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Comparative Studies on Tissue Reaction of Newly Sintered and Conventionally Sintered Hydroxyapatite

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Abstract

Comparative Studies on tissue reaction of newly sintered and conventionally sintered hydroxyapatite are carried out. Conventionally, hydroxyapatite sintering has been aimed at stiff, stable apatite due to chemical bonding with osseous tissue. However, direct bonding of sintered hydroxyapatite with bone may easily be destroyed under severe repeated loading just like osseointegration of metal because of differences in elastic moduli from each other. Therefore, development of stiff, stable hydroxyapatite makes no sense just as in the case of bioinert metal for biomechanical substitution. The merit in medical application of synthesized hydroxyapatite is to provide materials easily utilized and remodeled for surrounding living osseous tissue. Collagen and calcified substance are essential for bone remodeling. It is well known that the apatite phase found in living bodies exists as very small crystallites, which are bonded with organic, high-molecular polymers such as collagen. The biological apatite phase also contains bonded water, carbonate ions, and many other inorganic minor components. If it is possible to prepare an implant made of hydroxyapatite-collagen composite similar to biological apatite, the biological effects of the implant may be different from those of conventionally sintered hydroxyapatite [1-5]. Research on tissue reaction of conventionally sintered HA were carried out through co-operation between Research Institute for Ceramics Technology of the Italian National Research (IRTEC-CNR) and Experimental Surgery Division of "Rizzoli" Institutes of Bologna.

Introduction

From standpoint of the bone evolution as biological bioceramics stoichiometric as well as nonstoichiometric hydroxyapatite (HA) were sintered by high-pressure gas technique. After that they were implanted into dorsal muscles of dogs. Comparative studies on tissue reactions between biologically and conventionally sintered ceramics implanted were carried out. The definition of the vertebrates is a chordate having bony backbone i.e., vertebrae with different degree of ossification. Therefore, skeletal substances of connective tissue composed with the collagen, the cartilage and the bone are definitive substances of the vertebrates. The bone is mineralized fibrous collagenous connective tissues. At the initial stage of archetype vertebrates original skeletal substances were exoskeleton placoids which had been unified as calcified organs of the bone and tooth in

chondrichthyes. Moreover, in stage of prochordata organisms have placoids on the surface of derma, which is detected cartilage with sulfer of chondroitin by microanalyzer. The bone is the most important substance of the vertebrates. In evolution the origin of the bone is biological ceramics sintered in low temperature by enzyme with water, which are mineralized in nonstoichiometric condition. Sintering of calcium-deficient hydroxyapatite has already been reported, which bonded water at the calcium-deficient site, and was sintered up to fully dense bodies at 300°C under a pressure of 600 MPa[1]. In the presence of collagen in an aqueous phase, we tried to synthesize the hydroxyapatite by means of reaction between an aqueous solution of phosphoric acid and a calcium-hydroxide suspension. A diluted collagen solution was mixed with an aqueous solution of phosphoric acid and was poured slowly into a calcium-hydroxide suspended aqueous phase. No collagen was found in the mother liquid thus formed. All collagen in the solution was found to be collecting in the precipitate. Five hundred grams of commercially available collagen solution was diluted up to 8 liters and mixed with 0.6 mole of phosphoric acid. The CaO was crushed into fine powder and mixed with water. The Ca (OH) aqueous suspension thus formed was mixed, and the collagen-phosphoric acid mixed solution was added slowly. The precipitate was filtered and partly dried until suitable water content formed. Then, it was mounted in a metal capsule. The capsule was evacuated and sealed by welding. It was thin kept for 8 hrs at 200 MPa at 40°C. The resulting apatite-collagen composite was 1.75g/ml in density, 2 GPa in Young's modulus, and 6.5MPa in compression strength. The specimen could be cut by a knife, and was stable against immersion in water. The physical property of this type of complex may change according to composition and treating conditions. The sample was implanted in dogs and histologically evaluated.

Materials and Methods

From the standpoint of evolution the authors developed compact stoichiometric and nonstoichiometric HA as well as collagen-composed HA of compact type by high pressure sintering technique with water. Instead of the enzyme action of natural bone synthesis high pressure sintering technique with water were applied and sintered in 40-300 degree C. These biological bioceramics were sintered in National Institute for Research in Inorganic Materials and implanted into subcutis and muscles of dogs and monkeys as well as experimental evolutionary researches implanting into dorsal muscle of sharks were carried out in faculty of medicine, University of Tokyo.

(1) Comparative histopathological studies on conventional stoichiometric hydroxyapatite and new type hydroxyapatite sintered by high pressure gas technique

1) For a preliminary experiment, the following studies were carried out. Conventionally sintered stoichiometric porous hydroxyapatite plates and dense new type plates of stoichiometric and nonstoichiometric hydroxyapatite were implanted in the bone and muscle of a dog to compare histological tissue reactions for 8 weeks. For conventional stoichiometric hydroxyapatite, porous hydroxyapatite plate (40% porosity) made by ASAHI Optical Co. Ltd. was used. Dense new type of stoichiometric and nonstoichiometric hydroxyapatite were sintered in the National Institute for Research in Inorganic Materials. Undecalcified polished specimens for SEM and EPMA were made. They were then observed and compared.

2) Research on tissue reaction of conventionally sintered HA were carried out through co-operation between Research Institute for Ceramics Technology of the Italian National Research (IRTEC-CNR) and Experimental Surgery Division of "Rizzoli" Institutes of Bologna.

(2) Experiments on pressure sintering of apatite-collagen composite)

Five hundred grams of commercially available collagen solution (concentration 2 wt%, isoelectric point 9, pH3) were diluted up to 8 liters and mixed with 0.6 mole of phosphoric acid. The $CaCO_3$, 1 mole was kept at 900°C in air for 10 h. The CaO thus formed was crushed in a mortar into fine powder and mixed with 3 liters of water. The Ca(OH)2 aqueous suspension thus formed was mixed vigorously, and collagen-phosphoric acid mixed solution was slowly added at room temperature to the aqueous suspension. In this case, the mixing ratio of collagen to hydroxyapatite was 1 to 10. The precipitate thus formed was filtered and partly freeze-dried until the water content of the precipitate became suitable for sintering. Then, the precipitate was mounted in a metal capsule. The capsule was evacuated and sealed by welding, after which it was kept for 8 h at 200 MPa, 40°C.

The specimen thus formed was examined by both 5 MHz sound velocity measurement and compression strength measurement using INSTRON model 1123. The cross head speed was 0.5mm/min. during the measurement.

Sintered apatite-collagen composites were implanted in dorsal muscles of a dog; 8 weeks after implantation, specimens were recovered, prepared, and observed.

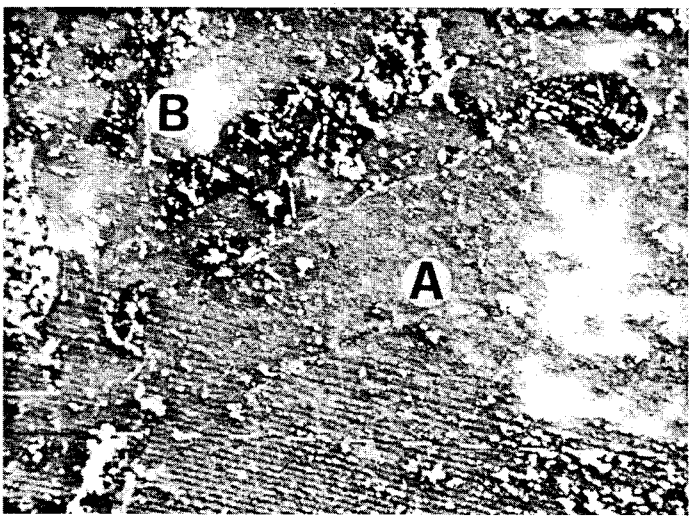
Results

Studies by SEM observation on comparison of conventional hydroxyapatite and new type hydroxyapatite sintered with high-pressure gas technique revealed the following:

Conventionally sintered porous hydroxyapatite was observed as amorphous in the cutting surface. Therefore, the grain could not be detected. Porous hydroxyapatite plate and newly formed bone fused well. However, conventionally sintered apatite plate did not fuse with fibrous tissue. In the interface between the porous apatite plate and fibrous tissue, Ca. 2μm space was observed, wherein some small spots of connection of tissue and apatite could be observed. On the contrary, dense apatite plates sintered with high-pressure gas technique fused with soft tissue firmly. Both new types of apatite (stoichiometric and nonstoichiometric) were observed constructed with ultrasmall grains by SEM. Nonstoichiometric apatite of high pressure gas technique was observed having very weak fusion with soft tissue. Almost all parts of the fusion were disrupted by artifact of specimen treatment.

The results of pressure sintering of the apatite-collagen composite were as follows:

After pressure treatment, the metal capsule made from lead was removed. In every run, the specimen was slightly yellow and solid. When the water content of a specimen during pressure treatment was about 10 wt%, for example, the resultant solid specimen was stable in air, but unstable in water. When the specimen was immersed in liquid water, it broke vigorously into small pieces. When the water content was near 50 vol%, the pressure-treated specimen was stable in liquid water. The solid feature of the specimen was unchanged during a period of one month of immersion in water at room temperature. The results were compared with apatite powder compact treated under the same conditions as those of the apatite-collagen composite, i.e., 200 MPa, 40°C, and 8 h of run duration, and the presence of liquid water. The apparent density of the apatite powder compact thus obtained was 2.1g/ml, and was hard and brittle. In this case, without collagen, the load-displacement curve was almost a straight line until it broke, while in the case of the collagen-apatite composite, the load-displacement line showed small and varying gradients, indicating large deformation of the specimen. These results coincided with the sound velocity measurement, from which 2 GPa of Young' modulus and 6.5 MPa in compression strength were calculated. The collagen-apatite composite could be cut with a razor blade. The average size of the apatite crystal was 10 nm in diameter and 40 nm in length.



The figure shows a slide-sample of an implant of a HA ceramic cylinder (made in a femur of rabbit) extracted after 6 months (after sacrifice of the animal). The implantation was made into mid-diaphiseal defect of the femur of 6 rabbits and the analyses gave substantially the same results. The HA cylinders were fired at 1240°C for 3 hours. The animals were allowed to move freely in its cage immediately after surgery. The implant (together with the surrounding bone and tissue) was removed after 6 months after implantation and fixed in a 10% formalin solution (adjusted to neutral pH by buffer mixture of sodium phosphate salts) and then embedded into methylmetacrylate. The specimen for instrumental examinations were sectioned parallely to the cylinder axis by a diamond circular saw (Leitz) to obtain sections with a thickness of about 50-100 μm. The sections were examined through a scanning electronic microscope (Cambridge). It is also visible the border of about 1-2 μm thick constituted with the *lamina limitans* material.

Discussion

New type hydroxyapatite apatite sintered with high-pressure gas technique proved to have excellent histocompatibility with fibrous and osseous tissue. Fusion of apatite to soft tissue is the most important property of any biomaterials. It was found that the stoichiometric hydroxyapatite became nonstoichiometric *in vivo*. On implantation of hydroxyapatite, it has been shown that the bond formation between the implanted hydroxyapatite and tissue of living bodies needs some induction period, about 4-5 weeks. In hard tissue of living bodies, it has been shown that the hydroxyapatite phase is complex in chemical composition. It is, therefore, doubtful that this induction period is long enough to change the implanted hydroxyapatite. It has been shown that stoichiometric hydroxyapatite is one of the thermodynamically stable phases of the system CaO-P2O5-H2O. But for nonstoichiometric hydroxyapatite, no stable field has been given. This means that the solubility of stoichiometric hydroxyapatite in aqueous solution is lower than other metastable phases such as nonstoichiometric hydroxyapatite. Due to this character, sintered stoichiometric hydroxyapatite can keep its shape long-term in living bodies, being an excellent material for implantation. From this experiment, it was found that stoichiometric hydroxyapatite sintered bodies change their composition to nonstoichiometric in living bodies. It seems important that the shape of implanted sintered bodies not be changed in spite of their compositions being changed. Sintering temperature suitable for hydroxyapatite is known to be 1200_100°C. Under a pressure of 200 MPa, a fully dense sintered specimen can be obtained at 800°C. Presence of water in the system lowers the sintering temperature of hydroxyapatite. In a stream of steam at 300°C and atmospheric pressure, grain

growth of hydroxyapatite can be found, and also a powder compact of hydroxyapatite shows small shrinkage in volume due to its sintering.

Previously, we reported that calcium-deficient hydroxyapatite, which has bonded water at the calcium-deficient site, sinters up to full density at 300°C under a pressure of 600 MPa. At that time, 300°C was the lowest limit to obtain a stable sintered specimen. At 200°C or lower, full density could not be obtained and strain remained in the pressed powder by treating it at 600 MPa. It was found that such an obtained specimen, placed in air, gradually broke into small pieces. When specimens with remaining strain were dipped into water at room temperature, they broke vigorously into small pieces within several seconds. The water content in these apatite specimens was determined. It was found that these specimens were intensely dried. The bonded water molecules in the calcium-deficient site had been partly lost. In the present experiment, therefore, wet apatite powder was used for the pressure treatment. The pressure-treated apatite specimen was thus obtained by pressure treatment at room temperature. It seemed obvious that in liquid water, the strain in the apatite phase due to the pressure treatment could be released at room temperature. This phenomenon led us to the further possibility of pressure-sintering of hydroxyapatite mixed with organic compounds at room temperature.

These results also suggest that the higher pressures make it possible to obtain a collagen-apatite composite of higher density, as well as higher strength at room temperature.

Resort to the use of ceramic hydroxyapatite (HA) as biomaterials is justified for application in bone, particularly in the maxillofacial and orthopaedic sectors, and also in dental and otorinolaringoiatric ones. Chemically speaking, the substance with this name corresponds to the stoichiometric formula $Ca_5(PO_4)_3OH$ (according to IUPAC standard rules) [in the following R-HA] taken as reference in biomedical field when speaking of substituted HA. In fact it belongs to the wider family of apatites where every ionic constituent can be totally or partially substituted by others with formation of apatitic solid solutions [in the following SS-HA, more or less crystalline]. Due to their very high insolubility in water (for values of pH $\geq$ 7) and its instability in acid environment, the apatites exist in practice only in the crystalline state. As concerns substitution that can occur in the particular case of SS-HA for biological use, PO_4^{3-} group can be substituted by *e.g.* CO_3^{2-} and HPO_4^{2-}; OH^- one by *e.g.* F^- (which confers higher stability to the crystalline lattice), but even by CO_3^{2-} (which on the contrary weakens it); Ca^{2+} by *e.g.* Na^+, K^+, Sr^{2+}, *etc* [6].

Fibroblasts grow very well on the surface of non-resorbable calcium-phosphates bioceramics, and particularly on SS-HA ones, on which they adhere with flat shape and with the tendency to enter the porosity if present [7,8]. This is particularly important in ossicular implants of HA bioceramic prosthesis (TORP or PORP). The implanted prosthesis tends to become covered with an epithelial tissue which is like to the natural one. Unfortunately, the amount of fibrous tissue formed all around the surface of the ossiculoplasty device is excessive and makes heavy it. This behavior was attributed to the presence of some small amorphous phase [9], however present in a sintered body.

Osteoblasts grow well on the surface of SS-HA bioceramics, however with a kinetic rate of growth slower than that of fibroblasts. Consequently, in some cases (as in the regeneration of the dental alveolar crest) to avoid a mixed growth of the two cellular species together, (apart from the use of specific growth and anti-growth factors) the zone of the soft tissue is separated with proper nanoporous membranes from that of the bone, giving rise to the so called *bone guided regeneration.*

On an implant of HA bioceramic, a deposition of a big amount of mineralised neoformed bone occur particularly in the wide and irregular cavities; many neoformed bone trabeculae adhere directly on the external surface. Only in some zones, a slight (apparently connective) inter-gap layer interposes between the surface of HA bioceramic and neoformed bone. A close contact between

neoformed bone and surface of the ceramic is however produced [10]. Many wide vascular cavities may be observed in the neoformed bone closely near the surface of HA bioceramics. Such cavities decrease their diameter in time until 12 months [11] on the basis of a decreased request of metabolic exchanges (due to lowering of cellular activities). At the end the HA ceramic surface may exhibit many indentations completely filled with new bone depositions (experiences on sheep femurs). Typical well formed osteocitary cavities appear all around HA bioceramics implants and even closely to the ceramic surface [12].
The goal was to accelerate the process of bone formation and to anchor the device to the bone as fastest as possible, also trying to obtain the greatest percentage of direct contact points too.

Conclusion

It is obvious that conventionally sintered HA above 1000 degree C is almost bioinert, so no tissue reaction can be observed in subcutis. As conclusion tissue reaction of biologically sintered HA is excellent to induce leukocytes hemopoiesis.

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