Effects of sintered apatite on bone regeneration in rabbits

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Abstract Apatite is considered to be effective for bone regeneration when it contains calcium carbonate and β -TCP. In the present study, we made 3 different apatite preparations, hydroxyapatite, hydroxyapatite containing calcium carbonate, and hydroxyapatite containing β -TCP, using spark plasma sintering and compared their abilities for osteoinduction. For elucidation of bone regeneration, evaluations are generally made after euthanasia of experimental animals. However, the present R_mCT[®] procedure allowed such evaluations while the animals were alive. Our results showed that sintered HAP containing β -TCP was more effective for guided bone regeneration than the other test materials.

Key words

Apatite,
Bone regeneration,
Calcium carbonate,
Micro-CT,
 β -tricalcium phosphate (β -TCP)

Introduction

One of the most significant accomplishments in the progress of ceramic technology in the 20th century was the development of bioactive ceramics that adhere to the bone and facilitate regeneration¹⁾. Among the various methods of producing and adjusting such ceramics, the effectiveness of spark plasma sintering (SPS) has been frequently reported²⁾, which is a newly-developed method of producing high-quality materials in shorter periods of time using electrical energy under high sintering pressure³⁾. With SPS it is possible to blend materials of different compositions to form a block and the method is especially excellent for producing calcium phosphate materials.

Hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$:HAP] is a major mineral constituent of human bone⁴⁾, whose properties of fast bone regeneration, direct bonding, and excellent biocompatibility with bone tissues

have been reported. It is used not only for bone regeneration, but also for a variety of bone defects in the oral cavity⁴⁾. In a study of rabbits that used sintered HAP blended with β -calcium phosphate [β - $\text{Ca}_3(\text{PO}_4)_2$: β -TCP], we found that SPS was effective for bone regeneration⁵⁾. Further, in this result β -TCP was characterized by osteo-conductivity and a high level of biodegradation. Meanwhile, another study found that the degradation rate of β -TCP was too fast for optimum bonding to bone, despite its high osteo-conductivity⁶⁾. In an attempt to overcome that deficit⁶⁾, an attempt was made to produce a ceramic material in which β -TCP was mixed with biophasic calcium phosphate ceramic at a weight ratio of 30% to control the rate of degradation. As a result, faster regeneration of bone tissue was attained and it was proposed as useful for repairing bone defects⁷⁾. In addition, it was reported that a sintered body, which has an altered structure, with a synthetic porous structure was effective for bone regeneration⁸⁾. In order to study bone regeneration, a long follow-up term with an individual animal is required. Arai^{9,10)}

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developed a limited cone beam X-ray CT system (R_mCT® Rigaku). The system is used for *in vivo* small animals imaging to observe the individual animals for long time and to compare the time course changes of various sintered bodies without considering individual differences. In the present study, we evaluated the time course changes of various sintered bodies prepared by SPS and placed in the tibias of rabbits using R_mCT® assessments.

Subjects and Methods

Experimental animals

Twenty-four Japanese white rabbits aged 5 weeks (male, body weight approximately 1 kg) were used after 14 days of monitoring. They were kept in rearing cages at a temperature of 24°C, with humidity at 55%, 1 atmospheric pressure, and light-dark cycles of 12 hours. The experimental protocol was approved by the Animal Experimentation Committee of Nihon University School of Dentistry at Matsudo and Matsumoto Dental University.

Materials

The following 3 test samples were prepared by SPS (Dr. Sinter® SPS515S, SPS, Sintech). A calcium phosphate (Sangi) base was mixed using a calcium phosphate mixture at the following weight ratios, then packed in a carbon cast 15 mm in diameter and processed by electric charging with SPS.

- (1) 100% calcium phosphate (HAP)
- (2) 60% calcium phosphate + 40% calcium carbonate (HAP + calcium carbonate)
- (3) 60% calcium carbonate + 40% β -TCP (HAP + β -TCP)

Procedures

All the experiments were performed under a sterile condition. General anesthesia was induced by injecting ketamine hydrochloride (Ketalar®) and Xylazine (Selactorl®) through the ear vein of each rabbit. After confirming the anesthetic effect, the operative site was shaved and then disinfected with iodine solution, after which 2% lidocaine was injected for local anesthesia of the operative site. Next, a longitudinal incision was made 10 mm distal from the knee joint on the medial side of the tibia to expose the bone surface and detach the periosteum. A hole 2.0 mm in diameter and 1.0 mm in depth was made perpendicular to the long axis of the cortical bone using a dental implanter (Bone Surgery System®,

Fig. 1 Schematic of implantation

A hole 2.0 mm in diameter and 1.0 mm in depth was made perpendicular to the long axis of the cortical bone.

Royal) with a #8 dental implant bar (2.0 mm in diameter) while infusing sterile physiological saline (Fig. 1). After washing the site with sterile physiological saline, the reconstructive material was placed and the wound sutured. Each rabbit received a single type of material.

Micro-CT

Images were obtained using a micro-CT device (R_mCT®) at 70 kv and 80 μ A. During the period of imaging, the animals were given general anesthesia in a manner similar to above and restrained on a fixed base, so as not to affect the images by body movement. The scan time is 17 s. The image reconstruction was carried out on personal computer using specially designed I-View® (J. Morita, Japan). Increases (CT value increases 5 green) and decreases (CT value decreases 5 red) of CT levels were processed into visual images using software prepared with C+ (Microsoft). The area was determined by measuring the images and excluding the effects of growth of the rabbits.

Patho-histological evaluations

The rabbits were sacrificed with CO₂ gas 90 days after the operation. The tibia was extirpated and fixed with 10% phosphate-buffered formalin, then dehydrated, penetrated with styrene monomer, embedded in polymer resin (Ohken-shouji), polymerized, and hardened. Ground specimens were prepared, and stained with hematoxylin and eosin for patho-histological examinations.

Fig. 2 Y-slice images of HAP the R_mCT®

At 14 days after placement, an area showing a lower level of radiation absorbance as compared to the cortical bone was present in the margin between the sintered HAP and cortical bone. In the images, the brightness was weak. At 30 days after placement, an area showing a lower level of radiation absorbance than the cortical bone was present in the margin between the sintered HAP and cortical bone. At 90 days after placement, an area demonstrating a greater level of absorbance than that seen at 30 days existed in the margin between the HAP and cortical bone.

Fig. 3 Y-slice images of HAP + calcium carbonate the R_mCT®

At 14 days after placement, an area showing a lower level of radiation absorbance as compared to the cortical bone was present in the margin between the HAP + calcium carbonate material and cortical bone. In the images, the brightness was weak. At 30 days after placement, an area showing a lower level of radiation absorbance than the cortical bone was present in the margin between the HAP + calcium carbonate material and cortical bone. At 90 days after placement, an area showing a greater level of radiation absorbance than that seen at 30 days after placement was present in the margin with the cortical bone.

Fig. 4 Y-slice images of HAP + β -TCP the R_mCT®

At 14 days after placement, an area showing a lower level of radiation absorbance as compared to the cortical bone was present in the margin between the HAP + β -TCP and cortical bone. In the images, the brightness was weak. At 30 days after placement, an area demonstrating a lower level of radiation absorbance as compared to the cortical bone was present in the margin with the cortical bone. At 90 days after placement, the margin between the material and cortical bone had a higher level of radiation absorbance than that seen at 30 days after placement.

Results

Micro-CT findings

(1) Sintered HAP

R_mCT® findings for the specimens are shown in

Fig. 2. At 14 days after placement, an area showing a lower level of radiation absorbance as compared to the cortical bone was present in the margin between the sintered HAP and cortical bone. In the images, the brightness was weak. At 30 days after placement,

Fig. 5 Y-slice images of HAP to the C⁺

Images demonstrating regeneration and absorption in the bone around the reconstructive HAP. At 14 days after placement, no great difference was observed. At 30 days after placement, bone regeneration was confirmed on the inner side (bone marrow side) of the material in the HAP. At 90 days after placement, a layer of bone regeneration was seen around the material in the HAP.

Fig. 6 Y-slice images of HAP + calcium carbonate to the C⁺

Images demonstrating regeneration and absorption in the bone around the reconstructive HAP + calcium carbonate. At 14 days after placement, no great difference was observed. At 30 days after placement, bone regeneration was confirmed on the inner side (bone marrow side) of the material in the HAP + calcium carbonate. At 90 days after placement, a layer of bone regeneration was seen around the material in the HAP + calcium carbonate.

Fig. 7 Y-slice images of HAP + β -TCP to the C⁺

Images demonstrating regeneration and absorption in the bone around the reconstructive HAP + β -TCP. At 14 days after placement, no great difference was observed. At 30 days after placement, regeneration was observed in both the inner and outer sides of the material in the HAP + β -TCP. At 90 days after placement, regeneration was confirmed in abundance on both the inner and outer sides.

an area showing a lower level of radiation absorbance than the cortical bone was present in the margin between the sintered HAP and cortical bone. At 90 days after placement, an area demonstrating a greater level of absorbance than that seen at 30 days existed in the margin between the HAP and cortical

bone. Further, the margin had become linear and was about $210\mu\text{m}$ in width.

(2) HAP + calcium carbonate

R_mCT[®] findings for the specimens are shown in Fig. 3. At 14 days after placement, an area showing

Fig. 8 Healing after implantation using the R_mCT®

At 14 days after placement no significant difference was observed, while there were significant differences at 30 and 90 days after placement.

a lower level of radiation absorbance as compared to the cortical bone was present in the margin between the HAP + calcium carbonate material and cortical bone. In the images, the brightness was weak. At 30 days after placement, an area showing a lower level of radiation absorbance than the cortical bone was present in the margin between the HAP + calcium carbonate material and cortical bone. Further, an area with the same level of radiation absorbance as the cortical bone was present immediately beneath the HAP + calcium carbonate. At 90 days after placement, an area showing a greater level of radiation absorbance than that seen at 30 days after placement was present in the margin with the cortical bone. In the margin, radiation absorbance was irregularly distributed in a width of about $390\mu\text{m}$.

(3) HAP + β -TCP

R_mCT® findings for the specimens are shown in Fig. 4. At 14 days after placement, an area showing a lower level of radiation absorbance as compared to the cortical bone was present in the margin between the HAP + β -TCP and cortical bone. In the images, the brightness was weak. At 30 days after placement, an area demonstrating a lower level of radiation absorbance as compared to the cortical bone was present in the margin with the cortical bone. At 90 days after placement, the margin between the

material and cortical bone had a higher level of radiation absorbance than that seen at 30 days after placement. Further, the margin had become linear and was about $30\mu\text{m}$ in width, which was the smallest width dimension of the 3 test materials.

(4) CT imaging increases

Images demonstrating regeneration and absorption in the bone around the reconstructive materials after placement are shown in Figs. 5, 6, and 7. At 14 days after placement, no great difference was observed among the material groups. At 30 days after placement, bone regeneration was confirmed on the inner side (bone marrow side) of the material in the HAP group, while regeneration was observed in both the inner and outer sides of the material in the HAP + β -TCP group. At 90 days after placement, a layer of bone regeneration was seen around the material in the HAP group, while bone regeneration was observed only on the inner or outer side in the HAP + calcium carbonate group. In the HAP + β -TCP group, regeneration was confirmed in abundance on both the inner and outer sides. Changes in the osteogenic areas around the materials are shown in Fig. 8. At 14 days after placement no significant difference was observed, while there were significant differences at 30 and 90 days after placement.

Fig. 9A HAP implantation after 90 days ($\times 20$)

New bone tissue was observed around the reconstructive material, and the area where the material and new bone tissue had direct contact covered about 30% of the material. A lamellar construction and Haversian canals were observed in the new bone tissue, demonstrating mature osseous tissue.

Fig. 9B HAP implantation after 90 days ($\times 100$)

The image that expands to $\times 100$ is shown. In the area where the reconstructive material and new bone tissue had no direct contact, fibrous connective tissue was observed on the surface of the material.

Patho-histological findings

(1) HAP

Figure 9 shows histological findings of the HAP group at postoperative 90 days. New bone tissue was observed around the reconstructive material, and the area where the material and new bone tissue had

direct contact covered about 30% of the material. A lamellar construction and Haversian canals were observed in the new bone tissue, demonstrating mature osseous tissue. In the area where the reconstructive material and new bone tissue had no direct contact, fibrous connective tissue was observed on the surface of the material.

Fig. 10A HAP + calcium carbonate implantation after 90 days ($\times 20$)

New bone tissue was observed around the reconstructive material, which had a lamellar construction and Haversian canals, demonstrating mature osseous tissue. However, the area where the reconstructive material and new bone tissue had direct contact was scarce. In most of the areas, fibrous connective tissue covered the surface of the material.

Fig. 10B HAP + calcium carbonate implantation after 90 days ($\times 100$)

The image that expands to $\times 100$ is shown. Dents that seemed to indicate bone defects due to resorption were found in abundance on the surface of the material.

(2) HAP + calcium carbonate

Figure 10 shows histological findings of the HAP + calcium carbonate group at postoperative 90 days. New bone tissue was observed around the reconstructive material, which had a lamellar construction and Haversian canals, demonstrating mature osseous tissue. However, the area where the reconstructive

material and new bone tissue had direct contact was scarce. In most of the areas, fibrous connective tissue covered the surface of the material. In addition, dents that seemed to indicate bone defects due to resorption were found in abundance on the surface of the material.

Fig. 11A HAP + β -TCP implantation after 90 days ($\times 20$)

New bone tissue was observed around the reconstructive material in direct contact with the bone.

Fig. 11B HAP + β -TCP implantation after 90 days ($\times 100$)

The image that expands to $\times 100$ is shown. A lamellar construction and Haversian canals were present in the new bone tissue, demonstrating mature osseous tissue.

(3) HAP + β -TCP

Figure 11 shows histological findings of the HAP + β -TCP group at postoperative 90 days. New bone tissue was observed around the reconstructive material in direct contact with the bone. Further, a lamellar construction and Haversian canals were present in the new bone tissue, demonstrating mature osseous tissue. In comparison to the HAP and

HAP + calcium carbonate groups, the reconstructive material and newly-formed bone tissue showed greater areas of contact.

Discussion

It has been reported that bioactive ceramics with properties that allow direct bonding with bone tissue

were effective for restoration and regeneration of bone tissue¹¹). Among the different types of bioactive ceramics, hydroxyl apatite and crystalline glass are well known. In clinical applications, the long-term safety of artificial materials has been ensured with confirmation that they are able to replace autologous bone. We previously reported that sintered apatite prepared using the same SPS method as in the present study was effective for bone regeneration if it contained β -TCP. In the present study, we investigated how such a sintered body was absorbed and replaced *in vivo*.

In the sintered HAP group, changes in CT levels were observed around the HAP material from 2 weeks after placement. Ozawa¹²) reported that osteoid tissue was formed in abundance between the material and bone at the second week after HAP placement. It was considered that the results in the present study indicated that osteoid tissue was formed similarly by the second week. The micro-CT levels at 30 days after placement indicated an area showing a lower level of radiation absorbance than the cortical bone present in the margin between the HAP material and cortical bone, similar to the findings after the second week. Accordingly, we considered that osteoid tissue was present between the sintered HAP and cortical bone. However, no bone formation was found in the CT images, though osteoid tissue was observed. At 90 days after placement, bone-like tissue was confirmed to be present around the HAP material. Additionally, bone-like tissue was similarly confirmed by the patho-histological findings at 90 days after placement that same as in the CT images. Ozawa suggested that bone tissue formation occurred earlier than physiological bone formation in areas where HAP materials used as an artificial bone coexisted with bone tissue and that the amount of newly formed bone tissue was large, while osteoid tissue transformed to bone tissue after 8 weeks¹²). It was also reported that bone formation changed to bone after 90 days in rabbits. These findings were confirmed by the results of the present study. Further, graphic changes in CT levels were observed, indicating that the bone tissue was constantly increasing.

In the HAP + calcium carbonate group, changes at 2 weeks after placement as shown by micro-CT findings were similar to those in the sintered HAP group. However, the CT levels at 30 days after placement showed that the areas with lower amounts of osteoid tissue and reduced CT levels were more

abundant as compared to those in the sintered HAP group. At 90 days after replacement, the amounts of guided osteoid tissue and areas demonstrating CT level decline were greater than those at 30 days. In both the CT and patho-histological findings, the margin between the material and cortical bone was the most vague among the 3 test materials, while the width of the margin was the greatest.

For development of bone reconstructive materials, attempts have been made to synthesize apatite materials that contain a small amount of calcium carbonate as bone apatite¹³), and a study reported that a uniform sintered body with relatively homogeneous crystal grain size and shape was obtained when calcium carbonate was sintered at 750°C¹⁴). Further, it was also found that half of the initial content of calcium carbonate remained in the apatite grid. On the basis of those findings, it was reported that an HAP + calcium carbonate material was more easily absorbed *in vivo* than sintered HAP¹⁵). In the present study, dents occurred more frequently along the edge of the HAP + calcium carbonate material than with the other materials, which was considered to be a result of differences in speed of resorption of the materials. Also, those findings suggested that apatite containing calcium carbonate is likely to be absorbed by osteoclasts *in vivo*¹⁵). The pores of the materials expanded after placement in the tissue, into which body fluid and cells easily invaded, which was considered to cause the margin with the surrounding bone tissue-like structures to become unclear. CT examinations in the present study revealed similar findings. In graphic images of the changes in CT levels, the bone tissue was constantly transformed from the second week in the HAP + calcium carbonate group, demonstrating a greater increase in bone-like tissue than in the HAP group.

The HAP + β -TCP group showed nearly the same changes in micro-CT findings at 2 weeks after placement as the other experimental groups. However, at 30 days after placement, a comparison with the sintered HAP and HAP + calcium carbonate groups confirmed an increase in CT levels on both the inner and outer sides of the material.

Ozawa¹⁶) reported that favorable bone formation and resorption of reconstructive material were seen with high purity β -TCP, and noted that the β -TCP-containing HAP and calcium carbonate-containing HAP materials were engulfed by the bone faster than the β -TCP and calcium carbonate without HAP materials. In the present study, bone-like tissue

was considered to be guided by β -TCP. At 90 days after placement, β -TCP observed abundant bone-like tissue on the outer side of the materials. Further, comparisons of the changes in CT levels between the periods of 14 to 30 days and 30 to 90 days after placement showed that the increase was larger in the period from 30 to 90 days. On the basis of these findings, we considered that the sintered HAP containing 40% β -TCP showed osteoinduction at an early stage. In addition, the amount of bone-like tissue was increased most in the HAP + β -TCP group.

Comparisons among the HAP + calcium carbonate, HAP + β -TCP, and sintered HAP groups revealed that the 2 former groups had higher osteoinduction, as shown by changes in CT levels. Further, the increase in bone-like tissue was higher in the HAP + β -TCP group as compared to the HAP + calcium carbonate group, which was considered to be due to osteoinduction of β -TCP. On the basis of these results, it was revealed that additional osteoinduction occurred when calcium carbonate or β -TCP was contained in HAP. Specifically, more bone was guided from the early stage when β -TCP was contained in HAP.

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References

- 1) Jarcho, M.: Calcium phosphate ceramics as hard tissue prosthetics. *Clin Orthop Rel* **157**: 259–278, 1981.
- 2) Nakahira, A., Tamai, M., Aritani, H., Nakamura, K. and Yamashita, K.: Biocompatibility of dense hydroxyapatite prepared using SPS process. *J Biomed Mater Res* **62**: 550–557, 2002.
- 3) Kasai, T., Ishikawa, K., Suzuki, K. and Yatani, H.: Initial evaluation of a ceramic form as a reconstructive material for bone defects. *Dent Mater J* **19**: 381–388, 2000.
- 4) Kohri, M., Miki, K., Waite, D.E., Nakajima, H. and Okabe, T.: *In vivo* stability of biphasic calcium phosphate ceramics. *Biomater* **14**: 423–429, 1993.
- 5) Arai, K., Matsune, K., Okamoto, H., Hirukawa, S., Kuramochi, H., Kiba, H., Kuboyama, A., Nishiyama, N., Nemoto, K. and Maeda, T.: A study about sintered apatite reproduction of osseous tissue. *Jpn J Ped Dent* **44**: 548–555, 2006. (in Japanese, English abstract)
- 6) Frayssinet, P., Trouillet, J.L., Rouquet, N., Azimus, E. and Autefage, A.: Osteointegration of macroporous calcium phosphate ceramics having a different chemical composition. *Biomater* **14**: 423–429, 1993.
- 7) Nery, E.B., LeGeros, R.Z., Lynch, K.L. and Lee, K.: Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/ β TCP in periodontal osseous defects. *J Periodontol* **63**: 729–735, 1992.
- 8) Chang, B., Lee, C., Hong, K., Youn, J., Ryu, H., Chung, S. and Park, K.: Osteoconduction at porous hydroxyapatite with various pore configurations. *Biomater* **21**: 1291–1298, 2000.
- 9) Arai, Y., Yamada, A., Ninomiya, T., Kato, T. and Masuda, Y.: Micro-computed tomography newly developed for *in vivo* small animal imaging. *Oral Radiol* **21**: 14–18, 2005.
- 10) Arai, Y., Tammisalo, E., Iwaki, K., Hashimoto, K. and Shinoda, K.: Development of a compact computed tomographic apparatus for dental use. *Dentomaxillofac Radiol* **28**: 245–248, 1999.
- 11) McAndrew, M.P., Gorman, P.W. and Lange, T.A.: Tricalcium phosphate as a bone graft substitute in trauma: Preliminary report. *J Orthop Trauma* **2**: 333–339, 1988.
- 12) Ozawa, S. and Kasugai, S.: Evaluation of implant materials (hydroxyapatite, glass-ceramics, titanium) in rat bone marrow stromal cell culture. *Biomater* **17**: 23–29, 1996.
- 13) Shirota, T., Ohno, K., Suzuki, K., Miyamoto, T., Matsui, Y. and Michi, K.: Effects of aging on bonds between hydroxyapatite and bone: Histological findings after placement in tibia of rats. *J Oral Sur* **38**: 41–47, 1992.
- 14) Nakano, Y.: Preparation of porous 8-calcium phosphate hardening body added with α -tricalcium phosphate and its material. *Dent Mater Appar* **19**: 65–79, 2000.
- 15) Orii, H., Sotome, S., Chen, J., Wang, J. and Shinomiya, K.: Beta-tricalcium phosphate (beta-TCP) graft combined with bone marrow stromal cells (MSCs) for posterolateral spine fusion. *J Med Dent Sci* **52**: 51–57, 2005.
- 16) Ozawa, M.: An experimental study on bone formation and resorption of high-purity β -TCP. *Biomed Mater* **13**: 167–175, 1995.