J. Mar. Biosci. Biotechnol. 2015, p. 58 - 70 Vol. 7, No. 2 [Research Paper]

http://dx.doi.org/10.15433/ksmb.2015.7.2.058 ISSN 2383-5400 (Online)

Bone Formation Effect of the RGD-bioconjugated Mussel Adhesive Proteins Composite Hydroxypropyl Methylcellulose Hydrogel Based Nano Hydroxyapatite and Collagen Membrane in Rabbits

Dong-Myong Kim[1,2*], Hyun-Cho Kim[2], Chang-Ho Yeun[2], Che-Hyun Lee[3], Un-Yun Lee[3], Hun-Yu Lim[3], Young-An Chang[3], Young-Dae Kim[3], Sung-Ju Choi[3], Chong-Suk Lee[3], Hyung Joon Cha[4]

[1]Lab. of Natural Resources Molecular Analysis, Seoul National University, Seoul 151-742, Republic of Korea
[2]Probiomimetic R&D Center, Purgo Ltd., Seongnam 463-760, Republic of Korea
[3]Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry, Seoul National University, Seoul 110-768, Republic of Korea
[4]Marine BioMaterials Research Center, Pohang University of Science and Technology, Pohang 790-784, Korea

(Received 14 December 2015, Revised 13 January 2016, Accepted 14 January 2016)

Abstract Injectable RGD-bioconjugated Mussel Adhesive Proteins (RGD-MAPs) composite hydroxypropyl methylcellulose (HPMC) hydrogels provide local periodontal tissue for bone filling in periodontal surgery. Previously we developed a novel type of injectable self-supported hydrogel (2 mg/ml of RGD-MAPs/HPMC) based porcine nano hydroxyapatite (MPH) 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 MPH and collagen membrane in rabbit calvarial defects. Eight male New Zealand rabbits were used and four circular calvarial defects were created on each animal. Defects were filled with different graft materials: 1) collagen membrane, 2) collagen membrane with MPH, 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 MPH 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 MPH, BBH and collagen membrane group at all healing periods. Within the limits of this study, collagen membrane with MPH was an effective material for bone formation and space maintaining in rabbit calvarial defects.

Keywords : bone formation, RGD-bioconjugated mussel adhesive protein, hydroxypropyl methylcellulose, hydrogel based nano hydroxyapatite, collagen membrane, calvaria

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 [1,2,3,4,5].

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 [5,6,7]. To avoid or minimize the complications related to autografts, re-

* Corresponding author
Phone: 82-10-8164-4524 Fax: 82-2-2649-4524
E-mail: hapyvirus@naver.com

searchers 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]. Porcine bone is being considered as an alternative to bovine bone [14,15,16,17,18]. Like demineralized bovine bone, demineralized porcine 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 (Fig. 1, 2) [19]. Demineralized porcine 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, porcine nano HA is more similar to human osseous tissue than synthetic HA is in chemical and histological structure [14,20,21,22,23,24]. Thus it has a higher biocompatiblity, better fusion on the trans plant site, and a high bond strength, and is being used

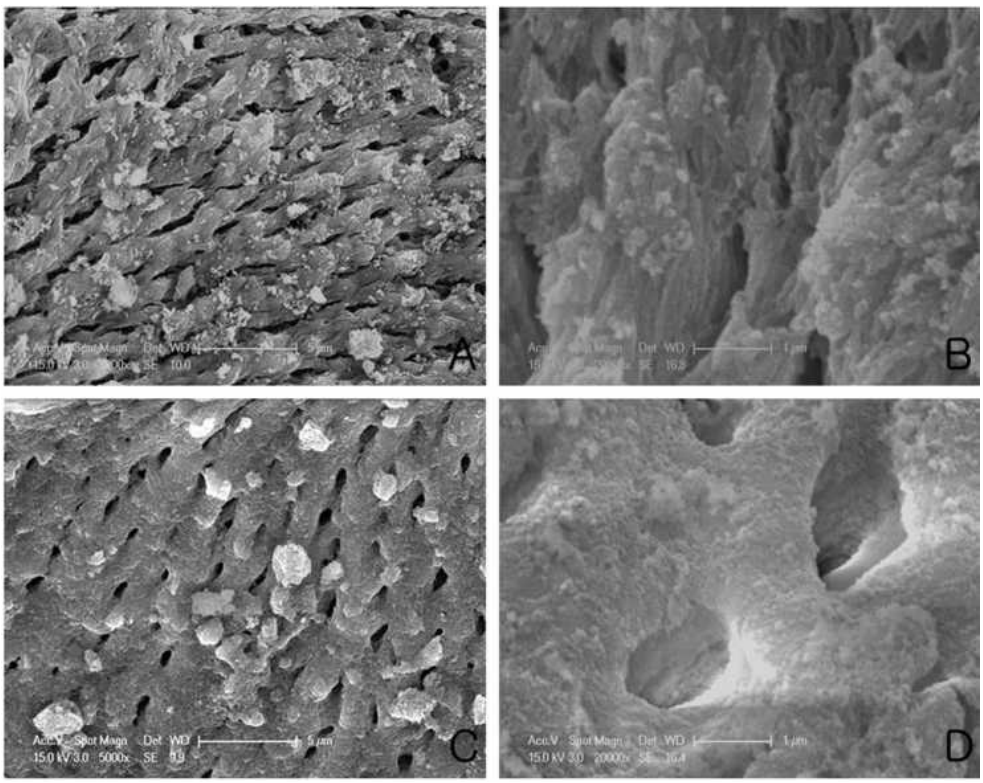

Figure 1. SEM images of BBH (A,B) and MPH (C,D). (A,C, X 5000; B,D, X 20000).

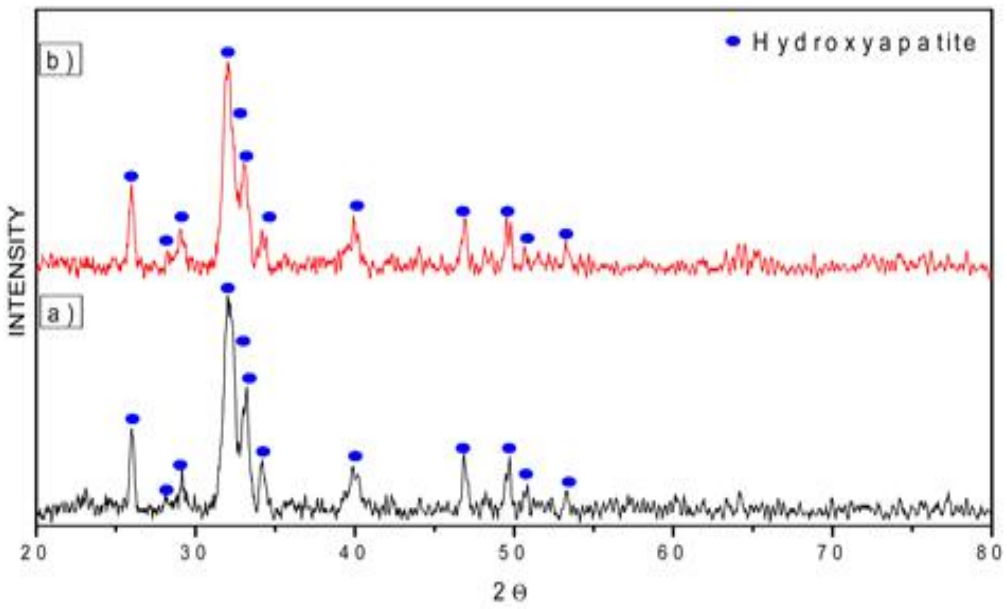

Figure 2. XRD charts of MPH (a) and BBH (b).

as an effective graft material for bone regeneration [16,25,26]. In clinical trials, promoting bone formation by covering the top of the graft materials with a membrane brings better results than using the graft materials alone [27]. 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 [28,29,30].

Mussel adhesive 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 bioadhesives from marine mussels. However, practical applications of Dopa-containing mussel adhesive proteins have been severely limited by uneconomical extraction and unsuccessful large-scale production [31]. Availability of large quantities of recombinant mussel adhesive proteins will enable to develop practical bioadhesives for diverse applications. Recent developed new hybrid types of mussel-inspired adhesive proteins might enable to realize this dream. Therefore, the researchers need to use several adhesive proteins simultaneously to develop bioadhesive materials with more practical and better properties, and based on these developed bioadhesives, they should find novel biological applications including gene and drug delivery, anti-biofouling coatings, medical device coatings, and surgical sealants [32,33,34].

Hwang *et al*. investigated the cell adhesion and spreading abilities of a new type of cell adhesion biomaterial, RGD-fp151 proteins, which is a fusion of the RGD motif to hybrid MAP fp151. They found that RGD-fp151 had the advantages of fp151, such as high production yield and simple purification, but also showed superior cell adhesion and spreading abilities under serum free conditions regardless of mammalian cell type compared with other widely used cell adhesion materials such as PLL and Cell-Tak [35,36].

Hydroxypropyl methylcellulose (HPMC) 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 swellability were greatly improved by the addition of HPMC 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 macroporous 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 RGD-bioconjugated mussel adhesive proteins (RGD-MAPs) composite HPMC hydrogel based porcine nano 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 RGD-MAPs/HPMC) based porcine nano hydroxyapatite (MPH) 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 MPH was up to 700 g bloom, and also no cytotoxic effects were observed [42,43,44].

In this study, MPH and bovine bone hydroxyapatite (BBH) covered by cross-linked resorbable collagen membrane were transplanted into calvarial 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 Seoul National University College of Medicine.

Materials

The graft materials used in this experiment included 0.25-1.0 mm particles of RGD-MAPs hydrogel based porcine nano hydroxyapatite (MPH, Probiomimetic R&D Center, Seoul, Korea) and 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 porcine-based 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 calvaria 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. MPH transplant group, collagen membrane: MPH 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 calvarial surgical site was depilated then disinfected with povidone iodine given infiltration anesthesia with 2% lidocaine (Lidocaine HCl, Huons, Seoul, Korea). The frontal bone was incised from the front portion to the back following the sagittal suture, and the calvaria 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 (Fig. 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.

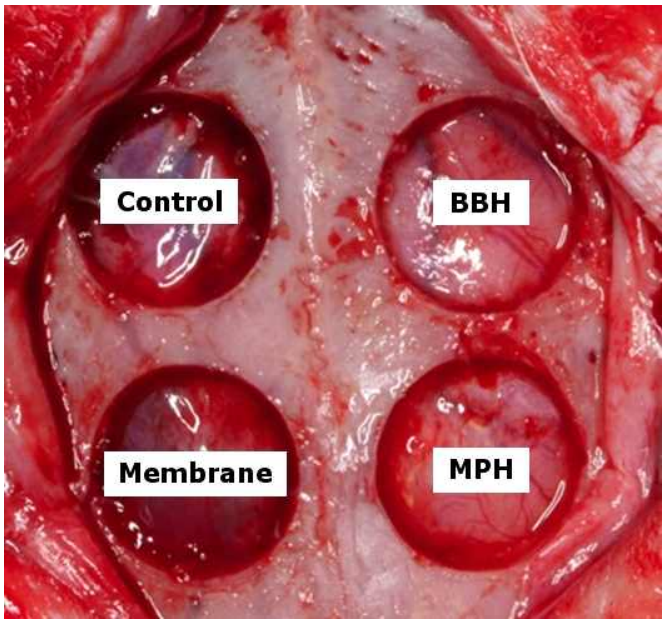

Figure 3. Schematic diagram showing the histometric analysis.

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 ㎛, 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 cybernetics, Silver Spring, Maryland, USA) (Fig. 4).

1) Total augmented area (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 (mm^2): The area of new bone on the defect site.

3) Residual particle (mm^2): The area of the remaining graft material on the defect site.

4. 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.

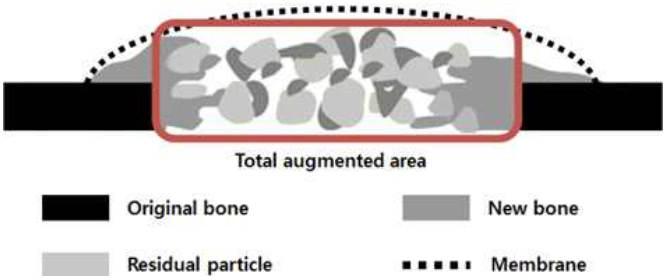

Figure 4. Calvarial defect formation and filled with bone mate rials.

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 bone were present in the center of the defect for some samples (Fig. 5).

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 (Fig. 6).

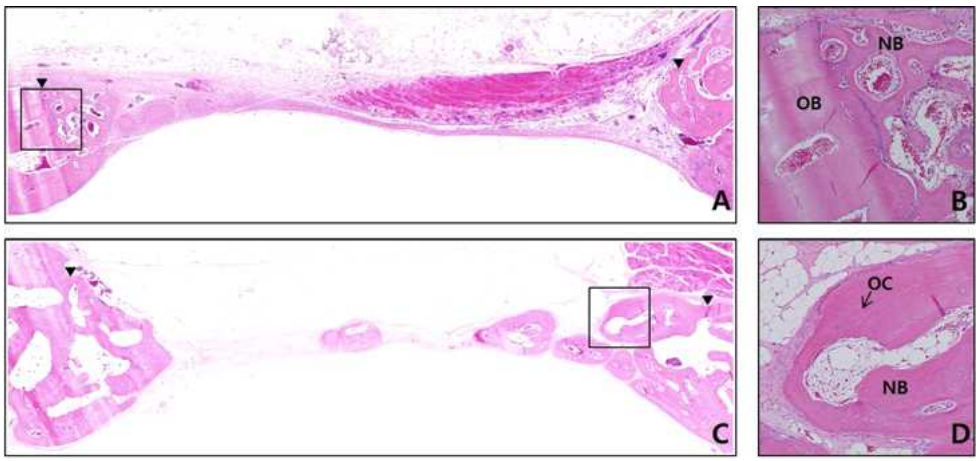

Figure 5. Transversal histologic presentation of control group at 2 weeks (A, B) and 8 weeks (C, D). Arrow head: defect margin, NB: new bone, OB: original bone, OC: osteocyte (H&E stain; original magnification: X 40 (A, C), X 100 (B, D)).

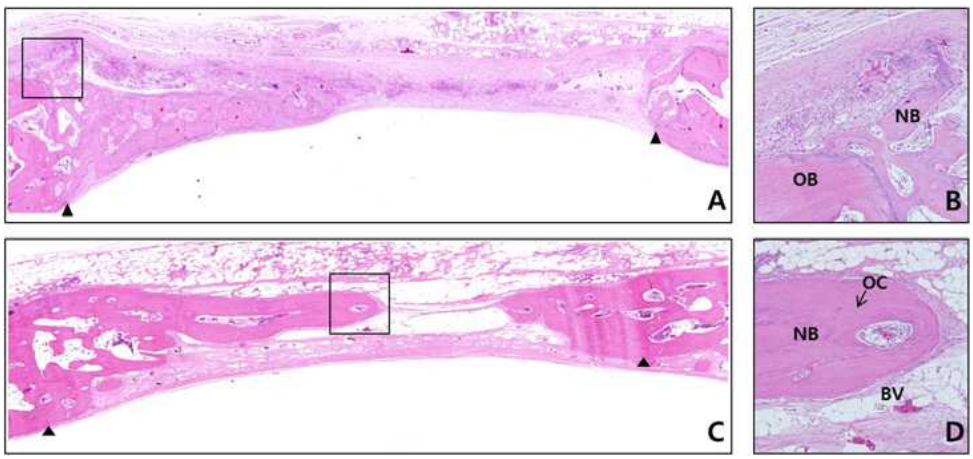

Figure 6. Transversal histologic presentation of membrane group at 2 weeks (A, B) and 8 weeks (C, D). Arrow head: defect margin, NB: new bone, OB: original bone, OC: osteocyte, BV: blood vessel (H&E stain; original magnification: X 40 (A, C), X 100 (B, D)).

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, re-

sulting 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 (Fig. 7).

4. MPH 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 (Fig. 8).

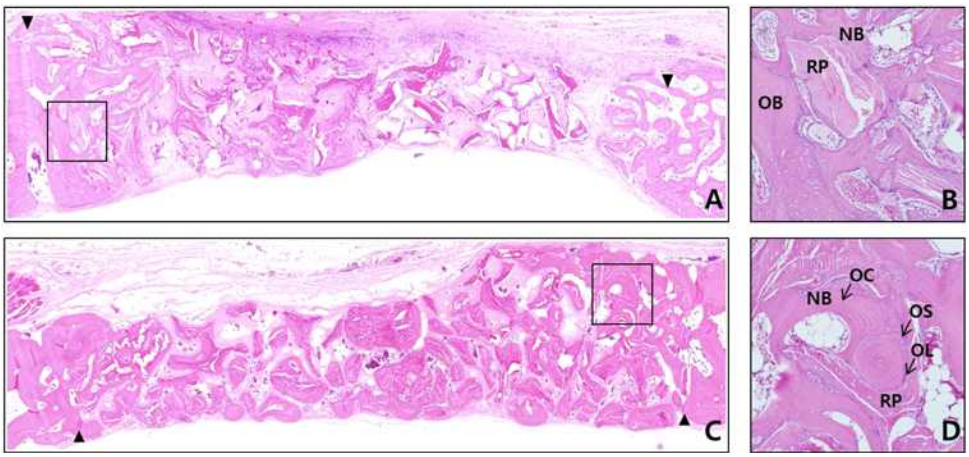

Figure 7. Transversal histologic presentation of BBH group at 2 weeks (A, B) and 8 weeks (C, D). Arrow head: defect margin, NB: new bone, OB: original bone, RP: residual particle, OC: osteocyte (H&E stain; original magnification: X 40 (A, C), X 100 (B, D)).

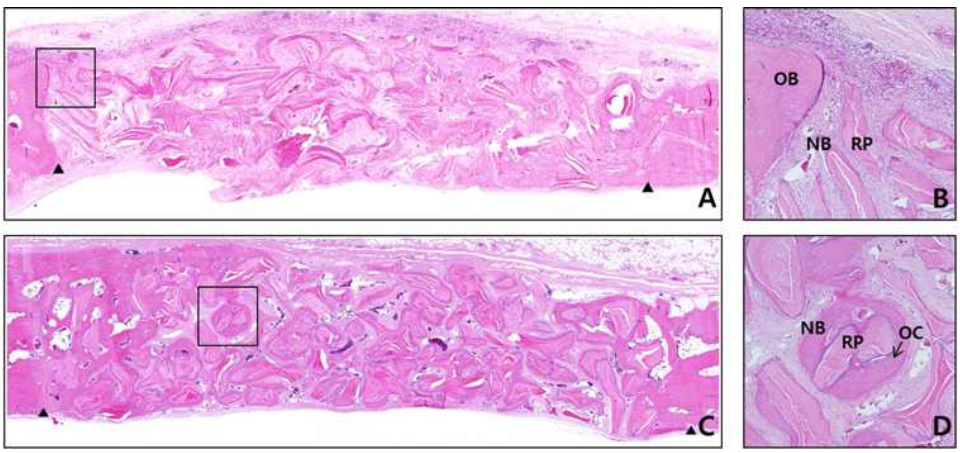

Figure 8. Transversal histologic presentation of MPH group at 2 weeks (A, B) and 8 weeks (C, D). Arrow head: defect margin, NB: new bone, OB: original bone, RP: residual particle, OC: osteocyte, OS: osteoblast, OL: Osteoblast lining (H&E stain; original magnification: X 40 (A, C), X 100 (B, D)).

5. Histomorphometrical observations

After two weeks and eight weeks, the MPH 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 MPH group, BBH group, membrane group. Both the control and experimental groups had no statistical significance

in tissue growth (Fig. 9).

The MPH, 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 MPH 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 (Fig. 10).

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

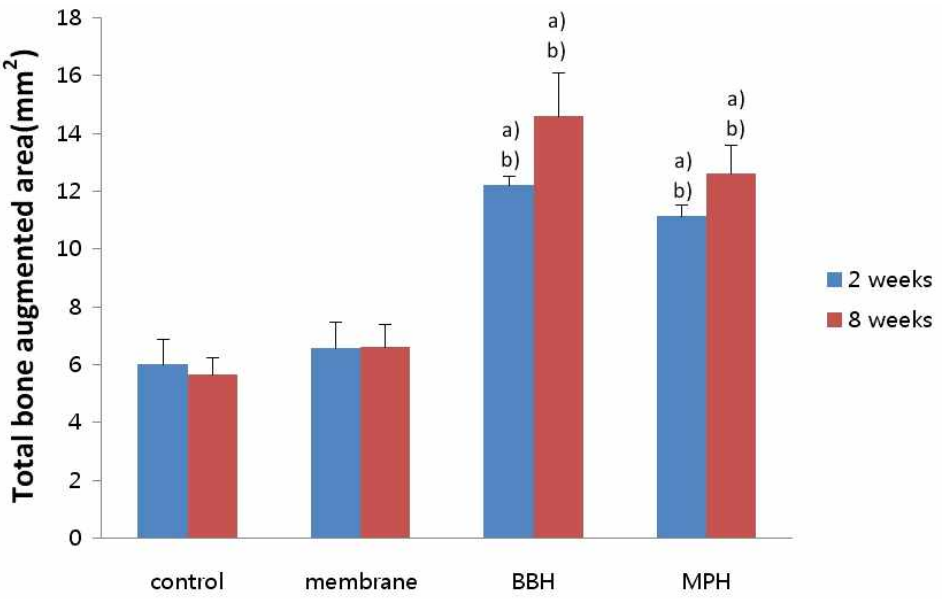

Figure 9. Total bone augmented area of histometric results at 2, 8 weeks. [a)]Significant statistical difference compared to control group at each week ($p < 0.05$). [b)]Significant statistical difference compared to membrane group at each week ($p < 0.05$).

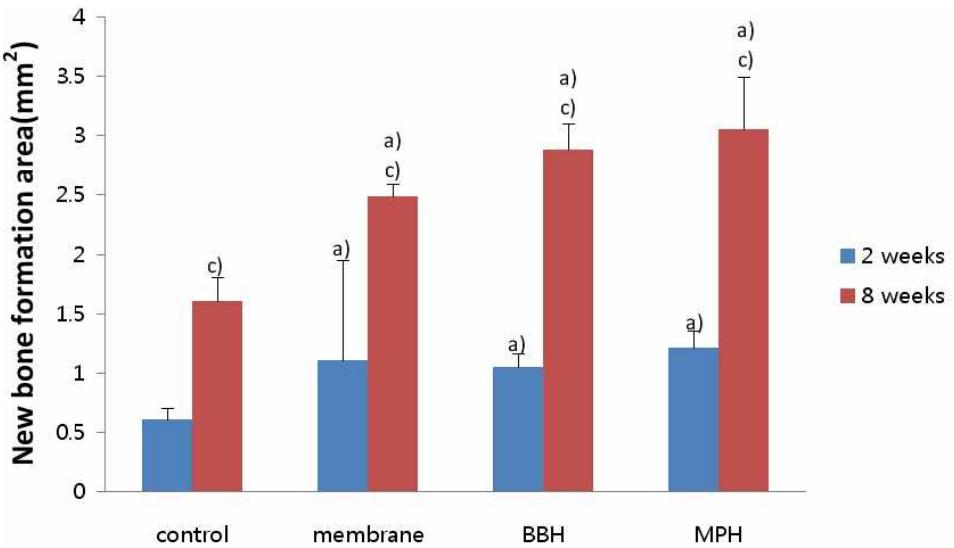

Figure 10. Total new bone formation of histometric results at 2, 8 weeks. [a)]Significant statistical difference compared to Control group at each week ($p < 0.05$). [c)]Significant statistical difference compared to 2 weeks ($p < 0.05$).

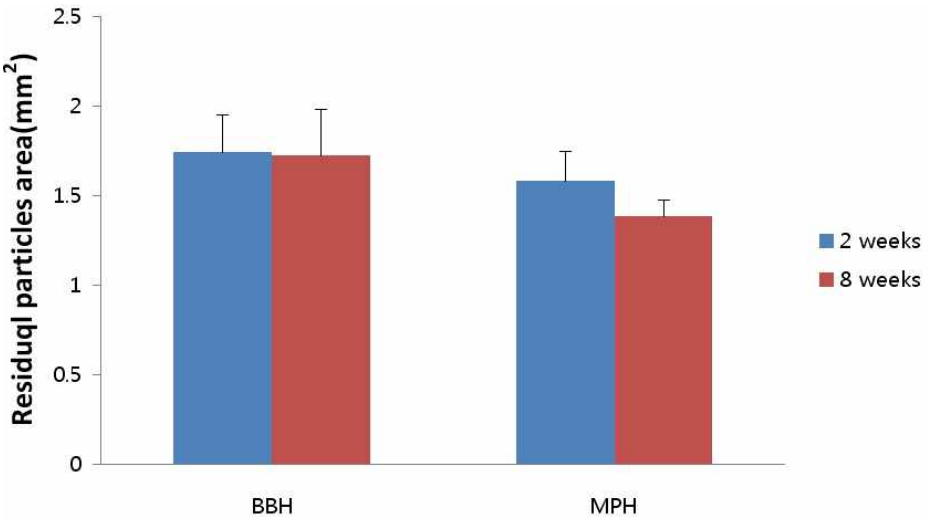

Figure 11. Residual particles area of histometric results at 2, 8 weeks.

Table 1. Histometric results after 2 and 8 weeks of healing (mm^2)

Parameters	Control	Membrane	BBH	MPH
2 weeks (n = 4)				
Augmented area	6.01 ± 1.75	6.56 ± 1.82	12.19 ± 0.66[a)b)]	11.12 ± 0.83[a)b)]
New bone area	0.60 ± 0.20	1.10 ± 0.16[a)]	1.05 ± 0.23[a)]	1.21 ± 0.28[a)]
Residual particles			1.74 ± 0.42	1.58 ± 0.34
8 weeks (n = 4)				
Augmented area	5.64 ± 1.17	6.61 ± 1.54	14.60 ± 2.97[a)b)]	12.61 ± 1.92[a)b)]
New bone area	1.60 ± 0.41[c)]	2.48 ± 0.22[a)c)]	2.88 ± 0.43[a)c)]	3.05 ± 0.89[a)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 formation capacity of MPH and BBH were compared by making four 8 mm wide defects divided into the control group, membrane group, BBH transplant group and MPH transplant group, and evaluating their abilities in bone formation and osseous tissue growth after two weeks and eight weeks. Although a critical size of 10-15 mm is normally used to test bone formation in bone defects [28,29,30], 8 mm defects have also been shown to be useful in evaluating the bone formation capacity of bone graft materials [45]. The bone defects of rabbit calvaria 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 [46]. 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 MPH 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 MPH 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 MPH 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.* [18] in 2005.

Porcine bone and bovine bone differ in micromorphological surface structure and chemical composition. At the micrometer scale, porcine 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 porcine bone and bovine bone into rabbit calvarial 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 porcine bone and bovine bone to rat calvarial defects and evaluated them eight weeks after, porcine 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 porcine bone was also greater than that of bovine bone [47]. This supports the data from this study that the amount of new bone formed by porcine nano hydroxyapatite is similar to that formed by bovine bone hydroxyapatite.

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 fibrillar 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 hydroxyapatite with a membrane as opposed to the membrane alone hinders the resorption of the bone graft material, limiting and furthermore interfering with osteogenesis [48], there was no statistically significant difference in bone formation between the membrane, MPH and BBH groups after two weeks and eight weeks in this study. The area of osseous tissue growth in the MPH 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 [49]. 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 have a slower resorbability than non cross-linked membranes, making them useful in bone regeneration [50]. However, using the membrane alone hinders space maintainence and osseous tissue growth. Since using a membrane in combination with bone graft material allows better space maintenance and tissue growth [51], 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 MPH 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 resorb-

able membranes and non cross-linked resorbable membranes. Further studies and comparison studies on membranes will be needed from many individuals.

Conclusions

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 RGD-MAPs hydrogel based porcine hydroxyapatite (MPH), the following conclusions were made:

1. MPH 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 maintainence is poor. For effective bone regeneration and osseous tissue growth, a bone graft should be used with the membrane.

Acknowledgements

This work was supported by the Marine Biotechnology Program (Marine BioMaterials Research Center) funded by the Ministry of Oceans and Fisheries, Korea.

References

1. Darby, I. 2011. Periodontal materials. *Aust. Dent. J.*, **56,** 107-118.
2. Sculean, A., Nikolidakis, D. and Schwarz F. 2008. Regene ration of periodontal tissues: combinations of barrier mem branes and grafting materials - biological foundation and preclinical evidence: a systematic review. *J. Clin. Periodo ntol.* **35,** 106-116.
3. Nkenke, E. and Stelzle, F. 2009. Clinical outcomes of sinus floor augmentation for implant placement using aut ogenous bone or bone substitutes: a systematic review. *Clin. Oral Implan. Res.* **20,** 124-133.
4. Chiapasco, M. and Zaniboni, M. 2009. Clinical outcomes of GBR procedures to correct peri-implant dehiscences and fenestrations: a systematic review. *Clin. Oral Implan. Res.* **20,** 113-123.
5. Wang, H. L., Greenwell, H. and Fiorellini, J. 2005. Period ontal regeneration. *J. Periodontol.* **76,** 1601-1622.
6. Hoexter, D. L. 2002. Bone regeneration graft materials. *J. Oral Implant.* **28,** 290-294.
7. Marx, R. E. 1994. Clinical application of bone biology to mandibular and maxillary reconstruction. *Clin. Plast. Surg.* **21,** 377-392.
8. Schwartz, Z., Somers A. and Mellonig J.T. 1998. Ability of commercial demineralized freeze-dried bone allograft to induce new bone formation is dependent on donor age but not gender. *J. Periodontol.* **69,** 470-478.
9. Reynolds, M. A., Aichelmann-Reidy, M. E., Branch-May s, G. L. and Gunsolley, J. C. 2003. The efficacy of bone replacement grafts in the treatment of periodontal osseous defects. *Annals of Periodontology/The American Academ y of Periodontology* **8,** 227-265.
10. Ducheyne, P. and Qiu, Q. 1999. Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. *Biomaterials* **20,** 2287-2303.
11. Pinholt, E. M., Bang, G. and Haanaes, H. R. 1991. Alveo lar ridge augmentation in rats by Bio-Oss. *Scand. J. Dent. Res.* **99,** 154-161.
12. Haas, R., Mailath, G., Dortbudak, O. and Watzek, G. 199 8. Bovine hydroxyapatite for maxillary sinus augmentatio n: analysis of interfacial bond strength of dental implants using pull-out tests. *Clin. Oral Implan. Res.* **9,** 117-122.
13. Kim, Y., Nowzari, H. and Rich, S. K. 2011. Risk of prion disease transmission through bovine-derived bone substitutes. *Clin. Implant Dent. R.* **15,** 120-130.
14. Kim, S. H., Shin, J. W. and Park, S. A. 2004. Chemical, structural properties, and osteoconductive effectiveness o f bone block derived from porcine cancellous bone. *J. Biomed. Mater. Res. B, Applied biomaterials* **68,** 69-74.
15. Park, J. W., Ko, H. J., Jang, J. H., Kang, H. and Suh, J. Y. 2012. Increased new bone formation with a surface magnesium-incorporated deproteinized porcine bone sub stitute in rabbit calvarial defects. *J. Biomed. Mater. Res. A* **100,** 834-840.
16. Pagliani, L., Andersson, P. and Lanza, M. 2012. A collage nated porcine bone substitute for augmentation at Neoss implant sites: a prospective 1-year multicenter case series study with histology. *Clin. Implant Dent. R.* **14,** 746-758.

17. Orsini, G., Scarano, A., Piattelli, M., Piccirilli, M., Caput i, S. and Piattelli, A. 2006. Histologic and ultrastructural analysis of regenerated bone in maxillary sinus augmenta tion using a porcine bone-derived biomaterial. *J. Periodo ntol.* **77,** 1984-1990.

18. Barone, A., Crespi, R., Aldini, N. N., Fini, M., Giardino, R. and Covani, U. 2005. Maxillary sinus augmentation: histologic and histomorphometric analysis. *Int. J. Oral Max. Impl.* **20,** 519-525.

19. Kim, D. M., Kim, H. J., Kang, H. C., Ahn, H. C., Kim, Y. M., Kim, H. S., Ahn, J. S., Jun, S. H., Choi, B. H. and Cha, H. J. 2014. Comparison with bone formation effect of different xenograft materials in calvarial bone defect in rabbits. *J. Korean Dent. Sci.* **114,** 78-83.

20. Kim, D. M., Kim, H. J., Kang, H. C., Kim, H. S. and Lee J. H. 2014. Development of advanced dental porcine cancellous bone graft with nano hydroxyapatite surface treatment (The Graft-HA NPs) and biocompatibility eval uation in calvarial bone defect in rabbit. *Biomater. Res.* **36,** 45-56.

21. Kim, D. M., Kim, H. J., Kang, H. C., Kim, H. S., Kim, D. H., Kim, K. I., Yun, S. Y., Han, K. H., Kim, J. Y. and Lee, J. H. 2014. A method to improve the biological activity of porcine bone graft materials by chemically attaching hydroxyapatite nanoparticles (HA NPs) with different shapes on the material surface for dental applic ations. *J. Biomed. Eng. Res.* **55,** 87-94.

22. Kim, D. M., Kim, H. J., Kang, H. C., Kim, H. S. and Lee, J. H. 2014. Method of funtionalize surface of bone graft with bone nano particle and bone graft having funti onalized surface by bone nano particle. *Kor. Patent.* No. 10-2014-0078335**,** 1-28.

23. Kim, D. M., Kim, H. J., Kang, H. C., Kim, H. S., Kim, D. H., Kim, K. I., Yun, S. Y., Han, K. H., Kim, J. Y. and Lee, J. H. 2014. A method for preparing a porcine bone graft with an excellent performance in cell adhesio n and bone formation using nano hydroxyapatite surface modification technology, and a porcine bone graft prepar ed thereby. *Kor. Patent.* No. 10-2014-0078335**,** 1-30.

24. Kim, D. H., Kim, K. I., Yoon, S. Y., Kim, H. J., Ahn, J. S., Jun, S. H., Kang, H. C., Pang, C. H., Kim, J. Y., Cha, H. J., Han, K. H., Kim, D. M. and Lee, J. H. 2015. Dental hetero-graft materials with nano hydroxyapatite su rface treatment. *J. Nanosci. Nanotechno.* **15,** 7942-7949.

25. Scarano, A., Piattelli, A. and Assenza, B. 2010. Porcine bone used in sinus augmentation procedures: a 5-year retrospective clinical evaluation. *J. Oral Maxil. Surg.* **68,** 1869-1873.

26. Scarano, A., Piattelli, A., Perrotti, V., Manzon, L. and Iezzi, G. 2011. Maxillary sinus augmentation in humans using cortical porcine bone: a histological and histomorp hometrical evaluation after 4 and 6 months. *Clin. Implant Dent. R.* **13,** 13-18.

27. Lundgren, A. K., Sennerby, L., Lundgren, D., Taylor, A., Gottlow, J. and Nyman, S. 1997. Bone augmentation at titanium implants using autologous bone grafts and a bioresorbable barrier. An experimental study in the rab bit tibia. *Clin. Oral Implan. Res.* **8,** 82-89.

28. Pripatnanont, P., Nuntanaranont, T. and Vongvatcharano n, S. 2009. Proportion of deproteinized bovine bone and autogenous bone affects bone formation in the treatment of calvarial defects in rabbits. *Int. J. Oral Maxillofac. Surg.* **38,** 356-362.

29. Xu, S., Lin, K. and Wang, Z. 2008. Reconstruction of calvarial defect of rabbits using porous calcium silicate bioactive ceramics. *Biomaterials.* **29,** 2588-2596.

30. Kim, D. M., Kim, H. J., Kang, H. C., Choi, J. H. and Assaf, A. G., 2014. Novel non-chemical cross-linking porcine deri ved collagen nanofibrous membrane (Biocover plus®) for the sustained degradation behavior of biomolecules imagi ng agents and for assisting the growth of human gingival cells. *Tissue Eng. Regen. Med.* **45**, 63-69.

31. Waite, J. H. and Tanzer, M. L. 1981. Polyphenolic substa nce of *Mytilus edulis*-novel adhesive containing L-DOP A and hydroxyproline. *Science.* **212,** 1038-1040.

32. Dove, J. and Sheridan, P. 1986. Adhesive protein from mussels: possibilites for dentistry, medicine, and industr y. *J. Am. Dent. Assoc.* **112,** 879-877.

33. Lee, H., Dellatore, S. M., Miller, W. M. and Messersmit h, P.B. 2007. Mussel-inspired surface chemistry for mult ifunctional coatings. *Science.* **318,** 426-430.

34. Kim, D. M., Kang, H. C., Ahn, J. S., Jun, S. H., Choi, B. H., Cha, H. J., Kim, J. E. and Choi, J. H. 2015. Formu lation of a smart released HPMC drug delivery system (Cell Binder®) containing Mussel adhesive proteins (MA Ps) and porcine bone graft (The Graft®) for use in the treatment of periodontal disease and tissue regeneration. *Tissue Eng. Regen. Med.* **46,** 67-73.

35. Hwang, D. S., Gim, Y., Yoo, H. J. and Cha, H. J. 2007. Practical recombinant hybrid mussel bioadhesive fp-151. *Biomaterials.* **28,** 3560-3568.

36. Hwang, D. S., Sim, S. B., and Cha, H. J. 2007. Cell adhesion biomaterial based on mussel adhesive protein fused with RGD peptide. *Biomaterials.* **28,** 4039-4046.

37. Colombo, P. 1993. Swelling-controlled release in hydrog el matrices for oral route. *Adv. Drug Deliver. Rev.* **11,** 37-57.

38. Doelker, E. 1986. Water-swollen cellulose derivatives in pharmacy. in: N.A. Peppas (Ed.). *Hydrogels in Medici ne and Pharmacy.* **2,** 115-160.

39. Brannon-Peppas, L. 1990. Preparation and characterizati on of cross-linked hydrophilic networks. *Absorbent Poly mer Technology.* **23,** 45-66.

40. Stankewich, C. J., Swiontkowski, M. F., Tencer. A. F., Yetkinler, D. N. and Poser, R. D. 1996. Augmentation of femoral neck fracture fixation with an injectable calciu m-phosphate bone mineral cement. *J. Orthop. Res.* 1**4,** 786-793.

41. Weiss, P., Gauthier, O., Bouler, J., Grimandi, G., and Daculsi, G. 1999. Injectable bone substitute using a hydr ophilic polymer. *Bone.* **25**, 67S-70S.

42. Kim, D. M., Kim, H. J., Kang, H. C., Ahn, H. C., Kim, Y. M., Kim, H. S., Ahn, J. S., Jun, S. H., Choi, B. H. and Cha, H. J. 2014. Comparison of Bone Repair Treated with mussel adhesive protein (MAPs) nanofibrous porcin e graft (Cell-Binder®) in rat calvaria. *J. Biomed. Eng. Res.* **58,** 58-66.

43. Kim, D. M., Kim, H. J., Kang, H. C., and Cha, H. J. 2015. Mussel adhesive protein-derived bone binder for preventing or treating periodontal disease and method for preparing the same. *Kor. Patent.* No. 10-2015-0061487**,** 1-37.

44. Kim, D. M., Kim, H. J., Kang, H. C., Ahn, H. C., Kim, Y. M., Kim, H. S., Ahn, J. S., Jun, S. H., Choi, B. H. and Cha, H. J. 2014. Histological study on the comparison of bone repair treated with novel mussel adhesive protein (MAPs) nanofibrous composited of porcine graft (Cell-Bin der®) or not with two different grafts (Bio-Oss® and The Graft®) in rat calvaria. *J. Periodontal Res.* **128,** 121-129.

45. Sohn, J. Y., Park, J. C. and Um, Y. J. 2010. Spontaneous healing capacity of rabbit cranial defects of various sizes. *J. Periodontal Implant Sci.* **40,** 180-187.

46. Misch, C. E. 1999. Contemporary Implant Dentistry, 2nd ed; Mosby, St. Louis. *Implant Dent.* **1,** 320-330.

47. Yoo, K. H., Shim, K. M., Park, H. J. and Choi, S, H. 2010. Effect of porcine cancellous bones on regeneration in rats with calvarial defect. *J. Life Sci.* **20,** 1207-1213.

48. Stavropoulos, A., Kostopoulos, L., Nyengaard, J. R. and Karring, T. 2003. Deproteinized bovine bone (Bio-Oss®) and bioactive glass (Biogran®) arrest bone formation wh en used as an adjunct to guided tissue regeneration (GT R): an experimental study in the rat. *J. Clin. Periodontol.* **30,** 636-643.

49. Sela, M. N., Kohavi, D., Krausz, E., Steinberg, D. and Rosen, G. 2003. Enzymatic degradation of collagen-guid ed tissue regeneration membranes by periodontal bacteri a. *Clin. Oral Implan. Res.* **14,** 263-268.

50. Charulatha, V. and Rajaram, A. 2003. Influence of differ ent crosslinking treatments on the physical properties of collagen membranes. *Biomaterials.* **24,** 759-767.

51. Becker, J., Al-Nawas, B., Klein, M. O., Schliephake, H., Terheyden, H. and Schwarz, F. 2009. Use of a new cross -linked collagen membrane for the treatment of dehiscen ce-type defects at titanium implants: a prospective, rando mized-controlled double-blinded clinical multicenter stu dy. *Clin. Oral Implan. Res.* **20,** 742-749.