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Effect of blood addition on the biocompatibility of calcium phosphate paste

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Abstract

The effect of blood addition on the biocompatibility and mechanical properties of calcium phosphate paste (CPP) has been examined. The addition of blood to the CPP increased the consistency and setting time in the malaxation operation; the specimen with blood addition possessed higher carbonate content and greater solubility into the acid but lower compressive strength, compared to the specimen without blood addition. Moreover, the immersion of CPP specimens with and without blood addition into the simulated body fluid showed no significant difference in conversion time of the chemical components (α-$Ca_3(PO_4)_2$ and $Ca_4O(PO_4)_2$) to the hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). These specimens were implanted into the tibiae of Japanese white rabbits. After the implantation for 12 weeks, the radiograph of CPP specimen with blood addition showed a clear regression of opacity, suggesting that the bioabsorbability and replacement with bone were promoted by the addition of blood; this effect proceeded significantly from surfaces to inside surfaces of CPP specimens as the amount of blood addition increased.

Keywords: Biomedical application; Composites; Porosity; Calcium phosphate paste; Mechanical properties

1. Introduction

The calcium phosphate paste (CPP) is now being used more extensively than polymethylmetacrylate (PMMA). The utilization of PMMA-based materials has the risk of monomer toxicity, and sometimes damage to cells due to the heat evolution during the stiffening. On the other hand, the utilization of CPP has advantages of (i) excellent biocompatibility and osteoconductivity, (ii) no heat evolution during the setting and (iii) restriction of incision area to a minimum. Thus, CPP is used for the restoration of bone defects formed by fractures or other causes.

When the CPP is implanted into the defect parts of bone, a certain amount of blood is inevitably released during the surgical operation. Such released blood seems to be utilized as a malaxation liquid for the setting of CPP. Relating to this, our previous experiments in vitro and in vivo revealed that the absorption of CPP and simultaneous replacement with bone are promoted by the addition of blood, regardless of the decrease in mechanical strength after stiffening.[1,2] Nevertheless, no systematical information on the properties of CPP with blood addition, e.g., setting conditions, mechanical strengths, biocompatibility, etc., has been available until now. Based on such background information, we describe the experimental results in vitro and in vivo, relating to the effect of blood addition on the properties of CPP.

2. Experimental procedures

2.1. Materials and fabrication of stiffened specimen

The materials used were CPP (BIOPEX-R®, Mitsubishi Materials Co. Ltd., Tokyo, Japan; CPP) and malaxation

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Table 1
Chemical compositions in the CPP powder and malaxation liquid

Chemical	Formula	Contents (%)
Powder		
α-Tricalcium phosphate	α-$Ca_3(PO_4)_2$	74.9
Tetracalcium phosphate	$Ca_4(PO_4)_2O$	18
Dicalcium phosphate dihydrate	$CaHPO_4{\cdot}2H_2O$	5
Hydroxyapatite	$Ca_{10}(PO_4)_6(OH)_2$	2
Magnesium phosphate	$Mg_3(PO_4)_2$	0.1
Liquid phase		
Sodium chondroitin sulfate	–	5
Disodium succinate	$(CH_2COONa)_2$	12
Sodium hydrogensulfite	$NaHSO_3$	0.3
Water	H_2O	82.7

liquid (BIOPEX-R® liquid). The chemical components of CPP and malaxation liquid are shown in Table 1. The CPP was composed of α-tricalcium phosphate (α-$Ca_3(PO_4)_2$; α-TCP), tetracalcium phosphate ($Ca_4(PO_4)_2O$; TECP), dicalcium phosphate dihydrate ($CaHPO_4{\cdot}2H_2O$), hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAp) and magnesium phosphate ($Mg_3(PO_4)_2$). The malaxation liquid consisted of sodium chondroitin sulfate, disodium succinate ($(CH_2COONa)_2$), sodium hydrogensulfite ($NaHSO_3$) and water (H_2O).

The starting CPP powder was mixed with malaxation liquid and human fresh blood. Two kinds of mixtures containing CPP, malaxation liquid and blood were prepared under the following conditions: (I) the amounts of powder and mixing liquid (malaxation liquid and blood) were 12 and 3.6 g, respectively, while the blood content was enhanced with decreasing amount of malaxation liquid; and (II) the amounts of powder and malaxation liquid were 12 and 3.6 g, respectively, while the total amount of liquid (malaxation liquid and blood) was enhanced with increasing blood content.

2.2. Evaluations

The consistency of the paste was evaluated as follows: 1 g of the CPP mixed with malaxation liquid and blood was promptly put on a glass plate, the glass plate with the mass of 120 g was gently laid on the paste, and then the longest and shortest sizes of paste were measured.[3] The setting time was measured as follows: the CPP mixed with malaxation liquid and blood was put in a ring with an inner diameter of 10 mm and a height of 3 mm and put into an incubator with the temperature of 37.0 ± 0.2 °C and the humidity of 100%, after mixing for 2 min.[3] The setting time was defined when a needle with a flush tip, having a diameter of 2 mm and a own weight of 300 g, made an impression on the surface of the specimen but the circular attachment failed to make a mark on it.

The total porosity (P) was measured by dividing the density (D) by the volume (V):

$$D = \frac{M}{V} \tag{1}$$

$$P = \left(1 - \frac{D}{D_{\mathrm{HAp}}}\right) \times 100 \tag{2}$$

where M is the mass of the stiffened paste in a plastic cylindrical container with an inner diameter of 7 mm and a height of 14 mm, and D_{HAp} is the theoretical density (3.16 g cm^{-3}) of HAp.

The compressive strength was measured using an Instron universal testing machine (Model 4466, Instron Co. Ltd., MA, USA) with a cross-head speed of 0.5 mm min^{-1}. The specimen was fabricated by immersing the stiffened paste with a diameter of 7 mm and a height of 14 mm into a volume of simulated body fluid (SBF)[4] at 37.0 ± 0.2 °C for the desired periods.

Crystalline phases were examined using a powder X-ray diffractometer (XRD; Model RINT 2400, Rigaku, Tokyo, Japan) with Cu Kα radiation (100 kV and 40 mA) at the scanning rate of 4° min^{-1}. The crystallinity of HAp was estimated using a reciprocal half-width (β) of the X-ray diffraction of HAp ($2\theta = 25.9°$); the scanning rate was 0.06° min^{-1}. The carbonate content of the sample was determined by an internal standard technique, using HAp with known amounts of carbonate for the calibration.

The solubility of the specimen in acid was examined on the basis of the Nancollas methods.[5] The sample was prepared via the procedures of (i) freeze–drying of each stiffened paste that had been kept immersed in the SBF for 7 days, (ii) pulverization of freeze–dried powder by agate mortar and pestle, and (iii) collection of powders after controlling particle sizes of 75 mesh sieve passage and 200 mesh sieve non-passage. The resulting powder (150 mg) was suspended in a diluted hydrochloric acid (HCl; 100 cm^3) maintained at 37.0 ± 0.2 °C and pH 5.5. A pH rise due to the partial resolution of paste in the HCl solution was kept at pH 5.5 by adding 0.1 mol dm^{-3} of HCl. The amount of HCl used for maintaining the pH at 5.5 was defined as the solubility in the acid.

The animal test was conducted using Japanese white rabbits. In order to avoid immunological rejection, the blood of rabbits themselves or self-supplied blood was used for the fabrication of specimens. Each specimen was then allowed to stand in a plastic cylindrical container with an inner diameter of 4.1 mm. A hole with a diameter of 4.2 mm was then made in the tibia by means of a drill; the specimen was implanted into the hole. Radiographs were then taken after the implantation for 2 and 12 weeks. After the implantation for a desired period between 2 and 12 weeks, the rabbits were sacrificed; a Villanueva bone stained and non-decalcified specimen was prepared and histologically examined.

3. Results

3.1. Fabrication of specimen with blood addition

Table 2 shows the consistency and setting time of CPP, together with the mixing conditions. Under the mixing

Table 2
Consistency and setting time of CPP with liquid, together with mixing conditions

	Powder (g)	Liquid			*P*/*L* (g/g)	Consistency (mm)	Setting time (min)
		Liquid (g)	Blood (g)	Total liquid (g)			
(I)							
Control	12	3.6	0	3.6	3.33	24	8
(i)	12	2.6	1.0	3.6	3.33	24	18
(ii)	12	1.0	2.6	3.6	3.33	25	58
(iii)	12	0	3.6	3.6	3.33	30	150
(II)							
Control	12	3.6	0	3.6	3.33	24	8
(i)	12	3.6	1.2	4.8	2.50	33	33
(ii)	12	3.6	2.4	6.0	2.00	44	41
(iii)	12	3.6	4.8	8.4	1.43	58	127

conditions of (I) and (II), the consistency and setting time increased with increasing amount of blood. The paste, which had been fabricated without malaxation liquid, was too loose; the setting time was as long as 150 min.

The effect of blood addition on the compressive strength of CPP specimen is shown in Fig. 1. The compressive strength of CPP specimen without blood addition (control) increased with increasing immersion time in the SBF and reached a maximum strength (78.8 ± 4.3 MPa) after 7 days. Although the compressive strengths of CPP specimens with blood addition also increased with increasing immersion time in the SBF, they were reduced by the addition of blood. For example, the compressive strength of CPP fabricated under the mixing conditions of (I)-(iii) was reduced down to 36.0 ± 3.2 MPa (3.6 g of the blood addition) after the immersion into the SBF for 7 days, which corresponded to 46% of the compressive strength of CPP specimen without blood addition; moreover, the compressive strengths of CPP specimens fabricated under the mixing conditions of (II)-(iii) was reduced down to about 10% (7.8 ± 1.1 MPa; the amount of blood, 4.8 g).

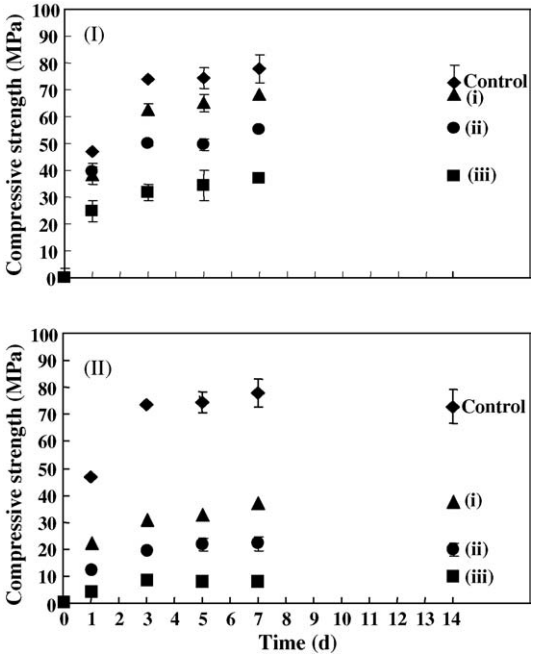

Fig. 1. Changes in compressive strengths of the CPP specimens with and without blood addition with immersion time in the SBF. Mixing conditions: (I) CPP, 12 g and total liquid (malaxation liquid + blood), 3.6 g. Control, no blood addition: (I)-(i) 1.0 g of blood addition; (I)-(ii) 2.6 g of blood addition; (I)-(iii) 3.6 g of blood addition. Mixing conditions: (II) CPP, 12 g and malaxation liquid, 3.6 g + blood. Control, no blood addition: (II)-(i) 1.2 g of blood addition; (II)-(ii) 2.4 g of blood addition; (II)-(iii) 4.8 g of blood addition. Note that the consistency was an average value of the longest and shortest sizes of the paste.

Fig. 2 shows the changes in compressive strengths of the CPP specimens with and without blood addition immersed into the SBF for 7 days as a function of total porosity. The compressive strengths of CPP specimens with blood addition, i.e., mixing conditions (I)-(i), (ii) and (iii), were deviated from the linear plots of the compressive strengths against the porosities of CPP specimens without blood addition; the strengths were lower than those of the CPP specimens without blood addition.

In order to determine the reason why the compressive strengths of CPP specimens were reduced by the addition of

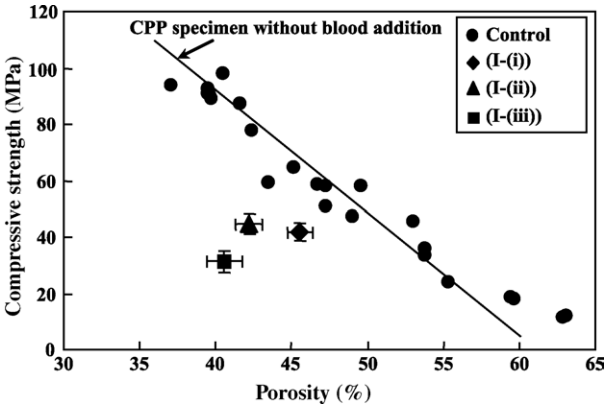

Fig. 2. Relationship between total porosities and compressive strengths of the CPP specimens with and without blood addition (mixing conditions (I)) after the immersion into SBF for 7 days. Note that all of the CPP specimens without blood addition (control) were fabricated by changing the *P*/*L* ratios from 1.0 to 4.2.

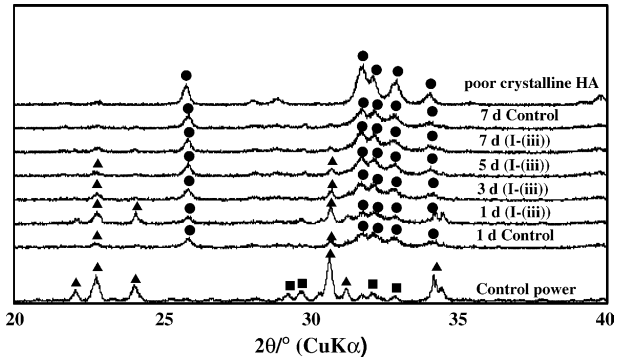

Fig. 3. XRD patterns of the CPP specimens with blood addition (mixing conditions (I)-(iii)) after the immersion into SBF. (●) HAp; (■) TECP; (▲) α-TCP.

blood, we examined crystalline phases of the CPP specimens with and without blood addition using the XRD. Typical XRD patterns of the CPP specimens with blood addition fabricated under the mixing conditions of (I)-(iii) are shown in Fig. 3. In both cases of the CPP specimens with and without blood addition, HAp increased with increasing immersion time in the SBF. X-ray diffraction intensities of CPP with blood addition were comparatively low but were similar to those of the CPP specimen without blood addition; no distinct delay in the conversion time of CPP to HAp was found to be caused by the addition of blood after the immersion into the SBF for 7 days.

In order to make clear the crystallinity of HAp and the carbonate content in the HAp, we examined changes in half-width of HAp ($2\theta = 25.9$), $1/\beta$,[6] and carbonate content, as a function of the amount of blood. Results are shown in Fig. 4. With increasing blood content, the $1/\beta$ value decreased, while the carbonate content increased.

Solubilities of CPP specimens with and without blood addition in HCl were examined. Results are shown in Fig. 5; the data on the HAp specimen fabricated by firing at 900 and 1200 °C are also plotted in the figure. Solubilities of the CPP specimens with and without blood addition into the HCl

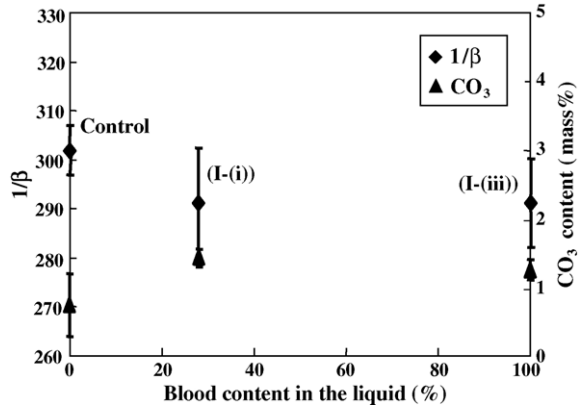

Fig. 4. Relationship between crystallinity ($1/\beta$) of HAp and the carbonate content of the CPP specimens with and without blood addition (mixing conditions (I)-(i) and (iii)).

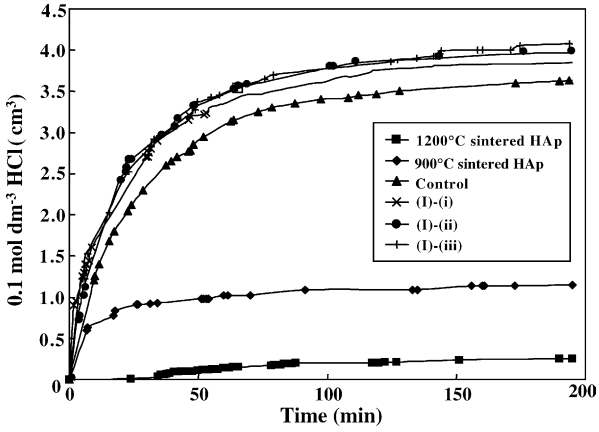

Fig. 5. Solubilities of CPP specimens with and without blood addition (mixing conditions (I)) in HCl together with those of the sintered HAp specimens.

solution were much higher than those of sintered HAp specimens; moreover, solubilities of CPP specimens with blood addition were higher than the solubility of the CPP specimen without blood addition.

3.2. Biocompatibility evaluation by animal test

The CPP specimens were implanted into Japanese white rabbits in order to examine the biocompatibility and osteoconductivity. Radiographs of the specimens implanted into the tibia of the rabbits are shown in Fig. 6. No significant changes in opacity of the CPP specimen without blood addition were found over the period of 2–12 weeks. On the other hand, a radiograph of the CPP specimens fabricated under the mixing conditions of II-(iii), i.e, 4.8 g of blood and 3.6 g of malaxation liquid, showed the clear regression of the opacity after the implantation for 12 weeks.

After the Villanueva bone staining, the histological examination was conducted using non-decalcified CPP specimens. Results are shown in Fig. 7. All of the specimens showed the presence of newly formed bone without any mediator on and near the surfaces of the CPP specimen after the implantation for 2 weeks. No distinct changes inside of the CPP specimen were found, after the implantation for 12 weeks. On the other hand, the distinct absorption of CPP specimen and simultaneous replacement with bone were observed near the surfaces of the specimen, especially in the case of 4.8 g of blood addition (conditions II-(iii)).

4. Discussion

4.1. Characteristics of the CPP specimen with blood addition

The preparation of the paste, which includes operations of adjusting, setting and stiffening, has to be completed within 20 min for the clinical utilization. The setting time of CPP increases with increasing blood content. In the case of the

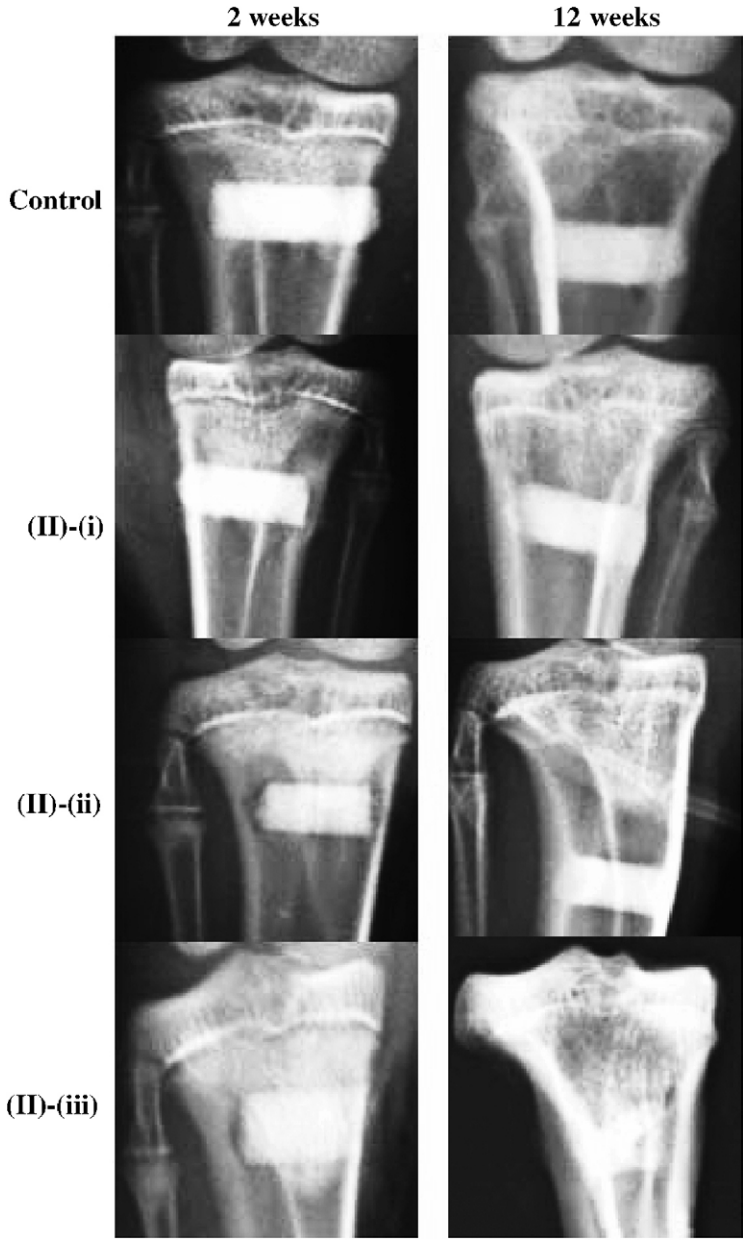

Fig. 6. Radiographs of the CPP specimen with and without blood addition (mixing conditions (II)).

P/*L* ratio of 3.33, for example, the setting time increases from 8 to 150 min with increasing amount of blood from 0 to 3.6 g (see Table 2; mixing conditions (I)). The initial stiffening, immediately after the mixing of CPP with malaxation liquid and blood, depends on a chelate reaction of calcium ions with succinic acid present in the malaxation liquid. An increase in setting time or the delay in setting with blood content may, therefore, be explained in terms of the retardation of chelate reaction due to the decrease in malaxation liquid.

The compressive strengths of CPP specimens with and without blood addition increase with immersion time in the SBF (Fig. 1). Compressive strengths of the CPP specimens with blood addition were, however, lower than those of the CPP specimens without blood addition, although the porosities of these specimens are similar to one another. This phenomenon may be clearly evidenced by plotting the compressive strengths of CPP specimens with and without blood addition against the porosity (Fig. 2). The lowering of compressive strengths due to the blood addition is explained by assuming that large pores are formed by the coalescence of gases given off from the blood and air entrapped during the mixing of loose paste with blood, because such large pores must act as the origin of failure.

XRD results revealed that the starting α-TCP and TECP disappear and that HAp forms after the immersion into the SBF solution (Fig. 3). The conversion route of components

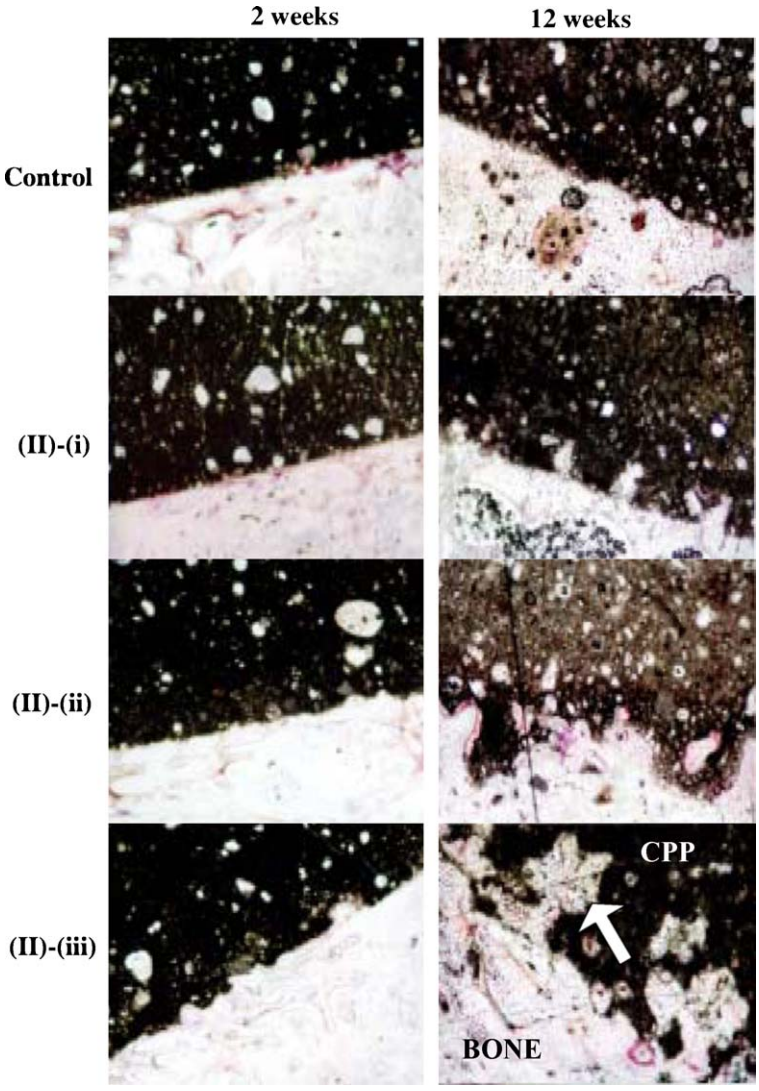

Fig. 7. Histological observation of the CPP specimens with and without blood addition (mixing conditions (II)). Note that that the CPP specimen with blood addition extended the absorption and bone formation from the surfaces to inside surfaces of the stiffened body (arrow mark), especially in the case of 4.8 g of blood addition (mixing conditions (II)-(iii)).

in the CPP to HAp during the immersion into the SBF can be expressed as follows:

$$2Ca_3(PO_4)_2 + Ca_4O(PO_4)_2 + H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 \quad (3)$$

$$2Ca_4O(PO_4)_2 + 2CaHPO_4{\cdot}2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 4H_2O \quad (4)$$

The small HAp crystals formed on the surfaces of CPP particles grow and become more entangled with each other with immersion time in the SBF, thereby enhancing the compressive strength. On the other hand, the decrease in crystallinity of HAp with increasing carbonate content indicates the formation of carbonate-containing apatite (Fig. 4). Increasing in the amount of carbonate-containing apatite may enhance the total porosity of the CPP specimen (Fig. 2), thereby enhancing the absorption of CPP and replacement with bone.[6,7] The increased absorption of CPP and replacement with bone may be, therefore, caused not only by the formation of larger amount of pores but also by the enhancement of solubility in HCl (Fig. 5).

4.2. Bioabsorbability of CPP with blood addition

The bioabsorbability and replacement with bone in vivo must be significantly affected by the preparation history of HAp.[8] Niwa and Hori[9] reported that the cell growth ability of HAp fired at 900 °C would be better than that of HAp fired at 1200 °C. This phenomenon is attributed to the crystallinity of HAp; the cell growth ability of poorer crystalline or smaller grains of HAp may promote the solubility of the body fluid.

Moreover, the crystallinity of HAp in the CPP is much poorer than that in the sintered specimen. The crystallinity

of HAp in CPP is similar to that of bones, and therefore, the cell growth ability of HAp formed in CPP must be better than that of a sintered specimen. As these data indicate, the lower crystallinity of HAp is favorable for the rapid absorption of CPP and bone replacement, although it is not effective for the enhancement of compressive strength.

The radiographs of the specimens implanted into the bones reveal that the CPP specimen without blood addition shows no distinct changes in opacity, whereas the CPP specimen with blood addition reveals the clear regression of the opacity after the implantation for 12 weeks (Fig. 6; conditions II-(iii)). In addition, the results of Villanueva bone staining suggest the excellent absorption of CPP specimen and replacement with bone (Fig. 7). We conclude from these results that the addition of blood is effective for the development of excellent bioabsorbable CPP and simultaneous replacement with bone. The carbonate, i.e., bone morphogenetic factor in the blood seems to be a driving force to induce the bones, and therefore, the replacement with bone may be promoted.

Longer setting time and decreased compressive strength of CPP specimen due to the blood addition have to be solved to allow clinical applications[10,11]; however, we consider that the CPP specimen with blood addition can be applied to the parts of bone defects, e.g., a radius which does not need high compressive strength but needs rapid absorption and bone replacement. One additional advantage may be that the infection is minimized when the self-supplied blood is clinically used.

As for the characteristics of cell growth, the blood addition may contribute to the cell growth, because it promotes the absorption of proteins, such as albumin on the surfaces.[12,13] In order to design the ideal bone-filling materials and to produce an artificial bone, one must control the bone creation ability by adding, for example, bone morphogenetic proteins (BMP). A further investigation is now being conducted in order to fabricate the ideal bone-filling material.

5. Conclusion

The effects of blood addition on the biocompatibility and mechanical properties of calcium phosphate paste (CPP) have been examined in this research. The results obtained are summarized as follows:

(1) The setting time and compressive strength of CPP specimen with blood addition were dependent on the blood content; however, the immersion of CPP specimens with and without blood addition into the simulated body fluid showed no significant difference in conversion time of the chemical components (α-$Ca_3(PO_4)_2$ and $Ca_4O(PO_4)_2$) to the hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) between them. The addition of blood to the CPP increased the consistency and setting time during the malaxation operation; the stiffened specimen with blood addition possessed higher carbonate content and solubility to the acid but lower compressive strength, compared to the specimen without blood addition.

(2) These specimens were implanted into the tibia of a Japanese white rabbit. The radiographs showed that no significant changes in absorption of CPP specimen without blood addition were observed for 12 weeks after the implantation. On the other hand, the radiograph of CPP specimen with blood addition showed the clear regression of opacity, suggesting that the bioabsorbability and replacement with bone were promoted by the addition of blood; this effect proceeded significantly from surfaces to center of CPP specimens as the amount of blood addition increased.

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