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Original

Effects of Heat Treatment of Hydroxyapatite on Osteoblast Differentiation

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Abstract: To develop hydroxyapatite that promotes early cure of postoperative tissue, we heat-treated hydroxyapatite and evaluated its pH-increasing effect and Ca release. We also evaluated the effect of heat-treated hydroxyapatite on osteoblast differentiation. (1) No marked change was observed regarding the surface morphology or structure of heat-treated hydroxyapatite on SEM. (2) When hydroxyapatite was immersed in physiologic saline, the saline was made more alkaline by heat-treated than non-heat-treated hydroxyapatite. (3) When hydroxyapatite was immersed in physiologic saline, more Ca ions were released from heat-treated than non-heat-treated hydroxyapatite. (4) X-ray diffraction analysis showed a peak of CaO, which is considered to explain the Ca ion release, in heat-treated hydroxyapatite. (5) When osteoblasts were cultured with hydroxyapatite, heat-treated hydroxyapatite prevented the decrease in the number of alkaline phosphatase-positive osteoblasts in the presence of non-heat-treated hydroxyapatite. Thus, heat-treated hydroxyapatite was suggested to promote early cure of postoperative tissue. Detailed analysis of *in vivo* effects of heat-treated hydroxyapatite is anticipated to make its clinical application possible.

Keywords: Alkaline phosphatase, Hydroxyapatite, Osteoblast

Introduction

Recently, various biological materials have been developed due to the advancement of material engineering, and new technologies have been introduced into the medical field. Particularly, hydroxyapatite-related materials are reported to have a high *in vivo* safety level, and are in wide clinical use as bone substitute or dental materials in dentistry[1-3]. In implant surgery, they are used as not only bone-filling materials but also implant surface-coating materials.

Implants coated with hydroxyapatite acquire osseointegration earlier than pure titanium implants, because while pure titanium implants are separated from bone by a slight gap filled by mucopolysaccharide proteins and are not in direct contact with bone on the microscopic level, Ca deposits on the interface between hydroxyapatite-coated implants and bone and are crystallologically integrated with hydroxyapatite, contributing to biointegration of the implants with bone[4,5]. In addition, coated hydroxyapatite is substituted by bone in remodeling, which is accomplished as osteoclasts resorb hydroxyapatite, and osteoblasts appear in the resultant space and form bone. Coating the implant surface with hydroxyapatite is considered to promote the remodeling of surrounding bone tissues[5-7]. It also has many clinical advantages such as that implants can be introduced without initial fixation or the presence of strong bone matrix at the recipient site[8].

Also, a treatment to induce osteoblast migration to bone defect sites by adding hydroxyapatite to an absorptive membrane is being developed. This method is characterized by the aggressive induction of bone formation by utilizing the osteogenic ability of hydroxyapatite, unlike conventional osteoplasty, which secures a space for osteoblasts to differentiate by blocking undifferentiated and highly proliferative mesenchymal cells[9,10]. This treatment, which requires no special clinical skill, is considered to be effective at sites where osteoplasty is difficult to perform.

From these previous reports, we considered that the development of hydroxyapatite, which not only has a high osteogenic ability but also promotes early cure of postoperative

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tissues, is important for improving the success rate of implant surgery. Tissues that have sustained surgical invasion become acidic (pH 5) due to anaerobic glycolysis. Therefore, neutralization of tissues at the surgical site is considered to promote wound healing. In this study, therefore, we prepared highly crystallized alkaline hydroxyapatite by heat-treating non-crystalline calcium phosphate with different Ca/P molar ratios. Furthermore, to evaluate the effects of non-crystalline calcium phosphate and crystallized hydroxyapatite on bone formation, we analyzed the pH-increasing effect and calcium-releasing behavior of heat-treated spherical hydroxyapatite. We also evaluated the effect of heat-treated hydroxyapatite on osteoblast differentiation. Alkaline phosphatase is an enzyme that is markedly expressed in osteoblasts, and it is considered to play an important role in bone calcification[11,12]. We analyzed the effect of heated-treated hydroxyapatite on osteoblast differentiation using alkaline phosphatase as an index.

Materials and Methods

Preparation of heat-treated hydroxyapatite

In this study, two types of spherical hydroxyapatite (mean particle size, 30 μm; Sekisui Kasei Co., Ltd.) with Ca/P molar ratios of 1.670 (100P) and 1.718 (114P) were evaluated. These hydroxyapatite preparations were treated in an electric furnace (SLC115, Selec) at 900°C for 6 hours and sterilized with ethylene oxide gas.

Osteoblast isolation and culture system

Primary osteoblasts were prepared from the calvariae of 2-day-old newborn ddY mice (male and female)[13]. The calvariae were collected aseptically, minced into 2-3 mm fragments, and treated in an enzyme solution containing collagenase and dispase with shaking for 10 minutes 5 times. Osteoblasts were obtained by collecting the cells released into the enzyme solution by centrifugation at 1,000 rpm for 10 minutes. These osteoblasts were cultured in minimum essential medium a modification (αMEM, Sigma) containing 10% fetal bovine serum (FBS, JRH Bioscience) on 10-cm dishs (Falcon) in a CO_2 incubator (37°C, 5% CO_2) until confluence, recovered by trypsin treatment, and stored at -80°C.

Scanning electron microscopy of the surface of hydroxyapatite

Several types of hydroxyapatite were treated by gold impregnation and examined under a scanning electron microscope (JSM-6360LA, JEOL). Also, 114P hydroxyapatite heat-treated at 900°C for 6 hours (114P/900°C/6h) was immersed in purified water for 1 hour, allowed to dry for 3 days, and examined by scanning electron microscopy.

pH measurement

100P hydroxyapatite heat-treated at 900°C for 6 hours (100P/900°C/6h) and 114P/900°C/6h were examined as samples, and hydroxyapatite without heat treatment (100P and 114P) was examined as a controls. After 0.6 g of each apatite was immersed in 80 ml of physiologic saline (Otsuka Pharmaceutical Co., Ltd.), it was placed in an incubator at 37°C, and the pH of the saline was measured with a pH meter (HM50S, TOA) after 1 hour, 1, 2, 3, and 4 weeks. The saline was not changed during the measurement period.

Measurement of Ca ion release

The quantity of Ca released from 114P/900°C/6h, which showed high alkalinity, was determined. The quantity of Ca release from non-heat-treated 114P was also determined as a control. Of each preparation, 0.6 g was immersed in 80 ml of physiologic saline (Otsuka) and incubated at 37°C. After 1, 2, 3, and 4 weeks, the saline was filtered (0.45-mm filter paper), and the Ca ion concentration was determined using an ion meter (IM-40S, TOA).

X-ray diffraction

X-ray diffraction by hydroxyapatite particles after various treatments was analyzed using an X-ray diffraction analyzer (JDX-3532, JEOL). The analytical conditions were: Tube voltage, 40 kV; tube current, 30 mA; and diffraction angle, 5-80°C.

Alkaline phosphatase staining

To 10-cm dishes (Falcon), 10 ml of αMEM containing 10% FBS was added as a medium, osteoblasts were seeded, and cultured in a CO_2 incubator (37°C, 5% CO_2) for 7 days. After confirming that cells had proliferated to confluence, 13 mg of each hydroxyapatite preparation (100P, 114P, 100P/900°C/6h, and 114P/900°C/6h) was added to a dish, and the cells were cultured for another 7 days. The cells were fixed with 10% formalin, stained for alkaline phosphatase, and examined grossly and microscopically[14].

Measurement of alkaline phosphatase activity

Osteoblasts were seeded onto 10-cm dishes, 10 ml of αMEM containing 10% FBS was added, and the cells were cultured for 7 days (37°C, 5%CO_2). After confirming that the cells had reached confluence, various hydroxyapatite preparations (100P, 114P, 100P/900°C/6h, and 114P/900°C/6h) were added at 13 mg/dish, the osteoblasts were cultured for 7 further days and recovered from the dishes by trypsin treatment. The recovered osteoblasts were homogenized in a sealed ultrasonic cell homogenizer (Bioruptor, COSMO BIO Co., Ltd.), and the alkaline phosphatase activity of the homogenate was measured.

The alkaline phosphatase activity was determined by the Bessey-Lowry method[15,16]. r-Nitrophenol phosphate was used as the substrate, and the amount of generated r-nitrophenol was determined with an absorption photometer (Nano Drop, Nano

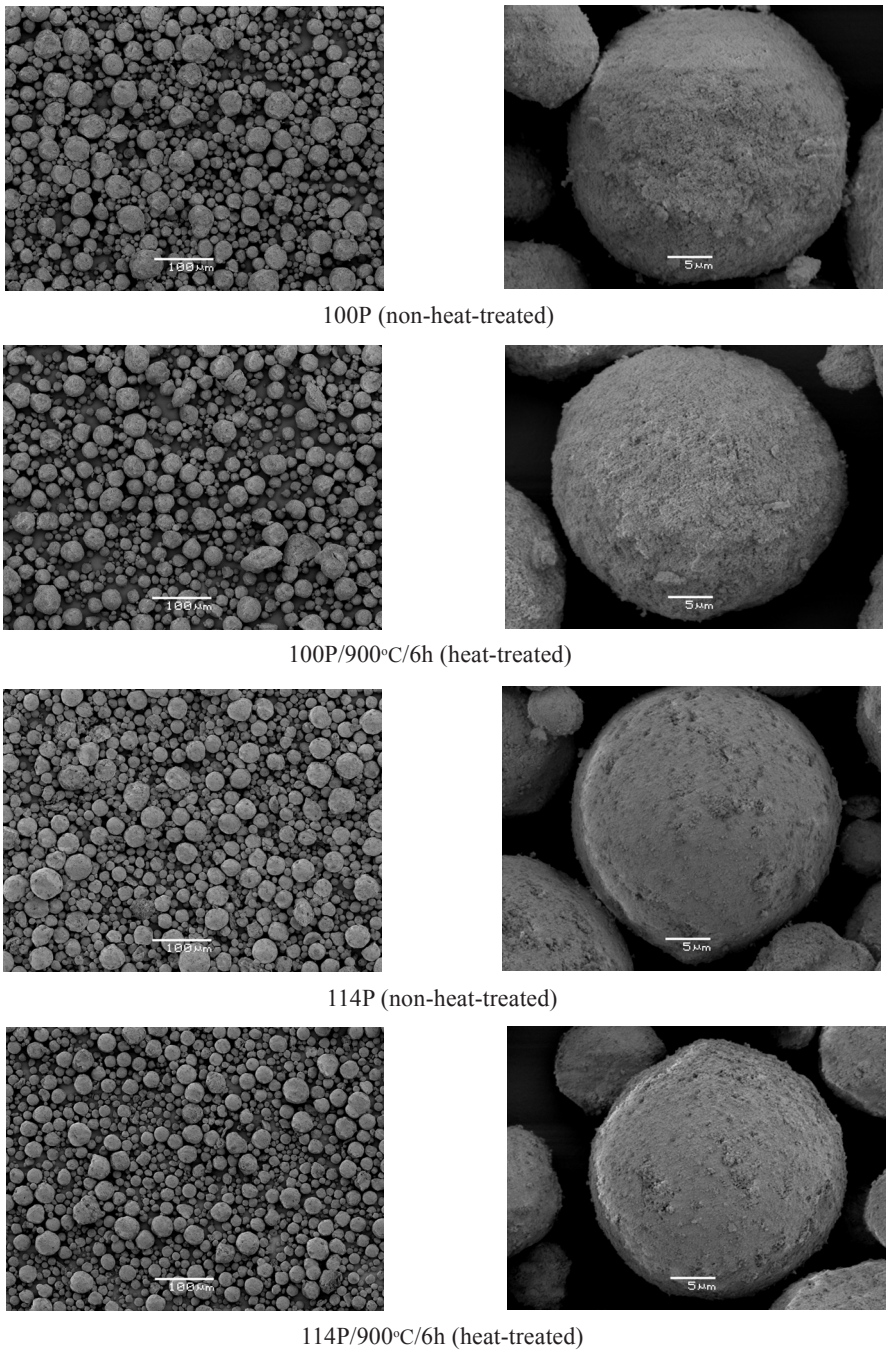


Fig. 1: Scanning electron microscopy of the surface of spherical hydroxyapatite
Various hydroxyapatite samples were impregnated with gold before and after heat treatment, and their surfaces were examined by scanning electron microscopy.

Drop Technologies) at a wavelength of 405 nm.

Statistics

Data are expressed as the mean ± SD.

Results

Observation of the surface of hydroxyapatite by SEM

SEM of various preparations of hydroxyapatite showed no marked difference in the morphology or surface structure (Fig. 1), but zonal patterns were observed on the surface of 114P/900°C/6h immersed in purified water (Fig. 2).

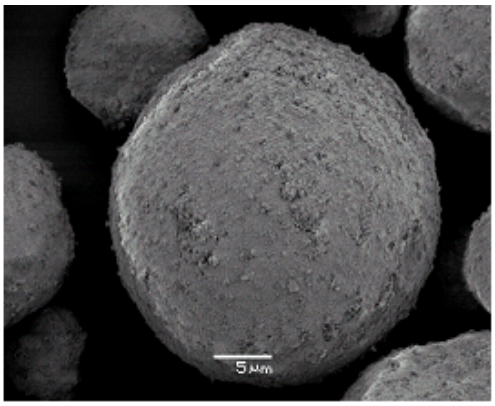
pH-increasing effect of heat treatment and serial changes

When the hydroxyapatite samples were immersed in physiologic saline, heat-treated hydroxyapatite (100P/900 °C/6h and 114P/900°C/6h) increased the pH of the saline compared with non-heat-treated hydroxyapatite after 1 hour incubation (Fig. 3). The pH of the saline decreased with time with all hydroxyapatite

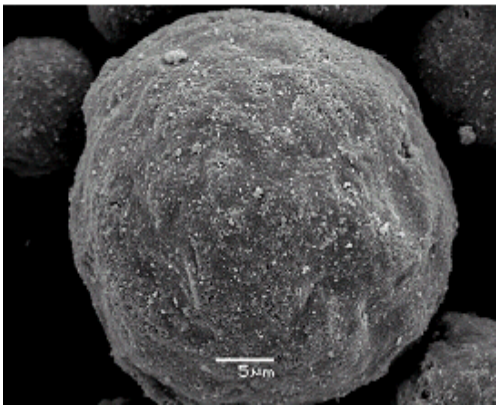
preparations after 1, 2, 3, and 4 weeks (Fig. 3).

Release of Ca ions from hydroxyapatite

The amount of Ca ions released into saline from heated-treated hydroxyapatite (114P/900°C/6h) was greater than that from non-heat-treated hydroxyapatite (114P) until after 3 weeks (Fig. 4). The mean Ca ion level in the saline from Week 1 to 3 was 6.86 ppm for 114P but 10.25 ppm for 114P/900°C/6h (Fig. 4).



114P (non-heat-treated)



114P/900°C/6h (heat-treated) (after immersion)

Fig. 2: Scanning electron microscopy of the surface of spherical hydroxyapatite after immersion in purified water

X-ray diffraction

Fig. 5 shows the results of X-ray diffraction analysis of heat-treated and non-heat-treated hydroxyapatite. The peak of CaO, which is considered to be the source of the released Ca ions, was noted only in 100P/900°C/6h and 114P/900°C/6h (Fig. 5, arrows). Next, the peak of CaO in 114P/900°C/6h disappeared after immersion in purified water (Fig. 6).

Observation of osteoblasts by alkaline phosphatase staining

Osteoblasts cultured with various kinds of hydroxyapatite were fixed and stained for alkaline phosphatase. The number of alkaline phosphatase-positive cells, which were stained blue, on

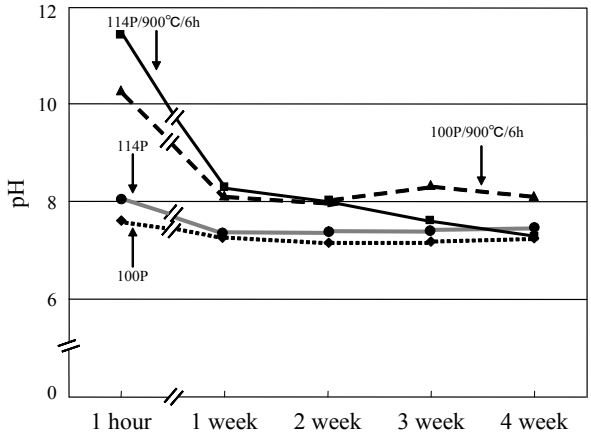


Fig. 3: Serial changes in the pH
The pH of the immersion fluid was measured using a pH meter after immersion of 100P/900°C/6h and 114P/900°C/6h as samples and 114p and 100P as controls. Experiments were repeated three times with similar results.

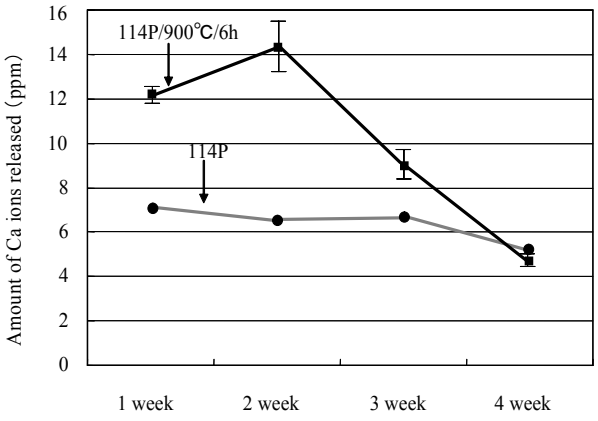


Fig. 4: Serial changes in Ca ion release
As a result of pH measurement, the amounts of Ca release from 114P/900°C/6h, which showed high alkalinity, as a sample and 114P as a control into the immersion fluid were measured using an ion concentration meter. Data are expressed as the mean ± SD of three samples. Experiments were repeated five times with similar results.

the dish decreased in the groups cultured with non-heat-treated 100P and 114P compared with no treatment control dish (Fig. 7). In the groups cultured with heat-treated 100P/900°C/6h and 114P/900°C/6h, the number of alkaline phosphatase-positive osteoblasts increased markedly compared with the groups cultured with 100P and 114P (Fig. 7).

The dishes in which osteoblasts were examined grossly by alkaline phosphatase staining were examined further by light microscopy. In the groups cultured with heat-treated 100P/900°C/6h and 114P/900°C/6h, larger numbers of alkaline phosphatase-positive cells were observed than in the groups cultured with non-heat-treated 100P and 114P. Particularly, the increase in alkaline phosphatase-positive cells was notable in the dish to which 114P/900°C/6h was added (Fig. 8).

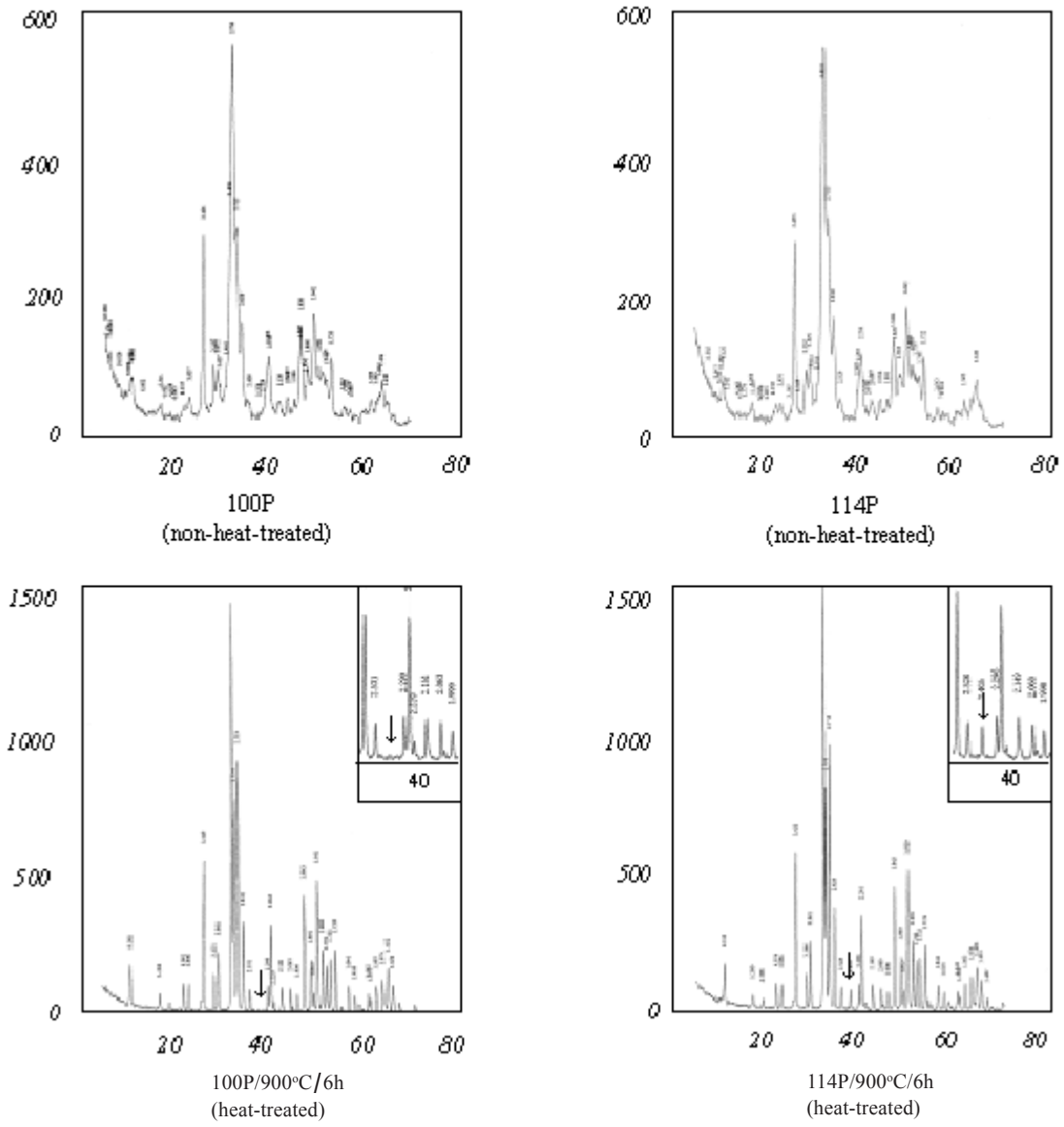


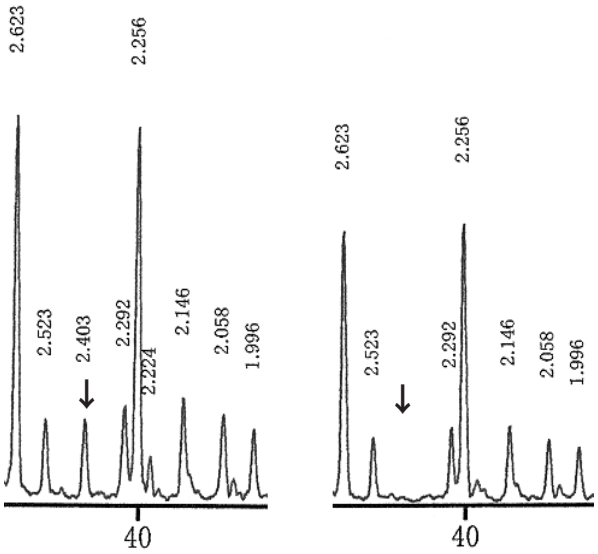
Fig. 5: X-ray diffraction of hydroxyapatite particles
X-ray diffraction analysis was performed with the 4 types of hydroxyapatite particles at a tube voltage of 40 kV, tube current of 30 mA, and diffraction angles of 5-80°C. The arrows show peaks of CaO.

Changes in the alkaline phosphatase activity of osteoblasts

In the groups cultured with non-heat-treated 100P and 114P, the alkaline phosphatase activity of osteoblasts decreased compared with the control group cultured without hydroxyapatite (Fig. 9). However, the alkaline phosphatase activity was higher in groups cultured with heat-treated 100P/900°C/6h and 114P/900°C/6h than in groups cultured with non-heat-treated 100P and 114P (Fig. 9).

Discussion

Usually, the healing course of sites of tooth extraction or minor surgery is divided into 4 periods. The first is the clot period until about 7 days after surgery, during which the surgical site is filled with a clot. Fibrin and neutrophils are observed in this period. This period is followed by the granulation tissue period, during which granulation tissue proliferates. After 1-3 weeks, the callus period begins as the bone matrix is calcified, and the cure period is reached after about 3 months with bone maturation. Thus, a long period is necessary for the natural repair of bone defects. Also, tissues that have sustained surgical insult have been reported to become acidic (pH 5) due to anaerobic glycolysis, and it is difficult to completely restore the wounded tissue to its original state in a short time. Recently, bone-induction techniques such as implantation immediately after tooth extraction, sinus lift and



114P/ 900°C/ 6h (heat-treated)

114P/ 900°C/ 6h (after immersion)

Fig. 6: X-ray diffraction after immersion of hydroxyapatite particles in purified water
The peak disappeared completely after immersion of 114P/ 900°C/ 6h in purified water.

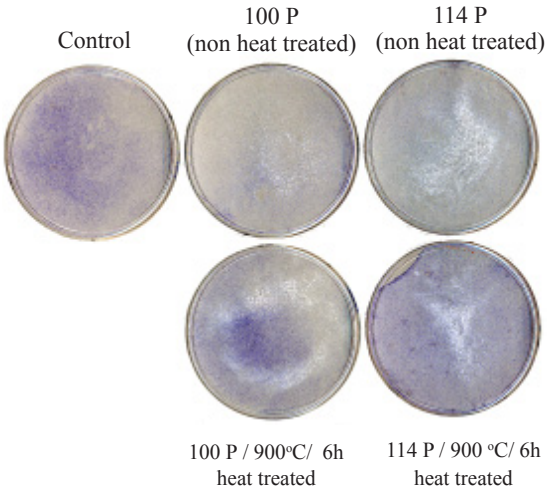
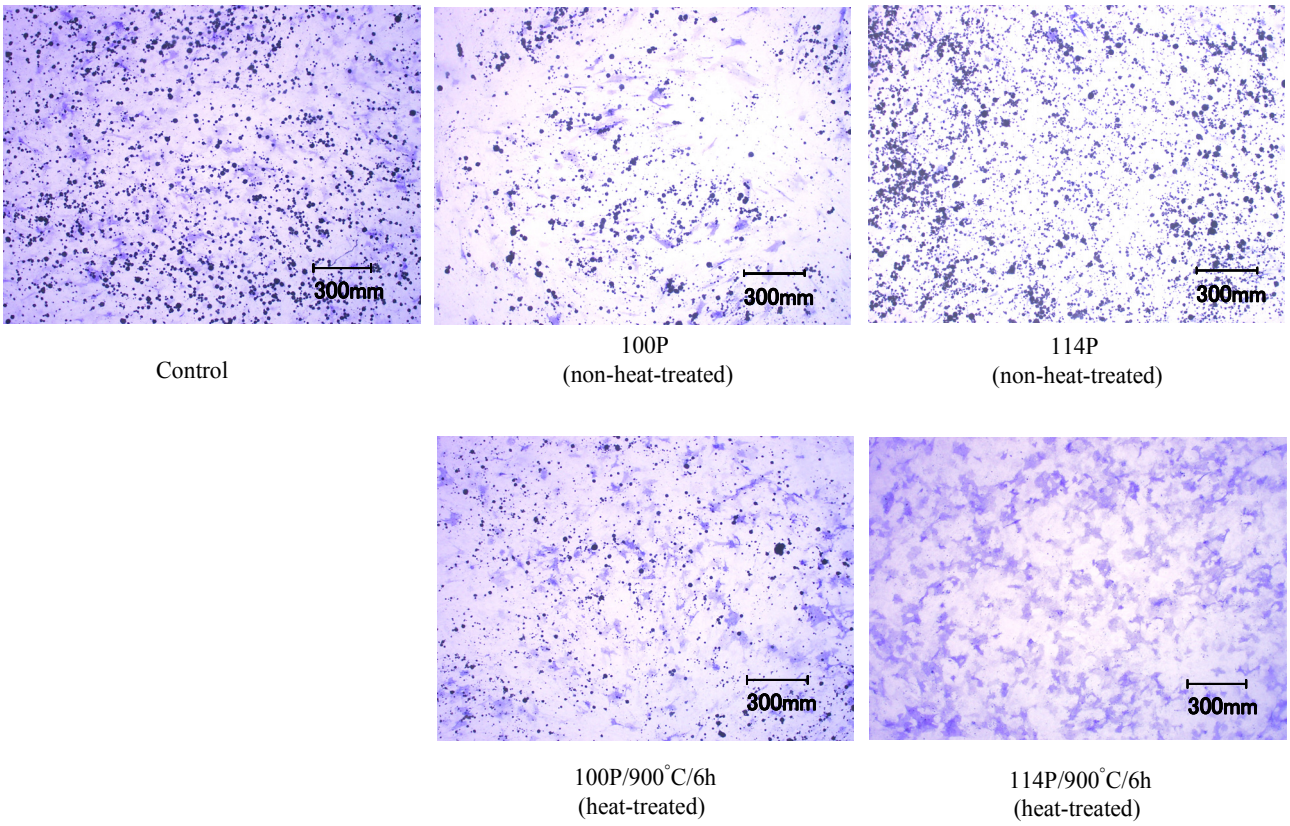


Fig. 7: Alkaline phosphatase staining of osteoblasts
Osteoblasts were seeded onto 10-cm dishes containing αMEM supplemented with 10% FBS and cultured for 7 days in an incubator. Hydroxyapatite (100P, 114P, 100P/900°C6h, and 14P/900°C6h) was added, the cells were cultured for 7 further days, stained for alkaline phosphatase, and examined grossly. Experiments were repeated five times with similar results.



Control

100P
(non-heat-treated)

114P
(non-heat-treated)

100P/900°C/6h
(heat-treated)

114P/900°C/6h
(heat-treated)

Fig. 8: Light microscopy by alkaline phosphatase staining of osteoblasts
Osteoblasts were seeded onto 10-cm dishes containing αMEM supplemented with 10% FBS and cultured for 7 days in an incubator. Hydroxyapatite (100P, 114P, 100P/900°C/6h, and 114P/900°C/6h) was added, the cells were cultured for 7 further days, stained for alkaline phosphatase, and examined microscopically. Experiments were repeated five times with similar results.

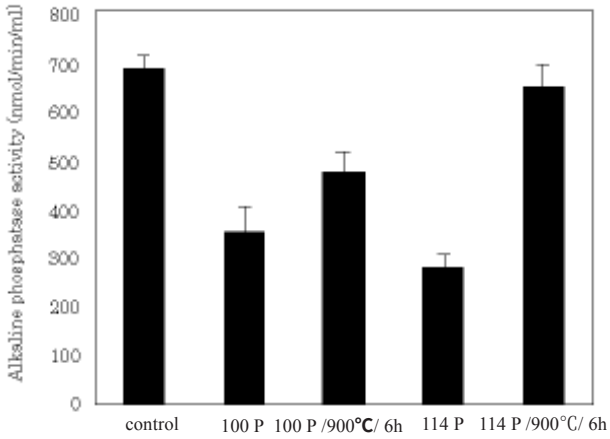


Fig. 9: Measurement of the alkaline phosphatase activity
Osteoblasts were seeded onto 10-cm dishes, 10 ml of αMEM containing 10% FBS was added, and the cells were cultured for 7 days. After confirming that the cells had reached confluence, various hydroxyapatite preparations (100P, 114P, 100P/900°C/6h, and 114P/900°C/6h) were added at 13 mg/dish, the osteoblasts were cultured for 7 further days and recovered from the dishes by trypsin treatment. The recovered osteoblasts were homogenized in a sealed ultrasonic cell homogenizer, and the alkaline phosphatase activity of the homogenate was measured. Data are expressed as the mean ± SD of three samples. Experiments were repeated five times with similar results.

guided bone regeneration (GBR) for the induction of new bone tissue at bone defects have been used frequently[17]. Although autologous bone grafting using iliac grafts has often been performed to repair large bone defects, efforts have been made to develop substitute artificial materials because of the serious surgical invasion of the donor site and limitation of the graft size. Among these materials, hydroxyapatite has been reported by many authors to have excellent biocompatibility and a high hard-tissue-forming ability[18-22]. Recently, a high surgical success rate and early postoperative recovery are demanded, but improvements in the surgical technique alone are insufficient to meet these demands. Artificial materials are expected to accelerate the healing process and improve the success rate of surgery by promoting bone formation.

In this study, therefore, we evaluated whether the early neutralization of tissue that has become acidic due to surgical invasion using a strong alkali and consequent activation of cells is effective for strengthening the healing power using hydroxyapatite. The properties of hydroxyapatite have been reported to change according to the temperature and time of heat treatment and Ca/P molar ratio, and hydroxyapatite treated at 800-900°C has been reported to exhibit an excellent bone-inducing ability[23-25]. The large particle size, good crystallinity, orderliness of the crystalline lattice, and low solubility are considered to provide heat-treated hydroxyapatite with its high bone-inducing ability. The pore size and pore rate of hydroxyapatite also affect bone formation. We, therefore, tentatively prepared heat-treated hydroxyapatite in which the Ca/P ratio was markedly changed from normal (1.67) and examined whether it has good bone-conduction and -induction abilities and is effective for accelerating the recovery of damaged tissues.

Heat treatment of hydroxyapatite increased the pH of the solution in which it was immersed. This effect was also greater as the heating temperature was higher. However, the pH decreased with time in all hydroxyapatite preparations. This suggests that the pH-increasing effect of heat-treated-hydroxyapatite was derived from the formation of compounds consisting of OH and Ca ions or ions in physiologic saline. Our group observed early new bone formation using hydroxyapatite heat-treated at 900°C for 24 hours, and reported that the maintenance of alkalinity with hydroxyapatite was a factor in this early new bone formation[26]. In this study, also, heat-treated hydroxyapatite retained a higher alkaline phosphatase activity in osteoblasts than non-heat-treated hydroxyapatite. These results are considered to support the early new bone formation-inducing effect of heat-treated hydroxyapatite.

In this report, Ca ion release from hydroxyapatite increased with the duration and temperature of heating. Also, Ca ion release from heat-treated was higher than that from non-heat-treated hydroxyapatite. On X-ray diffraction analysis in our study, a peak of CaO was noted in hydroxyapatite treated at 900°C (100P/900°C/6h and 114P/900°C/6h), which showed high Ca ion release, probably because excess CaO contained in the test hydroxyapatite was precipitated by heating at a high temperature for a long time. Also, the peak of CaO disappeared after immersion in purified water, probably because CaO escaped into water, and the zonal patterns observed on the surface of 114P/900°C/6h after immersion in purified water is considered to be the mark left by CaO precipitated on the surface by heat treatment but dissolved in water. The pH-increasing effect of heat-treated hydroxyapatite observed in this study may be explained by precipitated CaO.

Orii *et al.*[27] reported that, when osteoblasts were cultured in a medium containing a high level of Ca ions, the alkaline phosphatase activity increased rapidly when the formation of calcified materials began but decreased after the end of the calcification of bone tissue. In our experiment, the number of alkaline phosphatase-positive osteoblasts increased in the groups cultured with heat-treated 100P/900°C/6h and 114P/900°C/6h. The promotion of osteoblast differentiation by the heat-treated hydroxyapatite may have been caused by the high concentrations of Ca ions released from it. Detailed evaluation of the effects of Ca ion on alkaline phosphatase and protein levels in osteoblasts is necessary to clarify the mechanism of the promoting effect of heat-treated hydroxyapatite on osteoblast differentiation.

Hydroxyapatite has a high protein-adsorbent activity. When hydroxyapatite was added to cultures of osteoblasts, the alkaline phosphatase activity decreased compared with those in osteoblasts cultured without hydroxyapatite. This may have been a result of the adsorption of growth factor contained in FBS or other factors in culture medium by hydroxyapatite. Further evaluation of the

effect of heating of hydroxyapatite on its protein-adsorbing activity is also necessary. Furthermore, as phosphate ions are reported to modify osteoblast differentiation[28], phosphate ions derived from hydroxyapatite may have affected osteoblast proliferation or differentiation. Analysis of the effect of heat treatment on the release of phosphate ions from hydroxyapatite and the effect of these released phosphate ions on osteoblast differentiation will be necessary. In addition to these analyses, analysis of the *in vivo* effects of heat-treated hydroxyapatite is considered to be necessary. We would like to make the clinical application of heat-treated hydroxyapatite possible through these analyses.

Acknowledgments

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