JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 6 (1995) 340–347

Calcium phosphate cement: *in vitro* and *in vivo* studies of the α-tricalcium phosphate–dicalcium phosphate dibasic–tetracalcium phosphate monoxide system

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In this paper, calcium phosphate cement consisting of α-tricalcium phosphate (α-TCP), dicalcium phosphate dibasic (DCPD) and tetracalcium phosphate monoxide (TeCP) was investigated *in vitro* and *in vivo*. Measurements of compressive strength against soaking time in simulated body fluid (SBF) showed a rapid increase of the hardness for the first 7 days. The gained strength was retained up to 1 year and the maximal mean value was 94.7 (±14.4) MPa. X-ray diffraction (XRD) and scanning electron microscopy (SEM) presented precipitates of hydroxyapatite (HA) after mixing, also after soaking in SBF and after implantation in rat subcutaneous tissues. However, the conversion to HA happened in different ways between *in vitro* and *in vivo* exposures. Histologic examinations showed that the cement causes the same reactions at the interface with surrounding soft tissues as HA. The authors consider the cement to be a promising material as a bone substitute, bone cement or dental material, however, further studies in a paste form and in bone tissue environments are necessary.

1. Introduction

Calcium phosphate ceramics such as hydroxyapatite (HA) and tricalcium phosphate (TCP) have been used for bone replacement and augmentation due to good biocompatibility and osteoconductivity. They are usually applied in the form of blocks or granules. There is, however, difficulty in shaping blocks prior to surgery and in making them fit with the bone surface. These problems do not happen with granules, but secondary migration is often observed and a mechanical strength comparable to blocks cannot be expected. Recently, much attention has been paid to calcium phosphate cements, because they can be handled as a paste and set *in situ*, and they may overcome the practical disadvantages of blocks or granules [1–11].

Monma and Kanazawa [12] reported in 1976 that α-TCP powder sets in the presence of water. However, the high temperature (80 °C) and long time (2 h) necessary for the reaction hindered practical use of the system. Improvement to reduce the setting time at around 37 °C was performed by some researchers. One of the solutions was addition of organic acid into the mixing liquids, but acidity of the products sometimes caused adverse reactions *in vivo*. In 1985, Brown and Chew [13] advocated a new calcium phosphate cement system of a tetracalcium phosphate and dicalcium phosphate dibasic mixture, which sets without use of acid. After this, similar systems appeared. An α-TCP/DCPD system was presented by Monma [14, 15] in 1988, in which the setting occurs with water, producing octacalcium phosphate (OCP) or hydroxyapatite (HA). The authors developed a new cement based on Monma's report. In our system, an α-TCP/DCPD powder mixture was mixed with water containing sodium succinate, to control the setting time, and sodium chondroitine sulphate, for easy mixing. We reported that this cement has good biocompatibility with bone tissue, bonds directly to it, and resorbs *in vivo* [16, 17]. Recently, some improvements on this system were performed to obtain higher strength of the hardened body. In this paper, the results of *in vitro* and *in vivo* studies of the new cement are reported.

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2. Materials and methods

Cement powder consisting of α-TCP (75%), TeCP (20%) and DCPD (5%) was mixed for 1 min with water containing sodium succinate (12%) and sodium chondroitine sulphate (5%) to produce hardened materials. For an *in vitro* study, two kinds of hardened body were prepared. Columns of 7 mm in diameter and 14 mm in length were made at different powder/liquid ratios (P/L ratio), from 2.5 to 3.5 (w/w), and incubated in simulated body fluid (SBF) [18] for various periods up to 1 year, changing SBF weekly for the first month, and monthly thereafter. These columns were used to measure the compressive strength.The measurement of six materials was carried out in a wet condition for each combination of P/L ratio and soaking time. An Instron Universal Testing machine Type 1125 was used at a crosshead speed of 0.5 mm/min. Discs with a diameter of 10 mm and a thickness of 2 mm were also prepared at a P/L ratio of 3.0 for the second *in vitro* study. Three discs, soaked separately in 20 ml SBF, were kept in a reciprocal water bath at 37 °C for 2 weeks, 1, 2 and 3 months, changing the SBF weekly. All discs were weighed in dry condition both before and after soaking to evaluate the weight change in SBF. Weekly checking of Ca and P ion concentration is SBF took place only in the case of the three discs which had been kept for 3 months. After soaking for the scheduled periods, discs were dried completely and analysed with X-ray diffraction (XRD) and scanning electron microscopy (SEM).

In an *in vivo* study, ten Wistar rats received two discs each (the same kind as used in the *in vitro* study), in subcutaneous sockets created on their back under general anaesthesia with a muscular injection of Hypnorm^{R} (0.1 ml/100 g). Rats were sacrificed 1 (three rats), 2 (three) and 3 months (four) after implantation. The discs were retrieved with the surrounding tissues and fixed in 70% ethanol solution. One disc from each rat was dehydrated in a graded series of ethanol and embedded in PMMA. Serial thin sections of 10 μm thickness were cut and stained with basic fuchsine and methylene blue for microscopic examination. The other disc from each rat was carefully stripped of the surrounding soft tissues, dried and used for XRD and SEM analyses.

3. Results

Compressive strength of the cement against soaking time is shown in Fig. 1. For the first 7 days the values indicated a rapid rise in all P/L ratios, and after that did not change significantly up to 1 year. Cements with higher P/L ratios showed greater strength, however, the difference was not significant above P/L = 3.0. The maximal mean value of 94.7 (± 14.4) MPa was obtained for the materials with P/L = 3.5 after soaking for 1 year.

Weight changes of the discs after soaking are illustrated in Fig. 2, where the mean of gained weight is presented as a percentage of the original weight. The results show that the discs lost some weight after 2 weeks and 1 month soaking; on the other hand they gained weight after 2 and 3 months.

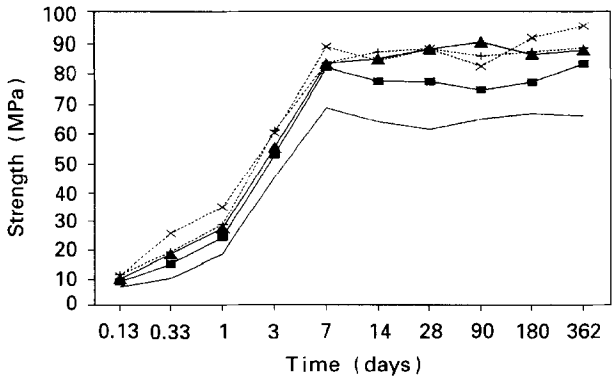

Figure 1 Compressive strength against soaking time. The values are the mean from six samples for each combination of P/L ratio and soaking time.—P/L = 2.5; —■ P/L = 2.8; —▲— P/L = 3.0; -+- p/L = 3.2; —×— P/L = 3.5.

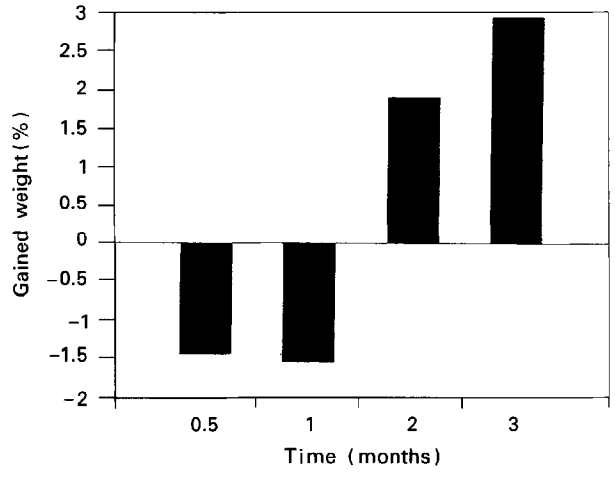

Figure 2 Weight change of the discs after soaking in SBF. The graph shows the mean of gained weights as a percentage of the original weight.

Ca ion concentrations in SBF after 1 week were 20–26 p.p.m. higher and P was 9–11 p.p.m. lower than the control SBF. Ca concentrations showed values close to the control on the second and third week and after that they were always lower than the control, showing a gradual decrease with soaking time. P concentrations showed an abrupt rise of several p.p.m. on the second week and maintained these values, lower than the control, until the end of the measurement (Fig. 3).

Fig. 4 shows SEM micrographs of the disc surfaces after soaking in SBF. It is clearly seen in these figures that the disc surfaces were covered with precipitates, and the morphological changes in them progressed as soaking time increased. The 2-week and 1-month samples showed petal form precipitates, which then united with each other on 2-month discs, forming an aggregate of oval or cylindrical grains with small flakes on the surface. Three-month samples had a large quantity of surface precipitates with fine flakes, which formed a flat and homogeneous surface.

XRD patterns of the control discs (before soaking) showed high α-TCP peaks, HA peaks and low TeCP peaks. The TeCP peaks became lower or disappeared on the XRD of samples subjected to 2-week soaking.

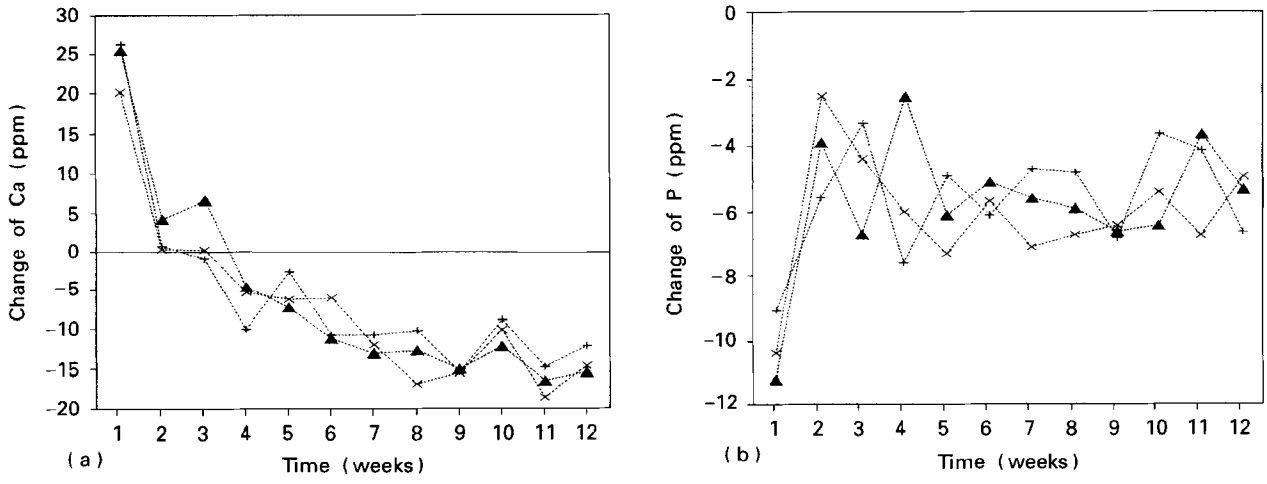

Figure 3 Ca (a) and P (b) concentrations in SBF with discs. Data are from three samples (▲, ×, +) soaked in SBF for 3 months.

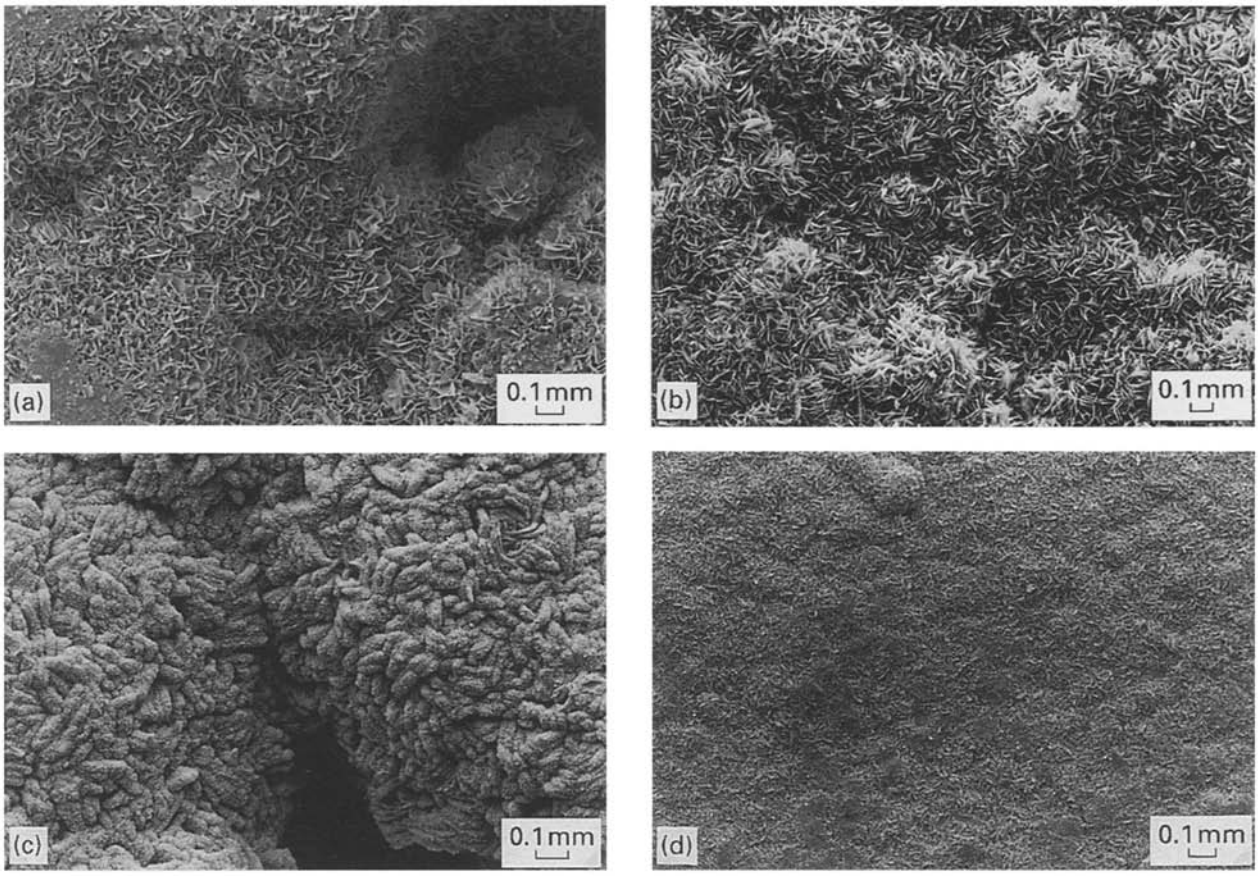

Figure 4 SEM photographs of the disc surface soaked in SBF for (a) 2 weeks; (b) 1 month; (c) 2 months and (d) 3 months.

As the soaking time increased, HA peaks became higher and α-TCP peaks, which disappeared after 2 months, became lower (Fig. 5).

SEM micrographs of the discs used in an *in vivo* study showed a different form of precipitates to those of an *in vitro* study. Precipitates appeared as a mass of spherical or polygonal grains several micrometres in diameter. They did not show a significant change in morphology and a noticeable increase in amount after 2 or 3 months of implantation. It is recognized in all implant periods that some parts of the disc surfaces, where precipitates were less, became more porous than the controls (Fig. 6).

Fig. 7 shows XRD patterns of discs implanted in rats. As in the *in vitro* study, the peaks for HA became higher and both α-TCP and TeCP peaks became lower as the implant period increased. However, TeCP could be seen in 2-month XRD patterns and α-TCP was still detected in 3-month XRD patterns.

Microscopically, all discs from different implant periods and rats showed similar reactions of surrounding tissues. Discs were surrounded by thin fibrous capsula, arranged parallel to the surface of the discs. Mild inflammatory reactions were seen, with multi-nucleated giant cells only on the rough surface of the discs. Resorption of the discs was not clearly recognized (Fig. 8).

4. Discussion

Reports on calcium phosphate cement are available in the literature and different formulations can be seen [1–11]. The authors developed a system based on Monma's report [14, 15], in which an α-TCP/DCPD mixture was mixed with water containing sodium succinate, for control of the setting time of 8–10 min, and sodium chondroitine sulphate, for smooth mixing. The results of animal experiments with this cement have been reported previously, where direct contact of

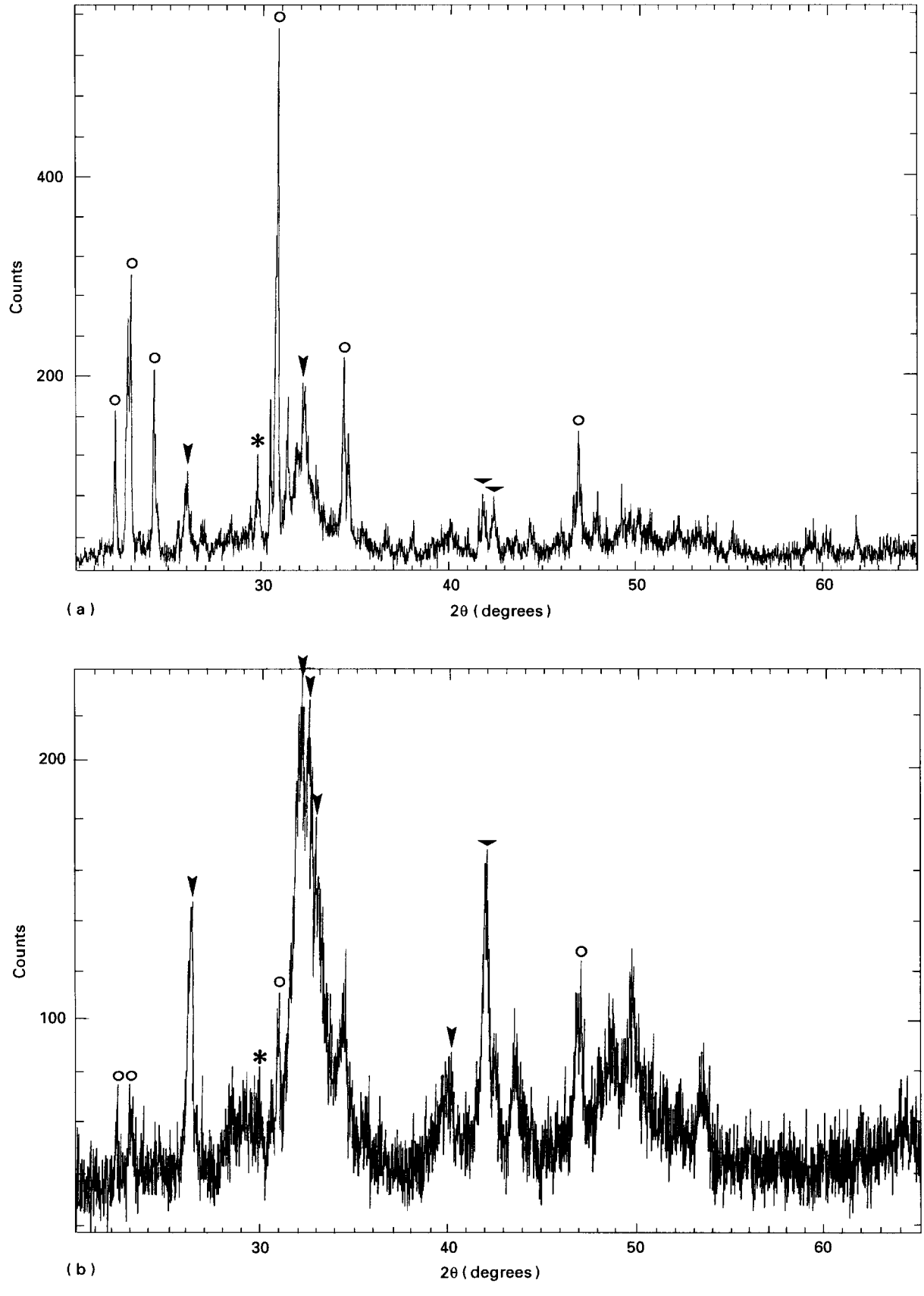

Figure 5 XRD patterns of discs from *in vitro* study. (a) Control disc (before soaking); (b) 2-week soaking; (c) 3-month soaking (○ α-TCP; ▼ HA; * TeCP; ▾ holder).

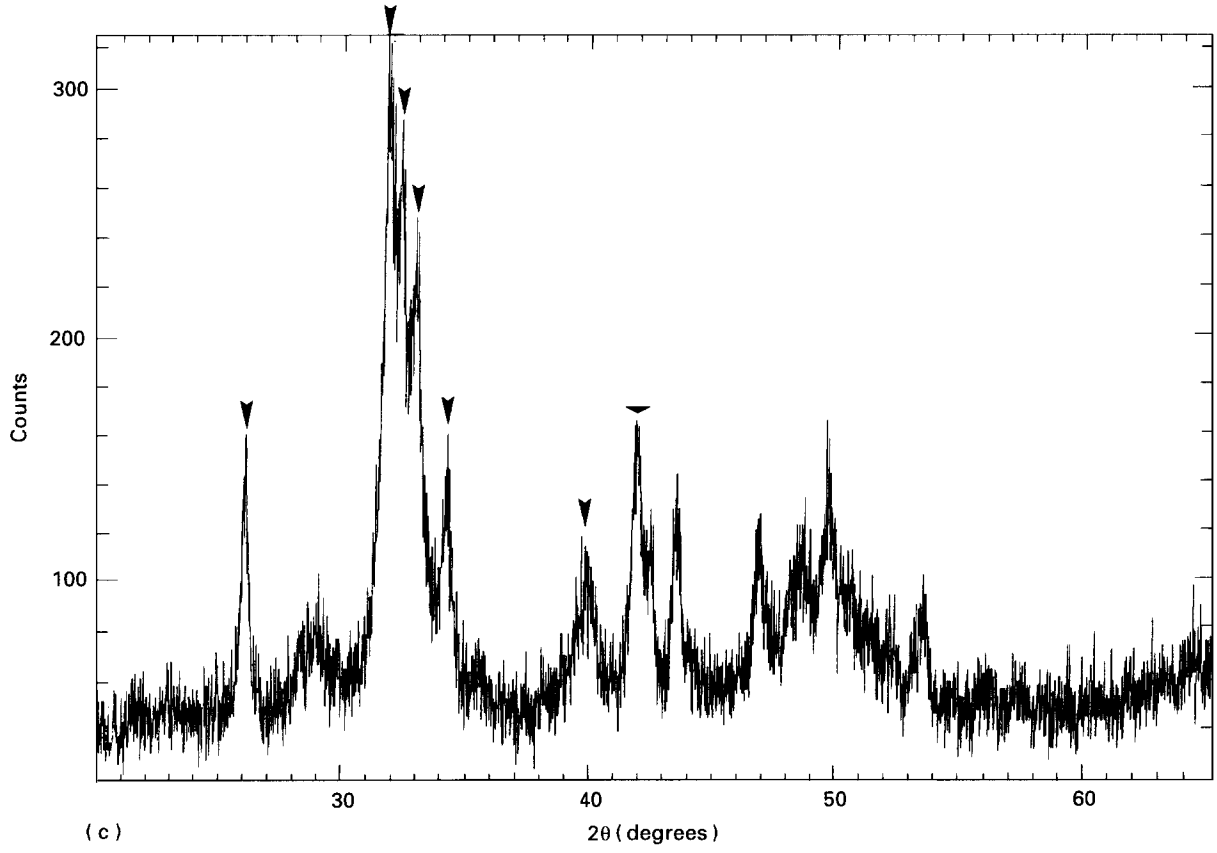

Figure 5 (continued)

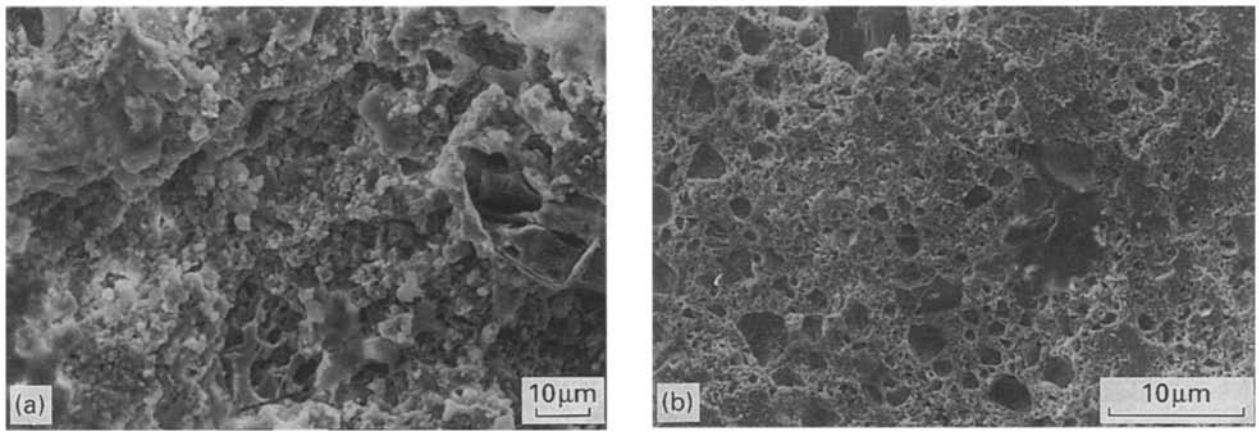

Figure 6 SEM photographs of *in vivo* discs. Significant difference was not seen among the three different implant periods. (a) HA precipitates on disc surface after 1-month soaking. (b) Surface roughness after 3-month soaking.

the bone to the cement and degradation of the cement were shown [16, 17].

Yamamoto *et al.* [19] investigated the same cement both *in vitro* and *in vivo* and reported that it showed good affinity with the surrounding tissues *in vivo*, it set, producing HA, and the compressive strength increased with soaking time in SBF. The maximal compressive strength in their experiments was 400 kg/cm^2. They concluded that the strength was equivalent to that of cancellous bone which is not enough for use in load-bearing areas. The authors attempted to increase the strength, and finally employed a higher sintering temperature for α-TCP granules, changing from 1250 to 1400 °C, and added TeCP to the cement powder. The final formula of the new system is shown in Table I, together with an old system for comparison. Preliminary mechanical tests showed a 30% increase in the maximum compressive strength.

In this study, compressive strength against soaking time in SBF showed significant increase during the first 7 days in all P/L ratios. After 7 days it did not change significantly and the maximal mean value of 94.7 (± 14.4) MPa was obtained for 3.5 P/L ratio materials after soaking for 1 year. Fig. 1 shows that the higher the P/L ratio, the greater compressive strength values obtained, however, a significant difference was not seen above a P/L ratio of 3.0. Bermúdez *et al.* [8] reviewed several calcium phosphate cements

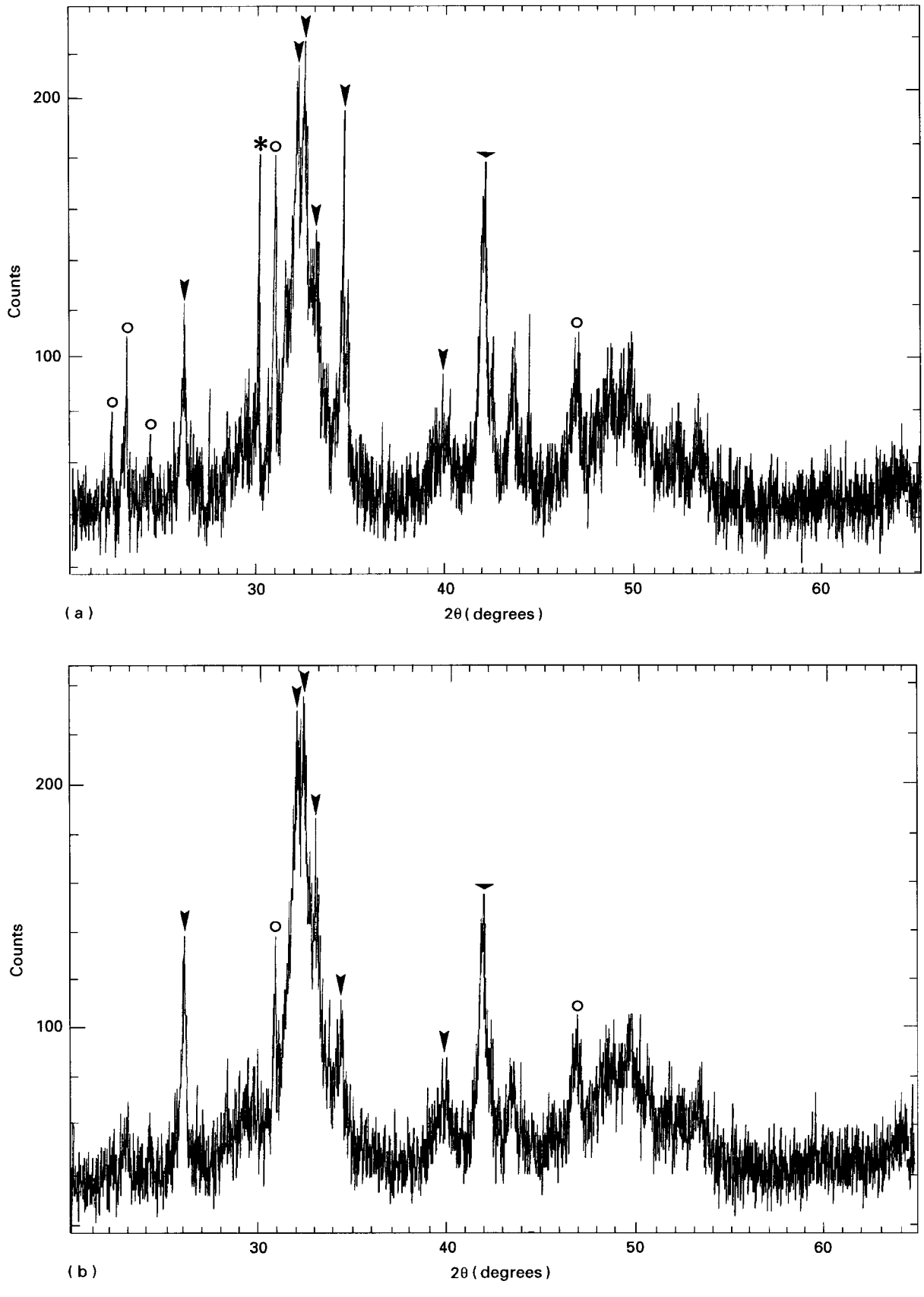

Figure 7 XRD patterns of discs in *in vivo* study. (a) 1-month implant; (b) 3-month implant (○ α-TCP; ▼ HA; * TeCP; ▾ holder).

in the literature and found that their compressive strength ranged from 9 (± 3) to 36 (± 1) MPa when stored for 24 h in water, air of 100% humidity or 0.9% NaCl solution. The values for our new cement soaked for 24 h in SBF vary from 18.3 (± 1) to 34.6 (± 1.4) MPa, depending on P/L ratios. Our cement has a strength corresponding to the previously reported data of other researchers. Though few data are available in the literature concerning long-term storage, it is important that the cements, as a bone substitute or bone cement, keep their strength for a long time, at least until incorporation with the surrounding

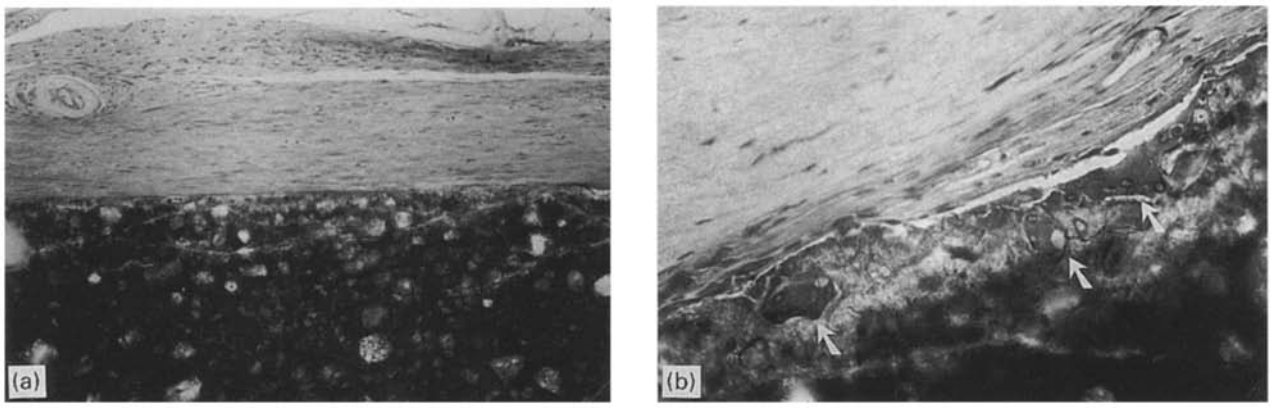

Figure 8 Light micrographs of implanted discs (dark part). Among three implant periods no difference was noticed. (a) Smooth surface of the disc (implanted for 1 month, stained with basic fuchsine and methylene blue, original magnification × 63). (b) Rough part of the disc surface where usually multi-nucleated giant cells (arrows) are seen and detachment of the fibrous capsula from giant cells is seen (implanted for 3 months, stained with basic fuchsine and methylene blue, original magnification × 400).

TABLE I Compositiion of the cement

	Old type (%)	New type (%)
Powder		
α-TCP	95	75
DCPD	5	5
TeCP		20
Liquid		
Water	78	83
Sodium chondroitin sulphate	10	5
Sodium succinate	12	12

bone tissue occurs. Our cement showed maintenance of its strength in SBF for up to 1 year.

The weight changes of discs shown in Fig. 2 indicates that dissolution of the discs is predominant over precipitation during the first stage of soaking. Ca ion concentration measurements in SBF showed results corresponding with this phenomenon. However, P ion concentration at 1 week showed lower values (9–11 p.p.m.) than in the control SBF. This may be caused by the presence of non-reacted TeCP, which is verified in the XRD patterns of the control discs. The disappearance of TeCP peaks after 2 weeks of soaking is proof of the dissolution or conversion of TeCP during the early soaking period. Furthermore, dissolution of TeCP could be the reason for the high values of Ca concentration after 1 week. Hence both Ca and P concentrations showed an abrupt change in their value on the second week, and the compressive strength did not change significantly after 2 weeks; the reaction of TeCP may be the significant contributor to the strength of the cement. After TeCP disappeared, HA precipitated constantly on the surface of the discs consuming Ca and P ions in SBF. In *in vivo* studies, HA precipitation on the discs was verified by XRD and SEM analyses. However, both the morphology and the amount were very different from *in vitro* studies. Precipitates were recognized as small grains of spherical or polygonal form. They did not show morphological changes and increased in amount after the longer implant periods. Furthermore, many holes, supposedly created by dissolution, were seen on the disc surfaces of all implant periods. These results indicate that dissolution is still active *in vivo* after 3 months. One of the reasons for the difference between *in vitro* and *in vivo* tests could be that the body fluid surrounding the disc is always changing *in vivo* as a result of circulation. A similar difference is shown in the report by Yamamoto *et al.* [20], who measured the strength of the cement (same cement as our old type) stored in SBF or implanted in rabbit subcutaneous tissues. In their study, the maximum strength *in vitro* was higher and showed up at an earlier stage of the experiment than *in vivo*. Since the strength of the cement is closely related to structural changes, as Nishimura *et al.* reported [21], the results show that the cement changes in different ways *in vitro* and *in vivo*. The most important finding here is the fact that the results of *in vitro* studies do not always reflect the phenomena *in vivo*, even if SBF is used.

Histological evaluation in this study showed that the discs were surrounded by thin, dense and fibrous capsula with little inflammatory reaction for all experimental periods. The fibrous capsula attached to the discs intimately at the smooth surfaces, and at the rough surface areas (very rare though) multi-nucleated giant cells appeared on the disc surface. Detachment of the capsula from giant cells was usually seen, which may be created during section preparations. It can be said that the reactions of the surrounding tissues seen in this study are, in general, the same as those seen when HA is implanted in soft tissues [22, 23]. Though the number of implants is limited in this study, the results suggest that the cement acts in the same way as HA does. This is to be expected, because the cement forms HA while hardening and HA precipitates on the surface *in vivo*. The authors consider that an experimental study is necessary with more implants and for longer implant periods. In addition, further studies should be carried out in bone tissue and with implants of paste to evaluate the usefulness of this cement as a bone substitute.

Acknowledgements

The authors wish to thank Mrs Sonja v.d. Meer, Mr Joop Wolke and Mr Jurren Koerts for their

assistance and support. The authors are also grateful to Professor A. Kotani (Shinshu University, Matsumoto, Japan) for his suggestions and help. In addition, one of us (Kenji Kurashina) has received a government grant form the Japanese Ministry of Education.

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Received 4 August
and accepted 17 October 1994.