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## The Cytocompatibility and Early Osteogenic Characteristics of an Injectable Calcium Phosphate Cement

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### ABSTRACT

In this study, the cytocompatibility and early osteogenic characteristics of rat bone marrow cells (RBMCs) on injectable calcium phosphate (CaP) cement (Calcibon) were investigated. In addition to unmodified CaP cement discs, 2 other treatments were given to the discs: preincubation in MilliQ and sintering at different temperatures. After primary culture, RBMCs were dropwise seeded on the discs and cultured for 12 days. The samples were evaluated in terms of cell viability, morphology (live and dead assays and scanning electron microscopy (SEM)), cell proliferation (deoxyribonucleic acid (DNA) analyses), early cell differentiation (alkaline phosphatase (ALP) activity), and physicochemical analyses (x-ray diffraction (XRD)). The live and dead, DNA, and SEM results showed that Calcibon discs without any additional treatment were not supporting osteoblast-like cells *in vitro*. There were fewer cells, and cell layers were detached from the disc surface. Therefore, different preincubation periods and sintering temperatures were evaluated to improve the cytocompatibility of the CaP cement. Preincubating discs in MilliQ for periods of 1, 4, 8, and 12 weeks resulted in the hydrolysis of  $\alpha$ -tri calcium phosphate (TCP) into an apatite-like structure with some  $\beta$ -TCP, as shown with XRD, but the material was not cytocompatible. Sintering the discs between 800°C and 1100°C resulted in conversion of  $\alpha$ -TCP to  $\beta$ -TCP with some hydroxyapatite and an increase in crystallinity. Eventually, the discs sintered at 1100°C achieved better cell attachment, more-abundant cell proliferation, and earlier differentiation than other sintered (600°C, 800°C, and 1000°C), preincubated, and unmodified specimens. On basis of our results, we conclude that *in vivo* results with CaP-based cements do not guarantee *in vitro* applicability. Furthermore, unmodified Calcibon is not cytocompatible *in vitro*, although preincubation of the material results in a more-favorable cell response, sintering of the material at 1100°C results in the best osteogenic properties. In contrast to *in vivo* studies, the Calcibon CaP cement is not suitable as a scaffold for cell-based tissue-engineering strategies.

### INTRODUCTION

CURRENTLY, THE USE OF autologous and allogeneous bone grafts is the method of choice for the treatment of large bone defects caused by trauma, disease, or tumor resection. However, both of these methods are associated with significant clinical problems such as donor site morbidity and the possible transfer of disease. In view of this, innovative bone-

engineering techniques have been introduced that include the use of a suitable scaffold in combination with mesenchymal stem cells.<sup>1-8</sup> Although the achieved results hold promise, the final goal (the safe and reliable healing of large bone defects) has not been accomplished. Current drawbacks of cell-based bone engineering are the low yield of cells with bone inducing capacities and the lack of standardized expansion and differentiation of progenitor cells.

As a consequence, reproducibility and predictability in human patients is low. Several solutions have already been suggested to solve this problem, such as the use of cell selection,<sup>9–11</sup> dynamic cell culture (flow perfusion system),<sup>12–16</sup> and encapsulation of cells in microspheres.<sup>17–20</sup> The final cell population has to be incorporated in a scaffold material that possesses intrinsic osteophilic properties. For bone purposes, the ideal scaffold material is considered to be bioactive, slowly biodegradable, moldable, and easy to apply. The scaffold material must also have a certain porosity, and the pores must have interconnectivity to allow tissue ingrowth and stabilization. The recently developed calcium phosphate (CaP) cements appear to fulfill these requirements, because they support bone growth (osteoconductive), are degradable because of osteoclastic remodeling, are injectable, can incorporate microspheres, and can be made micro- and macroporous to allow penetration of nutrients and clearance of waste.<sup>21–27</sup> Unfortunately, the results obtained with the culturing of osteoblastic cells on CaP cements are contradictory. This can be due to the composition of the starting powder used for the preparation of the CaP cement. For example, different CaP mixtures have been used, such as tetra calcium phosphate, dicalcium phosphate dehydrate, calcium pyrophosphate, tri calcium phosphate (TCP), which transform into materials composed of hydroxyapatite, carbonated apatite, and amorphous CaP during setting. However, calcium and phosphate ions can be released during this transformation process, which can be harmful during cell culturing. The size or geometry of the cement samples, manufacturing conditions, cell media changes, and total cell-medium volume, among other things, can enhance this effect. Evidently, no problems have been observed for the *in vivo* behavior of these CaP cement materials because the toxic products are sufficiently transported away during the initial phases of wound healing. At the same time, this phenomenon can explain the contradictory cell culture studies. For example, using the CaP cement Calcibon (Merck Biomaterial GmbH, Darmstadt, Germany), Hempel *et al.* found a lower cell proliferation and differentiation than with polystyrene cultures. The CaP scaffolds in this study were preincubated in simulated body fluid for 4 days.<sup>28</sup> In contrast to Hempel *et al.*, Oreffo *et al.* and Knabe *et al.* reported lower cell proliferation and greater cell differentiation on this material than with polystyrene cultures. Oreffo *et al.* preincubated the CaP scaffolds for 5 days in Ringer's solution, and Knabe *et al.* sterilized the scaffolds at 300°C for 3 h.<sup>28–30</sup>

Consequently, before the final use of CaP cement as scaffold material for a cell-based tissue-engineering approach, the cyto-compatibility of such a material has to be determined. In the current study, unmodified discs, together with discs preincubated in MilliQ and discs sintered at different temperatures, were used. These additional treatments can create phase transformations of the CaP cement, which can affect the cellular response.

## MATERIALS AND METHODS

### Substrates

Calcibon was used. The chemical composition of this cement is 62.5%  $\alpha$ -TCP, 26.8% dicalcium phosphate dihydrate, 8.9% calcium carbonate, and 1.8% precipitated hydroxyapatite. An aqueous solution of 1% disodium hydrogen phosphate dodecahydrate is used as the liquid component. The ideal liquid-to-powder ratio for clinical applications has been shown to be 0.35 mL/g. After mixing, the cement was immediately injected into a circular-shaped mould to ensure a standardized shape of the discs (cylinders of about 2.4 mm height and 6 mm diameter). The discs were removed from the molds after the cement had set. Histo-morphometrical analysis revealed that the total microporosity (pores < 1  $\mu$ m) of the cement was approximately 30% to 40%. In addition to the unmodified CaP cement discs, samples were preincubated (1, 4, 8, and 12 weeks) on a rotating plate in MilliQ, which was refreshed 3 times a week or sintered in a furnace for 2 h at 600°C, 800°C, 1000°C, or 1100°C.

The chemical composition of the samples was evaluated crushing the samples with a mortar until the discs were reduced to powder and then analyzing them using x-ray diffraction (XRD, Philips PW3710, Eindhoven, The Netherlands). The positions and intensities of the XRD peaks were used to identify the underlying structure (or phase) of the various treatments of the specimens.

### Cell isolation

Rat bone marrow cells (RBMCs) were isolated and cultured using the method described by Maniopoulos.<sup>31</sup> RBMCs were obtained from femora of male Wistar rats weighing between 120 g and 150 g. Femora were washed 3 times in culture medium minimal essential medium (MEM) (Gibco BRL, Life Technologies B.V., Breda, The Netherlands) with 0.5 mg/mL gentamycin and 3  $\mu$ g/mL Fungizone. Epiphyses were cut off and diaphyses flushed out with 15 mL complete culture medium  $\alpha$ -MEM supplemented with 10% fetal calf serum (Gibco), 50  $\mu$ g/mL ascorbic acid (Sigma Chemical Co., St. Louis, MO), 50  $\mu$ g/mL gentamycin, 10 mM sodium  $\beta$ -glycerophosphate (Sigma), and 10<sup>-8</sup> M dexamethasone (Sigma). Cells were incubated in a humidified atmosphere of 95% air, 5% carbon dioxide at 37°C. The medium was changed 3 times a week.

After 7 days of primary culture, cells were detached using trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25% w/v trypsin/0.02% EDTA). The cells were concentrated using centrifugation at 1500 rpm for 5 min and resuspended in a known amount of medium. Cells were counted using a Coulter counter (Beckman Coulter, Mijdrecht, The Netherlands) and resuspended in complete culture medium. A cell suspension of 50,000 cells per disc was used for the seeding and culturing experiments.

### Cell viability

Live and dead viability assays (Molecular Probes, Leiden, The Netherlands) ( $n = 2$  for all culture periods), consisting of washing with phosphate buffered saline (PBS), covering with a mix of calcein AM (1.0  $\mu\text{l}$ ) and ethidium homodimer (3.5  $\mu\text{l}$ ), incubating 30 to 45 min at 37°C, washing with PBS, and examining under a fluorescence microscope, were performed on days 0, 1, 2, 4, 8, and 12. Metabolically active cells were converting the calcein AM into green fluorescent calcein through intracellular esterases, where ethidium homodimer entered dead cells through damaged membranes to bind deoxyribonucleic acid (DNA). Green cells were viable, and red cells were considered to be dead.

### DNA analyses

DNA assays (Molecular Probes) ( $n = 6$  for all culture periods) were performed on days 0, 1, 2, 4, 8, and 12. Culture medium was removed, and discs were washed twice with PBS. One mL of MilliQ was added to each sample. Cells were harvested by placing the discs with inoculated cells in a 1.5-mL tube. One mL of MilliQ was added to each sample. Cell suspension was frozen to  $-80^\circ\text{C}$ , heated to 37°C, frozen to  $-80^\circ\text{C}$  again, and stored at  $-80^\circ\text{C}$  until usage.

A DNA standard curve was made using salmon testes DNA. One hundred  $\mu\text{l}$  of sample or standard were added to 100  $\mu\text{l}$  of Pico green working solution. The samples were incubated for 5 min at room temperature in the dark. After incubation, DNA was measured using a fluorescence microplate reader (Bio-Tek Instruments, Abcoude, The Netherlands) with excitation filter 365 nm and emission filter 450 nm.

### ALP activity

For the ALP assay (Sigma), the same samples from the DNA assay were used. Eighty  $\mu\text{l}$  of sample and 20  $\mu\text{l}$  of buffer solution (5 mM magnesium chloride, 0.5 M 2-amino-2-methyl-1-propanol) were added in a 96-well plate. One hundred  $\mu\text{l}$  of substrate solution (5 mM p-nitrophenylphosphate) was added to the well, and the plate was incubated for 1 h at 37°C. The reaction was stopped by adding 100  $\mu\text{l}$  of stop solution (0.3 M sodium hydroxide). For the standard curve, serial dilutions of 4-nitrophenol were added to final concentrations of 0 to 25 nM. The plate was read in an enzyme-linked immunosorbent assay reader (Bio-Tek Instruments) at 405 nm. ALP activity results were normalized to ng of DNA.

### Scanning electron microscopy

SEM analyses ( $n = 2$  for all culture periods) were performed on days 0, 1, 2, 4, 8, and 12. The samples were washed twice with PBS. Fixation was carried out for 10 min in 2% glutaraldehyde, and then substrates were washed twice with 0.1 M sodium-cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried using tet-

ramethyl silane. The specimens were sputter-coated with gold and examined and photographed using a Jeol 6310 SEM at an acceleration voltage of 10 kV. SEM was performed at the Microscopic Imaging Center of the Nijmegen Center for Molecular Life Sciences, The Netherlands.

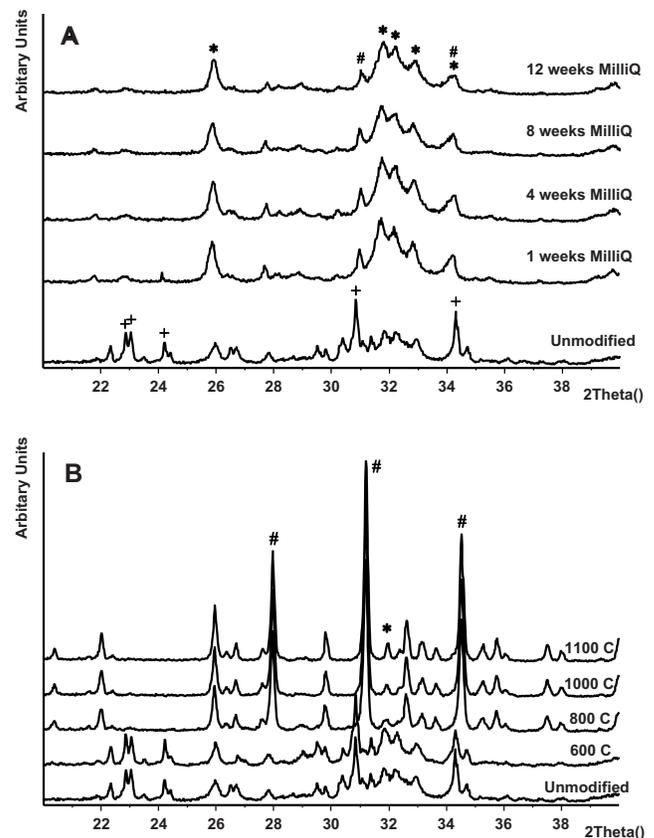
### Statistical analyses

Statistical analyses were performed using GraphPad Instat 3.05 software (GraphPad Software Inc., San Diego, CA) using one-way analyses of variance with a Tukey multiple comparison post test.

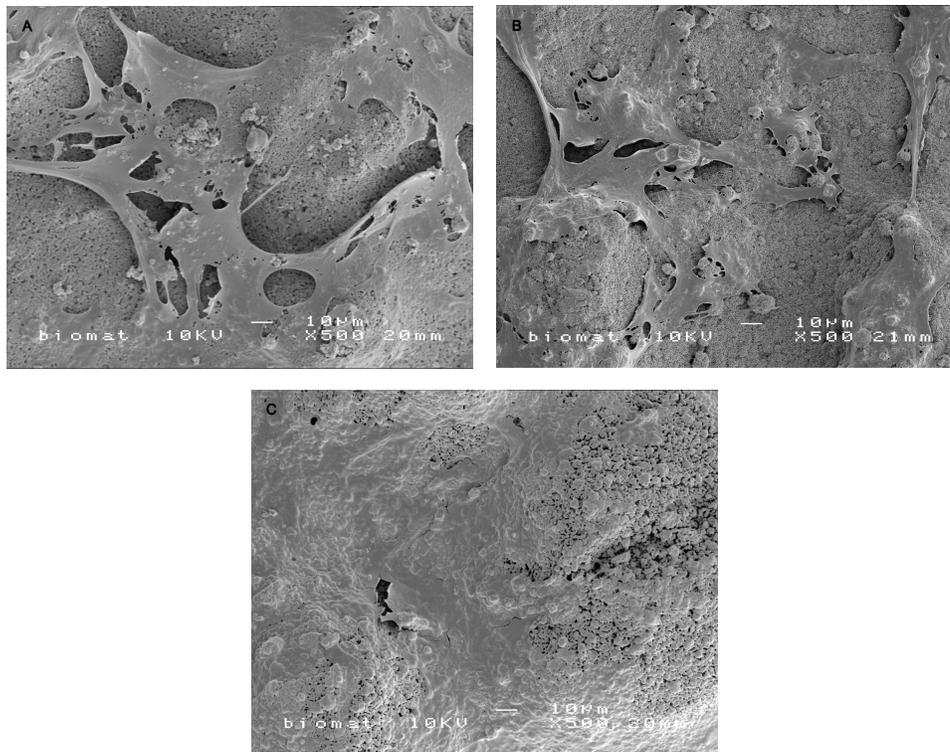
## RESULTS

### Unmodified Calcibon

The results of the XRD analyses (Fig. 1) demonstrated that the unmodified CaP cement discs were mainly composed of  $\alpha$ -TCP. These samples caused the pH of the culture



**FIG. 1.** X-ray diffraction (XRD) analyses of the calcium phosphate cement discs. The XRD powder patterns of (A) unmodified and discs preincubated for 1, 4, 8, and 12 weeks and (B) unmodified discs and discs sintered at 600°C, 800°C, 1000°C, and 1100°C are shown, respectively. (\* = apatite; # =  $\beta$ -tri calcium phosphate (TCP); + =  $\alpha$ -TCP).



**FIG. 2.** Scanning electron microscopy of (A) unmodified discs, (B) discs preincubated for 1 week, and (C) discs sintered at 1100°C after day 1 of culture.

medium to decrease, indicated by the change of color from red to yellowish. SEM images (Fig. 2A) revealed that cell layers detached from the disc surface. This started at day 1 and was observed throughout the entire culture period. The number of cells present on the surface also decreased over time. At day 8, only a few viable cells were observed on the disc surface with the live and dead assay (Fig. 3A). The results of the DNA analyses (Fig. 4) confirmed the results of live and dead and SEM by showing reduced DNA content, ending close to zero at day 12. Almost no ALP activity (Fig. 5) was measured during this culture period.

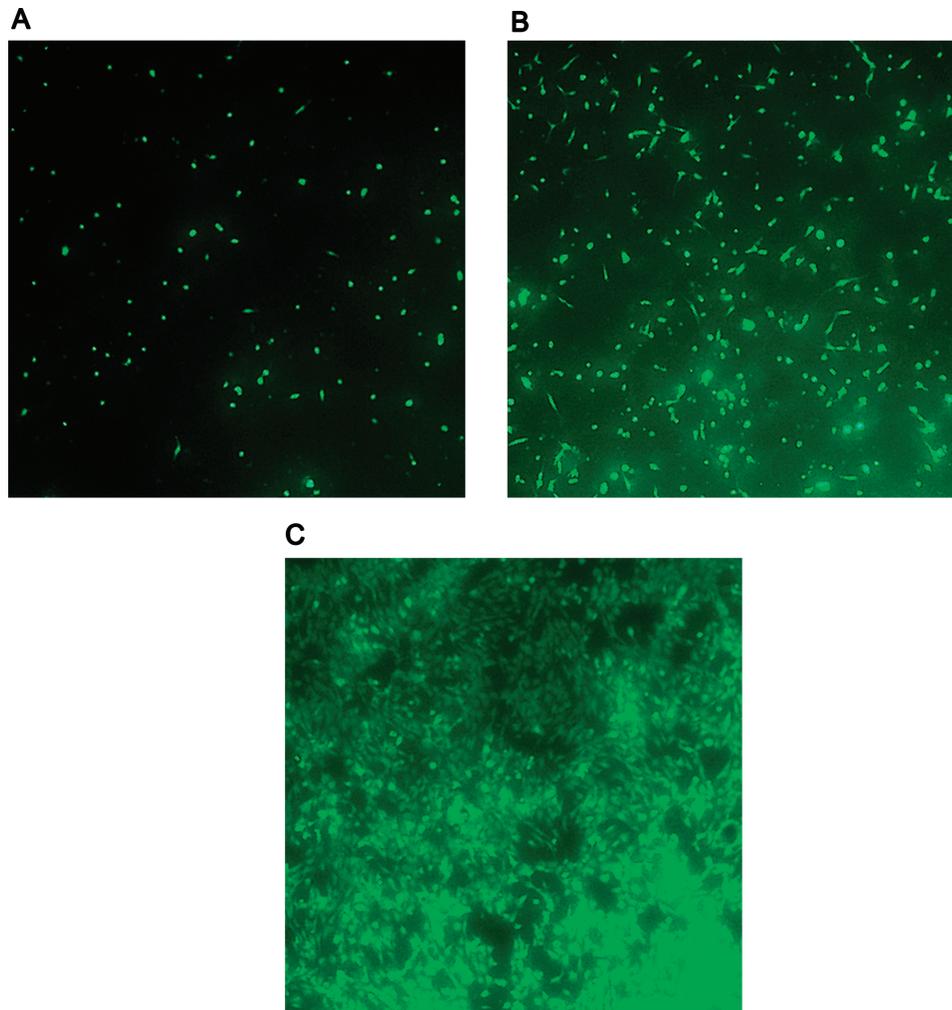
#### *Preincubated Calcibon*

The results of the XRD (Fig. 1A) demonstrated that preincubation of the CaP cement discs in MilliQ resulted in the hydrolysis of  $\alpha$ -TCP into an apatite-like structure with some  $\beta$ -TCP. These samples caused a decrease in pH of the culture medium, indicated by the change of color from red to yellowish. The duration (1, 4, 8, or 12 weeks) of preincubation resulted in no changes in physico-chemical properties. Cell attachment and viability results were comparable between the preincubation periods of 1, 4, 8, and 12 weeks (data not shown). SEM examination (Fig. 2B) revealed that cell layers were now only partially detaching from the disc surface and that more cells were attached to the surface than with the unmodified samples. This was already observed at day 1 but also throughout the culture period. However, live

and dead assays (Fig. 3B) displayed a reduced cell number after 8 days, although the cells were well spread on the surface of the discs. DNA levels (Fig. 4) remained the same as at the start of the experiment, but ALP activity (Fig. 5) was close to zero.

#### *Sintered Calcibon*

The results of the XRD analyses (Fig. 1B) demonstrated that the discs sintered at 600°C had the same chemical properties as the unmodified samples. However, sintering at 800°C, 1000°C, and 1100°C resulted in conversion of  $\alpha$ -TCP into  $\beta$ -TCP with some hydroxyapatite and in an increase of crystallinity (Fig. 1B). The 800°C samples (and to a lesser extent the 1000°C samples) caused a rapid increase in pH of the culture medium, indicated by the change of color from red to pink. The results of the live and dead and SEM analyses showed that osteoblast-like cells were not able to survive on the discs sintered at 800°C, whereas the cells on the discs sintered at 1000°C were viable and proliferating. Nonetheless, the discs sintered at 1100°C demonstrated better cell attachment and viability throughout the culturing period of 12 days than did those sintered at the other temperatures (600°C, 800°C, 1000°C) or preincubated or unmodified discs. This was confirmed using SEM examination (Fig. 2C), which showed well-spread cell layers, which were attached to the surface, and by the results of live and dead analyses (Fig. 3C) revealing the presence of live



**FIG. 3.** Live and dead assays of (A) unmodified, (B) discs preincubated for 1 week, and (C) discs sintered at 1100°C after 8 days of culture. Color images available online at [www.liebertpub.com/ten](http://www.liebertpub.com/ten).

cells over the complete surface area of the discs. DNA results (Fig. 4) showed that, from day 4, the amount of DNA was significantly greater than in the preincubated and unmodified samples; the difference was even greater after 8 and 12 days. In addition, ALP activity (Fig. 5) was observed and showed a bell-shaped curve for osteoblast differentiation, with peak activity at day 8. ALP activity at day 4 was close to the peak activity at day 8, indicating an early differentiation of the osteoblast-like cells. The increase in ALP activity started on the same day as cell seeding, also indicating earlier differentiation of osteoblast-like cells.

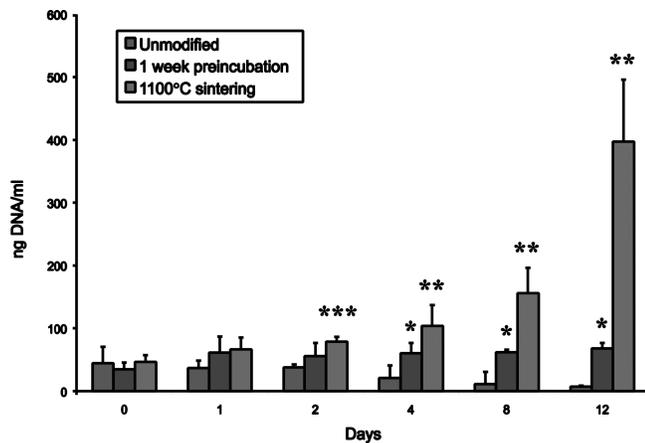
## DISCUSSION

The discrepancy in results of culturing cells on Calcibon CaP cement was the underlying justification for the current study, with specimens receiving different additional treatments. Our results indicated that the unmodified specimens were releasing calcium and phosphate ions in the medium,

creating an acidic environment for the osteoblast-like cells. As a consequence, the seeded cells were slowly dying and detached over time.

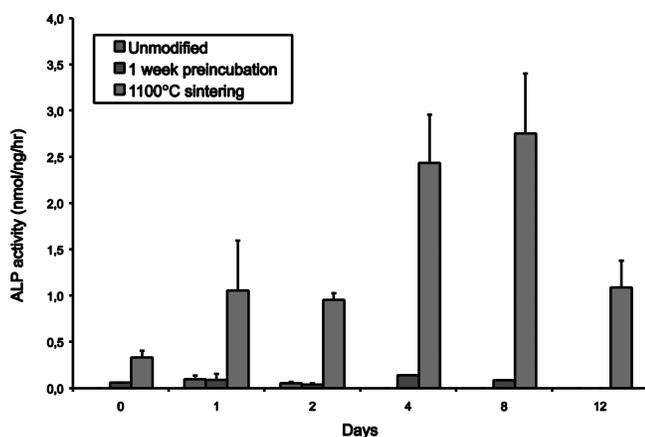
Preincubation of the CaP cement discs in MilliQ changed the chemical properties of the material. The originally present  $\alpha$ -TCP was hydrolyzed within 1 week of preincubation into an apatite-like structure with some  $\beta$ -TCP. These preincubated samples showed a little bit better cell viability than the unmodified CaP cement, but the material was still suppressing cell proliferation and differentiation of the osteoblast-like cells. The increase of acidity of the cell culture medium, in combination with the dissolution and re-precipitation of CaP phases of the discs, was hampering cell proliferation and differentiation of the osteoblast-like cells.<sup>32</sup>

On the other hand, XRD data showed that sintering the CaP cement discs between 800°C and 1100°C resulted in conversion of  $\alpha$ -TCP to  $\beta$ -TCP with some hydroxyapatite and in an increase in crystallinity. The high sintering temperature in which smaller crystallites grow to larger



**FIG. 4.** Deoxyribonucleic acid (DNA) (Pico green) assay of unmodified discs, discs preincubated for 1 week, and discs sintered at 1100°C until 12 days of culture. \*Significant differences ( $p < 0.05$ ) between preincubated and unmodified discs; \*\*a significant difference ( $p < 0.05$ ) between discs sintered at 1100°C and preincubated and unmodified samples; \*\*\*a significant difference ( $p < 0.05$ ) between discs sintered at 1100°C and unmodified samples.

crystallites explains this increase in crystallinity. The discs were sintered at a maximum temperature of 1100°C; otherwise the stable  $\beta$ -TCP would have been transformed into a more-reactive  $\alpha$ -TCP again.<sup>33</sup> The  $\beta$ -TCP phase of the sintered discs favors cell proliferation and differentiation *in vitro* significantly, creating a good scaffold material to support osteogenesis. Remarkably, the medium of the discs sintered at 800°C rapidly turned basic, in which osteoblast-like cells were unable to survive. The pH increase was also observed in the medium of the discs sintered at 1000°C but to a lesser extent. Eventually, the discs sintered at 1100°C were the most physicochemically stable, resulting in better cellular response of osteoblast-like cells than samples sintered at other temperatures and preincubated and unmodified



**FIG. 5.** Alkaline phosphatase (ALP) activity assay of unmodified discs, discs preincubated for 1 week, and discs sintered at 1100°C until 12 days of culture.

fied samples.<sup>34</sup> Although ALP activity on day 0 of the discs sintered at 1100°C was low, the discs apparently stimulated osteoblast differentiation already on the same day of cell seeding. Therefore, the elevated ALP activity in all measurements before day 8 can be explained. Furthermore, the immediate use of complete culture medium was necessary to direct the mesenchymal stem cells into the osteoblastic lineage. Previous research has indicated that the use of minimal medium during primary culture and complete medium during second culture would only delay differentiation of the osteoblast-like cells.

As mentioned before, different results have been reported regarding cell proliferation and cell differentiation of osteoblasts-like cells when cultured on Calcibon.<sup>28–30,35</sup> For example, Hempel *et al.* described that the toxic effects of unmodified CaP cement are due to physicochemically unstable discs and released phosphate and protons into the culture medium, resulting in a decrease in pH and an uptake of calcium by the specimens.<sup>30</sup> The decrease in pH, which was reported to affect cell attachment and behavior of the osteoblast-like cells *in vitro*, was also observed in our experiments with the unmodified and preincubated samples.<sup>36–38</sup> Its changed color, which went from red to yellowish, illustrated the alteration of the pH in the culture medium. This effect is the consequence of the presence of pH indicators in the  $\alpha$ -MEM solution. In contrast to Hempel *et al.*, Oreffo *et al.* and Knabe *et al.* reported lower cell proliferation and greater cell differentiation on Calcibon than with polystyrene cultures, although they were still able to culture the cells for an extended period.<sup>28,29</sup> Our results showed fewer cells on unmodified specimens over time. The alteration in pH and extracellular concentrations of calcium and phosphate were found to cause considerable stress for the osteoblast-like cells and ultimately resulted in severely limited cell growth and differentiation.<sup>30</sup> It was reported that this could be avoided by refreshing the medium daily, instead of every 2 to 3 days. In this way, cell viability can be maintained and cell proliferation and differentiation of the osteoblast-like cells improved.<sup>39</sup> In addition to the decrease in pH and the uptake of calcium by the specimens, the negative effects on osteoblast-like cells could be due to the particle size of the CaP cement. Pioletti *et al.* described that CaP cement particles smaller than 10  $\mu\text{m}$  have an effect on the viability and proliferation of osteoblast-like cells.<sup>40</sup>

Evidently, the observed acidity of Calcibon CaP cement in *in vitro* cell culture experiments does not hamper the final *in vivo* behavior of the material, because *in vivo* studies with Calcibon<sup>21,24,41</sup> describe excellent bone compatibility, as characterized by the fast deposition of new bone on the cement surface, which proves that the material is osteoconductive and suitable for clinical purposes. A reason for the excellent *in vivo* behavior might be vascularization, which provides a continuous flow around the inserted implant that can elute the toxicity of the CaP cement. This emphasizes that *in vitro* cell studies cannot be extrapolated implicitly to the *in vivo* situation, which Rosengren *et al.*, who found that

an initially cytotoxic scaffold *in vitro* could, over the long term, become biocompatible *in vivo*, described.<sup>42</sup>

## CONCLUSIONS

On basis of our results, we conclude that *in vivo* results with CaP-based cements do not guarantee their *in vitro* applicability. Furthermore, unmodified Calcibon is not cytocompatible *in vitro*; although preincubation of the material results in a more-favorable cell response, sintering of the material at 1100°C resulted in the best osteogenic properties. In contrast to *in vivo* studies, the Calcibon CaP cement is not suitable as a scaffold for cell-based tissue-engineering strategies.

## ACKNOWLEDGMENTS

We would like to thank the Dutch Technology Foundation (STW) applied science division of NWO and the technology program of the Ministry of Economic Affairs for their financial support in this project (NGT6205).

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