The Effects of Hydroxyapatite-Chitosan Membrane on Bone Regeneration in Rat Calvarial Defects

Jung-A Shin[1], Jung-Yoo Choi[1], Sung-Tae Kim[1], Chang-Sung Kim[1], Yong-Keun Lee[2],
Kyoo-Sung Cho[1], Jung-Kiu Chai[1], Chong-Kwan Kim[1], Seong-Ho Choi[1*]

1. Department of Periodontology, Research Institute for Periodontal Regeneration, Yonsei University, College of Dentistry
2. Department and Research Institute of Dental Biomaterials and Bioengineering, Yonsei University, College of Dentistry

ABSTRACT

Purpose: Recently, interest in chitosan has increased due to its excellent biological properties such as biocompatibility, antibacterial effect, and rapid healing capacity. On the other hand, hydroxyapatite is used as a bone substitute in the fields of orthopedics and dentistry. The hydroxyapatite-chitosan (HA-CS) complex containing hydroxyapatite nanoparticles was developed for synergy of both biomaterials. The objective of this study was to evaluate the effect of hydroxyapatite (HA)-chitosan (CS) membrane on bone regeneration in the rat calvarial defect.

Methods: Eight-millimeter critical-sized calvarial defects were created in 70 male Sprague-Dawley rats. The animals were divided into 7 groups of 10 animals and received either 1) chitosan (CS) 100% membrane, 2) hydroxyapatite (HA) 30%/CS 70% membrane, 3) HA 30%/CS 70%, pressed membrane, 4) HA 40%/CS 60% membrane, 5) HA 50%/CS 50% membrane, 6) HA 50%/CS 50%, pressed membrane, or 7) a sham - surgery control. The amount of newly formed bone from the surface of the rat calvarial defects was measured using histomorphometry, following 2- or 8- week healing intervals.

Results: Surgical implantation of the HA - CS membrane resulted in enhanced local bone formation at both 2 and 8 weeks compared to the control group. The HA - CS membrane would be significantly more effective than the chitosan membrane in early bone formation.

Conclusions: Concerning the advantages of biomaterials, the HA-CS membrane would be an effective biomaterial for regeneration of periodontal bone. Further studies will be required to improve the mechanical properties to develop a more rigid scaffold for the HA-CS membrane. *(J Korean Acad Periodontol 2009;39:213-222)*

KEY WORDS: bone regeneration; chitosan; hydroxyapatites.

INTRODUCTION

The main objective of periodontal treatment is not only to relieve symptoms but also to regenerate the destroyed tissues. Many methods have been introduced for regenerating damaged periodontal tissues. Guided tissue regeneration (GTR) therapy was introduced in the 1980s to achieve a repopulation of the periodontal ligament fibroblasts, and was shown to promote periodontal regeneration. The membrane barrier used in GTR should be histocompatible, biocompatible and have the capacity for space maintenance[1].

Absorbable membrane does not require a second surgical procedure, and membrane exposure is rare. Aquirre et al[2] showed new bone formation in bony defects using absorbable membrane. However, controlling the time of absorption is difficult and therefore could cause a localized inflammatory reaction. In addition to the above disadvantages, poor membrane stability in the wet state causes space loss between the tooth and the membrane, producing poor

clinical results[3]. An absorbable membrane should be used in GTR in places where exact initial closure is possible, since complete removal is difficult when exposed[4]. To solve this problem, many studies have been carried out on biodegradable membranes, and acceptable results were presented[5]. Theideal membrane should be absorbable and should not require removal after the tissue regenerates; it also should block tissue migration effectively and resist inflammatory reaction. Lastly, it must have space-maintaining capacity.

Recently, interest in chitosan has increased due to its excellent biological properties such as biocompatibility, antibacterial effect, and rapid healing capacity. Chitin and chitosan (poly—N—acetyl glucosaminoglycan), a carbohydrate biopolymer extracted from chitin, are the second most abundant natural biopolymers next to cellulose. Chitin is a primary structural component of the exoskeleton of arthropods (e.g. crustaceans), the cell wall of fungi, and the cuticle of insects. Chitin is a very stable polysaccharide and is a linear polymer of N—acetyl—D—glucosamine monomers joined in a $1,4\beta$ —glucosidic linkage. Chitosan is a derivative of chitin obtained by N—acetylating chitin. As with polymers in general, enzymes can hydrolyze chitin and chitosan. The most effective enzyme for this process is lysozyme[6,7].

Although the healing effects of chitin and chitosan on mammalian wounds have been known for centuries, it was not until the 1960s that the ingredients were documented[8].

Other studies since have suggested that chitosan enhances the formation of bone tissue and it could be used as the matrix of tissue engineering for gingiva[9]. Paik et al[10] reported that chitosan enhanced type Ⅰcollagen synthesis in the early stage, and facilitated differentiation into osteogenic cells in the human periodontal ligament fibroblasts in vitro. In addition, a chitosan/collagen sponge applied to one-wall intrabony defects surgically created in beagle dogs inhibited the apical migration of the epithelium and enhanced the growth of new bone and new cementum[11].

Another biomaterial of interest is hydroxyapatite, which is a major component of human bone. Hydroxyapatite is used as bone substitute in the fields of orthopedics and dentistry because of its good osteoconductivity, bioactivity and biocompatibility[12]. But hydroxyapatite is brittle and easy to fracture so it is difficult to mould into a specific shape. The hydroxyapatite—chitosan (HA—CS) complex, containing hydroxyapatite nano-particles, was therefore developed to overcome the original disadvantage of hydroxyapatite[13].

Although many materials are used to regenerate periodontal tissues, there is as yet no material that satisfies all conditions. This paper reports onthe fabrication of the HA—CS membrane in diverse proportion. The objective of this study was to evaluate bone regeneration capacity of HA—CS membrane in rat calvarial defects.

MATERIALS AND METHODS

This study included 70 male Sprague—Dawley rats (body weight 250—300 g) maintained in plastic cages in a room with a 12 hour day/night cycle and an ambient temperature of 21 ℃. The rats were allowed free access to water and standard laboratory fool pellets. Animal selection, management, surgical protocol, andpreparation followed the routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea(approval No.: 06—171).

Chitosan was dissolved in a 2% acetic acid solution, and then mixed with phosphoric acid solution. After $Ca(OH)_2$was added to this solution, HA was able to be synthesized. At that time, HA proportion was regulated, and 0:100, 30:70, 40:60, 50:50 (HA weight: CS weight) HA—CS composites were developed. After citric acid was added to each HA—CS composite, original solution for threading was developed. The HA—CS solution was filtered and threaded in a 10% NaOH sol-

ution and then washed and dried to develop the HA—CS membrane. Some of the membrane, HA 30%/CS 70 % and HA 50%/CS 50%, were pressed for advanced mechanical properties. The size of HA nanoparticles was 20–100 nm. All HA—CS membranes passed the cytotoxic test, MTT assay.

Eight—millimeter critical—sized calvarial defects were created in 70 male Sprague—Dawley rats. The animals were divided into 7 groups of 10 animals and received either 1) chitosan (CS) 100% membrane, 2) hydroxyapatite (HA) 30%/CS 70% membrane, 3) HA 30%/CS 70%, pressed membrane, 4) HA 40%/CS 60% membrane, 5) HA 50%/CS 50% membrane, 6) HA 50%/CS 50%, pressed membrane, or 7) a sham – surgery control.

The animals were sacrificed by CO_2 asphyxiation at 2 or 8 weeks post—surgery. The samples were fixed and decalcified and embedded in paraffin, stained with hematoxylin/eosin (H—E), and examined with an optical microscope. The sections were examined at 20x magnification for histometric evaluation. Measurements included % defect closure, % augmented bone area, and % new bone area (Fig. 1). Histometric recordings from the samples were used to calculate mean and standard deviations. We used one—way ANOVA, Least Significant Difference (LSD)method and paired t—test for statistical analysis.

RESULTS

1. Clinical observations

Wound healing was generally uneventful and appeared similar for all nonpressed—membrane experimental groups (Exp. 1, 2, 4 and 5). In some pressed—membrane experimental groups (Exp. 3 and 6), slight inflammatory reaction was observed at 2 weeks, but inflammation was not detected at 8 weeks. Material exposure or other complications of the surgical sites were not observed.

2. Histologic observations

1) Control

At 2 and 8 weeks post—surgery, defects filled with thin, loose connective tissue, with minimal new bone formation originating from the defect margins, were observed. The defect center had collapsed (Fig. 2).

2) Experimental groups

Inboth the chitosan—only and HA—CS membrane groups, the defects were filled with loose or dense, fibrous connective tissue, and limited new bone for-
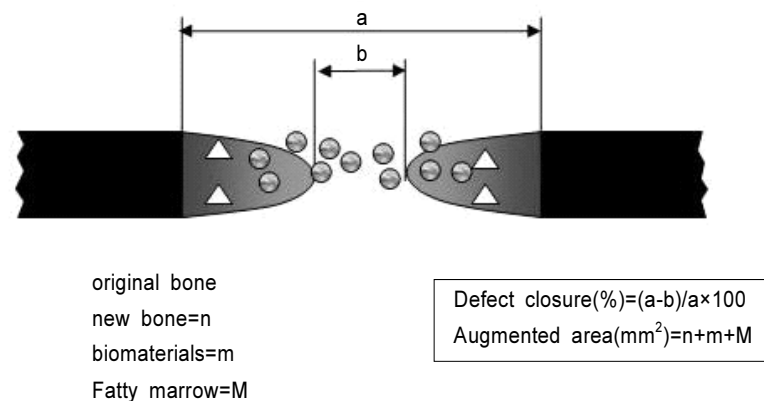

original bone
new bone=n
biomaterials=m
Fatty marrow=M

Defect closure(%)=(a-b)/a×100
Augmented area(mm^2)=n+m+M

Figure 1. Schematic drawings of calvarial osteotomy defect showing histometric analysis.
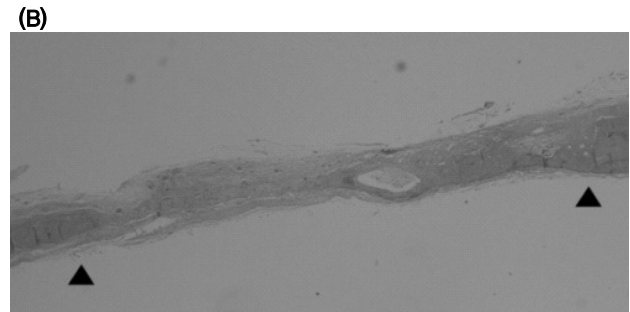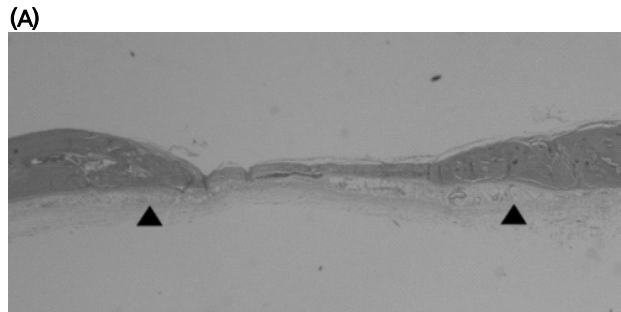
(A)

(B)

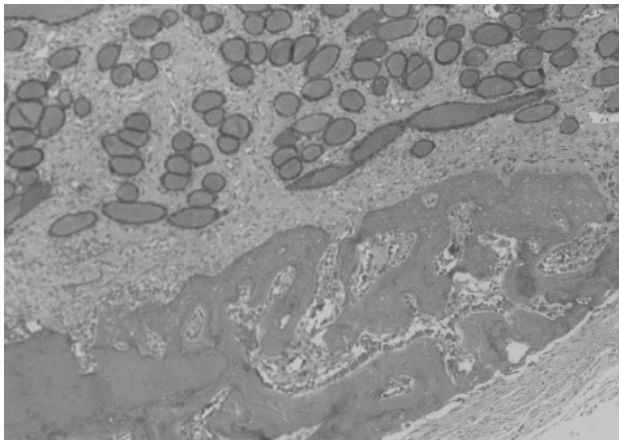**Figure 2.** *Control at (A) 2 and 8 (B) weeks postsurgery (X 20) (Arrow head: defect margin).*

Figure 3. *Exp–4: Hydroxyapatite 40%/ chitosan 60% membrane group, 2 weeks. A large number of residual chitosan fibers and hydroxyapatite particles were observed within the augmented area (H–E stain; original magnification X 100).*
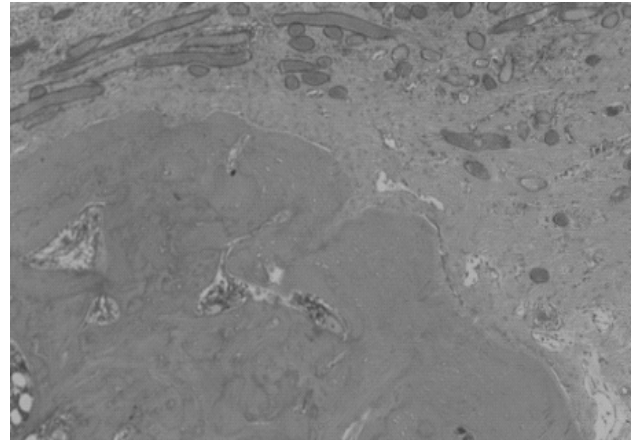
Figure 4. *Exp–6: Hydroxyapatite 50%/ chitosan 50%, pressed membrane group, 8 weeks. The membrane was widely absorbed away and a growth of new bony tissue is observed around the membrane (H–E stain; original magnification X 100).*
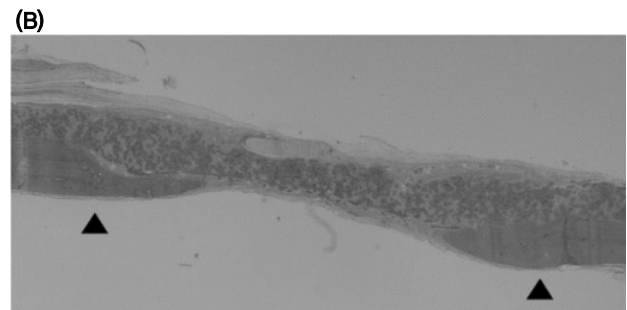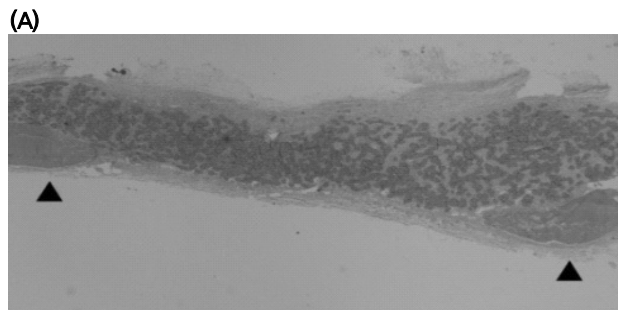
(A)

(B)

Figure 5. *Exp–1: chitosan membrane group at (A) 2 and 8 (B) weeks postsurgery. The defect is covered with fibrous tissue. (Arrow head: defect margin H–E stain; original magnification X 20).*
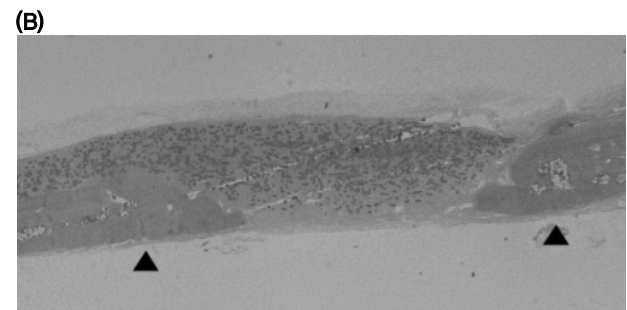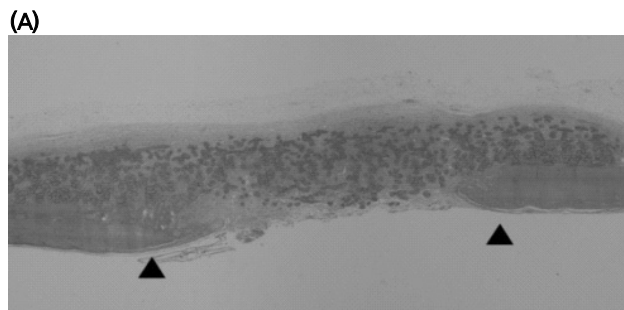
(A)

(B)

Figure 6. *Exp–2: Hydroxyapatite 30%/ chitosan 70% membrane group at (A) 2 and 8 (B) weeks postsurgery (Arrow head: defect margin H–E stain; original magnification X 20).*
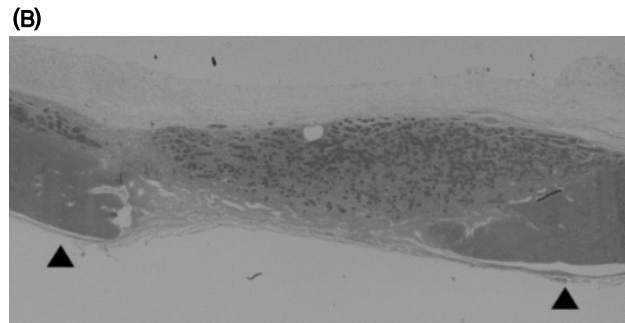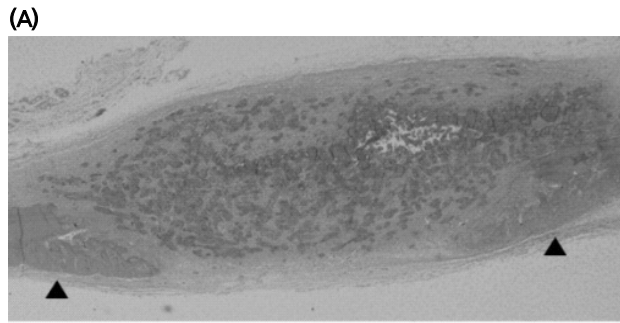
(A)

(B)

Figure 7. Exp-3: Hydroxyapatite 30%/ chitosan 70%, pressed membrane group at (A) 2 and (B) 8 weeks postsurgery. The augmented area was decreased after 8 weeks(Arrow head: defect margin H-E stain; original magnification X 20).

(A)

(B)

Figure 8. Exp-4: Hydroxyapatite 40%/ chitosan 60% membrane group at (A) 2 and (B) 8 weeks postsurgery(Arrow head: defect margin H-E stain; original magnification X 20).
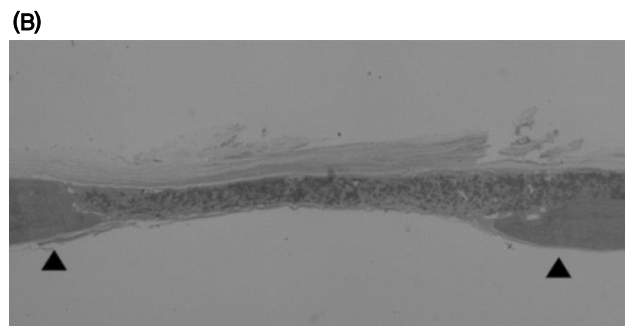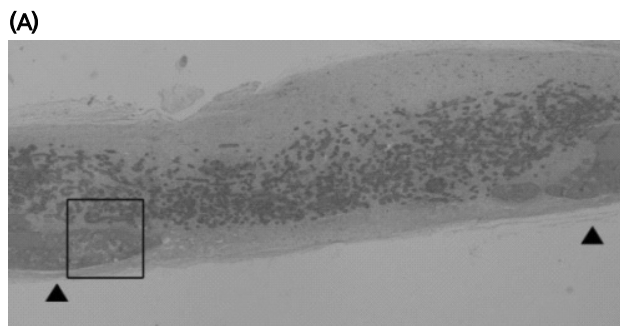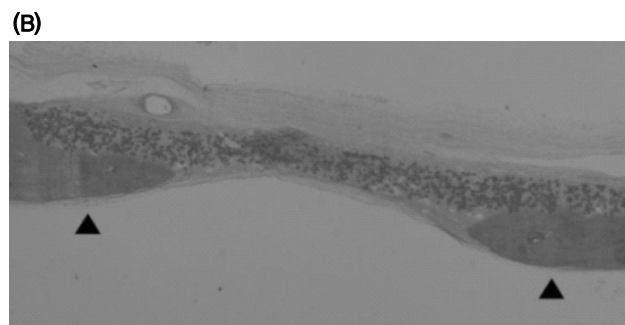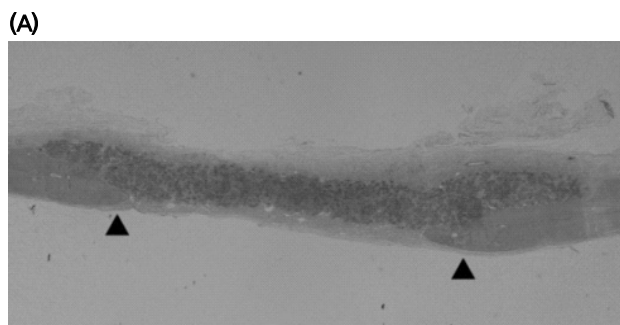
(A)

(B)

Figure 9. Exp-5: Hydroxyapatite 50%/ chitosan 50% membrane group at (A) 2 and (B) 8 weeks postsurgery(Arrow head: defect margin H-E stain; original magnification X 20).
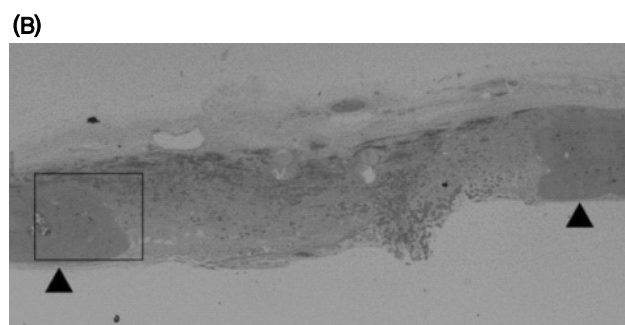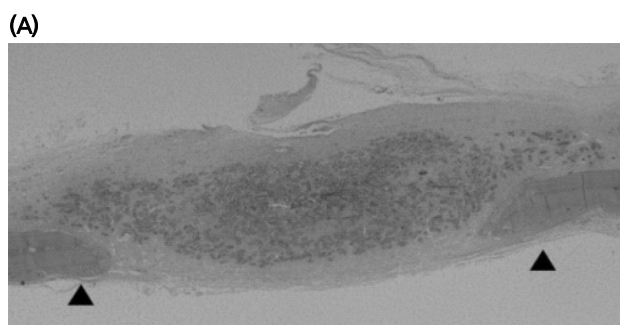
(A)

(B)

Figure 10. Exp-6: Hydroxyapatite 50%/ chitosan 50%, pressed membrane group at (A) 2 and (B) 8 weeks postsurgery (Arrow head:defect margin H-E stain; original magnification $\times$ 20).

mation was observed at the defect margins at 2 weeks. A large number of residual chitosan fibers and hydroxyapatite particles were observed within the new bone at 2 weeks (Fig. 3), but there appeared to be fewer of these at 8 weeks (Fig. 4). Irrespective of the hydroxyapatite and chitosan dose levels, all defect sites exhibited bone formation, and volume was increased. At 8 weeks, the appearance of the new bone was more lamellar than that observed at 2 weeks.

Membrane remnants are composed of chitosan fibers and hydroxyapatite particles. The membrane remnants were surrounded by connective tissue. As the HA dose of membrane increased, the size of membrane remnants had decreased at 8 weeks.

3. Histometric analysis

Three animals were excluded from the histometric analysis due to technical complications in the histologic processing. Only limited new bone formation was observed in the controls. Irrespective of the HA-CS dose, there were no significant differences in % defect closure at either 2 or 8 weeks post-surgery. There was also no statistically significant difference between

Table 1. Histometric Analysis of Augmented Area (Group eans ± Standard Deviation n=5, mm^2)

	2 weeks	8 weeks
Control(no membrane)[***]	0.23 ± 0.05	0.51 ± 0.09
Exp 1 (CS 100%)	12.74 ± 1.98[*]	15.06 ± 2.74[*]
Exp 2 (HA 30% / CS 70%)	14.84 ± 4.87[*]	10.38 ± 2.06[*¶]
Exp 3 (HA 30% / CS 70%, pressed)[***]	23.74 ± 2.84[*]	10.08 ± 2.06[*¶]
Exp 4 (HA 40% / CS 60%)	14.00 ± 3.02[*]	6.74 ± 0.92[*¶†ƒ]
Exp 5 (HA 50% + CS 50%)	17.60 ± 5.37[*]	6.45 ± 0.60[*¶†ƒ]
Exp 6 (HA 50% + CS 50%, pressed)[***]	25.94 ± 2.84[*]	6.88 ± 3.55[*¶†ƒ]

HA means hydoxyapatite and CS means chitosan.
[*]: Statistically significant difference compared to sham-surgery control group ($P < 0.05$)
[¶]: Statistically significant difference compared to Exp 1 group ($P < 0.05$)
[†]: Statistically significant difference compared to Exp 2 group ($P < 0.05$)
[ƒ]: Statistically significant difference compared to Exp 3 group ($P < 0.05$)
[***]: Statistically significant difference between 2 and 8 weeks ($P < 0.01$)

Table 2. Histometric Analysis of New Bone (Group Means ± Standard Deviation n = 5, mm^2)

	2 weeks	8 weeks
Control (no membrane)[***]	0.22 ± 0.05	0.49 ± 0.10
Exp 1 (CS 100%)[***]	1.53 ± 0.53[*]	2.88 ± 0.11[*]
Exp 2 (HA 30% + CS 70%)[***]	1.84 ± 0.34[*]	2.83 ± 0.26[*]
Exp 3 (HA 30% + CS 70%, pressed)	2.22 ± 0.46[*¶]	2.53 ± 0.59[*]
Exp 4 (HA 40% + CS 60%)	2.27 ± 0.48[*¶]	2.68 ± 0.14[*]
Exp 5 (HA 50% + CS 50%)	2.44 ± 0.14[*¶†]	2.64 ± 0.13[*]
Exp 6 (HA 50% + CS 50%, pressed)	2.46 ± 0.23[*¶†]	2.49 ± 0.09[¶]

HA means hydoxyapatite and CS means chitosan.
[*]: Statistically significant difference compared to sham-surgery control group ($P < 0.05$)
[¶]: Statistically significant difference compared to Exp 1 group ($P < 0.05$)
[†]: Statistically significant difference compared to Exp 2 group ($P < 0.05$)
[***]: Statistically significant difference between 2 and 8 weeks ($P < 0.01$)

the results at 2 and 8 weeks in terms of % defect closure. However, one-way ANOVA revealed that new bone and augmented areas did show significant differences in each healing interval ($p < 0.01$).

Tables 1 and 2show the results of histometric analysis. New bone deposition between 2 and 8 weeks was significantly different in the Exp. 1 and Exp. 2 groups (Fig. 5, 6). Augmented areas were significantly decreased from 2 weeks to 8 weeks post-surgery in the pressed-membrane group (Fig. 7, 10).

New bone formation for the 50% HA dose groups (Exp. 5 and Exp. 6) at 2 weeks post-surgery was significantly greater than for the HA 0% group (Exp. 1) or HA 30% group (Exp. 2). There were significant differences between 2 and 8 weeks in new bone formation in HA 0% group (Exp. 1) and HA 30% group (Exp. 2). But Exp. 5 and Exp. 6, which received the high HA dose, were not statistically significant.

DISCUSSION

Many absorbable membranes made of collagen or various kinds of polymers, including chitosan, have been developed to the present day, and many studies on their healing effects have been carried out [14-17].

Chitosan is known to accelerate cell migration and tissue maturation, leading to wound healing; therefore, many studies relating to this property are being undertaken in thefields of dentistry and orthopedic surgery. Chitosan could be adhesive to bioactive materials such as PDGF and BMP, and thus could be widely used clinically in addition to bone substitutes and barriers[18]. The nano type of hydroxyapatite can also be attached to chitosan fibers.

Nano-sized HP may have other special properties due to its small size and huge specific surface area. Webster et al[19] have demonstrated a significant increase in protein adsorption and osteoblast adhesion on the nano-sized ceramic. Studies have shown that nano-HA/chitosan composite scaffolds may serve as a good three-dimensional substrate for cell attachment in vitro and migration in engineered bone and periodontal tissue[20]. Some researchers experimented with a composite of chitosan and nano-HA paste, but it did not have porosity and could not be loaded with cells. Others have reported that fibrous scaffolds had a much greater surface-to-volume ratio than scaffolds with solid pore walls, which might have further increased protein adsorption capacity[21,22].

In this study, we attempted to show the clinical efficacy of a newly-formed fibrous hydroxyapatite-chitosan (HA-CS) membrane in a rat model. The experimental model used in this study has been shown to be effective for evaluating the potential for bone formation[23-25]. The rat calvarial defect model is convenient for examining bone regeneration because of its effective accessibility and lack of fixation requirements.

In our histometric analysis, the length and the area of the new bone formation were compared. Measurements were taken by using computer software, called Image Pro Plus. Specimens were obtained from the middle coronal section. The length of new bone formation was measured to compare the amount of cell migration. The more the cells migrate, the higher the possibility of bone union. As the cells' length growth increases, in considering the thickness, more bone formation could be predicted. Therefore, this could be regarded to be a good marker for the membrane's bone regenerative capacity. Many rat studies have shown a significant increase in the area and density of newly formed bone when water-soluble oligo-chitosan was applied to a calvarial defect[26] and chitosan reconstituted with absorbable collagen sponge has significant potential to induce the regeneration of bone in rat calvarial critical-sized defects[27]. Previous study showed augmented area, including new bone, of chitosan/absorbable collagen sponge group in 2 weeks post-surgery was 6.19 ± 2.03 mm^2 and 8 weeks

post-surgery was 4.84 ± 0.88 mm^2. All of experimental groups were enhanced augmented area both 2 and 8 weeks post-surgery in present study.

As the HA dose increased, there was more new bone formation in the early healing period, 2 weeks after surgery, while there was no significant difference as time went on. This suggests that HA nanoparticles may resorb quickly and induce new bone in the early stage of healing.

Ideally, the bone substitute should conduct or induce bone formation at the same time as it is completely resorbed and substituted by bone tissue. Evidence from previous studies suggests that HA resorption can be mediated by cells or by disintegration through the action of extracellular fluids[28,29]. Our histologic study also showed the presence of multinuclear giant cells in close contact with the HA and chitosan surface and bone formation adjacent to the particles. Liljensten et al[30]stated that even for HAs considered absorbable, the resorption process is slow and its finalization is not well determined.

Levels of wound healing and bone formation were similar in the non-pressed membrane experimental groups. But in some pressed-membrane experimental groups, inflammation was observed at 2 weeks after surgery but had subsided at 8 weeks. Andrade et al[31] studied denseand porous HA cylinders and observed the fibrous tissue development surrounding dense implants and the direct contact of the new bone formed in the porous implants. Takeshita et al[32] used dense HA granules (300 to 600μm) in bone defects created around osseointegrable implants and reported fibrous encapsulation of the granules. They concluded that dense HA granules negatively interfered with bone formation. Many other studies have reported improved bone-HA integration when the particles presented micro- and macropores. Our study also suggests that pressed-membrane with high proportion of HA particles in a unit area could cause inflammation in early healing period.

The augmented area of the pressed-membrane groups (Exp. 3 and Exp. 6) was significantly decreased after 8 weeks. As the HA dose increased, the augmented areas decreased at 8 weeks because the HA nanoparticles were absorbed earlier than the chitosan fiber.

From a histological view, it seems that the membrane lacked any major role as a scaffold. There was no statistical significance in defect closure that we could evaluate from length-growth of cells. Therefore, it appears that the HA-CS membrane does not stimulate cell migration to the center of defect. In summary, HA-CS membrane could collapse early in the healing period and seems to interfere in the formation of new bone in the central zone of surgical defects.

The pressed-membrane groups were developed primarily to overcome the weak mechanical properties of the conventional membranes and to be easy to handle. After the pressed-membrane absorbed water, however, there was no difference in handling from non-pressed, conventional membranes. Furthermore, pressed-membrane showed swollen shape at 2 weeks after surgery. To play a leading role in a scaffold, membranes should have improved mechanical properties in terms of water absorption.

In summary, surgical implantation of the HA-CS membrane resulted in enhanced local bone formation at both 2 and 8 weeks compared to the control group. The results suggest that the addition of HA accelerated new bone formation as seen in Table 3. Further studies will be required to improve the mechanical properties for development of a more rigid, pressed scaffold for the HA-CS membrane.

REFERENCES

1. Magnusson I, Batich C, Collins BR.New attachment formation following controlled tissue regeneration using biodegradable membranes. J Periodontol 1998;59:1-6.

2. Aquirre-Zorzano LA, Estefania-Cundin E, GilLozano J. Periodontal regeneration of infrabony defectsusing resorbable membrane: determinant of the healing response. An observational clinical study. Int J Periodontics Rest Dent 1999;19:363-371.

3. Zitzmann NU, Naef R and Schaurer P. Resorbable versus nonresorbable membranes in combination with Bio-Oss for guided bone regeneration. Int J Oral Maxillofac Implant 1997;12:844-852.

4. Becker W, Becker BD, Mellonig J. A prospective multi-center study evaluation periodontal regeneration for class II furcation invasions and intrabony defects after treatment with a bioabsorbable barrier membrane: 1-year results, J Periodontol 1996;67:641-649.

5. Hong SB, Kwon YH, Lee MS, Herr Y. Comparative study on tissue responses of 3 resorbable membranes in rats. J Korean Acad Periodontol 2002;32:475-488.

6. Amano K, Ito E. The action of lysozyme on partially deacetylate chitin. European J Biochem 1978;85:97-104.

7. Pangburn SH, Trescony PV, & Heller J. Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. Biomaterials 1982;3:105-108.

8. Reynolds, B.L. Wound healing III: Artificial maturation of arrested regenerate with an acetylated amino sugar. The American Surgeon 1960;26:113-117.

9. Kim OS, Chung HJ. Effects of chitosan on human gingival fibroblasts in vitro. J Korean Acad Periodontol 2002;32:235-247.

10. Paik JW, Lee HJ, Yoo YJ. The effects of chitosan on the human periodontal ligament fibroblasts in vitro. J Korean Acad Periodontol 2001;31:823-832.

11. Park JS, Choi SH, Moon IS. Eight-week histological analysis on the effect of chitosan on surgically created one-wall intrabony defects in beagle dogs. J Clin Periodontol 2003;30:443-453.

12. Salata LA, Craig GT, Brook IM. Bone healing following the use of hydroxyapatite or ionomericbone substitutes alone or combined with a guided bone regeneration technique: an animal study. Int J Oral Maxillofac Implants 1998;13:44-51.

13. Zhang C, Hu YY,Cui FZ, Zhang SM, Ruan DK. A study on a tissue-engineered bone using rhBMP-2 induced periosteal cells with a porous nano-hydroxyapatite/collagen /poly(L-lactic acid) scaffold. Biomed Mater 2006;2:56-62.

14. Wang HL, MacNeal RL, Shieh AT. Utilization of a resorbable collagen membrane in repairing gingival recession defects. Pract Periodont Aesthet Dent 1996;8:441-448.

15. Wang HL, O'Neal RB, Tomas CL. Evaluation of and absorbale collagen membrane in treating class II furcation defects. J Periodontol 1994;65:1029-1036.

16. Polson AM, Garrett S, Stoller NH. Guided tissue regeneration in human furcation defects after using biodegradable barrier. : A multi-center feasibility study. J Periodontol 1995;66:377-385.

17. Bouchard P, Giovannoli JL, Mattout C. Clinical evaluation of a bioabsorble regenerative material in mandibular class II furcation therapy. J Clin Periodontol 1997;24:511-518.

18. Jung UW, Song KY, Kim CS, Lee YK, Cho KS, Kim CK, Choi SH. Effects of a chitosan membrane coated with polylactic and polyglycolic acid on bone regeneration in a rat calvarial defect. Biomed Mater 2007;3:S101-105.

19. Webster TJ, Ergun C, Doremus RH. Enhanced functions of osteoblasts on nanophase ceramics. Biomaterials 2000;21:1803-1810.

20. Zhang YF, Cheng XR, Chen Y. Three-dimensional nano-hydroxyapatite/chitosan scaffolds as potential tissue engineered periodontal tissue. J Biomater Appl 2007;21:333-349.

21. Kong L, Gao Y, Cao W. Preparation and characterization of nano-hydroxyapatite/chitosan composite scaffolds. J Biomed Mater Res A 2005;75:275-282.

22. Guobao W, Peter X. Ma.Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. Biomaterials 2004;25:4749-4757.

23. Schmitz JP, Hollinger JO: The critical size defect as an experimental model for craniomandibulofacial nonunoins, Clin Orthop 1986;205:299-308.

24. Caton J, Mota L, Gandini L. Non-human primate models for testing the efficacy and safety of periodontal regeneration procedures. J Periodontol 1994;65:1143-1150.

25. Selvig KA. Discussion: Animal models in reconstructive therapy. J Periodontol 1994;65:1169-1172.

26. Jung UW, Suh JJ, Choi SH. The bone regenerative effects of chitosan on the calvarial critical size defect in Sprague-Dawley rats. J Korean Acad Periodontol 2000;30:851-868.

27. Pang EK, Paik JW, Kim SK. Effects of chitosan on human periodontal ligament fibroblasts in vitro and on bone formation in rat calvarial defects J Periodontol 2005;76:

1526-1533.

28. Oonishi H, Hench LL, Wilson J. Comparative bone growth behavior in granules of bioceramic materials of various sizes. J Biomed Mater Res 1999;44:31-43.

29. Manjubala I, Sivakumar M, Sureshkumar RV. Bioactivity and osseointegration study of calcium phosphate ceramic of different chemical composition. J Biomed Mater Res 2002; 63:200-208.

30. Liljensten E, Adolfsson E, Strid KG. Resorbable and non-resorbable hydroxyapatite granules as bone graft substitutes in rabbit cortical defects. Clin Implant Dent Relat Res 2003;5:95-101.

31. Andrade JC, Camilli JA, Kawachi EY. Behavior of dense and porous hydroxyapatite implants and tissue response in rat femoral defects. J Biomed Mater Res 2002;62:30-36.

32. Takeshita F, Ayukawa Y, lyama S. Histological comparison of early wound healing following dense hydroxyapatite granule grafting and barrier placement in surgically-created bone defects neighboring implants. J Periodontol 1997; 68:924-932.