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Bone marrow induced osteogenesis in hydroxyapatite and calcium carbonate implants

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In this experimental study, blocks of natural coral (calcium carbonate) and its structurally similar derivate in the form of hydroxyapatite (calcium phosphate) were implanted in rat latissimus dorsi muscle with autogenous bone marrow to compare their bone-forming capability. A block without marrow placed in the opposite latissimus muscle served as a control. The animals were killed at 3, 6 and 12 weeks and, in the hydroxyapatite group, also at 24 weeks. The sections were analysed histologically and histomorphometrically. Bone was found only in implants containing bone marrow. Bone formation was significantly ($p < 0.05$) higher in coral than in hydroxyapatite implants at 3 weeks (10.8% versus 4.8%) and at 12 weeks (13.7% versus 6.3%, bone/total original block area). At 12 weeks all the coral implants had lost their original structure, and the cross-sectional area of the block had diminished to 40% of the original area. © 1996 Elsevier Science Limited.

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In structure, the Pacific reef-building coral genus Porites resembles osteon-evacuated cortical bone[1]. It consists almost completely (99%) of calcium carbonate (aragonite) and can be used as a bone substitute after the remaining 1% of the organic material has been eliminated.

Natural coral (CC) can be converted into structurally similar porous hydroxyapatite (HA). Through a hydrothermal exchange reaction, the $CaCO_3$ skeleton of CC is changed to HA, $Ca_{10}(PO_4)_6(OH)_2$, which is the main mineral of bone[2]. The mean diameter of the pores is ca. 200 μm (190–230 μm)[3]. A pore size of over 100 μm was previously found to allow fibrovascular and bone tissue ingrowth, making direct integration with bone possible[4–6].

The basic difference between HA and CC is that CC is biodegradable and HA is not at all or only very little. The rate and mechanism of biodegradation of CC is not completely understood but it is assumed that the main factor is carbonic anhydrase, an enzyme that occurs in abundance in osteoclasts[7]. Both CC and HA are known to be osteoconductive, biocompatible and very inert[8–10]. They have been used clinically in dental[11–13], maxillo-facial[14–16], cranio-facial[17–20], and orthopaedic surgery[21–25].

Adding bone marrow to porous calcium ceramics induces membraneous bone formation in extraosseal sites. This has been shown with tricalcium phosphatase blocks implanted subcutaneously in rats[26], with HA blocks implanted intramuscularly in rabbits[27] and rats[28,29] and also with CC implanted subcutaneously in rats[29].

In modern surgery, prefabricated bone substitutes are needed for the reconstruction of bone defects or as augmentation materials. An implant with existing bone tissue before reconstruction could be used to facilitate integration with the recipient site. This study was designed to compare bone formation inside structurally similar CC and HA blocks in muscle tissue when implanted with autogenous marrow cells. The intramuscular site was chosen to evaluate bone formation in richly vascularized tissue without the direct influence of bone.

MATERIALS AND METHODS

The two implants derive from the same genus of coral, Porites. The materials used were Biocoral® (calcium carbonate, INOTEB, France), and Interpore 200® (hydroxyapatite, INTERPORE INTERNATIONAL, Irvine, California) which are similar in internal structure but differ in chemical constitution. Sixty-seven blocks measuring 3 × 3 × 6 mm were implanted.

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Table 1 Number of implanted blocks

Weeks	HA	HA contr	CC	CC contr
3	6	5(6)	6	6
6	5(6)	5(6)	6	6
12	7		6	
24	6			
animals	25		18	

HA, hydroxyapatite; CC, coral. Values in parenthesis indicate original number of implanted blocks. Three blocks were discarded because of technical failures in processing. Figures in last row are number of animals used.

Forty-three male Wistar rats weighing 250–350 g were used for the experiment. The animals were maintained on a standard dry laboratory diet and given water *ad libitum*. They were anaesthetized with an intraperitoneal injection of pentobarbital (50 mg kg^{-1}, Mebumat®, Orion, Finland) and were given procaine penicillin (300,000 IU kg^{-1}, Procapen®, Orion, Finland) intramuscularly before the operation. The numbers of animals and implanted blocks at different follow-up periods are given in *Table 1*. Autogenous bone marrow was harvested from the left femur through the knee joint. A parapatellar incision was cut and the patella shifted laterally. A hole was drilled into the intercondylar space and the marrow was sucked out with a 20 cm^{-3} syringe and 18-G needle. The bone marrow was mixed with 1 cm^{-3} of saline and the implant was immersed in the solution for 5 min. The left latissimus dorsi muscle was obtained through a dorsolateral incision. It was freed from its distal insertions and the implant was rolled inside the muscle. The muscle was sutured to prevent the implant from dislocating. Right latissimus dorsi muscle with an implant of the same material without bone marrow served as a control.

The animals were killed with an overdose of pentobarbital at 3, 6 and 12 weeks; those in the HA group also at 24 weeks. The muscle around the implant was dissected and the specimen was dehydrated in increasing concentrations of ethyl alcohol–water solution and embedded in methylmethacrylate. Longitudinal 5 μm-thick sections were cut in the centre of the block with microtome (Polycut S, Reichert-Jung, Nussloch, Germany) and stained with the Masson–Goldner method for histological and histomorphometric analysis.

The study followed the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes[30] and was approved by the ethical committee for animal experiments of Helsinki University Central Hospital.

The specimens were studied by ordinary light microscopy. The number of osteoblasts, osteocytes and giant cells and the amount of fat and bone marrow were evaluated on each sample. Special interest was focussed on the amount of macrophages and lymphocytes/neutrophils as signs of inflammation. The number of each cell type was scored from 0 to 3. The scoring system was only used as an aid to evaluation and the numbers are not presented here. The quality of bone was also assessed.

In quantitative histomorphometric analysis, a Leitz Diaplan light microscope was linked via video camera to a semiautomatic computerized analysis system (MOP Videoplan, Kontron, Munich, Germany). The entire section area was measured using ×150 magnification.

The variables to be measured were total implant cross-sectional area, bone area, fibrotic tissue area and the void area in pores. Three samples were excluded because processing failed.

Bone formation was compared using the total original cross-sectional area of the implant (before implantation) as a reference, not the true, resorbed area alone.

Values are given as means (±SEM). The Kruskal–Wallis nonparametric test was used for statistical analysis and $p < 0.05$ was considered significant.

RESULTS

Histology

None of the control implants showed bone formation. A giant cell reaction and invasion of macrophages were observed in both materials. In some of the CC implants small foreign-body type granulomas could be detected. No acute inflammation was seen in any of the specimens. The CC implant was successively deformed, whereas the size and form of the HA implants were unchanged. Practically all the pores in both control materials were filled with fibrovascular tissue at 3 weeks.

Bone was formed in all implants with bone marrow. At 3 weeks, abundant osteoblastic activity was seen directly on the surface of the pores. Bone formation had proceeded so far that scattered osteocytes and woven bone could be detected (*Figure 1a* and *b*). In both types of implants, numerous giant cells and macrophages were seen, but only in direct contact with the foreign material, never adjacent to new bone formation. Only scattered lymphocytes occurred, and no acute inflammation could be detected. The pores were filled almost completely with fibrovascular and bone tissue. At 6 weeks, bone marrow was also present, and both materials showed less osteoblastic activity than at 3 weeks. In some of the mature CC specimens, lamellar bone could also be seen. In the CC specimens there were fewer giant cells and macrophages than at 3 weeks, but in the HA specimens the number was unchanged. Some fibrovascular stroma, but no fat, could be seen in the pores in the periphery of the blocks of both groups.

At 12 weeks, bone marrow was still present in both types of material. The amount of mature bone had increased and there was less osteoblastic activity. In the CC specimens only a few giant cells or macrophages could be seen. In contrast, giant cells and macrophages were evident in the HA specimens in all areas where bone did not line the implant material. The CC implants were deformed and nearly all the coral material not lined by bone had been phagocytized. In CC blocks the innermost part of the implant resembled an ossicle (*Figure 1c* and *d*).

In the HA implants bone marrow was still present at

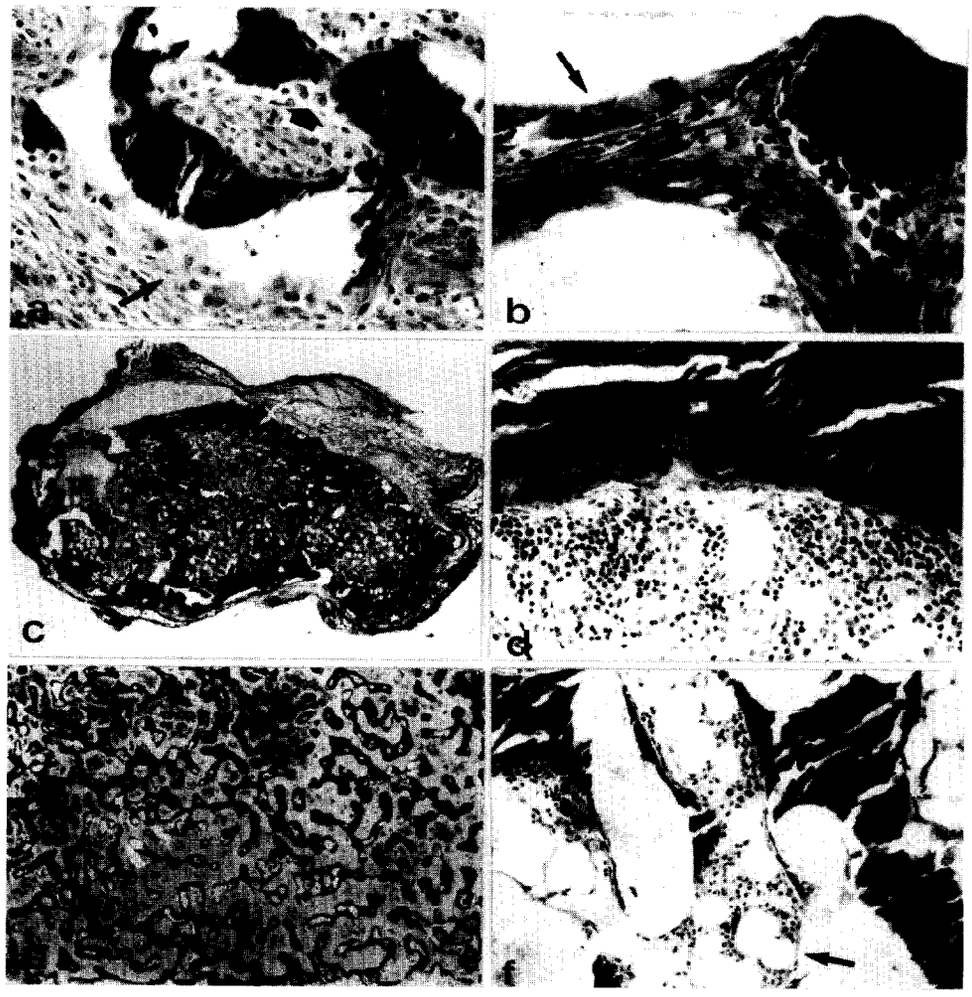

Figure 1 **a**, CC at 3 weeks: abundant osteoblastic activity (big arrow). Matrix (white) is partly phagocytized (arrow), ×200. **b**, HA at 3 weeks: giant cells and macrophages lying directly on matrix (arrow), ×260. **c**, CC at 12 weeks: implant has lost shape and form, bone marrow occupies central part and is surrounded by lamellar bond (dark) and matrix, ×80. **d**, Same specimen as in **c**: ostoeblastic activity and osteoid seam is still seen, ×400. **e**, HA at 24 weeks: form and shape of matrix (grey) is preserved, bone (dark) invades entire implant, ×80. **f**, Same specimen as in **e**: fat gradually replaces bone marrow (arrow), ×400.

24 weeks, although fat cells gradually invaded the pores and replaced the marrow. There was some osteoblastic activity, but most of the bone tissue was already quite mature, and contained abundant osteocytes. Some giant cells could still be detected. The size and form of the implant was unchanged (*Figure 1e* and *f*). No cartilage was detected in any implant. Bone formation did not show a centripetal pattern; instead bone was scattered all over the sample. New bone always appeared adjacent to the implant matrix.

Histomorphometry

At 3 weeks there was significantly more bone in CC than in HA blocks, 10.8 and 4.8%, respectively, of the original cross-sectional area (*Figure 2*). The CC implants were reduced to 87% of the original area (*Table 2*).

At 6 weeks the extent of bone formation had not changed significantly, the mean size of the CC blocks having decreased in the marrow group to 62% and in the control group to 61% of the original. The variation ranged from 88% to nil. In the control group one block was completely resorbed.

At 12 weeks the bone formation in the HA group was 6.3% and in the CC group 13.7%. The difference between the two materials was significant ($p = 0.032$). The increase was not statistically significant within either group when compared with 3 or 6 week samples.

The size of the CC blocks was reduced to 40% and all the blocks remained. At 24 weeks, 14.4% of bone was found in HA implants. Bone occupied 38% of the porous area (*Figure 3*).

DISCUSSION

In this study, more bone was produced in CC implants at 3 and 12 weeks than in HA, although at 12 weeks the CC blocks had been reduced to 40% of their original cross-sectional size. The absolute amount of bone did not increase significantly within either group after 3 weeks, but there was a trend of increased bone formation in HA implants at 24 weeks ($p = 0.051$), when the amount of bone was about the same as in CC

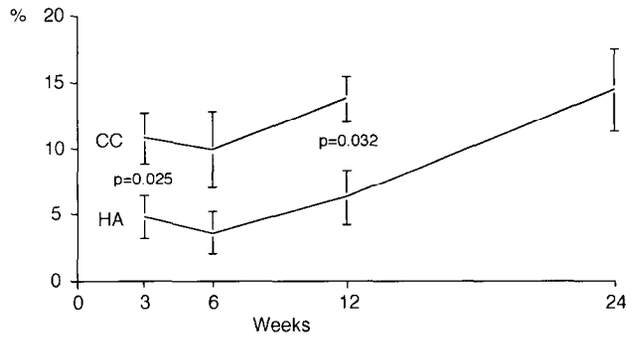

Figure 2 Bone ingrowth into CC and HA implants with bone marrow. Bone ingrowth in the implant is given as percentages of mean (±SEM) bone area related to original total cross-sectional area of implant. At 3 and 12 weeks the difference between materials is significant.

Table 2 Resorption of coral implants

Weeks	Mean (range)	
	CC (%)	control (%)
3	87 (92–84)	86 (93–77)
6	62 (84–19)	61 (88–0)
12	40 (60–20)	

Values are mean total cross-sectional areas in percentages related to original area before implantation. Maximum and minimum values are shown in parentheses.

implants at 12 weeks (14.4%/13.7%, *Figure 2*). As the structure of the two implants is similar, the difference in chemical constitution and in the resorption process of the CC matrix itself may affect bone formation.

Ohgushi *et al.* implanted CC and HA discs with bone marrow into subcutaneous pouches in rats. At 4 weeks, bone occupied 19.1% (HA) and 22.0% (CC) of the porous area[29]. Our results for intramuscular sites at 3 weeks are 13.4% (HA) and 20.1% (CC). Ohgushi *et al.* found bone only in blocks immersed with bone marrow, not in those without it. This is in accordance with our findings here and also with previous reports where porous ceramics have been used with bone marrow in extraosseal sites in rats[26,28]. Van Eeden and Ripamonti recently reported bone formation in porous HA implanted intramuscularly without any osteoinductive factor. Bone was found only in HA blocks but not in granular form. They used baboons in the experiment and postulated that the geometry of the implant is critical. Unfortunately, primates are seldom used in experimental work and further study is needed to explain the bone formation mechanism[31].

When HA implants without bone marrow have been placed in bone defects, bone ingrowth into HA has been variable. In orthognatic patients, Holmes *et al.* found 18% bone invasion (compared to total measured area) in a follow-up time of 4.7–16.4 (mean 9.3) months in HA implants[32]. Martin *et al.* placed HA blocks in cortical defects of dog humerus and radius. Bone ingrowth into pores increased from 52% at 16 weeks to 74% at 1 year (amount of bone relative to porous space). In the cancellous site, bone ingrowth was 38% at 4 weeks, then fell to 17% at 1 year[33]. It seems that the entire porous space of HA is seldom completely filled with bone[15].

Bone ingrowth into CC is more difficult to measure because of resorption. CC loses its internal porous structure very quickly, in our study after 6 weeks, and after that the bone does not actually invade the pores but replaces the matrix. When implants of CC have been used in posterior vertebral fusions in children or in animal experimental models in bone defects, new bone forms in previous implant sites[7,24]. Histology has been studied but quantitative data are insufficient.

An ideal bone substitute should resorb gradually after being invaded by bone and allow the new bone to remodel. HA allows bone ingrowth but it does not resorb significantly[3]. HA blocks and granules used in tibial plateau fractures and bone tumour surgery are well detectable in X-rays after many years[21,23]. The clinical use of HA is thus limited, because it lacks the ability to remodel the basic quality of living bone.

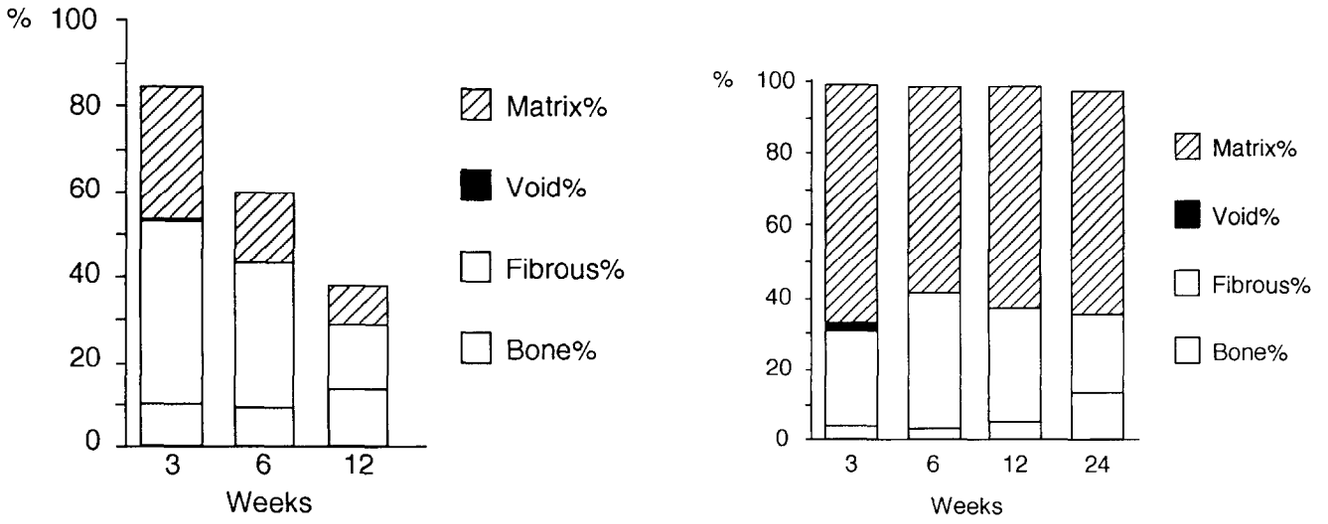

Figure 3 Tissue ingrowth into implants with bone marrow. Relative amounts of matrix and different tissue components and relative sizes of implants are presented for different follow-up periods. 100% is original total cross-sectional area of implant. Left side: coral implants; right side: hydroxyapatite implants.

In CC, the resorption rate may depend on the animal species used. When CC blocks were implanted in the cortex of the femur and tibia in pig and sheep, resorption at 1 month was 64 and 93%, respectively[34]; Naaman *et al.* observed complete resorption of CC granules at 24 weeks in connective tissue site in pigs. No inducing material was used and the blocks did not have contact with bone tissue[35]. In humans, degradation seems to be slower, 50% of the blocks used to fill the 10 mm cranial burr holes having resorbed completely at 1 year. With larger implants the resorption rate was much slower[20]. In vertebral fusions, small fragments of natural coral blocks were found after 1 year[24].

In our experiment the resorption varied widely, one block in the control group being already completely resorbed at 6 weeks, whereas the largest implant in the same group still retained 88% of its original size. The resorption pattern was noteworthy. At 12 weeks bone marrow occupied the central part of the implant and was surrounded by lamellar bone. The blocks were deformed and consisted of only 23% of matrix (originally 58%). Active bone formation continued and the implant resembled an ossicle (*Figure 1c*). It has previously been postulated that the main factor in the resorption process is carbonic anhydrase enzyme, abundant in osteoclasts[8]. The control implants did not show any bone formation but the average resorption rate was very similar in both coral groups. Giant cells were also found in the control specimens but whether these cells were osteoclasts or not could not be established.

When porous hydroxyapatite or natural coral are used as bone substitutes, quick and effective bone formation is advantageous, in particular for calcium carbonate. A bone substitute that resorbs before bone has filled the pores is useless. This favours the use of some inducing factor. At the moment autogenous bone marrow is the most readily available inducing material, and more use could be made of it in clinical applications to induce bone formation inside porous CC or HA implants.

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