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Development of carbonate-containing apatite/collagen composite for osteoconductive apical barrier material

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Abbreviated title: Ap/col composite for apical barrier

Abstract

The current report describes the properties of a new apical barrier material formulated from carbonate-containing apatite (CAp) and collagen. CAp particles of 50 nm were deposited on reconstituted collagen fibers. CAp/col with about 60wt% CAp (corresponding to apatite content of bone) was obtained after 1-day calcification. CAp content increased up to about 80wt% in a 15-day calcification reaction. CAp/col was composed of fine calcified collagen fibers. The crystallinity and Ca/PO_4 ratio of CAp were comparable to those of bone apatite. The mixture of CAp/col and saline reached a pH of about 9. The optimum powder-to-liquid ratio (P/L) to set into a root canal was determined to be 1.2. Furthermore, the mixture (P/L=1.2) condensed in a root canal was liquid permeable. Thus, the CAp/col was expected as an apical barrier material with osteoconductivity.

Keywords: carbonate-containing apatite; collagen; composite; osteoconductive apical barrier material

Introduction

In endodontic therapy to accomplish apical closure in teeth with open apices, calcium hydroxide has become commonly used. Gutta-percha also has become a common filling material used in root canals. In an effort to identify the best apical barrier material suitable for all situations, the efficiency of several materials has been evaluated: dentin chips (1,2), decalcified freeze-dried bone (DFDB) (3,4), true bone ceramic (TBC) (4), tricalcium phosphate (TCP) (5), hydroxyapatite (HAp) (6) and mineral trioxide aggregate (MTA) (7-11). However,

there are a number of drawbacks to these materials, such as bacterial contamination, antigenicity, quantity of supply, lifetime of the material, separation of particles from the apical foramen, and difficulty in manipulating the materials.

An ideal apical barrier material would have biological compatibility and material properties such as induction of cementogenesis, healing ability and biocompatibility. Currently, one of the best materials for an osteoconductive apical barrier consists of composites of apatite and collagen (Ap/col), because 1) apatite and collagen, respectively, are the major inorganic and organic components of dentin, cementum and bone, and 2) collagen alone has been used in wound dressing and adhesives in clinics due to its biological functions (12,13). Synthetic apatite compounds have a variety of applications as biomaterials due to their biocompatibility (14-16). Furthermore, once carbonate ions were incorporated into the lattice, CO_3-containing apatite (CAp) was distinguished for its excellent osteoconductivity (18-20). Therefore, there is growing interest in applications of this material. However, there have been no reports of applications of composite of CAp and collagen (CAp/col) in root canal treatments.

Therefore, we aimed to develop a CAp/col which is applicable to defects at the apical region of a root canal. To this end, 1) atelocollagen without antigenicity (21,22) was reconstituted, 2) apatite was deposited on the fibers using the enzymatic hydrolysis method (23-25), and 3) the entire fabrication and washing procedures were carried out under controlled stirring.

Materials and Methods

Construction of CO_3-containing apatite/collagen composite

Reagent grade chemicals and double distilled water (d-d-water) were used. All reactions were carried out at 37°C under stirring at 700 rpm. The direction of stirring was inverted every 5 seconds.

Atelocollagen (Cellmatrix Type-I A^{TM}, Nitta Gelatin, Osaka, Japan) was reconstituted with the addition of an equivalent amount of 200 mM triethanolamine hydrochloride buffer solution

(pH8.0) for 24 hours. The reconstituted collagen was cross-linked in 0.1% dimethylsuberimidate dihydrochloride (Nacalai Tesque, Kyoto, Japan) solution in the presence of 0.01% alkaline phosphatase (Type I-s, Sigma, Tokyo, Japan) and 0.01% egg-yolk phosvitine (Sigma) at pH 8.0 for 6 days. After cross-linking, the sediment was separated and soaked in 0.02% alkaline phosphatase-phosvitine solution for 3 hours. The sample was removed from the solution, washed with d-d-water and then put into a 6 mM calcium β-glycerophosphate solution for 20 hours. After each change of solution, the sample was thoroughly washed with d-d water using a tube-mixer and centrifuged at 3000 rpm for 10 minutes at room temperature. This process was considered one calcification cycle. The cycle was repeated 1-15 times (1-15 days of reaction) in order to explore the time to obtain a composite with apatite content suitable for apical barrier material. After each calcification reaction was terminated, calcified collagen was lyophilized for about 20 hours.

Analysis of the calcification rate

To analyze the calcification rate, apatite content of a composite of 1-15 days of reaction was determined thermo-gravimetrically (TG8120, Rigaku, Nagoya, Japan). Apatite content was calculated as amount (weight %) of the remainder of calcination at 1000°C and plotted as a function of reaction period. For the shorter reaction period, collagen was calcified for 2, 6 and 12 hours and calcified products were examined.

Characterization of CO_3-containing apatite/collagen composite

Lyophilized composite was characterized by optic microscopy, X-ray diffraction (XRD; RINT2500, 56 kV, 200 mA, CuKα; Rigaku), scanning electron microscopy (SEM; S4500, Hitachi, Japan), and Fourier transform infrared spectroscopy (FT-IR; FTIR8400S, Shimadzu, Kyoto, Japan). The Ca/PO_4 ratio of apatite was determined chemically: a known amount (around 4 mg) of composite was weighed, dissolved in 1 ml of 2 N HCl, and 100 ml of about 0.05 mM of apatite solution was prepared using d-d-water. Ca solution concentration was determined by ion chromatography (CCD-6A, Shimadzu) and PO_4 concentration was

determined absorptiometrically (UV-150-02, Shimadzu) using the phospho-molybdate method (26).

Properties of CO_3-containing apatite/collagen composite and saline mixture

To apply the composite as apical barrier material, the CAp/col was mixed with saline. The composite to liquid (powder to liquid (P/L)) ratio suitable to set into a root canal was explored, focusing on ease of manipulation and washout resistance from the root. The pH of the CAp/col and saline mixture was measured using a universal pH testing paper (Toyo Roshi, Nagoya, Japan). A liquid permeation test of the mixture was performed using ten human molar teeth with single root, which were removed: aliquot of the mixture (P/L=1.2) was set into the apical space of the root from the apex to a height about 2 mm. The remaining part of the root was filled with gutta-percha point (Zippere, Germany) and root canal sealer (Showayakuhinkakou, Japan) by the lateral condensation method, and then the top was covered with composite resin (Shofu, Kyoto, Japan). The root was soaked in India ink (Remel, USA) for 48 hours and the height of the stained region in the CAp/col was measured.

Results

The calcification of collagen fibers proceeded rapidly in the first 2 days: The amount of the deposit was about 30 wt% at 12 hours, about 60 wt% at 1 day and about 70 wt % at 2 days. After 2 days, the calcification proceeded gradually. The rate of calcification is shown in Figure 1, where apatite content (wt%) is plotted as a function of calcification period. The plot suggests that further increase in apatite content from 75wt% (at 7 days) was small. When the calcification reaction was terminated at 15 days, the amount of apatite was about 80wt%.

Reconstituted collagen fibers were fine, string-like under an optic microscope and they were loosely assembled (Fig.2A). The surface appeared smooth under SEM observation (Fig.2D). By soaking the collagen fibers in calcium β-glycerophosphate solution, particles around 50 nm in size deposited on the surface (Fig.2E1). Higher magnification of Fig.2E1 clearly showed the nano-particles on the collagen fiber (Fig.2E2). The calcified collagen fiber

was cotton-like under an optic microscope until 3 days of calcification (Fig.2B). The surface of collagen fibers was almost completely covered with the particles at 3 days (Fig.2F). After further reaction, calcified fibers formed small lumps. The lumps after 15 days of calcification (Fig.2C) were rather hard, but were easily unraveled. SEM observation showed that the lumps were composed of fine calcified fibers (Fig. 2E1, 2F). After a later stage of calcification, granulitic apatite deposited on the calcified collagen fibers (Fig. 2G). As shown by Fig. 2G, these granules were composed of elementary particles 50 to 100 nm in size.

In order to identify the deposit on the collagen fiber, composites at several calcification periods were analyzed by XRD and FT-IR. Figure 3 shows XRD patterns for the composites at 12 hours and 1, 3, 7 and 15 days of calcification along with reconstituted collagen. The composite of 12 hours of calcification showed broad but clear peaks at about 26 and 32 degrees in 2θ, along with several other small peaks. This observation suggested that the small granules shown in Fig. 2E consisted of apatite. The peaks became sharp and the number of peaks increased as the reaction progressed, confirming that the deposit was apatite. The broadness of the XRD peaks of CAp was comparable to that of dentin and bone-apatite.

FT-IR spectra of the composites after 1- and 7-day calcification are shown in Fig. 4. The lower image shows a spectrum of reconstituted collagen prior to calcification. By comparing these spectra, the bands at 1545, 1450, 1410, 1020 and 870 cm^{-1} were ascribed to those of apatite. According to previous IR studies (27-29), the bands at 1020 and 870 cm^{-1} were assigned to PO_4 and those at 1545, 1450 and 1410 cm^{-1} were assigned to CO_3 in the apatite lattice. It is probable that the other CO_3 band, at around 880-875 cm^{-1}, shifted to a slightly lower value and overlapped with the PO_4 band at 870 cm^{-1} (Fig. 4), because the frequency of a band changes depending on the content of CO_3 (28,29). CO_3 substitutes for both OH and PO_4 in apatite structure: The OH-substitution, defined as Type A, shows IR bands at around 1450, 1545 and 880 cm^{-1}; and the PO_4-substitution, defined as Type B, shows IR bands at around 1410, 1455 and 875 cm^{-1}. On the basis of the XRD and the FT-IR study, the deposit on the collagen fiber

was identified as CO_3-containing apatite, in which CO_3 substituted for both OH and PO_4. Chemical analysis showed that the Ca/PO_4 ratio of apatite was 1.70±0.05 (N=4).

The powder to liquid (P/L) ratio of 1.2 yielded a homogenous paste-like mixture, which was suitable to set into a root canal and to maintain within the root. When the P/L ratio was larger than 1.4, the mixture was too powder-rich to make a homogeneous mixture. At a P/L smaller than 1.0, the mixture was too soft to maintain its shape. The pH of the mixture was about 9, regardless of the P/L ratio. The CAp/col condensed in the root canal was liquid permeable. After 48 hours of soaking in Indian ink, the length of the Ap/col penetrated with the ink was 1.2±0.4mm (N=10) from the apex.

Discussion

Controlled stirring was important to construct and preserve the fine fibrous structure of collagen and the size of apatite deposited on the collagen fiber in the fabrication of fine CAp/col. When the fabrication was carried out without stirring, an agglomerate of composite was obtained, and it took several days to obtain a composite with 60 wt% apatite content (25). Fine collagen fibers formed *via* controlled stirring are expected to have a large surface area, which offered numerous nucleation sites (30,31). The apatite deposition rate under stirring was therefore faster than that without stirring. Thus, stirring was an effective means to obtain the composite in a shorter period. The XRD patterns and IR absorbance of CO_3 in 1- and 7-day calcifications were almost the same. This suggested that CAp was deposited with almost uniform composition during the reaction.

CAp was precipitated on collagen fibers whether or not carbonate was added as a reagent in the reaction. This was attributed to dissolved CO_2 in the air during the reaction. Since a buffer solution with pH 8.0 was used, CO_2 gas dissolution was promoted under stirring. The resulting CO_3 ions were incorporated into apatite lattice and substituted for PO_4. Therefore, the Ca/PO_4 ratio of apatite synthesized in this study was higher than that of stoichiometric HAp, 1.67. As a biomaterial, CAp with low crystallinity has advantage over stoichiometric HAp in

osteoconductivity (18-20). The stirring, in this sense, was favorable to form CAp without addition of any other chemical reagent.

One synthetic root-end filling material of note is MTA. Recent studies have shown promising results as a root-end filling material, such as sealing ability and stimulation of mineralization (7-11). Although MTA has chemical composition ($CaOSiO_2$, $CaOAl_2O_3$, $CaSO_42H_2O$) and crystallographic properties different from those of biological apatites, its bioactivity could be increased by immersing it in phosphate-containing solution to form Ca-deficient, poorly crystallized carbonated apatites (10,11). MTA is totally inorganic, with a dense structure, and does not contain collagen. Therefore, the present CAp/col composite is a constructive counterpoint of MTA. For further evaluation of the CAp/col, *in vivo* examination must be performed.

Hemostatic action of collagen fibers was also expected (13) for CAp/col as an apical barrier material, because 40 wt% of CAp/col was collagen. Furthermore, the CAp/col mixture filled into a root canal had liquid permeability. Blood and body fluid that penetrated the composite are supposed to loosen the calcified collagen fibers (32), thereby promoting dissolution of apatite. Dissociated Ca ions are expected to be a trigger of new hard tissue formation partly by activating calcium-dependent adenosine triphosphatase (33). Expansion of collagen matrix could lead to separation of apatite particles (32). To overcome this matter, we deposited apatite on the fibers using the enzymatic hydrolysis method (23-25). The superiority of this method was that alkaline phosphatase and phosvitine were cross-linked to collagen fibers so that apatite nucleated on the fiber. This may explain why the apatite crystals remained and accumulated on the fibers despite the stirring and mixing in aqueous solutions (Fig.2E-2G). Therefore, the apatite particles would remain within the composite after setting into the defect. Moreover, the pH of the mixture was around 9, which is not expected to be pungent to the surrounding tissue. Thus, the CAp/col was expected as an apical barrier material with osteoconductivity.

References

1. Tronstand L. Tissue reaction following apical pluging of the root canal with dentin chips in monkey teeth subjected to pulpectomy. Oral Surg 1978; 45: 297-304.
2. Safavi K, Horsted P, Pascon EA, Langeland K. Biologic evaluation of the apical dentin chip plug. J Endod 1985; 11: 18-24.
3. Hartwell GR, Marshal MC. Healing of furcations in primate teeth after repair with decalcified freeze-dried bone. J Endod 1993; 19: 357-61.
4. Yoshida T, Ito T, Saitoh T, Sekine I. Histlogical study on the use of freeze-dried allogenic dentin powder and True Bone Ceramics as an apical barrier. J Endod 1998; 24(9): 581-6
5. Heller AL, Koenigs IF, Brilliant DJ, Melfi CR, Driskell DT. Direct pulp capping of permanent teeth in primates using a resorbable form of tricalcium phosphate ceramic. J Endod 1975; 1:95-101.
6. Yoshida T. Experimental study on an apical barrier constructed of synthetic hydroxyapatite. J Gifu Dent Soc 1987; 14 : 309-28. (in Japanese)
7. Lee SJ, Monsef M, Torabinejad M. Sealing ability of a mineral trioxide aggregate for repair of lateral root perforation. J Endod 1993; 19(11): 541-4.
8. Gomes-Filho JE, de Faria MD, Bernabe PFE, Nery MJ, Otoboni-Filho JA, Dezan-Junior E, Costa MMTde M, Cannon M. Mineral trioxide aggregate but not light-cure mineral trioxide aggregate stimulated mineralization. J Endod 2008; 34(1): 62-5.
9. Tani-Ishii, N, Hamada N, Watanebe K, TujimotoY, Teranaka T, UmemotoT. Expression of bone extracellular matrix proteins on osteoblast cells in the presence of mineral trioxide. J Endod 2007; 33(7): 836-9.
10. Tay KCY, Loushine BA, Kapur R, Primus CM, Gutmann JL, Loushine RJ, Pashley DH, Tay FR. In Vitro Evaluation of a Ceramicrete-based Root-end Filling Material. J Endod 2007; 33(12): 1438-43.
11. Tay FR, Pashley DH, Rueggeberg FA, Loushine RJ, Weller RN. Calcium phosphate phase transformation produced by the interaction of the portland cement component of white

mineral trioxide aggregate with a phosphate-containing fluid. J Endod 2007; 33(11): 1347-51.

12. Kleinman HK, R.J. Klebe RJ, Martin GR. Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 1981; 88: 473-85.
13. Shoshan S, Finkelstein S. Acceleration of wound healing induced by enriched collagen solutions. J Surg Res 1960; 10: 485-91.
14. Aoki H. Science and medical application of hydroxyapatite. Tokyo: Takayama Press System Center Co. Inc., 1991. 137-63 p.
15. Rothstein SS, Paris DQ, Zacek MP. Use of hydroxyapatite for the augmentation of deficient alveolar ridges. J Oral Maxillofac Surg 1984; 42: 224-30.
16. Kenny EB, Lekovic V, Sa Ferreira JC, Han T, Dimitrijevic B, Carranz Jr. FA. Bone formation within porous hydroxyapatite implants in human periodontal defects. J Periodontal 1986; 57: 76-83.
17. Hasegawa M, Doi Y, Uchuda A. Osteoconduction and bioresorption of sintered carbonate apatite inplanted in rabbits. J Bone Joint Surg (Br) 2003; 85B: 142-7.
18. Cazalbou S, Combes C, Eichert D, Rey C, Glimcher MJ. Poorly crystalline apatites: evolution and maturation in vitro and in vivo. J Bone Miner Metab. 2004; 22:310-7.
19. Doi Y, Iwanaga H, Shibutani T., Moriwaki Y, Iwayama Y. Osteoclastic responses to various calcium phosphates in cell culture. J Biomed Mater Res 1999; 47: 424-33.
20. Barralet J, Akao M, Aoki H, Aoki H. Dissolution of dense carbonate apatite subcutaneously implanted in Wister rats. J Biomed Mater Res 2000; 49: 176-82
21. Schmitt FO, Levine L, Drake KM, Rubin AL, PfahlD, Davison PF. The antigenicity of trpocollagen. Proc Natl Acad Sci USA 1964; 51: 493-7.
22. DeLustro F, Condell RA, Nguyen MA, McPherson JM. A comparative study of the biologic and immunologic response to medical devices derived from dermal collagen. J Biomed Mater Res 1986; 20 : 109-20.
23. Banks E, Nakajima S, Shapiro LC, Tilevitz O, Alonzo JR, Chianell RR. Fibrous apatite

grown on modified collagen. Science 1977; 198: 1164-7.

24. Doi Y, Horiguchi T, Moriwaki Y, Kitago H, Kajimoto T, Iwayama Y. Formation of apatite-collagen complexes. J Biomed Mater Res 1996;31: 43-9.

25. Akita Y, Kawano S, Takenaka Y, Doi Y and Sekine I. Application of apatite/collagen complex to endodontic surgery. Jpn J Conserv Dent 2005;48: 835-49. (in Japanese)

26. Murphy J, Riley JP. A modified single solution method for the determination of phosphate in natural water. Anal Chim Acta. 1962; 27: 31-6.

27. Termine JD, Lundy DR. Spectra of some phosphate salts amorphous to X-ray diffraction. Calc Tiss Res 1974;15: 55-70.

28. Elliott JC. Structure and Chemistry of the Apatites and Other Calcium Orthophosphates. London: Elsevier, 1994. 215-8, 228, 230-4 p.

29. Fleet ME, Liu X. Local structure of channel ions in carbonate apatite. Biomaterials 2005; 26: 7548-54.

30. Katz EP. The kinetics of mineralization in vitro. I. The nucleation properties of 640A collagen at 25°C. Biochim Biophys Acta 1969; 194:121-9.

31. Jethi RK, Inlow CW, Wadkins CL. Studies of the mechanism of iological calcification. I. Kinetic properties of the in vitro calcification of collagen-containing matrix. Calc Tiss Res 1970; 6: 81-92.

32. Marouf HA, Quayle AA, Sloan P. In vitro and in vivo studies with collagen/hydroxy-apatite implants. Int J Oral Maxillofac Implants 1990; 5:148-54.

33. Seux D, Couble ML, Hartmann DJ, Gauthier JP, Magloire H. Odontblast-like cytodifferentiation of human dental pulp cells in vitro in the presence of a calcium hydroxide-containing cement. Arch Oral Biol 1991; 36:117-28.

Figure Legends:

Fig. 1. Calcification rate of composite, estimated by apatite content (wt%).

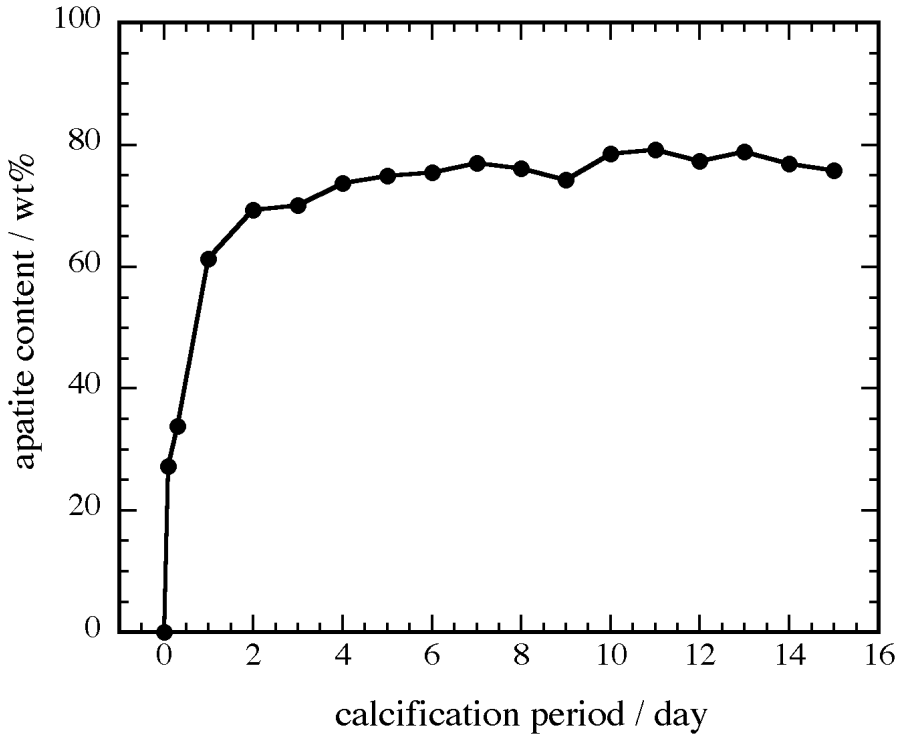
Fig. 2. Optical microscopic images of (A) reconstituted collagen and CAp/col after (B) 3 days and (C) 15 days of calcification. Composite became more solid, but was easily unraveled after 10-day calcification. Scale: 1 mm for (A), (B), and (C). SEM images of (D) reconstituted collagen and CAp/col after (E) 12 hours, (F) 3 days and (G) 15 days of calcification. (E_2) is higher magnification of (E_1).

Fig. 3. X-ray diffraction patterns of CAp/col: 12 hours and 1, 3, 7, and 15 days of calcification. Lower pattern is of reconstituted collagen prior to calcification.

Fig. 4. FT-IR spectra of reconstituted collagen and CAp/col after 1 day and 7 days of calcification. Absorption bands of CO_3 and PO_4 are indicated by *.

Figure

Fig.1


apatite content / wt%
calcification period / day
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20
40
60
80
100
0
2
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8
10
12
14
16

Fig.2

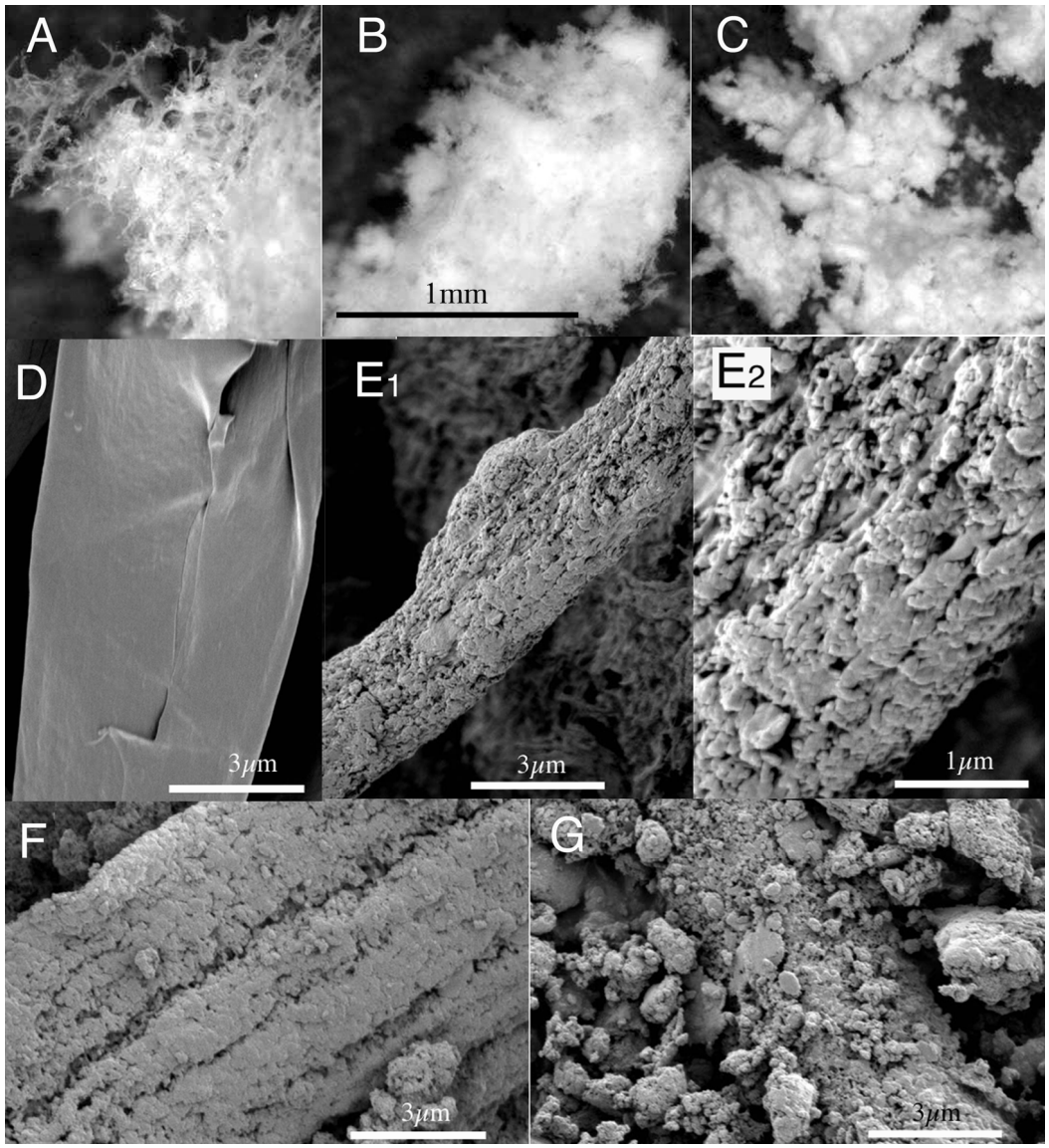
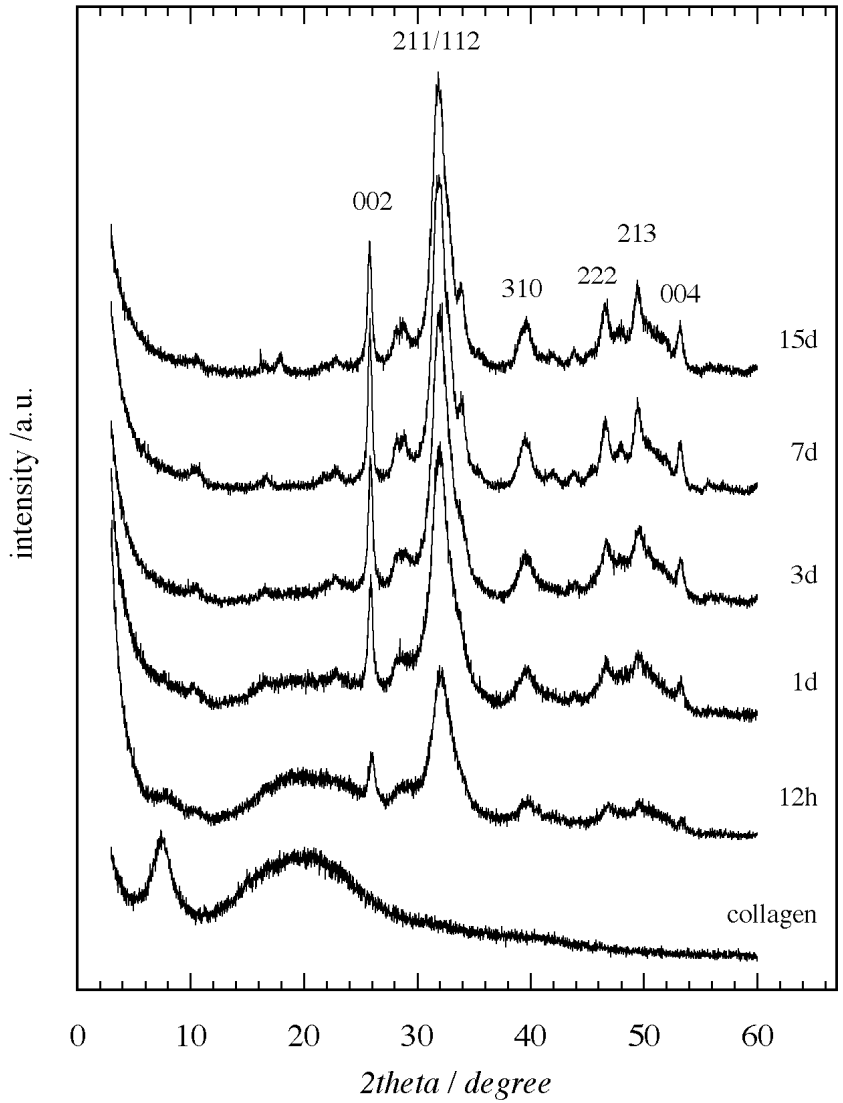


Fig.3


211/112
002
213
310
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collagen
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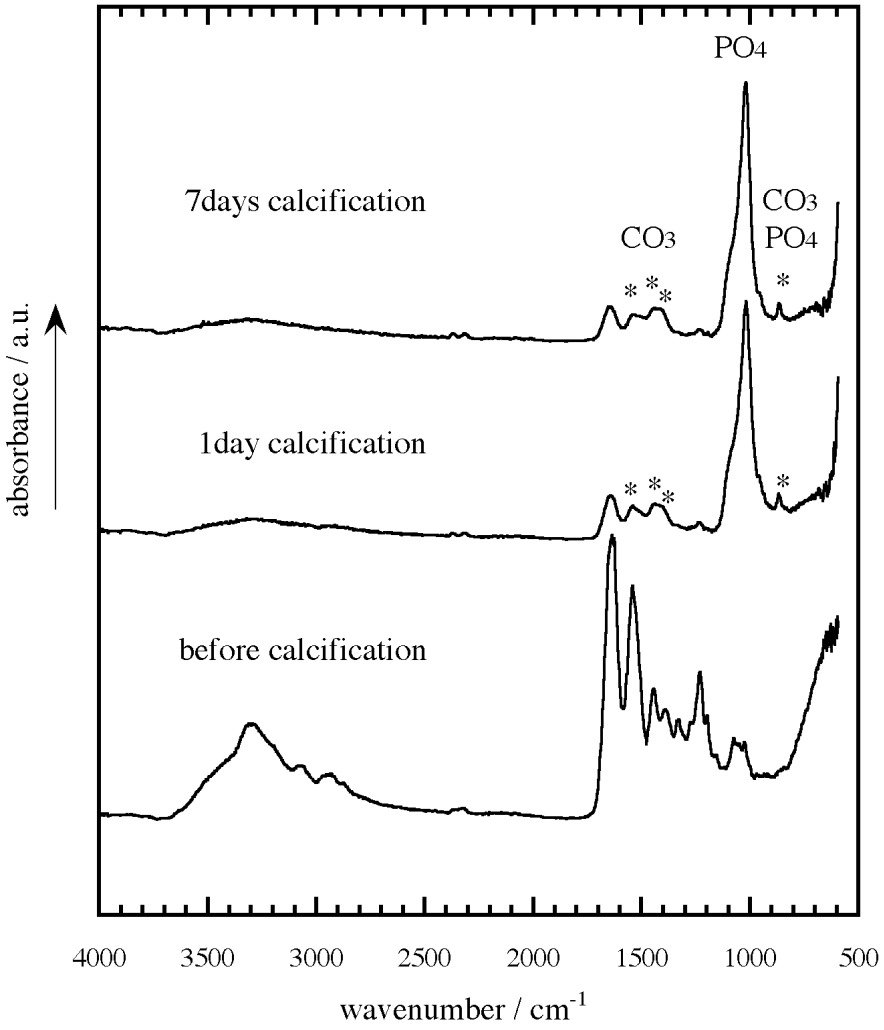
2theta / degree

Fig.4


PO4
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CO3
PO4
1day calcification
before calcification
absorbance / a.u.
4000
3500
3000
2500
2000
1500
1000
500
wavenumber / cm-1

Letter with Signatures

Prof. Kenneth M. Hargreaves, DDS, PhD
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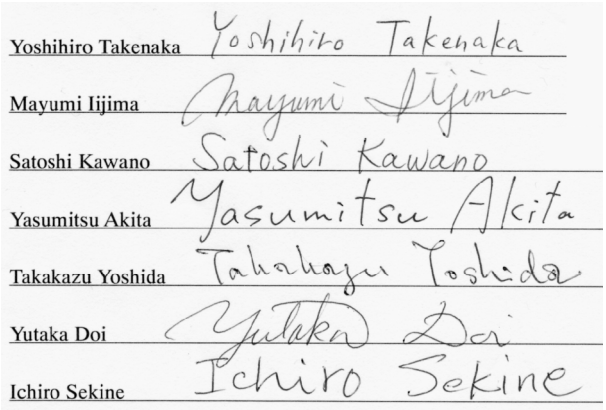
30 May 2008

Dear Prof. Kenneth Hargreaves,

We would like to submit our revised paper entitled " *Development of carbonate-containing apatite/collagen composite for osteoconductive apical barrier material* " to the *Journal of Endodontics* : Basic Research--Technology. The corresponding author is Dr. Mayumi Iijima (Asahi University School of Dentistry, 1851-1 Hozumi, Mizuho-city, Gifu 501-0296, iijima@dent.asahi-u.ac.jp). We revised the manuscript taking into all comments into account.

Sincerely Yours,



Optional Author Comments

Ref.: Ms. No. JOE 08-126
Development of carbonate-containing apatite/collagen for osteoconductive apical barrier material

Editor's comments:
1. Many of our readers have requested that articles discuss related papers recently published in the JOE. This helps the reader to understand new developments in a research field that may not be in an area of their expertise and complements periodic review articles that are a more extensive analysis of a research field. Accordingly, please consider discussing:
Gomes-Filho, J. E., M. D. de Faria, et al. (2008). "Mineral trioxide aggregate but not light-cure mineral trioxide aggregate stimulated mineralization." J Endod 34(1): 62-5.
Tani-Ishii, N., N. Hamada, et al. (2007). "Expression of bone extracellular matrix proteins on osteoblast cells in the presence of mineral trioxide." J Endod 33(7): 836-9.
Tay, K. C., B. A. Loushine, et al. (2007). "In Vitro Evaluation of a Ceramicrete-based Root-end Filling Material." J Endod 33(12): 1438-43.
We read those and other papers. We included statement about MTA in INTRODUCTION and DISCUSSION.

2. We have discovered that the section of the article with the greatest number of typos is the reference section. PLEASE NOTE that the official abbreviation for the Journal of Endodontics is: J Endod Please carefully review all references to ensure their accuracy.
References were checked and corrected.

3. Figures should be created/scanned and saved and submitted as either a TIFF (tagged image file format), an EPS (encapsulated PostScript) file, or a PPT (PowerPoint) file. Line art must have a resolution of at least 1200 dpi (dots per inch), and electronic photographs, radiographs, CT scans, and so on and scanned images must have a resolution of at least 300 dpi. Please note that artwork generated from office suite programs such CorelDRAW and MS Word and artwork downloaded from the Internet (JPEG or GIF files) cannot be used.
Original Figures were made using PhotoShop and KleidaGraph. Then, they were copied on PowerPoint file. We hope that all Figures are fine, but if the quality is not good, please let us know.

Associate Editor Comments: I agree with the reviewers and also recommend revision of this manuscript before publication. The introduction/background information needs to include the purpose for using an apical barrier and some discussion of MTA used as an apical barrier. I felt the gap in knowledge was identified and would be strengthened if information with regard to MTA barriers was included. I am not sure the manuscript can be significantly shortened as was recommended by one of the reviewers. I am afraid shortening the manuscript would delete some important background and discussion information. Both reviewers noted they would reduce the number of figures but did not indicate which figure or figures they would eliminate. All four seemed appropriate to me and each provided different information. With the number of revisions suggested by the reviewers, there is still a great deal of work to be done before this manuscript is

ready for publication. The subject area is of interest and the suggested changes can be made without altering the study design. This is the reason I suggest revision rather than rejection of this manuscript.
We appreciate you for supporting our manuscript. It was difficult to remove suggested Figure, because Figure 2E is important to show the texture of early deposit on collagen fiber. We added the explanation in text. We read papers concerning MTA. We included statement about MTA in INTRODUCTION and DISCUSSION.

Reviewers' comments:
Reviewer #1: Development of carbonate-containing apatite/collagen for osteoconductive apical barrier material

General Comments

The topic of this article is interesting and the authors have a good concept for creating a new apical barrier material. The authors have clearly spent a lot of time preparing this manuscript. These types of studies, which develop new types of biomaterials, are not very common and deserve support to advance the science and success of endodontic practice. Unfortunately, the written quality of English and use of phrases is poorly executed and needs improvement. Much of the text of this article is clumsy or confusing, and a lot of grammatical editing is required. In the material and methods the clarity of the text explaining the creation of materials could be improved. In the results section the authors begin with the first three paragraphs with a Figure number. This is not the correct format to write a results section. The authors should describe each of the results and then cite the figure to support their remarks. The discussion seems reasonable.
English had been checked by a native speaker of An English correction company before submission. We will ask another company for correction.
Text was rewritten.

The authors need to address the following specific comments:

Abstract
1. The authors should change the sentence structure to explain in the first sentence that: "The purpose of this article was to investigate the properties of a new apical barrier material formulated from Cap and collagen."
Sentence was corrected.
2. The second sentence should say "Cap particles of 50 nm were deposited on reconstituted collagen fibers.
Sentence was corrected.
3. In the fourth sentence, remove "reaction period" this is confusing.
Sentence was corrected.
4. The authors must remove "clinical use" from the final sentence since the materials were

not tested clinically.
Sentence was corrected.

Introduction

5. The end of the first sentence needs to be reworded to improve it's clarity as follows: ".calcium hydroxide have become common to help accomplish apical closure."
Sentence was corrected.

6. The second sentence should also be reworded to : "Gutta percha has become a common root canal filling material."
Sentence was corrected.

7. The third sentence should be reworded to: "To identify the best apical barrier material suitable for all situations."
Sentence was corrected.

8. Why "true" bone ceramic? Is bone ceramic not descriptive enough?
"True bone ceramic" is a trade name. It is manufactured by Koken Co. Ltd. (Tokyo, Japan).

9. Bacterial contamination is not from the material unless it has poor quality control, do you mean the ability of the material to prevent leakage?
This is concerned mainly when autologous dentin of infected root was used, because it is used in the process of treatment without disinfection.

10. In the final paragraph what is "atelocollagen"? Trpocollagen?
Atelocollagen is obtained by removing telopeptids from tropocollagen.

11. Why is the "controlled stirring" so prominent in the introduction and materials and methods? Isn't this expected from manufacturing?
Because the stirring method was contrived to prevent reconstituted collagen fibers from assembling into thick bundles. We found that reversing the stirring direction worked effectively. Thus, it was possible to obtain fine fibers of CAp and collagen composite.

Materials and Methods

12. For your materials suppliers, please include their name, city, state/country. This information is incomplete.
The information was supplied.

13. Please reference the spectrophotometer and ion-chromatography and other methods if possible to help readers reproduce your methods.
The reference concerning phosphate analysis was indicated. The analysis methods are mentioned briefly so as not over the limit of words.

14. Were your human premolar teeth obtained after institutional approval and patient consent? Please explain, how many teeth were used, the approval and consent process, and name the clinic they were obtained? Where the teeth extracted for routine dental treatment reasons following patient informed consent?
#14-1 Yes, they were. All the teeth used in this study were obtained in the dental clinic of Asahi University with all patients' consent and approval of Asahi University.

#14-2 Ten teeth were used.
#14-3 Yes, they were extracted for routine dental treatment reasons in Asahi University following patient informed consent.

Results

15. The citing of figures prior to the text is not reader-friendly. Please describe your results and then cite the figure numbers to support your text.

Text was edited as suggested.

16. Please leave a space between your numerals and days throughout your text.

Text was corrected as suggested.

17. Please remove Figure 2.E1 because it is of no usefulness.

Figure E is important to show the size and morphology of early deposits on collagen fiber. Explanation of Fig.E was added .

18. Please explain why: "The crystallinity was comparable to that of dentin and bone-apatite" How so?

The crystallinity represents the crystallographic quality of mineral, which is evaluated by the XRD profile. For example, sound enamel shows very sharp diffraction pattern, indicating that the crystallinity of enamel apatite is high. On the other hands, bone and dentine exhibit broad XRD patterns, indicating that the crystallinity of bone and dentin apatite is low. As shown by Figure 3, the XRD peaks of CAp were broad and they were similar to those of bone and dentin apatites.

19. Please explain how this was tested: ".. which was suitable to set into a root canal and to maintain it within the root."?

The composite to liquid (i.e., powder to liquid (P/L)) ratio suitable to set into a root canal was explored, focusing on easiness to manipulate and washout resistance. That is, it was tested if a mixture with certain P/W ratio can be filled easily into a root canal and if it does not flow out from the root, using removed human teeth.

Discussion

20. Why was the "Cap/col hemostatic action of collagen fibers was expected?"

Because, collagen fibers have hemostatic action and CAp/col composite contains collagen about 40wt%.

21. Please reference "Blood and body fluid that permeated into the composite probably loosen the calcified collagen fibers>"

Reference is indicated as (32).

22. Please reference: "This would be a trigger of new hard tissue formation."

Reference was cited as (33) along with additional explanation about it.

23. Please remove the comments about the materials being suitable for "clinical use" since this was never examined.
It was removed.

24. Please remove the final sentence about a future study, why is the in vivo study being report separately from this current article?
It was removed. Since there is limit of words, we could not include the in vivo experiment.

References
25. Please check the references for the correct spelling and abbreviations, there are several mistakes that need attention. See refs 1-4, for example what is "The Amer Assoc Endod"?
References were checked and corrected.

Figures
26. The figures seem clear, although Figures 1B and 1C could be more in focus, and Figure E1 seems unnecessary.
The composites shown in Fig.2A, 2B and 2C are not flat. This made focusing difficult.
Figure E is important to show the size and morphology of early deposits on collagen fiber. Explanation of Fig.E was added .

Reviewer #2: This paper describes the construction of a carbonate-containing apatite/collagen matrix for the proposed use in an apical barrier/root end closure technique. While this was an interesting article, please address the following points.

1. The introduction describes various techniques that have been used for treatment of teeth with open apices. The first sentence states that calcium hydroxide has become the basis for current apical closure techniques. No mention is made of MTA. MTA has been shown to be very effective and should be included in discussion.
We read several papers concerning MTA and included statement about MTA.

2. Line 10 page 3 proposes that composite of apatite and collagen is one of the best materials currently available for an osteoconductive apical barrier. Please justify this proposal with literature and describe why an osteoconductive barrier is needed in apical closure techniques. The successful formation of dentin and cementum have been shown with other apical closure techniques, but the formation of bone as a result of the type of barrier placed needs to be justified. Also, if you are expecting the formation of a "bone-like" barrier, please discuss the permeability and the potential for leakage as compared to a barrier with dentin and cementum or cementum alone.
#2-1 Because osteoconductive material could have a potential to promote an induction of cementum, the sooner recover would be the better.

#2-2 It is expected that a barrier is constructed by cementum and dentin or cementum.

3. Page 5 line 10. Please describe any tooth preparation that was completed (instrumentation, irrigation, etc.). How many teeth were used? The "bottom" is not appropriate terminology for a description of where the material was placed. Please use appropriate terminology. Page 5 line 12 - Please describe what root filling was used. Was sealer used?
#3-1 Ten teeth were used.
#3-2 The "bottom" was changed to "apex".
#3-3 The remaining part of the root was filled with gutta-percha point and Canals^{R} and the top was covered with composite resin.

4. Please describe what results are expected from placing this barrier in the apical 2mm of the canal. What type of hard tissue do you expect to form in the barrier (bone, cementum, dentin, etc.)? If you expect bone to form, please discuss the possibility of ankylosis. Describe how ankylosis would be prevented if this type of barrier is used.
We expect cementum, because we believe that recovery through increment of cementum is natural.

5. Page 7 line 27 - Please use appropriate grammar in this sentence.
English was corrected.

6. Page 7 line 50 - I'm not sure what "crammed" means. Please use better terminology.
It was changed to " filled into a root canal ".

7. Please place the following reference numbers in the correct JOE format: 2, 3, 5, 8, 11, 15, 16, 27.
They were corrected.

This article provides a good description of the production of a carbonate containing apatite collagen matrix. However, the article needs to site literature explaining why this type of barrier is expected to work in an apical closure technique. A hypothesis needs to be proposed as to how this barrier material will stimulate apical closure. The suggested changes in grammar and sentence construction will also strengthen the manuscript.
We expect from the CAp/col composite as an apical barrier material with biological functions, such as, enhancement of recovery through increment of surrounding cementum and healing ability, to promote natural and early recovery. Therefore, a biologically active material, which has the potential to induce cementum and to work as a barrier, is preferred.
A proposed hypothesis as to how this barrier material will stimulate apical closure: Blood and body fluid that penetrated into the composite probably loosen the calcified collagen fibers, thereby promoting dissolution of apatite. At the same time, hemostatic action of the composite is expected. CO3-containing apatite with low crystallinity is more soluble than well-crystallized apatite. Dissociated Ca ion would be a trigger of new hard tissue formation, because Ca ions are reported to

participate in the activation of calcium-dependent adenosine triphosphatase (33).