

Incorporation and biodegradation of hydroxyapatite–tricalcium phosphate implanted in large metaphyseal defects—An animal study

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Incorporation and biodegradation of hydroxyapatite(HA)–tricalcium phosphate(TCP) granules implanted in $5 \times 5 \times 5$ mm distal femoral metaphyseal defects created in 18 adult rabbits were studied. In two rabbits, the defects were left to heal spontaneously without any implant. Roentgenographic and histological study by light microscopy was done on silver nitrate stained undecalcified sections as well as haematoxylin–eosin stained decalcified sections. The synthetic HA-TCP was biocompatible and produced no adverse reactions. The implant was osteoconductive and allowed good new bone formation to occur, mainly from periphery to center, but mature trabeculae could be delineated only at 4-6 months. The HA-TCP biomaterial had very low biodegradability with marked amount of intact implant still present at final follow up. Bonding between implant and bone, though a close biological bond, was not uniformly strong. Rate of bone ingrowth was very slow and large areas of implant at center did not show new bone formation at 12 months.

Keywords: Bioactive ceramics, Bone substitutes, Hydroxyapatite, Metaphyseal defects, Tricalcium phosphate

Calcium hydroxyapatite and tricalcium phosphate are bioactive ceramics and belong to the calcium phosphate family¹⁻⁴. They have been widely studied as bone graft substitutes^{2,3,5,6}. Biological, biomechanical and histological studies have been done regarding their implantation into bone and revealed their excellent osteoconductive properties and biocompatibility⁷⁻¹⁶. Among the many areas of use of hydroxyapatite (HA) and tricalcium phosphate (TCP) as bone graft substitute, one of the important is their use to fill cavities after excision of benign bone tumors¹⁷⁻¹⁹.

But, bone ingrowth in osteoconductive materials is limited largely to periphery of implants which does not make it suitable for repair of large defects. Incorporation at the centre of implant and strength of larger bone defects filled with implant have not been

properly assessed. Most workers have studied the ceramic implant by inserting it in small defects which may heal even without any supplementation. The present study has been undertaken to evaluate the incorporation and biodegradation of HA-TCP composite in large metaphyseal defects of bone. This was done through radiological and histological analysis of experimentally created bone defects in adult white rabbits.

Materials and Methods

The study was carried out on a total of 20 adult Newzealand white rabbits, *Oryctolagus cuniculus* (Linnaeus, 1758) of which 18 (16 test and two controls) were available for follow up. All animals were maintained as per the norms laid down by the Committee for Control and Supervision of Experiments on Animals, at the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (Regd. No. 542/02/ab/CPCSEA). The experimental protocol was approved by the Institutional Animal Ethical Committee. All rabbits were aged more than 16 weeks and weighed more than 2 kg. Synthetic calcium phosphate biomaterial in

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the form of multiphasic hydroxyapatite-tricalcium phosphate granules was used as the implant. Biphasic/multiphasic calcium phosphate of varying HA to β -TCP weight ratios was obtained by sintering precipitated calcium deficient apatite (calcium/phosphorus ratio <1.67)¹⁹. The granules used had an average porosity of 30% and internal pore size of about 10 μ m and consisted of 60-70% HA and 20-30% TCP.

Following pre-operative antibiotics (Ceftriaxone sodium, 250 mg, ip), thiopentone sodium anaesthesia (15 mg as a 2.5% solution, ip) was administered to all rabbits. Glycopyrrolate in a dose of 0.1 mg ip was used as anaesthetic premedication. The distal femoral metaphysis was exposed by a lateral approach and a 5 $\times$ 5 $\times$ 5 mm defect was made in the metaphyseal region (greater than 1/3 the diameter of bone at that level). The defect was filled with HA-TCP granules in 18 rabbits and in two rabbits defects were left unfilled to heal spontaneously. Post-operative parenteral antibiotics were administered 6 hr and again after 12 hr postoperatively. The suture site was examined on day 3 and sutures removed on day 10. Animals were kept in separate cages and fed on the standard rabbit diet. The rabbits were followed by serial roentgenographs. The X-rays were studied according to the following criteria given by Uchida *et al*¹⁸:

- (i) The presence of a radioluscent line or halo around the implant in the immediate post implantation period was looked for and its change with passage of time noted (obliteration of halo would imply bonding of implant with bone);
- (ii) Change in the density of the implant on radiographic image with passage of time (implying incorporation of implant and new bone in growth); and
- (iii) Changes at the margin of the implant (implying biodegradation and remodeling).

Rabbits were sacrificed at different intervals as shown in Table 1 and subjected to histological study. Histological study was done by both haematoxylin-eosin staining of decalcified sections and Von-Kossa's silver nitrate staining (modified) on undecalcified sections for calcium deposits (the implant is stained as dark black clumps). The distal femur was separated and placed in 10% formaline for at least 72 hr for fixation. The specimen was cut into two parts transversely at the mid section of implant.

One part was decalcified and subjected to haematoxylin - eosin staining while the second part was subjected to Von - Kossa's Silver nitrate staining (modified) in undecalcified form for calcium deposits.

The specimens, meant for H & E staining were decalcified for 4-6 days in 10% formic acid. The specimens were dehydrated by passing for 12 hr in each of the graded concentration of ethanol separately and finally absolute alcohol. Alcohol was washed with xylene solution and specimens were embedded in paraffin wax. Sections of 5-8 μ m thickness were made by cutting them with microtomes and were transferred to xylene solution to remove paraffin. They were kept on bovine albumin coated slides. Sections were stained with H & E and mounted with Canada balsam. The specimens meant for Silver nitrate staining were washed in running tap water. They were then bulk stained with 2% silver nitrate and exposed to strong artificial light for 1 hr. After washing for 3 min in distilled water, these were placed in 5% sodium thiosulphate for fixation. These specimens were placed in 10% formic acid for 4-6 days to make them soft. They were washed and dehydrated in the same manner as described above followed by paraffin wax embedding and sectioning to 5-8 μ m sections using microtome. These were subjected to eosin counterstaining.

Results

In the post-operative period, wound infection and dehiscence occurred in 3 rabbits, all of them in the test group, necessitating secondary suturing. There were no signs of toxemia or local foreign body reaction in any rabbit.

Radiological evaluation

HA-TCP composite is radioopaque and was visible in the X-rays throughout the follow up period. No

Table 1—Time intervals of histological study of various rabbits

Period of follow up	No. of rabbits
A. Rabbits with defect filled with implant	
3 weeks	2
8 weeks	3
16 weeks	2
6 months	3
8 months	2
10 months	2
12 months	2
B. Control (rabbits)	
3 months	1
8 months	1
C. Mortality (Not available for follow-up)	2

radioluscent gap at the host bone-implant junction was delineated in our study in 13 of the 16 rabbits studied (Fig. 1b). In those cases (3 rabbits) where it was appreciated, the halo was not completely obliterated even at follow up, though it was much less prominent. Evidence of its slow obliteration was seen only after 4 months. There was evident periimplant osteoporosis in 6 rabbits upto 6 months follow up.

The outline and margins of the implant on serial X-rays were preserved upto 6 months and even upto 8 months in 2 rabbits. At 8-10 months of follow up the margins appeared hazy and blended with surrounding bone implying incorporation and new bone in growth, discernible radiologically (Fig. 1d). The radiodensity of implants appeared to increase in serial follow up at an average of 4 months after implantation, though in as many as 6 rabbits the increase could not be appreciated.

Based on roentgenographic evidence alone, there was no evidence of significant implant biodegradation even at final follow up. Evidence of incorporation of HA-TCP with bone could be seen in rabbits at variable time periods ranging from 4 months to 8 months (Fig. 1). There was no evidence of pathological fracture or articular surface collapse in any rabbit in the follow

up. In the control group, the defect (without implant) could not be delineated on X-rays and no further radiological followup was done.

Histological evaluation

The following aspects were observed in histology: (i) bonding between the implant and bone; (ii) extent of bone ingrowth into implant; (iii) signs of intact implant material still present at final follow up; and (iv) signs of periimplant inflammation and foreign body reaction.

The implant could be clearly observed as black clumps on silver nitrate staining. The detailed histologic results are shown in Table 2. The control rabbits (two) were studied histologically at 12 weeks and 8 months. At 12 weeks, well delineated cavity with little peripheral new bone formation was noted. At 8 months, the cavity contained fibrous tissue and marrow, but no new bone was seen in large areas of the cavity.

Histologic sections of test rabbits at 3 weeks did not show any new bone ingrowth into the implant, and no biodegradation of the implant was evident (Fig 2). Only marrow and connective tissue proliferation was noted. Good bonding between implant and host bone was noticed at 16 weeks, but only at the



Fig 1—Serial radiographs of rabbit followed up for 10 months. (a) X-ray before implantation and (b) 3 weeks post implantation. X-ray at 3 months (c) shows good incorporation with no radiolucence between implant and bone. X-ray at 10 months (d) shows good incorporation but intact implant still seen as radioopacity, implying poor biodegradability of HA-TCP even at 10 months

Table 2—Temporal sequence of events in histologic study of incorporation and biodegradation of implant

Histologic characteristic under study	Period of study				
	3 weeks	6 weeks	16 weeks	8 months	12 months
Connective tissue proliferation into implant	Present	Marked	Marked	Fibrosis present within as well as periphery of implant	Same as 8 months
New bone ingrowth at periphery of implant	Absent	Minimum	Present (especially at the cortical window)	Marked new bone formation	Marked
New bone at center of implant	Absent	Absent	Absent	Scattered new bone present but no trabeculae delineated	Increased amount of scattered new bone present (Fig. 4 and 5) compared to 8 months but large areas at center devoid of new bone
Marrow tissue proliferation	Present	Marked	Marked	Marked	Marked (Fig. 5)
Bonding between host bone and implant	—	Mesenchymal tissue interposition present in many places	Bone formation in close proximity to implant is observed in some fields at periphery	Bonding not uniformly strong. No connective tissue and cellular elements in some places denoting very good bonding (Fig. 3); whereas connective tissue interposition present in some places	Many new formed trabeculae extending into implant with no fibrous tissue interposition
Delineation of well formed trabeculae within implant	Absent	Absent	Present	Present	Present
Biodegradation	No evidence (Fig.2)	No evidence	Few histiocytes seen implying minimal evidence of implant removal by body	Minimal Large areas of intact implant visible, especially at center	Same as 8 months (Fig. 4)
Periimplant inflammatory reaction	Absent	Mild increase in plasma cells and lymphocytes	Same as at 6 weeks	Minimal increase seen	Absent

periphery . Histologic section showing excellent bonding at 8 months is shown in Fig. 3. But this bonding was not a uniformly strong one. No bone ingrowth was seen at center of implant even at 12 months. Scattered new bone and marrow proliferation was very evident throughout the implant, except the center (Fig. 4 and 5). Even at 12 months large amount of intact implant was still present in the created defect. (Fig. 4), implying incomplete and poor biodegradability of HA-TCP in large bone defects.

Discussion

Biological, histological and biomechanical studies regarding implantation of calcium hydroxyapatite and β -Tricalcium phosphate, either alone or in combination have revealed their excellent osteoconductive properties and their safety^{7-14,17-19}. But conflicting reports exit regarding their extent and time duration of incorporation and biodegradability.

Tricalcium phosphate has been shown to have a greater rate of biodegradation and better incorporation when compared to HA, but is more amorphous and friable^{15,17}. Daculsi¹⁰ applied the biphasic calcium phosphate concept to artificial bone and tried to obtain an optimum balance of the more stable HA and more soluble TCP for controlling gradual dissolution in body and seeding new bone formation. Uchida *et al*¹⁷ found better bone ingrowth into HA and TCP than with calcium aluminate. HA and TCP in ratio of 70: 30% was used in the present study.

Radiographically HA-TCP composites is radioopaque and was visible in the X-rays throughout the follow up period. Uchida *et al*¹⁸, in their study of 60 benign bone tumours treated by resection and curettage followed by implantation of calcium hydroxyapatite ceramic (CHA) noted a radioluscent halo around the implant in the post implantation period which was gradually obliterated. Also with time after

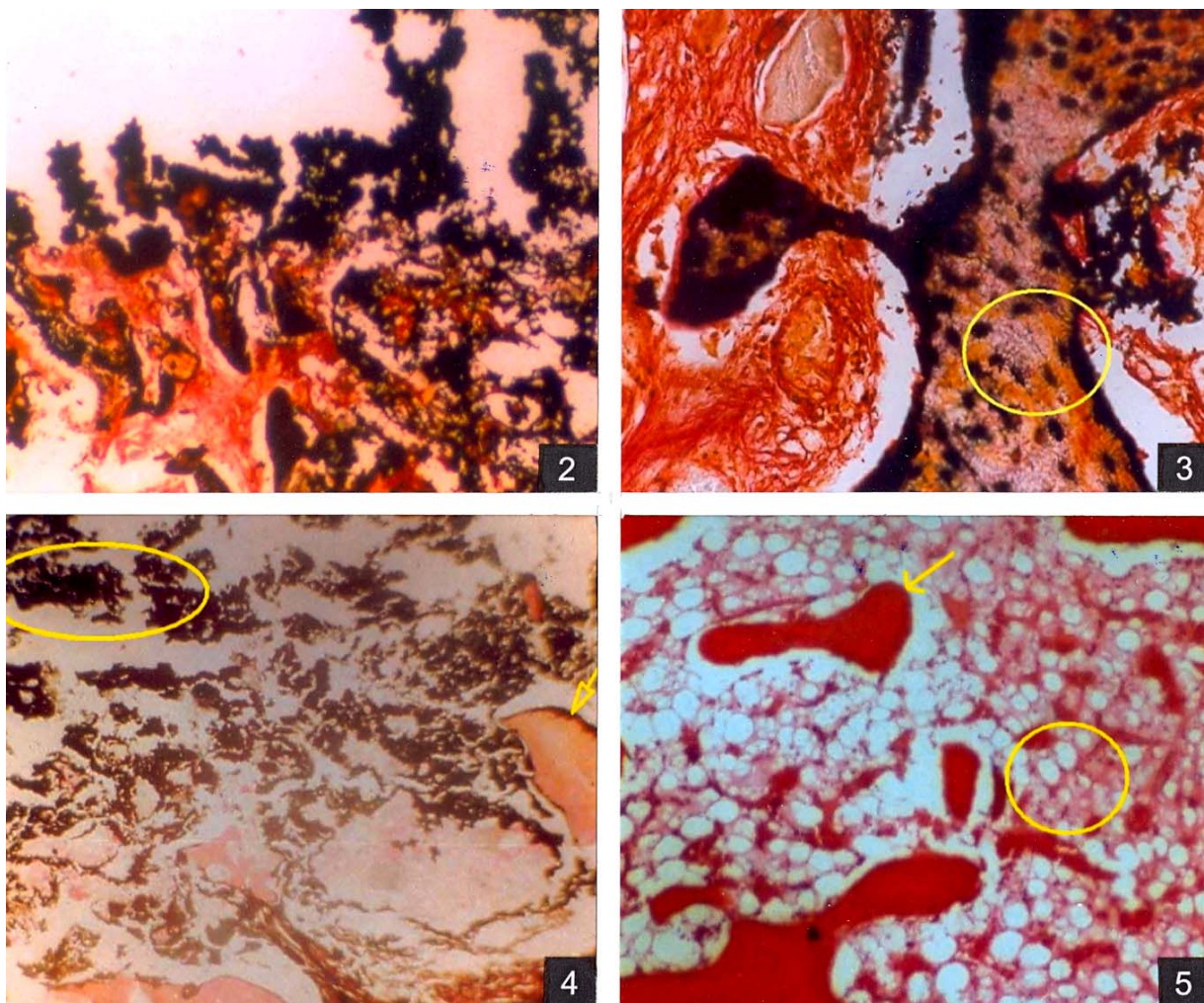


Fig 2-5—2: Photomicrograph showing the center of implant at 3 weeks post implantation. The scattered appearance is due to loss during processing and sectioning. The black clumps denote the implant. Modified Von Kossa's silver nitrate stain ($\times 40$); 3: Photomicrograph showing very good bonding between implant and host bone at periphery of the created defect at 8 months post implantation (marked by a circle). Silver nitrate stain ($\times 100$); 4: Photomicrograph at 10 months showing new bone in growth into implant (shown by arrow), but significant amount of intact implant still present (as seen within the circle). Silver nitrate stain ($\times 28$); 5: Photomicrograph at 12 months showing mature new bone (marked by arrow) and marrow (as seen within the circle) within the implant. H & E stain of decalcified bone-implant sections ($\times 400$).

In the present study, histological analysis showed HA-TCP as a good osteoconductive and bioactive material. The HA-TCP granules allowed good bone formation to occur, mainly from the periphery to the center. After 8-16 weeks new bone formation was visible in all microscopic fields at the periphery of implant and also scattered within the implant. This scattered new bone could be a result of osteogenic action of the bone marrow present within the cavity. Well defined trabeculae growing into the implant could be delineated only at 16 weeks.

HA and β -TCP have been shown to allow attachment, proliferation, migration and phenotypic

expression of bone cells leading to formation of new bone in direct apposition to the CaP biomaterial.⁴ Uchida *et al*¹⁸ had reported bone ingrowth in almost all pores of implant at 12 months; and Bucholz *et al*⁷ and Holmes *et al*^{13,14} had also reported complete implant incorporation. But unlike their reports, in the present study large areas at the center of implant were devoid of new bone even at the final follow up. That showed the inability of HA-TCP implant to act as an effective medium for bone formation when used in larger defects. That also confirmed previous observations that bone ingrowth in HA implants occurred from periphery to the center^{12,17-19}.

As regards the bioactivity of HA-TCP, though cellular elements and mesenchymal tissue interposition was found initially (8 weeks), new bone formation occurred in close apposition to the implant after the 16 weeks period as visualized in the microscopic fields, implying a strong biological bonding. But the bonding was not a uniformly strong one with fibrous tissue interposition present at some places even upto 10 months.

The present results showed no marked biodegradation of the HA-TCP biomaterial. Though good bone ingrowth occurred well into the implant, lot of intact implant material could be seen as black clumps in silver nitrate stained sections, especially at the center of implant. Holmes *et al.*,^{13,14} Uchida *et al.*¹⁸ and Yamamoto *et al.*¹⁹ had also noted only minimal biodegradation in their studies. Biodegradation in calcium phosphate materials has been suggested to be mainly cell mediated with histiocytes and multinucleated giant cells having been demonstrated^{4,19}. But the rate of resorption is too slow for the HA-TCP implant to be replaced significantly by new bone, thereby casting a doubt on the fracture strength of larger defects filled with these materials. The minimal biodegradation and the very slow rate of bone ingrowth make the HA-TCP used in granular form a poor option as a bone graft substitute in large metaphyseal defects.

No evidence of foreign body reaction to the HA-TCP composite was found in the present study in any rabbit. Though increase in plasma cell and lymphocyte count in the implant and periimplant area was seen in many histological sections between 8 week to 6 months, the increase was not marked. Fibrosis was evident within the implant and at bone-implant junction, even at final follow up. This could probably be due to inability to compactly fill the implant into the bone cavity under pressure. To conclude, HA-TCP used in large metaphyseal defects has a good incorporation with host bone only at the periphery; and has a poor biodegradability. It is probably not a good alternative to bone graft in large metaphyseal defects.

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