In vivo bioresorbability and bone formation ability of sintered highly pure calcium carbonate granules

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Calcium carbonate-based bone substitutes derived from natural coral exoskeleton (aragonite) are resorbed and remodeled faster than calcium phosphate-based substitutes. However, coral species with structures appropriate for use as bone substitutes are very limited. Therefore, it is important to evaluate potential of artificial calcium carbonate ceramics as a bone substitute. In this study, calcium carbonate granules with various porosities and pore sizes were prepared by sintering a highly pure (>99.98%) calcium carbonate powder (calcite), and their resorption properties and bone formation abilities were examined *in vivo* for the first time. The sintered calcium carbonate was resorbed faster than β -tricalcium phosphate, which has a similar structure. However, sintered calcium carbonate did not promote new bone formation during long-term implantation. Furthermore, both resorption and new bone formation were affected by the pore structure. The optimal structures of the artificially sintered calcium carbonate bone substitute were also discussed.

Keywords: Calcium carbonate, Sintered ceramics, Bioresorbable ceramics, *In vivo* test

INTRODUCTION

Calcium carbonate, either calcite or aragonite, is known to demonstrate biocompatibility and bone-bonding ability¹. Natural corals have been a source of bulk and porous calcium carbonate for its use as a bone substitute. Natural coral exoskeleton consists mostly of aragonitic calcium carbonate (95–97%); its porosity, depending on the species, varies from 20–80%². After treatments, such as protein removal and sterilization, natural corals are clinically used as bone substitutes (*e.g.*, Biocoral[®])². Numerous articles have discussed the physical and biological properties of coral-derived bone substitutes^{2–4}. For example, coral-derived aragonitic bone substitutes can promote bone regeneration in alveolar or periodontal bones^{5–8}, as well as long tubular bones⁹, resulting in good clinical outcomes. Culturing bone marrow stem cells in the presence of coralline aragonite can lead to faster bone regeneration^{10–13}. Several reports have suggested that Biocoral[®] promotes bone formation to an extent similar to the autologous bone¹⁴, while maintaining a low infection rate². Other researchers have reported that aragonitic bone substitutes are resorbed too fast depending on the implantation site or bone defect size¹⁵. Calcium carbonate phosphorylation, which is the hydrothermal treatment of calcium carbonate in a phosphate-containing solution to convert calcium carbonate to carbonated hydroxyapatite, has been shown to decrease the resorption rate^{3,4}. Furthermore, the resorption rate decreases with increasing phosphorylation, and

complete phosphorylation results in non-resorbable hydroxyapatite^{2,16}. A comparison of the bone-enhancing effects of phosphorylated and non-phosphorylated coralline aragonite shows no significant differences between the two¹⁷, suggesting that phosphorylation does not always lead to good clinical outcomes and optimal resorption, and may vary depending on the implantation site and bone defect size. In particular, reasonably rapid resorption and remodeling of bone substitutes are suitable for regenerating exoskeletal bones. For example, in guided alveolar bone regeneration slow bone substitute resorption could hinder dental implant placement, since the residual bone substitutes would not be able to osseointegrate with the titanium-based dental implants.

Due to the challenges associated with obtaining natural corals for bone substitution, there are significant incentives to develop a suitable sintered, bulk calcium carbonate product. However, the sinterability of commercial calcium carbonate powders is difficult due to its thermal decomposition at around 870–880 K under ambient atmospheric conditions. Coral species with a structure appropriate for use as bone substitutes are very limited¹⁸ and can only be harvested from particular areas of the ocean. Coral harvesting is costly and may damage the surrounding marine environment¹⁹. Several studies have focused on fabricating calcium carbonate blocks and bulk composites for use as biomaterials, such as calcite block from calcium hydroxide compact²⁰ and gypsum block²¹, interconnected porous calcite bridged by dicalcium phosphate dehydrate^{22,23}, calcium carbonate with phosphate glasses^{24,25}, calcium carbonate

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with gypsum²⁶⁾, with poly(lactic-glycolic acid)²⁷⁾, with poly(lactic acid)²⁸⁾, and calcium carbonate coating on titanium^{29,30)}, due to their high bioresorbability. Attempts have also been made to use porous or hollow calcium carbonate in biomedical applications^{23,31-34)}.

Very few studies have focused on sintered calcium carbonate (calcite). Calcium carbonate ceramics with up to 95 % density have been prepared in a CO₂ atmosphere; these ceramics were subsequently examined *in vitro* using simulated body fluid (SBF)³⁵⁾ or cultured cells, such as osteoblasts³⁶⁾ or epidermal cells³⁷⁾. However, to the best of our knowledge, there were no studies that focused on the *in vivo* properties of fabricated sintered calcium carbonate ceramics. We have found that highly pure and monodisperse calcium carbonate powder can be sintered without any sintering aid under flowing CO₂ at atmospheric pressure to fabricate porous calcium carbonate ceramics with 10–75% porosities. This work is the first attempt to investigate the *in vivo* properties of commercially fabricated calcium carbonate ceramics. Here we placed porous calcium carbonate granules into bone defects located in the calvaria of Sprague-Dawley (SD) rats, and then investigated the effects of pore size and porosity on biomaterial resorption and bone formation. Furthermore, based on the results of our *in vivo* experiments, optimal microstructural properties of calcium carbonate bone substitutes were also discussed.

MATERIALS AND METHODS

Preparation of test substances

Calcium carbonate powder was supplied by Shiraishi Central Laboratories (Hyogo, Japan). The chemical purity was higher than 99.99%, and the mean particle diameter was 150 nm. Test substance A, targeted to have 75% porosity and 50–300 µm diameter pores, was prepared by dispersing calcium carbonate (40% solid content) in water with a poly(acrylate) dispersant (Poiz 520, Kao, Tokyo, Japan) and methyl cellulose binder (Kanto Chemical, Tokyo, Japan), each relative to calcium carbonate powder. A forming agent (0.05% w/w with respect to the slurry, trimethyl ammonium lauryl sulfate, Emal TD, Kao) was then added, and whisked with using a hand mixer. The foam was positioned on filter paper and then placed into an oven at 363 K for consolidation, methyl cellulose gelation, and subsequent drying. The porous green body was debindered at 773 K for 16 h in air and sintered at 1,073 K for 1 h under flowing CO₂ at atmospheric pressure. The porosity was calculated to be 75% using the ideal density of calcite (2.711 g/cm³) and the apparent density. For the measurement of apparent density, the test piece was cut into 1×1×1.5 cm in size, and its weight was divided by apparent volume. Test substances B and C, targeted to have pores <10 µm in diameter and 50% and 10% porosity, respectively, were prepared by dispersing calcium carbonate in water, followed by centrifugation to form a compacted cake. The dried cake was calcined at 773 K in air for 16 h and identified as test substance B. Test substance C was prepared by additional heating

at 973 K for 1 h in flowing CO₂. The test substances were pulverized and sized between 1.0 to 2.0 mm before use. The porosities were determined from the mass difference before and after water infusion into cubes cut into 1×1×2 cm in size. Clinically used porous β-tricalcium phosphate (β-TCP) granules (Superpore®; porosity: 75%, pore diameter 50–300 µm, particle size: 1.0 to 2.0 mm; HOYA Technosurgical, Tokyo, Japan) were used as the control. Test substance A was prepared with the porosity, pore size, and grain size similar to the control.

Characterization of test substances

All test substances were examined with a scanning electron microscope (SEM; JSM-6330F, JEOL, Tokyo, Japan) to roughly estimate the diameters of pores and interconnections. The diameter ranges mean the largest and the smallest values among at least 10 observation points. The crystal phases of the specimens were determined using an X-ray diffractometer (XRD; Multiflex, Rigaku, Tokyo, Japan) operated at 40 kV and 20 mA. The diffraction angle was continuously scanned, from 2θ=10° to 60° at a scanning rate of 2°/min.

In vivo experiments

Male SD rats (12 weeks of age) were used for the study. The *in vivo* experiments were conducted with the approval of the Ethical Committee of *in vivo* experiments in Hamri (No. 18-121 and 19-044) in a perfectly authenticated facility accredited by AAALAC International, an international third-party accreditation organization that promotes humane treatment of animals in science.

The animals were anesthetized with isoflurane carried by a flow of pure oxygen. The rat scalps were shaved and disinfected with povidone iodine (Isodine®; Mundipharma, Tokyo, Japan) and ethanol. Following a skin incision along the midline of the calvarium, or along the medial line, the subcutaneous tissue and periosteum were avulsed. A bone defect 5.2 mm in diameter was made in the calvarium of each rat using a dental drill. The test substances were placed inside the bone defects of the rats in the experimental and control groups. After the surgery, the skin was disinfected with povidone iodine.

After 3 and 6 weeks of implantation, the rats were euthanized (*n*=6 or 7 for each group); the calvaria containing implanted material were removed, fixed in 10% normal buffered formalin, and dehydrated. After washing with water, samples were dehydrated with alcohol and embedded in paraffin. Sections 3.5 µm in average thickness were stained with hematoxylin-eosin (HE). The sections were observed with an optical microscope (CKX 41, Olympus, Tokyo, Japan). The percentage areas of new bone, fibrous tissue, and unresorbed materials were calculated and compared between the experimental and the control groups. Statistical significance was examined by the non-parametric Wilcoxon test.

RESULTS

Characterization of test substances

Figure 1 shows the SEM images of the test substances used. Test substance A and control had similar

Fig. 1 SEM images of test substances (a) A, (b) B, and (c) C, and (d) control.

Fig. 2 Powder XRD patterns of test substances (a) A, (b) B, and (c) C, and (d) control.

microstructures. Test substances B and C did not have pores larger than the orders of the grain size. The pore size of the test substances was determined from the SEM images. Figure 2 summarizes the powder XRD patterns of the test substances. Test substances A–C and the control showed peaks for calcite and β -TCP, respectively. The properties of the test substances are summarized in Table 1.

In vivo experiments using test substance A and control

Figure 3 shows the histological images of the calvaria implanted either with the test substance A (group A) or control at postoperative week 3. Only a small amount of fibrous tissue was observed in the cavity and vicinity of the granules (b and d) in the control, and the angulated morphology of the granules did not appear to change. In contrast, extensive formation of new bone and blood vessels was observed near the granules (c) in group A. The test substance A appeared to become rounded, suggesting a certain extent of resorption.

Figure 4 depicts the area percentages observed in group A and control samples at postoperative week 3. There was a significant difference was recognized in the amount of newly formed bone and unresorbed material

Fig. 3 Histological pictures of (a, b) test substance A-implanted thin sections (group A) and (c, d) control-implanted thin sections postoperative week 3.

NB, BV, UM, and FT represent new bone, blood vessel, unresorbed material, and fibrous tissue, respectively.

Table 1 Properties of test substances

	Test substance A	Test substance B	Test substance C	Control
Base material	CaCO ₃	CaCO ₃	CaCO ₃	β -TCP
Porosity [%]	75	50	10	75
Pore diameter [μ m]	100–300	<0.5	<0.2	100–300
Interconnection diameter [μ m]	<50	None	None	50–100

Fig. 4 Area percentages of new bone, unresorbed material, and fibrous tissue in test substance A-implanted thin sections (group A) and control-implanted thin sections postoperative week 3.
* $p < 0.05$

Fig. 6 Area percentages of new bone, unresorbed material, and fibrous tissue in (group A) and control-implanted thin sections postoperative week 6.
* $p < 0.05$

Fig. 5 Histological pictures of (a, b) test substance A-implanted thin sections (group A) and (c, d) control-implanted thin sections postoperative week 6.

between these two groups; however, the differences in the amount of fibrous tissue were not significant.

Figure 5 shows the histological images of the calvaria implanted either with the test substance A (group A) or control at postoperative week 6. In group A samples, neither the newly formed bone nor the remaining implanted substance was observed, suggesting an abrupt resorption of the implanted substance and new bone. In contrast, in the control-implanted steady bone formation had proceeded and implanted grain resorption was not remarkable.

Figure 6 depicts the area percentages observed in group A and control-implanted samples at postoperative week 6. There was a significant difference in the amount of newly formed bone and fibrous tissues between these two groups. Other area percentages and unresorbed materials did not show significant differences.

Fig. 7 Histological images of (a, c) test substance B-implanted thin sections (group B) and (b, d) test substance C-implanted thin sections (group C) postoperative weeks (a, b)3 and (c, d) 6, respectively.

In vivo experiments using test substances B and C

Figure 7 shows the histological images of the calvaria implanted with the test substance B and C (group B and C, respectively) postoperative weeks 3 and 6. Both groups showed similar changes with time, regardless of their porosity: the implanted granules became rounded on the corners, suggesting a certain extent of resorption; however, higher number of granules remained compared to group A (Fig. 3). In addition, the extent of new bone formation was smaller in groups B and C than in group A; however, unlike in the group A samples, implanted materials, as well as the newly formed bone, remained in group B and C samples even after 6 weeks (c, d).

DISCUSSION

The relationship between the pore structure and *in*

in vivo calcium carbonate behavior was investigated. Test substance A, with a pore structure similar to that of the control was resorbed more rapidly and promoted faster new bone formation and blood vessel formation than the control. These findings of faster resorption of calcium carbonate compared to β -TCP seems to be well established and generally accepted^{3,4,15,16}. Due to this rapid resorption, the calcium ion concentration around the implanted calcium carbonate is elevated. Previous studies demonstrated that calcium ions released from implanted materials promote osteoblast differentiation^{38,39} and endothelial cell migration^{40,41}. Rapid bone and blood vessel formation could be rationally explained to be a result of quick calcium carbonate resorption. These resorption rate, however, could vary depending on the pore size and porosity of the material^{2,9}. Resorption proceeds quickly when the pores are large and the porosity is high. In test substance A, the 75% porosity was high and a pore diameter of 300 μ m was large for sustainability. This combination of porosity and size persistently contributed to bone formation since the test substance had been resorbed along with the newly formed bones within 6 weeks after the surgery.

Given the faster resorption of calcium carbonate compared to that of β -TCP, either pore size or porosity, or both factors, should be lowered to guarantee sufficient longevity of the material. Following the results of test substance A, test substances B and C were prepared with pores of approximately <10 μ m. As shown in Fig. 7, the resorption was largely suppressed, resulting in the persistence of the implanted substance and the newly formed bone. In this case, the porosity of small pores appeared not to affect the resorption. The absence of large pores, larger than 50 μ m in diameter, did not allow the ingrowth of newly formed bone or blood vessels, which may be responsible for the slow bone formation rate.

Based on these results, we propose a guideline for an optimal microstructure of calcium carbonate granules for bone substitutes: it should have large pores (>100 μ m) and; thick walls, while maintaining a moderate porosity of 30–60%. This combination of porosity and microstructure should more closely resemble the structure of natural corals. Using excellent ceramic processing techniques, the goal of creating calcium carbonate bone substitutes with having tailored resorbability may be ultimately achieved.

CONCLUSIONS

Highly pure calcium carbonate granules with different pore sizes and porosity levels were implanted into the bone defects made in the cranial bones of SD rats. The implanted material resorption and bone/fibrous tissue formation were examined. Calcium carbonate was resorbed significantly faster than β -TCP, which has a similar pore structure, and promoting faster new bone formation. Although the resorption was found to be suppressed by eliminating large pores (>50 μ m diameter), these pores were found to be essential for new

bone ingrowth. An optimal pore structure of calcium carbonate granules for bone substitutes has been proposed, and will be studied in the future.

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CONFLICT OF INTEREST

The authors have no conflicts of interest directly relevant to the content of this article.

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