Evaluation of Biphasic Calcium Phosphate Cement in Rat Calvarial Model

Akiyoshi Sugawara1), Kenji Fujikawa1,2) and Shuichi Sato3)  

Abstract: Biphasic calcium phosphate (BCP) cement, consisting of an α-tricalcium phosphate-tetracalcium phosphate as the only solid phase and a calcium phosphate solution (Ca-P soln) as the liquid phase, was recently reported. The BCP cement provided sufficient handling and physical properties for clinical uses. Objective of this study was to evaluate the BCP cement when used for repairing artificially created bone defects in rat calvaria. The sample of BCP cement was prepared by mixing BCP powder with Ca-P soln at P/L of 3.0. A commercially available calcium phosphate cement, Biopex-R, was used as a positive control. Surgically created bone defects were filled with the BCP cement or the control cement. A defect with no filling material was used as a negative control. At 0 and 8 weeks after surgery, X-ray micro-CT image for each surgical site was taken. At 8 weeks after the surgery, the animals were sacrificed, and biopsies were obtained. Subsequently, the sections were subjected to HE and TRAP staining and examined under a microscope. After microscopic examination, computer-assisted histometric measurements of newly formed bone (NB) were performed using NIH image analysis. Defect closure (DC) was determined by measuring the distance between defect margin and new bone margin, and was expressed as a percentage of total defect width. The BCP cement kept original graft shape, and defects filled with the cement were mostly closed by NB. The control cement inadequately maintained original graft shape, and defects filled with the control cement were partially closed by NB. The negative control showed almost no bone formation in the defects. The DC rate of the BCP cement exhibited apparently larger than that of the control cement (p<0.05). These results were also confirmed by fluorescent labelling analysis and X-ray micro-CT observations. The results indicated that the BCP cement had osteoconductive potential for bone defects.

Key words: BCP cement, Bone defect, Defect closure

Materials and Methods

Materials used in this study are shown in Table 1. The powder phase of BCP cement was prepared by mixing a mixture of dicalcium phosphate anhydrous (DCPA) and calcium carbonate (CaCO3) in a furnace, and ground to a mono-modal size distribution. The compound is synthesized in a single manufacturing process, and ground to a mono-modal size distribution. This technology not only saves time and manufacturing costs, but also make it easier for regulatory agencies to validate the quality, assuring reproducible clinical outcome. The BCP cement provided good handling properties and desired hardening time, and it set to a sufficiently high strength for clinical use. In addition, during the setting reaction proceeded, the BCP cement mostly converted to hydroxyapatite (HA) as the final product, the main inorganic component of bone, within 24 hours of mixing.

The purpose of this study was to determine the histological aspects of BCP cement when used for repairing artificially created critical bone defects (5mm in diameter) in the calvaria of rats.
diluted 3.0-fold, 3.5-fold and 4.0-fold with H$_2$O, and then used as the cement liquid. Additionally, tri-sodium citrate anhydrous (Na$_3$Cit) in the powder phase and polyvinylpyrrolidone-k25 (PVP-k25) in the liquid phase were included as a water reducing agent and a washout resistance enhancer, respectively. The samples were prepared by mixing BCP (Ca/P=1.8) powder with the cement liquid at a P/L ratio of 3.0. The components and the setting reaction of the BCP cement are shown in Fig. 2. The total amount of Na$_3$Cit in the sample was adjusted to 1.5 mol/L. Na$_3$Cit achieved high zeta-potentials of apatite cement caused by adsorption of multiple charged citrate ions at the solid–liquid interface, enabling the effective dispersion of cement particles. Using the Na$_3$Cit solution decreased the viscosity, achieved injectability, and improved the strength of the cement paste when used as a bone graft material. In this study, the powder contained Na$_3$Cit and each liquid contained 0.75wt% of PVP-k25.

Table 1. Materials used in this study

| Materials | Manufactures |
|-----------|--------------|
| **Powder phase:** | Taihei Chemical Industrial Co. Ltd., Osaka, Japan |
| Tetracalcium Phosphate (TTCP): Ca$_4$(PO$_4$)$_2$O | Taihei Chemical Industrial Co. Ltd., Osaka, Japan |
| Dicalcium Phosphate Anhydrous (DCPA): CaHPO$_4$ | Taihei Chemical Industrial Co. Ltd., Osaka, Japan |
| α-Tricalcium Phosphate (α-TCP): Ca$_3$(PO$_4$)$_2$ | Taihei Chemical Industrial Co. Ltd., Osaka, Japan |
| Trisodium Citrate Anhydrous (Na$_3$Cit): Na$_3$(C$_3$H$_5$O(COO)$_3$) | Wako Pure Chemical Industries, Ltd., Osaka, Japan |
| Calcium Carbonate: CaCO$_3$ | BASF Japan Ltd., Tokyo, Japan |
| **Liquid phase:** | Wako Pure Chemical Industries, Ltd., Osaka, Japan |
| Polyvinylpyrrolidone-k25 (PVP-k25): (C$_6$H$_9$NO)$_n$ | Wako Pure Chemical Industries, Ltd., Osaka, Japan |
| Phosphoric Acid: H$_3$PO$_4$ | Nichi-Iko Pharmaceutical Co., Ltd. |
| Calcium Carbonate: CaCO$_3$ | Wako Pure Chemical Industries, Ltd., Osaka, Japan |
| Sterile Purified Water: H$_2$O | Nichi-Iko Pharmaceutical Co., Ltd. |

Figure 1. Schematic drawing and particle size distribution of BCP powder particles

The powder phase of BCP cement was a solid-solution of TTCP-α-TCP. BCP powder showed mono-modal particle distribution, and the median particle size of the powder was 9.96μm.

Figure 2. Components and setting reaction of the BCP cement

Three kinds of Ca-P soln., diluted 3.0-fold, 3.5-fold and 4.0-fold with H$_2$O, were used as the cement liquid. Na$_3$Cit in the powder phase and PVP-k25 in the liquid phase were included as a water reducing agent and a washout resistance enhancer, respectively. During the setting reaction proceeded, the BCP cement mostly converted to HA as the final product.
Akiyoshi Sugawara et al.: Evaluation of BCP Cement in Rat Carvaria

Surgically created bone defects were filled with the BCP or control cements. At 0, 4 and 8 weeks after surgery, X-ray micro-CT images (R-mCT2 system, Rigaku Co., Tokyo, Japan) for each surgical site were taken to confirm chronological changes of the grafted area (GA).

At 8 weeks after surgery, the animals were sacrificed, and 5 biopsies including the test materials were obtained for each experimental group. Histological preparation of the specimen was performed as follows. Biopsies were fixed in 10% neutralized-buffered formalin. Half of the specimens were decalcified using 10% EDTA for 10 days and embedded in paraffin. These decalcified samples were then sectioned through the sagittal plane across the cranium at central position of the defect, and then cut into approximately 8μm sections, and were stained with hematoxylin-eosin (HE) and tartaric acid resistant phosphatase (TRAP). We selected TRAP for the staining since it clearly stained osteoclast which resorbed residual bone (RB) and low crystalline HA and consequently converted to new bone. Histopathological features of each section were observed using an optical microscope (DM6000B, Leica Microsystems GmbH, Wetzlar, Germany).

Half of undecalcified specimens were prepared for fluorescent labelling analysis (FLA) by being embedded in methyl-metacrylate.

Histological Preparation of the Specimen

Histometric NIH image analysis

Sacrificed at weeks after surgery

Histological Preparation of the Specimen

Figure 3. Experimental procedures of this study
Each experimental material was tested in 9 weeks old male Fischer rats with an average body weight of 150 to 200g.

Preliminary experiments showed that the 3.5-fold Ca-P soln as the cement liquid and mixed at P/L ratio of 3.0°° gave the desired physical properties, such as hardening time, compressive strength, HA conversion or clinical applicability. A commercially available calcium phosphate cement, Biopex-R (Hoya Technosurgical Co., Tokyo, Japan), was used as a positive control. According to the product description, the powder phase was composed of α-TCP, TTCP, sodium hydrogen phosphate, HA and magnesium phosphate, and the liquid phase was composed of chondroitin sulfate sodium, disodium succinate, sodium hydrogen sulfite and water for injection. The control cement was mixed as following to the instructions. A defect with no filling material was used as a negative control.

Experiment procedures of this study are shown in Fig. 3. The study was permitted by the Animal Experimentation Committee at Nihon University School of Dentistry, and performed in the animal and cell culture laboratories at the Nihon University School of Dentistry. The experiments followed the “Guidelines for Animal Experimentation Committee at Nihon University School of Dentistry (AD14D015)”. Each experimental material was tested in 9 weeks old male Fischer rats with an average body weight of 150 to 200g. All experimental procedures on given animals were completed as aseptic as possible. The animals were pre-medicated by inhalation of isoflurane and then general anesthesia was induced by intraperitoneal injection of a mixture of 0.15 mg/kg butorphanol tartrate of body weight. The forehead of each rat was also locally anesthetized with an injection of 0.5 ml of 1:8-diluted lidocaine (Xylocaine, Astra Zeneca K.K., Osaka, Japan). Under the anesthesia, a 2cm incision was made in the sagittal plane across the cranium, and the full thickness flap was reflected to expose the calvarial bone. A standardized circular trans-osseous bone defect, approximately 5mm in diameter, was created on the cranium using a trephine drill bur with saline cooling°°° (Fig. 4-a). Two bone defects were created in each animal and filled with BCP cement (Fig.4-b), Biopex-R as the control cement or were left unfilled as negative control. Each filling materials was packed to approximately 1.5µl of the bone carrier and was injected into the defect area, and then the filled area was smoothed.

Histological Preparation of the Specimen

Each experimental material was tested in 9 weeks old male Fischer rats with an average body weight of 150 to 200g.
resin, ground to approximately 50 µm in thickness, and then examined using fluorescent microscopy (BX51-P, Olympus Co., Tokyo, Japan) for the presence and location of labeling agent on newly formed bone (NB). FLA clearly demonstrated the presence of NB at 8 weeks after surgery, since it spontaneously picked up the TC-fluorescent agent.

The amount of NB formed in each surgical site was evaluated quantitatively using NIH image analysis, which is an automated computer-assisted histometric imaging technique, for sections at a 25 × magnification. Measurements of defect closure (DC) were determined by measuring the distance between the defect margin and the ingrowing bone, at each side of the defect. The measurements are expressed as a percentage of the total defect width (Fig. 5). The boundary of original residual bone was determined by superimposing X-ray micro-CT image on the histological image. Histometric recordings from the samples were used to calculate the group mean (± SD). A student’s t-test was used to establish statistical significance (p<0.05).

Results

After the surgery, no apparent infection or complications were observed in any group. The results obtained from this study are shown below:

Histological observations

Negative control

In histological observation at 8 weeks after surgery, a negative control showed the defect was mostly covered with thin fibrous connective tissues (FCT) (Fig. 6). The shape of the defect appeared to have collapsed due to the physical pressure from surrounding tissue. A thin layer of newly formed woven bone, which was immature new alveolar bone, was observed in the portion adjacent to the RB. The above results were confirmed by FLA and micro-CT observations.

Control cement

Histological observation showed that the control cement filled in defects, it exhibited insufficient washout resistance, and also inadequately maintained the original graft shape and volume (Fig. 7). The GA was covered by relatively dense FCT, and partially NB formation had occurred adjacent to the defect margin of residual bone area (RA). Immature osseous tissues were also found in the GA. TRAP staining exhibited that osteoclasts (OCs), which resorbed RB and grafted material (GM) and led to converting to bone tissues, were scattered throughout the GM adjacent to RB. The GM did not completely unify with RB surface. In FLA, the fluorescent band corresponding to NB formation in the GA was almost the same as the results obtained in histological observations. These results were also identified by micro-CT observations, which also showed that the GM filling the defect did not adequately maintain the original graft shape and volume at 8 weeks after the surgery.

BCP cements

In general, all of the BCP cements showed good adaptability to the defect (Figs. 8-10). The cement also exhibited excellent washout resistance and maintained the original graft shape and volume. The GA was nearly completely closed with NB. NB was also observed above and under the GM where it attached the periosteum and subperiosteum. NB that formed under the GM was obviously thicker and more lamellar compared to that which formed above the GM. Some sections showed that osseous tissues extended into the GM. TRAP showed that the GM was apparently resorbed by OC and converted to NB. Natural lamellar bone was clearly found in the GA. Histological features exhibited that no apparent difference existed among 3.0-fold, 3.5-fold and 4.0-fold of BCP cements. According to FLA, NB formations were also showed above and under the GM, especially near the RB. These results were also confirmed by micro-CT observations. The micro-CT images also showed that BCP cements adequately maintained the original graft shape.
The control cement filled in defects, it exhibited insufficient washout resistance, and also inadequately maintained the original graft shape and volume. GA was covered by relatively dense FCT, and partially NB formation had occurred adjacent to the defect margin of RA. TRAP staining exhibited that OCs were scattered throughout the material adjacent to RB. In FLA, the fluorescent band corresponding to NB formation in the graft area was almost the same as the results obtained in histological observations. These results were also identified by micro-CT observations.

The cement showed good adaptability to the defect. The cement also exhibited excellent washout resistance, and maintained the original graft shape and volume. The GA was nearly completely closed by NB. NB was also observed above and under the GM where it attached the periosteum and subperiosteum.
Figure 9. BCP cement (3.5-fold)
The results observed in the section were almost same as other BCP cements. In general, the area of NB observed in the GA showed slightly larger compared to that observed in GA filled with other BCP cements.

Figure 10. BCP cement (4.0-fold)
The results observed in the section were almost same as other BCP cements. In general, the area of NB observed in the GA showed slightly smaller compared to that observed in GA filled with other BCP cements.
The cement also had sufficient physical properties and handling time resistance when immersed in water and physiological-like solution. Handling properties after mixing and showed superior washout resistance, were examined and handling properties, such as injectability, moldability and washout resistance, were compared to the conventional CPCs with improved physical properties and usability compared to OCs, osteoblasts, and their assembly. Previous studies have reported that conventional CPCs were excellent materials for use in reconstructive surgery of alveolar bone defects, such as periodontal bone defects and dental implant surgery, because most CPCs form low crystalline HA which is the main inorganic component of bone. On the other hand, CPCs still had some difficulties in maintaining the original shape at the defect site, because the washout resistance and the viscosity in the body fluid were insufficient. Most conventional CPCs also had flows in their hardening and formation of HA. Some commercially available CPCs had poor hardening times and showed inadequate physical properties for clinical use, even 24 hours after mixing. Even if CPCs could be used for bone generated from endochondral ossification, such as cranial bone, cheek bone or tubular bone, vertebrae bone or pelvic bone, most could not be used for bone generated from endochondral ossification, such as cranial bone, cheek bone or tubular bone, vertebrae bone or pelvic bone, mostly consisting of low crystalline HA which is the main inorganic component of bone. On the other hand, CPCs still had some defects, such as periodontal bone defects and dental implant surgery, because most CPCs form low crystalline HA which is the main inorganic component of bone. However, the GM with NB, compared to the original GA. The calcified area of BCP cements was obviously larger than that of the control cement at 8 weeks after surgery. The DC rates were, for the negative control (7.4±5.2), the control cement (75.3±11.7), and BCP cements (1.3-fold dilution=97.2±5.6, 1.3-5-fold=98.1±3.2, 1.4-0-fold=95±5.6) respectively (Table 2). The negative control showed slight NB formation. The control cement showed that NB formed more than half of the filled area. The defect filled with BCP cements was almost completely covered with newly formed bone. A student’s t-test revealed that each value of BCP cements showed no apparent difference among them. The DC rates of the BCP cement and the control cement showed significantly larger than that of the negative control (p<0.05). The rate of each BCP cement also exhibited apparently larger than that of the control cement (p<0.05). The above results indicated that BCP cement generally showed faster bone formation and replacement than the control cement.

**Discussion**

Previous studies have reported that conventional CPCs were excellent materials for use in reconstructive surgery of alveolar bone defects, such as periodontal bone defects and dental implant surgery, because most CPCs form low crystalline HA which is the main inorganic component of bone. On the other hand, CPCs still had some difficulties in maintaining the original shape at the defect site, because the washout resistance and the viscosity in the body fluid were insufficient. Most conventional CPCs also had flows in their hardening and formation of HA. Some commercially available CPCs had poor hardening times and showed inadequate physical properties for clinical use, even 24 hours after mixing. Even if CPCs could be used for bone generated from endochondral ossification, such as cranial bone, vertebrae bone or pelvic bone, most could not be used for intramembranous ossification, such as cranial bone, cheek bone or collagen bone. This is because the material did not have sufficient physical properties and shape integrity when applied to the defects. Some conventional CPCs were also slow to convert to natural bone by the remodeling system of basic multicellular units (BMU), consisting of OCs, osteoblasts, and their assembly.

As a response to the above reasons, it was developed the BCP cements with improved physical properties and usability compared to the conventional CPCs. Before this study, physical properties, such as diametral tensile strength, compressive strength, hardening time, and handling properties, such as injectability, moldability and washout resistance, were examined. The BCP cement showed excellent handling properties after mixing and showed superior washout resistance when immersed in water and physiological-like solution. The cement also had sufficient physical properties and handling time for clinical use and hardened rapidly in 7 to 10 minutes. Most commercialized CPCs, filled in defects but did not adequately maintain the original graft shape, and also had insufficient washout resistance and lost the original graft volume. This should be due to physical flow and chemical dissolution of the material components into bodily fluid.

The bone defects created in this study were a critical size that could not completely close and heal spontaneously. The BCP cement showed excellent washout resistance and sufficiently maintained the original graft shape due to the components and hardening mechanism of the material. All defects filled with the BCP cements showed that the GA was nearly completely covered by NB. The NB formation of BCP cements showed faster bone replacement than that of the control cement. These results suggested that the BCP cement should have excellent shape integrity and washout resistance, and also have superior osteoconductivity and bone replacement properties. The BCP cement showed NB was formed entirely above and under the material where the material attached to the periosteum and subperiosteum. NB that formed under the material was slightly thicker than that formed on the above-side, possibly due to differences in blood circulation.

Our previous studies reported that the BCP cement mostly converted to HA as the final product within 24 hours after mixing. HA crystals formed from the BCP cement had low-crystallinity and were nanometer-scale crystals similar to those found in bone. Here, we assume that the HA crystals could be easily resorbed by OC and converted to natural bone, which spontaneously occurred in bone remodeling. The BCP cement in the defect sites, mostly consisting of HA, firmly combined with RB due to the inorganic component of bone being made from the same material. Therefore, the cement filled in the defect and anchored tightly to the surface of RB which, in turn, increased the mechanical strength of the filling material and reduced its susceptibility to infections. The histological study of TRAP staining also showed that OC resorbed the BCP cement, should already have changed to HA, and gradually converted to natural bone. Therefore, we assumed that the GM of BCP cements, mostly consisting of low crystalline HA, should be resorbed by OC and rapidly converted to NB.

In this study, histometric measurements were performed using NIH image analysis. The DC rates of all BCP cements were over 95%, and the distribution of each data was not wide-spreading. Similar statistical tendency results were also shown in former studies that exhibited the DC rates using osteoinducible materials in rat calvarial defect model. The rate of the BCP cement was apparently larger than that of the control cement. The BCP and control cements were gradually resorbed by OC and converted to natural bone. However, the BCP cement consistently exhibited obviously faster (p<0.05) bone replacement than the control cement. These results suggested that the all the BCP cements should have excellent biocompatibility and osteoconductivity. Micro-CT results showed the cement maintained its original graft shape and adaptability through the entire experimental period, and also exhibited excellent washout resistance to bodily fluid when applied to bone defects. The results indicated that the BCP cement was successfully retained and had sufficient physical properties after the filling.

The control cement is a commercially prevalent and adequate material for clinical use already, so it is expected that the BCP cement also should be an effective and suitable material for bone defects.

**Acknowledgements**

The authors would like to give cordial thanks to Drs. Yoshinori Arai and Kazuya Honda, Department of Oral and Maxillofacial Radiology at Nihon University School of Dentistry, Mrs. Masumi Fujii and Miss. Ayaka Hirano, Frontier Science Co., for their technical supports.

**Conflict of Interest**

The author have declared that no COI exists.
References

1. Brown WE and Chow LC. Dental restoration cement pastes. US Patent: Patent No. 4518430, 1985
2. Dorozhkin SV. Self-setting calcium orthophosphate formulations. J Funct Biomater 4: 209-311, 2013
3. Schmitz JP, Hollinger JO and Milam SB. Reconstruction of bone using calcium phosphate bone cements: A critical review. J Oral Maxillofac Surg 57: 1122-1126, 1999
4. Sariibrahimoglu K, Wolke JG, Leeuwenburgh SC, Yubao L and Jansen JA. Injectable biphasic calcium phosphate cement as a potential bone substitute. J Biomed Mater Res B Appl Biomater 102B: 415-422, 2014
5. Tagaya M, Goto H, Iinuma M, Wakamatsu N and Tamura Y. Development of self setting Te-CP/α-TCP cement for pulpotomy. Dent Mater J 24: 555-561, 2005
6. Sugawara A. Method for controlling work time for forming shape of biphasic self-setting calcium phosphate. US Patent Application Publication: Pub. No. 15/123,533, 2016
7. Sugawara A. Method for controlling work time for forming shape of biphasic self-setting calcium phosphate. Japanese Patent Application: No. PCT/JP2015/001210, 2015
8. Hirayama S, Sugawara A, Fujikawa K, Okano H and Hirano A. Biphasic calcium phosphate based fast self-setting calcium phosphate cement (abstract 2368). J Dent Res (Spec Iss A) 180, 2015
9. Hirayama S, Iwai H, Sugawara A, Fujikawa K and Hirano A. Physical properties of biphasic calcium phosphate cements (abstract 3103). Jpn J Conserv Dent, Program, 103, 2016 (in Japanese)
10. Sugawara A, Fujikawa K, Koshi R, Ozawa Y, Kubota T, Yamamoto T and Sato S. Injection of biphasic calcium phosphate-based cement in rat calvarial model (abstract 3557). J Dent Res (Spec Iss A) 230, 2015
11. Tukune N, Sugawara A, Fujikawa K, Hirano A, Yamamoto T and Sato S. Evaluation of BCP cement when used for calvarial bone defect of rats (abstract 1925). J Dent Res (Spec Iss A) 184, 2017
12. Sugawara A, Yamamoto T, Fujikawa K, Hirano A, Koshi R and Sato S. Evaluation of TCP-TTCP cement in rat calvarial model (abstract 1926). J Dent Res (Spec Iss A) 184, 2017
13. Ozawa Y, Koshi R, Kubota T, Yamamoto T, Sugawara A, Fujikawa K and Sato S. A new generation bone substitute materials: Histopathological evaluation of biphasic calcium phosphate-based cement in rat calvarial model (abstract 2504). Jpn J Conserv Dent, Program, 156, 2016 (in Japanese)
14. Gbureck U, Barralet JE, Kerstin, Spatz K, Grover LM and Thull R. Ionic modification of calcium phosphate cement viscosity. Part I: Hypodermic injection and strength improvement of apatite cement. Biomaterials 25: 2187-2195, 2004
15. Kochi G, Sato S, Ebihara H, Hirano J, Arai Y and Ito K. A comparative study of microfocus CT and histomorphometry in the evaluation of bone augmentation in rat calvarium. J Oral Sci 52: 203-211, 2010
16. Pang EK, Im SU, Kim CS, Choi SH, Chai JK, Kim CK, Han SB and Cho KS. Effect of recombinant human bone morphogenetic protein-4 dose on bone formation in a rat calvarial defect model. J Periodontol 75: 1364-1370, 2004
17. Kim CS, Kim JI, Kim J, Choi SH, Chai JK, Kim CK and Cho KS. Ectopic bone formation associated with recombinant human bone morphogenetic protein-2 using absorbable collagen sponge and beta tricalcium phosphate as carriers. Biomaterials 26: 2501-2507, 2005
18. Jung UW, Choi SW, Pang EK and Kim CS, Choi SH and Cho KS. The Effect of varying the particle size of beta tricalcium phosphate carrier of recombinant human bone morphogenetic protein-4 on bone formation in rat calvarial defects. J Periodontol 77: 765-772, 2006
19. Chow LC, Takagi S, Costantino PD and Friedman CD. Self-setting calcium phosphate cements. Mater Res Soc Symp Proc 179: 3-24, 1991
20. Sugawara A, Nishiyama M, Kusama K, Moro I, Nishimura S, Kudo I, Chow LC and Takagi S. Histopathological reactions of calcium phosphate cement. Dent Mater J 11: 11-16, 1992
21. Sugawara A, Kusama K, Nishiyama S, Nishiyama M, Moro I, Kudo I, Takagi S and Chow LC. Histopathological reactions to calcium phosphate cement for bone filling. Dent Mater J 12: 691-698, 1993
22. Sugawara A, Kusama K, Nishimura S, Nishiyama M and Moro I. Histopathological reactions of a calcium phosphate cement root canal filler. J Hard Tissue Biol 4: 1-7, 1995
23. Fujikawa K, Sugawara A, Murai S, Nishiyama M, Takagi S and Chow LC. Histopathological reaction of calcium phosphate cement in periodontal bone defect. Dent Mater J 14: 45-57, 1995
24. Sugawara A, Fujikawa K, Kusama K, Nishiyama M, Murai S, Takagi S and Chow LC. Histopathological reaction to calcium phosphate cement for alveolar ridge augmentation. J Biomed Mater Res 61: 47-52, 2002
25. Fujikawa K, Sugawara A, Kusama K, Nishiyama M, Murai S, Takagi S and Chow LC. Fluorescent labeling analysis and electron probe microanalysis for alveolar ridge augmentation using calcium phosphate cement. Dent Mater J 21: 296-305, 2002
26. Takagi S, Chow LC, Hirayama S and Sugawara A. Premixed calcium-phosphate cement pastes. J Biomed Mater Res B Appl Biomater 67B: 689-696, 2003
27. Sugawara A, Fujikawa K, Takagi S, Chow LC, Nishiyama M and Murai S. Histopathological and cell enzyme studies of calcium phosphate cements. Dent Mater J 23: 613-620, 2004
28. Sugawara A, Fujikawa K, Takagi S and Chow LC. Histological analysis of calcium phosphate bone grafts for surgically created periodontal bone defects in dogs. Dent Mater J 27: 787-794, 2008
29. Sugawara A. Bone Regeneration Technology: Newly Revised Edition - Concept and Clinical Application of Bone Regeneration. Zenith Press, Tokyo, 2011. (in Japanese)
30. Sugawara A. Evidential Implantology. Zenith Press, Tokyo, 2013. (in Japanese)