J Mater Sci: Mater Med (2006) 17:807–813
DOI 10.1007/s10856-006-9839-9

Effect of calcium phosphate glass on bone formation in calvarial defects of Sprague-Dawley rats

Hyun-Ju Moon · Kyoung-Nam Kim · Kwang-Mahn Kim · Seong-Ho Choi · Chong-Kwan Kim · Kee-Deog Kim · Racquel Z. LeGeros · Yong-Keun Lee

Received: 13 December 2004 / Accepted: 21 October 2005

Abstract The purpose of this study was to investigate the bone regenerative effect of calcium phosphate glass *in vivo*. We prepared two different sizes of calcium phosphate glass powder using the system CaO-CaF_2-P_2O_5-MgO-ZnO; the particle size of the powders were 400 μm and 40 μm. 8 mm calvarial critical-sized defects were created in 60 male Sprague-Dawley rats. The animals were divided into 3 groups of 20 animals each. Each defect was filled with a constant weight of 0.5 g calcium phosphate glass powder mixed with saline. As controls, the defect was left empty. The rats were sacrificed 2 or 8 weeks after postsurgery, and the results were evaluated using radiodensitometric and histological studies; they were also examined histomorphometrically. When the bigger powders with 400 μm particle were grafted, the defects were nearly completely filled with new-formed bone in a clean healing condition after 8 week. When smaller powders with 40 μm particle were transplanted, new bone formation was even lower than the control group due to a lot of inflammatory cell infiltration. It was concluded that the prepared calcium phosphate glass enhanced the new bone formation in the calvarial defect of Sprague-Dawley rats and it is expected to be a good potential materials for hard tissue regeneration. The particle size of the calcium phosphate was crucial; 400 μm particles promoted new bone formation, while 40 μm particles inhibited it because of severe inflammation.

H.-J. Moon
Research Center for Orofacial Hard Tissue Regeneration, Yonsei University College of Dentistry, Seoul 120-752, Korea; Department and Research Institute of Dental Biomaterials and Bioengineering, Yonsei University College of Dentistry, Seoul 120-752, Korea

K.-N. Kim · K.-M. Kim · Y.-K. Lee (✉)
Research Center for Orofacial Hard Tissue Regeneration, Yonsei University College of Dentistry, Seoul 120-752, Korea; Brain Korea 21 Project for Medical Science, Yonsei University, Seoul 120-752, Korea; Department and Research Institute of Dental Biomaterials and Bioengineering, Yonsei University College of Dentistry, Seoul 120-752, Korea

S.-H. Choi · C.-K. Kim
Research Center for Orofacial Hard Tissue Regeneration, Yonsei University College of Dentistry, Seoul 120-752, Korea; Brain Korea 21 Project for Medical Science, Yonsei University, Seoul 120-752, Korea; Department of Periodontics, Yonsei University College of Dentistry, Seoul 120-752, Korea

K.-D. Kim
Department of Oral and Maxillofacial Radiology, Yonsei University College of Dentistry, Seoul 120-752, Korea

R. Z. LeGeros
Department of Biomaterials and Biomimetics, New York University College of Dentistry, New York, NY 10010, USA

Introduction

The ultimate goal of periodontal therapy is the regeneration of periodontal tissue which has been lost due to destructive periodontal disease [1]. This requires new bone formation and new cementum formation accompanied by newly inserted functionally oriented fibers at a tooth site previously exposed to the oral environment. Many investigations about transplantation techniques for the treatment of periodontal disease have been made in order to induce the formation of new bone as well as new cementum [2]. There are autogeneous, allogenic, xenogenic and alloplastic bone grafts. Autogenous bone grafts, by using intraoral or an extraoral donor site, provide very favorable results to achieve periodontal

regeneration. However, autografts require additional surgical procedures and a large amount of donor tissue is often hard to obtain [3, 4]. Allografts have problems with unreliable graft incorporation, immune response and possible disease transmission. For these reasons, efforts have been made to investigate various other kinds of alloplastic bone-graft materials. Alloplastic bone-graft materials include calcium phosphates, calcium carbonate, bioglass and glass-ceramics, and polymers [5, 6]. Among them, calcium phosphates have been received the most attention and they are widely used because of their good biocompatibility and osteointegrative properties [7, 8].

Synthetic hydroxyapatite is one of the calcium phosphates and it can be anchored to bone by the establishment of a physicochemical bond with living tissue [9]. It has been under intensive research over the past 20 years as a leading candidate of the calcium phosphates, and it has been used clinically in various forms due to its osteoconductive properties. Also, many investigators reported that hydroxyapatite resulted in attachment gain and pocket reduction in clinical studies [10, 11]. In contrast, there have been reports of no new bone formation and no attachment gain were observed around the hydroxyapatite and tricalcium phosphate implants in human histological studies [12, 13].

The use of a rigid hydroxyapatite implant for skeletal reconstructions is associated with potential long-term interference with mechanical stress and strain in load-bearing areas. In addition, hydroxyapatite as well as tricalcium phosphate are not adequate for the filling of large bony defects because they can not be replaced completely by new bone. Therefore, the clinical application of the calcium phosphates has been restricted due to its poor absorbability and brittleness. Consequently, a degradable material that can eventually be replaced by bone tissue is needed for bone repairs [8].

The similarities in composition of the calcium phosphate glass to bone and teeth make it an ideal candidate for hard tissue substitution. The first proposed application of the calcium phosphate glass to the biomaterials was for dental restorative materials such as the crown because of their excellent castability as well as their adequate mechanical strength [14, 15].

The purpose of this study was to evaluate the bone regenerative effect of calcium phosphate glass in the system of CaO-CaF_2-P_2O_5-MgO-ZnO, which is characterized by having a very low Ca/O ratio of 0.6. It was already reported that calcium phosphate glass has shown a high dissolution rate in buffer solutions, and they have good bioactivity after an exposure to either simulated body fluid or fetal bovine serum [16]. The promotion of a bone-like tissue formation and an enhanced alkaline phosphatase activity in vitro by calcium phosphate glass has also been observed [17]. In this study, we used calcium phosphate glass as a bone-graft material for bone regeneration on calvarial defects of Sprague-Dawley rats.

Materials and methods

Sample preparation

Calcium phosphate glass in the system of CaO-CaF_2-P_2O_5-MgO-ZnO was prepared with a Ca/P ratio of 0.6 by using raw materials such as $CaCO_3$, CaF_2, H_3PO_4, MgO, and ZnO. The molar ratio of CaO/CaF_2 was fixed to 9. Both MgO and ZnO were added at 1% weight percentages. Mixed batches were dried for 12 h at 80°C, then calcined for 1 h at 450°C, and finally melted in a platinum crucible at 1250°C. After the glasses were melted in a kanthal super furnace, they were poured onto the graphite plate at room temperature. Asquenched glasses were then rendered to two different sized-powders by grinding them in an alumina mortar and this was followed by a ball milling. The mean particle size of the smaller powders was 40 μm, while that of the bigger powder was 400 μm as is shown in Fig. 1.

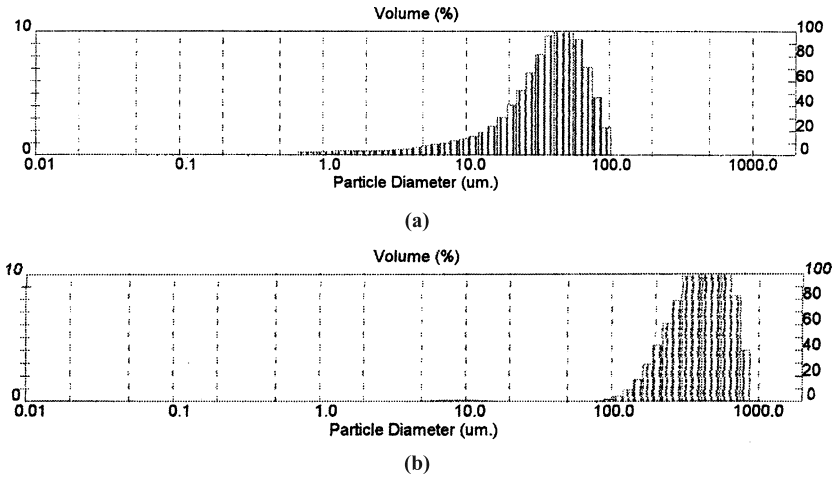


Fig. 1 Particle size distribution of the prepared calcium phosphate glasses having a mean particle size of (a) 40 μm and (b) 400 μm, respectively.

Animals

Sixty male Sprague-Dawley rats (20 per group), weighing 200 to 250 g, were used for this experiment. Each five animals were caged with water and food supplied *ad libitum.* Animal selection and management, surgical protocol, and preparation followed routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

Operative procedure

The animals were anesthetized with an intramuscular injection (5 mg/kg body weight) consisting of ketamine hydrochloride (Ketalar, Yuhan Co., Seoul, Korea). During surgery, routine infiltration anesthesia was applied at the surgical site using 2% lidocaine (Kwangmyung Pharm., Seoul, Korea) mixed with epinephrine at a ratio of 1:100,000.

The surgical site was shaved and scrubbed with iodine. An incision was made in the sagittal plane across the cranium. A full-thickness flap including the periosteum was reflected, exposing the calvarial bone. Then, a critical-sized (8 mm in diameter) circular and transosseous defect was created on the cranium using a saline-cooled trephine drill. Extreme care was used to avoid injury to the dura mater [18].

Each defect was filled with a constant weight of 0.5 g calcium phosphate glass powder mixed with saline. Smaller-sized powders were implanted for the experimental group 1, while the bigger-sized powders were transplanted for the experimental group 2. As controls, the defect was left empty. The incisions were closed with absorbable sutures.

Analysis

2 or 8 weeks after postsurgery, the animals were sacrificed by CO_2 asphyxiation; they were operated and the craniums were carefully dissected free of soft tissue. The craniums were immediately placed into vials and fixed in 10% neutral-buffered formalin for 10 days. All samples were decalcificated in EDTA-HCl for 7 days, and embedded in paraffin. 3 μm thick coronal sections through the center of the circular defects were stained with hematoxylin and eosin. After a conventional microscopic examination, length and area of the new bone formation were measured using a computer-assisted Image-Pro Plus System (Media Cybernetics, Silver Spring, MD, USA). Length and area of the new bone formation were expressed in mm and mm^2, respectively.

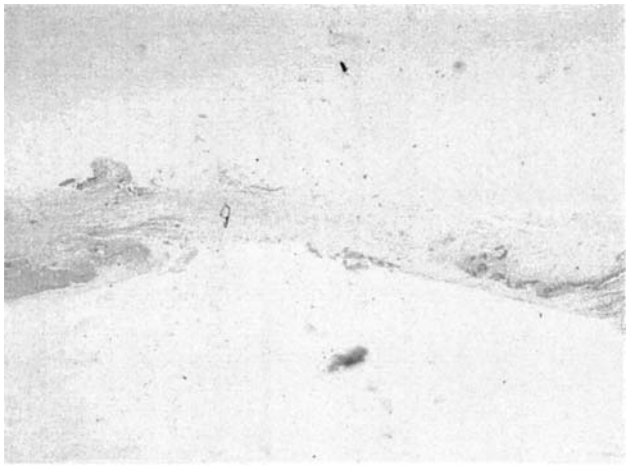
The statistical significant differences of the results between the experimental and control groups were analyzed using an ANOVA with a Kruskal-Wallis test at a level of 0.05. The significant differences among the sacrification period were analyzed with a Mann-Whitney test at a same level.

Results

Histological observations

After 2 and 8 weeks in the control group, thin connective tissue was abundant at the defect site, as is shown in Fig. 2 and 3. Also, there was a minimal quantity of new bone formation originating from the defect margins. The defect center was collapsed.

In the experimental group 1 after 2 weeks, the center of defect was also filled with loose connective tissue, as is shown in Fig. 4. A little new bone formation adjacent to the defect margins and a host bond-to-new bone interface was obvious. Inflammatory cell infiltration was observed in the connective tissue as well new bone growth. No grafted materials could be observed. At 8 weeks time, a similar pattern to the 2-week observations was observed; however, a lot of inflammatory cell infiltration was observed at the defect site, as is shown in Fig. 5.



(a) X20

(b) X200

Fig. 2 Representative microphotographs of the H-E stained control group at 2 weeks postsurgery with magnification of (a) X20 and (b) X200.

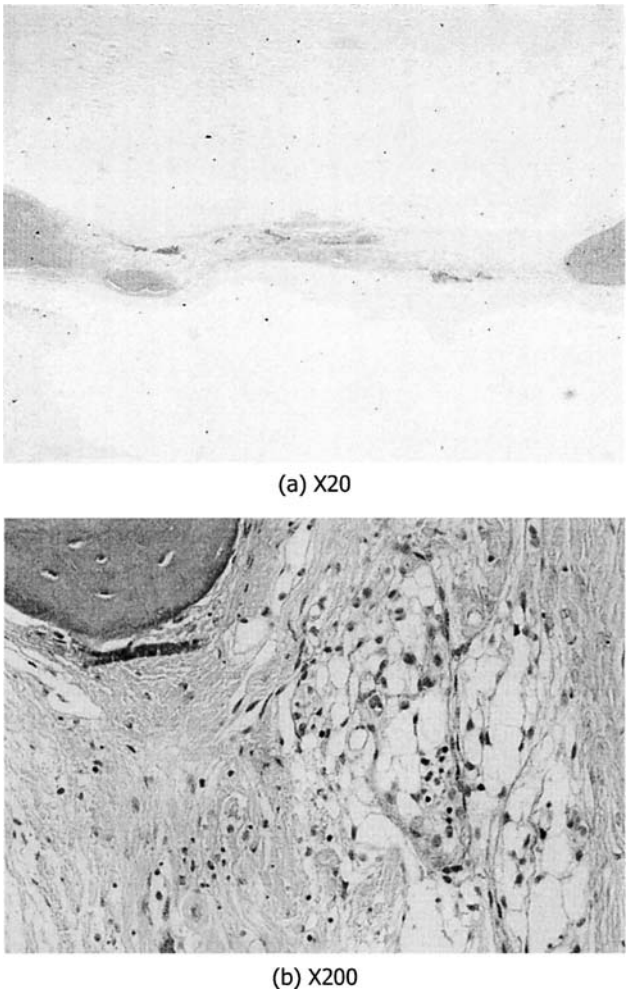


Fig. 3 Representative microphotographs of the H-E stained control group at 8 weeks postsurgery with magnification of (a) X20 and (b) X200.

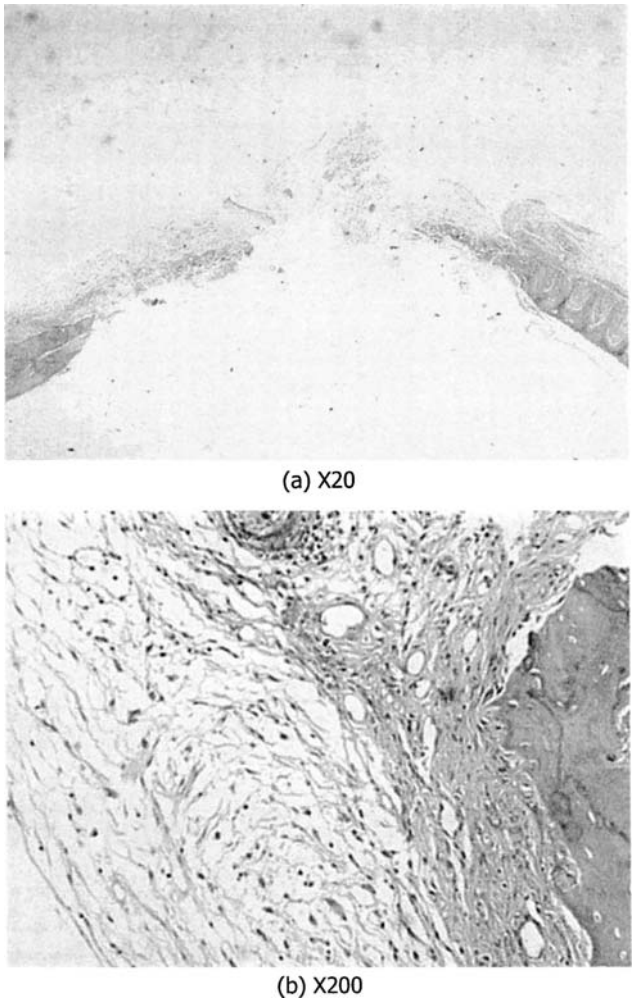


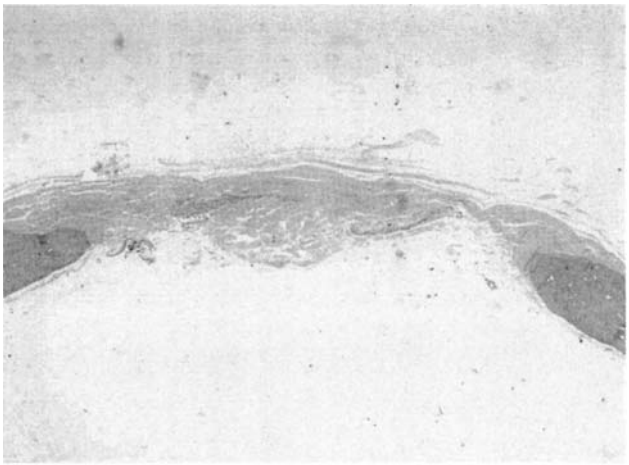
Fig. 4 Representative microphotographs of the H-E stained experimental group 1 at 2 weeks postsurgery with magnification of (a) X20 and (b) X200.

In the experimental group 2, 2 weeks after the surgery, there were dense, fibrous connective tissues at the defect site, as is shown in Fig. 6. A large number of residual calcium phosphate glass particles were present within the fibrous connective tissue at the defect site. More abundant new bone formation was observed than in either the control or experimental group 1. Inflammatory cell infiltration was minimal at the defect site. At 8 weeks, the histological observations were similar to the 2-week observations as shown in Fig. 7. Residual calcium phosphate glass particles are fewer in number and the quantity of new bone was greater than that observed at 2 weeks.

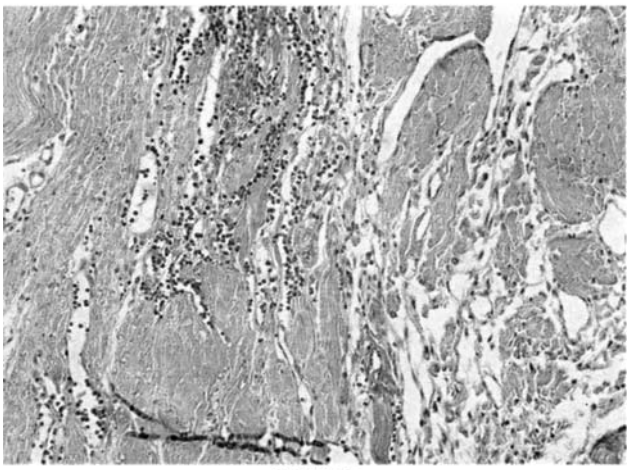
Histomorphometric analysis

The results of histomorphometric analysis are shown in Table 1 and Table 2. At 2 weeks postsurgery, mean length of new bone for the control, the experimental group 1 and experimental group 2 amounted to 0.93 mm, 1.47 mm, and 1.75 mm. At 8 weeks postsurgery, the corresponding values are 1.66 mm, 1.42 mm, and 2.38 mm. In all groups, both length and area of new bone increase with increasing the grafting duration. There are significant differences between the control group and experimental group 2 both at 2 and 8 weeks ($p < 0.05$), while there are no significant differences between the control group and experimental group 1 ($p > 0.05$). In all groups, the length of new bone was increased with an increasing grafting period, and the differences are significant ($p < 0.05$). However, the length of new bone was decreased in the experimental group 1 and this is without a significant difference ($p > 0.05$).

At 2 weeks postsurgery, the mean area of new bone for the control, experimental group 1 and experimental group 2 amounted to 0.28 mm^2, 0.43 mm^2, and 0.96 mm^2. At 8 weeks postsurgery, the corresponding values are 0.86 mm^2, 0.41 mm^2, and 1.53 mm^2. A similar pattern for the length of new bone was observed. As is shown in Table 2, a much

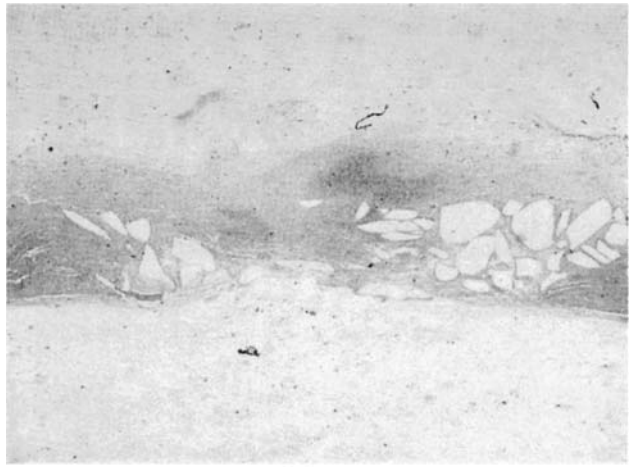


(a) X20

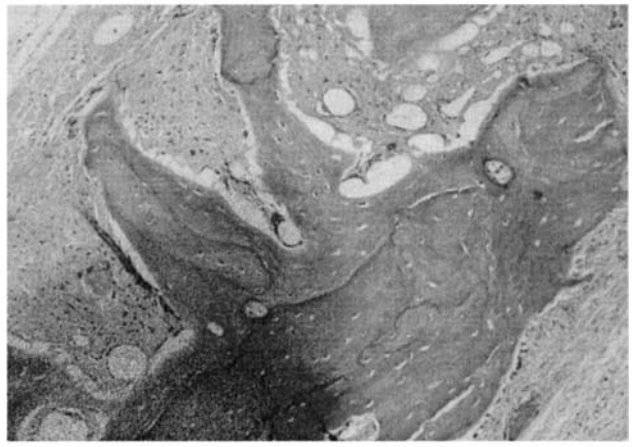


(b) X200

Fig. 5 Representative microphotographs of the H-E stained experimental group 1 at 8 weeks postsurgery with magnification of (a) X20 and (b) X200.



(a) X20



(b) X200

Fig. 6 Representative microphotographs of the H-E stained experimental group 2 at 2 weeks postsurgery with magnification of (a) X20 and (b) X200.

greater difference between the experimental group 1 and 2 was observed.

Discussion

The objective of this study was to evaluate the bone regenerative effect of non-crystalline calcium phosphate glass in a system of CaO-CaF_2-P_2O_5-MgO-ZnO. In addition, we compared the effects of the particle size of the calcium phosphate glass on the bone regeneration. We used an 8 mm calvarial critical-sized defect model in the rat and calcium phosphate glass powder was used at either a mean particle diameter of 40 μm or 400 μm.

In this study, we used a so-called "critical-sized defect", which implies that the defect does not heal by itself during the lifetime of the animal. In addition, we used calvarial defects that had previously been proven to be good models for investigating the effects of bone-graft materials. Freeman and Turnbull [19] were the first to attempt the study of critical-sized defects in rat calvaria. Tagaki and Urist [20] reported that 8 mm calvarial defects in six-month-old Sprague-Dawley rats were reduced to 5 mm after four weeks and no further healing was noted after 12 weeks. The rat calvarial defect, compared with other experimental bone defects, is a convenient model for studying bone regenerative materials because of its lack of fixation requirements [18]. In addition, the Sprague-Dawley rats defects are both reproducible and native and induced healing processes have been well characterized [21]. Also, 8 mm trephine calvarial defects in rats have been shown to be critical-sized defects [18, 19, 22, 23].

Various calcium phosphates, such as hydroxyapatite, tricalcium phopshate, and biphasic calcium phosphate as well as bioglass have been widely used as bone-graft materials for healing of bone defects. They generally bond to surrounding osseous tissue and enhance bone tissue

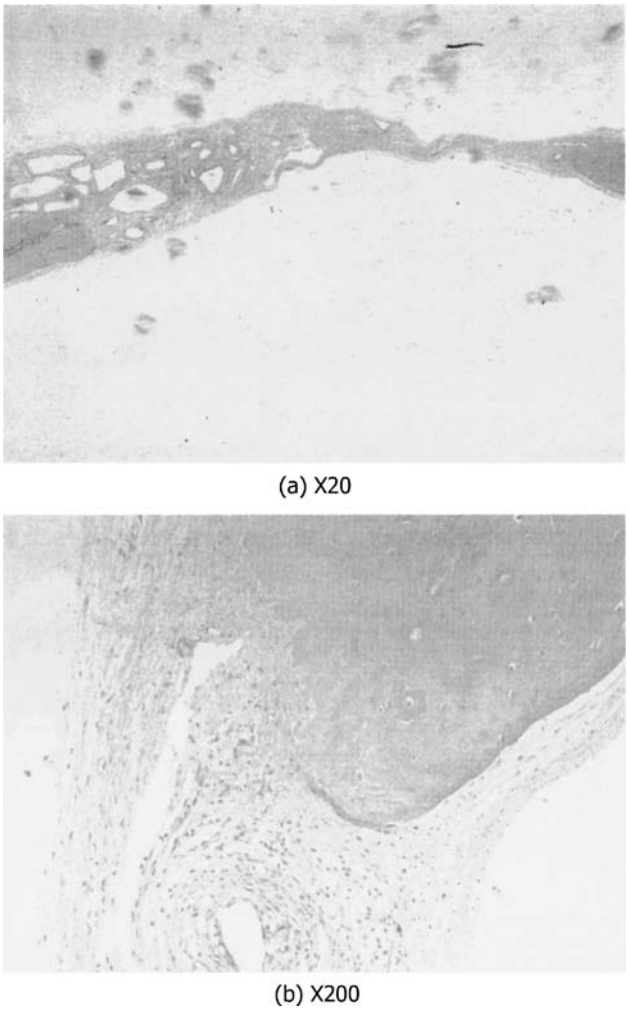


Fig. 7 Representative microphotographs of the H-E stained experimental group 2 at 8 weeks postsurgery with magnification of (a) X20 and (b) X200.

formation [24]. However, they are not adequate for the filling of large bony defects due to their slow degradation rate [25].

In this study, we used calcium phosphate glass as a bone-graft material for bone regeneration on calvarial defects of Sprague-Dawley rats. The calcium phosphate glass was first developed as a dental crown material since it is easily fused and has a low viscosity in a molten state [14]. The results of *in vitro* tests have shown that the calcium phosphate glass is highly biodegradable and bioactive in either simulated body fluid or fetal bovine serum [16]. It was also observed that the alkaline phosphatase activity was significantly more enhanced by the calcium phosphate glass at 10–18 days after incubation than those of experiment group without the calcium phosphate glass ($p < 0.05$). In addition, bone-like tissue formation was promoted on the calcium phosphate after 7 days and thereafter [17].

Table 1 Histomorphometric analysis for the length of the new bone formed

Group	2 weeks	8 weeks
Control group	0.93 ± 0.07 mm	1.66 ± 0.14 mm $^{+}$
Experimental group 1	1.47 ± 0.37 mm	1.42 ± 0.18 mm
Experimental group 2	1.75 ± 0.10 mm*	2.38 ± 0.13 mm $^{*\#+}$

* : Significant difference from the control group ($p < 0.05$)
#: Significant difference from the experimental group 1 ($p < 0.05$)
$+$: Significant difference between 2 and 8 weeks ($p < 0.05$)

Effects of the particle size of the bone-graft materials on the new bone formation are well-documented and have been shown with different materials in various compositions [26, 27]. Mankani et al. [28] demonstrated the particle size of biphasic calcium phosphate plays a crucial role in the extent of new bone formation. They transplanted various sizes, 44-2000 μm, of biphasic calcium phosphate after mixing it with cultured human bone marow stromal cells, into subcutaneous pockets and then harvested it over a 10-weeks period. They observed a quadratic, unimodal relationship between the size of the spherical particles and bone formation. There was a peak in bone formation at 100–250 μm and no bone was formed below a threshold of 44 μm. Bruijn et al. [29] also reported the important role of the particle size on the bone formation. They subcutaneously implanted hydroxyapatite granules, these particles were either smooth granules 210–300 μm or rough granules 500–700 μm, in nude mice for 4–10 weeks. The smooth granules allowed for new bone formation, but there was no bone formed around the rough granules.

In our present study, the bigger calcium phosphate glass powder group at 2 weeks showed a clear healing condition. Only minimal inflammatory cell infiltration was observed at the defect site. The calcium phosphate glass powder was partially resorbed and new bone was formed with plenty of dense and fibrous connective tissues. At 8 weeks, the calcium phosphate glass was almost completely resorbed and the defect was nearly completely filled with new-formed bone. In a study reported by Kamakura [30], new bone was formed in the upper side of the defects as well as at the defect margin and the dura mater both in the bigger powder group, while bone formation was restricted within

Table 2 Histomorphometric analysis for the area of the new bone formed

Group	2 weeks	8 weeks
Control group	0.28 ± 0.04 mm^2	0.86 ± 0.09 mm^{2+}
Experimental group 1	0.43 ± 0.20 mm^2	0.41 ± 0.08 mm^{2*}
Experimental group 2	0.96 ± 0.11 mm$^{2*\#}$	1.53 ± 0.18 mm$^{2*\#+}$

* : Significant difference from the control group ($p < 0.05$)
: Significant difference from the experimental group 1 ($p < 0.05$)
$+$: Significant difference between 2 and 8 weeks ($p < 0.05$)

the defect margin and dura mater both in the control and smaller groups as reported by Kamakura [30]. These results by Kamakura indicated that the bigger calcium phosphate glass powder is biodegradable *in vivo* and the bigger grafted powder might act to increase osteoconductive activity into the free space and among particles to promote bone regeneration.

Conclusion

In this study, the critical size defects were surgically produced in the calvarial bone of Sprague-Dawley rats using an 8 mm trephine bur. In order to compare the effect of the particle size of the calcium phosphate glass on the bone regeneration, calcium phosphate glass powders with mean particle size of 40 μm and 400 μm were grafted onto the calvarial critical-sized defects in Sprague-Dawley rats. When the bigger powders, 400 μm, was grafted, the defects were almost completely filled with new-formed bone in a clean healing condition after 8 weeks. When the small powder, 40 μm, was grafted, new bone formation was even lower than the control group due to the presence of lot of inflammatory cell infiltration. It was concluded that the prepared calcium phosphate glass enhanced the new bone formation in the calvarial defect of Sprague-Dawley rats and that it has a good potential for use as a hard tissue regeneration material. The particle size of the calcium phosphate was crucial; 400 μm promoted new bone formation, while 40 μm inhibited bond formation because of severe inflammation.

Acknowledgements This work was supported by grant No. R13-2003-13 from the Medical Science and Engineering Research Program of the Korea Science & Engineering Foundation.

References

1. H.A. ZANDER, *J Periodontol* **47** (1976) 261.
2. S.G. JEFFREY, *Comp Cont Educ Dent* **18** (1997) 1013.
3. I. AUKHIL, *J Perio Res.* **18** (1983) 643.
4. M. R. DRAGOO, *J Periodontol* **44** (1973) 599.
5. B. J. SAMUEL, *Int J Periodot Rest Dent.* **17** (1997) 359.
6. R. A. YUKNA, *J Periodontol* **64** (1994) 2.
7. K. KURASHINA, *Biomaterials* **23** (2002) 407.
8. J. DONG, *ibid* **23** (2002) 4493.
9. M. JARCHO, *J Bioeng* **1** (1977) 78.
10. M. L. RABALIS, *J Periodontol* **52** (1981) 680.
11. R. A. YUKNA, *ibid* **65** (1994) 177.
12. S. J. FROUM, *ibid* **53** (1982) 719.
13. B. S. MOSKOW, *ibid* **54** (1983) 455.
14. Y. ABE, *Bioceramics* **1** (1988) 181.
15. S. KIHARA, *J Am Ceram Soc.* **67** (1984) C100.
16. R. Z. LEGEROS, *J Mater Sci.* **39** (2004) 5577.
17. Y. K. LEE, *J Biomed Mater Res.* **69 A** (2004) 188.
18. J. P. SCHMITZ, *Clin Orthop Rel Res.* **205** (1986) 299.
19. E. FREEMAN, *J Perio Res.* **8** (1973) 229.
20. K. TAGAKI, *Ann Surg.* **196** (1982) 100.
21. L. J. MARDEN, *J Biomed Mater Res.* **28** (1994) 1127.
22. C. BOSCH, *J Craniofac Surg.* **9** (1998) 310.
23. J. P. SCHMITZ, *Acta Anat* **138** (1990) 185.
24. P. DUCHEYNE, *J Biomed Mater Res.* **19** (1985) 273.
25. F. B. BAGAMBISA, *J Oral Maxillofac Implants* **5** (1990) 217.
26. S. G. JEFFREY, *Comp Cont Educ Dent.* **18** (1997) 1013.
27. O. GAUTHIER, *Biomaterial* **19** (1998) 133.
28. M. H. MANKANI, *Biotech Bioeng* **72** (2001) 96.
29. J. D. BRUIJN, Abstract of the 28th annual meeting of the Biomaterials Society, 427, 2002.
30. S. KAMAKURA, *J Dent Rer.* **78** (1999) 1682.