold standard because of its superior osteogenic potential but has a few limitations such as rapid resorption and donor-site morbidity[14]. Allogenic and xenogenic bone graft materials also show immunologic problems[15]. Therefore, many synthetic materials have been developed. Biphasic calcium phosphate (BCP), which consists of less-soluble hydroxyapatite

(HA) and highly soluble beta-tricalcium phosphate (β-TCP), is quickly becoming the preferred material, as its structure and composition are similar to those of bone[16]. Further, it exhibits good biocompatibility, bioactivity, and osteoconductivity[15] and the HA/β-TCP ratio can be readily varied, allowing one to control the chemical properties of BCP, including the resorption and bone substitution processes[14, 17]. As previously reported[18], osteobl astic bone formation is related to osteoclastic activity, and Yamada et al.[19] suggested that the solubility of BCP influences osteoclastic activity. The increased solubility at high β-TCP contents (within certain limits) promotes osteoclastic resorption and consequently is expected to enhance bone regeneration. However, as the content of soluble β-TCP is increased, the mechanical strength of BCP decreases. Mechanical stability is also an essential characteristic of bone-substitute materials because such materials should be able to function as a scaffold for osteoconductive proliferation and support the overlying membrane[2, 20]. A tradeoff between resorption and mechanical stability is the key to the use of BCP, and the property to be enhanced can be determined based on the specific application in mind.

It had been suggested previously that BCP with a HA/β-TCP ratio of 20/80 is comparable to autogenous bone in terms of the resorption rate and amount of new bone formed[23]. Highly soluble BCP is superior with respect to osteoconductivity, even though it results in poor space maintenance and shows low mechanical strength after rapid resorption. The aim of this study was to evaluate the bone regenerative capacity of highly soluble BCP (HA/β-TCP ratio of 20/80) with and without a chemically cross-linked porcine collagen membrane based on bone healing, material biodegradation, and the area of total augmentation using a localized rabbit calvarial defect model. The hypothesis was that a chemically cross-linked porcine collagen membrane in combination with highly soluble BCP would result in optimized bone regeneration, as the mechanically strong and slowly resorbing membrane would compensate for the rapid resorption of BCP.

Ⅱ. Materials and methods

1. Materials

The following four materials were used in this study:

1) Cross-linked porcine collagen membrane (CM): A chemically cross-linked porcine collagen membrane (Collagen membrane-P®, Genoss, Suwon, South Korea) derived from the type I collagen of porcine tendon. Type I porcine collagen fibers were purified and dispersed in an acidic solution (pH 2.5), neutralized, freeze-dried, and finally sterilized by γ-irradiation.

2) Cross-linked bovine collagen membrane (CM-B): A chemically cross-linked bovine collagen membrane (Collagen membrane®, Genoss, Suwon, South Korea) derived from type I and III collagens of bovine tendon. The

manufacturing process was identical to that for the CM sample.

3) Non-cross-linked collagen membrane (NCM): A non-cross-linked collagen membrane (Bio-gide®, Geistlich Pharma AG, Wolhusen, Switzerland) derived from porcine dermis and consisted of type I and III collagens.

4) Biphasic calcium phosphate (BCP): The biphasic calcium phosphate (New Bone®, Genoss, Suwon, South Korea) sample used was composed of 20% HA and 80% β-TCP with a macropore size range from 200-400 ㎛ and total porosity around 70-80% (macroposity: 70-75%, microposity: 80%). The particle size of the BCP ceramic was typically 500-1000 ㎛. Further, the macro- and micropores in the ceramic were highly interconnected.

2. In-vitro study

1) Scanning electron microscopy (SEM) of CM sample

Scanning electron microscopy (SEM; S-3000N, Hitachi, Tokyo, Japan) was performed on the CM sample. The thickness of the membrane and its morphology were observed. Further, SEM images of every surface were taken for close examination.

2) Tensile strength test

Dry and wet (soaked in phosphate buffered saline(PBS) for 24 h) CM and CM-B samples with dimensions of 30 × 10 mm were tested for tensile strength. The membranes were placed on a universal testing machine and stretched at 70 mm/min until the point of breakage. The cell load was 1,000 N, and 3 specimens of each membrane type were tested.

3) Enzymatic degradation test

For the degradation test, CM, CM-B, and NCM samples were tested using 50 U/mL of collagenase at 100 rpm and 37℃ for 24 h. The remaining samples were collected, lyophilized, and weighed to determine the dry weight after enzymatic degradation (Wd). The relative weight proportion (%) was defined as Wd/Wi × 100 (Wi: initial weight).

3. In-vivo study

1) Animals

A total of ten 16-weeks-old New Zealand white male rabbits, weighing 3.0-3.5 kg, were used in this study. The animals were housed in separate cages under standard laboratory conditions and were fed a standard diet. The animal selection, management, preparation, and surgical protocols were approved by the International Animal Care and Use Committee (Yonsei University Health System, Seoul, Korea; permission number 2014-0060) in compliance with the applicable ethical guidelines and regulations of Yonsei Medical Center.

2) Surgical procedure

The surgical site was shaved and disinfected with povidone iodine (Povidin, Firson, Cheonan, Chungcheongnam-do, Korea) while keeping the animal on a surgical table under general

anesthesia using ketamine hydrochloride (10mg/kg; Ketalar, Yuhan, Suwon, Korea) and xylazine (2mg/kg; Rompun, Bayer Korea, Suwon, Korea). An incision was made in the sagittal plane across the cranium, and a full-thickness flap was elevated to expose the calvarial bone under infiltration anesthesia with 2% lidocaine (2% lidocaine hydrochloride-epinephrine 1:100,000, Kwangmyung Pharm aceutical). Four circumfere ntial bony defects were created on the sagittal plane with an 8-mm-diameter trephine bur under copious saline irrigation. Each defect was randomly assigned to one of the following 4 groups (Fig. 1):

(1) BCP- CM group: The defect was filled with 0.1 g of BCP (HA/β-TCP: 20/80) and covered with a piece of the chemically cross-linked porcine collagen membrane.
(2) BCP group: The defect was filled with 0.1 g of BCP (HA/β-TCP: 20/80) particles only.
(3) CM group: The defect was covered with a piece of the chemically cross-linked porcine collagen membrane only.
(4) C (control) group: The defect was filled with a blood clot.

After the defects had been made, the periosteum was sutured using a resorbable suture material (4-0 vicryl, Johnson and Johnson Int., USA) to immobilize the membranes. The skin was also sutured using the same material. The animals were sacrificed post-operation at 2 weeks (n = 5) and 8 weeks (n = 5) by an intravenous injection of phenobarbital (100 mg/kg).

3) Histologic processing

Block sections with the surgical sites were removed and trimmed after the animal had been sacrificed. The samples were fixed with 10% neutral formalin for 10 days and demineralized with 5% nitric acid for 5 days. Next, the demineralized tissue was embedded in paraffin. Serial sections with a thickness of 5 ㎛ were cut through the center of the circumferential defects along the coronal plane and stained with

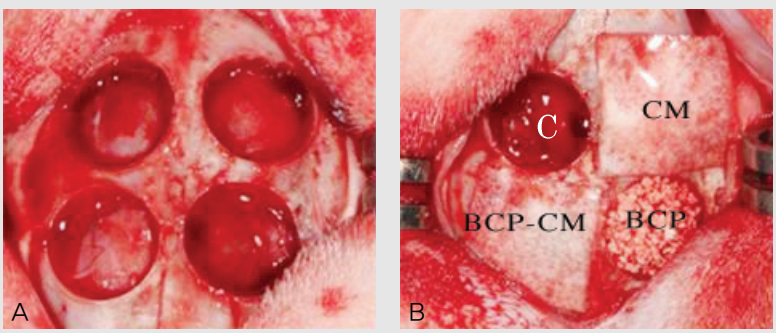

Fig. 1. Clinical photographs of the experimental sites. (A) Four circumferential defects with an 8-mm-diameter were created on 10 New Zealand white rabbits' calvaria. Full thickness of the bone was carefully removed. (B) Four different groups were randomly allocated (C: control, CM: a chemically cross-linked porcine collagen membrane, BCP: biphasic calcium phosphate with 20% hydroxyapatite (HA) and 80% β-tricalcium phosphate (β-TCP), BCP-CM: BCP covered with CM).

hematoxylin-eosin and Goldner's Masson trichrome for histologic observations and histomorphometric analysis.

4) Evaluation methods

(1) Clinical observations: The animals were carefully observed for inflammation or complications at the surgical sites. They were also inspected at the time of sacrifice.

(2) Histologic and histomorphometric analyses: For the histologic analyses, all the specimens were examined using a binocular microscope (Leica DM LB; Leica Microsystems, Wetzlar, Germany). Images of all the specimens were captured, reconstructed to obtain a sectional view, and saved (cellSens Standard 1.11; Olympus Corporation, Center Valley, PA, USA). Following the microscopic examination, computer-assisted histomorphometric measure ments of the calvarial defects were performed with the aid of an image processing program (Photoshop CS6; Adobe Systems, San Jose, CA, USA). The following parameters were measured for each histologic section (Fig. 2):

a. Area of total augmentation (TA) [㎟]: Total area below the covered membrane, the lateral boundaries of the defect, and the dura-mater inferiorly. This consisted of the sum of the area of new bone, the residual materials, fibrous tissue, adipose tissue, and the fibrovascular and marrow tissues between the defect margins.
b. Area of new bone (NB) [㎟]: Area of newly formed bone within the defect.
c. Area of residual material (RM) [㎟]: Area of the remaining graft material that was not substituted with new bone.

(3) Statistical analysis: Statistical analysis was performed using a commercially available software program (SPSS 18.0, SPSS, Chicago, IL). Histomorphometric records of the calvarial defects were used to calculate the mean and standard deviation (SD) values for the four groups, namely, the BCP-CM, BCP, CM, and C groups. The Kruskal-Wallis test was used to analyze the differences between the groups at each time point, while the differences between the various parameters for the two healing periods (2 and 8 weeks) within the same experimental group were analyzed using the Mann-Whitney U test. Statistical significance was assumed when $P < 0.05$.

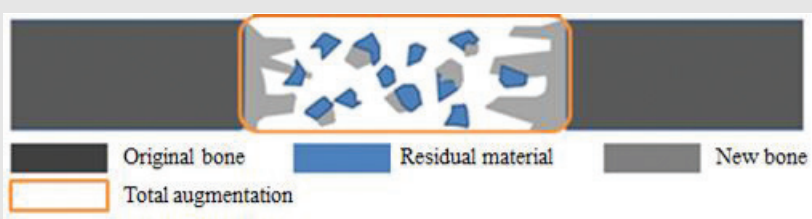

Fig. 2. A schematic diagram of histomorphometric analysis.

Ⅲ. Results

1. In-vitro study

1) Scanning electron microscopy (SEM) of CM sample

The thickness of the CM sample was determined to be 300 ㎛. Further, it was observed that the CM sample exhibited a dense and compact surficial morphology. In the cross-sectional view, irregular macro- and micropores were seen roughly interconnected between the densely compacted collagen fibrils (Fig. 3).

2) Tensile strength test

The tensile strength of the CM sample was approximately 80 N/㎟ under dry conditions. This was lower than that of the CM-B sample (115 N/㎟). Under wet conditions, that is, after being soaked in PBS, the CM sample showed a tensile strength of 28 N/㎟, which was slightly higher than that of the CM-B sample (18 N/㎟) (Fig. 4).

3) Enzymatic degradation test

The results of the enzymatic degradation test indicated that, during the first 12 h of immersion in collagenase, the CM (n=5) and CM-B (n=5)

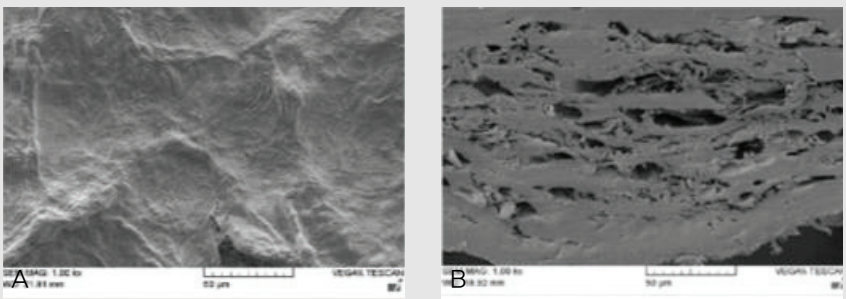

Fig. 3. Scanning electron microscopy (SEM) findings for the chemically cross-linked porcine collagen membrane. (A) A dense and comsurficial morphology. (B) Irregular macro- and micro pores were roughly interconnected between densely compacted collagen fibrils in the cross sectional view.

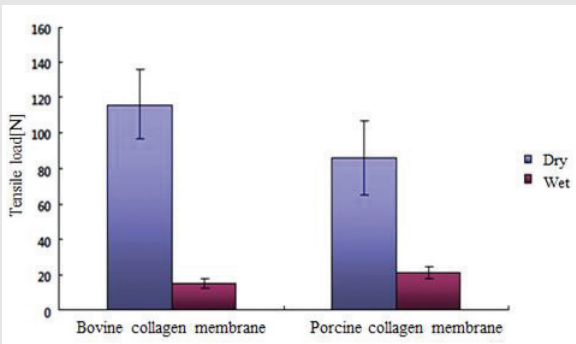

Fig. 4. Tensile strength test. The tensile strength of the CM sample (80N/㎟) is lower than that of CM-B (115N/㎟) sample under dry conditions. When the membranes were compared under wet conditions, the tensile strength of CM (28N/㎟) sample was slightly higher than that of CM-B (18N/㎟) sample (CM: the chemically cross-linked porcine collagen membrane, CM-B: the chemically cross-linked bovine collagen membrane).

samples exhibited relatively slow degradation while the NCM (n=5) samples exhibited fast degradation. During the next 12 h, the CM sample exhibited faster degradation compared to the CM-B sample, which continued to show delayed degradation, retaining more than 80% of its initial weight. On the other hand, the NCM sample was completely degraded during first 4 h. A significant difference in relative weight was observed between the two chemically cross-linked collagen membranes (CM: 20.02 ± 0.05% and CM-B: 78.92 ± 0.10%) and the non-cross-linked one (NCM: 3.4 ± 0.05%) after 24 h of degradation by collagenase (Fig. 5).

2. In-vivo study

1) Clinical findings

Wound healing was uneventful and no specific inflammatory process was observed in any of the samples. In a 2-weeks animal specimen, at one of the defect sites in the CM group, a membrane was exposed. This sample was excluded from the analysis.

2) Histologic findings

(1) BCP-CM group: In the 2-weeks specimens, filopodia-shaped new bone stretched from the defect margins towards the defect center. New bone apposition was observed and many capillaries were present in the area where the BCP particles had been resorbed. The membrane remained intact and was separated from the inner and outer connective tissue. The

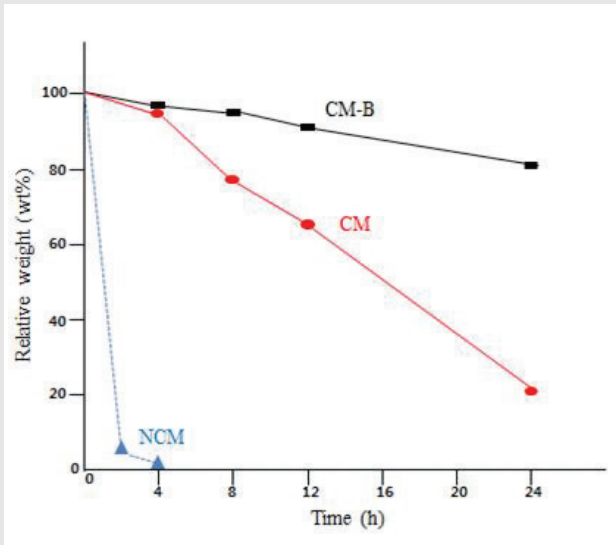

Fig. 5. Enzymatic degradation test. During the first 12 hours, the CM and CM-B samples exhibited relatively slow degradation while the NCM sample exhibited fast degradation. During the next 12 hours, the CM sample exhibited faster degradation compared to the CM-B sample, while the NCM sample was completely degraded during first 4 hours (CM: the chemically cross-linked porcine collagen membrane, CM-B: the chemically cross-linked bovine collagen membrane, NCM: the non-cross linked porcine collagen membrane).

defect was augmented by the BCP particles and protected by the membrane. In the 8-weeks specimens, the new bone had matured, with its area being greater. This was because bone had not only formed from the bottom of the defect but was also in close contact with the particle surfaces as well as within the interparticle spaces, in contrast to the case for the 2-weeks specimens. The area where the particles had been resorbed was occupied by pre-matured new bone while bone marrow was present in the matured new bone. The membrane had gradually denatured, exhibiting a loose stratified structure. The number of small blood vessels below the membrane was higher in the area of contact with the newly formed bone. The augmented area was stably preserved by the membrane barrier (Fig. 6).

(2) BCP group: The pattern of new bone formation was similar to that in the case of the BCP-CM group: filopodia-shaped new bone with small capillaries was formed near the defect margin and in close contact with the BCP particles. In contrast to the BCP-CM group, however, the upper border of the defect, which did not have a barrier membrane, was covered with fibrous connective tissue in the case of the 2-weeks specimens, while the augmented area was maintained to a relatively high degree and contained BCP particles. In the 8-weeks specimens, connective tissue and capillaries filled the upper area of the defect and adipose tissue was present over the connective tissue without boundaries. New bone was formed in the area where the particles had been resorbed. However, most of the remaining particles were surrounded by connective tissue, and bone-to-

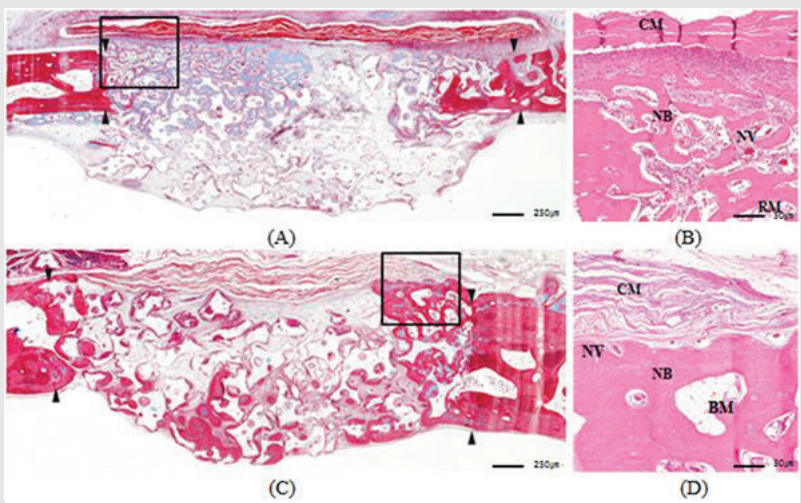

Fig. 6. Transversal histologic sections of BCP-CM group at 2 weeks (A and B) and 8 weeks (B and D). Arrow head: defect margin, NB: new bone, CM: a chemically cross-linked porcine collagen membrane, RM: residual material, BM: bone marrow, NV: new vessel (A and C: Goldner's Masson trichrome stain; B and D: hematoxylin and eosin stain).

particle contact decreased. The augmented area was relatively depressed as the connective and adipose tissues occupied the center of the defect (Fig. 7).

(3) CM group: In the 2-weeks specimens, a limited amount of new bone with capillaries was observed at the lateral borders while the center of the defect was occupied by non-mineralized fibrous tissue. The defect was semi-preserved by the lower border of the membrane. In the 8-weeks specimens, bony islets from the bottom were connected to each other, forming a bridge. Further, the previously formed new bone had matured and contained bone marrow. Compared to the case for the 2-weeks specimens, the collagen membrane had been partially resorbed. However, the defect area was mostly maintained, despite the partial resorption of the membrane (Fig. 8).

(4) C group: In the 2-weeks specimens, new bone formation was similar to that in the CM group, while the center of the defect was pressed and flattened. The defect was almost filled with loose fibrous tissue, and linear adipose tissue clusters were present over the connective tissue. In the 8-weeks specimens, the new bone had matured and increased in area. Further, a bony bridge had been formed by the islets on the defect bottom. The defect space was almost filled with loose fibrous tissue and adipose tissue fenestrations, with the defect becoming concave owing to shrinkage (Fig. 9).

3) Histomorphometric findings

The results of the histomorphometric measurements are listed in Tables 1-3. A

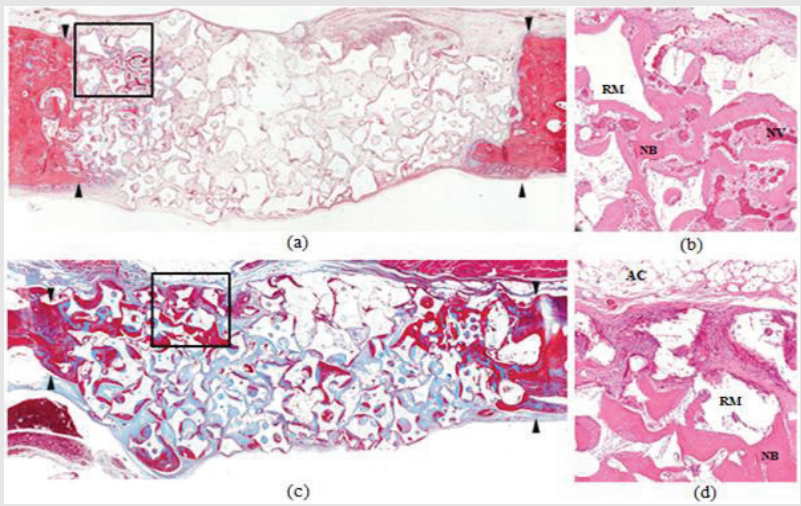

Fig. 7. Transversal histologic sections of BCP group at 2 weeks (A and B) and 8 weeks (C and D). Arrow head: defect margin, NB: new bone, RM: residual material, AC: adipocyte, NV: new vessel, CT: connective tissue (A and C: Goldner's Masson trichrome stain; B and D: hematoxylin and eosin stain).

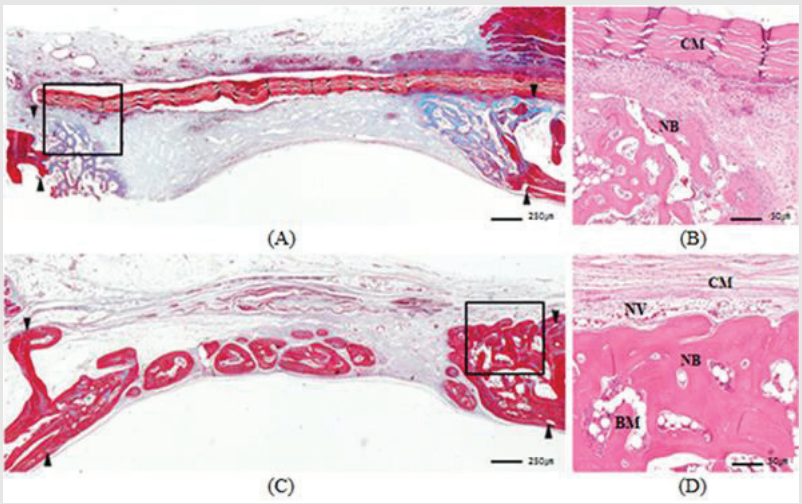

Fig. 8. Transversal histologic sections of CM group at 2 weeks (A and B) and 8 weeks (C and D). Arrow head: defect margin, NB: new bone, CM: a chemically cross-linked porcine collagen membrane, BM: bone marrow, NV: new vessel (A and C: Goldner's Masson trichrome stain; B and D: hematoxylin and eosin stain).

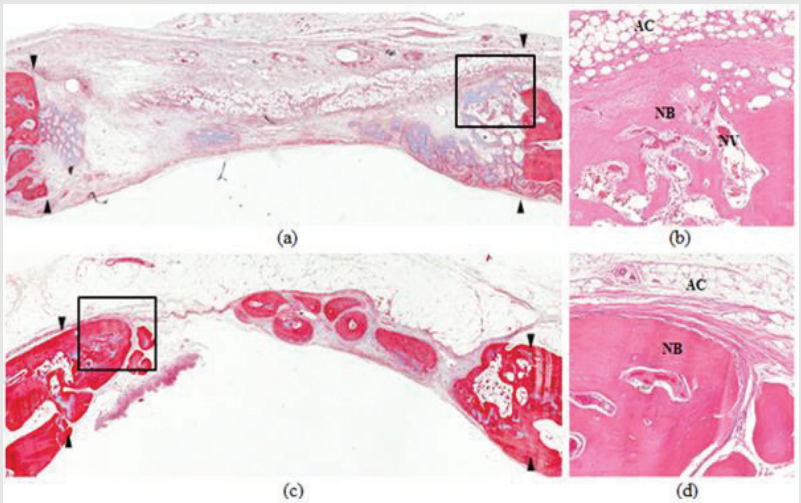

Fig. 9. Transversal histologic sections of control group at 2 weeks (A and B) and 8 weeks (C and D). Arrow head: defect margin, NB: new bone, AC: adipocyte, NV: new vessel (A and C: Goldner's Masson trichrome stain; B and D: hematoxylin and eosin stain).

significantly higher degree of NB was observed in the two BCP-treated groups (BCP-CM: 5.35 ± 2.42 ㎟ and BCP: 3.91 ± 1.47 ㎟) at 2 weeks as compared to that in the CM (1.89 ± 0.82 ㎟) and control (1.79 ± 1.18 ㎟) groups. Similar results were obtained at 8 weeks, with NB being significantly different between the BCP-grafted groups (BCP-CM: 7.33 ± 1.90 ㎟ and BCP: 7.71 ± 1.26 ㎟) and BCP non-grafted groups (CM: 2.10 ± 1.03 ㎟ and control: 2.97 ± 0.95 ㎟). Moreover, bone formation increased significantly from 2 to 8 weeks only in the BCP-

CM and BCP groups (Table 1). Within the augmented area, at 2 weeks, the TA value of the BCP-CM group was the highest (27.12 ± 3.99 ㎟), followed by those of the BCP (21.97 ± 2.27 ㎟), CM (11.90 ± 2.20 ㎟), and control (7.40 ± 2.21 ㎟) groups. At 8 weeks as well, the TA value of the BCP-CM group (25.75 ± 1.82 ㎟) was the highest, followed by those of the BCP (22.48 ± 1.10 ㎟), CM (9.01 ± 3.52 ㎟), and control (8.20 ± 1.46 ㎟) groups. At both 2 and 8 weeks, the TA values for the treatment groups of BCP-CM and BCP were significantly higher than those for the CM and control groups (Table 2). Finally, RM, the area of the remaining BCP, between 2

Table 1. Area of new bone formation (NB) (㎟; Group Mean ± SD; n = 5)

	2weeks	8weeks
Control	1.79±1.18	2.97±0.95
CM	1.89±0.82	2.10±1.03
BCP	3.91±1.47[a)]	7.71±1.26[b),c)]
BCP-CM	5.35±2.42[a)]	7.33±1.90[b),c)]

CM: a chemically cross-linked porcine collagen membrane ,
BCP: biphasic calcium phosphate with 20% hydroxyapatite (HA) and 80% β-tricalcium phosphate (β-TCP),
[a)] Significantly different from control group at 2 weeks postoperatively (P < 0.05).
[b)] Significantly different from control group at 8 weeks postoperatively (P < 0.05).
[c)] Significant difference in the same experimental group between 2 and 8 weeks postoperatively (P < 0.05).

Table 2. Area of total augmentation (TA) (㎟; Group Mean ± SD; n = 5)

	2weeks	8weeks
Control	7.40±2.21	8.20±1.46
CM	11.90±2.20	9.01±3.52
BCP	21.97±2.27[a)]	22.48±1.10[b)]
BCP-CM	27.12±3.99[a)]	25.75±1.82[b)]

CM: a chemically cross-linked porcine collagen membrane,
BCP: biphasic calcium phosphate with 20% hydroxyapatite (HA) and 80% β-tricalcium phosphate (β-TCP),
[a)] Significantly different from control group at 2 weeks postoperatively. (P < 0.05).
[b)] Significantly different from control group at 8 weeks postoperatively. (P < 0.05).

Table 3. Area of residual materials (RM) (㎟; Group Mean ± SD; n = 5)

	2weeks	8weeks
BCP	6.71±1.14	5.19±1.15
BCP-CM	6.31±1.37	4.53±0.63

CM: a chemically cross-linked porcine collagen membrane,
BCP: biphasic calcium phosphate with 20% hydroxyapatite (HA) and 80% β-tricalcium phosphate (β-TCP),
There was no significant difference in BCP and BCP-CM groups between 2 and 8weeks.

and 8 weeks was not significantly different for the BCP-CM and BCP groups (Table 3).

Ⅳ. Discussion

The present study was designed to evaluate the combined effects of a chemically cross-linked porcine collagen membrane and highly soluble BCP (HA/β-TCP ratio of 20/80) on localized bone regeneration. Within the limitations of this study, it was observed that 1) the use of BCP with and without a barrier membrane results in greater bone formation and that the new bone is appositional with respect to the bone-substitute particles and 2) a chemically cross-linked porcine collagen membrane in combination with BCP results in the largest augmented area both at 2 and 8 weeks, with the membrane acting as a barrier and leading to superior space maintenance.

The rabbit calvarial defect model has been used widely to evaluate the bone regeneration ability of biomaterials based on the amount of bone marrow generated, even though New Zealand white rabbits have a fast metabolism[24]. Although the critical size defect (CSD) has been reported to range from 10 to 15 mm[25, 26], it is not possible to create multiple defects with the suggested CSD on the rabbit calvarium. Sohn et al.[27] reported that, even though a diameter of 8 mm is smaller than the suggested CSD, four defects with a diameter of 8 mm are suitable for evaluating the early healing phase (i.e., 2 or 4 weeks) as well as the late healing phase (i.e., 8 weeks) in the rabbit calvarial model. Based on these findings, in the present study, the two healing phases were set to correspond to 2 and 8 weeks. Further, each defect was positioned as far as 2 mm from the other defects to prevent the influx of materials into the defects belonging to the other groups.

The purpose of the bone-substitute material used would vary with the type of defect. For example, in a well-contained defect such as an extraction socket, the rapid formation of new bone is more important than space maintenance, when considering the graft material to be used, because bone healing may be delayed because of the residual materials, while the space maintena nce of the graft material is more critical in a severely damaged site[28]. The chemical properties of BCP can be altered relatively easily by changing the HA/β-TCP ratio: the solubility of BCP, which is followed by the formation of new bone in the interspace between bone and the BCP particles[19]. A high β-TCP content accelerates the resorption of BCP and promotes new bone formation but lowers the mechanical stability of BCP[29]. However, it is necessary to keep the β-TCP content high to ensure new bone formation, with the lowered stability being an undesirable side effect.

The results of histomorphometric analysis showed the NB and TA values of the BCP-grafted groups (BCP-CM and BCP) were higher both after 2 and 8 weeks. On the other hand, there was no significant difference between the CM and control groups with regards to the NB and TA values for both experimental periods. These

results confirmed that BCP grafting has a positive effect on new bone formation and volume augmentation. However, there was a significant difference in NB of the two BCP-grafted groups between 2 and 8 weeks, but there was no difference in RM. These results suggest that BCP dissolved in the early healing phase, and so new bone formation was influenced by BCP resorption, although it was not directly proportional to this resorption in the late healing phase. This can be explained by the possibility of BCP exhibiting osteoinductivity. Although the osteoinductivity of BCP was suggested to be not inherent, the critical geometry of BCP, in addition to its macro- and microporous structures, could result in the successful trapping of ions, osteogenic cells, and osteogenic proteins such as BMPs, thereby providing a suitable microenvironment for osteoinduction. In particular, the provision of calcium and phosphate ions by the dissolution of soluble β-TCP could contribute to a favorable environment for bone formation[30]. However, since the mechanism of osteoinduction by BCP ceramics is still unclear, further researches are needed to elucidate it. From a histologic viewpoint, both the BCP-CM group and the BCP group showed rapid new bone growth, with the bone having a filopodia-like shape. Further, the resorption of materials from the defect margins was observed in the early healing phase (i.e., at 2 weeks). The formation of new bone, in which BCP particles were resorbed (i.e., at the defect margins and in the vicinity of the BCP particles), was noticeable; this may be attributed to β-TCP resorption. It can be presumed that osteoclastic resorption on the surfaces of the BCP particles is followed by osteoblastic activity and consequently an increase in the NB value. This result is in accordance with that of a previous report[31], wherein it was reported that BCP undergoes rapid resorption in the early healing phase and that the higher the β-TCP content, the faster the resorption process is. Even though it is not clear whether the amount of new bone formed is directly proportional to the β-TCP content31), in this study, new bone formation was associated with the resorption of the β-TCP in BCP, with BCP dissolving to high degree during the early healing phase. Therefore, it can be concluded that space maintenance in the initial stage is critical when highly soluble BCP is used as the bone-substitute material. In addition small vessels were observed among the newly formed bone; this was also in keeping with a previous study, which suggested that the rate of vascularization is significantly related to successful material-guided defect repair[29].

In terms of the TA value, there was no significant difference between the BCP-grafted groups (BCP-CM and BCP) after 2 and 8 weeks of healing, as determined by the histomor phometric analysis. On the other hand, based on various bone-healing characteristics, it was observed that, in the case of BCP-CM, the defects were preserved by the collagen membrane, in contrast to the case for BCP?in the latter case, the defects exhibited a gradual depression at 8 weeks, owing to the penetration of connective tissue and the aggregation of

adipose tissue. Over the experimental periods, the TA value increased owing the formation of new bone and decreased because of material resorption and the invasion of outer cells such as connective and adipose tissues. In the case of the BCP-CM group, the collagen membrane continued to act as a barrier for 8 weeks even though its structure weakened. Further, osseous proliferation occurred near the BCP particles as well as within the space between them, and the space was well maintained by the collagen membrane. On the other hand, in the case of the BCP group, the adjacent connective and adipose tissues invaded and occupied the space where the new bone could grow, resulting in the defects becoming depressed and the TA value decreasing. Successful mineralization was observed in the areas where there was contact between bone and the test material; however, the encapsulation of the BCP particles by connective tissue probably inhibited the formation of new bone[29], as observed after 8 weeks. These phenomena were also observed in the cases of the BCP non-grafted groups (CM and control). The original shape of the defect as well as the defect space was maintained in the case of the CM group, with osteogenic proliferation from the margins and bottom of the defect being observed. Beneath the collagen membrane, new bone growth with vascular formation along the lower border of the membrane was observed in the early healing phase. Further, the defect was gradually closed from the bottom because of the formation of a bony bridge at 8 weeks. On the other hand, in the case of the control group, fibrous connective and adipose tissues aggregated rapidly and occupied the defect space, resulting in the defect becoming depressed at 2 weeks. At 8 weeks, the complete collapse and severe shrinkage of the defect was observed, and the defect became completely concave, even though bone was formed at the defect margins and bottom. These results confirmed that a durable barrier membrane can prevent the unwanted invasion of cells and protect the defect space. Further, the membrane must act as a barrier long enough to allow bone regeneration to progress.

Collagen is used widely for manufacturing barrier membranes. The stability of these membranes is of great importance, because native collagen membranes usually degrade within 2-4 weeks[13], and if membrane resorption occurs within 4 weeks of placement, there may not be enough time for bone and periodontal ligament cells to fill the defect beneath the membrane[32]. Further, the membrane must have an interconnected three-dimensional architecture in order to act as a barrier[33]. Various cross-linking techniques can be used to alter the structure of collagen membranes, affecting their mechanical strength and stability. Membrane biocompatibility is another factor to consider, as it has been reported that cross-linking techniques such as those requiring the use of ultraviolet radiation or glutaraldehyde increase membrane toxicity. Thus, there may be a tradeoff relationship between membrane stability and mechanical strength and membrane biocompati bility and tissue integration. Of the various cross-

linking techniques available, it has been reported that chemical cross-linking results in membranes with a moderately high degradation time (4-24 weeks) and suitable biocompatibility, including desirable degrees of tissue integration and vascularization and fewer foreign body reactions[13]. In an in-vitro study, the experimental collagen membrane exhibited a dense and compact structure after being chemically cross-linked. The density of the membrane structure is influenced by the extent of cross-linking and, in turn, affects the mechanical strength[34]. An enzymatic degradation test was performed to simulate oral conditions on the cross-linked membranes of porcine and bovine collagen. After 12h, the cross-linked collagen membranes had barely degraded while a non-cross-linked collagen membrane had degraded completely. The improved stability in the former case was attributable to the cross-linking process. However, there were differences in the degradation times of the two cross-linked membranes (CM and CM-B). Despite the difference in their degradation rates, the tensile strength of CM under wet conditions was slightly higher than that of CM-B. Types of I and III collagen of porcine and bovine origin are the most commercially available collagen membranes[7] and the mechanical properties can be adjustable. Further, too long a degradation period can increase the risk of exposure, resulting in a lower degree of tissue integration[13]. Thus, this chemically cross-linked porcine collagen membrane shows high resistance to enzymatic action and enhanced mechanical strength. From a histologic viewpoint, during the in-vivo analysis of the BCP-CM and CM groups, after 2 weeks, the collagen membrane protected the defect space and remained intact despite the rapid resorption of BCP in the case of the BCP-CM group and the empty space in the case of the CM group. Further, a defect depression was not observed, confirming the mechanical stability of the collagen membrane. At 8 weeks, the collagen membrane preserved the defect from the adjacent connective tissue. Further, the membrane also preserved the osteogenic environment, allowing for cell-to-cell contact and cell-matrix interactions, which are essential for new bone formation[19]. In addition to exhibiting improved mechanical properties, the collagen membrane allowed a large number of capillaries and appositional new bone to form beneath its lower border after 2 weeks in the case of the BCP-CM group. It has been suggested that vascular formation is evidence of good tissue integration[35]. Thus, the biocompatible collagen membrane integrated well with the existing osteogenic environment and had a positive effect on bone formation with vascularization. At 8 weeks, a greater number of vessel and matured new bone with increased bone marrow was observed in contact with the collagen membrane in the case of the BCP-CM and CM groups. Bone marrow is a living soft tissue that contains important components, such as vessels, osteoblasts, osteoclasts, and mesenchymal stem cells, which can be replaced by new mineralized bone[36]. Thus, based on these results, it can be concluded that the experimental collagen

membranes remain mechanically strong in the early healing phase and exhibit sufficient durability till the late healing phase (i.e., till 8 weeks), aiding the formation of new bone while exhibiting good biocompatibility.

In this study, two materials, namely a bone substituent and a barrier membrane, were used in combination for GBR. The solubility of BCP could be attributed to the β-TCP content, where an increased β-TCP content improves the solu bility of BCP and leads to its rapid resorption. In this study, β-TCP dissolution and the subsequent formation of new bone occurred rapidly in the early healing phase. The membrane-supporting capacity of BCP can decrease with its dissolution and this can lead to the partial depression of the defect. Therefore, in the early healing phase, the mechanical stability of the collagen membrane used should be high enough to protect the defect, when using a conventional bone-substitute material with a high initial resorption rate. The rate of BCP dissolution may decrease with time. Thus, in the late healing period, the collagen membrane must act as a barrier to prevent soft tissue infiltration. Hence, prolonged membrane stability, induced through cross-linking, and high biocompatibility are important after the initial healing period.

In conclusion, highly soluble BCP with a high β-TCP content dissolved rapidly in the early healing period, leading to new bone formation and volume augmentation. The chemically cross-linked porcine collagen membrane tested in this study was found to be biologically safe and showed good mechanical strength and prolonged stability. Within the limitations of the study, it can thus be concluded that the combined use of a chemically cross-linked porcine collagen membrane and highly soluble BCP leads to improved localized bone regeneration. However, further studies are needed to directly compare the effects of native collagen membranes and the one tested in this study while using the same BCP material. In addition, the effects of different BCP content ratios and the same membrane on the healing pattern should also be elucidated.

Ⅴ. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ⅵ. AUTHOR CONTRIBUTIONS

You-Kyoung Kim and Yin-Zhe An contributed equally to this work.

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